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Présentée par

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Propriétés pharmacologiques et caractérisation physico-chimique de la propolis et gelée royale

Soutenue le 07/02/2020 devant le jury composé de :

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Pharmacological properties and phytochemical characterization of propolis and royal jelly

Soukaïna El-Guendouz

Thesis submitted to the Faculty of science Dhar El Mahraz in partial fulfillment of the requirement for the degree of Doctor in biology

This thesis is dedicated …

To my beloved parents whom I treasure …

To my brothers and sister I love…

To all my family that I respect …

To the dear soul of my grandmother…

You have encouraged and supported me in moments of doubt and fatigue.
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Abstract

The hive is an unparalleled source of natural substances, where bees produce various products with a high benefit for human health. Propolis and royal jelly (RJ) are two of the most fascinating bee products possessing numerous biological activities. In order to evaluate local Moroccan products and contribute to their valorization, this thesis was devoted to study two Moroccan bee products: propolis and royal jelly (RJ). To go through this objective, the thesis was structured according to the results of eleven scientific publications and one book chapter. Various axes of research were drawn. First of all, available scientific data of Mediterranean propolis and RJ were reviewed and we have concluded that in Morocco there was limited scientific research regarding those two bee products. Afterwards, samples of propolis and RJ were gathered from different regions of Morocco and were subjected to extensive investigations regarding their biological activities and chemical composition. Besides, an attempt to introduce the propolis in different applications for diverse purposes was implemented.

The evaluation of the biological properties of Moroccan propolis revealed it high polyphenol contents and a large spectrum of biological activities including antioxidant, antibacterial, anti-quorum sensing, anti-biofilm, anti-acetylcholinesterase, antidiabetic, anti-inflammatory, anti-tyrosinase and anti-xanthine oxidase. The results showed differences between the activities among the propolis samples and we suggested that the geographic origin was the main factor responsible on those differences. On the basis of this findings, we tested the effect of propolis coupled with magnetite nanoparticles (MNPs) on adherence capacity of *Staphylococcus aureus* (*S. aureus*) on experimental catheter and it was possible to conclude that the tested nanocomposite 'MNPs / propolis' reveals a high potential to prevent or disrupt the formation of *S. aureus* biofilms as compared to the control. Moreover, this nanostructure (MNPs / propolis) was coupled to the antibiotic 'chloramphenicol' and was tested to be used as pharmaceutical agent (drug delivery system) to face various bacterial infections caused by *S. aureus.* This experimentation allowed us to show that, the tested nanocarrier (MNPs /propolis / chloramphenicol) inhibit the bacterial growth and has the capacity to disrupt and protrude bacterial cell wall. In other field, propolis was tested for its capacity to prevent oxidation of oil/water (O/W) emulsion and the results attests that at the concentration of 0.02 and 0.04%, propolis was more effective on preventing the oxidation during the storage, especially when the lipid phase was almond oil, as compared to the thyme waste extract and butylated hydroxyanisole (BHA). Moreover, the volatiles composition of Moroccan propolis evaluated by GC (gas chromatography) and GC/MS (gas chromatography coupled to mass spectrometry) revealed the presence of two clusters: one with samples mainly constituted by *β*-eudesmol, cedrol and *n*-tricosane, and the second one dominated by *ar*-curcumene.

On the other hand, the reviews done of data found in literature on RJ makes clear the situation of the RJ investigation, especially those of Mediterranean origin being limited in very few countries. Nevertheless, accessible data demonstrates it high water, protein and sugar contents, besides its richness in lipids, vitamins, minerals and phenols. Investigations of volatiles in RJ are scarce. In the few

references found determining the composition of volatile fractions of RJ, we can report that several factors are responsible for differences in the general composition such us honeybee species, harvesting time, regions, storage conditions and extraction methods. Only one report was found regarding the volatile constituents of Mediterranean RJ, and reported it to be dominated by carboxylic acids and some newly identified compounds such as 2-hydroxyhexanoic acid, phenylacetic acid, pentanoic acid and 2-methylbutanoic acid. The biological properties of RJ were mainly exploited in medicine through various protocols for the benefit of human health.

Regarding Moroccan RJ, it was rich in protein and 10-HDA (10-hydroxy-2-decenoic acid) and its biological capacities were compared with samples from Spain and Portugal. The results have shown that, except the powder form, RJ from Morocco was less effective as compared to the ones from Portugal and Spain, this could be related to the harvesting method which is mainly traditional in Morocco, as well as storage condition, besides the geographic origin. While, the chemical composition of volatiles disclose that it was dominated by linolenic acid, 2-decenoic acid and octanoic acid in variable amounts.

Overall, those findings highlighted the important potential of two bee products originated from Morocco, and underline the necessity to explore them more, in the intention to increase the scientific information in an attempt to standardize those products and contribute for its valorization.

Résumé

La ruche est une source incomparable de substances naturelles, où les abeilles produisent divers produits dotés d'effets thérapeutiques. La propolis et la gelée royale (GR) sont parmi les produits apicoles les plus fascinants possédant de nombreuses activités biologiques. Afin d'évaluer les produits Marocains locaux et contribuer à leur valorisation, cette thèse était consacrée à l'étude de ces deux produits constituant un trésor caché et une merveille naturelle. Pour atteindre cet objectif, différents axes de recherche ont été élaborés, afin de structurer cette thèse en fonction des résultats de onze publications scientifiques et un chapitre de livre.

D'emblée, notre étude a été dédiée à une révision détaillée des données scientifiques disponibles dans la littérature, concernant la propolis et la GR méditerranéennes á pair de laquelle nous avons conclu que les recherches scientifiques concernant ces deux produits sont très peu nombreuses. Par la suite, les échantillons de la propolis et de la GR ont été collecté de différentes régions du Maroc et ont fait l'objet d'une études approfondies en ce qui concerne leurs activités biologiques et leurs composition chimique. En outre, une tentative visant à introduire la propolis dans différentes applications à diverses fins a été mise en œuvre.

L'évaluation des propriétés biologiques de la propolis Marocaine a révélé une teneur élevée en polyphénols et un large spectre d'activités biologiques : antioxydante, antibactérienne, anti-quorum sensing, anti-biofilm, anti-acétylcholinestérase, antidiabétique, anti-inflammatoire, anti-tyrosinase et anti-xanthine oxydase. Les résultats ont montré des différences entre les activités des échantillons de la propolis ce qui a suggéré que l'origine géographique pourrait être principalement le responsable de cette différence. En se basant sur ces résultats, nous avons testé l'effet de la propolis couplée aux nanoparticules de magnétites (MNP) sur la capacité d'adhérence de *Staphylococcus aureus* (*S. aureus*) sur un cathéter expérimental et il était possible de conclure que ce nano-composite testé 'MNP / propolis' a un potentiel élevé de prévention ou de perturbation de la formation de biofilms de *S. aureus* par rapport au control. De plus, cette nanostructure (MNP / propolis) couplée à l'antibiotique 'chloramphénicol' a été testée pour être utilisée en tant qu'agent pharmaceutique (système de libération de médicaments) pour faire face à diverses infections bactériennes causées par *S. aureus.* Cette expérience nous a permis de montrer que le nano-porteur testé (MNP / propolis/ chloramphénicol) inhibe la croissance bactérienne et pouvait perturber et faire saillir la paroi cellulaire bactérienne. Dans autres domaines, la propolis a été testée pour sa capacité d'empêcher l'oxydation de l'émulsion huile / eau (H / E) et les résultats montrent qu'à la concentration de 0.02 et 0.04%, la propolis était plus efficace à prévenir l'oxydation au cours du stockage, en particulier lorsque la phase lipidique était l'huile d'amande, par rapport à l'extrait de déchets de thym, mais aussi à l'hydroxyanisolène butylé (BHA). De plus, la composition de la partie volatiles de la propolis Marocaine a été évaluée par analyse GC (chromatographie en phase gazeuse) et GC / MS (chromatographie en phase gazeuse couplée à la spectrométrie de masse). Les résultats ont révélé la présence de deux principaux groupes : un groupe renfermant des échantillons

constitués principalement de *β*-eudesmol, de cédrol et de *n*-tricosane et un second dominé par l'*ar*-curcumène.

En revanche la recherche bibliographique sur les données trouvées dans la littérature sur la GR montre clairement que la situation de la recherche scientifique focussant sur la GR, en particulier celles d'origine méditerranéenne, est limitée dans très peu de pays. Néanmoins, les données accessibles montrent sa teneur élevée en eau, en protéines et en sucre, ainsi que sa richesse en lipides, vitamines, minéraux et phénols. Les rapports sur la composition de la partie volatiles de la GR sont rares. Les quelques références qui déterminent la composition des fractions volatiles de la GR, montrent que plusieurs facteurs sont responsables des différences dans la composition générale, telles que la race des abeilles, le temps de la récolte, les régions, les conditions de stockage et les méthodes d'extraction. Un seul rapport a été trouvé concernant la composition de la fraction volatile des GR Méditerranéenne, indiquant qu'elle était dominée par les acides carboxyliques et certains nouveaux composés identifiés tels que l'acide 2-hydroxyhexanoïque, l'acide phénylacétique, l'acide pentanoïque et l'acide 2-méthylbutanoïque. Les propriétés biologiques de la GR ont été principalement exploitées en médecine selon différents protocoles au profit de la santé humaine.

En ce qui concerne la GR Marocaine, notre étude révèle sa richesse en protéines et en 10-HDA (acide 10-hydroxy-2-décénoïque) et ses capacités biologiques ont été comparées à des échantillons provenant d'Espagne et du Portugal. Les résultats ont montré qu'à l'exception des échantillons sous forme de poudre, la GR du Maroc était moins efficace que celle du Portugal et de l'Espagne, ce qui pourrait être lié à la méthode de récolte, qui est principalement traditionnelle au Maroc, ainsi qu'aux conditions de stockage en plus de l'origine géographique. La composition chimique des substances volatiles indique qu'il était dominé par l'acide linolénique, l'acide 2-décénoïque et l'acide octanoïque en quantités variables.

En général, ces résultats ont mis en évidence le potentiel important de deux produits apicoles originaires du Maroc soulignant la nécessité de les explorer davantage, dans l'intention d'accroître les informations scientifiques pour tenter de les normaliser et de contribuer à leur valorisation.

Resumo

A colmeia é uma fonte incomparável de produtos naturais, onde as abelhas produzem vários produtos com altos benefícios para a saúde humana. A própolis e a geleia real (GR) são dois dos mais fascinantes produtos apícolas que possuem inúmeras atividades biológicas. Com o objetivo de avaliar os produtos Marroquinos locais e sua valorização, esta tese foi dedicada ao estudo de dois produtos apícolas Marroquinos: a própolis e a geléia real (GR). Para percorrer este objetivo, a tese foi estruturada de acordo com os resultados de onze publicações científicas e um capítulo do livro. Vários eixos de pesquisa foram elaborados. Em primeiro lugar, os dados científicos disponíveis da própolis mediterrânea e da GR foram revist os e concluímos que, em Marrocos, havia pouca pesquisa científica sobre esses dois produtos apícolas. Posteriormente, amostras de própolis e da GR foram coletadas em diferentes regiões do Marrocos e submetidas a extensos estudos sobre sua composição química e atividades biológicas. Além disso, foi realizada uma tentativa de introduzir a própolis em diferentes aplicações para diversos fins.

A avaliação das propriedades biológicas da própolis marroquina mostrou que tem um alto conteúdo de polifenóis e possui um amplo espectro de atividades, incluindo atividades antioxidantes, antibacterianos, inibição do quorum sensing, anti-biofilme, anti-acetilcolinesterase, antidiabética, anti-inflamatória, anti-tirosinase e anti-xantina oxidase. Os resultados mostraram que houve diferenças de atividades entre as amostras de própolis e sugeriram que a origem geográfica foi o principal fator responsável por essas diferenças. Esses resultados permitiram testar a própolis acoplada a nanopartículas de magnetita (MNPs) e foi possível constatar que este nanocompósito (própolis / MNPs) revelou um alto potencial para prevenir ou interromper a formação de biofilmes de *Staphylococcus aureus*. Além disso, essa nanoestrutura (própolis / MNPs) acoplada ao antibiótico cloranfenicol foi testada com a finalidade de uso como sistemas de entrega de de fármacos contra diferentes cepas de *S. aureus*, e os resultados mostraram que ela inibiu o crescimento bacteriano e tem a capacidade de romper e sobressair a parede celular. Em outro lado, a própolis foi testada quanto à sua capacidade de impedir a oxidação de emulsão óleo / água (O/A) e os resultados atestam que na concentração de 0,02 e 0,04% a própolis foi mais eficaz na prevenção da oxidação durante o armazenamento, especialmente quando a fase lipídica foi óleo de amêndoa, em comparação com o extrato de resíduos de tomilho e 2 e 3 terc-butil-4-hidroxianisol (BHA).

Outrossim, a composição volátil da própolis marroquina avaliada por CG (cromatografia gasosa) e GC/EM (cromatografia gasosa acoplada a espectrometria de massas) revelou a presença de dois aglomerados: um gropo de amostras constituídas principalmente por *β*-eudesmol, cedrol *e n*-tricosano, e o segundo dominado por *ar*-curcumene.

A revisão feita na GR, originária do Mediterrâneo, deixa clara a situação da investigação sobre a GR sendo limitada em poucos países. No entanto, dados acessíveis demonstram que ela tem alto teor de água, proeínas e açúcar além de sua riqueza em lipídios, vitaminas, minerais e fenóis. A investigação de

voláteis no GR é escassa. Nas poucas referências encontradas determinando a composição das frações voláteis da GR, podemos relatar que vários fatores são responsáveis por diferenças na composição geral, como espécies de abelhas, época de colheita, regiões, condições de armazenamento e métodos de extração. Por outro lado, apenas um trabalho foi encontrado sobre seus constituintes voláteis e relatou que é dominada por ácidos carboxílicos e alguns novos compostos identificados, como ácido 2-hidroxi-hexanóico, ácido fenilacético, ácido pentanóico e ácido 2-metilbutanóico. As propriedades biológicas do GR foram principalmente exploradas na medicina usando vários protocolos para vários fins em benefício da saúde humana.

Em relação a GR marroquina, era rica em proteínas e 10-AHD (ácido 10-hidroxi-2-decenóico) e suas capacidades biológicas foram comparadas com amostras da Espanha e Portugal e os resultados mostraram que, exceto a forma de pó, o GR de Marrocos foi menos eficaz em comparação com os de Portugal e Espanha, isso pode estar relacionado ao método de colheita que é principalmente tradicional e às condições de armazenamento em Marrocos, além da origem geográfica. Enquanto, A composição química dos voláteis revela que foi dominada pelo ácido linolênico, ácido 2-decenóico e ácido octanóico em quantidades variáveis.

No geral, essas descobertas destacaram o importante potencial de dois produtos apícolas originários de Marrocos e destacam a necessidade de explorá-los mais, com a intenção de aumentar as informações científicas na tentativa de padronizar esses produtos e contribuir para sua valorização.

ملخص

خلية النحل مصدر لا مثيل له من المواد الطبيعية ، حيث ينتج النحل منتجات مختلفة ذات فائدة عالية لصحة الإنسان. يعتبر العكبر وغذاء ملكات النحل من أكثر منتجات النحل المهمة التي تمتلك العديد من الأنشطة البيولوجية. من أجل تقييم المنتجات المغربية المحلية والمساهمة في تثمينها، تم تكريس هذه الأطروحة لدراسة منتجين مغربيين من منتجات النحل: العكبر وغذاء ملكات النحل. لتحقيق هذا الهدف، تم تنظيم الأطروحة وفقا لنتائج أحد عشر منشورا علميا وفصلا واحدًا من كتاب علمي. محاور بحث مختلفة وضعت. أولا، تمت مراجعة البيانات العلمية المتاحة فيما يتعلق بالعكبر وغذاء ملكات من البلدان المنتمية للبحر الأبيض المتوسط وتم استنتاج أنه في المغرب، البحث العلمي محدود جدا فيما يتعلق بهذين المنتجين. بعد ذلك، تم جمع عينات من العكبر وغذاء ملكات النحل من مناطق مختلفة من المغرب وتم إخضعها لدراسات واسعة النطاق فيما يتعلق بأنشطتها البيولوجية والتركيب الكيميائي، بالإضافة إلى ذلك، تم تنفيذ محاولة لإدخال العكبر في تطبيقات متعددة في مجالات مختلفة.

كشف تقييم الخواص البيولوجية للعكبر المغربي عن احتوائه على نسبة عالية من البوليفينول ومجموعة كبيرة الأنشطة البيولوجية: كمضاد للأكسدة، مضاد للجراثيم، مضاد ل " quorum sensing"، له القدرة علا مكافحة تشكيل الأغشية الحيوية من طرف البكتيريا، مضاد للأسيتيل كولينيستراز، مضاد للسكري، مضاد للالتهابات، مضاد تيوزيناز و "الزانثين أكسيداز". إنطلاقا من هذه النتائج لوحظ تواجد اختلافات في جميع الأنشطة البيولوجية لعينات العكبر المغربي الشيء الذي يرجح أن الأصل الجغرافي يمكن أن يكون العامل الرئيسي المسؤول عن تلك الاختلافات. بناءً على هذه النتائج، اختبرنا تأثير العكبر مرفق مع جسيمات المغنتيت النانوية (MNPs) على قدرة تمسك بكتيريا المكورات العنقودية الذهبية *(Staphylococcus aureus)* على قسطر تجريبي و تم استنتاج أن المركب النانوي الذي تم اختباره "MNPs / عكبر" لديه قدرة عالية لمنع أو تعطيل تكوين الأغشية الحيوية من طرف بكتيريا المكورات العنقودية الذهبية. علاوة على ذلك، تم ربط هذه البنية النانوية (MNPs / عكبر) بالمضاد الحيوي "الكلورامفينيكول" وتم اختبارها لاستخدامها كعامل دوائي (نظام تسليم الدواء) لمواجهة الالتهابات البكتيرية المختلفة التي تسببها المكورات العنقودية الذهبية. سمحت لنا هذه التجربة بإظهار أن النانوتركيبة المختبرة (MNPs / propolis / chloramphenicol) تحول دون نمو البكتيريا ولديها القدرة على التأثير سلبيا على جدار الخلية البكتيرية. في مجال آخر، تم اختبار قدرة العكبر على منع أكسدة مستحلب زيت في ماء (O / W) ، وتشير النتائج إلى أن العكبر بتركيز 0.02 و 0.04 ٪ كان أكثر فعالية في الوقاية من الأكسدة طولى مدة التخزين، خاصة عندما يكون المكون الدهني للمستحلب مكون من زيت اللوز،هذا مقارنة مع مستخلص مخلفات الزعترو هيدروكسيانيسول بوتيلاتي (BHA). علاوة على ذلك، تمت دراسة مكونات الزيت الطيارة المستخرجة من العكبر المغربي من خلال تحليل GC (كروماتوجرافيا الغاز) و / GC MS(كروماتوجرافيا الغاز مرفقة بقياس الطيف الكتلي). كشفت النتائج عن وجود مجموعتين رئيسيتين مختلفتين: المجموعة الأولى تحتوي على عينات تتكون أساسًا من *β*-eudesmol -، *n*- cedrol و tricosane و الثانية يغلب عليها *ar*-curcumene .

من ناحية أخرى ، توضح المراجعات التي أجريت للبيانات الموجودة في المؤلفات والمراجع العلمية المتوفرة التي تتطرق إلى دراسة غذاء ملكات النحل أن الأبحاث المنجزة، خاصة تلك التي تخص غذاء ملكات النحل المنحدر من بلدان البحر الأبيض المتوسط، محدودة في عدد قليل جدًا من البلدان. من خلال البيانات التي يمكن الوصول إليها توضح أن عذاء ملكات النحل يحتوي على نسبة عالية من الماء، البروتين والسكر، إلى جانب ثرائه بالدهون، الفيتامينات، المعادن وأيضا المركبات الفينولية. تقاريرالبحوث فيما يتعلق بالمواد الطيارة في عذاء ملكات النحل نادرة جدا. استنادا على المراجع القليلة الموجودة التي تحدد تركيبة هذه الأخيرة، يمكننا استنتاج أن عدة عوامل مسؤولة عن الاختلافات في تركيبها العام، مثل نوع نحل العسل، المناطق المنحدرة منها العينات، ظروف التخزين، وقت وطرق إستخراج العينات. تم العثور على تقرير واحد فقط فيما يتعلق بمكونات المواد الطيارة لغذاء ملكات النحل المنحدرمن بلدان البحرالأبيض المتوسط، حيث اتضح أنه يهيمن عليها الأحماض الكربوكسيلية وبعض المركبات التي تم تحديدها حديثا مثل حمض 2-هيدروكسي هكسانويك، وحمض فينيل أسيتيك، حمض البنتانويك وحمض 2-ميثيل بوتانيك. من جهة أخرى تم استغلال الخصائص البيولوجية لغذاء ملكات النحل بشكل رئيسي في الطب عن طريق بروتوكولات مختلفة لخدمة صحة الإنسان.

فيما يتعلق بغذاء ملكات النحل من أصل مغربي، أظهرت الدراسات التي تم القيام بها أنه غني بالبروتينات و10 -HDA (حمض هيدروكسي -2 دينوكويك) أيضا، تمت مقارنة خواصه البيولوجية بالخواص البيولوجية لعينات من إسبانيا والبرتغال. أظهرت النتائج أنه، باستثناءالعينات اللتي على شكل مسحوق، كان غذاء ملكات النحل المغربي أقل فعالية بالمقارنة مع العينات البرتغالية والإسبانية. في حين أن دراسة التركيب الكيميائي للمواد الطيارة كشفت أنه متكونعموما من حمض اللينولينيك، حمض دينيويك وحمض الأوكتانويك بتراكيز مختلفة.

بشكل عام، سلطت هذه النتائج الضوء على أهمية نوعين من منتجات نحل العسل من المغرب، حيث تم التأ كيد على ضرورة إخضاعهما لدراسات مكثفة بشكل أكبر، بهدف زيادة المعلومات العلمية حولهما في محاولة لتوحيد تلك المنتجات والمساهمة في تثمينها.

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List of abbreviations

10-HDA	10-hydroxy-2-decenoic acid
2D-PAGE	Two-dimensional polyacrylamide gel electrophoresis
A.D	Anno Domini
AAPH	2,2’-Azobis (2-methylpropionamidine) dihydrochloride
ABTS	2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid)
AC	Aberrant crypt foci
AChE	Acetylcholinesterase
AD	Alzheimer's disease
ADP	Ddenosine 5’-diphosphate
ALT	Alanine aminotransferase
AMP	5’-monophosphate
AST	Aminotransferase
ATCI	Acetylthiocholine iodide
ATP	Adenosine triphosphate
AUC	Area under the curve
B.C	Before Christ
BHA	Butylhydroxyanisole
BHI	Brain-heart infusion
BHT	Butylhydroxytoluene
BMD	Bone mineral density
BSAE	Bovine Serum Albumin Equivalents
C6-HSL	N-hexanoylhomoserine lactone
CAE	Caffeic acid equivalent
CAPE	Caffeic acid phenethyl ester
CAT	Superoxide catalase
CCD	Central composite design
CCl_4	Carbon tetrachloride
CDDP	cisplatin
CFU	Colony forming units
CLo	Chloramphenicol
COX	Cyclooxygenase
CsA	Cyclosporine
CZE	Capillary Zone Electrophoresis
DNP	2,4-dinitrophenylhydrazine
DPD	dihydropyrimidine dehydrogenase
DPPH	2,2-diphenyl-1-picrylhydrazyl
DTNB	5,5′-dithiobis(2-nitrobenzoic acid)
EAC	Ehrlich ascites carcinoma
EDTA	Ethylenediaminetetraacetic acid
EE	Eriodictyol equivalent
EEAC	Catechin equivalent antioxidant capacity
EEP	Ethanolic extract of propolis
ES	Spain
EUCAST	European Society of Clinical Microbiology and Infectious Diseases
FAE	Ferulic acid equivalent
FDA	Food and Drug Administration
FIDs	Flame ionization detectors
FL	Fluorescein
FRAP	Ferric Reducing Antioxidant Power
FTIR	Fourier Transform InfraRed spectroscopy
GA	Gallic acid
GABA	Gamma amino butyric acid
GAGs	Glycosaminoglycans

GC-MS	Gas Chromatography-Mass Spectrometry
GRAS	Generally recognized as safe
GSH-Px	Glutathione peroxidase
GST	Glutathione S-transferase
H_2O_2	Hydrogen peroxide
HAIs	Healthcare associated infections
HCT 116	Human colorectal cancer
HDL	High-density lipoprotein
HepG2	Human liver cancer
HPLC	High-performance thin-layer chromatography
HSV-1	Herpes simplex virus
IC_{50}	Half maximal inhibitory concentration
IHC	International Honey Commission
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IL	Interleukine
IR	Infrared
IRI	Irinotecan
LB	Luria-Bertani
LC-MS	Liquid Chromatography-Mass Spectrometry
LOX	Lipoxygenase
LPS	Lipopolysaccharide
m/z	Mass/charge ratio
MA	Morocco
MAP	Modified atmosphere packaging
MBC	Minimum bactericidal concentration
MCa	transplantable mammary carcinoma
MDA	Malondialdehyde
MIC	Minimum inhibitory concentration
MN	Micronucleus
MNPs	Magnetite nanoparticles
MRJPs	Major royal jelly proteins
MRSA	*Staphylococcus aureus methicillin-resistant*
MSSA	*Staphylococcus aureus methicillin-susceptible*
MSTFA	N-methyl-N-(trimethylsilyl) trifluoroacetamide
NADH	Nicotinamide adenine dinucleotide disodium salt hydrate
NBT	Nitroblue tetrazolium
NE	Not Evaluated
NI	Not Indicated
NIMs	Nano-in-microparticles
NO	Nitric oxide
NOS_2	Nitric oxide synthase
NPs	Nanoparticles
O/W	Oil-in-water
OA	Oleic acid
OAS	Oral allergy syndrome
OD	Optical density
ORAC	Oxygen radical absorbance capacity
P	Propolis
PBS	Phosphate buffered saline
PDMAB	p-dimethylaminobenzaldehyde
PDS	Propolis-based dietary supplement
PGE_2	Prostaglandin E_2
PMS	Phenazine methosulfate
PNPG	p-Nitrophenyl-β-D-glucopyranoside

PPO	Polyphenol oxidase
PT	Portugal
PUFAs	polyunsaturated fatty acids
Py-GC/MS	Pyrolysis coupled to gas chromatography and mass spectrometry analysis
PZQ	Praziquantel
QE	Quercetin equivalent
QS	Quorum sensing system
RJ	Royal jelly
ROS	Reactive oxygen species
RSM	Response surface methodology
SCI	Spinal cord injury
SDS-PAGE	Sodium dodecyl polyacrylamide gel electrophoresis
SEM	Scanning electron microscope
SLNs	Solid lipid nanoparticles
SOD	Superoxide dismutase
SPM	Suspended particulate matter
SPSS	Statistical Package for the Social Sciences
T	Tartrazine
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid-reactive substances
TE	Trolox equivalent
TEAC	Trolox Equivalent Antioxidant Capacity
TMAH	Tetramethylammonium hydroxide solution
TNF-α	Tumor necrosis factor-alpha
TPC	Total phenol content
TSHR Ab	Thyroid-stimulating hormone receptor antibodies
TWE	Thyme waste extract
UHPLC-LTQ/ORBITRAP/MS/MS	Ultrahigh Performance Liquid Chromatography-Linear Ion Trap-High Resolution Orbitrap Mass Spectrometry System
UPGMA	Unweighted pair group method with arithmetic averages
UVA	Wave ultraviolet
UVB	Short wave ultraviolet B
v/v	volume/ volume
V/W	volume/weight
WGO	wheat germ oil
XO	Xanthine oxidase
XRD	X-ray diffraction
β-CD	β-cyclodextrin

Preface

Allopathic medicine is facing several criticisms or even rejections, due to its countless side effects, where the severity varies from a drug to another. Traditional medicine being more natural and much less invasive has been the goal of many researchers in order to apply it as an alternative or complementary medicine. This recent interest shift increased greatly the consumption of natural products, from where the renewed interest in the properties of natural products including bee products.

Toolbox validated by science, Apitherapy, whose roots can be traced back more than 6000 years to medicine in ancient Egypt, is currently known as alternative or complementary medicine to prevent or treat ailments and promote healing using bee products. It is considered as a precious chance which it is up to us to seize and develop, for a less expensive pharmacopoeia, with much lower side effects. Hive is source of a variety of precious and valuable natural products including honey, propolis, royal jelly (RJ), bee venom, bee bread, bee pollen and wax. They have been used for therapeutic and medicinal purposes due to the various bioactive molecules they contain. Propolis and RJ are two natural products with health-promoting bioactive compounds, who have been known from centuries for their enormous potentialities on human health. Propolis is a resinous substance used by bees to seal holes in hives, smooth out the internal walls making the hive defensible and as a barrier against external invaders and diseases, thermal insulation, humidity and wind. Whilst, RJ is secreted by young worker honeybees and serves as the most important part of honeybee larvae diet, playing a major role in caste differentiation and development of the queen honeybee.

Currently, many studies worldwide, are targeted toward investigating the health benefits and pharmacological properties of bee products, especially propolis and RJ. In Morocco, with the 'Plan Maroc Vert' strategy, much attention has been given to apiculture and exploration of bees-product. Nevertheless, beekeeping in Morocco is less developed and still confined to honey. Moreover, scientific information's are very scarce on the quality of national bee products as compared to international available information. Hence, to pursue the international development of bee products, an extensive scientific investigation regarding chemical composition and biological properties as well as potential applications is mandatory for the valorization of the Moroccan hive products.

Thereupon, this thesis aim was to investigate propolis and RJ from different regions in Morocco regarding their chemical composition, biological properties and possible application for possible typification and thus to contribute on ensuring the quality and safety necessary for a commercial purpose, which could be a serious challenge for a globally competition. To achieve this goal the following approach was used and thirteen chapters were developed.

The thesis is divided in three major Sections which in turn are split up into chapters. In propolis Section there are nine Chapters, in one of them thyme waste was included in the aim to compare this natural product with propolis. In RJ Section (section 2) there are three Chapters. Section 3, provides a

main conclusion and summarizes the most important accomplishment of the preceding Chapters, as well as future perspective taking into account the main contributions of this work.

Regarding the propolis Section, first of all, available information about propolis from countries surrounding Mediterranean Sea were gathered and a review of the different aspects involving this bee product, from historical information and physical characteristics, to chemical composition and biological activities besides their various applications, was performed and was displayed in Chapter 1. Afterward, for Chapter 2, samples of propolis were gathered from different regions in Morocco and were extracted with an adequate method then were subjected to preliminary screening of biological activities, including anti-oxidant, ant-inflammatory and antibacterial through various experimental designs. Chapter 3 describes the activity of Moroccan propolis on diverse enzymes with important health actions. Since antimicrobial and antioxidant activities were found to be important for those propolis samples, Chapters 4 and 5 reported the applications of magnetite nanoparticles functionalized together with propolis as possible nano-antibiotics with the objective to provide responses that could overcome to the increase of microorganisms' resistance to the antibiotics. In Chapter 6 it is described the utilization of propolis as antioxidant in emulsions O/W for diverse applications, mainly cosmetics, in order to replace the synthetic ones. In Chapter 7, the principle is the same of that reported in the Chapter 6, but propolis was replaced by thyme waste obtained after steam distillation in local producers of essential oils in Portugal. In Chapter 8, it is compared the results of those obtained using propolis and thyme waste. In chapter 9, we reported the volatile composition of Moroccan propolis.

Because the rareness and difficulty of finding and collecting RJ samples in Morocco, as well as its sensibility to storage condition, the respective Section has only three Chapters. The Chapter 10 represents a review of the chemical composition and biological activities of RJ from the Mediterranean area. While, Chapter 11, display available data concerning the chemical composition of the volatile part of RJ. Whereas, Chapter 12 is devoted to a general research study of volatiles components and biological properties of sample of RJ from Morocco, and then the results were compared with samples from the Mediterranean area.

To be aforementioned that, the work presented in this thesis, is an adapted form from eleven scientific papers and one book chapter, each one corresponds to one Chapter, ten of them have been previously published in referred Journals including the chapter book, and two are already submitted being under review. While, because of the limitation of volume the thesis, the appendices report two research articles underlining the effect of combined honey and propolis administration on urine volume and electrolytes in animal models, as well as a preliminary screening of the protective effect of propolis against chromium-induced nephrotoxicity in rats. Moreover, supplementary details of each Chapter are described in the Appendices.

List of publications:

SECTION 1

1. **Soukaina El-Guendouz,** Badiaa Lyoussi and Maria G. Miguel. Insight on propolis from Mediterranean countries chemical composition, biological activities and application fields. Chemistry & Biodiversity, 16 (7), **2019.** Doi: 10.1002/cbdv.201900094.
2. **Soukaïna El-Guendouz,** Smail Aazza, Badiaa Lyoussi, Vassya Bankova, Milena Popova, Luís Neto, Leonor Faleiro and Maria G. Miguel. Moroccan propolis: a natural antioxidant, anti-bacterial and anti-biofilm against *Staphylococcus aureus* with no induction of resistance after continuous exposure. Evidence-Based Complementary and Alternative Medicine. (4), 19, **2018.** Doi: 10.1155/2018/9759240.
3. **Soukaïna El-Guendouz**, Smail Aazza, Badiaa Lyoussi, Maria D. Antunes, Maria L. Faleiro Maria G. Miguel. Anti-acetylcholinesterase, antidiabetic, anti-inflammatory, anti-tyrosinase and anti-xanthine oxidase activities of Moroccan propolis. International Journal of Food Science and Technology. 51 (8), 1762-1773, **2016**. Doi: 10.1111/ijfs.13133.
4. **Soukaïna El-Guendouz**, Smail Aazza, Badiaa Lyoussi, Vassya Bankova , João P. Lourenço, Ana M. Rosa Costa, José F. Mariano, Maria G. Miguel and Maria L. Faleiro. Impact of biohybrid magnetite nanoparticles and Moroccan propolis on adherence of methicillin resistant strains of *Staphylococcus aureus*. Molecules. 21(9), 1208, **2016**. Doi: 10.3390/molecules21091208.
5. **Soukaïna El-Guendouz,** Badiaa Lyoussi, João P. Lourenço, Ana M. Rosa Costa, Maria G. Miguel, Cristina Barrocas Dias, Ana C. Manhita, Luísa Jordão, Isabel Nogueira, Maria L. Faleiro. Magnetite nanoparticles functionalized with propolis against methicillin resistant strains of *Staphylococcus aureus*. Journal of the Taiwan Institute of Chemical Engineers. 102, 25-33, **2019.**
6. **Soukaïna El-Guendouz,** Smail Aazza, Badiaa Lyoussi, Nessrine Majdoub, Vassya Bankova, Milena Popova, Sara Raposo, Maria D C Antunes, Maria Graça Miguel. Effect of poplar-type propolis on oxidative stability and rheological properties of O/W emulsions. Saudi Pharmaceutical Journal, 26 (8), 1073-1082, **2018**. Doi: 10.1016/j.jsps.2018.05.017.
7. **Soukaïna El-Guendouz,** Smail Aazza, Susana Anahi Dandlen, Nessrine Majdoub, Badiaa Lyoussi, Sara Raposo, Maria Dulce Antunes, Vera Gomes and Maria Graça Miguel Antioxidant activity of thyme waste extract in O/W emulsions. Antioxidants. *8* (8), 243. **2019**. https://doi.org/10.3390/antiox8080243.
8. **Soukaïna El-Guendouz,** Smail Aazza, Susana Anahi Dandlen, Nessrine Majdoub, Badiaa Lyoussi, Sara Raposo, Vera Gomes, Maria Graça Miguel. Natural antioxidants in emulsions O/W. **2019** (submitted).
9. **Soukaïna El-Guendouz,** Badiaa Lyoussi, Maria Graça Miguel, A. Cristina Figueiredo. Characterization of volatiles from Moroccan propolis samples. Journal of Essential Oil Research. 27-33, **2018**. Doi*:* 10.1080/10412905.2018.1520748.

SECTION 2

10. **Soukaïna El-Guendouz,** Badiaa Lyoussi and Maria G. Miguel. Journal of Apicultural research Insight in chemical composition and biological properties of Mediterranean Royal jelly. **2019** (submitted).
11. Maria G. Miguel and Soukaïna El-Guendouz. 'Volatile Compounds of Royal Jelly'. In Bee Products - Chemical and Biological Properties; Alvarez-Suarez, J.M. (Eds.). Springer International Publishing. pp. 191-198, **2017**.
12. **Soukaïna El-Guendouz**, Alexandra M. Machado, Smail Aazza, Badiaâ Lyoussi, M. Graça Miguel, M. Conceição Mateus and A. Cristina Figueiredo. Chemical characterization and biological properties of royal jelly samples from the Mediterranean area. Natural product communications, 15(2): 1–13, **2020**. DOI: 10.1177/1934578X20908080.

APPENDICES

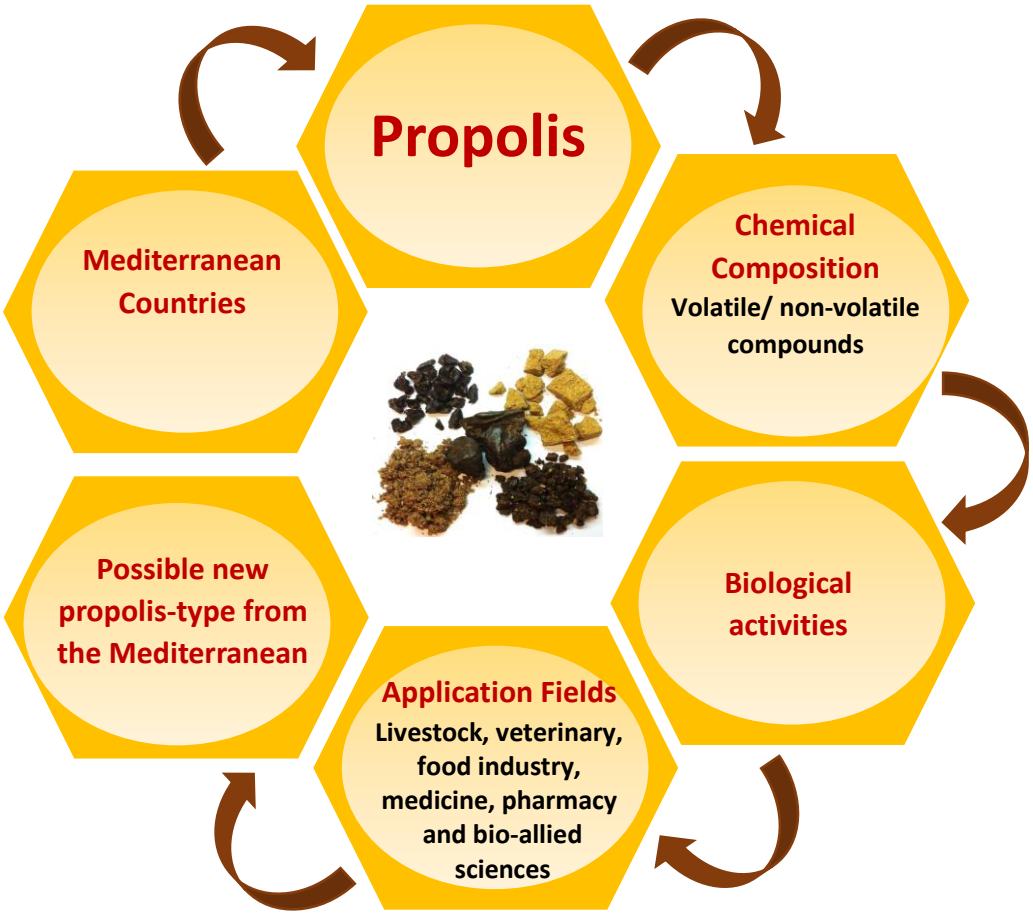
13. **Soukaïna El-Guendouz**, Noori Al-Waili, Smail Aazza,Youssef Elamine, Soumia Zizi, Thia Al-Waili, Ali Al-Waili, Badiaa Lyoussi. Antioxidant and diuretic activity of co-administration of Capparis spinosa honey and propolis in comparison to furosemide. Asian Pacific Journal of Tropical Medicine. 10 (10), 974-980, **2017**. https://doi.org/10.1016/j.apjtm.2017.09.009.
14. **Soukaïna El-Guendouz**, Soumia Zizi, Youssef Elamine and Badiaa Lyoussi. Preliminary screening of the possible protective effect of propolis against chromium-induced nephrotoxicity in animal model. Veteranary World, **2020** (accepted).

SECTION 1

Propolis

Chapter 1

Insight on propolis from Mediterranean countries: chemical composition, biological activities and application fields



Insight on propolis from Mediterranean countries: chemical composition, biological activities and application fields*♠

Abstract

This review updates the information upon the chemical composition of propolis from all Mediterranean countries as well as their biological properties and applications. The non-volatile fraction of propolis was characterized by the presence of phenolic acids and their esters, and flavonoids. Nevertheless, in some countries, diterpenes were also present: Sicily (Italy), Croatia, Malta, Creta (Greece), Tukey, Cyprus, Egypt, Lybia and Algeria. The volatile fraction of propolis was characterized by the presence of benzoic acid and its esters, mono- and sesquiterpenes, being the oxygenated sesquiterpene ß-eudesmol characteristic of poplar propolis, whereas the hydrocarbon monoterpene α-pinene has been related with the presence of conifers. Regardless the chemical composition, there are common biological properties attributed to propolis. Owing to these attributes, propolis has been target of study for applications in diverse areas, such as food, medicine and livestock.

*Adapted from 'Insight on propolis from Mediterranean countries: chemical composition, biological activities and application fields'. Soukaïna El-Guendouz, Badiaa Lyoussi and Maria G. Miguel. Chemistry & Biodiversity, 16 (7), 2019. DOI: 10.1002/cbdv.201900094.

♠ Detailed information's about the chemical composition the non-volatile and volatile parts of propolis from different Mediterranean Sea countries, its biological properties as well as the fields of application are displayed in appendices A.

1.1. Introduction

Nowadays, there is an increased awareness on the part of consumers of the harmful effect of synthetic products, used in different field, on human health. Hence, the use of product and compounds from natural source has highly increased. In this framework, extracts from natural origins takes special attention from the food and pharmaceutical industries. Results of scientific research collected regarding bee products revealed a far-reaching ability of this 'boon' of nature with several nutraceutical applications, from daily used product such as creams and shampoo to foods and medical treatment. In this context, in the last years, we assist to a strong renewed interest in the properties of natural products including bee products.

The hive is unparalleled source of natural products, where bees produce various products with a high benefit for human health. It is unquestionable that, the most popular bee-product is honey, it has been largely used in medical treatment, while, the other hive products such as, bee-pollen, propolis, royal jelly (RJ) and bee venom have been less involved in medical applications.[1,2,3] Propolis is a resinous substance gathered by honey bees, from different part of melliferous plants, mixes with their enzymes and used for the construction and maintenance of their hives. Propolis is known as a source of bioactive compounds with enormous biological and pharmacological potentialities as well as the capacity to prevent and treat numerous diseases. Thus, the exploration of bee products regarding their chemical composition and biological capacities is of high importance to their standardization thus promoting their potential applications and therefore their commercialization.

1.2. Propolis in history

Among the various insect species existed on our planet, none is found more attractive than the honey bee regarding their importance for our environment, economy as well as our health. One of the oldest forms of animal life still in existence from the Neolithic Age are honey bees. Honey bees and their products like propolis, bee wax, pollen, royal jelly, as well as honey, had been known and used even in antiquity and the Middle Ages, they can be traced back to 13,000 B.C.[4,5]

The term "propolis" is derived from the Greek joining two words pro- ('in front of' or 'at the entrance to') and -polis ('community' or 'city') which means a substance that is for or in defense of the bee-hive.[1] There is a long history of the use of propolis, and it continue until today in homemade remedies. It was used by mankind since the ancient times, extensively employed by several civilizations as antiseptic and a raw material for numerous preparations such as perfumes, health foods and beverages.[6,7] It was well known in ancient Egypt, to the

priests who has monopolized medicine, chemistry and art of mummifying corpses.[2] Various Greek and Roman physicians, including Aristoteles, Dioscorides, Hippocrates, Pliny, and Galen, described several preparations of propolis for different purposes. The Greeks have involved propolis in the formulation of a perfume called "polyanthus" as a main ingredient, besides, olibanum, styrax and aromatic herbs.[8] Hence, the Greek doctor Discorides, in 1st century A.D, considered that propolis is originating from Styrax trees and declare it having medical properties.[8] Furthermore, the Roman scholar Plinius (23-79 A.D) hypothesized, that its originating from buds of different trees such as willow, poplar, elm, reed and many other plants. He declares in his 35 volumes 'Natural History' to know propolis as glue in the hive and reported it to be used for several medical applications.[9] In addition, the ancient Jews in history considered "tsori", which means propolis, as a medicine.[2,10] The Arabs also, knows about propolis. Doctor Abu Ali Ibn Sina (Avicenna) distinguished two kind of wax in his well-known work 'The Canon of Medicine (Al Qanûn fi Al-Tibb)', the clean wax and the black wax. The clean one was the comb wells where the bees rear the brood and store the honey, while, the black one, with a strong smell, has the characteristics to eliminate the spikes of the bolts and the stakes, also it rarefied, cleans and soaks.[11] In the Middle Ages, in folk Georgian medicine, ointments with propolis were used to cure oral and pharyngeal infections as well as dental caries.[5] In addition, the medicine book "The Carbadini", published in the 13th century, the author suggests that propolis is good against dental decay.[5,11] In the early history in Europe, propolis was mentioned in the herbal literatures, in John Gerard's herbal book, 'The History of Plants' (1597), where he mentioned the use of "the resin or clammy substance' which comes from the black poplar tree buds, for healing ointments in the treatment of inflammations and wounds.[10] The use of propolis was not exclusive for medical applications, Stradivari, Amati and others, a violin builders, used propolis as constituent of their violin varnish.[13] Thereafter, between the seventeenth and twentieth century's, propolis which was considered as a 'drug', becomes very popular in Europe especially for its anti-bacterial activity.[14] The beginning of the 20th century (1908) assisted the first research work about propolis composition indexed by chemical abstracts.[15] As reported by Toreti et al.,[15] only after one hundred and nine years from the first scientific publication, the number of publications on propolis reached 3880 in journals. [15] Nowadays, the global interest in propolis research have been renewed, it was appreciated for its wide spectrum of therapeutic applications, which make it a potential candidate in several scenarios.

1.3. General overview on propolis

Honey bees '*Apis mellifera*', native in many parts of Europe, Africa and the Middle East; collects resin from buds and leaves of numerous tree species like birch, poplar, pine, alder, willow and palm.[14] Material actively secreted by plants and droplets appearing on the trunks or limbs of trees as well as resin from a few tropical flowers are also collected by honeybee.[16] This resin is gathered by fragmenting leaves using their mandibles (mouthparts) or by direct collection from plant surface.[16] Once collected, bees then carry this resin on their mandibles to the site in the hive where it will be mixed with wax and their salivary and enzymatic secretions, it becomes what beekeepers know as propolis.[2,17] The fact of collecting this plant resin to use it inside their hive on the behalf of protecting colony against pathogens and parasite is a "social immunity" which characterizes many social insects.[16] Furthermore, it was also supported that the presence of this resin in the nest have a large effect in the immune defense of individual bees, improving colony health and fitness.[18]

In general, honeybees foragers, collects the resins from droplets appearing on the bark of the trunks or limbs of trees from the surfaces of some fruits or more typically on the vegetative apices, with the privilege of young leaves and vegetative buds.[19] Mainly, the collection goes through four stages, starting from fragmentation of the particle of the gathered resin with their mandibles; pass it to the forelegs; then transfer it to the middle leg; and finally pass it to the corbicula of the hind leg on the same side.[2] A sequence of the four steps is repeated until there is a maximum of resin loaded on both corbicula. This process takes from seven to fifteen minutes going up to an hour depending on the weather.[20] Once full load, bees-foragers returns to the colony to deposit the collected resin and the nestmates bees 'cementing bees' unload the resin which can takes from 15 minutes to seven hours or even overnight.[19] Then cementing bees smoothes the resin, using their mandibles in a manner that is similar to that of wax construction, to become 'PROPOLIS'.[20] Bees-foragers usually are not older than 15 days old,[8] while it was observed that cementing bees are older with atrophied wax glands.[19] Even if the availability of resin foragers is from May to November in temperate regions, it seems that the collect of resin depend on the seasonal factor, in general it is made in late summer (end of June) trough autumn, when the honey flow is greatly decreased.[20] Foraging for resins was reported to be occurs between 10 am and 3:30 pm during sunny days, as resin is easy to gather under high temperatures.[19,20] It was supposed that the period of collect is due to some inherent change in foraging behavior in late summer, not by climatic conditions being that some bees could be induced to collect resin on any time in the season.[2] Apparently, all cited factor which interfere on resin collection are interrelated to those involved in the use of propolis by the

bees.[2] Moreover, it is conceivable that the fact of collecting resin in late summer is a result of the lower pollen and nectar foraging.[19]

Its noteworthy that not all bees collect the resin (exp: *Apis cerana* and *Apis florea*), and those who collect resin don't do it on the same degrees, it depends on the needs of the colony, some species and races rely very little on the substance such as colonies of *Apis dorsata* which use resin periodically to invigorate the site of comb attachment on a branch, while others use resins and propolis extensively such as '*Apis mellifera*'.[16] Stingless bee species '*Melipona* spp.', native to tropical countries, collect also the plant exudates and combine it with soil elements from where the name of 'geopropolis'.[123]

In Morocco, there are three predominant breeds of bees. They are differentiated mainly by their color. Two of them, the "*Apis mellifira intermissa*" tellien (North West Atlas), and the "*Apis mellifira major*" (North Rif), are black. The last, "*Apis mellifira sahariensis*" (South-East Atlas), is golden yellow.[20,21]

Inside de hive, propolis, have different functions, it is deposed in a thin layer on the internal walls of their hive to cover surfaces, seal holes and cracks, close gaps in the hive to reduce the air currents and/or reducing size of the entrance for securing the entrance, thus for easier defense[11]. Moreover, propolis is used to embalm killed invader insects such as the wax moths '*Galleria mellonella*' as well as '*Achroia grisella*' ,' *Acherontia atropos*', '*Vespa vulgaris*', '*V. crabro*', and small snails '*Limacida*, *Arionida*', they cover the carcass, being too heavy to carry outside, with propolis and wax, hence ensuring sterile conditions inside the hive that protects them from pathogenic microorganisms which may invade the bee colony.[16]

1.4. Physical characteristics

Propolis known also by 'bee glue' is identified as a dark-colored resinous and sticky substance.[22] The physical appearance of propolis varies widely, it is usually lipophilic in nature, its texture is variable among the samples from hard and brittle, to, soft, pliable, gummy, and very sticky.[22] Propolis is characterized by a pleasant aromatic smell and divers color ranging from light yellow/yellow green to red and to dark brown. It was also reported the existence of a transparent propolis.[13] In a very low temperature propolis is hard and brittle, but under a temperature of 25 °C to 45 °C it becomes soft, pliable and sticky, while, with increasing temperature, above of 45 °C, it transformed to a very sticky and gummy material, which becomes liquid at temperatures <60 °C depending on the sample origin.[22] Considering the characteristics of propolis, cited above obviously it is not easily extracted. The common suitable

solvent used for propolis extraction are water, methanol, ethanol, chloroform, dichloromethane, ether, and acetone with the privilege of water and alcohol.[13]

Figure 1.1. a) Moroccan crude propolis samples;
b) hydroalcoholic extracts of Moroccan propolis

1.5. Mediterranean propolis

Generally, raw propolis is mainly composed of resin and vegetal balsam polysaccharides, and proteins. However, it is originated from resin material of different plant species whereby it has a chemical diversity and different raw appearance which varies considerably with the geographical origin owing to the local flora. Many studies revealed different types of propolis around the world with different chemical composition according to the plants available around the hive, the geography and climatic conditions of the harvesting site[23].

According to the previous review,[7] seven main types of propolis have been reported depending on their major constituents and plant origin: poplar, birch, green (alecrim) Brazilan, red, "Clusia", "Pacific" and Mediterranean. Poplar propolis (resin source is from *Populus* species) can be found in the temperate regions of Europe and North America, non-tropical regions of Asia, China and New Zealand. In this kind of propolis, flavones, flavonones, cinnamic acids and their esters are the typical components.[7,24] Birch propolis from Russia (resin source is *Betula verrucose*) where the main compounds are flavones and flavonols different from those found in poplar type.[7,24] Green propolis from Brazil (Baccharis spp.) is mainly composed by prenylated phenylpropanoids, caffeoylquinic acids and diterpenes.[7,24] In red propolis found in Brazil, Cuba and Mexico arising from *Dalbergia* spp. predominates isoflavonoids, neoflavonoids, pterocarpans and lignans.[7,24-27] *Clusia* propolis originating from Cuba and Venezuela arising from flowers of *Clusia* spp. is rich in prenylated benzophenones.[7,24]

Pacific propolis from Okinawa, Taiwan, Indonesia (*Macaranga tanariu*) is rich in *C*-prenyl-flavanones.[7,24] Mediterranean propolis from Sicily, Greece and Malta in which the plant source belongs to the Cupressaceae family[28], present the diterpenes (mainly acids of labdane type) as the major compounds.[7,29-32]

In this work we will not deal with propolis from all over the world, instead, we are going to focus on the comparison of available data of propolis originating from Mediterranean Sea countries regarding its chemical composition of volatile and non-volatile part, biological properties and fields of application. It must be emphasized that in the literature it was found many papers reporting the chemical and biological application of propolis in different area, many more than those reported in the tables below, but we did not mention them all due to the lack of any information on the geographic origin of propolis used. To be noted also that papers reporting commercial propolis with unknow geographic origin will not be included. Moreover, from Tables A.1, A.2, A.3 and A.4 (see appendices), it is clear that there are some countries where the propolis research area is more developed than others, obviously from the number of papers in relation with propolis found, notably Italy, Croatia and Turkey from southern European coast of the Mediterranean Sea.

1.6. Chemical composition of Mediterranean propolis

1.6.1. Non-volatile constituent of propolis

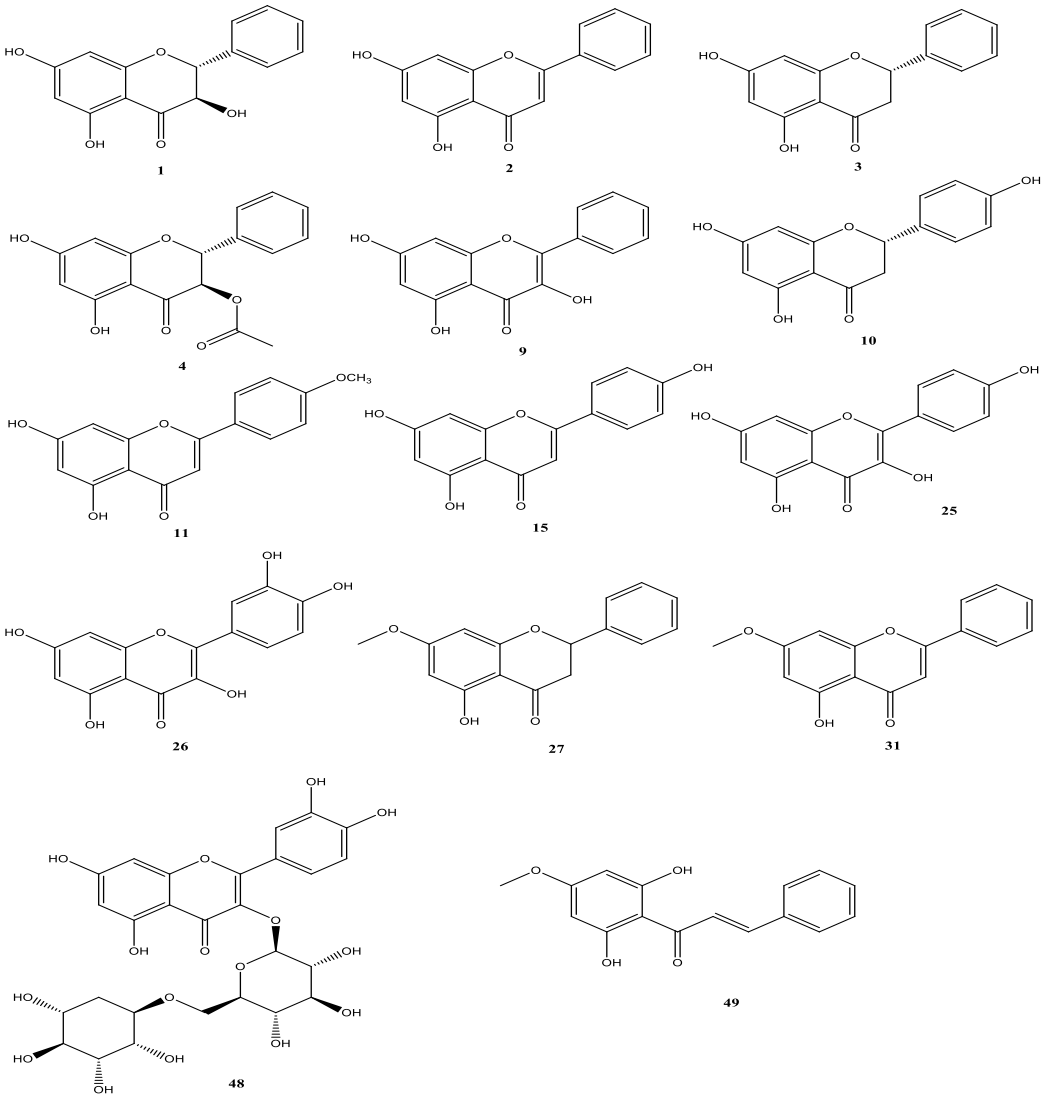
In the last decades, propolis has become the subject of several studies carried out all over the world. However, the wide application of propolis in modern medicine has drawn growing attention to its chemical composition which is crucial for understanding its biological activities. Many authors have shown that the observed effects could be the result of the synergistic effect of its complex constituents, itself are dependent on different parameters such the botanical origin of propolis samples. [33,34]

The Mediterranean, an eco-region with a common sea that links three continents, is remarkable with the richness of its biodiversity. Twenty-two countries and territories border on the Mediterranean belonging to the Southern European, Levantine and Northern African Coasts; hence, the chemical compositions of propolis may vary between the different regions. As far as we found, in Table A.1, we summarized the chemical composition of non-volatile part of Mediterranean propolis.

1.6.1.1. Southern European Coast

Hydro-alcoholic extracts of propolis from Spain were analyzed regarding their chemical composition. Kumazawa et al.[35] reported that East Andalusian ethanolic extract of propolis (EEP) was mainly composed by flavonoids (Figure 1.2) such as pinobanksin (**1**), chrysin (**2**), pinocembrin (**3**), and pinobanksin-3-acetate (**4**) and phenolic acids (Figure 1.3) such as caffeic acid (**5**), *p*-coumaric (**6**) and ferulic (**7**) acids. Caffeic acid phenethyl ester (CAPE) (**8**) was also mentioned to be present in Spanish propolis samples.[35-37] Volpi and Bergonzini[38] declares

that propolis from Spain contain as a main bioactive compound flavonoids such as pinocembrin (**3**), galangin (**9**), naringenin (**10**), acacetin (**11**) and chrysin (**2**). Also, Vivar-Quintana et al.[37] in a study in which they determined the phenolic acids present in crude propolis from Galicia and Castilla y León, Northern Spain, they found the same phenolic acids already cited by Kumazawa et al.[35] Volpi and Bergonzini[38] and Sawaya et al.[36] declares that propolis from Spain contain as main bioactive compounds flavonoids such as pinocembrin (**3**), galangin (**9**), naringenin (**10**), acacetin (**11**) and chrysin (**2**). In the other hand, in propolis samples originating from La Alcarria and Nerpio it was also reported the presence of hydrocarbons and long chain fatty acids.[39] To be concluded that whatever the geographical origin of Spanish propolis, flavonoids and phenolic acids were present in almost all tested samples.



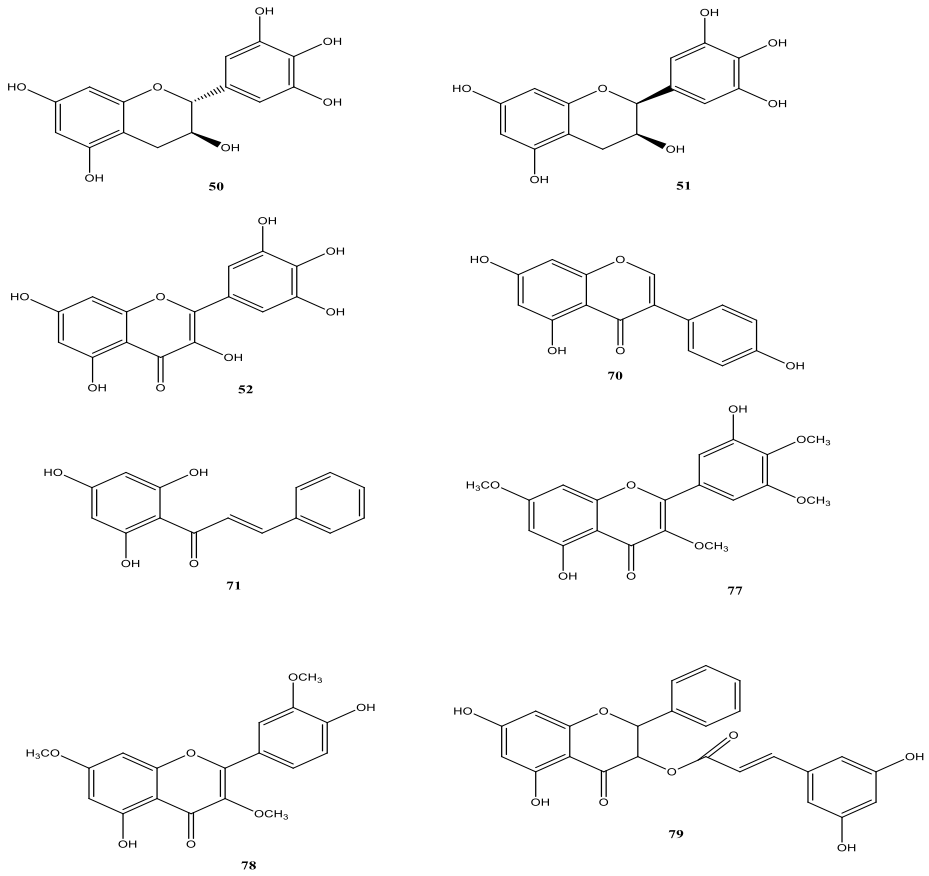


Figure 1.2. Examples of flavonoids and their derivatives present in Mediterranean propolis.

Data regarding the chemical composition of propolis from France are scarce. Boisard et al.[40,41] declared that phenolic acids esters [benzyl caffeate (**12**) and prenyl caffeate (**13**)] and flavonoids [pinobanksin-3-acetate (**4**), pinocembrin (**3**), chrysin (**2**), and galangin (**9**)] were the most abundant constituents of French propolis. Caffeic acid (**5**), *p*-coumaric acid (**6**), ferulic acid (**7**) and isoferulic acid (**14**) were also detected in those propolis samples. The study of Chasset et al.[42] was in concordance with[40,41] finding about the composition of French propolis, where they investigated propolis from different areas in France through high-performance thin-layer chromatography and mass spectrometry (HPTLC/MS). Regardless the collection area of propolis in France, phenolic acids and flavonoids predominated in the extracts. Nevertheless the individual flavonoids were not with the same abundance in all extracts and apparently without any correlation with the collection site. For example, galangin was present in both continental and Mediterranean samples.[42]

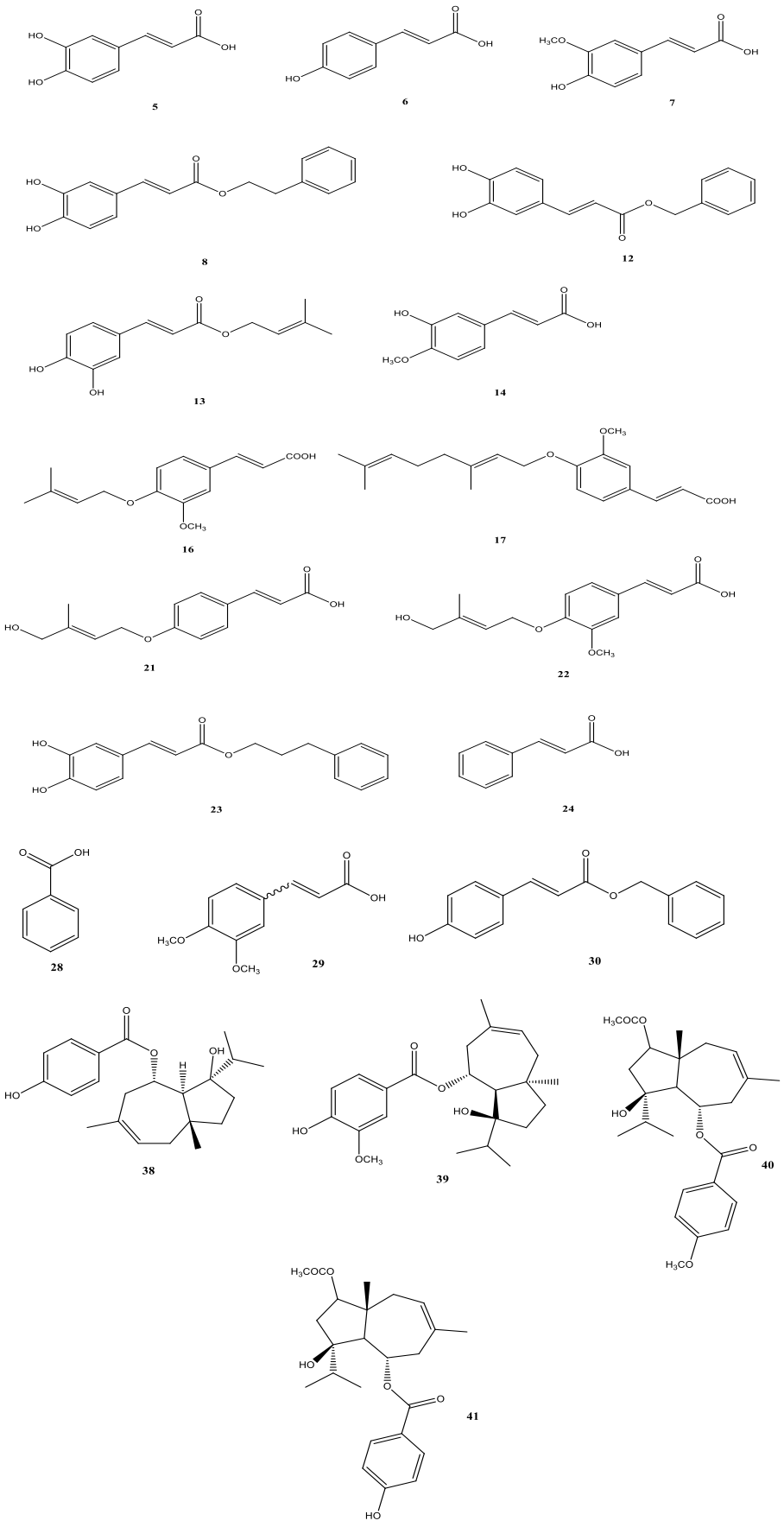


Figure 1.3. Examples of phenolic acids and their derivatives present in Mediterranean propolis.

Several studies have demonstrated the existence of flavonoids as main components of Italian propolis.[30,38,43--48] The analysis of ethanolic extracts of propolis by several authors has showed the presence of pinocembrin (**3**), galangin (**9**), naringenin (**10**), chrysin (**2**), and apigenin (**15**) (Figure 1.2) as common flavonoids found in all analyzed samples. While Bankova et al.[29] found also in propolis samples originating from Piedmont, Emilia Romagna, and Sicily, benzyl caffeate (**12**) and phenethyl caffeate (**13**) (Figure 1.3). Nevertheless, it is noteworthy the presence of diterpenic acids (53 %) in the sample from Sicily, not found in the remaining propolis samples, in contrast to the finding,[46] in which diterpenes were not detected in the Italian island. Phenolic acids were also found in Italian propolis and the main present ones were *p*-coumaric acid (**6**), ferulic acid (**7**) and caffeic acid (**5**). Some phenolic acid esters, such as CAPE (**8**) were reported to be included on the chemical composition of Italian propolis (Figure 1.3).[45,47,48] From above, the authors concluded that all samples assayed from central Italy were of poplar origin. However, it is not clear that the Mediterranean propolis, characterized by the presence of diterpenes, is typical of the Mediterranean islands, since Gardini et al. [46] did not find them in Sicilian propolis. More recently, Taddeo et al.[49] have reported the presence of new phenolic acid derivatives [boropinic acid (**16**) and 4'-geranyloxyferulic acid (**17**)] (Figure 1.3), and coumarin derivatives [umbelliferone (**18**), 7-isopentenyloxycoumarin (**19**) and auraptene (**20**)] (Figure 1.4) in Italian propolis, and Fiorito et al.[50] declared to have found two new cinnamic acids [4-(3'-hydroxymethyl-3'-methylallyloxy)acid (**21**) and 4-(3'-hydroxymethyl-3'-methylallyloxy-3-methoxycinnamic acid (**22**)] (Figure 1.3) in propolis originating from Italy.

Slovenian propolis was characterized by different phenolic acids and flavonoids as previously reported.[51] Its analysis through liquid chromatography-mass spectrometry (LC/MS) have shown the presence of various bioactive compounds namely *p*-coumaric (**6**), ferulic (**7**) and caffeic (**5**) acids, caffeic acid benzyl ester (**12**) and caffeic acid cinnamyl ester (**23**), cinnamic acid (**24**) (Figure 1.3), pinobanksin (**1**), apigenin (**15**), kaempferol (**25**), pinobanksin-3-*O*-acetate (**4**), chrysin (**2**), galangin (**9**), pinocembrin (**3**) (Figure1.2). Therefore, the authors concluded that the phenolic profile of Slovenian propolis is comparable to that of European propolis.[51,52] More recently, a group of research has reported that propolis samples from Slovenia, Croatia and France, mainly reflected patterns characteristic of blue-type propolis rich in flavonoids and with low quantity of phenolic acids.[53]

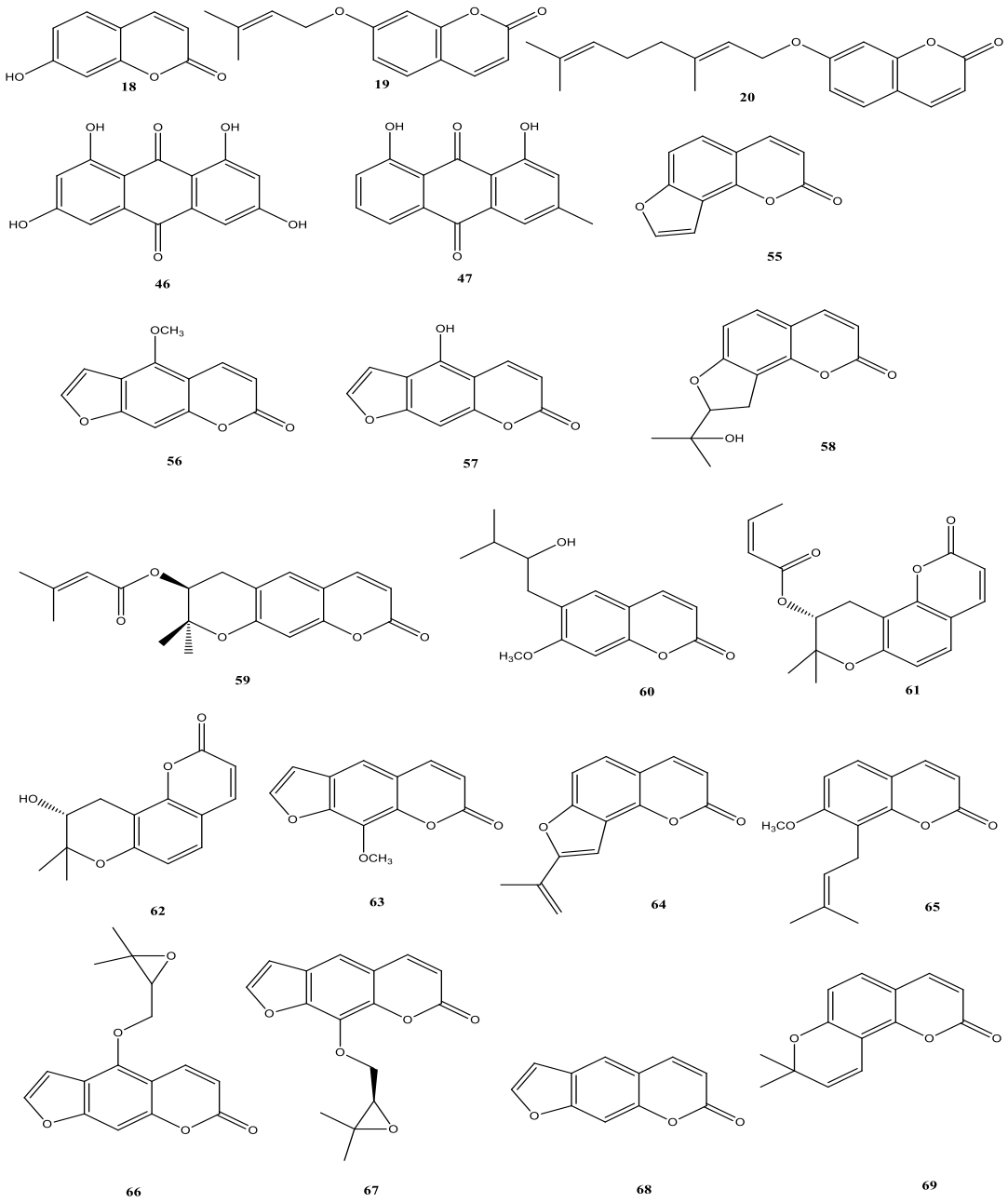


Figure 1.4. Examples of anthraquinones (**18**-**20)** and coumarins present in Mediterranean propolis.

Ethanolic extracts of propolis from several locations in Croatia, analyzed by several methods such as GC/MS, two-dimensional thin-layer chromatography, HPLC-thin-layer chromatography among others, had relatively high amounts of flavonoids besides the presence of phenolic acids. It was found that the main flavonoids found were pinocembrin (**3**), pinobanksin (**1**), pinobanksin-3-*O*-acetate (**4**), chrysin (**2**), quercetin (**26**), kaempferol (**25**) pinocembrin-7-methyl ether (**27**) and galangin (**9**) (Figure 1.2), while benzoic (**28**), ferulic (**7**), caffeic (**5**), 3,4-dimethoxycinnamic (**29**), cinnamic (**21**) acids were the most abundant phenolic

acids.[54-64] However, other authors demonstrated that Croatian propolis contain phenolic acid esters such as CAPE (**8**), benzyl caffeate (**12**), benzyl coumarate (**30**) and cinnamic acid esters.[64-66] On the another hand, Sobočanec et al.[67] have reported the presence of flavonoids only in propolis samples from Continental Croatia. To be noted that in propolis samples from Lokrum Island and Trogir it was reported the presence of low diterpene content.[61]

Regarding Bosnia and Herzegovina, as far as we know, only a study effectuated by Barbarić et al.[68] through HPLC, reported the presence of ferulic (**7**), *p*-coumaric acids (**6**), tectochrysin (**31**), pinocembrin (**3**) and chrysin (**2**) as main common components of propolis.[68] Not so far, in Albania, Kujumgiev et al.[69] have previously reported the presence of phenolic acid ester (53 %) and flavonoids (38%) in propolis samples.

Investigation of the silylated ethanolic extracts of propolis from Malta analyzed by GC/MS has revealed to possess the typical Mediterranean chemical profile being rich in diterpene compounds. Isocupressic (**32**), communic (**33**), pimaric (**34**) and imbricatoloic acid (**35**), together with totarol (**36**) and 13-*epi*-torulosal (**37**) were found in all samples. Mono- and sesquiterpenyl esters of substituted benzoic acids were also detected [ferutinin (**38**), teferin (**39**), 2-acetoxy-6-*p*-methoxybenzoyljaeschkeanadiol (**40**) and 2-acetoxy-6-*p*-hydroxybenzoyljaeschkeanadiol (**41**)] (Figure 1.5).[70] In 2012, Popova et al.[28] concluded that the diterpenic profile of the previous studied Maltese propolis was similar to the profile of the resin of *Cupressus sempervirens* and consider that cypress is the principal plant source of this propolis. Later on,[71] have reported totarol (**36**) to be the most abundant diterpenoid in Maltese propolis.

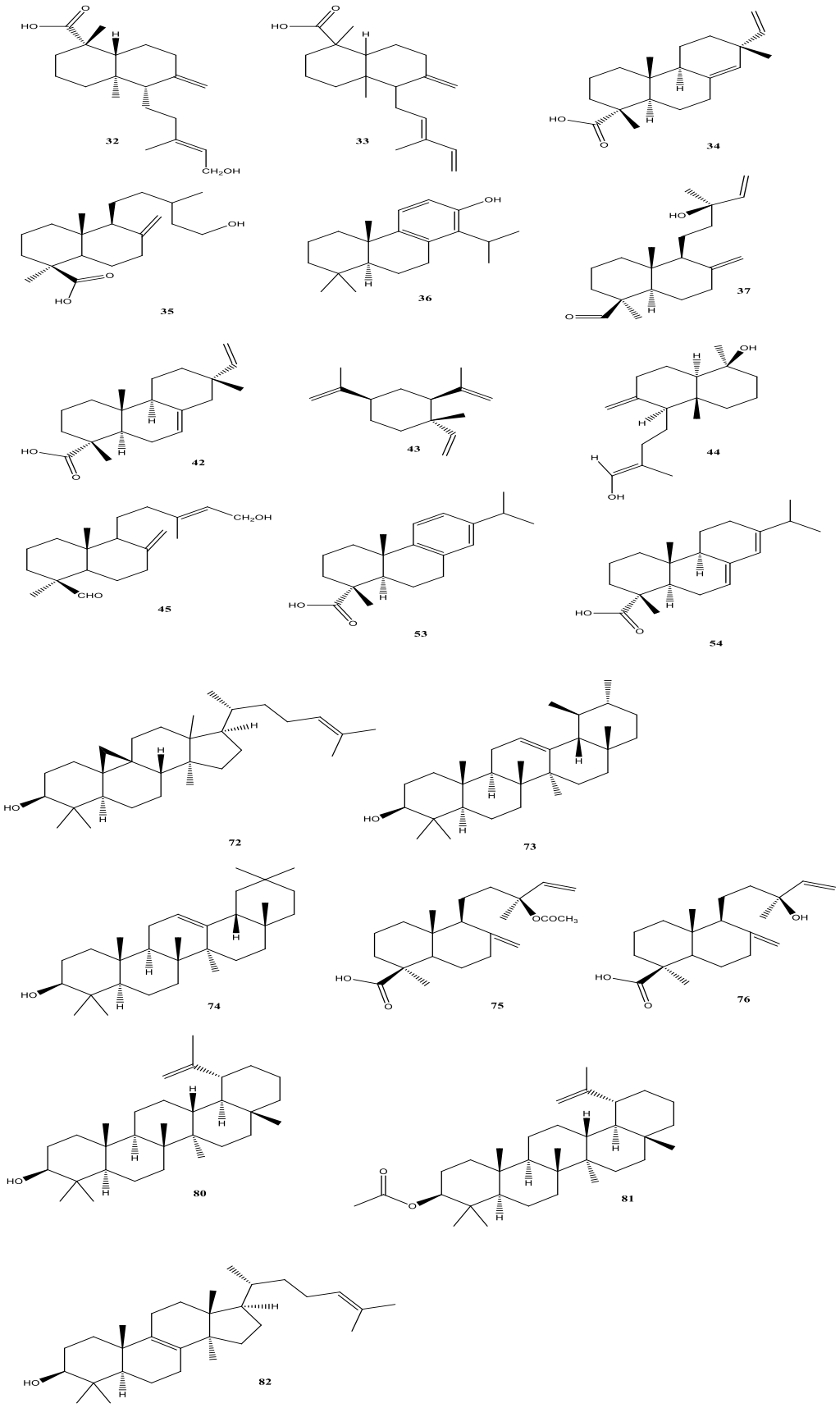


Figure 1.5. Examples of terpenes and their derivatives present in Mediterranean propolis.

In ethanolic extract of propolis from Greece, it was reported a significant amount of flavonoids [mainly chrysin (**2**), pinocembrin (**3**), pinobanksin-3-*O*-acetate (**4**), galangin (**9**), and apigenin (**15**)] (Figure1.2), and low amounts of phenolic acids and their esters.[31,64,72--75] Terpenes [mainly totarol (**36**), isopimaric acid (**42**), *β*-elemene (**43**), agathadiol (**44**), pimaric acid (**34**), isoagatholal (**45**) and isocupressic acid (**32**)] (Figure 1.5) were also found with the exception of propolis from Nigrita that had high amounts of flavonoids and low amounts of phenolic acids and their esters, and without diterpenic compounds.[74] In addition, in the recent study of Kasiotis et al.[76] it was not reported the presence of diterpenes in samples originating from Crete, Kos, and Amorgos (Greece), in the opposite of the finding of Popova et al.,[30] where they have reported a relative high amount of this group of compounds in Crete samples. In propolis samples from Continental Greece (West North of Macedonia (Kastoria) as well as in Dodecanese islands (Rhodes), Lagouri et al.[77] did not detect diterpenes. Kalogeropoulos et al.[31] found anthraquinones, mainly emodin (**46**) and chrysophanol (**47**) (Figure 1.4), in propolis samples from Greece.

In other countries of Southern European Coast, such as Turkey, chemical composition of propolis has widely investigated by several authors. Quercetin (**26**), rutin (**48**), chrysin (**2**), pinocembrin (**3**), pinostrobin chalcone (**49**) and pinobanksin (**1**) and its derivatives were the most repeated flavonoids found in various simples of propolis from Turkey using different method of analysis such as gas chromatography-mass spectrometry (GC/MS), high-performance liquid chromatography (HPLC) and ultrahigh-performance liquid chromatography with a linear ion trap-high resolution *Orbitrap mass spectrometry* system (UHPLC-LTQ/orbitrap/MS/MS),[73,78--95] while Erdogan et al.[96] have reported the presence of other flavonoids such as gallocatechin (**50**), epigallocatechin (**51**) and myricetin (**52**) in Anatolian propolis submitted to a pressurized liquid extraction. Other authors did not report the presence of flavonoids in Turkish propolis originating from Hatay, Ankara-Kazan and Mugla-Marmaris,[97-100] at the same time they declared that terpenes such as *α*- and *β*-eudesmol, isopimaric acid (**42**), dehydroabietic acid (**53**) and abietic acid (**54**) (Figure 1.5) were included in the bioactive compounds of tested propolis samples. Notwithstanding, the same authors reported the presence of phenolic acids and their esters, although some authors [101-105] described that aromatic acids and aromatic acid esters were only present with low concentration in propolis from Elazig Province. Even though, and whatever the method used to determine the chemical composition of Turkish propolis, it was reported to be rich of phenolic acids, mainly *p*-coumaric (**6**), ferulic (**7**) benzoic and caffeic (**5**) acids (Figure 1.2).[79,81--83,95,99,106,107] Regarding terpenes, an amount ranging between 0.15 and 27.47 % was revealed in Turkish

propolis.[73,79,108] To be noted that the presence of diterpenes could not depend on the nearness to the Mediterranean Sea, because[108] described a typical poplar propolis from Izmir (Eagean Sea belonging to the Mediterranean basin) and Mediterranean type of propolis from Adana (Mediterranean Sea). Nevertheless Çelemli et al.[73] reported relatively high amounts of totarol (diterpene) in propolis from the Aegean region of Turkey.

Çetin et al.[109] in their investigations on propolis from Kayseri, they affirmed the presence of cinnamic acid (**24**) (Figure 1.2*)* and their esters with a high concentration. Coumarins were also reported in Turkish propolis from Hakkari, by Bayram et al.[90] in their recent study, where twenty-six coumarins were detected including angelicin (**55**), bergapten (**56**), bergaptol (**57**), columbianetin (**58**), decursin (**59**), isogeijerin (**60**), jatamansin (**61**), lomatin (**62**), methoxsalen (**63**), oroselone (**64**), osthole (**65**), oxypeucedanin (**66**), prangenin (**67**), psoralen (**68**) and seselin (**69**) (Appendix A, Table A.1).

1.6.1.2. Levantine Coast

Likewise, in Greece, authors have investigated propolis from Cyprus and have signaled the presence of anthraquinones [emodin (**46**): 1.8--4.08 mg/g EEP)] and flavonoids mainly chrysin (**2**), galangin (**9**), pinocembrin (**3**); pinobanksin (**1**) and pinobanksin-3-*O*-acetate (**4**).[31,61] The presence, with high concentration, of isocupressic acid (**32**), isopimaric acid (**42**), imbricatoloic acid (**35**), agathadiol (**44**), totarol (**36**), 13-*epi*-torulosal (**37**), abietic acid (**54**), dehydroabietic acid (**53**) (Figure 1.5) were also mentioned.[31,61] Noureddine et al.[110] explored propolis from South of Lebanon and have concluded that bioactive compounds present in the tested extracts showed a similarity to those of southern European coast, concerning the presence of flavonoids and phenolic acids. It was reported that flavonoids were more abundant than phenolic acid including rutin (**48**), quercetin (**26**), genistein (**70**), kaempferol (**25**), chrysin (**2**), pinocembrin (**3**) and galangin (**9**).[110]

1.6.1.3. Northern African Coast

Egyptian propolis analyzed by GC/MS was a typical poplar propolis type as reported by Abd El Hady and Hegazy [111] but with few differences such as the presence of di- and triterpenes but at very low concentrations. Samples from Upper and East Nile Delta in Egypt were mainly characterized by the presence aliphatic acids (lactic acid, succinic acid, stearic acid, oleic acid, palmitic acid), phenolic acids [benzoic (**28**), ferulic (**7**), caffeic (**5**), 3,4-dimethoxycinnamic (**29**), cinnamic (**21**) and *p*-coumaric acids (**6**)] (Figure 1.3), flavonoids [pinocembrin chalcone (**71**), pinocembrin (**3**) pinobankasin-3-acetate (**4**)] (Figure 1.2) and di- and triterpene, in lower

amounts, were also detected (e.g., cycloartenol (**72**), α-amyrin (**73**), triterpenes of β-amyrin type (**74**)] (Figure 1.5).[111,112] Other authors have reported the presence of phenolic acids and di- and triterpenes in Egyptian propolis samples who also reported to find low concentration of aliphatic acids. The flavonoids found by those authors were mainly catechol, catechin and esculetin.[113] In Libya, it was reported that propolis from different localities present the diterpenes as main bioactive compounds. Phytochemical analysis of the Libyan propolis display the presence of compounds belonged to phenolic, flavonoids, terpenoids, phytosterols and coumarins.[114,115] Siheri et al.[116] have isolated three diterpenes [13-*epi*-torulosal (**37**), acetyl-13-*epi*-cupressic acid (**75**) and 13-*epi*-cupressic acid (**76**)] (Figure 1.7*)* from Libyan propolis with antiprotozoal effect.

As far as we found, studies regarding the chemical composition of propolis from Tunisia are very scarce. HPLC analysis of Tunisian propolis collected in Menzel Mhiri showed the presence of chrysin (**2**), galangin (**9**), tectochrysin (**31**), pinocembrin (**3**), pinobanksin (**1**) and phenolic ester like dimethylallyl caffeate (**13**), phenylethyl caffeate (**8**) among others. Myricetin 3,7,4',5'-tetramethyl ether (**77**) and quercetin 3,7,3'-trimethyl ether (**78**) (Figure 1.2) were also found in Tunisian propolis sample.[117]

Some authors reported that diterpenic acids and especially a compound with M=322 (hydroxyditerpenic acid), constituted the main compounds of hydro-alcoholic extracts of propolis from Algeria. Flavonoids were also detected in the same extract mainly pinobanksin (**1**) and pinobanksin 3-*O*-acetate (**4**).[74] Furthermore, flavonoids such as chrysin (**2**), apigenin (**15**), kaempferol (**25**), galangin (**9**), pinobanksin (**1**), pinobanksin-3-acetate (**4**), and pinobanksin-3-(*E*)-caffeate (**79**) among others were detected in alcoholic extracts of propolis from Algeria (Figure 1.2). Those samples were also rich on di- and triterpenic compounds. [118,119] In contrast, in Graikou et al.[61] finding, it was reported that Algerian propolis belong to a group of propolis with relatively high amounts of flavonoids and low diterpene content. In conclusion, the results obtained by diverse researcher teams reveal a co-existence of diterpenic-rich propolis and propolis rich in flavonoids.

Few investigations were done with propolis from Morocco regarding its chemical composition and it was concluded that in summary samples are characterized by high flavonoid contents including chrysin (**2**), pinocembrin (**3**), and pinobanksin-3-acetate (**4**), galangin (**9**), naringenin (**10**) and apigenin (**15**) (Figure 1.2) .[120-122] Popova et al.[121] reported the presence of di- and triterpenes in Moroccan propolis such as isocupressic acid (**32**) and imbricatoloic acid (**35**) (Figure 1.5), among others. Phenolic esters were also among the principal groups of bioactive compounds found in Moroccan propolis.[122]

From above, we can conclude that the reported chemical composition of propolis from different countries of Mediterranean basin is heterogenous. It is possible to find diterpenes, independent on their levels, in propolis samples from Italy (Sicily), Croatia, Malta, Greece, Turkey, Cyprus, Egypt, Libya, Algeria and Morocco (Appendix A,Table A.1). However, not all samples studied in these areas presented diterpenes. For example, there are studies showing the presence of diterpenes in propolis samples collected in the island of Crete (Greece) and other ones where this does not occur. The same is true for other Greek islands in the Aegean Sea and on the eastern coast of the country. In some propolis samples from Turkey, Italy and Libya, coumarins were detected, whereas in some samples from Greece and Cyprus anthraquinones were also reported. In some samples of Malta, Greece, Egypt, Algeria and Morocco, triterpenes were also detected (Appendix A, Table A.1).

1.6.2 Volatile constituent of propolis

Identification of volatile oils in propolis can give precious information about propolis origin, although they account for only a minor portion of propolis constituents, it gives particular aromas to propolis from different origins, considered as a possible quality criterion for propolis freshness and plays an important role in propolis characterization because they are known to possess valuable biological activities, especially antibacterial, which contribute to the understanding its biological activity.[123]

Propolis volatiles have been studied by several researchers around the world, recently reviewed by Bankova et al. and Miguel et al.[123,124] Likewise to the non-volatile part, volatile components of propolis depend on a number of factors, such as site of collection, bee type, local flora, and also the method used for isolation and analysis. Regarding propolis volatiles from Mediterranean basin, only few studies were found and are reported in Appendix A, Table A.2. The chemical pattern of the volatile part of propolis is very complex, presenting different classes of compounds (aldehydes, ketones, alcohols, esters, terpenes, acids, etc.).[124]

1.6.2.1. Southern European Coast

In France there is no recent studies that targets propolis volatiles. Clair[125] have reported the oxygen-containing sesquiterpene, in particular β-eudesmol (**83**) (Figure 1.6), as a major group constituent of propolis volatile oils from France. Likewise, in Italy, researchers have found that the main bioactive compounds detected were oxygen-containing sesquiterpene such as δ-cadinene (**84**), γ-cadinene (**85**), α-muurolene (**86**), β-eudesmol (**83**), τ-cadinol (**87**) and α-cadinol (**88**) (Figure 1.6). Benzoic acid (**28**) (Figure 1.4) and its esters were only found with a

significant amount in Italian propolis.[126,127] It is interesting to note that, Pellati et al.[127] have concluded that volatile composition of the Italian propolis indicates a close relationship with bud exudates of *Populus* species.

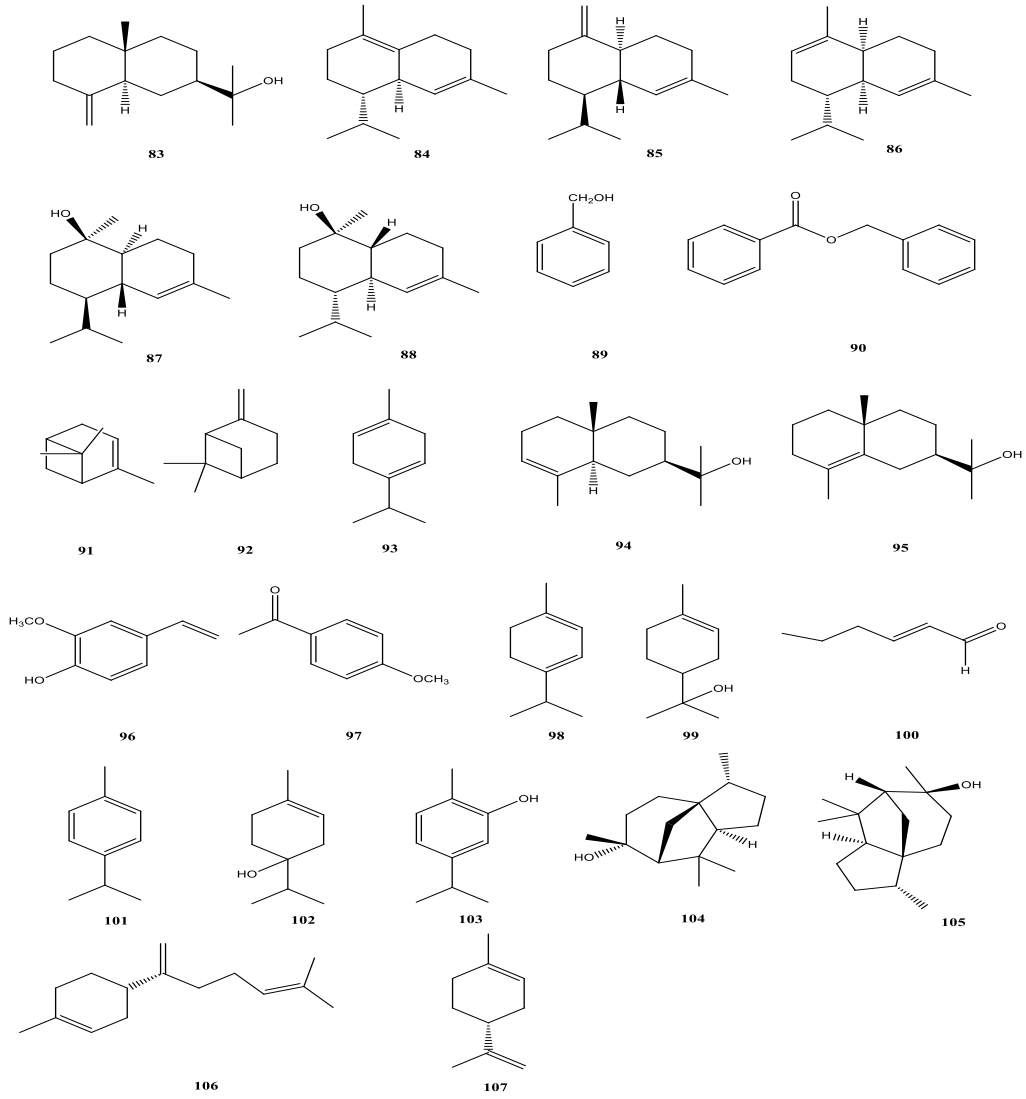


Figure 1.6. Examples of volatiles present in Mediterranean propolis

The major volatile components of Croatian (Dalmatia) propolis identified included terpenes (30%), while in other region (Slavonia) benzyl alcohol (**89**), benzoic acid (**28**) and benzyl benzoate (**90**) were predominant (49 %). α-Pinene (**91**), β-pinene (**92**), γ-terpinene (**93**), α-muurolene (**86**), γ-cadinene (**85**) and δ-cadinene (**84**) were also identified as components of propolis.[128] Jerković et al.[65] have also identified these volatile compounds in addition to α-eudesmol (**94**), β-eudesmol (**83**), γ-eudesmol (**95**), benzyl benzoate (**90**), and 4-vinyl-2-methoxyphenol (**96**) among other components, depending on the collection zone of propolis. Moreover, the procedure of extraction, simultaneous distillation extraction or headspace solid-phase microextraction, of propolis volatiles also influenced the amounts of the compounds, but

not the qualitative profile of volatiles of Croatian propolis. In addition, Jerković et al.[65] also found a correlation between the presence of some volatiles with some phenolic compounds: eudesmol isomers and CAPE although not biosynthetically related, they are present and characteristic of black poplar propolis.[124] The presence of β-eudesmol is even the major constituent of propolis volatiles from other European countries, such as France, Hungary and Northern Italy.[124,125,127]

Steam distillation for 4 h, followed by extraction with ether/pentane 1:1 of Albanian propolis have revealed the presence of hydrocarbons such as heptacosane, tricosane, nonacosane, heneicosane, pentacosane and hentriacontane. Cadinene (10.5 %) and methoxyacetophenone (**97**) were also detected.[129] Results of total profile of the volatile constituents of Greek propolis reveal the predominance of terpenoids against aromatic compounds where α-pinene (**91**) represented more than 45 % of the total components found.[130] On another hand, in Yildirim et al. finding,[131] for Turkish propolis sample from South Eastern Anatolia (Malatya), monoterpenes, mainly α-terpinene (**98**) and α-terpineol (**99**), were the most abundant constituents. Some authors[132] explored the volatile compounds of propolis sample from Turkey by headspace-solid-phase microextraction coupled with GC/MS, and have identified oxygenated hydrocarbons, oxygenated sesquiterpenes, aromatic alcohols and esters as the main aroma-active constituent in propolis simple from North Eastern Anatolia (Turkey).

1.6.2.2. Northern African Coast

Headspace GC/MS analysis if propolis from different locations in Tunisia showed the presence of different compounds in all propolis samples. Propolis volatiles were dominated by monoterpene hydrocarbons being α-pinene (**91**) the major compound, representing more than 90 % the total propolis volatiles.[133]

The volatile fraction of propolis samples collected in different locations in Algeria, analyzed through GC/MS have revealed a distinct composition dominated with 2-hexenal (**100**), myristic acid, linoleic acid, spathulenol isooctane, linoleic acid, undecane, hexadecane, *p*-cymene (**101**), palmitic acid, 4-terpineol (**102**), carvacrol (**103**), and α-cedrol (**104**) dominated.[134]

Information upon the volatiles' composition of propolis from the countries surrounded by the Mediterranean Sea is lower when compared to the non-volatile profile, even though it allows to observe the following trend: in propolis from poplar type, sesquiterpenes, particularly, β-eudesmol is always present in relative high amounts, whereas the monoterpenes such as α-pinene are mainly present in the Mediterranean propolis type, maybe due to the plant source

used by bees which are mainly constituted by coniferous species, as for example, *Cupressus sempervirens* L.[124]

1.7. Biological activities of propolis from Mediterranean countries

Propolis, selected as a natural product, is showing a large spectrum of therapeutic properties due to its richness of different bioactive compounds.[135] Indeed, several studies have been carried out on propolis over the world in view to explore more and more its valuable biological activities. However, in this section, we reviewed those properties particularly for propolis from the Mediterranean countries that are also depicted in Appendix A, Tables A.1 and A.3. as well as Table A.4.

1.7.1. Antioxidant activity

The involvement of oxidative stress is believed to be responsible for the occurrence of diseases such as diabetes, cancer, inflammation, cardiovascular and many others. As well documented, antioxidants are able to prevent free radicals' generation, scavenging them and promoting their decomposition.[32] In fact, antioxidant effect of propolis has been widely studied through many assays in Mediterranean countries.

1.7.1.1. Southern European Coast

In Spain, several authors have investigated propolis from different location for its antioxidant capacities.[35,136-140] Those authors state the *in vitro* antioxidant capacity of Spanish propolis through several methods [2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Ferric Reducing Antioxidant Power (FRAP), Trolox Equivalent Antioxidant Capacity (TEAC)] and radical-scavenging effect on hydroxyl radicals antioxidant activity assay and the results revealed a significant correlation between the phenolic composition and the antioxidant activity. The activities found were dependent on the amounts of phenolic compounds, which, in turn they were dependent on the harvesting method, solvent of extraction as well as the method of extraction. The methods used were the same reported for French propolis leading to same conclusion as reported for Spanish one.[40]

Ethanolic extract of propolis samples from several region in Italy also presented an important antioxidant activity, which manifests in the preventing the lipid peroxidation, scavenging effect on superoxide anion, DPPH and inhibiting the xanthine oxidase activity.[46,141--144] These authors showed a potential correlation between antioxidant activity and polyphenolic content. Moreover,[144] have examined the antioxidant activity of propolis with and without CAPE and declare that propolis with CAPE was more active than propolis without CAPE, while CAPE alone was more efficient than galangin regarding its antioxidant activity,

from where the similarity to the results of Spanish researchers on phenolic compounds, being the bioactive component responsible of the antioxidant activity of propolis.

Slovenian ethanolic extract of propolis was screened for its chemical and antioxidant activity and was found that antioxidant properties were related to the total phenolic contents. Propolis also found to responsible on changes in the levels of antioxidative proteins and proteins involved in ATP synthesis in *Saccharomyces cerevisiae.*[51,52,145,146]

Different propolis extracts including Croatian, Greek and Cyprus (Levantine Coast) ones were shown to possess a variable antioxidant capacity due to its richness in bioactive compounds.[31,51,62,65-67,77,146-147] Jug et al.[148] have found propolis from Dalmatia and Zagreb, Croatia, to be excellent as antioxidant agent based on the results of the antioxidant tests assayed.

Accordingly, extract of propolis, using different solvent of extraction, from different province of Turkey exhibited an effective antioxidant ability seeing different assays overall DPPH, total antioxidant activity, ferric ions (Fe^{3+}) and cupric ions (Cu^{2+}) reducing abilities, ABTS, H_2O_2 scavenging and metal chelating activities.[23,35,76,80,85,96,99,106,135,149-155] Furthermore this biological property of Turkish propolis was confirmed through other assay such as heat-induced oxidation of an aqueous emulsion system of β-carotene and linoleic acid assay where the total antioxidant activity increased with the increasing amount of extracts added to linoleic acid emulsion.[80] Also, a screening method for antioxidant activity used reversed Phase HPTLC-DPPH free radical assay.[89] In general, the antioxidant capacity of different Turkish propolis extracts was assessed to be positively correlated with their total polyphenol content. In agreement with these findings, a higher reducing power ability was observed among Algerian and Moroccan (Northern African Coast) propolis samples suggesting the most contributing role of phenolic compounds to this activity.[24,156-160] As noted by the reviewed articles, the authors were agreeing about crucial role of propolis as antioxidants source.

1.7.2. Antimicrobial activity

1.7.2.1. Southern European Coast

Antimicrobial activity is another valuable propriety of propolis. Ethanolic and propylene glycol extracts of propolis from Spain was reported to have antimicrobial activity that acts mainly on Gram-positive bacteria and yeasts, showing a positive correlation with flavonoid content and the bactericide properties.[137,140] Other authors disclosed that propolis from Roma and Apulia (Italy) and Barcelona (Spain) exhibit a fungistatic and fungicidal activity on fungi isolated from Venezuelan patients including *Candida albicans*, *Candida krusei*, *Candida guilliermondii* and *Candida tropicalis.*[140,161,162]

Benzyl caffeate, *p*-coumaric acid and pinocembrin were predominant in French propolis sample and showed a great effectiveness against tested pathogens exhibiting an important antibacterial and antifungal activities against *Staphylococcus aureus; Escherichia coli*, and *Candida albicans.*[163] More recently, Boisard et al.[41] have found the same properties of aqueous and methanolic extracts of poplar propolis, where they found a selective Gram-positive antibacterial activity, in particular against *Staphylococcus aureus* and several of its *methicillin-resistant* (MRSA) and *methicillin-susceptible* (MSSA) strains.[41] Moreover, Amoros et al.[164] have shown that 80 % ethanol extract of propolis from Renne, France, possess an antiviral effect against Herpes simplex virus (HSV-1). The authors also investigate the anti-HSV-1 activity of the abundant flavonoids identified in French propolis comparing the results with those from the ethanol extract of crude propolis. The authors have found that propolis was more active than its individual compounds attributing such results to the synergic effect of propolis flavonoids.

Propolis from different place in Italy showed good antimicrobial activity against *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis*, *Staphylococcus aureus*, *Candida albicans*, *Streptococcus pyogenes*, *Escherichia coli*, *Proteus mirabilis* and *Pseudomonas aeruginosa* but not against Enterobacteriaceae.[44,165-167] Propolis from Piedmont region, where hybrids of poplar, alders, poplars, hazels and pine trees dominated, had a strong antibacterial activity against almost all strains of *Staphylococcus aureus* tested.[166] Likewise, Bisio et al.[44] investigated samples from the same region in Italy and have attributed the bacteriostatic and bactericidal properties of propolis found against Streptococcus pyogenes strains, to the predominance of pinocembrin and galangin in the studied samples. Later, Scazzocchio et al.[167] and Gil et al.[168] have examined the antimicrobial activity and synergistic effect with some antibiotics of ethanolic extract of propolis against bacterial clinical isolates of *Staphylococcus* spp. and *Streptococcus* spp. and demonstrated the positive action of propolis against different virulence factors of the tested strains and reports the efficiency of application of propolis along with ineffective antibacterial drugs, due to antibiotic resistance, during early stages of infection. On the other side, other authors have shown that propolis originating from Italy possess a good antibiofilm activity against *Pseudomonas aeruginosa* and could be used as adjuvant in the therapy against *Pseudomonas aeruginosa* chronic infection.

Extracts of propolis collected at Dolenjska, Slovenia, were more effective against Gram-positive bacteria than against fungi and Gram-negative bacteria tested such as *Salmonella* and *Escherichia coli*, except *Campylobacter* and declared that this different action could be

associated with the total content of phenolic compounds present in the tested propolis samples which act through a synergistic manner.[51]

Croatian propolis, originating from continental and near the Mediterranean Sea, presented a robust antimicrobial capacity against *Escherichia coli*, *Staphylococcus aureus*, *Candida albicans*, *Aspergillus niger*, and *Bacillus subtilis* which correlated well with polyphenol and flavonoid content.[54,61,62] However, Mašek et al.[66] explored typical Croatian poplar type of propolis rich in aromatic acids and flavonoids with very low content of terpenes and observed a great difference in chemical composition of different extracts, but they did not find a clear correlation between chemical composition and biological activity.

Antimicrobial effect of propolis extracts from Malta was also reported. According to Popova et al.[70] all Maltese propolis samples tested were active against *Staphylococcus aureus* but only those with high concentrations of terpenyl esters showed antifungal activity against *Candida albicans*, while they were ineffective against *Escherichia coli*.

Hydroalcoholic extracts of Greek (Nigrita) and Turkish (Bursa) propolis rich in flavonoids and esters of caffeic and ferulic acids were reported to have a significant antibacterial and weak to moderate antifungal activity.[74] At the same time, one new 2,3-dihydroflavone derivative, 7-*O*-prenylstrobopinin, and 25 known diterpenes and phenolic compounds were identified in the butanol extract of Greek propolis from Preveza region, which showed a good antimicrobial activity against Gram (+/-) bacteria and fungi.[72] In another experiment, ethanol extracts of propolis were tested for antimicrobial activities against ten *Paenibacillus larvae* isolates, a spore forming bacteria which cause one of the most severe bacterial diseases affecting the larvae of honey bees and responsible for colony loss. The results of this study revealed a strong susceptibility of the *P. larvae* to the tested propolis.[169] Besides, it was revealed that volatiles from Greek propolis also possess antimicrobial activity against human pathogenic agents.[130]

1.7.2.2. Levantine Coast

Several authors have reported that propolis from Greece (Southern European Coast) and Cyprus to be very rich in terpene and aromatic compounds (flavonoids, phenolic acids and esters) and declared that this class of bioactive compound participate largely on the antibacterial and antifungal activities.[61] Moreover, Kalogeropoulos et al.[30] found that propolis extract from different places in Greece (Southern European Coast) and Cyprus exhibited similar antimicrobial activity, inhibiting Gram positive pathogens and fungi growth, and not affecting

several lactic acid bacteria. Those propolis extracts were more efficient in large spectrum of microorganisms as compared to nisin, a food grade antibiotic.[30]

Propolis from several region of Turkey has been widely exanimated for its antibacterial and antifungal properties against different $Gram^+$ and $Gram^-$ bacteria, clinical, instant soups and foodborne isolates. Table .A.1(Appendix A) shows the different study done on propolis from Turkey. All report of antimicrobial activity of Turkish propolis displayed a convergent pattern.[78-83,91,93,95,98,105,149,150,169-190] Practically, all tested samples exhibited an antifungal and antibacterial performance. In general, antimicrobial activity varied depending on the chemical composition and origin of propolis sample, dosage of propolis, the extraction solvents and incubation period, for all test microorganisms. Withal, some authors have reported that propolis showed a good antibacterial ability against Gram positive *cocci* such as *Staphylococcus aureus, beta hem. Streptococcus* and a limited activity against Gram negative bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa*, and yeast (*Candida albicans*).[78,171,189] Other research team reported that *C. albicans* was the least sensitive microorganism toward Turkish propolis.[98] Furthermore, in the Silici et al.[190] study, it was found that propolis sample gathered by *Apis mellifera caucasica* showed higher antibacterial activity than the one gathered by *Apis mellifera anatolica* and *Apis mellifera carnica*. The antibiofilm activity, embodied in the inhibition of biofilm formation and the reduction of established biofilm of propolis was approved also against clinical isolates.[81] Additionally, the antiparasital effect of propolis from Kayseri, Adana, Hatay, and Bursa provinces, Turkey, was screened against *Leishmania promastigote*, and it was found to be effective presenting a promising activity for drug discovery and the development of novel antileishmanial agents.[106,191,192] Also, propolis from Trabzon, Turkey, was found to possess amoebicidal, as well as cysticidal properties for *Acanthamoeba trophozoites* and cysts.[186] As well, Sahin et al.[193] affirmed that propolis showed an inhibitory potential against the zinc metalloenzyme carbonic anhydrase. Moreover, Turkish propolis was able to inhibit crucial enzymes such as acetylcholinesterase, xanthine oxidase and urease.[194] According to the authors, propolis originating from Turkey could be considered as natural product to be involved in clinical medicine and food industry.

1.7.2.3. Northern African Coast

Exploring the biological properties of Egyptian propolis from different locations, there are studies which allowed to conclude that propolis from Egypt showed a variation in the antimicrobial activity according to its origin and had a concentration-dependent activity.[111,112,163,195-197] Elbaz and Elsayad II[198] compared the antimicrobial effect of Egyptian

propolis *vs.* New Zealand one on *Streptococcus mutans* and *Lactobacilli* in saliva and have found that the hexane fraction of the commercial New Zealand propolis possessed the strongest antimicrobial action, while ethanolic extract was more potent on *Streptococcus mutans* than on *Lactobacilli.* In other report, propolis was checked for its synergetic effect and was characterized by a capacity of preventing the microbial growth alone and has synergistic effect when used with honey or ethanol. This activity was depending on propolis geographical origin.[199] More recently, Mokhtar et al.[200] highlighted the potential antiprotozoal activity of Egyptian propolis extract as a potent natural alternative for metronidazole in treatment of blastocystosis.

In Libya, propolis from Tukra and Al'Aquriyah, showed an important activity against *Trypanosoma brucei,* which is the aetiologic agent of sleeping sickness, and *Leishmania donovani,* which causes visceral leishmaniasis *Trypanosoma brucei.*[116] Later on, the authors,[114] supported the previous finding regarding antiparasitic activity of Libyan propolis: antitrypanosomal, antileishmanial, anti-*Mycobacterium marinum,* anti-*Plasmodium falciparum* and anti-*Crithidia fasciculata*.

The anticariogenic and antibiofilms activities of the ethanol extracts of propolis from Monastir, Tunisa were also reported. The tested samples were able to inhibit cancer cell proliferation, cariogenic bacteria and oral biofilms formation. The excellent activities found was attributed to specific bioactive compounds of the Tunisian propolis.[201]

Antimicrobial activity due to the presence of diterpenic compounds, was also reported in propolis samples from Algeria, particularly against bacteria.[74] It was found that propolis samples from Tiaret, Tlemcen, Sidi bel Abbes and Mascara, Algeria, were active against Gram positive bacteria (*Staphylococcus aureus*, *Bacillus subtilis* and *Bacillus cereus*), and inactive against Gram negative bacteria (*Pseudomonas aeruginosa*, *E. coli*).[202,203] In Soltani et al.[118] study, it was demonstrated the immunomodulatory activity and the bactericidal activity of Algerian propolis against important bacterial pathogens in sea bream, and was suggested that propolis could be a source of new natural biocides and/or immunomodulators in aquaculture practice. In addition, it is noteworthy that caffeic acid derivatives from propolis from Beni Belaïd area had the capacity to inhibit Stromelysin-1 enzyme enrolled in collagenolytic and elastolytic cascades, leading to cutaneous intrinsic and extrinsic aging.[204]

Finally, Moroccan propolis from different regions exhibited antibacterial activity[122]. On the same insight of the results reported in this review, the authors related this activity to the bioactive compounds present in Moroccan propolis samples.[122]

From above, we can conclude that propolis from Mediterranean basin is mainly active on Gram+ bacteria and less effective regarding Gram- bacteria and yeast, as reported for other propolis with different geographical origins. Regardless the diversity of the chemical profile of propolis, generally all of them present antimicrobial activity. The role of propolis is maintaining the hive free of intruders, including microorganisms. For this reason, bees have to use the material that is available near the hives with the purpose of keeping them in healthy conditions. The plant material available for bees is different and changes according to the region but the biological properties must be effective to preserve the healthy bees.

1.8. Application fields of propolis from Mediterranean Basin

As previously presented propolis from countries of the Mediterranean basin showed a great heterogeneity regarding its chemical composition and possesses a wide range of pharmacological potentials. This is attributed to the various origins of propolis, collect seasons, surrounding plants, genetic of bees, harvesting and extraction methods of propolis as already aforementioned.

Propolis applications for various purposes such human medicine, quality of life, cosmetic, food industry and aquaculture as well as in livestock farming, with a high interest will be presented and developed in this section. In many cases, the application of propolis in the diverse fields were sustained in the fact that propolis possesses antioxidant and/or antimicrobial properties.

Several investigations have been done by researchers of the Mediterranean basin either using *in vitro* or *in vivo* assays (animal models) to find out possible applications of propolis from the Mediterranean countries in different fields. In this section, for all countries belonging to the Southern European, Levantine and Northern African Coasts, it will be reported three main fields of propolis application which are the following: medicine/pharmacy and bio-allied sciences; food industry and improvement of livestock.

The principal biological properties of propolis included anti-inflammatory, antioxidant, antimicrobial, anticancer, antidiabetic, as can be read in Appendix A, Table A.4 and described in this section. Considering that the inflammation could be due to the oxidation processes; because inflammatory process induces oxidative stress and reduces cellular antioxidant capacity, leading to several diseases like diabetes, heart diseases, and even cancer, among other pathologies; the antioxidant, anti-inflammatory and antimicrobial activities of propolis are included in subsection of medicine/pharmacy and bio-allied sciences. The subsection of food industry will present all studies done with propolis from the Mediterranean countries with the

purpose of preservation of food quality, although, in the third subsection (improvement of livestock), we gathered all investigation found in literature regarding growth, performance and improvement of livestock as well as some possible veterinary application.

1.8.1. Southern European Coast

1.8.1.1. Application in medicine, pharmacy and bio-allied science

Spanish propolis was tested for medical application by Lisbona et al.[205] who examined if the oral supplementation with propolis samples from Granada would be able to diminish oxidative aggression and free radical's generation associated to aging. In this study, the authors have found that diets supplemented with propolis in rats' models increased the glucose level, cholesterol concentrations and reduced the protein peroxidation, thus reducing the oxidative stress associated with aging. According to the authors these results could be the results of a synergic effect of different propolis components.

Recently, hydroalcoholic extract of blended propolis from France was tested in combination with cranberry regarding its capacity to reduce the mobility and biofilm formation of uropathogenic *E. coli* (UPEC) responsible of urinary tract infections.[206] In this study, it was reported that propolis potentiates strongly the effect of cranberry on UPEC, representing a promising strategy to prevent recurrent urinary tract infections. Some authors have screened the ethanol extract of propolis from west France for its photoprotective properties, and have shown that propolis exhibit an important photoprotective effect comparable to that of the positive control (homosalate). Thus, it can be used as a natural sunscreen agent.[207]

For its anticancer activity, propolis from Italy was also investigated. In a first report, Scifo et al.[208] checked the possible association of propolis and resveratrol together or with vinorelbine (anticancer drug) at low concentration in the therapy of prostate cancer, and have found that propolis provoked an apoptosis-like while resveratrol generates necrosis-like cell death for androgen-resistant prostate cancer cell line DU145. Thus, propolis and resveratrol could be used as natural therapy in low concentration with vinorelbine for cancer treatments. On the same insight, Borrelli et al.[209] have investigated the anticarcinogenesis action of propolis extract without CAPE and CAPE alone and concluded that CAPE may be the responsible of the anticancer activity of propolis. Other authors reported a protective action of propolis in cartilage alteration resulted from joint diseases, and have shown that propolis acted by an anti-inflammatory mechanism by reducing the key molecules released during anti-inflammatory events as nitric oxide (NO) and glycosaminoglycans (GAGs).[210]

Rossi et al.[211,212] have investigated propolis and some of its components such as CAPE and galangin, regarding their anti-inflammatory activity on lungs homogenates and J774 macrophages. The COX-1 and COX-2 inhibitory activity of propolis was concentration-dependent. These authors have investigated propolis with/without CAPE and have found that propolis extract with CAPE was less potent than propolis extract deprived of CAPE, they also found that galangin had inhibitory activity on COX but lower than that of propolis or CAPE, suggesting that both CAPE and galangin participated to the anti-inflammatory activity of propolis.

Still in Italy, Rassu et al.[213] used propolis as a 'nanocarrier' for the formulation of solid lipid nanoparticles (SLNs) intended for topical nasal drug delivery, not only its frequent application in otolaryngology, but also for its properties in antibacterial and antiviral infections and inflammation. The *in vitro* and *ex vivo* permeation tests performed for the drug was compared with that obtained from the aqueous solution of diclofenac (anti-inflammatory drug), several formulations were done and they found that, the drug is released faster from the formulation containing 25/1 (propolis/drug, w/w) although, it more slowly crosses the lipid membranes of the biological membrane, slowing the absorption. Thus, the drug and flavonoids would remain in the surface of the mucosa, and deploy their therapeutic effects.

A trial to develop a new topical mucoadhesive formulation containing propolis for possible application in the stomatological field was assessed by Ceschel et al[214] In this study, the authors did the formulation of a mucoadhesive topical gel containing propolis, and the *in vitro* and *in vivo* tests performed showed that propolis components mainly CAPE were able to get through the *in vitro* porcine buccal mucosa, moreover, the formulated gel also presented an adequate comfort, a non-irritancy and a good acceptance by the volunteers.[214]

For the purpose of exploiting its antioxidant properties, propolis was screened by Gregoris et al.[215] for a possible use in natural sunscreens formulation for sunburn. Gregoris et al.[215] have found that propolis had a good broad spectrum UVB and UVA photoprotection, and concluded that it could be a brilliant candidate on formulation of sunscreen based natural products.

Oršolić et al.[216] (Appendix A, Table A.4) have examined the effect of Croatian propolis and some of its polyphenolic derivative compounds, such as caffeic acid, and CAPE, quercetin, chrysin and naringenin on the metastatic capacity, immune reaction, apoptosis and necrosis of transplantable mammary carcinoma (MCa) of CBA mouse and it was found that propolis and its components were cytotoxic to tumor cells, they were able to induce apoptosis and necrosis, to inhibit tumor growth, to prolong survival of mice, and led to many ameliorations in several

immunological parameters, such as the production of lymphocyte activating factor by peritoneal macrophages and the efficacy of those macrophages to kill tumor cell and responses of lymphocytes to mitogen.[216-220] The established results suggest that propolis exhibit a noteworthy antimetastatic potential and act as immunomodulatory agent. This finding presents propolis as a potential product for the control of tumor growth, referring its activity to a possible synergetic effect of its phenolic compounds. The same group of research has investigated Croatian propolis and some of its derivatives for its antitumoral capacity against the development of Ehrlich ascites tumor (EAT) alone of combined to an anticancer drug irinotecan or cisplatin. The combination of propolis with the anticancer drugs inhibited the growth of EAT cells and increased survival of bearing tumor animals. Moreover, propolis and its derivatives deteriorate toxic and genotoxic effects led by anticancer drugs to normal cells without influencing the irinotecan cytotoxicity in tumor cells.[220-223]

Further, propolis and its phenolic compounds exhibited a protector effect on experimental animals from the lethal effect of irradiation.[224-226] As a conclusion to those results, the authors suggested that propolis could be an adjunct to anticancer drugs to boost their antitumor activity and decrease the post chemo/radiotherapeutic deteriorated reactions as well as its possible use as natural therapeutic drugs to prevent or treat psoriatic complications. Furthermore, Sobočanec et al.[227] have investigated also the effect of propolis in combination with an anticancer drug (5-Fluorouracil) in mice injected with transplantable 4T1 mammary carcinoma, and have observed, on same insight to the other investigators[228,229] that propolis boost the effect of anticancer drugs by prolonging the suppressive effect of 5FU on tumor growth, and reducing the number of metastasis and decreased dihydropyrimidine dehydrogenase (DPD) protein level.

Besides, Oršolić et al.,[224,225,230] Benković et al.,[226,231,232] and Lisičić et al.[233] have investigated the protective effect of Croatian propolis on psoriatic lesion regression and side effects resulted by radiation and/or chemotherapy such as cytopenias in animal models. Those authors showed that the topically application of propolis improve psoriatic-like skin lesions by suppressing functional activity of macrophages and ROS production.[230]

In addition, the possible amelioration of different damage in diabetic animals by propolis was studied by Oršolić et al.[228,229] on diabetic mice model. Treatment with propolis leads to a raise of body weight, hematological and immunological parameters of blood, 100 % survival of diabetic mice, diminution of lipid peroxidation in liver, kidney and brain tissue and reduction of DNA damage in peripheral lymphocytes. Data found by Oršolić et al.[229,230] demonstrated that propolis exhibited an oxidative and anti-inflammatory potential and could be used in strategies for the prevention and treatment of diabetes mellitus.

Other researchers,[234] more recently, checked different propolis samples from Croatia regarding their anti-aggregatory activity on platelet aggregation, and demonstrated that propolis even in low concentrations presented an important anti-aggregatory potential, suggesting that propolis supplementation may influence platelet aggregation, which can play crucial role in thrombus formation responsible of stroke or heart attacks. Moreover, Barbarić et al.[68] studied propolis samples from Bosnia and Hercegovina and Croatia regarding its cytotoxic effects on cervical tumor cell line (HeLa), and they found that it exhibited an antiproliferative and cytotoxic effect.

In Albania, Meto et al.[235] examined the possible positive effect of propolis on the inflamed pulpal tissue after pulpotomy in piglets and have found that propolis possessed a large anti-inflammatory and regenerative effect, suggesting that it could be used clinically.

The antitumoral propriety of propolis from Greece and its isolated diterpenes was also tested on different human solid tumor cell lines, human promyelocytic leukemia cell line (ECACC) and human skin fibroblast strain AG01523c. Propolis revealed to be an important antitumor natural product due to cytostatic activity, especially that of manol which exhibited a promising profile as an antiproliferative agent.[236]

Moreover, Kalogeropoulos et al.[237] elaborated a method for the encapsulation of Greek propolis extract in β-cyclodextrin (β-CD) cavity in the intention of raising the solubility of its components and use for medical application. As a result of this investigation, they found that the biodisponibility of propolis compounds was size-dependent, small molecules were more efficient in encapsulation, while their release from the β-CD was more difficult than 'bigger' molecules. The release of compounds from encapsulated propolis depends on their chemical properties and their relative abundance in the matrix.

Propolis from Turkey has been found to have a wide spectrum of biological applications. Several trials for the application of propolis as anticancer agent and related side effects using different cancer cell lines were assessed.[23,86,88,106,238-246]

Some authors[23,88,239,240,242-245] and more recently Ozdal et al.[106] have tested propolis from different locations in Turkey for their anticarcinogenic and antimitotic effect against human breast carcinoma, hepatocellular carcinoma, prostate adenocarcinoma, colon adenocarcinoma, HL-60 myeloid leukemia, lung carcinoma and mammary adenocarcinoma cells line. Those authors concluded that the tested propolis exhibited a noteworthy capacity to inhibit the cellular growth of tumor and induced apoptosis. This high potential of propolis as antitumoral agents was related to its constituents especially its phenolic content. Moreover, Onbas et al.[242] have formed a microencapsulation by complex coacervation of propolis and

screened its anti-inflammatory and cytotoxic potential on cancer cells, and have found that this complex has same activity as compared with propolis in free form, and suggested its possible use in the industry for the formulation of natural supplement.

On the same insight, protective effect of Turkish propolis has been widely investigated by various research groups. The experimentations done by some authors[87,104,109,151,152,245-253] aimed to explore the hepatoprotective, radioprotective, chemopreventive, cytoprotective, hepatoprotective as well as antigenotoxic and genotoxic potential of Turkish propolis for medical purpose. It was shown that propolis exhibited an important preventive effect enhancing the disturbance caused by different harmful agents including liver injury, DNA damage in fibroblast cells, vascular endothelial cells damage and the adverse effects on biochemical and hematological parameters. In addition, Kolankaya et al.[251] have explored the protective effect of propolis on serum lipid and liver enzymes against alcohol induced oxidative stress in male rats and have found that propolis from Bursa, Turkey, has a protective role against degenerative diseases and against alcohol-induced oxidative stress an animal model. Agca et al.[252] affirmed that Turkish propolis from Bingöl region, could be used in the prevention and treatment of neuronal impairments, including malignant tumors and neurodegenerative disorders associated with excessive astrocytic activation, as a result of the investigation done on rat brain astrocytes. Contradictory, Eraslan et al.[253] declared that the evaluation of Turkish propolis from Erzurum led to the conclusion that the tested sample was not able to completely eliminate the free radicals and the other adverse effects generated in rats treated with sodium fluoride. In the same field, Ertürküner et al.[254] explored the effects of Turkish propolis on endotoxin-induced uveitis on male Wistar albino rats and have revealed that propolis can act as anti-inflammatory agent and could be used for the treatment of ophthalmic diseases. Besides, propolis from Turkey showed immunomodulatory, antioxidant and anti-inflammatory activities in animal models.[84,255-257] Also, it was significantly effective in healing of burned skin wounds in rat as a cream as compared to silver sulfadiazine.[258] It is noteworthy that, in stomatological field, propolis from Rize, Turkey, was found to have a favorable effect on the dentin bond strength of the tested self-etch adhesive to coronal denti when compared with other irrigation solutions.[259]

1.8.1.2. Application in food industry

Another area of the application of propolis is in the food industry for the prevention of foods from microbials and oxidative agents. Thus, several researches have been done. Luis-Villaroya et al.,[260] in Spain, characterized a propolis-based dietary supplement (PDS) and screened its possible use as a natural additive in apple juice. The purpose of this study was to

assess propolis in combination with mild heat for the inactivation of *E. coli* O157:H7 cells in apple juice, without impacting its hedonic acceptability. The results of those authors revealed that in the presence of propolis and mild heat, the heating time required for the inactivation of *E. coli* cells cycles decreased by more than 40 times. In apple juice, the thermal treatment required to achieve the goal inactivation level also decreased by, at least, 4 times. Furthermore, propolis was sensorially acceptable. Because of its favorable antioxidant and antimicrobial properties, and considering this successful study on its incorporation into apple juice, propolis can be an excellent candidate to be used for preserving different foods.[261]

Propolis could be used in alimentary industries as a natural preservative in dairy beverages according to the results of Thamnopoulos et al.[261] In this experiment, the authors added Greek propolis extract solubilized in glycerol to extended shelf-life milk, artificially contaminated with *Listeria monocytogenes*, and explored its antilisterial effect during improper storage. The use of propolis with glycerol was effective reducing the growth of the pathogen in milk stored under improper refrigeration.[261]

Turkish propolis have been tested for its capacity to preserve the quality of star ruby grapefruit, sweet cherry and fresh shibuta (*Barbus grypus*) during the storage, regarding chemical, microbiological, storage life and sensory quality.[262-264] It was found that treatment of star ruby grapefruit and fresh shibuta with propolis before storage protected the sensory quality, fungal decay and microbiological growth, which resulted in a prolongation of the product's shelf-life.[263,264] In the opposite, Çandir et al.[262] declared that propolis adversely affected sensory quality and stem color of cherries, even if it was effective in preventing fungal decay. Furthermore, Silici et al.[190] and Koç et al.[94] examined the antifungal activity of Turkish propolis in fruit juices against mould and yeast isolated from spoiled fruit juices and affirmed that it was a good antimicrobial agent. Also, propolis appears to be useful as a natural preservative for the foods prone to microbial spoilage instead of chemical preservatives according to Sagdic et al.[265] and Silici and Karaman.[92] Torlak and Sert.[266] screened the antibacterial effectiveness of chitosan–propolis coated polypropylene films against foodborne pathogens and declared that propolis is a promising antimicrobial agent for the food packaging applications. On the other hand, the authors[100,267,268] have established also the effect of propolis on interior egg quality, weight loss, hatchability, chick performance and its antimicrobial activity on egg-shells. The eggs sprayed with propolis presented lower weight lost, a reduced bacterial activity and an improvement on interior egg quality during storage.[100,268] From those results the authors underlined the benefit of Turkish propolis on the prevention of foods during storage.

1.8.1.3. Improvement of livestock

In the fish farming field, Italian propolis was tested for its possible advantageous effect on fish eggs mortality caused by mycosis, also its effect on characteristics of fish muscle fibers was evaluated.[269] Fish fed propolis has shown a reduction of mortality of fish eggs and more rapid muscular growth, which can be of high importance to develop the field of fish farming.

Propolis application was included also in poultry farming. Seven et al.[101,270] have published research exploring the effect of propolis-supplementation (originating from Elazig and Kayseri Province (Turkey) on basal diet of laying hens, exposed to heat stress induced oxidative damage, on several parameter including feed intake, body weight, body weight gain, feed conversion rate, nutrient digestibility, lying performance, egg production and qualities, biochemical indicators and antioxidant enzyme activities.[101,270,271] They concluded that supplementation of propolis attenuated heat stress-induced oxidative damage. In addition, it was been demonstrated that propolis could be added to laying hens diet as alternative to antibiotic for the treatment of adverse effects of heat stress.[101,102] The same experimentation was done on Japanese quail with dietary addition of propolis. Denli et al.[272] and Seven et al.[273] have shown that propolis supplementation improved feed conversion ratio, weight gain, feed efficiency, carcass weight, serum Ca level and antioxidant status. From above, we can conclude that propolis could be used as a natural substitute for antibiotics in poultry diets. Not only in poultry, but also in pigs and cattle farming, propolis was introduced. Yildirim et al.[32] and Çam et al.[274] explored the effect of propolis on dermatophytosis tuberculosis infection on cattle or pigs. In the dermatophytosis case, propolis effect was better than the use of Whitfield's ointment alone, because the application of propolis together with Whitfield's ointment was highly effective, while propolis showed a limited effect on the development of tuberculosis infection in pigs. On the same insight, Fuat Gulhan et al.[275] studied the influence of propolis on microbiologic, biochemical parameters, growth performance and antioxidant status in blood Rainbow trout exposed to cypermethrin 'pesticide', and it leads to a positively changes on the fillet quality and some biochemical and microbiologic functions of fishes. Also, propolis decreased the negative effects of oxidative stress induced by Long-term low flow on fish trout.[276]

1.8.2. Levantine Coast

1.8.2.1. Application in medicine, pharmacy and bio-Allied science

Regarding the Levantine coast there is scarce investigations regarding the propolis application, which can be due to the few countries number representing this area. In Lebanon,

Noureddine et al.[110] studied the antiproliferative and proapoptotic activity of Lebanese propolis and have found similar activity to those aforementioned regarding its cytotoxicity and antiproliferative effect.

1.8.2.2. Application in food industries field

In Cyprus some authors have tested the use of propolis from Cyprus on the post-harvest quality of pomegranate with/without modified atmosphere packaging (MAP) and have found that propolis, not only, significantly influence the maintenance of fruit, weight, juice content and visual quality and marketability, but also controlled gray mold development and slowed the occurrence of chilling injury.[277]

1.8.3. Northern African Coast

1.8.3.1. Application in medicine, pharmacy and bio-allied science

Propolis in Egypt was subjected to different *in vivo* investigations, such as antitumor, antibacterial and antiparasitic activities. Many authors have investigated the antitumoral capacity of Egyptian propolis against different human cancer cells. El-Khawaga, el al.[278] and Badr et al.[279] have investigated the effect of propolis from Egypt against tumor in mice induced by Ehrlich ascites carcinoma (EAC) cell lines and have revealed that, not only, it acts as an antiproliferative and immunostimulatory agent, but also, it induces apoptosis processes.

Other authors explored the possible protective effect of propolis against side effects of some anticancer drugs such as doxorubicin and cisplatin, which leads to nephrotoxicity, reproductive toxicity, hepatotoxicity and genotoxicity.[280,281] In those authors finding, propolis defeat the toxic effect induced by doxorubicin and cisplatin including alleviating testicular functions, improving the histological manifestation of hepatic, renal and testicular disorder. Propolis protected animals from anticancer drug side effects without affecting its antitumor capacity.[280,281]

The results found by several research teams[278--282] supported the data cited above for other propolis originated from the countries of the Mediterranean Sea, which were characterized by their high antitumor capacity. Beyond that, Elbaz et al.[282] have constructed a new formulated propolis-loaded nano-in-microparticles (NIMs) for enhancing its anticancer property and oral delivery against human liver cancer (HepG2) and human colorectal cancer (HCT 116) cells. This research highlighted that propolis-loaded NIMs induced more cytotoxic effect on HepG2 cells than HCT-116 cells, present three-fold higher therapeutic efficiency than free propolis and leads to the apoptosis of HepG2 cells, moreover, it increases propolis solubility with a controlling release profile in different gastrointestinal tract environments. On

the same insight, and more recently, Elhakim et al.[283] using a propolis sample from El Aliubiya region, constructed a sensitive electrochemical sensor containing silver nanoparticles/propolis for microRNAlet-7a detection and have been successfully tested on human serum samples, hepatocellular carcinoma patients and human liver cancer cells. This created biosensor could be used to large-scale miRNAs detection[283] which may be involved in therapeutic application.[283]

On the other hand, propolis from Egypt, revealed a noteworthy capacity for the impairment of the anti-inflammatory score and displayed a noticeable curative effect against biological disorder appeared in mouse conalbumin-induced asthma model.[284] In addition, propolis from Egypt exhibited an antiparasitic capacity in animal model against two human pathogenic parasite 'schistosomiasis and cryptosporidium'. Such was evidenced through the reduction of hepatic granuloma number and their lymphocytic infiltration and aggregation accompanied by restoring the synthesis of plasma proteins and alleviating the state of oxidative stress resulted from schistosomiasis infection. The same study evidenced the reduction of oocysts shedding and modulation of the leukogram, serum proteins and histopathological changes in ileum associated with *Cryptosporidium* infection.[285,286] It is noteworthy that propolis used along with Praziquantel (PZQ: drug used to treat several types of parasitic worm infections) as an adjunct, potentiate its activity in mice models.[285]

Regarding stomatological field, the use of Egyptian propolis contributed on the maintaining of the cleanliness of dental fixtures and/or treating recurrent candidiasis as a complementary and alternative treatment, especially in elders and immuno-compromised patients, it was the conclusion of Gomaa et al.[287] which investigated the possible use of propolis in the dentistry field for blocking oral *Candida* cells adhesion and, therefore, preventing their colonization.

Other use of propolis is its incorporation as 'natural product' in textile materials for the production of cotton textile with antibacterial activity for biomedical applications.[288] It was found that utilization of propolis generated a cotton textile with superior antibacterial activity, water repellent, ease of care characteristics and UV protection.

As far as we found, in Tunisia, there was only one report that states the possible application of propolis from Mahares, a south suburb of Sfax, on the nephrotoxicity damage and the change of biochemical parameter resulted from exposition to cobalt on animal model.[289] Like reported previously in this review for other propolis samples from Mediterranean countries, Tunisian propolis has the ability to control the cobalt-induced nephrotoxicity in experimental rats and their progeny. Garoui et al.[289] have related this

capacity for quenching free radicals, which increased the antioxidant status and metal-chelating abilities of propolis, mainly those rich in flavones and esters compounds.

In Algeria, propolis was tested for its protective effect against peroxidative damage in heart mitochondria and oxidative stress in kidney induced by administration of doxorubicin in rats model, and revealed a high capacity to decrease peroxidative damage in the heart mitochondria, to restore the renal functions and to reduce the whole toxic effect of doxorubicin.[291,292] As a conclusion, Alyane et al.[290] and Boutabet et al.[291] declared that antioxidants from natural sources, such as propolis, may be useful in the protection of cardio/nephrotoxicity in patients who receive doxorubicin, especially during an anticancer treatment.

Other authors have found similar results as Ertürküner et al.[254] for propolis from Algeria, which showed immunomodulatory and anti-inflammatory effect in animals with uveitis induced by α-tropomyosin.[292]

Mouse et al.[120] did a screening of the antitumor potential of Moroccan propolis extracts, from Beni Mellal region, in P815 tumor-bearing mice and have found that the treatment with propolis have the capacity to reduce the tumor volume as compared with the control.[120] Mountassir et al.[293] have investigated samples from the same region regarding its possible antinociceptive activity and have conclude that propolis activated both central and peripheral mechanisms to elicit the analgesic effect in tested animals.[293] Hydro-alcoholic extracts of propolis from Salé region in Morocco, have been investigated for its protective effect in proteinuria, crystalluria, nephrotoxicity and hepatotoxicity induced by ethylene glycol ingestion.[294] The authors have also evaluated the protective effect of hydro-ethanolic extract of propolis against paracetamol-induced liver and kidney damage in rats models.[295] The results of this application joined the previous results cited in this review, for the potential protective effect of propolis on hepatotoxicity and nephrotoxicity, moreover, its showed that propolis from Morocco has potential to treat and prevent urinary calculus, crystalluria and proteinuria.

1.8.3.2. Application in food industries

Successful experiments have been performed by Ali et al.[296] regarding the use of propolis originating from Cairo (Egypt) on the shelf-life and different quality criteria of fresh oriental sausage. Those authors have concluded that propolis offered a longer shelf-life, decreased the thiobarbituric acid value and total volatile base nitrogen.

1.8.3.3. Improvement of livestock and veterinary

In poultry models, propolis from Giza Province (Egypt) was tested for its beneficial effects on controlling the oxidative stress induced by paraquat injection to turkey poults. Abass et al.[297] stated that propolis could improve turkey immunity and performance, particularly under inflammation and oxidative stress, since it modeled the biochemical and immunological disorders led by paraquat.

Besides, in veterinary medicine, for its antibacterial effect against *Pasteurella multocida* (*P. multocida*), causing pasteurellosis, a most important health problem in rabbit, propolis was tested. Nassar et al.[398,299] evaluated the capacity of propolis alone or along with inactivated *P. multocida* vaccine on rabbits challenged with a virulent strain of *P. multocida*. They have found that the use of propolis improved general health condition, liver and kidney functions in addition to reduction of the severity of adverse clinical signs, mortality rates, and histopathological disorder.[298,299]

In Egypt, propolis was used in other areas, such as in aquaculture for improving the growth performance and productivity of fish. In this context, on Nile tilapia fish, Abd-El-Rhman[300] evaluated the efficacity of propolis on the growth performance and the immunostimulant and resistance to *Aeromonas hydrophila*, while Kandiel et al.[301] explored the effectiveness of the supplementation of propolis in the dietary feed to crush the genotoxic and endocrine disturbing effects caused by malathion polluted water. In short, propolis acted positively on the growth, immunity and resistance of tested fish against *A. hydrophila*. Moreover, it minimized the health hazardous of malathion, such as genotoxicity and endocrine disruption, besides its high nutritional value.

1.9. Propolis allergy

The use of propolis has been studied for further application in food industry, medicine, cosmetics, stomatology as well as fish and poultry farming, being regarded as not representing side-effects, and, therefore, harmless. However, allergic reactions have been described in several case reports. In Spain, Fernández et al.[302] have reported three cases in beekeepers of occupational contact sensitization to propolis, with varied features, and considered that propolis a direct and an airborne contact allergen. Allergic contact stomatitis in two cases caused by propolis administrated for therapeutic purposes, were signaled by the same group of research Fernández et al.[303] and Cabanillas et al.[304] Fernández et al.[303] declared that the compounds 3-methyl-2-butenyl caffeate, phenylethyl caffeate, benzyl salicylate and benzyl cinnamate found in propolis composition, are main causes of this allergy. In Italy, Pasolini et al.[305]

described an allergic contact cheilitis with chronicity induced by repeated contact with propolis contained in homemade honey. Likewise, Giusti et al.[306] have examined the frequency and the features of contact sensitization to topical products containing propolis in children and have concluded that propolis showed high sensitization rate at the pediatric age. On the same insight, Hay and Greig.[307] described a case report having an acute oral mucositis with ulceration as a result of using propolis-containing lozenges. Moreover, it was reported that propolis is a strong sensitizer and well-known in apiarists as an occupational allergic eczematous contact dermatitis,[308] also declaring that propolis is contraindicated in patients with an allergic predisposition, being a potent allergic agent. Fernández et al.[303] and Menniti-Ippolito et al.[308] affirmed that the allergenic action seems to be due to caffeic acids derivates. Bellegrandi et al.[309] in a case report, reported an allergic contact dermatitis (cheilitis and stomatitis) in HIV-infected patients provoked by propolis.

1.10. Conclusion

Propolis from all countries of the Mediterranean Sea is constituted by the presence of phenolic acids and their esters and flavonoids, nevertheless in some countries the presence of diterpenes were also detected [Sicily (Italy), Croatia, Malta, Crete (Greece), Turkey, Cyprus, Egypt, Libya, Algeria and Morocco], which led to the introduction of a new type of propolis (Mediterranean-type propolis). However, in some of those places, the terpenes were not detected. For example, in Crete there were found works in which diterpenes were not found in contrast to other ones in which diterpenes were present in relative high amounts. Other examples include the description of propolis from some Aegean islands or from the Eastern Coast of Greece in which diterpenes could be detected or not, depending on the research team. The very few works on propolis from some Mediterranean countries may explain the absence of the Mediterranean propolis, as happened for Spain and France. In contrast, Turkish propolis is extensively studied when compared to the remaining countries bordered by the Mediterranean Sea. Despite the volatile constituents of propolis of this region are almost unexplored, it was possible to conclude that the volatile fraction was also dependent on the plant source, for example in poplar type propolis, β-eudesmol is always present, whereas α-pinene, in considerable amounts, is mainly present in propolis collected from places where conifers predominate. Regarding the propolis application, it is clear that it was mostly in the medicine and bio-allied sciences, through many *in vitro* assays and preclinical investigation (*in vivo*) revealing important targets and general mechanisms of action of propolis from Mediterranean countries.

1.11. Propolis in the Moroccan scenario:

The apiarian potentialities in Morocco are paramount but, remains under exploited. Unfortunately, propolis is less popular in Morocco as compared to honey, thus the harvesting is mainly traditional, through scarping off the bee glue present on the comb frames and in the bee hive box from where the contamination of propolis with pieces of wood, wax and other debris.

However, in the last few years, especially with the Green Morocco strategy, 'Plan Maroc Vert', much attention has been given to apiculture and exploring more and more bees-product.[310] Therefore, we assist to a newest way of propolis collection, a kind of traps, grid nets from mainly from plastic, which are placed on top of the beehive, allowing bees to seal the gaps with propolis.[310]

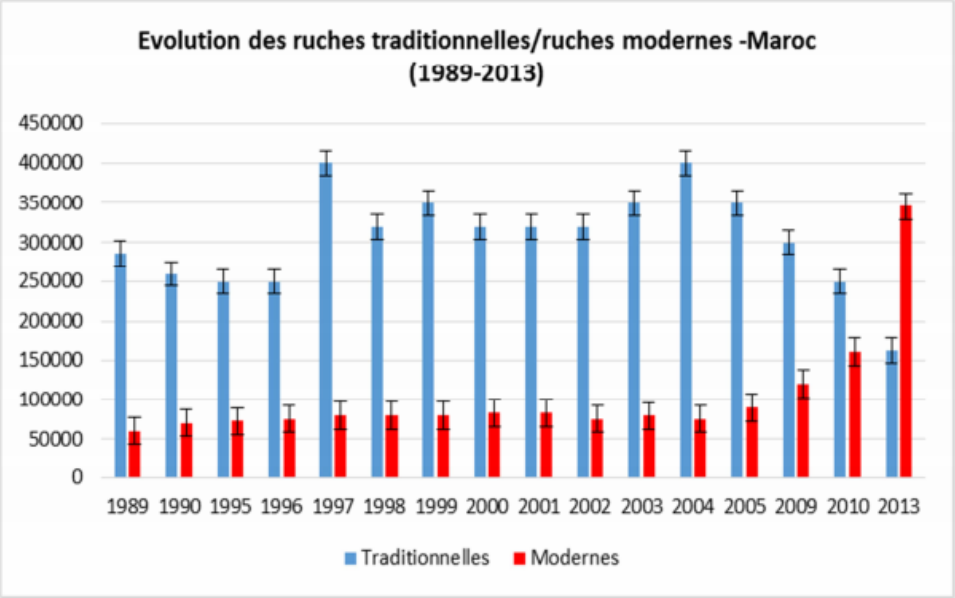


Figure 1.7 Evolution of traditional hives vs modern hives (1989-2013) [310]

Nevertheless, the scientific production about propolis originating from Morocco is limited in some papers where it was investigated the biological properties of propolis from different location in Morocco including antioxidant, anti-inflammatory, antimicrobial, anticancer activities as reported below. A general view of the chemical composition of propolis was reported. Hence, this section was devoted to explore biological and pharmacological properties of Moroccan propolis from different geographic origins.

References

[1] J. Bryan, P. Redden, C. Traba, 'The mechanism of action of Russian propolis ethanol extracts against two antibioticresistant biofilm-forming bacteria', Lett. Appl. Microbiol. 2016, 62, 192–198.

[2] Y. Park, M. Ikegaki, S. M. de Alencar, F. F. Moura, 'Evaluation of Brazilian propolis by both physicochemical methods and biological activity', Honeybee Sci. 2000, 21, 85–90.

[3] Fujita, T., Kozuka-Hata, H., Ao-Kondo, H., Kunieda, T., Oyama, M., Kubo, T., 2013. Proteomic analysis of the royal jelly and characterization of the functions of its derivation glands in the honeybee. J. Proteome Res. https://doi.org/10.1021/pr300700e

[4] G. A. Burdock, 'Review of the biological properties and toxicity of bee propolis (propolis)', Food Chem. Toxicol. 1998, 36, 347–363.

[5] A. G. Hegazi, 'Propolis: an overview', J. Bee Informed. 1998, 5, 22–23.

[6] S. Bogdanov, 'Propolis: composition, health, medicine: A Review', Bee Prod. Sci. 2014, 1–40.

[7] A. C. de Groot, 'Propolis: a review of properties, applications, chemical composition, contact allergy, and other adverse effects', Dermatitis 2013, 24, 263–282.

[8] Bogdanov Stefan, B.V., 2011. Propolis: Origin, Production, Compostion, in: The Propolis Book: Chapter 1.

[9] Andrzej K. Kuropatnicki, Ewelina Szliszka, and Wojciech Krol . Historical Aspects of Propolis Research in Modern Times. Evid Based Complement Alternat Med. 2013; 2013: 964149. doi: 10.1155/2013/964149.

[10] Crane E, 1997. The past and present importance of bee products to man, in: Bee Products—Properties, Applications, and Apitherapy, New York, pp. 1–13.

[11] J. Fearnley, 'Bee propolis: natural healing from the hive (nature's remedies)', Souvenir Press. London, UK, 2001.

[12] Gerard J, 1597. The Herball or Generall Historie of Plants, UK. ed. London.

[13] Wagh, V.D., 2013. Propolis: A wonder bees product and its pharmacological potentials. Adv. Pharmacol. Sci. https://doi.org/10.1155/2013/308249

[14] Castaldo Stefano, C.F., 2002. Propolis, an old remedy used in modern medicine. Fitoterapia 73, S1–S6.

[15] Toreti, V.C., Sato, H.H., Pastore, G.M., Park, Y.K., 2013. Recent progress of propolis for its biological and chemical compositions and its botanical origin. Evidence-based Complement. Altern. Med. https://doi.org/10.1155/2013/697390

[16] Tobergte, D.R., Curtis, S., 2013. The bees of the world, Journal of Chemical Information and Modeling. https://doi.org/10.1653/0015-4040(2002)085[0290:FMBLZH]2.0.CO;2

[17] Bankova, V.S., de Castro, S.L., Marcucci, M.C., 2000. Propolis: recent advances in chemistry and plant origin. Apidologie. https://doi.org/10.1051/apido:2000102.

[18] Borba, R.S., Klyczek, K.K., Mogen, K.L., Spivak, M., 2015. Seasonal benefits of a natural propolis envelope to honey bee immunity and colony health. J. Exp. Biol. https://doi.org/10.1242/jeb.127324

[19] Simone-Finstrom, M., Spivak, M., 2010. Propolis and bee health: the natural history and significance of resin use by honey bees. Apidologie. https://doi.org/10.1051/apido/2010016.

[20] Cornuet, J., Daoudi, A., Mohssine, E.H., Cornuet, J., Daoudi, A., Mohssine, E.H., Fresnaye, J.F., 1988. Etude biométrique de populations d'abeilles marocaines. Apidologie.

[21] WahidaLoucif, M.A., 2015. An Insightful Molecular Analysis Reveals Foreign Honeybees Among Algerian Honeybee Populations (*Apis mellifera* L.). J. Data Mining Genomics Proteomics. https://doi.org/10.4172/2153-0602.1000166

[22] Salatino, A., Teixeira, É.W., Negri, G., Message, D., 2005. Origin and chemical variation of Brazilian propolis. Evidence-based Complement. Altern. Med. https://doi.org/10.1093/ecam/neh060

[23] Ahangari Z, Naseri M, Vatandoost F. Propolis: Chemical Composition and Its Applications in Endodontics. *Iran Endod J*. 2018;13(3):285–292. doi:10.22037/iej.v13i3.20994

[24] V. Bankova, 'Chemical diversity of propolis and the problem of standardization', J.

Ethnopharmacol. 2005, 100, 114–117.

[25] A. Daugsch, C. S. Moraes, P. Fort, Y. K. Park, 'Brazilian red propolis - Chemical composition and botanical origin', Evid. Based Complement. Alternat. Med. 2008, 5, 435–441.

[26] M. C. Fernández, O. Cuesta-Rubio, A. R. Perez, R. M. De Oca Porto, I. M. Hernández, A. L. Piccinelli, L. Rastrelli, 'GCMS determination of isoflavonoids in seven red cuban propolis samples', J. Agric. Food Chem. 2008, 56, 9927– 9932.

[27] C. Lotti, M.C Fernandez, A. L. Piccinelli, O. Cuesta-Rubio, I. M. Hernández, L. Rastrelli, 'Chemical constituents of red Mexican propolis', J. Agric. Food Chem. 2010, 58, 2209– 2213.

[28] M. Popova, B. Trusheva, S. Cutajar, D. Antonova, D. Mifsud, C. Farrugia, V. Bankova, 'Identification of the plant origin of the botanical biomarkers of Mediterranean type propolis', Nat. Prod. Commun. 2012, 7, 569–570.

[29] V. Bankova, M. Popova, S. Bogdanov, A. G. Sabatini, 'Chemical composition of European propolis: Expected and unexpected results', Z. Naturforsch. –Sect. C J. Biosci. 2002, 57, 530–533.

[30] M. P. Popova, I. B. Chinou, I. N. Marekov, V. S. Bankova, 'Terpenes with antimicrobial activity from Cretan propolis', Phytochemistry 2009, 70, 1262–1271.

[31] N. Kalogeropoulos, S. J. Konteles, E. Troullidou, I. Mourtzinos, V. T. Karathanos, 'Chemical composition, antioxidant activity and antimicrobial properties of propolis extracts from Greece and Cyprus', Food Chem. 2009, 116, 452– 461.

[32] M. G. Miguel, 'Chemical and biological properties from the Western countries of the Mediterranean basin and Portugal', Int. J. Pharm. 2013, 5, 403–409.

[33] J. M. Sforcin, R. O. Orsi, V. Bankova, 'Effect of propolis, some isolated compounds and its source plant on antibody production', J. Ethnopharmacol. 2005, 98, 301–305.

[34] B. Bueno-Silva, S. M. Alencar, H. Koo, M. Ikegaki, G. V. J. Silva, M. H. Napimoga, P. L. Rosalen, 'Anti-inflammatory and antimicrobial evaluation of neovestitol and vestitol isolated from Brazilian red propolis', J. Agric. Food Chem. 2013, 61, 4546–4550.

[35] S. Kumazawa, J. S. Bonvehí, C. Torres, A. Mok-Ryeon, F. J. O. Bermejo, 'Chemical and functional characterisation of propolis collected from East Andalusia (Southern Spain)', Phytochem. Anal. 2013, 24, 608–615.

[36] A. C. H. F. Sawaya, P. V. Abdelnur, M. N. Eberlin, S. Kumazawa, M. R. Ahn, K. S. Bang, N. Nagaraja, V. Bankova, H. Afrouzan, 'Fingerprinting of propolis by easy ambient sonic-spray ionization mass spectrometry', Talanta 2010, 81, 100–108.

[37] A. M. Vivar-Quintana, M. I. González-Martín, I. Revilla, E. V. Betances-Salcedo, 'Determination and quantification of phenolic acids in raw propolis by reversed phase high performance liquid chromatography. Feasibility study for the use of near infrared spectroscopy', J. Apic. Res. 2018, 57, 648–656.

[38] N. Volpi, G. Bergonzini, 'Analysis of flavonoids from propolis by on-line HPLC-electrospray mass spectrometry', J. Pharm. Biomed. Anal. 2006, 42, 354–361.

[39] C. García-Viguera, W. Greenaway, F. R. Whatley, 'Composition of propolis from two fifferent Spanish regions', Z. Naturforsch. C 1992, 47, 634–637.

[40] S. Boisard, A. M. le Ray, J. Gatto, M. C. Aumond, P. Blanchard, S. Derbré, C. Flurin, P. Richomme, 'Chemical composition, antioxidant and anti-AGEs activities of a French poplar type propolis', J. Agric. Food Chem. 2014, 62, 1344–1351.

[41] S. Boisard, A. M. le Ray, A. Landreau, M. Kempf, V. Cassisa, C. Flurin, P. Richomme, 'Antifungal and antibacterial metabolites from a French poplar type propolis', Evid. Based Complement. Alternat. Med. 2015, 2015, 319240.

[42] T. Chasset, T. T. Häbe, P. Ristivojevic, G. E. Morlock, 'Profiling and classification of French propolis by combined multivariate data analysis of planar chromatograms and scanning direct analysis in real time mass spectra', J. Chromatogr. A 2016, 1465, 197–204.

[43] A. Aliboni, A. D'Andrea, P. Massanisso, 'Propolis specimens from different locations of central Italy: Chemical profiling and gas chromatography-mass spectrometry (GC/MS) quantitative analysis of the allergenic esters benzyl cinnamate and benzyl salicylate', J. Agric. Food Chem. 2011, 59, 282–288.

[44] K. Bosio, C. Avanzini, A. D'Avolio, O. Ozino, D. Savoia, '*In Vitro* activity of propolis against Streptococcus pyogenes', Lett. Appl. Microbiol. 2000, 31, 174–177.

[45] S. Fabris, 'Antioxidant properties and chemical composition relationship of Europeans and Brazilians propolis', Pharmacology 2013, 04, 46–51.

[46] S. Gardini, D. Bertelli, L. Marchetti, R. Graziosi, D. Pinetti, M. Plessi, G. L. Marcazzan, 'Chemical composition of Italian propolis of different ecoregional origin', J. Apic. Res. 2018, 57, 639–647.

[47] G. Papotti, D. Bertelli, L. Bortolotti, M. Plessi, 'Chemical and functional characterization of Italian propolis obtained by different harvesting methods', J. Agric. Food Chem. 2012, 60, 2852–2862.

[48] F. Pellati, F. P. Prencipe, D. Bertelli, S. Benvenuti, 'An efficient chemical analysis of phenolic acids and flavonoids in raw propolis by microwave-assisted extraction combined with high-performance liquid chromatography using the fused-core technology', J. Pharm. Biomed. Anal. 2013, 81–82, 126–132.

[49] V. A. Taddeo, F. Epifano, S. Fiorito, S. Genovese, 'Comparison of different extraction methods and HPLC quantification of prenylated and unprenylated phenylpropanoids in raw Italian propolis', J. Pharm. Biomed. Anal. 2016, 129, 219–223.

[50] S. Fiorito, F. Epifano, P. De Medina, V. A. Taddeo, S. Genovese, 'Two novel cinnamic acid derivatives from honey and propolis', J. Apic. Res. 2016, 55, 228–229.

[51] A. Mavri, H. Abramovic, T. Polak, J. Bertoncelj, P. Jamnik, M. S. Smole, B. Jeršek, 'Chemical properties and antioxidant and antimicrobial activities of slovenian propolis', Chem. Biodiversity 2012, 9, 1545–1558.

[52] T. Petelinc, T. Polak, L. Demšar, P. Jamnik, 'Fractionation of phenolic compounds extracted from propolis and their activity in the yeast Saccharomyces cerevisiae', PLoS One 2013, 8, e56104.

[53] D. Milojković Opsenica, P. Ristivojević, J. Trifković, I. Vovk, D. Lušić, Ž. Tešić, 'TLC fingerprinting and pattern recognition methods in the assessment of authenticity of poplar-type propolis', J. Chromatogr. Sci. 2016, 54, 1077– 1084.

[54] I. Kosaleć, M. Bakmaz, S. Pepeljnjak, 'Analysis of propolis from the continental and Adriatic regions of Croatia', Acta Pharm. 2003, 53, 275–285.

[55] M. Medić-Šarić, I. Jasprica, A. Smolcic, A. Mornar, 'Optimization of chromatographic conditions in thin layer chromatography of flavonoids and phenolic acids', Croat. Chem. Acta 2004, 77, 361–366.

[56] I. Jasprica, A. Smolčić-Bubalo, A. Mornar, M. Medić-Šarić, 'Investigation of the flavonoids in Croatian propolis by thin-layer chromatography', J. Planar Chromatogr.–Mod. TLC 2004, 17, 95–101.

[57] J. Cvek, M. Medić-Šarić, I. Jasprica, A. Mornar, 'Highperformance thin-layer chromatographic analysis of the phenolic acid and flavonoid content of Croatian propolis samples', J. Planar Chromatogr.–Mod. TLC 2007, 20, 429– 435.

[58] M. Medić-Šarić, V. Rastija, M. Bojić, Ž. Maleš, 'From functional food to medicinal product: Systematic approach in analysis of polyphenolics from propolis and wine', Nutr. J. 2009, 8, 33.

[59] P. Ristivojević, F. L. Andrić, J. D. Trifković, I. Vovk, L. Z. Stanisavljević, Ž. L. Tešić, D. M. Milojković-Opsenica, 'Pattern recognition methods and multivariate image analysis in HPTLC fingerprinting of propolis extracts', J. Chemom. 2014, 28, 301–310.

[60] B. Anđelković, L. Vujisić, I. Vučković, V. Tešević, V. Vajs, D. Gođevac, 'Metabolomics study of Populus type propolis', J. Pharm. Biomed. Anal. 2017, 135, 217–226.

[61] K. Graikou, M. Popova, O. Gortzi, V. Bankova, I. Chinou, 'Characterization and biological evaluation of selected Mediterranean propolis samples. Is it a new type?', LWTFood Sci. Technol. 2016, 65, 261–267.

[62] I. T. Gajger, I. Pavlovic, M. BoBojic, I. Kosalec, S. Srecec, T. Vlainic, J. Vlainic, 'The components responsible for the antimicrobial activity of propolis from Continental and Mediterranean regions in Croatia', Czech J. Food Sci. 2017, 35, 376–385.

[63] L. Safti, C. Z. Persuri, E. Fornal, T. Pavlesic, S. K. Paveli, 'Targeted and untargeted LC–MS polyphenolic profiling and chemometric analysis of propolis from different regions of Croatia', J. Pharm. Biomed. Anal. 2019, 165, 162–172.

[64] V. A. Isidorov, L. Szczepaniak, S. Bakier, 'Rapid GC/MS determination of botanical precursors of Eurasian propolis', Food Chem. 2014, 142, 101–106.

[65] I. Jerković, Z. Marijanović, P. M. Kuś, C. I. G. Tuberoso, 'Comprehensive study of Mediterranean (Croatian) propolis peculiarity: Headspace, volatiles, anti-varroa-treatment residue, phenolics, and antioxidant properties', Chem. Biodiversity 2016, 13, 210–218.

[66] T. Mašek, N. Perin, L. Racané, M. Cindrić, H. Čipčić Paljetak, M. Perić, M. Matijašić, D. Verbanac, B. Radić, J. Šuran, K. Starčević, 'Chemical composition, antioxidant and antibacterial activity of different extracts of poplar yype propolis', Croat. Chem. Acta 2018, 91, 81–88.

[67] S. Sobočanec, V. Šverko, T. Balog, A. Šarić, G. Rusak, S. Likić, B. Kušić, V. Katalinić, S. Radić, T. Marotti, 'Oxidant/ antioxidant properties of Croatian native propolis', J. Agric. Food Chem. 2006, 54, 8018–8026.

[68] M. Barbarić, K. Mišković, M. Bojić, M. B. Lončar, A. SmolčićBubalo, Z. Debeljak, M. Medić-Šarić, 'Chemical composition of the ethanolic propolis extracts and its effect on HeLa cells', J. Ethnopharmacol. 2011, 135, 772–778.

[69] A. Kujumgiev, I. Tsvetkova, Y. Serkedjieva, V. Bankova, R. Christov, S. Popov, 'Antibacterial, antifungal and antiviral activity of propolis of different geographic origin', J. Ethnopharmacol. 1999, 64, 235–240.

[70] M. Popova, B. Trusheva, D. Antonova, S. Cutajar, D. Mifsud, C. Farrugia, I. Tsvetkova, H. Najdenski, V. Bankova, 'The specific chemical profile of Mediterranean propolis from Malta', Food Chem. 2011, 126, 1431–1435.

[71] E. J. Zammit, K. B. Theuma, S. Darmanin, M. Muraglia, M. T. Camilleri-Podesta, J. A. Buhagiar, J. Calleja-Agius, M. Z. Adami, M. Micallef, C. Franchini, P. Schembri-Wismayer, 'Totarol content and cytotoxicity varies significantly in different types of propolis', Res. J. Pharm. Biol. Chem. Sci. 2013, 4, 1047–1058.

[72] E. Melliou, I. Chinou, 'Chemical analysis and antimicrobial activity of Greek propolis', Planta Med. 2004, 70, 515– 519.

[73] O. G. Celemli, F. Hatjina, L. Charistos, A. Schiesser, A. Özkirim, 'Insight into the chemical composition of Greek propolis: differences and similarities with Turkish propolis', Z. Naturforsch. C 2013, 68, 429–438.

[74] M. Velikova, V. Bankova, K. Sorkun, S. Houcine, I. Tsvetkova, A. Kujumgiev, 'Propolis from the Mediterranean region: chemical composition and antimicrobial activity', Z. Naturforsch. C 2000, 55, 790–793.

[75] M. P. Popova, K. Graikou, I. Chinou, V. S. Bankova, 'GC/MS profiling of diterpene compounds in mediterranean propolis from Greece', J. Agric. Food Chem. 2010, 58, 3167–3176.

[76] K. M. Kasiotis, P. Anastasiadou, A. Papadopoulos, K. Machera, 'Revisiting Greek propolis: Chromatographic analysis and antioxidant activity study', PLoS One 2017, 12, e0170077.

[77] V. Lagouri, D. Prasianaki, F. Krysta, 'Antioxidant properties and phenolic composition of Greek propolis extracts', Int. J. Food Prop. 2014, 17, 511–522.

[78] A. Temiz, A. Şener, A. Ö. Tüylü, K. Sorkun, B. Salih, 'Antibacterial activity of bee propolis samples from different geographical regions of Turkey against two foodborne pathogens, Salmonella enteritidis and Listeria monocytogenes', Turk. J. Biol. 2011, 35, 503–511.

[79] M. Popova, S. Silici, O. Kaftanoglu, V. Bankova, 'Antibacterial activity of Turkish propolis and its qualitative and quantitative chemical composition', Phytomedicine 2005, 12, 221–228.

[80] N. Mercan, I. Kivrak, M. E. Duru, H. Katircioglu, S. Gulcan, S. Malci, G. Acar, B. Salih, 'Chemical composition effects onto antimicrobial and antioxidant activities of propolis collected from different regions of Turkey', Ann. Microbiol. 2006, 56, 373–378.

[81] G. A. Doganli, 'Phenolic content and antibiofilm activity of propolis against clinical MSSA strains', Rec. Nat. Prod. 2016, 10, 617–627.

[82] R. Aliyazıcıoglu, H. Sahin, O. Erturk, E. Ulusoy, S. Kolayli 'Properties of phenolic composition and biological activity of propolis from Turkey', Int. J. Food Prop. 2011, 16, 277– 287.

[83] O. Koru, F. Toksoy, C. H. Acikel, Y. M. Tunca, M. Baysallar, A. G. Uskudar, E. Akca, T. A. Ozkok, K. Sorkun, M. Tanyuksel, B. Salih, '*In vitro* antimicrobial activity of propolis samples from different geographical origins against certain oral pathogens', Anaerobe 2007, 13, 140– 145.

[84] I. Seven, T. Aksu, P. T. Seven, 'The effects of propolis and vitamin C supplemented feed on performance, nutrien utilization and carcass characteristcs in broilers exposed to lead', Livest. Sci. 2012, 148, 10–15.

[85] P. Ristivojević, I. Dimkić, E. Guzelmeric, J. Trifković, M. Knežević, T. Berić, E. Yesilada, D. Milojković-Opsenica, S Stanković, 'Profiling of Turkish propolis subtypes: Comparative evaluation of their phytochemical compositions, antioxidant and antimicrobial activities', LWT-Food Sci. Technol. 2018, 85, 367–379.

[86] Y. Ozkul, S. Silici, E. Erogˇlu, 'The anticarcinogenic effect of propolis in human lymphocytes culture', Phytomedicine 2005, 12, 742–747.

[87] Z. Dogˇanyigˇit, F. Ö. Küp, S. Silici, K. Deniz, B. Yakan, T. Atayoglu, 'Protective effects of propolis on female rats', histopathological, biochemical and genotoxic changes during LPS induced endotoxemia', Phytomedicine 2013, 20, 632–639.

[88] M. Tartik, E. Darendelioglu, G. Aykutoglu, G. Baydas, 'Turkish propolis supresses MCF-7 cell death induced by homocysteine', Biomed. Pharmacother. 2016, 82, 704– 712.

[89] E. Guzelmeric, P. Ristivojević, J. Trifković, T. Dastan, O. Yilmaz, O. Cengiz, E. Yesilada, 'Authentication of Turkish propolis through HPTLC fingerprints combined with multivariate analysis and palynological data and their comparative antioxidant activity', LWT-Food Sci. Technol. 2018, 87, 23–32.

[90] N. E. Bayram, K. Sorkun, G. C. Öz, B. Salih, G. Topçu, 'Chemical characterization of 64 propolis samples from hakkari, Turkey', Rec. Nat. Prod. 2018, 12, 569–581.

[91] S. Silici, A. N. Koc, 'Comparative study of *in vitro* methods to analyse the antifungal activity of propolis against yeasts isolated from patients with superficial mycoses', Lett. Appl. Microbiol. 2006, 43, 318–324.

[92] S. Silici, K. Karaman, 'Inhibitory effect of propolis on patulin production of penicillium expansum in apple juice', J. Food Process. Preserv. 2014, 38, 1129–1134.

[93] A. Uzel, K. Sorkun, Ö. Önçagˇ, D. Çogˇulu, Ö. Gençay, B. Salih, 'Chemical compositions and antimicrobial activities of four different Anatolian propolis samples', Microbiol. Res. 2005, 160, 189–195.

[94] A. N. Koç, S. Silici, F. Mutlu-Sariguzel, O. Sagdic, A. Koc, 'Antifungal activity of propolis in four different fruit juices', Food Technol. Biotechnol. 2007, 45, 57–61.

[95] S. Silici, S. Kutluca, 'Chemical composition and antibacterial activity of propolis collected by three different races of honeybees in the same region', J. Ethnopharmacol. 2005, 99, 69–73.

[96] S. Erdogan, B. Ates, G. Durmaz, I. Yilmaz, T. Seckin, 'Pressurized liquid extraction of phenolic compounds from Anatolia propolis and their radical scavenging capacities', Food Chem. Toxicol. 2011, 49, 1592–1597.

[97] M. Kartal, S. Kaya, S. Kurucu, 'GC/MS analysis of propolis samples from two different regions of Turkey', Z. Naturforsch. C 2002, 57, 905–909.

[98] M. Kartal, S. Yildiz, S. Kaya, S. Kuruku, G. Topçu, 'Antimicrobial activity of propolis samples from two different regions of Anatolia', J. Ethnopharmacol. 2003, 86, 69–73.

[99] A. O. Sarikaya, E. Ulusoy, N. Öztürk, M. Tunçel, S. Kolayli 'Antioxidant activity and phenolic acid constituents of chestnut (Castania sativa Mill.) honey and propolis', J. Food Biochem. 2009, 33, 470–481.

[100] N. Sahinler, A. Gül, G. Çopur, 'Chemical composition and preservative effect of Turkish propolis on egg quality durig storage', Asian J. Chem. 2009, 21, 1877–1886.

[101] P. T. Seven, 'The effects of dietary Turkish propolis and vitamin C on performance, digestibility, egg production and egg quality in laying hens under different environmental temperatures', Asian-Australas. J. Anim. Sci. 2008, 21, 1164–1170.

[102] I. Seven, P. T. Seven, S. Silici, 'Effects of dietary Turkish propolis as alternative to antibiotic on growth and laying performances, nutrient digestibility and egg quality in laying hens under heat stress', Rev. Med. Vet. 2011, 162, 186–191.

[103] P. T. Seven, I. Seven, M. Yilmaz, Ü. G. Şimşek, 'The effects of Turkish propolis on growth and carcass characteristics in broilers under heat stress', Anim. Feed Sci. Technol. 2008, 146, 137–148.

[104] H. Türkez, M. I. Yousef, F. Geyikoglu, 'Propolis protects against 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced toxicity in rat hepatocytes', Food Chem. Toxicol. 2012, 50, 2142–2148.

[105] T. Özen, A. Kiliç, O. Bedir, Ö. Koru, K. Sorkun, M. Tanyuksel, S. Kiliç, Ö. Gençay, O. Yildiz, M. Baysallar, '*In vitro* activity of Turkish propolis samples against anaerobic bacteria causing oral cavity infections', Kafkas Univ. Vet. Fak. Derg. 2010, 16, 293–298.

[106] T. Ozdal, G. Sari-Kaplan, E. Mutlu-Altundag, D. Boyacioglu, E. Capanoglu, 'Evaluation of Turkish propolis for its chemical composition, antioxidant capacity, anti-proliferative effect on several human breast cancer cell lines and proliferative effect on fibroblasts and mouse mesenchymal stem cell line', J. Apic. Res. 2018, 57, 627–638.

[107] N. Duran, M. Muz, G. Culha, G. Duran, B. Ozer, 'GC/MS analysis and antileishmanial activities of two Turkish propolis types', Parasitol. Res. 2011, 108, 95–105.

[108] K. Sorkun, B. Süer, B. Salih, 'Determination of chemical composition of Turkish propolis', Z. Naturforsch. C 2001, 56, 666–668.

[109] E. Çetin, M. Kanbur, S. Silici, G. Eraslan, 'Propetamphosinduced changes in haematological and biochemical parameters of female rats: Protective role of propolis', Food Chem. Toxicol. 2010, 48, 1806–1810.

[110] H. Noureddine, R. Hage-Sleiman, B. Wehbi, A. H. FayyadKazan, S. Hayar, M. Traboulssi, O. A. Alyamani, W. H. Faour, Y. El-Makhour, 'Chemical characterization and cytotoxic activity evaluation of Lebanese propolis', Biomed. Pharmacother. 2017, 95, 298–307.

[111] F. K. Abd El Hady, A. G. Hegazi, 'Egyptian propolis: 2. Chemical composition, antiviral and antimicrobial activities of East Nile Delta propolis', Z. Naturforsch. C 2002, 57, 386–394.

[112] A. G. Hegazi, F. K. Abd El Hady, 'Egyptian propolis: 3. Antioxidant, antimicrobial activities and chemical composition of propolis from reclaimed lands', Z. Naturforsch. 2002, 57, 395–402.

[113] S. A. Sohaimy, S. H. D. Masry, 'Phenolic Content, antioxidant and antimicrobial activities of Egyptian and Chinese propolis', J. Agric. Sci. 2014, 14, 1116–1124.

[114] W. Siheri, T. Zhang, G. U. Ebiloma, M. Biddau, N. Woods, M. Y. H., C. J. Clements, J. Fearnley, R. A. E. Ebel, T. Paget, S. Muller, K. C. Carter, V. A. Ferro, H. P. de Koning, D. G. Watson, 'Chemical and antimicrobial profiling of propolis from different regions within Libya', PLoS One 2016, 11, e0155355.

[115] N. Labyad, B. Doro, N. S. Elmarbet, M. M. Aluonsy, M. Kahmasi, 2016, 'Phytochemical antioxidant and antimicrobial study of Libyan propolis ethanolic extract', Int. J. Herb. Med. 2016, 4, 1–4.

[116] W. Siheri, J. O. Igoli, A. I. Gray, T. G. Nasciemento, T. Zhang, J. Fearnley, C. J. Clements, K. C. Carter, J. Carrurhers, R. Edrada-Ebel, D. G. Watson, 'The isolation of antiprotozoal compounds from Libyan propolis', Phytother. Res. 2014, 28, 1756–1760.

[117] I. Martos, M. Cossentini, F. Ferreres, F. A. Tomas-Barberan, 'Flavonoid composition of Tunisian honeys and propolis', J. Agric. Food Chem. 1997, 45, 2824–2829.

[118] E. K. Soltani, R. Cerezuela, N. Charef, S. Mezaache-Aichour, M. A. Esteban, M. M. Zerroug, 'Algerian propolis extracts: Chemical composition, bactericidal activity and *in vitro* effects on gilthead seabream innate immune responses', Fish Shellfish Immunol. 2017, 62, 57–67.

[119] A. L. Piccinelli, T. Mencherini, R. Celano, Z. Mouhoubi, A. Tamendjari, R. P. Aquino, L. Rastrelli, 'Chemical composition and antioxidant activity of Algerian propolis', J. Agric. Food Chem. 2013, 61, 5080–5088.

[120] H. Ait Mouse, M. TilaouiI, A. Jaafari, L. Ait M'barek, R. Aboufatima, C. Abderrahmane, A. Zyad, 'Evaluation of the *In Vitro* and In Vivo anticancer properties of Moroccan propolis extracts', Braz. J. Pharmacogn. 2012, 22, 558– 567.

[121] M. Popova, B. Lyoussi, S. Aazza, D. Antunes, V. Bankova, G. Miguel, 'Antioxidant and α-glucosidase inhibitory properties and chemical profiles of Moroccan propolis', Nat. Prod. Commun. 2015, 10, 1961–1964.

[122] S. Touzani, N. Al-Waili, N. El Menyiy, B. Filipic, A. Pereyra, I. El Arabi, W. Al-Waili, B. Lyoussi, 'Chemical analysis and antioxidant content of various propolis samples collected from different regions and their impact on antimicrobial activities', Asian Pac. J. Trop. Med. 2018, 11, 436–442.

[123] M. G. Miguel, A. C. Figueiredo, 'Propolis and geopropolis volatiles', in Alvarez-Suarez J. (Ed.), Bee Products - Chemical and Biological Properties. Springer, Cham, 2017, 113–136.

[124] V. Bankova, M. Popova, B. Trusheva, 'Propolis volatile compounds: chemical diversity and biological activity: a review' Chem. Cent. J. 2014, 8, 28.

[125] G. P. L. Clair, 'The study of propolis essential oil', Riv. Ital. EPPOS 1981, 63, 168–170.

[126] M. Corsi, 'Les huilles essentielles de la propolis', in: Meletinov C (Ed.) XXVIIIth International Congress of Apiculture. Apimondia, Acapulco, Mexico, 1981, p. 407– 411.

[127] F. Pellati, F. P. Prencipe, S. Benvenuti, 'Headspace solidphase microextraction-gas chromatography-mass spectrometry characterization of propolis volatile compounds', J. Pharm. Biomed. Anal. 2013, 84, 103–111.

[128] I. Borcic, A. Radonic, K. Grzunov, 'Comparison of the volatile constituents of propolis gathered in different regions of Croatia', Flavour Fragrance J. 1996, 11, 311– 313.

[129] V. Bankova, R. Christov, S. Popov, O. Pureb, G. Bocari, 'Volatile constituents of propolis', Z. Naturforsch. C 1994, 49, 6–10.

[130] E. Melliou, E. Stratis, I. Chinou, 'Volatile constituents of propolis from various regions of Greece - Antimicrobial activity', Food Chem. 2007, 103, 375–380.

[131] Z. Yildirim, S. Hacievliyagil, N. O. Kutlu, N. E. Aydin, M. Kurkcuoglu, M. Iraz, R.1177 Durmaz, 'Effect of water extract of Turkish propolis on tuberculosis infection in guinea-1178 pigs' Pharmacol. Res. 2004, 49, 287–292.

[132] E. E. Hames-Kocabas, D. Betul, U. Atac, D. Fatih, 'Volatile composition of Anatolian propolis by headspace-solidphase microextraction (HS-SPME), antimicrobial activity against food contaminants and antioxidant activity', J. Med. Res. 2013, 7, 2140–2149.

[133] A. Jihene, I. J. Karoui, A. Ameni, M. Hammami, M. Abderrabba, 'Volatile compounds analysis of Tunisian propolis and its antifungal activity', J. Biosci. 2018, 6, 115– 131.

[134] N. Segueni, F. Khadraoui, F. Moussaoui, 'Volatile constituents of Algerian propolis', Ann. Biol. 2010, 1, 103–107.

[135] I. Gülçin, E. Bursal, M. H. Şehitoğlu, M. Bilsel, A. C. Gören, 'Polyphenol contents and antioxidant activity of lyophilized aqueous extract of propolis from Erzurum, Turkey', Food Chem. Toxicol. 2010, 48, 2227–2238.

[136] E. Betances-Salcedo, I. Revilla, A. M. Vivar-Quintana, M. I. González-Martín, 'Flavonoid and antioxidant capacity of propolis prediction using near infrared spectroscopy', Sensors 2017, 17, E1647.

[137] J. S. Bonvehí, A. L. Gutiérrez, 'The antimicrobial effects of propolis collected in different regions in the Basque Country (Northern Spain)', World J. Microbiol. Biotechnol. 2012, 28, 1351–1358.

[138] J. S. Bonvehí, A. L. Gutiérrez, 'Antioxidant activity and total phenolics of propolis from the basque country (Northeastern Spain)', J. Am. Oil Chem. Soc. 2011, 88, 1387– 1395.

[139] S. M. Osés, A. Pascual-Maté, M. A. López-Diaz, T. M. LópezDiaz, M. T. Sancho, 'Bioactive properties of honey with propolis', Food Chem. 2016, 196, 1215–1223.

[140] I. Revilla, A. M. Vivar-Quintana, I. González-Martín, O. Escuredo, C. Seijo, 'The potential of near infrared spectroscopy for determining the phenolic, antioxidant, color and bactericide characteristics of raw propolis', Microchem. J. 2017, 134, 211–217.

[141] S. Buratti, S. Benedetti, M. S. Cosio, 'Evaluation of the antioxidant power of honey, propolis and royal jelly by amperometric flow injection analysis', Talanta 2007, 71, 1387–1392.

[142] S. De Marco, M. Piccioni, R. Pagiotti, D. Pietrella, 'Antibiofilm and antioxidant activity of propolis and bud poplar resins versus Pseudomonas aeruginosa', Evid. Based Complement. Alternat. Med. 2017, 2017, 5163575.

[143] E. Gregoris, R. Stevanato, 'Correlations between polyphenolic composition and antioxidant activity of Venetian propolis', Food Chem. Toxicol. 2010, 48, 76–82.

[144] A. Russo, R. Longo, A. Vanella, 'Antioxidant activity of propolis: role of caffeic acid phenethyl ester and galangin', Fitoterapia 2002, 73, S21–S29.

[145] T. Cigut, T. Polak, L. Gašperlin, P. Raspor, P. Jamnik, 'Antioxidative activity of propolis extract in yeast cells', J. Agric. Food Chem. 2011, 59, 11449–11455.

[146] I. Jasprica, M. Bojic, A. Mornar, E. Besic, K. Bucan, M. Medic-Saric, 'Evaluation of antioxidative activity of Cro atian propolis samples using DPPH and ABTS·+ stable free radical assays', Molecules 2007, 12, 1006–1021.

[147] V. Katalinić, S. Radić, D. Ropac, R. Mulić, A. Katalinić, 'Antioxidative activity of propolis from Dalmatia (Croatia)', Acta Med. Croat. 2004, 58, 373–376.

[148] M. Jug, M. Zovko, C. K. Ci, I. Kosalec, 'Modulation of antioxidant, chelating and antimicrobial activity of poplar chemo-type propolis by extraction procures', LWT-Food Sci. Technol. 2014, 57, 530–537.

[149] F. Karadal, N. E. Onmaz, S. Abay, Y. Yildirim, S. Al, I. Tatyuz, A. Akcay, 'A study of antibacterial and antioxidant activities of bee products: Propolis, pollen and honey samples', Ethiop. J. Heal. Dev. 2018, 32, 116–122.

[150] O. Yildiz, F. Karahalil, Z. Can, H. Sahin, S. Kolayli, 'Total monoamine oxidase (MAO) inhibition by chestnut honey, pollen and propolis', J. Enzyme Inhib. Med. Chem. 2014, 29, 690–694.

[151] İ. Seven, B. G. Baykalir, P. T. Seven, G. Dağoğlu, 'The ameliorative effects of propolis against cyclosporine A induced hepatotoxicity and nephrotoxicity in rats', Kafkas Univ. Vet. Fak. Derg. 2014, 20, 641–648.

[152] Ö. Saral, O. Yildiz, R. Aliyazicioğlu, E. Yuluğ, S. Canpolat, F. Öztürk, S. Kolayli, 'Apitherapy products enhance the recovery of CCL 4-induced hepatic damages in rats', Turk. J. Med. Sci. 2016, 46, 194–202.

[153] İ. Kızılpınar Temizer, A. Güder, Ç. Ömür Gençay, 'Botanical origin and antioxidant activities of propolis from the Irano-Turanian region', Istanbul J. Pharm. 2017, 47, 107– 111.

[154] D. Özkök, S. Silici, 'Antioxidant activities of honeybee products and their mixtures', Food Sci. Technol. 2017, 26, 201–206.

[155] S. Misir, Y. Aliyazicioglu, S. Demir, I. Turan, S. O. Yaman, O. Deger, 'Antioxidant properties and protective effect of Turkish propolis on t-BHP-induced oxidative stress in foreskin fibroblast cells', Indian J. Pharm. Educ. 2018, 52, 94–100.

[156] Z. Mouhoubi-Tafinine, S. Ouchemoukh, A. Tamendjari, 'Antioxydant activity of some algerian honey and propolis', Ind. Crops Prod. 2016, 88, 85–90.

[157] Y. M, Boufadi, J. Soubhye, A. Riazi, A. Rousseau, M. Vanhaeverbeek, J. Nève, K. Z. Boudjeltia, P. Van Antwerpen, 'Characterization and antioxidant properties of six Algerian propolis extracts: Ethyl acetate extracts inhibit Myeloperoxidase activity', Int. J. Mol. Sci. 2014, 15, 2327–2345.

[158] S. Narimane, S. Akkal, B. Ozcelik, S. Rhouati, E. Demircan, A. Salah, B. Özçelik, R. Salah, 'Correlation between antioxidant activity and phenolic acids profile and content of Algerian propolis: Influence of solvent', Pak. J. Pharm. Sci. 2017, 30, 1417–1423.

[159] A. Ouamani, B. Bencharki, D. Nacera, L. Hilali, 'Antioxidant Activities of Propolis collected from Different Regions of Morocco', Int. J. Sci. Eng. 2017, 8, 2229–5518.

[160] M. G. Miguel, O. Doughmi, S. Aazza, D. Antunes, B. Lyoussi, 'Antioxidant, anti-inflammatory and acetylcholinesterase inhibitory activities of propolis from different regions of Morocco', Food Sci. Technol. 2014, 23, 313– 322.

[161] C. Cafarchia, N. De Laurentis, M. A. Milillo, V. Losacco, V. Puccini, 'Antifungal activity of Apulia region propolis', Parassitologia 1999, 41, 587–590.

[162] J. Milagros, G. Marielsa, B. P. Gilberto, 'Fungistatic and fungicidal activity ethanolic propolis.

[163] A. G. Hegazi, F. K. Abd El Hady, F. A. M. Abd Allah, 'Chemical composition and antimicrobial activity of European propolis', Z. Naturforsch. C 2000, 55, 70–75.

[164] M. Amoros, C. M. O. Simōes, L. Girre, F. Sauvager, M. Cormier, 'Synergistic effect of flavones and flavonols against herpes simplex virus type 1 in cell culture. Comparison with the antiviral activity of propolis', J. Nat. Prod. 1992, 55, 1732–1740.

[165] P. Dolci, O. I. Ozino, 'Study of the *In Vitro* sensitivity to honey bee propolis of *Staphylococcus aureus* strains characterized by different sensitivity to antibiotics', Ann. Microbiol. 2003, 53, 233–243.

[166] L. Drago, B. Mombelli, E. De Vecchi, M. C. F. L. Tocalli, M. R. Gismondo, '*In Vitro* antimicrobial activity of propolis dry extract', J. Chemother. 2000, 12, 390–395.

[167] F. Scazzocchio, F. D. D'Auria, D. Alessandrini, F. Pantanella, 'Multifactorial aspects of antimicrobial activity of propolis', Microbiol. Res. 2006, 161, 327–333.

[168] M. Gil, M. Gonzalez, O. Orlandi, K. Ugas, G. Nicita, E. Perozo, 'Actividad bacteriostática y bactericida de extractos etanólicos de propóleos venezolanos y europeos sobre *Escherichia coli* y *Staphylococcus aureus'*, MedULA 2016, 23, 4–11.

[169] A. Özkırım, Ö. G. Çelemli, A. Schiesser, L. Charistos, F. Hatjina, 'A comparison of the activities of Greek and Turkish propolis against Paenibacillus larvae', J. Apic. Res. 2014, 53, 528–536.

[170] N. Keskin, S. Hazir, K. H. C. Baser, M. Kürkçüoglu, 'Antibacterial activity and chemical composition of Turkish propolis', Z. Naturforsch. C 2001, 56, 1112–1115.

[171] A. Ugur, T. Arslan, 'An *In Vitro* study on antimicrobial activity of propolis from Mugla Province of Turkey', J. Med. Food. 2004, 7, 90–94.

[172] M. Özcan, O. Sagdic, G. Ozkan, 'Antibacterial effects of Turkish pollen and propolis extracts at different concentrations', Arch. Lebensmittelhyg. 2004, 55, 39–40.

[173] M. Özcan, 'Inhibition of Aspergillus parasiticus NRRL 2999 by pollen and propolis extracts', J. Med. Food. 2004, 7, 114–116.

[174] A. Kilic, M. Baysallar, B. Besirbellioglu, B. Salih, K. Sorkun, M. Tanyuksel, '*In Vitro* antimicrobial activity of propolis against methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant Enterococcus faecium', Ann. Microbiol. 2005, 55, 113–117.

[175] M. Özcan, A. Ünver, D. A. Ceylan, R. Yetişir, 'Inhibitory effect of pollen and propolis extracts', Food Nahrung. 2004, 48, 188–194.

[176] H. Apaydin, T. Gümüş, 'Inhibitory effect of propolis (bee gum) against *Staphylococcus aureus* bacteria isolated from instant soups', J. Tekirdag Agric. Fac. 2018, 15, 67– 75.

[177] F. Mutlu Sariguzel, E. Berk, A. N. Koc, H. Sav, G. Demir, 'Antifungal activity of propolis against yeasts isolated from blood culture: *In Vitro* evaluation', J. Clin. Lab. Anal. 2016, 30, 513–516.

[178] A. E. Akca, G. Akca, F. T. Topçu, E. Macit, L. Pikdöken, I. Ş. Özgen, 'The comparative evaluation of the antimicrobial effect of propolis with chlorhexidine against oral pathogens: An *In Vitro* study', BioMed Res. Int. 2016, 2016, 3627463.

[179] S. Arslan, H. Ozbilge, E. G. Kaya, O. Er, '*In Vitro* antimicrobial activity of propolis, BioPure MTAD, sodium hypochlorite, and chlorhexidine on Enterococcus faecalis and Candida albicans', Saudi Med. J. 2011, 32, 479–483.

[180] S. Sonmez, L. Kirilmaz, M. Yucesoy, B. Yücel, B. Yilmaz, 'The effect of bee propolis on oral pathogens and human gingival fibroblasts', J. Ethnopharmacol. 2005, 102, 371– 376.

[181] I. Peker, G. Akca, C. Sarikir, M. Toraman Alkurt, I. Celik, 'Effectiveness of alternative methods for toothbrush disinfection: An *In Vitro* study', Sci. World J. 2014, 2014, 726190.

[182] F. Özan, Z. Sümer, Z. A. Polat, K. Er, U. Özan, O. Deger, 'Effect of mouthrinse containing propolis on oral microorganisms and human gingival fibroblasts', Eur. J. Dermatol. 2007, 1, 195–201.

[183] O. Erkmen, M. M. Özcan, 'Antimicrobial effects of Turkish propolis, pollen, and laurel on spoilage and pathogenic food-related microorganisms', J. Med. Food. 2008, 11, 587–592.

[184] A. N. Koç, S. Silici, F. Kasap, H. T. Hörmet-Öz, H. MavusBuldu, B. D. Ercal, 'Antifungal activity of the honeybee products against Candida spp. and Trichosporon spp.', J. Med. Food. 2011, 14, 128–134.

[185] N. Topcuoglu, F. Ozan, M. Ozyurt, G. Kulekci, '*In vitro* antibacterial effects of glass-ionomer cement containing ethanolic extract of propolis on Streptococcus mutans', Eur. J. Dermatol. 2012, 6, 428–433.

[186] A. Topalkara, A. Vural, Z. Polat, M. I. Toker, M. K. Arici, F. Ozan, A. Cetin, '*In vitro* amoebicidal activity of propolis on Acanthamoeba castellanii', J. Ocul. Pharmacol. Ther. 2007, 23, 40–45.

[187] E. Basim, H. Basim, M. Özcan, 'Antibacterial activities of Turkish pollen and propolis extracts against plant bacterial pathogens', J. Food Eng. 2006, 77, 992–996.

[188] S. Silici, M. Ünlü, G. Vardar-Ünlü, 'Antibacterial activity and phytochemical evidence for the plant origin of Turkish propolis from different regions', World J. Microbiol. Biotechnol. 2007, 23, 1797–1803.

[189] S. Silici, N. A. Koç, D. Ayangil, S. Çankaya, 'Antifungal activities of propolis collected by different races of honeybees against yeasts isolated from patients with superficial mycoses', J. Pharmacol. Sci. 2005, 99, 39–44.

[190] S. Silici, N. A. Koc, F. M. Sariguzel, O. Sagdic, 'Mould inhibition in different fruit juices by propolis', Arch. Lebensmittelhyg. 2005, 56, 87–90.

[191] O. Hatice, K. E. Gunduz, S. Albayrak, S. Sibel, 'Antileishmanial activities of ethanolic extract of Kayseri propolis', Afr. J. Microbiol. Res. 2010, 4, 556–560.

[192] G. Duran, N. Duran, G. Culha, B. Ozcan, H. Oztas, B. Ozer, '*In Vitro* antileishmanial activity of Adana propolis samples on Leishmania tropica: A preliminary study', Parasitol. Res. 2008, 102, 1217–1225.

[193] H. Sahin, R. Aliyazicioglu, O. Yildiz, S. Kolayli, A. Innocenti, C. T. Supuran, 'Honey, polen, and propolis extracts show potent inhibitory activity against the zinc metalloenzyme carbonic anhydrase', J. Enzyme Inhib. Med. Chem. 2011, 26, 440–444

[194] N. Baltas, O. Yildiz, S. Kolayli, 'Inhibition properties of propolis extracts to some clinically important enzymes', J. Enzyme Inhib. Med. Chem. 2016, 31, S52–S55.

[195] A. G. Hegazi, F. K. Abd El Hady, 'Egyptian propolis: 1- Antimicrobial activity and chemical composition of upper Egypt propolis', Z. Naturforsch. C 2001, 56, 82–88.

[196] A. El-Bassuony, S. AbouZid, 'A new prenylated flavanoid with antibacterial activity from propolis collected in Egypt', Nat. Prod. Commun. 2010, 5, 43–45.

[197] M. F. Ghaly, S. M. Ezzat, M. M. Sarhan, 'Use of propolis and ultragriseofulvin to inhibit aflatoxigenic fungi', Folia Microbiol. 1998, 43, 156–160.

[198] G. A. Elbaz, I. I. Elsayad, 'Comparison of the antimicrobial effect of Egyptian propolis vs. New Zealand propolis on Streptococcus mutans and Lactobacilli in saliva', Oral Health 2012, 10, 155–160.

[199] N. Al-Waili, A. Al-Ghamdi, M. J. Ansari, Y. Al-Attal, K. Salom, 'Synergistic effects of honey and propolis toward drug multi-resistant *Staphylococcus aureus*, Escherichia coli and Candida albicans isolates in single and polymicrobial cultures', Int. J. Med. Sci. 2012, 9, 793–800.

[200] A. B. Mokhtar, E. K. El-Gayar, E. S. Habib, '*In Vitro* antiprotozoal activity of propolis extract and cysteine proteases inhibitor (phenyl vinyl sulfone) on blastocystis species', J. Egypt. Soc. Parasitol. 2016, 46, 261–272.

[201] B. Kouidhi, T. Zmantar, A. Bakhrouf, 'Anti-cariogenic and anti-biofilms activity of Tunisian propolis extract and its potential protective effect against cancer cells proliferation', Anaerobe 2010, 16, 566–571.

[202] M. Benhanifia, W. M. Mohamed, Y. Bellik, H. Benbarek, 'Antimicrobial and antioxidant activities of different propolis samples from north-western Algeria', Int. J. Food Sci. Technol. 2013, 48, 2521–2527.

[203] M. Benhanifia, K. Shimomura, I. Tsuchiya, S. Inui, S. Kumazawa, W. Mohamed, L. Boukraa, M. Sakharkar, H. Benbarek, 'Chemical composition and antimicrobial activity of propolis collected from some localities of Western Algeria', Acta Aliment. 2014, 43, 482–488.

[204] N. Segueni, A. Alabdul Magid, M. Decarme, S. Rhouati, M. Lahouel, F. Antonicelli, C. Lavaud, W. Hornebeck, 'Inhibition of stromelysin-1 by caffeic acid derivatives from a propolis sample from Algeria', Planta Med. 2011, 77, 999– 1004.

[205] C. Lisbona, J. Díaz-Castro, M. J. M. Alférez, I. M. Guisado, R. Guisado, I. López-Aliaga, 'Positive influence of a natural product as propolis on antioxidant status and lipid peroxidation in senescent rats', J. Physiol. Biochem. 2013, 69, 919–925.

[206] J. C. Dunyach-Remy, J. P. Lavigne, A. Sotto, 'Propolis potentiates the effect of cranberry (Vaccinium macrocarpon) in reducing the motility and the biofilm formation of uropathogenic Escherichia coli', PLoS One 2018, 13, e0202609.

[207] C. Couteau, M. Pommier, E. Paparis, L. J. M. Coiffard, 'Photoprotective activity of propolis', Nat. Prod. Res. 2008, 22, 264–268.

[208] C. Scifo, V. Cardile, A. Russo, R. Consoli, C. Vancheri, F. Capasso, A. Vanella, M. Renis, 'Resveratrol and propolis as necrosis or apoptosis inducers in human prostate carcinoma cells', Oncol. Res. 2004, 14, 415–426.

[209] F. Borrelli, A. A. Izzo, G. Di Carlo, P. Maffia, A. Russo, F. M. Maiello, F. Capasso, N. Mascolo, 'Effect of a propolis extract and caffeic acid phenethyl ester on formation of aberrant crypt foci and tumors in the rat colon', Fitoterapia 2002, 73, S38–S43.

[210] V. Cardile, A. Panico, B. Gentile, F. Borrelli, A. Russo, 'Effect of propolis on human cartilage and chondrocytes', Life Sci. 2003, 11, 1027–1035.

[211] A. Rossi, A. Ligresti, R. Longo, A. Russo, F. Borrelli, L. Sautebin, 'The inhibitory effect of propolis and caffeic acid phenethyl ester on cyclooxygenase activity in J774 macrophages', Phytomedicine 2002, 9, 530–535.

[212] A. Rossi, R. Longo, A. Russo, F. Borrelli, L. Sautebin, 'The role of the phenethyl ester of caffeic acid (Caffeic acid phenethyl ester) in the inhibition of rat lung cyclooxygenase activity by propolis', Fitoterapia 2002, 73, S30– S37.

[213] G. Rassu, M. Cossu, R. Langasco, A. Carta, R. Cavalli, P. Giunchedi, E. Gavini, 'Propolis as lipid bioactive nanocarrier for topical nasal drug delivery', Colloids Surf. B 2015, 136, 908–917.

[214] G. C. Ceschel, P. Maffei, A. Sforzini, S. Lombardi Borgia, A. Yasin, C. Ronchi, '*In Vitro* permeation through porcine buccal mucosa of caffeic acid phenetyl ester (caffeic acid phenethyl ester) from a topical mucoadhesive gel containing propolis', Fitoterapia 2002, 73, S44–S52.

[215] E. Gregoris, S. Fabris, M. Bertelle, L. Grassato, R. Stevanato, 'Propolis as potential cosmeceutical sunscreen agent for its combined photoprotective and antioxidant properties', Int. J. Pharm. 2011, 405, 97–101.

[216] N. Oršolić, I. Bašić, 'Immunomodulation by water-soluble derivative of propolis: A factor of antitumor reactivity', J. Ethnopharmacol. 2003, 84, 265–273.

[217] N. Oršolić, L. Šver, S. Terzić, Z. Tadić, I. Bašić, 'Inhibitory effect of water-soluble derivative of propolis and its polyphenolic compounds on tumor growth and metastasizing ability: A possible mode of antitumor action', Nutr. Cancer 2003, 47, 156–163.

[218] N. Oršolić, A. H. Knežević, L. Šver, S. Terzić, I. Bašić, 'Immunomodulatory and antimetastatic action of propolis and related polyphenolic compounds', J. Ethnopharmacol. 2004, 94, 307–315.

[219] N. Oršolic, L. Šver, S. Terzić, I. Bašić, 'Peroral application of water-soluble derivative of propolis (WSDP) and its related polyphenolic compounds and their influence on immunological and antitumor activity', Vet. Res. Commun. 2005, 29, 575–593.

[220] N. Oršolic¨, N. Car, D. Lisičic¨, V. Benkovic¨, A. H. Kneževic¨, D. Đikić, J. Petrik, 'Synergism between propolis and hyperthermal intraperitoneal chemotherapy with cisplatin on Ehrlich ascites tumor in mice', J. Pharm. Sci. 2013, 102, 4395–4405.

[221] N. Oršolić, V. Benković, D. Lisičić, D. Dikić, J. Erhardt, A. H. Knezević, 'Protective effects of propolis and related polyphenolic/flavonoid compounds against toxicity induced by irinotecan', Med. Oncol. 2010, 27, 1346–1358.

[222] N. Oršolić, I. Bašić, 'Water-soluble derivative of propolis and its polyphenolic compounds enhance tumoricidal activity of macrophages', J. Ethnopharmacol. 2005, 102, 37–45.

[223] V. Benkovic, A. Horvat Knezevic, G. Brozovic, F. Knezevic, D. Dikic, M. Bevanda, I. Basic, N. Orsolic, 'Enhanced antitumor activity of irinotecan combined with propolis and its polyphenolic compounds on Ehrlich ascites tumor in mice', Biomed. Pharmacother. 2007, 61, 292–297.

[224] N. Oršolić, I. Bašić, 'Antitumor, hematostimulative and radioprotective action of water-soluble derivative of propolis (WSDP)', Biomed. Pharmacother. 2005, 59, 561– 570.

[225] N. Oršolić, V. Benković, A. H. Knežević, N. Kopjar, I. Kosalec, M. Bakmaz, Ž. Mihaljević, K. Bendelja, I. Bašić, 'Assessment by survival analysis of the radioprotective properties of propolis and its polyphenolic compounds', Biol. Pharm. Bull. 2007, 30, 946–951.

[226] V. Benković, N. Orsolić, A. Horvat Knežević, S. Ramić, D. Dikić, I. Bašić, N. Kopjar, 'Evaluation of the radioprotective effects of propolis and flavonoids in gamma-irradiated mice: The alkaline comet assay study', Biol. Pharm. Bull. 2008, 31, 167–172.

[227] S. Sobočanec, T. Balog, A. Šari, Ž. MačAk-Šafranko, M. Štroser, K. Žarković, N. Žarković, R. Stojković, S. Ivanković, T. Marotti, 'Antitumor effect of Croatian propolis as a consequence of diverse sex-related dihydropyrimidine dehydrogenase (DPD) protein expression', Phytomedicine 2011, 18, 852–858.

[228] N. Oršolić, D. Sirovina, M. Z. Končić, G. Lacković, G. Gregorović, 'Effect of Croatian propolis on diabetic nephropathy and liver toxicity in mice', BMC Complementary Altern. Med. 2012, 12, 117.

[229] N. Oršolić, D. Sirovina, G. Gajski, V. Garaj-Vrhovac, M. Jazvinšćak Jembrek, I. Kosalec, 'Assessment of DNA damage and lipid peroxidation in diabetic mice: Effects of propolis and epigallocatechin gallate (EGCG)', Mutat. Res. - Genet. Toxicol. Environ. Mutagen. 2013, 757, 36–44.

[230] N. Oršolić, J. Skurić, D. Crossed, D. Signikić, G. Stanić, 'Inhibitory effect of a propolis on di-n-propyl disulfide or n-hexyl salycilate-induced skin irritation, oxidative stress and inflammatory responses in mice', Fitoterapia 2014, 93, 18–30.

[231] V. Benković, A. Knežević, D. Đikić, D. Lisičić, N. Oršolić, I. Bašić, N. Kopjar, 'Radioprotective effects of quercetin and ethanolic extract of propolis in gamma-irradiated mice', Phytomedicine, 2009, 60, 129–138.

[232] V. Benković, A. H. Knezevic, N. Orsolic, I. Basic, S. Ramic, T. Viculin, F. Knezevic, N. Kopjar, 'Evaluation of radioprotective effects of propolis and its flavonoid constituents: *In vitro* study on human white blood cells', Phytother. Res. 2009, 23, 1159–1168.

[233] D. Lisičić, V. Benković, D. Đikić, A. S. Blažević, J. Mihaljević, N. Oršolić, A. H. Knežević, 'Addition of propolis to irinotecan therapy prolongs survival in Ehrlich ascites tumor-bearing mice', Cancer Biother. Radiopharm. 2014, 29, 62–69.

[234] M. Bojić, A. Antolić, M. Tomičić, Ž. Debeljak, Ž. Maleš, 'Propolis ethanolic extracts reduce adenosine diphos phate induced platelet aggregation determined on whole blood', Nutr. J. 2018, 17, 52.

[235] A. Meto, A. Meto, B. Bimbari, K. Shytaj, M. Ozcan, 'Antiinflammatory and regenerative effects of Albanian propolis in experimental vital amputations', Eur. J. Prosthodont. Restor. Dent. 2016, 24, 145–151.

[236] H. Pratsinis, D. Kletsas, E. Melliou, I. Chinou, 'Antiproliferative activity of Greek propolis', J. Med. Food. 2010, 13, 286–290.

[237] N. Kalogeropoulos, S. Konteles, I. Mourtzinos, E. Troullidou, A. Chiou, V. T. Karathanos, 'Encapsulation of complex extracts in cyclodextrin: An application to propolis ethanolic extract', J. Microencapsulation 2009, 26, 603– 613.

[238] Y. Aliyazicioglu, O. Deger, E. Ovali, Y. Tekelioglu, Y. Barlak, I. Hosver, S. C. Karahan, 'Effects of Turkish pollen and propolis extracts on respiratory burst for K-562 cell lines', Int. Immunopharmacol. 2005, 5, 1652–1657.

[239] H. Erhan Erogˇlu, Y. Özkul, A. Tatlisen, S. Silici, 'Anticarcinogenic and antimitotic effects of Turkish propolis and mitomycin-C on tissue cultures of bladder cancer', Nat. Prod. Res. 2008, 22, 1060–1066.

[240] H. Seda Vatansever, K. Sorkun, S. Ismet Deliloğlu Gurhan, F. Ozdal-Kurt, E. Turkoz, O. Gencay, B. Salih, 'Propolis from Turkey induces apoptosis through activating caspases in human breast carcinoma cell lines', Acta Histochem. 2010, 112, 546–556.

[241] İ. Turan, O. Değer, Y. Aliyazicioğlu, S. Demir, K. Kilinç, A. Sümer, 'Effects of Turkish propolis on expression of hOGG-1 and NEIL-1', Turk. J. Med. Sci. 2015, 45, 804–811.

[242] R. Onbas, A. Kazan, A. Nalbantsoy, O. Yesil-Celiktas, 'Cytotoxic and nitric oxide inhibition activities of propolis extract along with microencapsulation by complex coacervation', Plant Foods Hum. Nutr. 2016, 71, 286–293.

[243] M. Uçar, O. Değer, Y. Barlak, 'Effect of turkish propolis extracts on expression of voltage-gated sodium channel nav 1.5 and 1.7 α-isoforms in PC-3 human prostate cancer cells', Trop. J. Pharm. Res. 2016, 15, 2093–2097.

[244] M. Uçar, O. Değer, A. Y. Gerigelmez, S. Cengiz, Y. Barlak, E. Ovalı, 'Effect of Turkish pollen and propolis extracts on caspase-3 activity in myeloid cancer cell lines', Trop. J. Pharm. Res. 2016, 15, 2445–2449.

[245] I. Turan, S. Demir, S. Misir, K. Kilinc, A. Mentese, Y. Aliyazicioglu, O. Deger, 'Cytotoxic effect of Turkish propolis on liver, colon, breast, cervix and prostate cancer cell lines', Trop. J. Pharm. Res. 2015, 14, 777–782.

[246] S. Demir, Y. Aliyazicioglu, I. Turan, S. Misir, A. Mentese, S. O. Yaman, K. Akbulut, K. Kilinc, O. Deger, 'Antiproliferative and proapoptotic activity of Turkish propolis on human lung cancer cell line', Nutr. Cancer. 2016, 68, 165– 172.

[247] Y. Ozkul, H. E. Eroglu, E. Ok, 'Genotoxic potential of Turkish propolis in peripheral blood lymphocytes', Pharmazie 2006, 61, 638–640.

[248] Y. Aliyazicioglu, S. Demir, I. Turan, T. Cakiroglu, I. Akalin, O. Deger, A. Bedir, 'Preventive and protective effects of Turkish propolis on H2O2-induced DNA damage in foreskin fibroblast cell lines', Acta Biol. Hung. 2011, 62, 388–396.

[249] C. Yalcin, Y. Aliyazicioglu, S. Demir, I. Turan, Z. Bahat, S. Misir, O. Deger, 'Evaluation of the radioprotective effect of Turkish propolis on foreskin fibroblast cells', J. Cancer Res. Ther. 2016, 12, 990–994.

[250] E. Darendelioglu, G. Aykutoglu, M. Tartik, G. Baydas, 'Turkish propolis protects human endothelial cells *In Vitro* from homocysteine-induced apoptosis', Acta Histochem. 2016, 118, 369–376.

[251] D. Kolankaya, G. Selmanog˘lu, K. Sorkun, B. Salih, 'Protective effects of Turkish propolis on alcohol-induced serum lipid changes and liver injury in male rats', Food Chem. 2002, 78, 213–217.

[252] C. A. Agca, A. A. Tykhomyrov, G. Baydas, V. S. Nedzvetsky, 'Effects of a propolis extract on the viability of and levels of cytoskeletal and regulatory proteins in rat brain astrocytes: an *In Vitro* study', Neurophysiology 2017, 49, 261–271.

[253] G. Eraslan, M. Kanbur, S. Silici, 'Evaluation of propolis effects on some biochemical parameters in rats treated with sodium fluoride', Pestic. Biochem. Physiol. 2007, 88, 273–283.

[254] S. P. Ertürküner, E. Y. Saraç, S. S. Göçmez, H. Ekmekçi, Z. B. Öztürk, İ. Seçkin, Ö. Sever, K. Keskinbora, 'Anti-inflammatory and ultrastructural effects of Turkish propolis in a rat model of endotoxin-induced uveitis', Folia Histochem. Cytobiol. 2016, 54, 49–57.

[255] G. Girgin, T. Baydar, M. Ledochowski, H. Schennach, D. N. Bolukbasi, K. Sorkun, B. Salih, G. Sahin, D. Fuchs, 'Immunomodulatory effects of Turkish propolis: Changes in neopterin release and tryptophan degradation', Immunobiology 2009, 214, 129–134.

[256] I. Seven, T. Aksu, P. Tatli Seven, 'The effects of propolis on biochemical parameters and activity of antioxidant enzymes in broilers exposed to lead-induced oxidative stress', Asian-Australas. J. Anim. Sci. 2010, 23, 1482–1489.

[257] Y. Barlak, O. Değer, M. Uçar, T. N. Çakiroğlu, 'Effects of Turkish propolis extract on secretion of polymorphonuclear elastase following respiratory burst', Turk. J. Biol. 2015, 39, 194–201.

[258] M. C. Han, A. S. Durmus, E. Karabulut, I. Yaman, 'Effects of Turkish propolis and silver sulfadiazine on burn wound healing in rats', Rev. Med. Vet. 2005, 12, 624–627.

[259] E. Kalyoncuoglu, N. Gönülol, Ö. E. Demiryürek, E. Bodrumlu, 'Effect of propolis as a root canal irrigant on bond strength to dentin', J. Appl. Biomater. 2015, 18, 13.

[260] A. Luis-Villaroya, L. Espina, D. García-Gonzalo, S. Bayarri, C. Pérez, R. Pagán, 'Bioactive properties of a propolis-based dietary supplement and its use in combination with mild heat for apple juice preservation', Int. J. Food Microbiol. 2015, 205, 90–97.

[261] I. A. I. Thamnopoulos, G. F. Michailidis, D. J. Fletouris, A. Badeka, M. G. Kontominas, A. S. Angelidis, 'Inhibitory activity of propolis against Listeria monocytogenes in milk stored under refrigeration', Food Microbiol. 2018, 73, 168– 176.

[262] E. E. Çandir, A. E. Özdemir, E. M. Soylu, N. Sahinler, A. Gül, 'Effects of propolis on storage of sweet cherry cultivar aksehir napolyon', Asian J. Chem. 2009, 21, 2659–2666.

[263] A. E. Özdemir, E. E. Çandir, M. Kaplankiran, E. M. Soylu, N. Şahinler, A. Gül, 'The effects of ethanol-dissolved propolis on the storage of grapefruit cv. Star Ruby', Turk. J. Agric. For. 2010, 34, 155–162.

[264] M. Duman, E. Ozpolat, 'Effects of water extract of propolis on fresh shibuta (Barbus grypus) fillets during chilled storage', Food Chem. 2015, 189, 80–85.

[265] O. Sagdic, S. Silici, H. Yetim, 'Fate of Escherichia coli and E. coli O157:H7 in apple juice treated with propolis extract', Ann. Microbiol. 2007, 57, 345–348.

[266] E. Torlak, D. Sert, 'Antibacterial effectiveness of chitosanpropolis coated polypropylene films against foodborne pathogens', Int. J. Biol. Macromol. 2013, 60, 52–55.

[267] A. Aygun, D. Sert, G. Copur, 'Effects of propolis on eggshell microbial activity, hatchability, and chick performance in Japanese quail (Coturnix coturnix japonica) eggs', Poult. Sci. 2012, 91, 1018–1025.

[268] G. Copur, O. Camci, N. Sahinler, A. Gul, 'The effect of propolis egg shell coatings on interior egg quality', Arch. Gefluegelkd. 2008, 72, 35–40.

[269] S. Velotto, C. Vitale, E. Varricchio, A. Crasto, 'Effect of propolis on the fish muscular development and histomorphometrical characteristics', Acta Vet. Brno. 2010, 79, 543–550.

[270] P. T. Seven, S. Yilmaz, I. Seven, I. H. Cerci, M. A. Azman, M. Yilmaz, 'Effects of propolis on selected blood indicators and antioxidant enzyme activities in broilers under heat stress', Acta Vet. Brno. 2009, 78, 75–83.

[271] D. Ozkok, K. M. Iscan, S. Silici, 'Effects of dietary propolis supplementation on performance and egg quality in laying hens', J. Anim. Vet. Adv. 2013, 12, 269–275.

[272] M. Denli, F. Okan, A. N. Uluocak, 'Effect of dietary supplementation of herb essential oils on the growth performance, carcass and intestinal characteristics of quail (Coturnix coturnix japonica)', South African J. Anim. Sci. 2004, 34, 174–179.

[273] P. T. Seven, A. S. Arslan, M. Özçelik, Ü. G. Şimşek, İ. Seven, 'Effects of propolis and royal jelly dietary supplementation on performance, egg characteristics, lipid peroxidation, antioxidant enzyme activity and mineral levels in Japanese quail', Eur. Poult. Sci. 2016, 80, 16.

[274] Y. Çam, A. N. Koç, S. Silici, V. Günes, H. Buldu, A. C. Onmaz, F. F. Kasap, 'Treatment of dermatophytosis in young cattle with propolis and whitfield's ointment', Vet. Rec. 2009, 165, 57–58.

[275] M. Fuat Gulhan, A. Duran, Z. Selamoglu Talas, S. Kakoolaki, S. M. Mansouri, 'Effects of propolis on microbiologic and biochemical parameters of rainbow trout (Oncorhynchus mykiss) after exposure to the pesticide', Iran. J. Fish. Sci. 2012, 11, 490–503.

[276] G. T. Kelestemur, P. T. Seven, S. Yilmaz, 'Effects of dietary propolis and vitamin E on growth performance and antioxidant status in blood of juvenile rainbow trout, Oncorhynchus mykiss (Teleostei: Salmoniformes) under different flow rates', Zoologica 2012, 29, 99–108.

[277] İ. Kahramanoğlu, M. Aktas, Ş. Gündüz, 'Effects of fludioxonil, propolis and black seed oil application on the postharvest quality of 'Wonderful', pomegranate', PLoS One 2018, 13, e0198411.

[278] O. A. Y. El-Khawaga, T. A. Salem, M. F. Elshal, 'Protective role of Egyptian propolis against tumor in mice', Clin. Chim. Acta 2003, 338, 11–16.

[279] M. Badr, N. Edrees, A. Abdallah, 'Anti-tumor effects of Egyptian propolis on Ehrlich ascites carcinoma', Vet. Ital. 2011, 47, 341–350.

[280] A. A. Tohamy, E. M. Abdella, R. R. Ahmed, Y. K. Ahmed, 'Assessment of anti-mutagenic, anti-histopathologic and antioxidant capacities of Egyptian bee pollen and propolis extracts', Cytotechnology. 2014, 66, 283–297.

[281] S. M. Rizk, H. F. Zaki, M. A. M. Mina, 'Propolis attenuates doxorubicin-induced testicular toxicity in rats', Food Chem. Toxicol. 2014, 67, 176–186.

[282] N. M. Elbaz, I. A. Khalil, A. A. Abd-Rabou, I. M. El-Sherbiny, 'Chitosan-based nano-in-microparticle carriers for enhanced oral delivery and anticancer activity of propolis', Int. J. Biol. Macromol. 2016, 92, 254–269.

[283] H. K. A. Elhakim, S. M. Azab, A. M. Fekry, 'A novel simple biosensor containing silver nanoparticles/propolis (bee glue) for microRNA let-7a determination', Mater. Sci. Eng. C 2018, 92, 489–495.

[284] W. K. El-Aidy, A. A. Ebeid, A. El R. M. Sallam, I. E. Muhammad, A. T. Abbas, M. A. Kamal, S. S. Sohrab, 'Evaluation of propolis, honey, and royal jelly in amelioration of peripheral blood leukocytes and lung inflammation in mouse conalbumin-induced asthma model', Saudi J. Biol. Sci. 2015, 22, 780–788.

[285] T. Y. Mahmoud, S. M. Rizk, A. S. Maghraby, A. A. Shaheen, 'Propolis enhances the effectiveness of praziquantel in experimental schistosomiasis: biochemical and histopathological study', Parasitol. Res. 2014, 113, 4513–4523.

[286] H. Soufy, N. M. El-Beih, S. M. Nasr, T. H. Abd El-Aziz, F. A. M. Khalil, Y. F. Ahmed, H. A. A. Abou Zeina, 'Effect of Egyptian propolis on cryptosporidiosis in immunosuppressed rats with special emphasis on oocysts shedding, leukogram, protein profile and ileum histopathology', Asian Pac. J. Trop. Med. 2017, 10, 253–262.

[287] O. M. Gomaa, A. S. Gaweesh, 'Variation in adhesion and germ tube formation of oral Candida using Egyptian propolis', Can. J. Microbiol. 2013, 59, 197–203.

[288] S. Sharaf, A. Higazy, A. Hebeish, 'Propolis induced antibacterial activity and other technical properties of cotton textiles', Int. J. Biol. Macromol. 2013, 59, 408–416.

[289] E. M. Garoui, A. Troudi, H. Fetoui, N. Soudani, T. Boudawara, N. Zeghal, 'Propolis attenuates cobalt induced-nephrotoxicity in adult rats and their progeny', Exp. Toxicol. Pathol. 2012, 64, 837–846.

[290] M. Alyane, L. Benguedouar, W. Kebsa, H. N. Boussenane, H. Rouibah, M. Lahouel, 'Cardioprotective effects and mechanism of action of polyphenols extracted from propolis against doxorubicin toxicity', Pak. J. Pharm. Sci. 2008, 21, 201–209.

[291] K. Boutabet, W. Kebsa, M. Alyane, M. Lahouel, 'Polyphenolic fraction of Algerian propolis protects rat kidney against acute oxidative stress induced by doxorubicin', Indian J. Nephrol. 2011, 21, 101–106.

[292] K. Touri, H. Belquendouz, O. Medjeber, Z. Djeraba, K. Lahmar, C. Touil-Boukoffa, 'Propolis modulates NOS2/ arginase-1 pathway in tropomyosin-induced experimental autoimmune uveitis', Inflammopharmacology 2018, 26, 1293–1303.

[293] M. Mountassir, S. Chaib, Y. Selami, H. Khalki, A. Ouachrif, S. Moubtakir, R. Aboufatima, A. Zyad, A. Benharref, A. Chait, 'Antinociceptive activity and acute toxicity of Moroccan black propolis', Int. J. Engine Res. 2014, 3, 2393–2397

[294] N. El Menyiy, N. Al Waili, M. Bakour, H. Al-Waili, B. Lyoussi, 'Protective effect of propolis in proteinuria, crystaluria, nephrotoxicity and hepatotoxicity induced by ethylene glycol ingestion', Arch. Med. Res. 2016, 47, 526–534.

[295] N. El Menyiy, N. Al-Waili, A. El Ghouizi, W. Al-Wail, B. Lyoussi, 'Evaluation of antiproteinuric and hepato-renal protective activities of propolis in paracetamol toxicity in rats', Nutr. Res. Pract. 2018, 23, 1976–1457.

[296] F. H. F. Ali, G. G. M. Kassem, O. Atta-Alla, 'Propolis as a natural decontaminant and antioxidant in fresh oriental sausage', Vet. Ital. 2010, 46, 167–172.

[297] A. O. Abass, N. N. Kamel, W. H. Khalifa, G. F. Gouda, M. A. F. El-Manylawi, G. M. K. Mehaisen, M. M. Mashaly, 'Propolis supplementation attenuates the negative effects of oxidative stress induced by paraquat injection on productive performance and immune function in Turkey poults', Poult. Sci. 2017, 96, 4419–4429.

[298] S. A. Nassar, A. H. Mohamed, H. Soufy, S. M. Nasr, K. M. Mahran, 'Immunostimulant effect of Egyptian propolis in rabbits', Sci. World J. 2012, 2012, 901516.

[299] S. A. Nassar, A. H. Mohamed, H. Soufy, S. M. Nasr, 'Protective effect of Egyptian propolis against rabbit pasteurellosis', BioMed Res. Int. 2013, 2013, 163724.

[300] A. M. M. Abd-El-Rhman, 'Antagonism of Aeromonas hydrophila by propolis and its effect on the performance of Nile tilapia, Oreochromis niloticus', Fish Shellfish Immunol. 2009, 27, 454–459.

[301] M. M. M. Kandiel, A. M. El-Asely, H. A. Radwan, A. A. Abbass, 'Modulation of genotoxicity and endocrine disruptive effects of malathion by dietary honeybee pollen and propolis in Nile tilapia (Oreochromis niloticus)', J. Adv. Res. 2014, 5, 671–684.

[302] S. G. Fernández, E. A. Alemán, B. E. García Figueroa, E. G. Fagoaga, J. M. Olaguibel Rivera, A. I. Tabar Purroy, 'Direct and airborne contact dermatitis from propolis in beekeepers', Contact Dermatitis 2004, 50, 320–321.

[303] S. G. Fernández, E. L. Luaces, S. E. Madoz, E. A. Alemán, M. A. Apiñániz, A. I. Tabar Purroy, 'Allergic contact stomatitis due to therapeutic propolis', Contact Dermatitis 2004, 50, 321.

[304] M. Cabanillas, V. Fernández-Redondo, J. Toribio, 'Allergic contact dermatitis to plants in a Spanish dermatology department: A 7-year review', Contact Dermatitis 2006, 55, 84–91.

[305] G. Pasolini, D. Semenza, R. Capezzera, R. Sala, C. Zane, R. Rodella, P. Calzavara-Pinton, 'Allergic contact chellitis induced by repeated contact with propolis-enriched honey', Contact Dermatitis 2004, 50, 322–323.

[306] F. Giusti, R. Miglietta, P. Pepe, S. Seidenari, 'Sensitization to propolis in 1255 children undergoing patch testing', Contact Dermatitis 2004, 51, 255–258.

[307] K. D. Hay, D. E. Greig, 'Propolis allergy: A cause of oral mucositis with ulceration', Oral Surg. Oral Med. Oral Pathol. 1990, 70, 584–586.

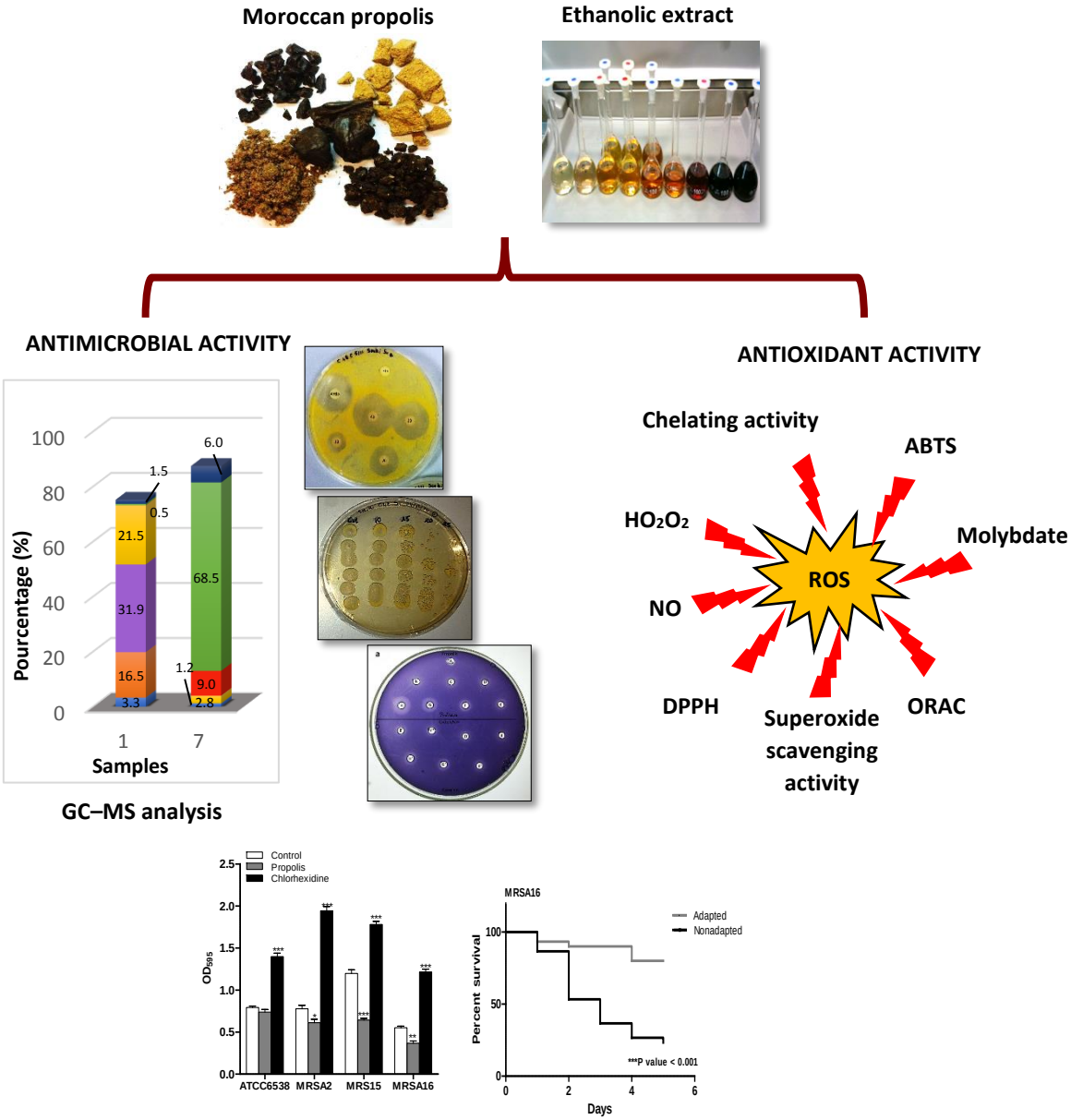
[308] F. Menniti-Ippolito, G. Mazzanti, C. Santuccio, P. A. Moro, G. Calapai, F. Firenzuoli, A. Valeri, R. Raschetti, 'Surveillance of suspected adverse reactions to natural health products in Italy', Pharmacoepidemiol. Drug Saf. 2008, 17, 626–635.

[309] S. Bellegrandi, G. D'Offizi, I. J. Ansotegui, R. Ferrara, E. Scala, R. Paganelli, 'Propolis allergy in an HIV-positive patient', J. Am. Acad. Dermatol. 1996, 35, 644.

[310] A. Moujanni, A. K. Essamadi, A. Terrab, ,L'apiculture au Maroc: focus sur la production de miel' Int. J. Inn. App St. 2017, 20, 52-78.

Chapter 2

Moroccan propolis: a natural antioxidant, anti-bacterial and anti-biofilm against *Staphylococcus aureus* with no induction of resistance after continuous exposure



Moroccan propolis: a natural antioxidant, anti-bacterial and anti-biofilm against *Staphylococcus aureus* with no induction of resistance after continuous exposure*♠

Abstract

This study was performed to evaluate the total phenols, flavonoids, and antioxidant activities of twenty-four propolis samples from different regions of Morocco. In addition, two samples were screened regarding the antibacterial effect against four *Staphylococcus aureus* strains. Gas chromatography coupled to mass spectra (GC-MS) analysis was done for propolis samples used in antibacterial tests. The minimum inhibitory and minimum bactericidal concentration (MIC, MBC) were determined. The potential to acquire the resistance after sequential exposure of bacterial strains and the impact of adaptation to propolis on virulence using the *Galleria mellonella* were evaluated. Additionally, the effects of propolis extract on the bacterial adherence ability and its ability to inhibit the quorum sensing activity were also examined. Among the twenty-four extracts studied, the samples from Sefrou, Outat el Haj, and the two samples marketed in Morocco were the best for scavenging DPPH, ABTS, NO, peroxyl, and superoxide radicals as well as in scavenging of hydrogen peroxide. A strong correlation was found between the amounts of phenols, flavonoids, and antioxidant activities. Propolis extract at the MIC value (0.36 mg/mL) significantly reduced ($p < 0.001$) the virulence potential of *S. Aureus* ATCC 6538 and the MRSA strains without leading to the development of resistance in the sequence of continuous exposure. It was able to impair the bacterial biofilm formation. The results have revealed that sample 1 reduces violacein production in a concentration dependent manner, indicating inhibition of quorum sensing. This extract has as main group of secondary metabolites flavonoids (31.9%), diterpenes (21.5%), and phenolic acid esters (16.5%).

*Adapted form 'Moroccan propolis: a natural antioxidant, anti-bacterial and anti-biofilm against *Staphylococcus aureus* with no induction of resistance after continuous exposure'. Soukaïna El-Guendouz, Smail Aazza, Badiaa Lyoussi, Vassya Bankova, Milena Popova, Luís Neto, Maria Leonor Faleiro, and Maria da Graça Miguel. Evidence-Based Complementary and Alternative Medicine. Volume 2018, Article ID 9759240, 19 pages.

♠ Detailed information's about the chemical composition of propolis samples used for microbiological assays are displayed in appendix B.

2.1. Introduction

Reactive oxygen species (ROS) have a dual role in living beings. In adequate concentrations, they have a beneficial effect because they may constitute as defense against infectious agents, or being involved in some cellular signaling systems. Nevertheless, at higher concentrations, they damage cell structures (membranes) and biomolecules (lipids, nucleic acids, and proteins), leading to the known oxidative stress and consequently to the development of several age-dependent diseases (cancer, arteriosclerosis, arthritis, and neurodegenerative diseases, among others).[1] The above shows that the organisms produce ROS, if not adequately controlled, through antioxidant systems; they are responsible for deleterious effects in the body. Such antioxidant systems may be endogenous [vitamin A, enzyme cofactors (Q10), nitrogen compounds (uric acid), and peptides (glutathione), enzymes (glutathione peroxidase, catalase, superoxide dismutase, glutathione reductase, and glucose-6-phosphate dehydrogenase)] or supplied through the diet (exogenous).[2] Compounds present in food with potential antioxidant activity include vitamins C, E, and K, phenols (phenolic acids, flavonoids, thymol, and carvacrol, among others), and carotenoids.[2,3] Thus, antioxidants, mainly those originating from natural products, are of a high important for the human health.

Elseways, *Staphylococcus aureus* (Gram positive bacterium) is both a human commensal and pathogen. This bacterium mainly colonizes the anterior nares of the nose; nevertheless, the remaining part of the respiratory tract, as well as the gastrointestinal tract, skin, perineum, vagina, and axillae, may also be target of colonization.[4] *S. aureus* is one of the main causes of hospital-acquired infections, whenever the patients are submitted to intravenous drugs or under treatment with enteral feedings or dialysis, in postoperative surgical wounds in decubitus ulcers or in indwelling catheters, and almost always associated with a compromised defense mechanism.[5-8] In addition to the nosocomial infections, its incidence is increasing also in the community.[9] Moreover, this bacterium has a noteworthy potential to develop resistance to antibiotics. Methicillin resistant *S. aureus* (MRSA), which are resistant to β-lactam class of antibiotics (penicillins, cephalosporins, and carbapenems), are causing severe nosocomial infections.[8,10]

Equally important to mention that, bacterial pathogenic cells that adhere closely to an abiotic or biotic surface are responsible for serious infections, because such biofilm matrix and phenotypic characteristics of the bacteria confer resistance to the action of antimicrobial agents. Furthermore, such biofilm may develop either on living tissues or on inert surfaces of medical devices, leading to infections in urogenital, gastrointestinal, respiratory tracts and eyes, and wounds generally difficult to treat.[11,12]

Besides, intercellular communication within biofilms has been attributed to the quorum sensing system (QS) which is mediated by diffusible small signaling molecules.[12,13] Bacteria in biofilms are resistant to disinfectants and antibiotics leading to persistent infections, due to their higher virulence. To find compounds which are able to interfere with the bacterial QS system will regulate the virulence of bacterial pathogens, regardless of multidrug resistance.[13]

Propolis, a natural resinous substance constituted by resin (50%), wax (30%), essential oils (10%), pollen (5%), and other substances (5%), such as debris, minerals, and organic compounds, possess antimicrobial, anti-inflammatory, antioxidant, antidiabetic, spasmolytic, anaesthetic, anticancer, and immunomodulatory effects.[14]

The antioxidant activity of propolis extracts and/or their constituents has been deeply evaluated worldwide, including in the Mediterranean basin: Algeria;[15-17] Croatia;[18,19] Cyprus;[20] Egypt;[21,22] Greece;[20] Italy;[23-25] Macedonia;[26] Serbia;[27] Slovenia;[28] Spain;[29,30] and Turkey,[31,32] using several methodologies: antioxidant; reducing power; chelating activity; capacity for scavenging the DPPH, ABTS, peroxyl [oxygen radical absorbance capacity (ORAC)] and NO free radicals; capacity for scavenging the superoxide anion radical; and capacity for scavenging hydrogen peroxide. Very few works on antioxidant activity of Moroccan propolis were found.[33,34] Otherwise, the *in vitro* antimicrobial activity of propolis against *S. aureus* and MRSA strains has been reported;[15,35-44] however in Moroccan propolis such information is scarce or even absent.

The aim of the present chapter was to evaluate propolis as a natural product regarding the antioxidant activity, measured through the capacity for scavenging diverse free radicals (DPPH, ABTS, superoxide, NO, and peroxyl); capacity for reducing ion metals or chelating activity of hydro-alcoholic extracts of propolis was assessed, allowing to determine by which mechanisms they act as antioxidants, at the same time, the *in vitro* antibacterial activity of hydro-alcoholic extracts of Moroccan propolis against *S. aureus* strains, mainly MRSA clinical isolates, as well as the effect of the adaptation of the *S. aureus* strains on the susceptibility to propolis extract, and its influence on the adherence of those microorganisms to surfaces. The capacity for preventing this adherence reveals to be of particular interest because the adherent bacterial cells (biofilm bacteria) are more resistant to antibiotics than planktonic cells. In addition, the ability of propolis extract to inhibit the QS system, diminishing the virulence of bacterial pathogens, was also screened.

2.2. Experimental

2.2.1. Chemicals

Iron (II) chloride anhydrous and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox®) were purchased from Fluka Chemicals (Buchs, Switzerland). Thiobarbituric acid, ferulic acid, DNP (2,4-dinitrophenylhydrazine), quercetin, sulphuric acid (H2SO4), potassium hydroxide (KOH), ABTS (2,2'-azino bis(3thylbenzothiazoline-6-sulphonic acid), fluorescein sodium salt, sodium nitrite ASC reagent, and eryodictiol were purchased from Fluka Biochemika, Sigma-Aldrich, Steinheim, Germany. Acetic acid, potassium dihydrogen phosphate (KH_2PO4), dipotassium hydrogen phosphate (K2HPO4), and trichloroacetic acid were purchased from VWR, Leuven, Belgium. Phenazine methosulfate (PMS), nicotinamide adenine dinucleotide disodium salt hydrate (NADH), ferrozine iron reagent hydrate; AAPH [2,2'-Azobis (2-methylpropionamidine) dihydrochloride]; ethylenediaminetetraacetic acid (EDTA), and potassium persulfate were purchased from Acros organics, New Jersey, USA. Nitrotetrazolium blue chloride and trichloroacetic were purchased from Sigma Aldrich Chemie, Steinheim, Germany. Phosphate buffered saline tablets (PBS) were from Fisher Scientific, New Jersey, USA. Sodium nitroprussiate dehydrate, 2,2'-diphenyl-1-picrylhydrazyl (DPPH), and potassium hexacyanoferrate were from Riedel-de Haën, Sigma-Aldrich, Seelze, Germany. Griess reagent system was purchased from Promega Corporation, Madison, USA. Calcium chloride, Folin Ciocalteu's phenol reagent, $AlCl_3$, di-potassium hydrogen phosphate anhydrous (K_2HPO_4), and hydrogen peroxide (H_2O_2) were purchased from Panreac Quimica, Montcada i Reixac, Barcelona, Spain. Potassium dihydrogen phosphate (KH_2PO_4) and Na_2CO_3 were purchased from Riedel de Haen (Seelze, Germany, Riedel-de-Haën Laboratory Chemicals, Germany). Potassium chloride (KCl) was purchased from Analar Normapur, Geldenaaksebaan, Leuven, Belgium. Ammonium heptamolybdate tetrahydrate PA was purchased from PRONALAB, LISBOA, Portugal. The medium brain-heart infusion (BHI), Luria-Bertani (LB), and bacteriological agar type E were purchased from Biokar Diagnostics (Beauvais, France). Crystal violet was from Merck, Germany. *Chromobacterium violaceum* CV026 (HgR, cvil:Tn5 xylE, KanR and spontaneous resistance StrR) was a gift from Professor Mondher EL Jaziri, Plant Biotechnology Laboratory, Université Libre de Bruxelles, Belgium.

2.2.2. Propolis extraction

Hydro-alcoholic extracts of twenty-four propolis samples, originating from different location in Morocco (Table 2.1), were obtained, as described by El-Guendouz et al.,[45] with slight modification. One gram of each propolis sample was chopped into small pieces and

extracted by maceration using 30 mL of 70% ethanol and maintained for one week at 37°C under agitation (200 rpm). The resulting solution was filtered under vacuum. A clear solution, without further purification, was used for successive analyses. First of all, polyphenol contents and antioxidant activities were screened for all samples (twenty-four) while only two samples were used for microbiological study (one among the most active regarding the antioxidant activity and polyphenol contents and the second among the weaker samples regarding the same parameters).

2.2.3. Determination of different phenolic groups

2.2.3.1. Total phenol content

The total polyphenol content in propolis samples was determined using the method of Gülçin et al..[46] Hydro-alcoholic extracts (25 µL) were mixed with 125 µL of Folin-Ciocalteu reagent (0.2 N) and 100 µL of 7.5% Na_2CO_3, and the absorbance was measured at 760 nm after 2 h of incubation at room temperature. The total polyphenol content was expressed as mg per g of Ferulic Acid equivalents using a calibration curve.

2.2.3.2. Total flavones and flavonol content

The amounts of flavones and flavonols in extracts were determined according to the method Miguel et al.,[47] with minor modification. An amount of 100 µL of Al_2Cl_3 (20%) was added to 100 µL of extract, and after one hour at room temperature the absorbance was measured at 420 nm. Total flavones and the flavonols content were calculated as quercetin equivalents (mg per g) using a calibration curve.

2.2.3.3. Total flavanones and dihydroflavonol contents

The total quantification of flavanone and dihydroflavonol compounds was performed as described by some authors.[48] Briefly, 75 µL of sample or standard and two milliliters of DNP solution (one-gram DNP in two milliliters 96% sulfuric acid, diluted to 100 mL with methanol) were heated at 50°C for 50 min. After cooling at room temperature, the mixture was diluted to 10 mL with 10% KOH in methanol (w/v). A sample of one milliliter of the resulting solution was added to 10 mL methanol and diluted to 50 mL with methanol. Absorbance was measured at 486 nm.

2.2.4. Antioxidant activity

2.2.4.1. DPPH free radical scavenging activity

Scavenging of the DPPH radical was assayed following the method of Aazza et al.,[49] with some modification. Solutions with different extract concentrations were prepared and 25 µL of each solution was added to 150 µL of DPPH solution (63.4 µM) and 125 µL of 96% ethanol. Absorbance measurements were read at 517 nm, after 30 min of incubation time at room temperature. Reduction of the amount of the DPPH radical present was measured using a decrease in the absorption value at 517 nm. The extract concentration providing 50% inhibition (IC_{50}) was calculated using a graph of the scavenging effect percentage against the extract concentration. The scavenging effect percentage was calculated from the formula: [(A_0-A_1/A_0) x 100], where A_0 is the absorbance of a negative control (blank sample containing the same amount of solvent of extraction and DPPH solution) and A_1 is the absorbance of the sample. The percentage was plotted against the extract concentration, and IC_{50} values were determined (concentration of extract able to scavenger 50% of the DPPH free radical).

2.2.4.2. ABTS free radical-scavenging activity

Determination of the ABTS radical scavenging activity was carried out as reported by Aazza et al..[50] Briefly, the ABTS radical was generated by reaction of a 7 mM ABTS aqueous solution with $K_2S_2O_8$ (2.45 mM) in the dark for 16 h and adjusting the absorbance at 734 nm to 0.7 at room temperature. Samples (25 µL) were added to 275 µL of ABTS and the absorbance at 734 nm was read after 6 min. The capability to scavenge the $ABTS^+$ was calculated using the formula: ABTS scavenging activity (%) = [(A_0-A_1)/A_0] × 100 (%), where A_0 is the absorbance of the control (ethanol 70% instead sample) and A_1 is the absorbance in the presence of the sample. The sample concentration providing 50% inhibition (IC_{50}) was obtained by plotting the inhibition percentage against extracts concentrations.

2.2.4.3. Evaluation of total antioxidant capacity by phosphomolybdenum

The antioxidant activities of samples were evaluated by the phosphomolybdenum method as described by Prieto et al.[51] and expressed relative to that of ascorbic acid. Briefly, an aliquot of 50 µL of propolis sample was mixed with one milliliter of the reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). Ethanol 70% was used as blank instead of propolis solution. The reaction mixture was vortex-mixed and let to stand in a water bath at 95°C for 90 min. The tubes were cooled to room temperature and the absorbance

was measured at 695 nm. The experiment was conducted in triplicate and values are expressed as mg equivalents of ascorbic acid per gram of propolis.

2.2.4.4. Nitric oxide scavenging activity

The nitric oxide (NO) scavenging activity was measured according to Ho et al..[52] In this method 50 µL of different concentration of sample was added to 50 µL of 10 mM sodium nitroprusside in PBS into a 96-well plate and the plate was incubated at room temperature for 90 min. Finally, an equal volume of Griess reagent was added to each well and the absorbance was read at 532 nm. Several concentrations of samples were made and the percentage inhibition calculated from the formula $[1 - (A_{sample} - A_{sample\ blank})/(A_{control} - A_{control\ blank})]x100$, where $(A_{sample} - A_{sample\ blank})$ is the difference in the absorbance of a sample, with or without 10 mM sodium nitroprusside, and $(A_{control} - A_{control\ blank})$ is the difference in the absorbance of the PBS control, with or without 10 mM sodium nitroprusside. The sample concentration providing 50% inhibition (IC_{50}) was obtained by plotting the inhibition percentage against extracts concentrations.

2.2.4.5. Chelating metal ions

The degree of chelating of ferrous ions by the propolis samples was evaluated according to Miguel et al..[53] Briefly, 0.1 mL of different concentration of samples was incubated with 0.05 mL of $FeCl_2.4H_2O$ (2 mM). The addition of 0.2 mL of 5 mM ferrozine initiated the reaction, and after10 min, the absorbance at 562 nm was measured. An untreated sample served as the control. The percentage of chelating ability was determined according to the following formula: $[(A_0-A_1)/A_0\ x100]$, in which A_0 is the absorbance of the control and A1 is the absorbance of propolis sample. The values of IC_{50} were determined as reported above.

2.2.4.6. Scavenging ability of superoxide anion radical

Scavenging ability of superoxide anion radical was evaluated as previously reported by Aazza et al..[54] Superoxide anions were generated in a nonenzymatic PMS-NADH system by oxidation of NADH and assayed by reduction of nitroblue tetrazolium (NBT). The superoxide anion was generated in 300 µL of phosphate buffer (19 mM, pH=7.4) containing NBT (43 µM) solution, NADH (166 µM) solution, and different concentrations of propolis. The reaction was started with the addition of PMS solution (2.7 µM) to the mixture. The reaction mixture was incubated at room temperature for 10 min and the absorbance reading was performed at 560 nm in a microplate reader (Tecan Infinite M200, Tecan, Austria). The percentage of inhibition was calculated using the following equation: Inhibition = $[(A_0-A_1)/A_0]\ x100$ (%), where A_0 is

the absorbance of the control (without sample) and A_1 is the absorbance in the presence of the sample. Tests were carried out in triplicate. The sample concentration providing 50% inhibition (IC_{50}) was obtained by plotting the inhibition percentage against extracts concentrations.

2.2.4.7. Hydrogen peroxide scavenging capacity

The ability of the propolis extracts to scavenge hydrogen peroxide was determined according to the method of Ruch et al.,[55] with slight modification. A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (50 mM, pH=7.4). Fifty µL of each propolis concentration was added to a hydrogen peroxide solution (0.6 mL, 40mM), one milliliter of phosphate buffer (50 mM, pH=7.4), and 1650 µL of distilled water. Absorbance of hydrogen peroxide at 230 nm was determined 20 minutes later against a blank solution containing the phosphate buffer without hydrogen peroxide. Tests were carried out in triplicate and the values of IC_{50} were determined as reported above.

2.2.4.8. Scavenging ability of peroxyl radicals by the oxygen radical absorbance capacity (ORAC)

An ORAC assay is based on the capacity of antioxidants in a sample to quench peroxyl radicals generated from thermal decomposition of AAPH. The ORAC method used fluorescein (FL) as the fluorescent probe following the method of Ou et al..[56] An amount of 0.414 g of AAPH was dissolved in 10 mL of phosphate buffer 75 mM (pH 7.4) and kept in an ice bath. A solution of fluorescein (0.00419 mM) was prepared in phosphate buffer and kept in the dark at 4°C. A new concentration (8.16×10^{-5} mM) was prepared before reactions. A Trolox standard (0.02 M) was prepared in phosphate buffer and diluted to 50, 25, 12.5, and 6.25 µM. As the ORAC assay is extremely sensitive, the samples must be diluted (1/1,000; 1/10,000) before analysis to avoid interference. An amount of 150 µL of a fluorescein working solution, 25 µL of sample, and either a blank (Milli-Q water) or a standard were mixed on a plate, which was then covered with a lid and incubated in a preheated (37°C) microplate reader for 10 min. An amount of 25 µL of AAPH solution was then added to each well. The microplate was shaken for 10 s and then the fluorescence was read every minute for two hours at an excitation wavelength of 485 nm and an emission wavelength of 527 nm. The net area under the curve (AUC) of the standards and samples was calculated. A standard curve was obtained by plotting Trolox concentrations against the average net AUC of two measurements for each concentration. Final ORAC values were calculated using a regression equation between the Trolox concentration and the net AUC and are expressed as µmol of Trolox/g propolis.

2.2.4.9. Reducing power assay

The reducing power was determined according to the method described by Rajibul et al.,[57] with slight modifications. The extracts (50 µL) were mixed with 500 µL of 0.2M sodium phosphate buffer (pH=6) and 500 µL of 1% potassium ferricyanide (K_3CN_6). The mixture was incubated at 50°C for 20 min. After 500 µL of 10% trichloroacetic acid was added, the mixture was centrifuged for 10 min at 3000 rpm. 500 µL of the supernatant liquid was mixed with 500 µL of distilled water and 100 µL of 0.1% ferric chloride. The absorbance of the mixture was measured at 700 nm.

2.2.5. Antimicrobial activity

After the screening of polyphenol content and the antioxidant activities of all propolis samples, only two samples were chosen for microbiological test (samples 1 and 7) based on the previous test performed.

2.2.5.1. GC-MS analysis of propolis extract

GC-MS analysis was done for the two samples (1 and 7) chosen for the antimicrobial assays. The analysis was performed with a Hewlett–Packard gas chromatograph 5890 series II Plus linked to a Hewlett–Packard 5972 mass spectrometer system equipped with a 30 m long, 0.25 mm i.d., and 0.5 µm film thickness HP5-MS capillary column. The work conditions were the same as previously reported.[58] Semi-quantification was carried out by internal normalization with the area of each compound. The addition of individual areas of the compounds corresponds to 100% area. Compound identification was performed using commercial libraries and comparison of mass spectra and retention times of reference compounds.

2.2.5.2. Bacterial strains and growth conditions

The bacterial strains were cultivated in BHI. For solid media, agar was added at 1.5 %, w/v. The bacterial strains of *S. aureus* used in this study are indicated in Table 2.5. Bacteria were stored in BHI supplemented with 25 % (v/v) glycerol at -80°C. Prior to use, bacteria were transferred to fresh BHI agar plates and incubated at 37°C.

2.2.5.3. Disc diffusion method

A paper disc diffusion assay to assess the preliminary antibacterial activity of two propolis samples (most active (1) and the less active (7) samples regarding their total phenol content) was performed according to Al-Abbadi et al..[59] *S. aureus* cultures were incubated overnight

before the OD_{600} was adjusted to 0.3-0.4. Hundred microliters of this bacterial culture were then spread on BHI agar plates and left to dry at room temperature for a few minutes. Then sterile filter paper discs were placed over the plate and propolis extracts at concentration of 0.24, 0.49, 0.73, and 0.98 mg/mL were loaded onto the discs. Diluted ethanol 70% was used as a negative control and the chloramphenicol disc (30 µg/mL) as a positive control. The plates were incubated at 37°C for 24 hours. After the incubation time the inhibition zones were determined. Three biological and three technical replicates (N=9) were done.

2.2.5.4. Determination of the minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of propolis samples (1 and 7) was determined using a microdilution method according to Apolónio et al..[60] Concentrations of propolis were 0.24; 0.36; 0.49; 0.61; 0.73; 0.98; and 1.22 mg/mL. Each *S. aureus* strain was cultivated in BHI agar plates for 24 h at 37°C. A loop of isolated bacterial colonies from each plate was inoculated into 10 mL of BHI broth, and the culture was incubated overnight in a shaking water bath at 37°C, to prepare a suspension with a turbidity of 0.5 of the McFarland scale (10^8 CFU/mL). 100 µL of the overnight bacterial culture was centrifuged, and the pellet was resuspended with the same volume of medium supplemented with the appropriate propolis concentration, then 20 µL of the bacterial suspension was inoculated into each well of a flat-bottom microplate (Greiner, Labortechnik, Frickenhausen, Germany) previously filled with 180 µL of BHI supplemented with propolis at the proper concentration. Wells containing the culture medium supplemented with ethanol 70% or chloramphenicol (30 µg/mL) were included, as control. Negative control was constituted by the culture in BHI and no propolis added. Three biological and three technical replicates for each strain were used (N = 9). The growth was followed by spectrophotometry (OD) in a microplate reader (Tecan Infinite M200, Tecan, Austria) at optical density of 600 nm. The MIC value was considered as the lowest concentration of propolis that inhibits the visible bacterial growth (no increase in the OD after 24–48 h). The lowest concentration that did not allowed the recovery of cells in agar plates was considered the minimum bactericidal concentration (MBC).

2.2.5.5. Effect of the adaptation of S. aureus strains on the susceptibility to propolis extract and antibiotics

According to the disc diffusion and MIC value results the remaining microbiological tests will be done only with sample 1. Adaptation procedure (successive passage) was performed as described by Apolónio et al..[60] The tested concentrations of propolis (sample 1) were 0.12; 0.24; 0.36; 0.49; and 0.61 mg/mL. Each bacterial strain was cultivated on BHI agar plates at

37°C during 24 h. 10 mL of BHI broth was inoculated with a loop of isolated colonies from each plate, and the culture incubated overnight. 300 µL of the overnight bacterial culture was centrifuged (2,790 x g, 5 min at 4°c) and the pellet was resuspended in 300 µL BHI supplemented with 0.12 mg/mL of propolis extract and then inoculated into 10 mL of BHI with the same concentration of propolis extract. The bacterial culture was incubated for 24 h in a shaking water bath at 37°C and then was transferred sequentially at the same concentration of propolis extract for 4 days. The bacterial growth at each successive passage was followed by spectrophotometry at OD_{600} nm. Following the four days of sequential passages, bacteria were transferred to BHI with higher concentration of propolis extract. Agar disc diffusion was performed each day of the sequential passages of each concentration. The purpose was to evaluate the bacteria susceptibility to antibiotics and eventual development of antibiotic resistance (gentamicin, erythromycin, chloramphenicol, and tetracycline). These procedures were repeated until bacteria cease to grow. Culture with no propolis extract was used as control in parallel. Three independent replicates were performed.

2.2.5.6. Influence of propolis extract on adherence

The influence of propolis extract (sample 1) on adherence of *S. aureus* was performed according to Adrião et al.[61] with slight modifications. Briefly, 200 µL of an overnight culture (OD = 0.4-0.5), prepared as described above, was centrifuged and the pellet was resuspended with medium supplemented with propolis extract at the MIC value (0.36 mg/mL), chlorhexidine 0.2% (positive control), and BHI only (negative control). The prepared culture was inoculated into each well of a flat-bottom microplate and left to adhere for 30 min at room temperature in a flow cabinet. After incubation nonadherent cells and residues of media component were removed by washing the wells with sterile PBS. Wells were air dried for 15 min. The adherent cells were heat-fixed at 80°C for 30 min, stained with 0.1% crystal violet, and kept for 10 min to react. The wells were washed again before the dissolution of the stain by addition of ethanol–acetone (80:20), and the OD was determined with a microplate reader at 595 nm.

2.2.5.7. Screening of propolis extract for anti-QS properties

This assay was performed as described by Norizan et al.[62] with some modifications. *Chromobacterium violaceum* CV026 was cultured on LB agar plates at 30°C during 24 h. LB broth was inoculated from each plate, and the culture was incubated overnight in a water bath at 30°C. Eight mL of the overnight culture was adjusted to OD_{600} of 1.2 and transferred into 40 mL of warm molten LB agar, followed by the addition of N-hexanoylhomoserine lactone (C6-HSL) at final concentration of 0.12 µg/mL. The agar mixture

was mixed and poured into the Petri dish and left to solidify inside a flow cabinet for one hour. Small wells were made on the solidified agar plate with the bottom of a Pasteur pipette. Wells were filled with 40 µL of propolis extract at the concentration 0.24; 0.36; 0.49; 0.61; 0.73; 0.98; and 1.22 mg/mL. The plate was incubated for 24 hours at 30°C to check for the violacein inhibition. The positive control for the QS inhibition in this assay was (+)-catechin and ethanol 70% diluted was used as the negative control.

2.2.5.8. Influence of propolis extract on virulence

The effect of adaptation of propolis (sample 1) on virulence of *S. aureus* strains was determined using larvae of the Greater Wax Moth *Galleria mellonella* (Lepidoptera: Pyralidae) according to Schrama et al..[63] The insects weighing between 250 and 350 mg were reared during the first and second instars with pollen and wax then with an artificial diet containing a mixture of glycerol, honey, water, dry dog food, and wheat bran. It is noted that the development of the insects was carried out in the dark at 30°C. *S. aureus* strains were previously adapted to subinhibitory propolis concentration (0.24 mg/mL) for three successive passages. Each *S. aureus* culture (10^8 CFU mL^{-1}) was centrifuged at 2,790 x g for 5 min at 4°C, then the bacterial cells were resuspended in one milliliter of sterile PBS. First larvae were superficially disinfected with ethanol at 70% v/v and then were infected with 10 µL of the initial suspension (10^6 CFU) by injection on the second right proleg using a 50 µL microsyringe. Larvae were placed in sterile Petri dishes at 37°C and death checked every 24 h for 5 days. Death was considered when no response to touch was observed. Three independent experiments were carried out. PBS was injected to the insect control.

2.2.6. Statistical analysis

Data were analyzed by ANOVA using the SPSS 23.0 program (Inc., Chicago, IL, USA). Statistical significance was set as $p<0.05$; when the analysis was statistically significant, Tukey's post hoc test was done. The survival data were treated using GraphPad Prism 5 statistical software.

2.3. Results and Discussion

2.3.1. Phenols

Table 2.1 depicts the phenol content of Moroccan propolis samples. Samples 17, 23, and 1 had the highest concentrations of total phenols (265.37, 264.80, and 205.82 mg ferulic acid equivalent (FAE)/g, respectively), total flavones and flavonols (129.60, 80.97, and 53.50 mg QE/g, respectively), and total dihydroflavonols and flavanones (6.67, 15.95, and 7.69 mg EE/g,

respectively). Among these samples, propolis extract 1 had the lowest concentrations of total phenols, flavones, and flavonols, whereas propolis extract 17 had the lowest concentration of dihydroflavonols and flavanones (Table 2.1). Shortly, samples 17 and 23 had similar amounts of total phenols, but sample 17 had higher flavones and flavonols and lower concentrations of flavanones and dihydroflavonols than sample 1. The polyphenol content of these samples is superior to those described by Miguel et al..[47] In this case, 91.22 mg caffeic acid equivalent (CAE)/g was the best value found among the 14 samples of propolis collected at different places of Morocco. With the exceptions of samples 24, 21, 7, and 12 with the lowest concentrations of total polyphenols (7.06-9.53 mg FAE/g) and those with the highest concentrations of the same metabolites, the remaining samples had similar amounts of phenols to those reported for the neighbor country, Algeria, 10–47 mg gallic acid (GA)/g,[17] and superior to the majority of samples for Morocco (0.7–65.7 mg CAE/g)[47]. Lower amounts of flavonoids were also reported by some authors.[17,47] When compared to the results presented in the present work, the highest concentration of total flavonoids detected by these authors did not surpass 35 mg QE/g for Moroccan propolis or 30 mg QE/g for Algerian propolis.

Table 2.1. Phenols, flavones and dihydroflavonols contents of hydro-alcoholic extracts of Moroccan propolis harvested at different places.

Sample	Region	City	Surrounding plants	Phenols (mg Eq Ferulic Acid / g de propolis)	Flavones (mg Eq Quercetin / g de propolis)	Dihydroflavonols (mg Eq Eriodictyol / g de propolis)
1	Fez-Boulemane	Sefrou	*Olea, Pinus, Quercus, Juniperus, Rosmarinus, Cistus, Lavandula and Pistacia.*	205.82±0.47^{b}	53.50±0.36^{c}	7.69±0.04^{b}
2	Gharb-Chrarda-Beni-Hsen	Moulay.Bouselhame	*Vaccinium*	16.92±0.47^{l}	2.90±0.36^{jkl}	1.41±0.04^{m}
3*	Gharb-Ghrada-Beni-Hsen Meknes-Tafilalet Tadla-Azilal	Sidi Slimane Khenichat Khenifra Beni mellal	*Citrus ,Zizifus , Silybum , and Ceratonia*	33.01±0.47^{i}	10.57±0.36^{g}	2.75±0.04^{h}
4	Meknes-Tafilalet	Khenifra	*Ceratonia*	13.07±0.47^{m}	2.63±0.36^{kl}	1.48±0.04^{m}
5	Meknes-Tafilalet	Khenifra	*Ceratonia*	21.95±0.47^{k}	4.29±0.36^{ij}	2.08±0.04^{k}
6	Doukkala-Abda	Oualidia	*Eucalyptus*	14.85±0.47^{m}	3.21±0.36^{jk}	1.80±0.04^{l}
7	Souss-Massa-Draa	Zagora	*Thymus, Euphorbia and citrus*	7.80±0.47^{no}	0.85±0.36^{mno}	0.92±0.04^{o}
8	Rabat-Sale-Zemmour-Zaer	Rabat	*Eucalyptus, Quercus and Juniperus*	57.62±0.47^{f}	12.05±0.36^{f}	2.51±0.04^{i}
9	Gharb-Ghrada-Beni-Hsen	Moulay .Bouselham	*Vaccinium*	13.30±0.47^{m}	1.72±0.36^{lmn}	1.71±0.04^{l}
10	Marketed in Morocco	Marketed in Morocco	Marketed in Morocco	144.07±0.47^{c}	23.44±0.36^{d}	5.03±0.04^{e}
11	Taza-Al Hoceima-Taounate	Taza	*Olea, Pinus, Quercus, Juniperus, Rosmarinus, Cistus, Lavandula and Pistacia*	20.97±0.47^{k}	2.01±0.36klm	1.80±0.04^{l}
12	Unknown	Unknown	Unknown	9.53±0.47^{n}	0.16±0.36^{o}	1.13±0.04^{n}
13	Gharb-Chrada-Beni-Hsen	Zeggouta	*Olea, Ceratonia, Chamaerops, Euphorbia, Juniperus, Quercus, Pinus, Pistacia, Thuya, and Eucalyptus*	39.20±0.47^{h}	5.59±0.36^{i}	2.14±0.04^{jk}
14	Gharb-Chrada-Beni-Hsen	Sidi Slimane	*Eucalyptus*	63.33±0.47^{e}	12.26±0.36^{f}	4.17±0.04^{f}
15	Rabat-Sale-Zemmour-Zaer	Temara	*Olea, Ceratonia, Chamaerops, Euphorbia, Juniperus, Quercus, Pinus, Pistacia, Thuya, and Eucalyptus*	57.86±0.47^{f}	9.93±0.36^{gh}	5.27±0.04^{d}
16	Gharb-Chrada-Beni-Hsen	Kenitra	*Olea, Ceratonia, Chamaerops, Euphorbia, Juniperus, Quercus, Pinus, Pistacia, Thuya, and Eucalyptus*	53.61±0.47^{g}	8.92±0.36^{h}	4.26±0.04^{f}
17	Fez Boulemane	Outat el Haj	*Olea, Pinus, Quercus, Juniperus, Rosmarinus, Cistus, Lavandula and Pistacia*	265.37±0.47^{a}	129.60±0.36^{a}	6.67±0.04^{c}
18	Grand-Casablanca	Casablanca	*Thymus -euphorbia – Citrus*	24.04±0.47^{j}	4.09±0.36^{j}	1.74±0.04^{l}
19	Rabat-Sale-Zemmour-Zaer	Tifelt	*Ricinus- chardon- Vaccinium -eucalyptus - Marrubium and Mentha*	14.30±0.47^{m}	2.05±0.36^{klm}	2.21±0.04^{jk}
20	Fez -Boulmane	Sefrou	*Bupleurum and Thymus*	72.33±0.47^{d}	18.12±0.36^{e}	3.26±0.04^{g}
21	Guelmim-Es Semara	Tantan	*Arganier, Acacia, and Euphorbia*	7.83±0.47^{no}	0.61±0.36^{mn}	2.29±0.04^{j}
22	Fez -Boulmane	Moulay Acoub	*Olea, Pinus, Quercus, Juniperus, Rosmarinus, Cistus, Lavandula and Pistacia*	56.75±0.47^{f}	12.66±0.36^{f}	4.14±0.04^{f}
23	Marketed in Morocco	Marketed in Morocco	Marketed in Morocco	264.80±0.47^{a}	80.97±0.36^{b}	15.95±0.04^{a}
24	Souss-Massa-Draa	Tiznit	*Citrus, Arganier, Acacia, and Euphorbia*	7.06±0.47^{o}	1.17±0.36^{mno}	1.74±0.04^{l}

Values in the same column followed by the same letter are not significant different ($p < 0.05$) by the Tukey's multiple range test. *The beehives were displaced between the regions reported above, according to the information of the beekeeper.

2.3.2. Antioxidant Activity

The antioxidant activity was checked through diverse methods: capacity for scavenging diverse free radicals (DPPH, ABTS, superoxide, NO, peroxyl), or chelating activity. The results are presented in Table 2.2. The capacity for reducing ion metals is depicted in Figure 2.1.

The results will be only compared with those from Mediterranean origin found in articles listed in the ISI Web of Knowledge.[15-17, 27, 28, 31, 34, 47, 50, 64-70] Among all propolis extracts, sample 1 had the best total antioxidant activity (48.95 meq Ascorbic Acid Equivalent /g) (Table 2.2), higher than those already reported for Moroccan propolis.[34] This sample, along with samples 10, 17, and 23, presented the lowest IC_{50} values for the assays that measure the ability for scavenging ABTS and DPPH free radicals, that is, those that showed the best capacity for scavenging such free radicals. Samples 13-16 also had significant higher capacity for scavenging the ABTS free radicals (Table 2.2).

Table 2.2. Antioxidant activity of hydro-alcoholic extracts of Moroccan propolis harvested at different places, measured through distinct methods, and expressed in IC_{50} (mg/mL), and Trolox equivalent (TE) for ORAC assay and mg Equivalent ascorbic acid/g sample (mg Eq A.Asc/g). (Mean ± standard error).

	DPPH (mg/mL)	ABTS (mg/mL)	Molybdate (mg Eq A.Asc/g)	NO (mg/mL)	Chelating (mg/mL)	Superoxide (mg/mL)	H_2O_2 (mg/mL)	ORAC (μmol TE/g)
1	0.022±0.044^{a}	0.014±0.123^{a}	48.92±0.04^{m}	0.310±0.195^{abc}	2.429±0.291^{fgh}	0.810 ±0.233^{abc}	0.086±0.021^{a}	1357.150±33.793^{k}
2	0.605±0.044^{fg}	0.310±0.123^{ab}	29.57±0.0^{g}	5.194±0.195^{h}	1.157±0.291^{abcde}	3.413 ±0.233^{f}	ND	681.336±33.793^{ab}
3	0.772±0.044^{g}	0.215±0.123^{ab}	32.57±0.04^{h}	2.360±0.195^{e}	1.151±0.291^{abcd}	5.359 ±0.233^{gh}	0.373±0.021^{fg}	821.727±33.793^{cdefg}
4	0.400±0.044^{de}	0.182±0.123^{ab}	24.69±0.04^{d}	5.230±0.195^{h}	1.583±0.291^{cdef}	5.466 ±0.233^{gh}	0.778±0.021^{gh}	691.713±33.793^{abc}
5	0.541±0.044^{ef}	0.189±0.123^{ab}	28.16±0.04^{f}	3.758±0.195^{g}	1.865±0.291^{defg}	4.807 ±0.233^{g}	0.608±0.021^{gh}	720.261±33.793^{abcd}
6	0.343±0.044^{d}	0.388±0.123^{ab}	16.73±0.04^{b}	3.615±0.195^{fg}	2.288±0.291^{efgh}	ND	0.479±0.021^{h}	698.592±33.793^{abc}
7	1.935 ±0.044^{i}	2.085±0.123^{d}	9.40±0.04^{a}	15.072±0.195^{l}	1.325±0.291^{abcdef}	ND	ND	652.953±33.793^{a}
8	0.356±0.044^{d}	0.321±0.123^{ab}	20.48±0.04^{c}	1.269±0.195^{d}	2.879±0.291^{gh}	1.139 ±0.233^{bcd}	ND	801.962±33.793^{bcdefg}
9	0.290±0.044^{d}	0.144±0.123^{ab}	33.57±0.04^{h}	3.056±0.195^{efg}	1.563±0.291^{bcdef}	1.684 ±0.233^{cde}	0.436±0.021^{gh}	848.374±33.793^{defgh}
10	0.024±0.044^{a}	0.014±0.123^{a}	39.39±0.04^{h}	0.372±0.195^{abc}	0.606±0.291^{abc}	0.253 ±0.233^{a}	0.168±0.021^{bc}	1072.133±33.793^{j}
11	0.386±0.044^{de}	0.260±0.123^{ab}	26.22±0.04^{e}	3.429±0.195^{fg}	1.204±0.291^{abcde}	1.919±0.233^{de}	0.928±0.021^{gh}	954.108±33.793^{hij}
12	1.160±0.044^{h}	0.595±0.123^{b}	21.34±0.04^{c}	12.087±0.195^{j}	1.074±0.291^{abcd}	ND	ND	707.142±33.793^{abc}
13	0.237±0.044^{bcd}	0.058±0.123^{a}	33.64±0.04^{h}	2.425±0.195^{e}	0.251±0.291^{a}	0.723 ±0.233^{ab}	0.463±0.021^{h}	875.555±33.793^{fgh}
14	0.101±0.044^{abc}	0.057±0.123^{a}	35.01±0.04^{i}	0.795±0.195^{bcd}	2.790±0.291^{gh}	0.506 ±0.233^{ab}	0.214±0.021^{cd}	881.777±33.793^{ghi}
15	0.107±0.044^{abc}	0.017±0.123^{a}	33.54±0.04^{h}	1.344±0.195^{d}	0.716±0.291^{abc}	0.571 ±0.233^{ab}	0.124±0.021^{ab}	926.763±33.793^{ghi}
16	0.101±0.044^{abc}	0.044±0.123^{a}	35.32±0.04^{i}	0.822±0.195^{bcd}	1.548±0.291^{bcdef}	0.459 ±0.233^{ab}	0.254±0.021^{de}	918.095±33.793^{ghi}
17	0.013±0.044^{a}	0.009±0.123^{a}	37.80±0.04^{j}	0.025±0.195^{a}	6.037±0.291^{i}	0.323 ±0.233^{ab}	ND	1723.289±33.793^{l}
18	0.687±0.044^{fg}	0.557±0.123^{b}	20.73±0.04^{c}	2.985±0.195^{ef}	0.441±0.291^{ab}	5.863 ±0.233^{gh}	1.856±0.021^{gh}	750.594±33.793^{abcdef}
19	0.638±0.044^{fg}	0.279±0.123^{ab}	40.20±0.04^{kl}	6.162±0.195^{i}	0.963±0.291^{abcd}	2.497 ±0.233^{e}	0.473±0.021^{h}	736.201±33.793^{abcde}
20	0.260±0.044^{cd}	0.259±0.123^{ab}	27.58±0.04^{f}	1.053±0.195^{cd}	7.473±0.291^{j}	4.880 ±0.233^{g}	0.315±0.021^{ef}	1005.958±33.793^{ij}
21	3.110±0.044^{j}	2.768±0.123^{e}	40.89±0.04^{l}	13.788±0.195^{k}	3.054±0.291^{h}	ND	ND	630.392±33.793^{a}
22	0.077±0.044^{ab}	0.049±0.123^{a}	40.27±0.04^{kl}	0.742±0.195^{abcd}	10.495±0.291^{k}	1.137 ±0.233^{bcd}	0.290±0.021d^{e}	851.107±33.793^{efgh}
23	0.007±0.044^{a}	0.002±0.123^{a}	39.71±0.04^{kl}	0.194±0.195^{ab}	5.464±0.291^{i}	0.213 ±0.233^{a}	0.048±0.021^{a}	1616.654±33.793^{l}
24	1.793±0.044^{i}	1.265±0.123^{c}	23.43±0.04^{d}	12.290±0.195^{j}	0.881±0.291^{abcd}	ND	ND	647.116±33.793^{a}

Values in the same column followed by the same letter are not significant different ($p<0.05$) by the Tukey's multiple range test. ND: not detected at the concentrations tested

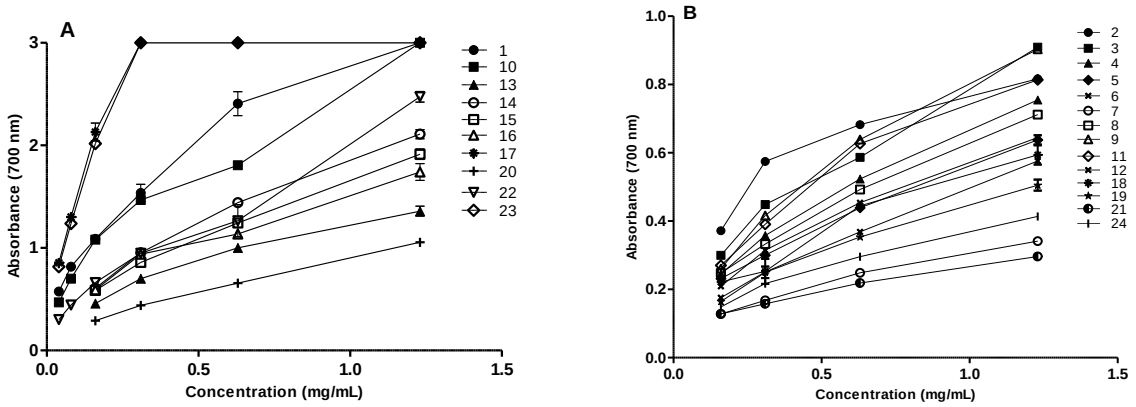


Figure 2.1. The reducing power of the hydro-alcoholic extracts of propolis from different areas of Morocco. (a) Group of samples with the best reducing power; (b) group of samples with the lower reducing power.

In the DPPH and ABTS assays, the lowest IC_{50} values (0.007–0.024 mg/mL and 0.002–0.044 mg/mL, respectively) which correspond to the best activities are within the range found for propolis samples from Morocco[47]. For the DPPH method, Algerian propolis extracts had lower activity (IC_{50} values ranging from 32.3 to 600 μg/mL).[15] In the same work, these authors reported that the purified compounds had higher activity than the extracts, being kaempferol and phenetyl-(E)-caffeate those possessing the best activities, with IC_{50} of 4.2 and 9.9 μg/mL, respectively. The same authors found a direct correlation between the amount of phenols, including flavonoids, and the capacity for scavenging the free radicals. For scavenging about 60% of DPPH free radicals,[17] reported that it was necessary at least 100 μg/mL of ethanolic extract of propolis from Algeria. Like Piccinelli et al.,[15] Benhanifia et al.[17] also found a remarkable correlation between phenol content and antioxidant activity, measured through the capacity for scavenging DPPH free radicals, but absent in the capacity for reducing ferric ions. The authors[16] found as IC_{50} value, 25 μg/mL, for an ethanolic extract of propolis from Algeria, closer to those reported in the present work.

When the antioxidant activity was evaluated through the capacity for scavenging ABTS free radicals, beyond those samples with the highest activity for scavenging DPPH free radicals (1, 10, 17, and 23), other ones were equally good scavengers of ABTS, such as 13-16 (Table 2.2). These samples did not possess as high amounts of phenols or flavonoids as the remaining samples. These results reveal the importance of some compounds and/or their functional groups present in the extracts on the activity and that show behavior somehow different in the presence of DPPH or ABTS free radicals.[64, 65]

The capacity for scavenging DPPH and ABTS, cheap and simple methods, can give information about the antiradical activity of samples; nevertheless, they are not natural radicals,

whereby the evaluation of the capacity of samples for scavenging ROS produced by the organisms is with a high interest. The most important reactive molecules include superoxide and hydrogen peroxide. Samples 10 (IC_{50} = 0.253 mg/mL) and 23 (IC_{50} = 0.213 mg/mL) had the best capacity for scavenging superoxide radical anions, poorer when compared to the best IC_{50} values found for samples from Morocco (IC_{50} = 0.15 mg/mL), previously reported,[34] and markedly poorer to those reported by Gülçin et al.[31] for propolis samples from Turkey, with IC_{50} = 9.89 μg/mL. Mavri et al.[28] also evaluated the capacity of Slovenian propolis for scavenging superoxide anion radicals, showing that 0.02 mg/mL of propolis phenols present in the extracts were able to scavenge between 6.8 and 21% of superoxide anion radicals, depending on these percentages of the ethanol: water ratio of the extraction solvent.

The capacity for scavenging hydrogen peroxide was significantly higher for samples 1 (IC_{50} = 0.086 mg/mL) and 23 (IC_{50} = 0.048 mg/mL), being such values about tenfold higher than those reported by[31] for Turkish samples (6.54 μg/mL), and therefore with poorer ability for scavenging H_2O_2. Potkonjak et al.[27] determined the antioxidant activity of commercial propolis extracts from Serbia through direct current polarography, expressed in H_2O_2 equivalent (the volume of H_2O_2 that corresponds to 1.0 mmol/L H_2O_2 decrease). The values found by the authors ranged from 1.71 to 8.00 μL. Once the unities are different, it is not possible to compare the results obtained by[27] with the values obtained in the present work. The capacity of samples 1, 10, and 23 for scavenging superoxide and hydrogen peroxide not only prevents the generation of more reactive species, such as hydroxyl radicals, but also avoids the interaction between ROS and biomolecules.[66]

The best capacity for scavenging peroxyl free radicals, measured through the method called ORAC, that measures the capacity for scavenging peroxyl radicals, was detected for samples 17 (1723.289 μmol TE/g) and 23 (1616.654 μmol TE/g), inferior to the maximal observed for Moroccan propolis.[47] The best antiradical capacity of those samples (17 and 23) is revealed to be important since they may prevent the propagation of lipid peroxidation caused by the peroxyl radicals.[67]

Only sample 17 was significantly better as NO scavenger (IC_{50} = 0.025 mg/ml) when compared to the remaining samples, whilst sample 13 presented the best chelating activity (IC_{50} = 0.251 mg/ml) (Table 2.2). The capacity for scavenging NO radicals was better than those found by[34] for Moroccan propolis (IC_{50} = 0.08-2.90 mg/mL). The best chelating activity found in the present work was inferior to that previously reported (IC_{50} = 0.13 mg/mL) also for Moroccan propolis[34] and significantly inferior to those found for Turkish samples (0.012 mg/mL),[31] but better when compared to the Slovenian propolis (2 mg/mL).[28] The capacity of

sample 17 for nitric oxide quenching can avoid the generation of peroxynitrite, in the presence of superoxide anion radicals, that irreversibly binds to proteins impairing cellular function.[68]

The presence of iron ions and hydrogen peroxide may trigger the formation of the most reactive hydroxyl radicals, through the Fenton reaction; therefore, the absence of these ions as well as other transition metal ions, such as copper, is important. Sample 13 is revealed to be the most active in what concerns the capacity for chelating iron ions.[68]

Two main groups may be considered in terms of capacity for reducing Fe^{3+} to Fe^{2+} by propolis extracts (Figures 2.1(a) and 2.1(b)). Samples 1, 10, 13, 14, 15, 16, 17, 20, 22, and 23 are in group one (Figure 2.1(a)), being those with the best reducing capacity (for the higher concentration used, the absorbance was ≥ 1, at λ=700 nm); and the remaining samples belong to group two, that is, those with significant lower reducing activities (Figure 2.1(b)). It is noteworthy to refer that with the exception of samples 20 and 22, which were introduced in the first group, the remaining samples belonging to the same group are also those with the best capacity for scavenging ABTS free radicals. Such may partly be explained by the mechanism involved in the reaction between ABTS free radicals and compounds, which is predominantly based in single electron transfer mechanism, that is, the same mechanism that occurred during the reduction of Fe^{3+} to Fe^{2+}. At least two factors may have contributed to the lower number of samples (1, 10, 17, and 23) with relative high capacity for scavenging DPPH free radicals when compared to the number of samples with significant higher capacity for scavenging ABTS free radicals: (a) the mechanisms involved in the reaction between DPPH and compounds are a mix of single electron transfer and hydrogen atom transfer mechanisms; and (b) the reaction between extracts, constituted by several compounds, and DPPH is less than the total reactivity of individual compounds.[65]

A negative correlation between IC_{50} values (DPPH, ABTS, NO, and superoxide) and phenol content ($p<0.01$) was found, which means higher activity with higher concentrations of phenols. The same correlation was observed between the same values and the amounts of flavonoids (flavanone, di-hydroflavonols, flavones, and flavonols) (Table 2.3). A positive correlation was observed between the values for ORAC test and the concentrations of phenols, independent on the type, meaning an increase of the capacity for scavenging free peroxyl radicals and phenols. The same was observed between the amounts of phenols and the total antioxidant activity determined by the molybdate method, although there is higher correlation between the values and the amounts of flavanones and dihydroflavonols (Table 2.3). A positive correlation between IC_{50} values and chelating activity was found. In this case, it seems that phenols, independent on the type, have a negative effect on the chelating activity, impairing it.

The chelating efficiency of phenols on Fe^{2+} depends on the number of hydroxyl groups on the benzene ring as well as on the hydroxyl substitution in the ortho position.[69]An absence of correlation between chelating activity and phenol amounts was already reported by Aazza et al.[50] for commercial honeys for Morocco. Although the presence of high levels of polyphenol in cocoa extracts, Andújar et al.[69] also found an absence of ability of samples for chelating Fe^{2+} ions. The results found in the present work suggest that the observed chelating activity can be assigned to compounds other than phenols.

Table 2.3. Spearman correlation coefficients among total phenols, and flavonoids and antioxidant activities

	Total phenol	**Flavone+Flavonol**	**Flavanone+Dihydroflavonol**
Total phenol	1	0.965**	0.857**
Flavone+Flavonol	0.965**	1	0.849**
Flavanone+Dihydroflavonol	0.857**	0.849**	1
Molybdate	0.458*	0.440*	0.705**
DPPH	-0.855**	-0.828**	-0.773**
ABTS	-0.691**	-0.634**	-0.645**
Chelating assay	0.359*	0.415*	0.394*
Nitric oxide	-0.951**	-0.941**	-0.851**
Hydrogen peroxide	0.735**	-0.807**	-0.920**
ORAC	0.868**	0.791**	0.778**
Superoxide scavenging	-0.675**	-0.567**	-0.737**

* Correlation is significant at the $p < 0.05$ level. ** Correlation is significant at the $p < 0.01$ level.

2.3.3. Antimicrobial properties

2.3.3.1. Anti-staphylococcal activity of propolis

The results of the antibacterial activity of propolis extracts against *S. aureus* strains are indicated in Table 2.4. Our results showed a visible inhibition zone, particularly sample 1, at all concentrations tested against the four strains. The highest inhibition zone (27.00 ± 1.52 mm; 25.67 ± 0.57 mm; 31.67 ± 1.73 mm and 32.33 ± 1.51 mm) was recorded from sample 1 at 0.98 mg/mL, higher than chloramphenicol (26.00 ± 2.51 mm; 19.00 ± 0.57mm; 24.33 ± 0.01mm and 24.67 ± 2.29mm) for MRSA strains, while sample 7 showed a lower inhibition zone (14.33±0.57 mm; 16.33±4.93 mm; 16.00±1.01 mm; and 17.67±2.88 mm) at the same concentrations. At concentration of 0.24 mg/mL and 0.49 mg/mL, the inhibition zones were very small <20mm for sample 1 and <10 mm for sample 7 (data not shown). Ethanol (negative control) did not show antibacterial activity, as expected. The diameters of inhibition zones were increased with respect to the volume of the extracts.

Table 2.4. Susceptibility of *S. aureus* strains to propolis extracts using disc diffusion method. Minimum inhibitory (MIC) and minimum bactericidal concentrations MBC) of propolis extracts.

Strains	Origin	Source	0.73 mg/mL	0.98 mg/mL	Chloramphenicol	Ethanol	MIC (mg/mL)	MBC (mg/mL)
Sample 1								
S. aureus ATCC 6538	Wound	American Type Culture Collection	22.33±0.57	27.00±1.52	26.00±2.51	--	0.36	0.98
MRSA2	Clinical	UAlg, CBMR. Portugal	19.67±1.15	25.67±0.57	19.00±0.57	--	0.36	1.22
MRSA15	Clinical	UAlg, CBMR, Portugal	26.67±1.51	31.67±1.73	24.33±0.01	--	0.36	1.22
MRSA16	Clinical	UAlg, CBMR, Portugal	25.67±0.05	32.33±1.51	24.67±2.29	--	0.36	1.22
Sample 7								
S. aureus ATCC 6538	Wound	American Type Culture Collection	12.33±2.30	14.33±0.57	25.00±3.08	--	ND	ND
MRSA2	Clinical	UAlg, CBMR. Portugal	14.00±4.35	16.33±4.93	22.67±2.04	--	ND	ND
MRSA15	Clinical	UAlg, CBMR, Portugal	15.00±1.01	16.00±1.01	25.67±1.52	--	ND	ND
MRSA16	Clinical	UAlg, CBMR, Portugal	15.67±0.57	17.67±2.88	21.33±1.52	--	ND	ND

--: No effect; ND: not determined

The best activity found for sample 1 was confirmed through the values of MIC and MBC found. Propolis sample 1 showed a MIC value of 0.36 mg/mL and the MBC ranged from 0.98 to 1.22 mg/mL for *S. aureus* tested strains. No differences were observed between the MIC values of sample 1 (0.36 mg/mL) for all strains tested. However, the MBC values determined for *S. aureus* MRSA2, MRSA15, and MRSA16 were 1.22 mg/mL, except for *S. aureus* ATCC 6538, which was 0.98 mg/mL. In contrast, the susceptibility of *S. aureus* strains to sample 7 was not proved. The concentrations tested did not affect the bacterial growth.

The differences found in the activities between samples 1 and 7 may result for their distinct chemical composition. In Figure 2.2, it is possible to see that sample 7 was predominantly constituted by sugars and their derivatives (68.5%), whereas sample 1 had as main group of secondary metabolites, the flavonoids, diterpenes, and phenolic acid esters.[45] The high percentage of sugars is mainly constituted by monosaccharide.

Sample 7 had also triterpenes, particularly sterols represented particularly by lanosterol lupeol (3.5%), (3-β-OH) (2.9%), lanosterol (3-α-OH) (1.2%), and α-amyrin (0.4%). The diterpenes were the third abundant group in the propolis component and mainly represented by imbricataloic acid, totarol, and dehydroabietic acid (1.3%, 1.2%, and 0.3%, respectively) (Appendix B). This propolis sample had also fatty acids [hexadecanoic acid (2.8%), octadecanoic acid (1.9%), octadecenoic acid (0.5%), and tetracosanoic acid (0.8%)] and aromatic acids [benzoic acid (1.2%)].

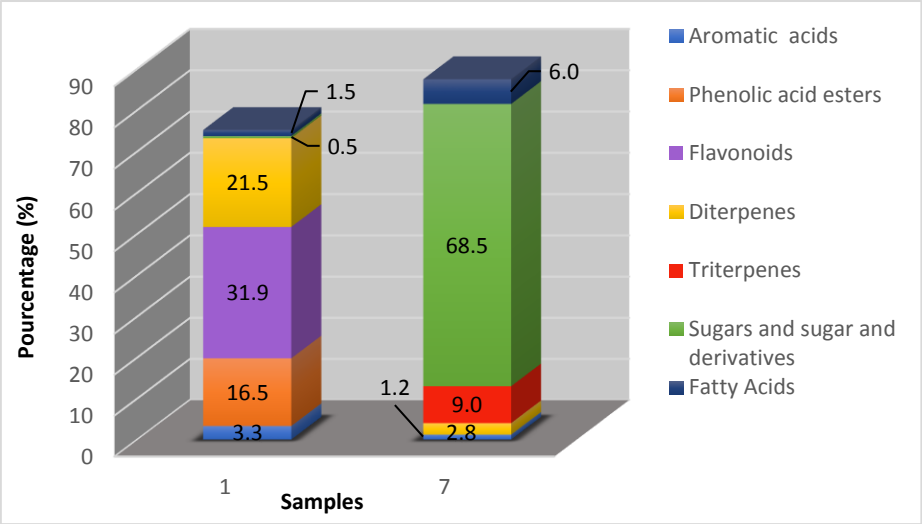


Figure 2.2. Group of compounds present in propolis samples 1 and 7.

The values of MIC found for sample 1 are higher than those reported for ethanolic extracts of propolis from Taiwan and Iran where MIC values ranged from <3.75 to 60 µg/mL and 205 µg/mL, respectively[70,71] and lower than that from Canada (MIC = 2.74 mg/mL) for *S. aureus* strains.[72] The antimicrobial activity of propolis extracts has been attributed to a synergism effect of phenol compounds, including caffeic acid and caffeic acid phenetyl ester, and flavonoids.[40] However, in sample 1, diterpenes predominate, mainly isocupressic acid.[58] The results obtained may, therefore, be attributed to diterpenes along with phenols, by either synergism or antagonism. The antistaphylococcal activity of Mediterranean propolis, from Malta, was better than Bulgarian poplar propolis.[73] The authors, also, attributed this better activity to the possible presence of relative high amounts of diterpenes in Maltese propolis.

2.3.3.2. Adaptation to subinhibitory concentrations of propolis extract on the induction of resistance to propolis extract or antibiotics

In order to evaluate the impact of sequential exposure of *S. aureus* strains to propolis on developing resistance against this natural product, the effect of the adaptation of *the S. aureus* strains on the susceptibility to propolis extract was investigated. *S. aureus* ATCC 6538 was able to overcome just four passages at the MIC value (0.36 mg/mL) of propolis extract after the sequential passages at 0.12, 0.24, and 0.36 mg/mL, but failed to overcome the following lethal concentration of 0.49 mg/mL, after the first passage (Table 2.5). Adapted cells of MRSA15 and MRSA16 strains were able to overcome just four passages of the concentration 0.49 mg/mL after the same sequential passages. The MRSA 2 cells were able to overcome the four passages at 0.49 and 0.73 mg/mL. According to the EUCAST breakpoint tables (2016), the four *S. aureus* strains showed a susceptible profile to chloramphenicol, gentamicin,

tetracycline, and erythromycin after the adaptation with different concentration of propolis (data not shown).

Table 2.5. Impact of continued exposure of *Staphylococcus aureus* strains to increasing concentrations of propolis*

Passages	Concentration of propolis sample (mg/mL)				
	0.12	**0.24**	**0.36**	**0.49**	**0.73**
***Staphylococcus aureus* ATCC 6538**					
1°	72.44±21.87[a]	42.81±1.41[a]	40.24±9.81[a]	14.22±0.43	NA
2°	36±10.72[a]	50.88±7.26[a]	61.33±10.00[a]	NG	
3°	87.17±61.12[a]	100.52±47.22[a]	57.33±15.19[a]		
4°	24.71±6.5[a]	103.68±16.5[a]	74.35±18.23[a]		
***Staphylococcus aureus* methicillin-resistant 2 (MRSA 2)**					
1°	114.09±35.26[a]	131.95±24.28[a]	5.33±0.91[a]	26.35±3.37[a]	35.92±18.65[a]
2°	139.7±45.72[a]	99.90±4.81[a]	48.49±15.95[b]	39.26±3.66[a]	8.98±3.02[a]
3°	75.38±13.02[a]	86.15±16.15[a]	55.75±24.95[b]	46.59±4.55[a]	31.46±26.68[a]
4°	105.17±21.68[a]	119.59±28.23[a]	33.20±3.82[ab]	83.55±20.67[b]	0.73±0.45[a]
***Staphylococcus aureus* methicillin-resistant 15 (MRSA 15)**					
1°	123.65±40.98[a]	125.40±15.72[b]	30.74±0.48[a]	32.74±27.93[a]	NA
2°	100.45±58.78[a]	124.64±9.27[b]	39.02±84.66[a]	27.13±7.29[a]	
3°	110.22±48.25[a]	123.99±36.06[b]	33.38±9.14[a]	18.38±15.47[a]	
4°	62.66±2.06[a]	65.66±11.69[a]	42.03±12.93[a]	0.59±0.40[a]	
***Staphylococcus aureus* methicillin-resistant 16 (MRSA 16)**					
1°	129.79±14.65[b]	43.57±6.84[a]	46.80±21.55[a]	43.22±8.18[c]	NA
2°	113.68±13.42[b]	96.39±13.47[b]	45.30±17.68[a]	22.09±10.66[bc]	
3°	57.71±7.81[a]	66.98±6.86a[b]	38.99±4.94[a]	21.60±9.03[b]	
4°	92.88±28.63[ab]	57.50±15.60[a]	83.80±29.52[a]	0.28±0.25[a]	

*Fold change in growth at each passage is indicated as the ratio between the OD at T_{24} and the OD at T_0 (ODT_{24}/ODT_0), **NG**–No Growth. **NA**–Not Applied. Data are representative of three independent replicates. For each strain, data in the column with different letters are significantly different ($p < 0.05$).

Beyond the antimicrobial activity of propolis extracts against *Staphylococcus* sp. and other microorganisms, they have also been reported to potentiate the action of antibiotics.[74] Propolis is widely used as ingredient in chewing gums, toothpastes and oral sprays, shampoos, soaps, and cosmetics. The repeated utilization of these products by people leads to a continuous exposure of microorganisms, including pathogens when an infectious disease is declared, to the subinhibitory concentrations of propolis. With this vast utilization of propolis under several forms, it is very important to clarify the impact of this repeated exposure of pathogens to propolis and their constituents on the development of resistance, to either propolis or their components and antibiotics. This sort of studies has been carried out by some authors[60,75,76] but with essential oils and/or their main components and controversial results have been reported. In the present work, it is clear that the sequential exposure to sublethal doses of

propolis from Morocco did not result in the development of resistance to the propolis itself or to antibiotics (chloramphenicol and tetracycline for all strains; an erythromycin for *S. aureus* ATCC6538 and MRSA 2). With such results, care must be taken with the frequent utilization of these kinds of natural products associated with some antibiotics, in the presence of particular MRSA strains.

2.3.3.3. Influence of propolis extract on adherence

The results of the impact of propolis on the adherence ability of *S. aureus* strains are illustrated in Figure 2.3. The adherence of the cells of ATCC 6538 strain was not affected by propolis exposure in comparison to the MRSA strains whose adherence was significantly impaired. In contrast, the exposure to chlorhexidine (0.2%, w/v) not only did not inhibit the adherence of all the *S. aureus* strains, but have boosted it significantly ($p < 0.001$) in comparison with the control. This finding is particularly interesting since a decrease in the susceptibility of clinical isolates of *S. aureus* has been reported.[77, 78] The adherence inhibition of MRSA strains by propolis was strain dependent, and the adherence of MRSA15 was particularly affected ($p < 0.001$) in comparison with the control. The adherence of MRSA16 was also impaired by the propolis extract ($p < 0.01$), but the adherence inhibition of the strain MRSA2 strain was lower ($p < 0.05$).

The ability to adhere of eight *S. aureus* strains was impaired in the presence of several concentrations of ethanolic extracts of propolis from Romania.[12] The prevention of biofilm formation promoted by propolis was also found for other microorganisms being also such property dose-dependent.[12,79,80] The geographic origin of propolis is another factor responsible for the variability of inhibition of biofilm formation, that is, dependent on the chemical composition. Those authors related the activities found with the chemical amounts of several types of phenols present in propolis samples. In contrast to the results reported by these authors, in the present work the presence of propolis extract did not impair the adherence of *S. aureus* ATCC6538 cells; nevertheless, the diverse strains of MRSA cells were inhibited to form biofilm in the presence of propolis of the same origin. Diterpenes along with phenols, the main group of compounds of our extract of propolis (sample 1), may have a role in the activity found. Bacterial biofilm formation is linked to the quorum-sensing system (QS), a cell-cell communication system that modulates gene expression and works in a cell density dependent mode.[81] In *staphylococci*, the QS mechanism comprises the accessory gene regulator (agr) locus that controls the production of a small auto-inducing signal peptide (AIP);[82] how

propolis impairs biofilm formation by disrupting the QS system in *S. aureus* needs further investigation.

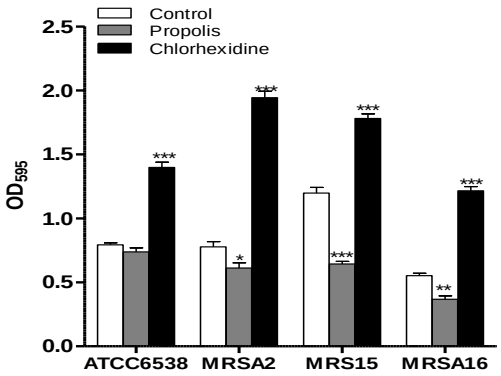


Figure 2.3. Impact of propolis extract (sample 1) at the MIC value (0.36 mg/mL) on the adherence ability of four *Staphylococcus aureus* strains. Data are the mean of three independent experiments (n = 12). Error bars represent the standard deviation. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, statistically significant when compared with the control.

2.3.3.4. Screening of propolis extract for anti-quorum sensing properties

The anti-QS activity of propolis extract was determined by assessing the violacein production by C. violaceum and the results are shown in Figures 2.4(a) and 2.4(b). Loss of the purple pigment in C. violaceum indicates the inhibition of QS by the propolis extract. Control wells containing catechin and ethanol were included. The seven concentrations 0.24; 0.36; 0.49; 0.61; 0.73; 0.98; and 1.22 mg/mL showed halo zone on the purple background. Therefore, the highest inhibition zone (15.43±1.57mm) was observed for the concentration 1.22 mg/mL followed by 10.5±1.00 mm for the concentration 0.98 mg/mL. No inhibition was apparent with ethanol (Figure 2.4(a)).

Figure 2.4(b) showed no growth inhibition zone at all tested concentration of propolis; this demonstrates that propolis extract does not affect the growth of *C. violaceum* CV026.
QS inhibitory activity was already reported for distinct propolis samples from the United States, being such activities dependent on the geographic origin of propolis.[83] The authors considered that pinocembrin present in poplar propolis was a potential active compound on the QS inhibitory activity. However, in South African propolis,[84] reported that only caffeic acid had anti-quorum sensing property, being the remaining compounds present in propolis, including pinocembrin and their derivatives, unable to inhibit the violacein production, and therefore without anti-quorum sensing activity.

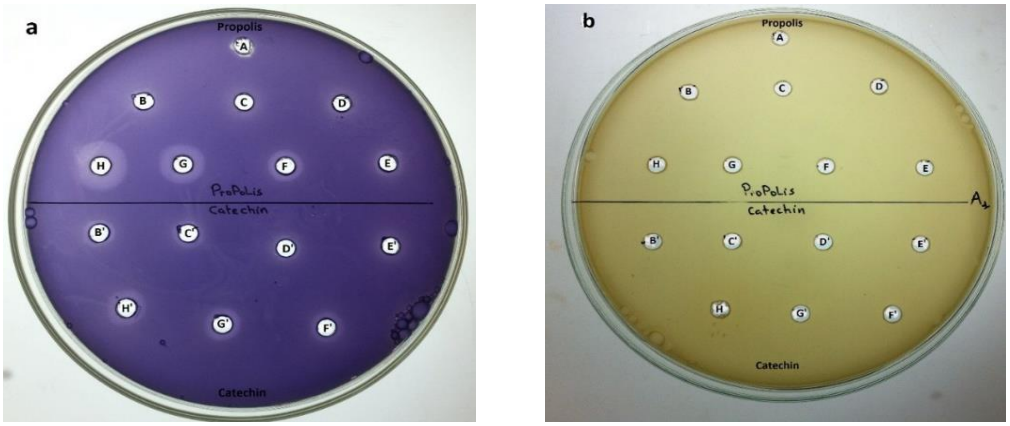


Figure 2.4. Anti-QS properties of propolis and catechine. a) N-hexanoylhomoserine lactone (C6-HSL) at 0.12 µg/mL was added to the culture medium b) No addition of C6-HSL to the culture medium was done. In each well was added (**A**): ethanol 70%; (**B**): propolis at 0.24 mg/mL; (**C**): propolis at 0.36 mg/mL; (**D**): propolis at 0.49 mg/mL; (**E**): propolis at 0.61mg/mL; (**F**): propolis at 0.73 mg/mL; (**G**): propolis at 0.98 mg/mL; (**H**): propolis at 1.22 mg/mL. (**B'**): (+)-catechin at 0.24 mg/mL; (**C'**): (+)-catechin at 0.36 mg/mL; (**D'**): (+)-catechin at 0.49 mg/mL; (**E'**): (+)-catechin at 0.61mg/mL; (**F'**): (+)-catechin at 0.73 mg/mL; (**G'**): (+)-catechin at 0.98 mg/mL; (**H'**): (+)-catechin at 1.22 mg/mL. This assay was conducted in three independent triplicates (N=9).

Other studies showed that an isoprenyl caffeate-rich fraction of manuka propolis was particularly an active QS inhibitor.[13] These authors found that 1 mg dry weight/disc or at least 0.450 mg/mL of that fraction of manuka propolis completely inhibited violacein production, concluding the authors that the presence of isoprenyl caffeate was very important on the activity detected, because the activities found by other authors[85,86] in distinct propolis samples were drastically inferior to those found by them. In the present work, isocupressic acid (8.1%), a diterpene, was the major compound of Moroccan propolis, immediately followed by pinocembrin (7.4%). Both compounds alone or along with other compounds present in the sample may have been responsible for the activity found.

2.3.3.5. Influence of propolis extract on virulence

The impact of adaptation on virulence of *S. aureus* strains was evaluated with *the G. mellonella* model. Adapted MRSA 15 and MRSA 16 strains submitted to subinhibitory propolis concentration were injected in *G. mellonella* larvae, and their survival was monitored (Figure 2.5). Differences in the ability of MRSA 15 and MRSA 16 to cause the death of the larvae were observed. It was found that larvae injected with MRSA 15 adapted cells presented 100% of survival in comparison with 43% of survival of insects injected with nonadapted cells ($p < 0.001$). MRSA15 was the less virulent strain in contrast to MRSA 16 that caused a mortality of 20% of larvae injected with nonadapted cells by the end of the experiment; also, significant differences ($p < 0.001$) in mortality of insects were observed between adapted and

nonadapted cells. Larvae injected with PBS achieved a 100% survival until the end of the experiment.

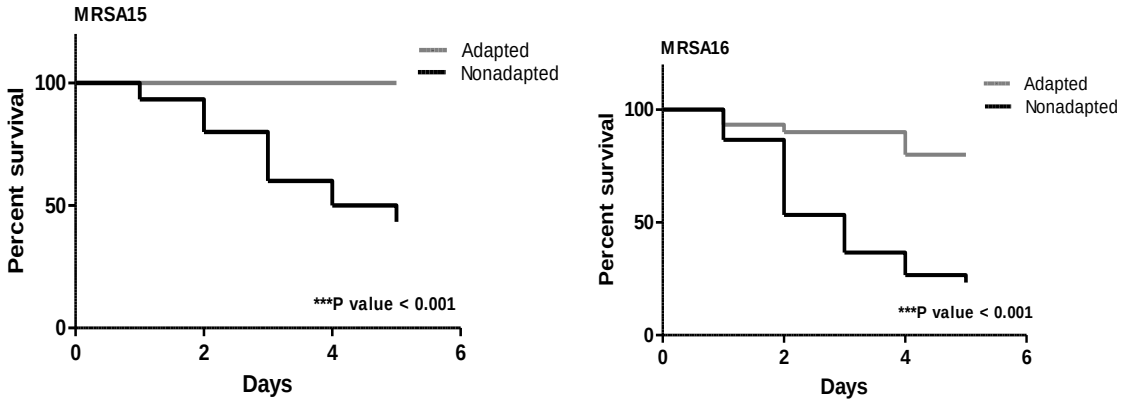


Figure 2.5. Kaplan–Meier survival curves for insects after injection with adapted cells (three passages at subinhibitory propolis concentration (0.24 mg/mL)) and non-adapted cells (three passages in BHI only, control). Test was done with sample 1. Data are the mean of three independent experiments (n = 30). Larvae injected with PBS showed a 100 % over the experiment.

The *G. mellonella* model has been used to evaluate the virulence potential of several human pathogens, including *S. aureus* and MRSA strains.[60,87] This choice to use this model was also based on the fact that propolis has practically no toxic effect on the insect,[88] although other studies had demonstrated the opposite, particularly for Chinese and Egyptian propolis extracts.[89] The results of the present work show that sequential exposure of MRSA strains to subinhibitory propolis concentration affected their virulence. Such data evidence that Moroccan propolis with diterpenes as main group of secondary metabolites has capacity for combating MRSA strains diminishing their virulence potential, inhibiting the formation of biofilm.

2.4. Conclusion

The screening of different Moroccan propolis samples regarding their antioxidant activities has revealed different antioxidant activities; these differences can be attributed to the compounds with antioxidant capacity in each propolis sample. On the other hand, we report in this work the efficiency of one of the most active propolis samples, as antibacterial agent against *Staphylococcus aureus* strains. Propolis did not induce the development of resistance to antibiotics, inhibit the biofilm formation, possess an anti-quorum sensing, and diminished the virulence of *S. aureus* for all tested strains. This finding, illustrates the diversity of propolis and presents propolis as an important source of exogenous source of compounds with antioxidant anti-bacterial capacities that can be highly involved in the prevention and treatment of several human diseases.

References

[1] M. Valko, C.J. Rhodes, J. Moncol, M. Izakovic, M. Mazur, 'Free radicals, metals and antioxidants, in oxidative stress-induced cancer', Chem-Biol. Interact. 2006, 160, 1-40.

[2] M. Carocho, I. C. F. R. Ferreira, 'A review on antioxidants, prooxidants and related controversy: Natural and synthetic compounds, screening and analysis methodologies and future perspectives', Food Chem Toxicol. 2013, 51, 15-25.

[3] M.G. Miguel, 'Antioxidant activity of medicinal and aromatic plants. A review', Flavour Frag. J. 2010, 25, 291-312.

[4] H. F. L. Wertheim, D. C. Melles, M. C. Vos, W. Van-Leeuwen, A. Van-Belkum, H. A. Verbrugh, J. L. Nouwen, 'The role of nasal carriage in *Staphylococcus aureus* infections', Lancet. Infect. Dis. *2005*, 5, 751–762.

[5] D. N. Frank, L. M. Feazel, M. T. Bessesen, C. S. Price, E. N. Janoff, N. R. Pace, 'The human nasal microbiota and *Staphylococcus aureus* carriage', PLoS. ONE. 2010.

[6] M. J. Rybak, K. L. LaPlante, 'Community-associated methicillin-resistant *Staphylococcus aureus*: a review', Pharmacotherapy. 2005, 25, 74-85.

[7] H. F. Wertheim, M. C. Vos, A. Ott, A. van-Belkum, A. Voss, J. A. Kluytmans, P. H. van-Keulen, C. M Vandenbroucke-Grauls, M. H. Meester, H. A. Verbrugh, 'Risk and outcome of nosocomial *Staphylococcus aureus* bacteraemia in nasal carriers versus non-carriers', Lancet. 2004, 364, 703–705.

[8] K. Plata, A. E. Rosato, G. Węgrzyn, '*Staphylococcus aureus* as an infectious agent: overview of biochemistry and molecular genetics of its pathogenicity', Acta. Biochim. Pol. 2009, 56, 597-612.

[9] H. F. Chambers, F. R. DeLeo, 'Waves of resistance: *Staphylococcus aureus* in the antibiotic era', Nat. Rev. Microbiol. *2009*, 7, 629-641.

[10] C. M. Huang, C. H. Chen, D. Pornpattananangkul, L. Zhang, M. Chan, M. F. Hsieh, L. Zhang, 'Eradication of drug resistant *Staphylococcus aureus* by liposomal oleic acids', Biomaterial. 2011, 32, 214-221.

[11] H. McCarthy, J. K. Rudkin, N. S. Black, L. Gallagher, E. Neill, J.P. O'Gara, 'Methicillin resistance and the biofilm phenotype in *Staphylococcus aureus*', Front Cell Infect Mi. 2015.

[12] T. Stan, L. Mărutescu, M.C. Chifiriuc, and V. Lazăr, 'Anti-pathogenic effect of propolis extracts from different Romanian regions on *Staphylococcus* sp. Clinical strains', Rom Biotechnol Lett. 2016, 21, 11166-11175.

[13] A.T. Gemiarto, N.N. Ninyio, S.W. Lee, J. Logis, A. Fatima, E.W. Chan, and C.S. Lim, 'Isoprenyl caffeate, a major compound in manuka propolis, is a quorum sensing inhibitor in *Chromobacterium violaceum*', Antonie. Van. Leeuwenhoek. 2015, 108, 491-504, 2015.

[14] R. Silva-Carvalho, F. Baltazar, C. Almeida-Aguiar, 'Propolis: A Complex Natural Product with a Plethora of Biological Activities That Can Be Explored for Drug Development' Evid Based. Complement. Alternat. Med. 2015, 29. http://dx.doi.org/10.1155/2015/206439.

[15] A. L. Piccinelli, T. Mencherini, R. Celano, Z. Mouhoubi, A. Tamendjari, R. Aquino, L. Rastrelli, 'Chemical composition and antioxidant activity of Algerian propolis', J. Agric. Food Chem. 2013, 61, 5080-5088.

[16] A. Rebiai, and T. Lanez, '*In vitro* evaluation of antioxidant capacity of Algerian propolis by spectrophotometrical and electrochemical assays', Int. J. Pharm. 2011, 7, 113-11.

[17] M. Benhanifia, W. M. Mohamed, Y. Bellik, and H. Benbarek, 'Antimicrobial and antioxidant activities of different propolis samples from north-western Algeria', J. Food Sci. Technol. 2013, 48, 2521-2527.

[18] S. S. Anec, V. Sverko, T. Balog, A. Saricä, G. Rusak, S. Likicä, B. Kusić, V. Katalinić, S. Radić, and T. Marotti, 'Oxidant/antioxidant properties of Croatian native propolis' J. Agric. Food Chem. 2006, 54, 8018−8026.

[19] V. Katalinić, S. Radić, D. Ropac, R. Mulić, A. Katalinić, 'Antioxidative activity of propolis from Dalmatia (Croatia)', Acta. Med. Croat. 2004, 58, 373-376.

[20] N. Kalogeropoulos, S. J. Konteles, E. Troullidou, I. Mourtzinos, T. Vaios, and V. T. Karathanos, 'Chemical composition, antioxidant activity and antimicrobial properties of propolis extracts from Greece and Cyprus', Food. Chem. 2009, 116, 452–461.

[21] A. G. Hegazi, F. K. A. El-Hady, 'Egyptian propolis: 3. Antioxidant, antimicrobial activities and chemical composition of propolis from reclaimed lands', Z. Naturforsch. 2002, 57, 395-402.

[22] S. A. El Sohaimy, S. H. D. Masry,'Phenolic content, antioxidant and antimicrobial activities of Egyptian and Chinese propolis', Am.-Eurasian J. Sustain. 2014, 14, 116-1124.

[23] G. Papotti, D. Bertelli, B. L. Laura, M. Plessi, 'Chemical and functional characterization of Italian propolis obtained by different harvesting methods', J. Agric. Food Chem. 2012, 60, 2852−2862.

[24] S. Fabris, M. Bertelle, O. Astafyeva, E. Gregoris, R. Zangrando, A. Gambaro, G. P. P. Lima, R. Stevanato,'Antioxidant properties and chemical composition relationship of European and Brazilian propolis', Pharmacol. Pharm. 2013, 4, 46-51.

[25] E. Gregoris, R. Stevanato, 'Correlations between polyphenolic composition and antioxidant activity of Venetian propolis', Food Chem. Toxicol. 2010, 48, 76–82, 2010.

[26] V. Lagouri, D. Prasianaki, F. Krystalli, 'Antioxidant properties and phenolic composition of Greek propolis extracts', Int. J. Food. Prop. 2013, 17, 511-522.

[27] N. I. Potkonjak, D. S. Veselinovic, M. M. Novakovic, S. Z. Gorjanovic, L. L. Pezo, D. Z. Suznjevic, 'Antioxidant activity of propolis extracts from Serbia: A polarographic approach,' Food Chem. Toxicol. 2012, 50, 3614–3618.

[28] A. Mavri, H. Abramovic, T. Polak, J. Bertoncelj, P. Jamnik, S. S. Mozina, B. Jersek, 'Chemical properties and antioxidant and antimicrobial activities of Slovenian propolis', Chem. Biodivers. *2012*, 9, 1545-1558.

[29] J. S. Bonvehı, and A. L. Gutierrez, 'Antioxidant activity and total phenolics of propolis from the Basque country (Northeastern Spain)', J. Am. Oil Chem.' Soc. Society. 2011, 88, 1387–1395.

[30] S. Kumazawa, J. S. Bonvehí, C. Torres, A. Mok-Ryeond, F. J. O. Bermejo, 'Chemical and functional characterisation of propolis collected from East Andalusia (Southern Spain)', *Phytochem. Analysis*. 2013, 24, 608-615.

[31] I. Gülçin, E. Bursal, M. H. Sehitoğlu, M. Bilsel, A. C. Gören, 'Polyphenol contents and antioxidant activity of lyophilized aqueous extract of propolis from Erzurum, Turkey', Food Chem. Toxicol. 2010, 48, 2227–2238.

[32] R. Aliyazıcıoglu, H. Sahin, O. Erturk, E. Ulusoy, S. Kolayli, 'Properties of phenolic composition and biological activity of propolis from Turkey', Int.J. Food. Prop. 2013, 16, 277–287.

[33] S. Aazza, B. Lyoussi, D. Antunes, M. G. Miguel, 'Physicochemical characterization and antioxidant activity of 17 commercial Moroccan honeys', Int. J. Food Sci. Nutr. *2*014, 65, 449-57. Doi: 10.3109/09637486.2013.873888.

[34] M. Popova, B. Lyoussi, S. Aazza, D. Antunes, V. Bankova, G. Miguel, 'Antioxidant and alpha-glucosidase inhibitory properties and chemical profiles of Moroccan propolis', *Nat.* Prod. Commun. 2015, 10, 1961-1964.

[35] A. Kilic, M. Baisallar, B. Besirbellioglu, B. Salih, K. Sorkun, M. Tanyuksel, '*In vitro* antimicrobial activity of propolis against *methicillin-resistant Staphylococcus aureus* and *vancomycin-resistant Enterococcus faecium*', Ann. Microbiol. 2005, 55, 113-117.

[36] S. Pepeljnjak, I. Kosalec, 'Galangin expresses bactericidal activity against multiple-resistant bacteria: MRSA, Enterococcus spp. and *Pseudomonas aeruginosa*', FEMS. Microbiol. Lett. 2004, 240, 111-116.

[37] R. Raghukumar, L. Vali, D. Watson, J. Fearnley, V. Seidel, 'Antimethicillin-resistant *Staphylococcus aureus (MRSA)* activity of 'Pacific propolis' and isolated prenylflavanones', Phytother. Res. 2010, 24, 1181-1187.

[38] R. M. Darwish, R. J. A. Fares, M. H. A. Zarga, I. K. Nazer, 'Antibacterial effect of Jordanian propolis and isolated flavonoids against human pathogenic bacteria', Afr. J. Biotechnol. 2010, 9, 5966-5974.

[39] N. Vera, E. Solorzano, R. Ordonez, L. Maldonado, E. Bedas-Carrasbure, M. I. Isla, 'Chemical composition of Argentinean propolis collected in extreme regions and its relation with antimicrobial and antioxidant activities', Nat. Prod. Commun. 2011, 6, 823-827.

[40] S. Boisard, A. M. Le Ray, A. Landreau, M. Kempf, V. Cassisa, C. Fluri, P. Richomme 'Antifungal and antibacterial metabolites from a French poplar type propolis', Evid. Based. Complement. Alternat. Med. 2015, 10. http://dx.doi.org/10.1155/ 2015/319240.

[41] L. C. Pamplona-Zomenhan, B.C. Pamplona, C. B. DaSilva, M. C. Marcucci, L. M. J. Mimica, 'Evaluation of the *in vitro* antimicrobial activity of an ethanol extract of Brazilian classified propolis on strains of *Staphylococcus aureus*', Braz. J. Microbiol. 2011, 42, 1259-1264.

[42] A. Astani, S. Zimmermann, E. Hassan, J. Reichling, K. H. Sensch, P. Schnitzler, 'Antimicrobial activity of propolis special extract GH2002 against multidrug-resistant clinical isolates', Pharmazie. 2013, 68, 695-701.

[43] R. D. Wojtyczka, A. Dziedzic, D. Idzik, M. Kepa, R. Kubina, A. Kabala-Dzik, J. Smolerí-Dzirba, J. Stojko, M. Sajewicz, T. J. Wasik, 'Susceptibility of *Staphylococcus aureus* clinical isolates to propolis extract alone or in combination with antimicrobial drugs', Molecules. 2013, 18, 9623-9640.

[44] A. A. Saddiq, A. M. Abouwada, 'Effect of propolis extracts against methicillin-resistant *Staphylococcus aureus*', Main Group Chem. 2016, 15, 75-86.

[45] S. El-Guendouz, S. Aazza, B. Lyoussi, M. D. Antunes, M. L. Faleiro, M. G. Miguel, 'Anti-acetylcholinesterase, antidiabetic, anti-inflammatory, antityrosinase and antixanthine oxidase activities of Moroccan propolis', Int. J. Food. Sci. Tech. 2016, 51, 1762-1773.

[46] I. Gülçin, S. Beydemir, I. G. Sat, Ö. İ. Küfrevioğlu, 'Evaluation of antioxidant activity of cornelian cherry (*Cornus mas L.)*', Acta Aliment. 2005, 34, 193-202.

[47] M. G. Miguel, O. Doughmi, S. Aazza, D. Antunes, and B. Lyoussi, "Antioxidant, anti-inflammatory and acetylcholinesterase inhibitory activities of propolis from different regions of Morocco,' Food Sci. Biotechnol. 2014, 23, 313-322.

[48] M. Popova, V. Bankova, D. Butovska, 'Validated methods for the quantification of biologically active constituents of poplar-type propolis', Phytochem. Anal. 2004, 15, 235–240.

[49] S. Aazza, B. Lyoussi, M. G. Miguel, 'Antioxidant activity of some Morrocan hydrosols', J. Med. Plants Res. 2011, 5, 6688-6696.

[50] S. Aazza, B. Lyoussi, C. Megías, I. Cortés-Giraldo, J. Vioque, A. C. Figueiredo, M. G. Miguel, 'Anti-oxidant, anti-inflammatory and anti-proliferative activities of Moroccan commercial essential oils', Nat. Prod. Commun. 2014, 587-594.

[51] P. Prieto, M. Pineda, M. Aguilar, 'Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E', Anal. Biochem. 1999, 269, 337–341.

[52] S. C. Ho, Y. L. Tang, S. M. Lin, Y. F. Liew, 'Evaluation of peroxynitrite-scavenging capacities of several commonly used fresh spices', Food. Chem. 2010, 119, 1102–1107.

[53] M.G. Miguel, S. Nunes, S. A. Dandlen, A. M. Cavaco, M. D. Antunes, 'Phenols and antioxidant activities of hydro-alcoholic extracts of propolis from Algarve, South of Portugal', Food. Chem. Toxicol. 2010, 48, 3418-3423.

[54] S. Aazza, B. Lyoussi, M.G. Miguel, 'Antioxidant and antiacetylcholinesterase activities of some commercial essential oils and their major compounds', Molecules. 2011, 16, 7672-7690.

[55] R. J. Ruch, S. J. Cheng, J. E. Klaunig, 'Prevention of cytotoxicity and inhibition of intracellular communication by antioxidant catechins isolated from Chinese green tea', Carcinogenesis. 1998,10, 1003-1008.

[56] B. Ou, M. Hampsch-Woodill, R. L. Prior, 'Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe', J. Agric. Food. Chem. 2001, 49, 4619- 4626.

[57] A. L. Rajibul, S. K. Ismail, R. Nayan, A. B. Naznin, 'Antioxidant activity of Indian propolis and its chemical constituents', Food. Chem. 2010, 122, 233–237.

[58] S. El-Guendouz, S. Aazza, B. Lyoussi, V. Bankova, J. P. Lourenço, A. M. Costa, J. F. Mariano, M. G. Miguel, M. L. Faleiro, 'Impact of biohybrid magnetite nanoparticles and Moroccan propolis on adherence of Methicillin resistant strains of *Staphylococcus aureus*', Molecules. 2016, 21, 1208.

[59] A. A. Al-Abbadi, I. H. Ghabeish, M. A. Ateyyat, A. D. Hawari, S. A. Araj, 'A comparison between the anti-microbial activity of native propolis and the anti-microbial activity of imported ones against different health microbes', Jordan J Biol Sci, vol. 8, pp. 65 – 70, 2015.

[60] J. Apolónio, M. L. Faleiro, M. G. Miguel, L. Neto, 'No induction of antimicrobial resistance in *Staphylococcus aureus* and *Listeria monocytogenes* during continuous exposure to eugenol and citral', FEMS. Microbiol. Lett. 2014, 354, 92–101.

[61] A. Adrião, M. Vieira, I. Fernandes, M. Barbosa, M. Sol, R. P. Tenreiro, L. Chambel, B. Barata, I. Zilhao, G. Sharma, S. Perni, S. J. Jordan, P. W. Andrew, M. L. Faleiro, 'Marked intra-strain variation in response of *Listeria monocytogenes* dairy isolates to acid or salt stress and the effect of acid or salt adaptation on adherence to abiotic surfaces', Int. J. Food. Microbiol. 2008, 123, pp. 142–150.

[62] S. N. M. Norizan, Y. Wai-Fong, C. Kok-Gan, 'Caffeine as a potential quorum sensing inhibitor,' Sensors. 2013, 13, 5117-5129.

[63] D. Schrama, N. Helliwell, L. Neto, M. L. Faleiro, 'Adaptation of *Listeria monocytogenes* in a simulated cheese medium: effects on virulence using the *Galleria mellonella* infection model', Lett. Appl. Microbiol. 2013, 56, 421–427.

[64] J. Badanai, C. Silva, D. Martins, D. Antunes, M.G. Miguel, 'Ability of scavenging free radicals and preventing lipid peroxidation of some phenols and ascorbic acid', J. Appl. Pharm., 2015, 5, 34-41.

[65] K. M. Schaich, X. Tian, J. Xie, 'Reprint of "Hurdles and pitfalls in measuring antioxidant efficacy: a critical evaluation of ABTS, DPPH, and ORAC assay', J. Funct. Foods, 2015, 18, 782-796.

[66] P. Sharma, A. B. Jha, R. S. Dubey, M. Pessarakli, 'Reactive Oxygen Species, Oxidative Damage, and Antioxidative Defense Mechanism in Plants under Stressful Conditions', J. Bot. 2011, 26. Doi:10.1155/2012/217037.

[67] M. S. Brewer, 'Natural Antioxidants: Sources, Compounds, Mechanisms of Action, and Potential Applications' Compr. Rev. Food. Sci. F. .2011, 10. Doi: 10.1111/j.1541-4337.2011.00156.x.

[68] A. Weidinger, A. V. Kozlov, 'Biological Activities of Reactive Oxygen and Nitrogen Species: Oxidative Stress versus Signal Transduction', Biomolecules. 2015, 5, 472-484. Doi:10.3390/biom5020472.

[69] I. Andújar, M. C. Recio, R. M. Giner, J. L. Rios, "Cocoa polyphenols and their potential benefits for human health," Oxid. Med. Cell. Longev. 2012, Article ID 906252. doi:10.1155/2012/906252, 2012.

[70] L. C. Lu, Y. W. Chen, C. C. Chou, 'Antibacterial activity of propolis against *Staphylococcus aureus*', Int. J. Food. Microbiol., 2005, 102, 213-220.

[71] T. S. J. Kashi, R. K. Kermanshadi, M. Erfan, E. V. Dastjerdi, Y. Rezaei, F. S. Tabatabaei, 'Evaluating the *in vitro* anti-bacterial effects of Iranian propolis on oral microorganisms', Iran. J. Pharm. Res. 2011, 10, 363-368.

[72] M. M. Rahman, A. Richardson, M. Sofian-Azirun, 'Antibacterial activity of propolis and honey against *Staphylococcus aureus* and *Escherichia coli'*, Afr. J. Microbiol. Res. 2010, 4, 1872-1878.

[73] M. Popova, B. Trusheva, D. Antonova, S. Cutajar, D. Mifsud, C. Farrugia, I. Tsvetkova, H. Najdenski, V. Bankova, 'The specific chemical profile of Mediterranean propolis from Malta' Food. Chem. 2011, 126, 1431-1435.

[74] M. C. Marcucci, 'Propolis: chemical composition, biological properties and therapeutic activity' Apidologie. 1995, 26, 83-99.

[75] M. McMahon, I. Blair, J. Moore, D. A. McDowell, 'Habituation to sublethal concentrations of tea tree oil (*Melaleuca alternifolia*) is associated with reduced susceptibility to antibiotics in human pathogens', J. Antimicrob. Chemother. vol. 59, pp. 125–127, 2007.

[76] K. A. Hammer, C. F. Carson, T. V. Riley, 'Effects of Melaleuca alternifolia (tea tree) essential oil and the major monoterpene component terpinen-4-ol on the development of single and multistep antibiotic resistance and antimicrobial susceptibility', Antimicrob. Agents. Chemother. 2012, 56, 909-915.

[77] J. C. McNeil, E. Y. Kok, J. G. Vallejo, J. R. Campbell, K. G. Hulten, E. O. Mason, S. L. Kaplan, 'Clinical and molecular features of decreased chlorhexidine susceptibility among nosocomial *Staphylococcus aureus* isolates at Texas Children's Hospital' Antimicrob. Agents. Chemother. 2016, 60, 1121–1128, 2016. Doi:10.1128/AAC.02011-15.

[78] K. Hardy, K. Sunnucks, H. Gil, S. Shabir, E. Trampari, P. Hawkey, M. Webber, 'Increased usage of antiseptics is associated with reduced susceptibility in clinical isolates of *Staphylococcus aureus'* mBio. 2018, 9, 9:e00894-18.

[79] T. Stan, L. Marutescu, M. C. Chifiriuc, C. Mateescu, V. Lazăr, 'Study of the antimicrobial and antibiofilm activity of romanian propolis', Bio Res App Chem. 2013, 3, 541-550.

[80] G. F. Helaly, A. A. A. El-Aziz, F. I. Sonbol, T. E. El-Banna, N. L. Louise, 'Dexpanthenol and propolis extract in combination with local antibiotics for treatment of Staphylococcal and Pseudomonal wound infections', Arch. Clin. Microbiol. 2011, 2, 4-3. Doi: 10:3823/235.

[81] D.G. Davies, M.R. Parsek, J.P. Pearson, B. H. Iglewski, J.W. Costerton, E.P. Greenberg, 'The involvement of cell-to-cell signals in the development of a bacterial biofilm' Science. 1998, 295-298.

[82] M. Otto, 'Coagulase-negative *staphylococci* as reservoirs of genes facilitating MRSA infection,' Bioassays, vol. 35, 4–11, 2013.

[83] M. A. Savka, L. Dailey, M. Popova, R. Mihaylova, B. Merritt, M. Massek, P. Le, S. R. M. Nor, M. Ahmad, A. O. Hudson, V. Bankova, 'Chemical composition and disruption of quorum sensing signalling in geographically diverse United States propolis', Evid Based. Complement. Alternat. Med. 2015. Article ID 472593.

[84] D. Kasoto, A. Ahmad., W. Chen, S. Combrinck, A. Viljoen, 'HPTLC-MS as an efficient hyphenated technique for the rapid identification of antimicrobial compounds from propolis', Phytochem. Lett. 2015, 11, 326-331.

[85] Z. Bulman, P. Lee, A. O. Hudson, M. A. Savka, 'A novel property of propolis (bee glue): anti-pathogenic activity by inhibition of *N*-acyl-homoserine lactone mediated signaling in bacteria', J. Ethnopharmacol. 2011, 138, 788–797.

[86] L. E. Lamberte, E. C. Cabrera, and W. L. Rivera, 'Activity of the ethanolic extract of propolis (EEP) as a potential inhibitor of QS mediated pigment production in *Chromobacterium violaceum* and virulence factor production in *Pseudomonas aeruginosa*', Philipp. Agric. Sci. 2011, 94, 14–22.

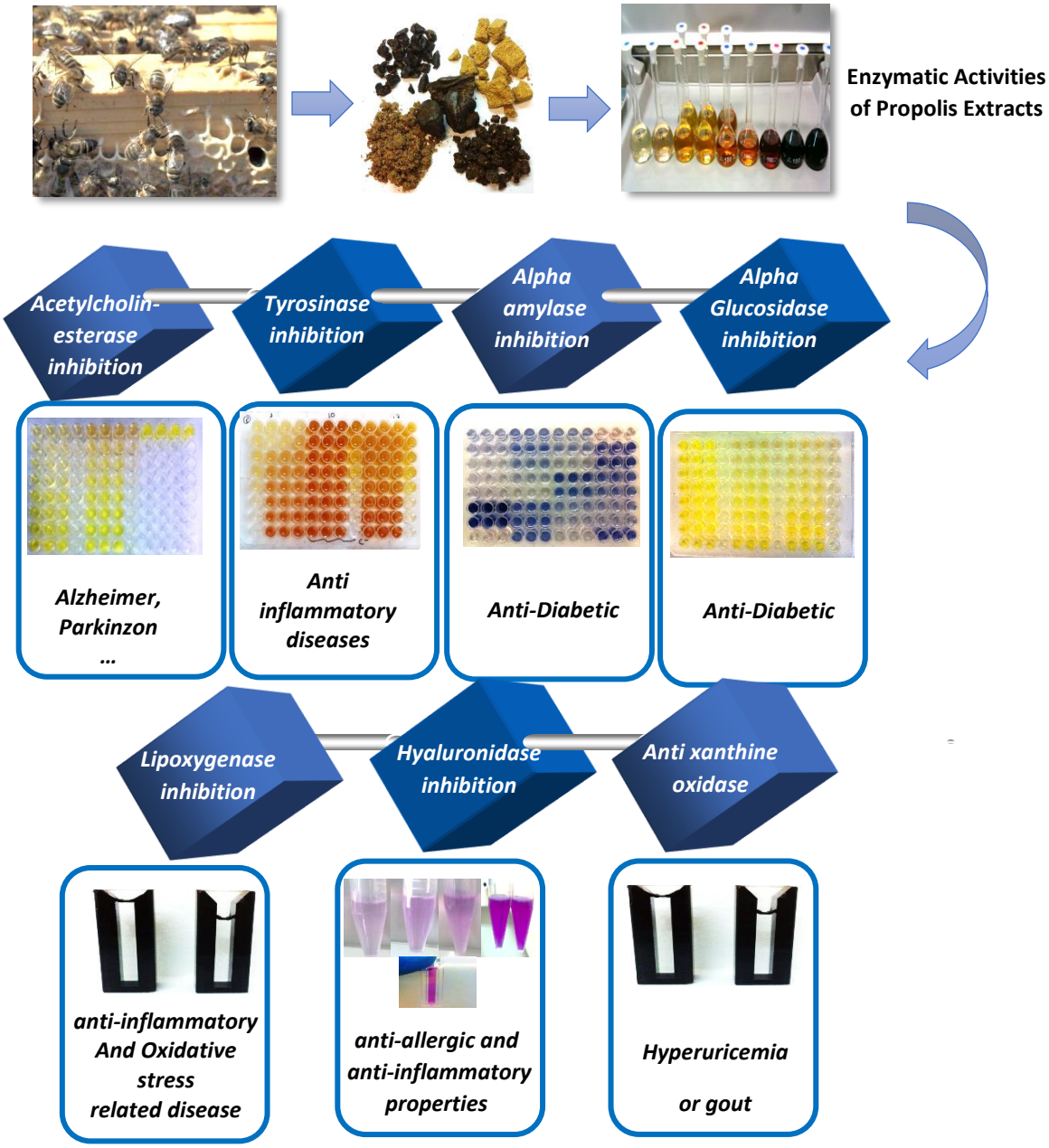
[87] A. P. Desbois, P. J. Coote, 'Wax moth larva (*Galleria mellonella*): an *in vivo* model for assessing the efficacy of antistaphylococcal agents', J. Antimicrob. Chemother. 2011, 66, 1785–1790.

[88] G. A. Burdock, 'Review of the biological properties and toxicity of bee propolis', Food. Chem. Toxicol. 1998, 36, 347-363.

[89] R. E. Sanad, K. M. Mohanny, 'Toxicological and biological effects of propolis and three plant extracts on the greater wax moth, Galleria mellonella L.', Egypt. J. Biol. Pest. Co. 2015, 25, 213-219.

Chapter 3

Anti-acetylcholinesterase, antidiabetic, anti-inflammatory, anti-tyrosinase and anti-xanthine oxidase activities of Moroccan propolis



Anti-acetylcholinesterase, antidiabetic, anti-inflammatory, anti-tyrosinase and anti-xanthine oxidase activities of Moroccan propolis*

Abstract

Biological properties of Moroccan propolis have been scarcely studied. In the present work, the total phenols and flavonoids from 21 samples of propolis collected in different places of Morocco or 3 supplied in the market were determined, as well as the *in vitro* capacity for inhibiting the activities of acetylcholinesterase, α-glucosidase, α-amylase, lipoxygenase, tyrosinase, xanthine oxidase and hyaluronidase. The results showed that samples 1 (region Fez-Boulemane, Sefrou city) (IC_{50} = 0.065, 0.006, 0.020, 0.050, 0.014 mg/mL) and 23 (marketed) (IC_{50} = 0.018, 0.002, 0.046, 0.037, 0.008 mg/ mL) had the best *in vitro* capacity for inhibiting the a-amylase, a-glucosidase, lipoxygenase, tyrosinase and xanthine oxidase activities, respectively. A negative correlation between IC_{50} values and concentration of phenols, flavones and flavanones was found. These activities corresponded to the generally higher amounts of phenols and flavonoids. In the same region, propolis samples have dissimilar phenol content and enzyme inhibitory activities.

*****Adapted from 'Anti-acetylcholinesterase, antidiabetic, anti-inflammatory, anti-tyrosinase and anti-xanthine oxidase activities of Moroccan propolis', Soukaïna El-Guendouz, Smail Aazza, Badiaa Lyoussi, Maria D. Antunes, Maria L. Faleiro & Maria G. Miguel. International Journal of Food Science and Technology, Volume 51, Issue 8, 2016.

3.1. Introduction

Propolis is a plant product–resinous mixture with a broad spectrum of biological properties, collected by the *Apis mellifera* bee from the bud and exudates of various plants and transformed in the presence of bee enzymes.[1,2] The wide application of propolis in modern medicine and the increasing demand for it, due to its health benefits and use in cosmetic and food products, have drawn growing attention to its chemical composition, which is susceptible to the geographical location, collection site, botanical origin,[3] bee species[4] and the availability of water and climate.[5] Flavonoids, phenylpropanoids, terpenoids, stilbenes, lignans, coumarins and their prenylated derivatives, alkaloids and iridoids are some groups of compounds identified in propolis worldwide. This chemical diversity is related to the bioactivity and potential uses of this product.[6]

The biological properties attributed to the propolis of different parts of the world have been the target of studies, and very recently, Silva-Carvalho et al.[7] compiled the recent published information about antibacterial and antifungal, antiviral, anti-inflammatory, antioxidant, immunomodulatory and antitumor activities of propolis.

Alzheimer's disease is a progressive, neurodegenerative disease that is clinically characterised by a progressive loss of cognitive abilities. The pathophysiology of that disease involves defective beta-amyloid protein metabolism, abnormalities of glutamatergic, adrenergic, serotonergic and dopaminergic neurotransmission and the potential involvement of inflammatory, oxidative and hormonal pathways.[8,9] For the treatment of Alzheimer's disease, acetylcholinesterase (AChE) inhibitors are included, which increase the availability of acetylcholine at cholinergic synapses, although the modest benefits were obtained.[9] Some natural products of plant origin have revealed to be effective acetylcholinesterase inhibitors – of them some were used as medicines, such as some alkaloids (galantamine). Other groups of compounds have been also checked and some flavonoids have also revealed the ability for inhibiting this enzyme.[10] Only very few works regarding the capacity of propolis extracts to inhibit acetylcholinesterase have been published.[2,8]

Postprandial hyperglycaemia plays an important role in the development of type 2 diabetes mellitus, and one strategy used to stabilise postprandial plasma glucose is the utilisation of specific enzyme inhibitors, such as α-glucosidase and pancreas α-amylase inhibitors.[11] Natural bioactive compounds with the capacity to delay the glucose absorption by the inhibition of α-glucosidase or α-amylase activities and consequently to moderate the excess

postprandial blood glucose level have been searched, including those present in the propolis from different geographical origins: China,[12] Morocco[13] and Brazil.[14]

The inhibition of lipoxygenase has been considered as an indicator of anti-inflammatory and antioxidant activities. 5-Lipoxygenase is the key enzyme of leukotriene biosynthesis, and it has been the target for many inhibitors for trying to find potential drugs for combating a variety of inflammation- and hypersensitivity-based human diseases including asthma, arthritis, bowel diseases such as ulcerative colitis and Crohn's disease and circulatory disorders such as shock and myocardial ischaemia.[15] Propolis[2] and particularly some components (e. g. caffeic acid phenethyl ester) have been reported as lipoxygenase inhibitors with antioxidant properties.[16, 17, 18]

Hyaluronidase is an enzyme that depolymerises the polysaccharide hyaluronic acid in the extracellular matrix of connective tissue, and it is involved in allergic reactions and inflammation.[19] Hyaluronidase inhibitors may have therefore anti-allergic and anti-inflammatory properties. Inhibition of hyaluronidase by propolis from diverse geographical origins such as Portugal[20] and China and Brazil[21,22] has been reported, and therefore, propolis is considered as a potential anti-inflammatory natural product.

Tyrosinase (monophenol or *o*-diphenol, oxygen oxidoreductase), also known as polyphenol oxidase (PPO), is a copper-containing monooxygenase. This enzyme is a key in melanin biosynthesis, which involves in the colour formation of mammalian hair and skin and prevents the skin from damage by ultraviolet.[23, 24] Propolis from diverse origins (Australia, Brazil, China, Hungary, Japan, Ukraine and Uruguay) has been described to inhibit the tyrosinase activity.[25]

The enzyme xanthine oxidase catalyses the oxidation of hypoxanthine and xanthine to uric acid. Overproduction of uric acid, termed hyperuricaemia, is the underlying cause of gout. During the reoxidation of xanthine oxidase, molecular oxygen acts as an electron acceptor, producing superoxide radical and hydrogen peroxide.[26] During this process, beyond the production of uric acid, there is also the production of reactive oxygen species that contribute to the oxidative stress which is involved in several pathological processes such as inflammation, atherosclerosis, cancer, ageing, among other ones.[26]

Propolis extracts and their components have the ability to inhibit xanthine oxidase, either *in vitro* or *in vivo* systems, which may be considered as potential agent against gout and oxidation (the production of superoxide radical and hydrogen peroxide is hampered).[27, 28, 29]

Only very few works have focused on Moroccan propolis. One of the few studies concerning propolis extracts from this country reported *in vitro* and *in vivo* anticancer properties,[30] whereas antioxidant, anti-inflammatory (the inhibition of lipoxygenase activity) and anti-acetylcholinesterase activities of fourteen propolis samples from diverse regions of Morocco were evaluated by Miguel et al.[2] and α-glucosidase and α-amylase inhibitory activities were reported by Popova et al. [13] from the propolis collected in five diverse locations of that country.

The main goal of the present work was to evaluate the inhibitory activities of 21 propolis samples of diverse geographical origins of Morocco on acetylcholinesterase, lipoxygenase, α-glucosidase and α-amylase activities, previously studied but with fewer number of propolis samples.[2,13] At the same time, other enzyme inhibitory activities, such as xanthine oxidase and hyaluronidase, enzymes also involved in inflammatory processes, were evaluated, as well as the inhibitory activity of tyrosinase by the same samples. This work may contribute to ascertain the importance or not of the harvesting places on the enzyme inhibitory activities. In addition, three samples purchased from the market were also the target of study, due to the increasing demand and sell of propolis by Moroccan people.

3.2. Experimental

3.2.1. Chemicals

Acetylthiocholine iodide (ATCI), AChE (type VI-S) from the electric eel Electrophorus electricus, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), hyaluronidase from bovine testes, sodium tetraborate, alpha-amylase, alpha-glucosidase, 5-lipoxygenase from soya bean, linoleic acid, mushroom tyrosinase, L-DOPA, xanthine oxidase, xanthine and methanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). *p*-Nitrophenyl-β-D-glucopyranoside (PNPG), boric acid, ascorbic acid, Tris–HCl and hydrochloric acid (HCl) were purchased from Merck, Darmstadt, Germany. Starch, potassium iodide, iodine and Na_2CO_3 were purchased from Riedel de Haen (Seelze, Germany), Riedel-de-Haën Laboratory chemicals, Germany. Calcium chloride, Folin-Ciocalteu's phenol reagent and $AlCl_3$ were purchased from Panreac Química, Montcada i Reixac, Barcelona, Spain. Acetic acid, PDMAB (*p*-dimethylaminobenzaldehyde), potassium dihydrogen phosphate (KH_2PO_4), dipotassium hydrogen phosphate (K_2HPO_4), sodium dihydrogen phosphate (NaH_2PO_4) and disodium hydrogen phosphate (Na_2HPO_4) were purchased from VWR (Leuven, Belgium). Ferulic Acid, DNP (2,4-dinitrophenylhydrazine), quercetin, H_2SO_4 and KOH and eriodictyol were purchased from Fluka Biochemika (Sigma-Aldrich, Steinheim, Germany).

3.2.2. Propolis extraction

Twenty-four propolis samples were provided by diverse Moroccan beekeepers or purchased from the market. The harvesting places and their coordinates, climate data as well as the surrounding plants of the beehives are given in table 2.1 (Chapter 2). Extracts were obtained as reported by Miguel et al.[2] with slight modifications. Briefly, one gram of propolis was chopped into small pieces and extracted by maceration using 30 mL of 70% ethanol and maintained for 1 week at 37 °C under agitation (200 rpm). The resulting solution was filtered under vacuum. For the successive analyses, a clear solution, without further purification, was used to prepare serial dilutions with the concentrations of 33.33, 16.66, 8.33, 4.17, 2.08, 1.04, 0.52 and 0.26 mg mL^{-1}.

3.2.3. Determination of different phenolic groups

3.2.3.1. Total phenol content

The total polyphenol content in propolis samples was determined using the method of Gülcin et al..[31] Hydroalcoholic extracts (25 µL) were mixed with 125 µL of Folin-Ciocalteu's phenol reagent (0.2 N) and 100 µL of 7.5% Na_2CO_3, and the absorbance was measured at 765 nm after 2 h of incubation at room temperature. The total polyphenol content was expressed as mg ferulic acid equivalents per mL (mg FAE mL^{-1}) using a calibration curve.

3.2.3.2. Flavones and flavonol content

The amounts of flavones and flavonols in the extracts were determined according to the method of Miguel et al.[32] with a minor modification. An amount of 100 µL of $AlCl_3$ (20%) was added to 100 µL of extract, and after 1 h at room temperature, the absorbance was measured at 420 nm. Total flavone and the flavonol contents were calculated as mg quercetin equivalents per mL (mg QE mL^{-1}) using a calibration curve.

3.2.3.3. Flavanone and dihydroflavonol contents

The quantification of total flavanones and dihydroflavonols was performed as described by Popova et al.[33] with slight modifications. Briefly, 75 µL of sample and 2 mL of DNP (2,4-dinitrophenylhydrazine) solution (1 g DNP in 2 mL 96% sulphuric acid, diluted to 100 mL with methanol) were heated at 50 °C for 50 min. After cooling at room temperature, the mixture was diluted to 10 mL with 10% KOH in methanol (w/v). A sample (1 mL) of the resulting solution was added to 10 mL methanol and diluted to 50 mL with methanol. The absorbance was measured at 486 nm.

3.2.4. Enzymatic activities

3.2.4.1. Inhibition of acetylcholinesterase

Acetylcholinesterase degrades the substrate acetylcholine into acetic acid and thiocholine, which interacts with 5,5′-dithiobis-2-nitrobenzoic acid (DTNB). Accumulation of 5-thio-2-nitrobenzoic acid was measured at 405 nm. The acetylcholinesterase inhibition assay was adapted from the method of Mata et al..[34] About 25 μL of sample, 425 μL of Tris–HCl buffer (0.1 M, pH 8) and 25 μL of enzyme (0.28 U mL^{-1}) were added, and then the mixture was agitated and incubated for 15 min at room temperature. After this period, 75 μL of substrate (0.005 g of iodine acetylcholine in 10 mL of buffer) and 475 μL of 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) (0.059 g in 50 mL of buffer) were then added. The absorbance of sample (Abs_{sample}) was read after 30 min and compared with the absorbance of the control ($Abs_{control}$) in which the sample was replaced by buffer. Inhibition percentage of enzyme was calculated as follows: $[(Abs_{control} - Abs_{sample})/Abs_{control}] \times 100$. The analyses were carried out in triplicate. The inhibition percentage of enzyme was plotted against propolis extract concentration (w/v), and IC_{50} values were determined (the concentration of propolis extract able to inhibit 50% of acetylcholinesterase).

3.2.4.2. Inhibition of α-amylase

α-Amylase inhibition assay was carried out by a modified starch iodine.[35] The total assay mixture consisting of 100 μL sodium phosphate buffer (0.02 M, pH 6.9 containing 6 mM sodium chloride), 50 μL of α-amylase (0.02 units) and propolis was incubated at 37 °C for 10 min. After the incubation, 200 μL of soluble starch (1%, w/v) was added to each test tube and the mixture was reincubated for 20 min at 37 °C. About 300 μL of 10% HCl was added to stop the enzymatic reaction, followed by the addition of 300 μL of iodine reagent (5 mM I_2 and 5 mM KI), and after that, 8 mL of distilled water was added. The absorbance was read at 620 nm. Sample, substrate and blank were undertaken under the same conditions. Each experiment was done in triplicate. The inhibition percentage of the enzyme was calculated using the following formula:

% of α-amylase inhibition: $$\left[1 - \frac{[(A^{-}_{control}) - (A^{+}_{control})] - (A_{sample})}{(A^{-}_{control}) - (A^{+}_{control})}\right] \times 100$$

where $A^{-}_{control}$ denotes the absorbance of 100% enzyme activity (ethanol 70% with enzyme), $A^{+}_{control}$ denotes the absorbance of 0% enzyme activity (ethanol 70% without enzyme), and A_{sample} denotes the absorbance of sample. And then, the IC_{50} values were compared.

3.2.4.3. Inhibition of α-glucosidase

The yeast α-glucosidase inhibitory activity of the 24 propolis samples was determined by a modified version of the method described by Popova et al..[13] Briefly, a mixture of 25 μL of different concentrations of the samples, 30 μL of yeast α-glucosidase (2.4 U mL^{-1}) and 100 mM phosphate buffer (pH 6.8) was incubated in 96-well plates at room temperature for 10 min. After pre-incubation, 100 μL of 0.5 mM *p*-nitrophenyl-β-D-glucopyranoside (PNPG) solution in phosphate buffer was added to each well. The reaction mixtures were incubated at 37 °C for 30 min, and then 80 μL of sodium carbonate solution (0.4 mM) was added to stop the reaction. The absorbance was recorded with a microplate reader at 405 nm. The control had 25 μL of 70% ethanol instead of test samples. The inhibition percentage of the enzyme was calculated, and the IC_{50} values were compared. The analyses were carried out in triplicate.

3.2.4.4. Inhibition of lipoxygenase

The inhibition of lipoxygenase was carried out as previously reported by Frum & Viljoen.[36] The reaction was initiated by the addition of 5 μL 5-lipoxygenase solution (0.054 g in 1 mL borate buffer 0.005%, Tween 0.1 M, pH 9) to 937 μL borate buffer, 10 μL sample and 50 μL linoleic acid (0.001 M). The enzymatic reactions were performed in the absence or in the presence of propolis extracts and their kinetics were compared. The inhibition percentage of the enzyme was calculated, and the IC_{50} values were compared. The analyses were carried out in triplicate.

3.2.4.5. Inhibition of tyrosinase

The inhibitory action of propolis on tyrosinase was evaluated as previously described by El-Hady et al.,[37] but with the slight modifications. In short, 140 μL phosphate buffer (50 mM, pH 6.5), 25 μL of sample and 60 μL of mushroom tyrosinase solution (100 U mL^{-1}) were added to a 96-well microplate. The assay mixture was incubated at 25 °C for 40 min. Following incubation, 60 μL of 5 mM L-DOPA solution was added and the amount of dopachrome produced in the reaction mixture was determined spectrophotometrically at 492 nm in a microplate reader. The percentage of inhibition of tyrosinase activity was calculated and the IC_{50} value was determined.

3.2.4.6. Inhibition of xanthine oxidase

Xanthine oxidase activity was determined by measuring the formation of uric acid from xanthine. Xanthine oxidase inhibiting activity was conveniently measured with a modified assay according to Umamaheswari et al..[38] The assay mixture consisted of 100 μL of the

sample, 350 μL of phosphate buffer (pH 7.5, 50 mM) and 45 μL of xanthine oxidase enzyme solution (0.4 units mL^{-1} in phosphate buffer, pH 7.5), which was prepared immediately before use. After pre-incubation at room temperature for 15 min, the reaction was initiated by the addition of 330 μL of 0.150 mM xanthine solution (substrate). The assay mixture was incubated for 30 min. The reaction was stopped by adding 100 μL of 1 M HCl, and the absorbance was measured at 290 nm using an UV/VIS spectrophotometer. The assay was done in triplicate, and the IC_{50} values were calculated from the percentage of inhibition.

3.2.4.7. Inhibition of hyaluronidase

Hyaluronidase inhibition was determined by measuring the amount of *N*-acetylglucosamine separated from sodium hyaluronate as described by Sahasrabudhe & Deodhar.[39] Bovine hyaluronidase solution (150 μL) (7900 units mL^{-1}) dissolved in acetate buffer (pH 3.6) was mixed with 25 μL of propolis extracts and incubated for 30 min at 37 °C. After 30 min, 50 μL of calcium chloride (12.5 mM) was added to the reaction mixture and again incubated for 30 min at 37 °C. After this period, 250 μL sodium hyaluronate (1.2 mg mL^{-1}) was added and again incubated at 37 °C for 1 h 30 min. After incubation, 50 μL of 0.4 M sodium hydroxide and 100 μL of 0.6 M sodium borate were added to the reaction mixture and incubated in the boiling water bath for 3 min. After cooling in ice, 1.5 mL of PDMAB (*p*-dimethylaminobenzaldehyde) solution (4 g PDMAB dissolved in 50 mL of 10 M HCL and 350 mL of glacial acetic acid) was added to the reaction mixture. The absorbance was measured at 585 nm. The assay was done in triplicate.

3.2.5. Statistical analysis

Statistical analysis was performed with the Statistical Package for the Social Sciences (SPSS) 23.0 software (SPSS Inc., Chicago, IL, USA). Statistical comparisons were made with one-way analysis of variance followed by Tukey's multiple comparisons. The level of significance was set at $P < 0.05$. Correlations between phenol and flavonoid contents and enzyme inhibitory activities were achieved by Spearman's correlation coefficient (r) at a significance level of 95%. Hierarchical cluster analysis was used to investigate the similarities and dissimilarities between the 24 propolis samples with respect to the total phenols, flavones and flavonols, flavanones and dihydroflavonols content and the capacity to inhibit xanthine oxidase and α-amylase activities. In 9 samples, due to its low inhibitory activity on α-glucosidase, lipoxygenase, acetylcholinesterase and tyrosinase, the IC_{50} values were impossible to determine in some assays. In this case, only 15 samples were submitted to hierarchical cluster

analysis, to investigate the similarities and dissimilarities between the 15 propolis samples with respect to the total phenols, flavones and flavonols, flavanones and dihydroflavonols contents and the capacity for inhibiting those enzymes. In any case, for the classification purposes, the Ward's minimum variance method was used; the squared Euclidean distance was used as the dissimilarity measure for Ward's method.

3.3. Results and discussion

3.3.1. Phenols

The concentrations of total phenols, flavones and flavonols, and flavanones and dihydroflavonols found for propolis samples collected in several places of Morocco are presented in Table 3.1 The results reveal a great variability in concentrations of all groups of phenols. Concerning total phenols, the concentrations ranged from 0.236 mg, in sample 24, to 8.846 mg FAE mL^{-1} in sample 17. Sample 23 (8.827 mg FAE mL^{-1}) had a concentration similar to that of sample 17. Total phenol concentrations of samples 1 and 10 were 6.861 and 4.802 mg FAE mL^{-1}, respectively. The amounts of flavones and flavonols were always superior to those of flavanones and dihydroflavonols (Table 3.1). As reported for total phenols, the concentrations of flavones and flavonols were higher in the sample 17 (4.320 mg QE mL^{-1}), immediately followed by the samples 23 (2.699 mg QE mL^{-1}) and 1 (1.784 mg QE mL^{-1}). Concerning dihydroflavonols, the sample 23 had the highest amount of this group of compounds (0.532 mg EE mL^{-1}) and not sample 17, as found for total phenols and flavones and flavonols, immediately followed by samples 1 (0.257 mg EE mL^{-1}) and 17 (0.222 mg EE mL^{-1})

Table 3.1. Concentration of total phenols, flavonols, flavones, flavanones and dihydroflavonols in propolis extracts

Samples	Phenols (mg ferulic acid equivalent (FAE)/mL extract)	Flavonoides (mg quercetin equivalent (QE)/mL extract)	Dihydroflavones (mg eriodictyol equivalent (EE)/mL extract)
1	6.861±0.016^{b}	1.784±0.012^{c}	0.257±0.001^{b}
2	0.564±0.016^{l}	0.097±0.012^{jkl}	0.047±0.001^{m}
3	1.101±0.016^{i}	0.353±0.012^{g}	0.092±0.001^{h}
4	0.436±0.016^{m}	0.088±0.012^{kl}	0.050±0.001^{m}
5	0.732±0.016^{k}	0.143±0.012^{ij}	0.070±0.001^{k}
6	0.465±0.016^{m}	0.107±0.012^{jk}	0.060±0.001^{l}
7	0.260±0.016^{no}	0.028±0.012^{mno}	0.031±0.001^{o}
8	1.921±0.016^{f}	0.402±0.012^{f}	0.084±0.001^{i}
9	0.443±0.016^{m}	0.057±0.012^{lmn}	0.057±0.001^{l}
10	4.802±0.016^{c}	0.781±0.012^{d}	0.168±0.001^{e}
11	0.699±0.016^{k}	0.067±0.012^{klm}	0.060±0.001^{l}
12	0.318±0.016^{n}	0.006±0.012^{o}	0.038±0.001^{n}
13	1.307±0.016^{h}	0.186±0.012^{i}	0.071±0.001^{jk}
14	2.113±0.016^{e}	0.409±0.012^{f}	0.139±0.001^{f}
15	1.929±0.016^{f}	0.331±0.012^{h}	0.176±0.001^{d}
16	1.787±0.016 ^{g}	0.297±0.012^{h}	0.0142±0.001^{f}
17	8.864±0.016^{a}	4.320±0.012^{a}	0.222±0.001^{c}
18	0.802±0.016^{j}	0.0136±0.012^{j}	0.058±0.001^{l}
19	0.477±0.016^{m}	0.069±0.012^{klm}	0.074±0.001^{k}
20	2.429±0.016^{d}	0.604±0.012^{e}	0.109±0.001^{g}
21	0.261±0.016^{no}	0.020±0.012^{mn}	0.077±0.001^{j}
22	1.892±0.016^{f}	0.0427±0.012^{f}	0.138±0.001^{f}
23	8.827±0.016^{a}	2.699±0.012^{b}	0.532±0.001^{a}
24	0.039±0.016^{o}	0.039±0.012^{mno}	0.058±0.001^{l}

Values with the same letter are not significantly different (P<0.05) by the Tukey's multiple range test

3.3.2. Inhibition of acetylcholinesterase

A possible strategy for the treatment of Alzheimer's disease, which is characterised by a decline in cognitive function and mental atrophy, may include the inhibition of acetylcholinesterase, the fundamental enzyme in the breakdown of acetylcholine by the termination of the nerve impulse transmission at cholinergic synapses.[2] Several propolis samples had similar and relatively high ability for inhibiting acetylcholinesterase: samples 1, 10, 14, 15, 16, 17, 21, 22 and 23 (Table 3.2). Although an inverse correlation between IC_{50} values and total phenols and flavonoids was observed ($P < 0.01$) (Table 3.3), which is representative of the importance of these groups of compounds for the inhibition of acetylcholinesterase activity, sample 8 had higher concentration of phenols than sample 21, but had lower activity. According to Miguel et al.,[2] propolis samples collected in Moulay Bousselham, Sidi Sliman, Taza, Sefrou and Kenitra were not able to inhibit acetylcholinesterase, which was in contrast to the results obtained in the present study (Table 3.2). The importance of phenols and flavonoids present in Moroccan propolis samples was already reported.[2] In addition, the acetylcholinesterase inhibitory activity was also reported by Chen et al.[9] for Chinese propolis, because water-soluble extracts, consisted of mainly

flavonoids, significantly inhibited the acetylcholinesterase activity in the hippocampus of adult male Kunming mice compared to control mice given with normal saline solution. In spite of these findings by both Chen et al.[9] and Miguel et al.,[2] other group of compounds present in propolis samples has a role in the inhibitory activity of acetylcholinesterase, owing to the relatively good activity observed for sample 21 rather than the relatively low concentration of phenols and flavonoids.

Table 3.2. Enzyme inhibitory activities (IC_{50} = mg mL^{-1}) of hydroalcoholic extracts of Moroccan propolis harvested at different places

Sample	Acetylcholinesterase	α-Amylase	α-Glucosidase	Lipoxygenase	Tyrosinase	Xanthine oxidase
1	0.009±0.051^{i}	0.065±0.097^{l}	0.006±0.016^{ef}	0.020±0.019^{n}	0.050±0.192^{j}	0.014±0.052^{i}
2	-	2.020±0.097^{f}	-	0.463±0.019^{cde}	0.374±0.192^{ij}	0.250±0.052^{defg}
3	0.535±0.051^{fg}	2.066±0.097^{ef}	0.056±0.016^{cdef}	0.406±0.019^{efg}	-	0.245±0.052^{defg}
4	0.446±0.051^{g}	2.730±0.097^{c}	0.068±0.016^{cd}	0.653±0.019^{a}	0.971±0.192^{hi}	0.237±0.052^{defg}
5	0.199±0.051^{h}	3.304±0.097^{b}	0.103±0.016^{c}	0.568±0.019^{b}	3.824±0.192^{ab}	0.279±0.052^{def}
6	1.453±0.051^{c}	2.417±0.097^{d}	0.062±0.016^{cde}	0.646±0.019^{a}	2.444±0.192^{d}	0.585±0.052^{c}
7	2.034±0.051^{b}	1.263±0.097^{hi}	-	-	-	0.753±0.052^{b}
8	0.188±0.051^{hi}	1.355±0.097^{hi}	0.032±0.016^{def}	0.478±0.019^{cd}	1.595±0.192^{fg}	0.362±0.052^{de}
9	0.087±0.051^{hi}	2.144±0.097^{def}	0.027±0.016^{def}	0.441±0.019^{def}	1.844±0.192^{ef}	0.245±0.052^{defg}
10	0.007±0.051^{i}	0.170±0.097^{l}	0.007±0.016^{ef}	0.086±0.019^{m}	0.451±0.192^{hij}	0.031±0.052^{i}
11	1.341±0.051^{cd}	1.249±0.097^{hi}	0.018±0.016^{def}	0.642±0.019^{a}	-	0.179±0.052^{efgh}
12	1.212±0.051^{d}	2.853±0.097^{c}	-	0.505±0.019^{c}	4.286±0.192^{a}	0.381±0.052^{d}
13	0.648±0.051^{f}	3.632±0.097^{a}	0.746±0.016^{a}	0.372±0.019^{gh}	-	0.162±0.052^{fgh}
14	0.002±0.051^{i}	0.855±0.097j	0.019±0.016^{def}	0.239±0.019^{k}	0.295±0.192^{j}	0.219±0.052^{defg}
15	0.024±0.051^{i}	1.484±0.097^{gh}	0.027±0.016^{def}	0.152±0.019^{l}	0.612±0.192^{hij}	0.076±0.052^{hi}
16	0.007±0.051^{i}	1.450±0.097^{gh}	0.025±0.016^{def}	0.256±0.019^{jk}	3.600±0.192^{bc}	0.125±0.052^{fgh}
17	0.003±0.051^{i}	0.146±0.097^{l}	0.014±0.016^{def}	0.065±0.019^{mn}	0.110±0.192^{j}	0.017±0.052^{i}
18	0.123±0.051^{hi}	2.338±0.097^{de}	0.155±0.016^{b}	0.574±0.019^{b}	-	0.619±0.052^{bc}
19	0.871±0.051^{e}	0.524±0.097^{k}	0.176±0.016^{b}	0.324±0.019^{hi}	2.288±0.192^{de}	0.279±0.052^{def}
20	0.160±0.051^{hi}	0.493±0.097^{k}	0.015±0.016^{def}	0.312±0.019$_{ij}$	1.047±0.192^{g}h	0.279±0.052^{def}
21	0.009±0.051^{i}	3.471±0.097^{ab}	-	0.425±0.019^{defg}	-	3.116±0.052^{a}
22	0.013±0.051^{i}	1.080±0.097^{ij}	0.038±0.016^{def}	0.253±0.019^{jk}	3.173±0.192^{c}	0.252±0.052^{defg}
23	0.013±0.051^{i}	0.018±0.097^{l}	0.002±0.016^{f}	0.046±0.019^{mn}	0.037±0.192^{j}	0.008±0.052^{i}
24	3.555±0.051^{a}	1.692±0.097^{g}	-	0.393±0.019^{fg}	-	0.391±0.052^{d}

–: without activity. Values in the same column followed by the same letter are not significantly different ($P < 0.05$) by Tukey's multiple range test.

Table 3.3. Spearman's correlation coefficients between total phenols and flavonoids and enzyme inhibitory activities

	Total phenol	Flavone+Flavonol	Flavanone+Dihydroflavonol
Total phenol	1	0.965**	0.858**
Flavone+Flavonol	0.965**	1	0.850**
Flavanone+Dihydroflavonol	0.858**	0.850**	1
Acetylcholinesterase	-0.638**	-0.648**	-0.750**
α-Amylase	-0.663**	-0.653**	-0.631**
α-Glucosidase	-0.729**	-0.663**	-0.687**
Lipoxygenase	-0.714**	-0.697**	-0.863**
Tyrosinase	-0.698**	-0.634**	-0.568*
Xanthine oxidase	-0.718**	-0.666**	-0.674**

*Correlation is significant at the $P < 0.05$ level. **Correlation is significant at the $P < 0.01$ level.

3.3.3. Inhibition of α-amylase and α-glucosidase

Pancreatic α-amylase is an enzyme, such as α-glucosidase, of dietary carbohydrate digestion in humans. Inhibitors of α-amylase may be effective in retarding carbohydrate digestion and glucose absorption and, therefore, in suppressing postprandial hyperglycemia.[40]

α-Glucosidase is located in the brush border surface of the intestinal cell and catalyses the final step in the digestive process of carbohydrates, releasing absorbable monosaccharides. This process is responsible for the increased blood glucose levels.[12] When that enzyme is inhibited, the liberation of D-glucose from dietary complex carbohydrates can be retarded and may delay the digestion and absorption of carbohydrates and restrain postprandial hyperglycaemic episode.[12]

Concerning α-amylase, samples 1 and 23 had the lowest IC_{50} values (0.065 and 0.018 mg mL^{-1}, respectively), although not significantly different to those found for samples 10 and 17 (0.17 and 0.146 mg mL^{-1}, respectively) (Table 3.2). Such as observed for α-glucosidase, the α-amylase inhibitor activity of propolis extracts, expressed as IC_{50} values, is inversely correlated with total phenols and flavonoid concentrations ($P < 0.01$) (Table 3.3). Caffeoylquinic acid derivatives as well as flavonoids from Brazilian propolis have been reported as being able to inhibit both α-glucosidase and α-amylase activities and, therefore, with hypoglycaemic property.[14,40]

Sample 23 (geographical origin unknown) was the best inhibitor of α-glucosidase (IC_{50} = 0.002 mg mL^{-1}), immediately followed by the samples 1 (Sefrou) and 10 (geographical origin unknown) (IC_{50} = 0.006 and 0.007 mg mL^{-1}, respectively) (Table 3.2). Such as reported for the inhibitory activities reported above, also in this case, the activity was inversely correlated with the amounts of total phenols and flavonoids (Table 3.3). The inhibitory activity of propolis extracts against α-glucosidase was already reported in other propolis extracts of different geographical origins.[12,13] Although two samples of propolis studied by Popova et al.[13] were from the same city of those reported in the present work (2 and 9: Moulay Bousselhame; 8: Rabat), their activities were different. For the sample from Moulay Bousselham, the inhibitory activities, expressed as IC_{50} values, of α-glucosidase and α-amylase were 0.07 and 0.52 mg mL^{-1}, respectively, while in the present work such IC_{50} values for α-glucosidase inhibitory activities ranged from without activity (sample 2) up to 0.027 mg mL^{-1} (sample 9). Concerning α-amylase inhibitory activities, IC_{50}values were 2.0–2.1 mg mL^{-1} for samples 2 and 9, respectively. For the sample from Rabat, Popova et al.[13] found 0.010 and 0.090 mg mL^{-1} as IC_{50} values for α-glucosidase and α-amylase inhibitory activities,

respectively, that is lower than those observed for sample 8 (0.032 and 1.36 mg mL^{-1}) (Table 3.2). The lowest IC_{50} values found in the present work are similar to those reported by Zhang et al., [12] for aqueous ethanol extracts of Brazilian propolis. The hypoglycemic activity of propolis when mixed with mulberry leaf extract was already reported by Murata et al.[41] after the administration to patients with type 2 diabetes.

3.3.4. Inhibition of lipoxygenase

After the oxidation of arachidonic acid, 5-lipoxygenase is responsible for the formation of the proinflammatory leukotrienes. Linoleic acid is also a substrate for this enzyme, which is enzymatically converted by 5-lipoxygenase to a conjugated diene, which results in a continuous increase in absorbance at 234 nm.[42]

Sample 1 had the lowest IC_{50} value (0.020 mg mL^{-1}) – so that it had the ability to inhibit 5-lipoxygenase – immediately followed by the sample 17 and sample 23 (IC_{50} = 0.065 and 0.046 mg mL^{-1}, respectively) (Table 3.2). These values are significantly lower than those previously reported by Miguel et al.[2] for Moroccan propolis extracts from different origins. Although the samples possess the same harvesting place (Moulay Bousselham, Rabat, Sidi Sliman, Taza, Sefrou and Kenitra) as the samples investigated by Miguel et al.[2], only that from Rabat had higher IC_{50} value. A negative correlation was observed between the IC_{50} values and the concentration of total phenols and flavonoids (Table 3.3), particularly flavanones and dihydroflavonols. This type of correlation was already reported for Moroccan propolis[2]. Mirzoeva & Calder[17] found that propolis and its components caffeic acid phenethyl ester (CAPE), caffeic acid (CA), quercetin and naringenin significantly suppressed the lipoxygenase pathway of arachidonic acid metabolism during inflammation *in vivo*. However, CAPE was the most potent modulator of the arachidonic acid metabolism among the propolis components studied by the authors. However, according to Araújo et al.,[43] the anti-inflammatory activity of propolis seems to be particularly associated with the presence of flavonoids, especially galangin and quercetin. These flavonoids have been shown to inhibit the activity of lipoxygenase, among other enzymes and factors responsible for inflammation processes: inhibition of cyclooxygenase, reduction in the levels of PGE_2 as well as in the release and expression of the induced isoform cyclooxygenase-2 (COX-2). In the present work, the compounds were not identified; nevertheless, the correlation between the inhibitory activity and flavanones and dihydroflavonol contents was strong compared to the remaining correlations between phenol content and inhibitory activity, which may suppose to have higher importance of those kinds of compounds in the lipoxygenase inhibitory activity.

3.3.5. Inhibition of tyrosinase

The tyrosinase inhibition by propolis samples is depicted in Table 3.2 Samples 1 and 23 were the most active against this enzyme, with IC_{50} values of 0.050 and 0.037 mg mL^{-1}, respectively. These values were not significantly different (Table 3.2). An inverse correlation was observed between the inhibition activity presented as IC_{50} values and the concentrations of total phenols and flavonoids, demonstrating that these compounds have an important role in the inhibition of tyrosinase (Table 3.3). Some flavonoids have demonstrated to be able to inhibit mushroom tyrosinase by the chelation of active sites. Examples include the chelation reactions between the vicinal 3′,4′-dihydroxyl group of catechin and the Cu^{2+} on tyrosinase, or still the structure of 3-hydroxy-4-keto moiety in some flavonoids, such as quercetin.[44] Other studies revealed that caffeic acid phenethyl ester suppresses melanogenic enzyme expression, but not via a direct inhibition of tyrosinase activity itself.[45]

3.3.6. Inhibition of xanthine oxidase

The inhibitory activity expressed as IC_{50} values is presented in Table 3.2. Without significant differences ($P < 0.05$), the propolis samples 1, 10, 17 and 23 possessed the lowest IC_{50} values (0.014, 0.031, 0.017 and 0.008 mg mL^{-1}, respectively) and therefore are considered as the best ones for inhibiting xanthine oxidase activity (Table 3.2). As reported for the other inhibitory enzyme activities, there was an inverse correlation between IC_{50} values and the amounts of phenols and flavonoids (Table 3.3). According to Cos et al.,[26] flavonoids possess the capacity for inhibiting xanthine oxidase – mainly those showing the hydroxyl groups at C-5 and C-7 and the double bond between C-2 and C-3 – and found that the presence of a hydroxyl group at C-3 would slightly decrease the inhibitory activity. Yoshizumi et al.[28] reported that propolis from China with relatively high amounts of chrysin, galangin and caffeic acid phenethyl ester possessed higher inhibitory activity than the Brazilian propolis with higher amounts of *p*-coumaric acid and artepillin C and also concluded that a continuous intake of propolis could be effective for the prevention and the treatment of gout and hyperuricaemia.

3.3.7. Inhibition of hyaluronidase

Figure 3.1 presents the percentage of inhibition of hyaluronidase obtained from Moroccan propolis extracts when at a final concentration of 33.33 mg mL^{-1}. Samples 1, 8, 10, 14, 17, 20 and 22 had percentages of inhibition higher than 80%, whereas the samples 7, 12 and 21 even at those relatively high concentrations (33.33 mg mL^{-1}) did not present the ability for inhibiting the hyaluronidase enzyme.

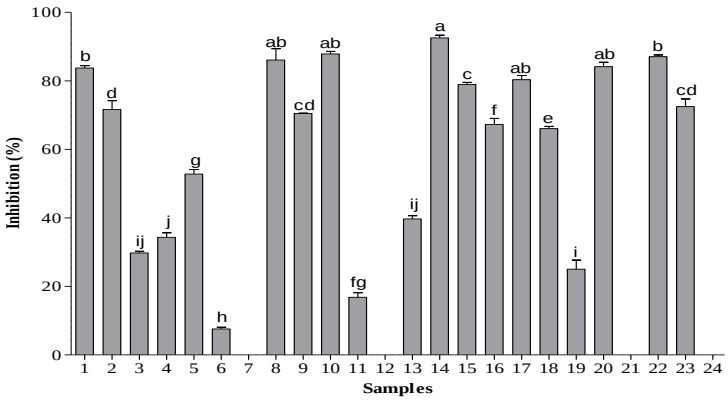


Figure 3.1. Percentage of hyaluronidase inhibition obtained from propolis extracts (33.33 mg/mL) collected at different places of morocco. Bars represent standard deviations (n=3). In many cases, such bars may not be visible due to the very low standard deviations. Values with the same letter are not significantly different (P<0.05) by the Tukey's multiple range test.

The capacity of Portuguese propolis extracts for inhibiting hyaluronidase was already reported.[7] The values found by these authors were also dependent on the place of harvesting, such as observed by us in the present work. Silva et al.[7] did not find a correlation between phenol contents and activity, suggesting that other components present in the propolis extracts, such as proteins and vitamins, might also be involved in the activity.

3.3.8. Statistical analysis

A combination of cluster analysis using Ward's technique (Figure 3.2a) revealed the presence of three principal clusters with respect to the phenol, flavone, flavonol, flavanone, dihydroflavonol content, α-amylase and xanthine oxidase inhibitory activities of 24 samples. Samples belonging to cluster 1 included propolis sample 1 (region Fez-Boulemane), 10 (purchased from the market), 17 (region Fez-Boulemane) and 23 (purchased from the market). These samples had the highest concentration of all types of phenols as well as the highest inhibitory activities of xanthine oxidase and α-amylase. Cluster 2 included samples 2–7, 9, 11-13, 18, 19, 21 and 24, which possessed the lowest activities as well as the lowest amounts of all types of phenols. The harvesting places for these propolis samples are widespread all over the country. Samples 8 (region Rabat), 14 (region Gharb), 15 (region Rabat), 16 (region Gharb), 20 and 22 (both from Fez-Boulemane) possessed intermediate concentrations of phenols and inhibitory activities. Some of these samples (14 and 16) were collected in regions of Gharb in which other samples were also harvested and had lower amounts of phenols and lower activities (samples 9 and 13 from cluster 2) and therefore belonged to the cluster 2. Samples in cluster 3 (samples 20 and 22, which were harvested in Fez-Boulemane region) and the samples 1 and 17 in cluster 1 had the best inhibitory activities as well as the highest phenol content (Tables 3.1 and 3.2).

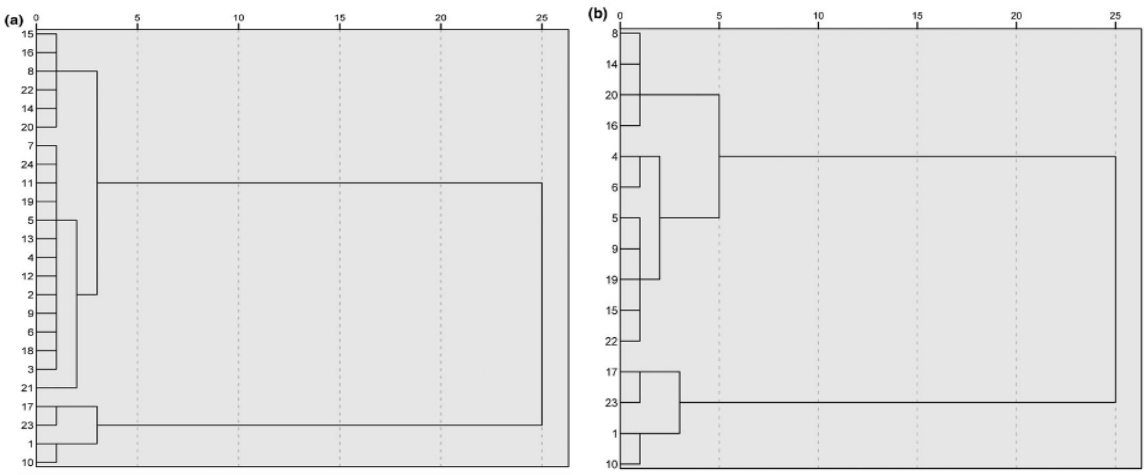


Figure 3.2. (a) Dendrogram obtained from the cluster analysis of 24 samples of propolis from Morocco with respect to the total phenols, flavones and flavonols, flavanones and dihydroflavonols contents and the capacity to inhibit xanthine oxidase and α-amylase. Samples were clustered using Ward's technique with the squared Euclidean distance measure. (b) Dendrogram obtained from the cluster analysis of 15 samples of propolis from Morocco with respect to the total phenols, flavones and flavonols, flavanones and dihydroflavonols contents and the capacity to inhibit acetylcholinesterase, α-glucosidase, lipoxygenase and tyrosinase. Samples were clustered using Ward's technique with the squared Euclidean distance measure.

Three principal clusters with respect to the phenol, flavone, flavonol, flavanone, dihydroflavonol contents and α-glucosidase, lipoxygenase, acetylcholinesterase and tyrosinase inhibitory activities (Figure 3.2b) were found for 15 samples. Samples 1, 10, 17 and 23 belonged to cluster 1; samples 4–6, 9, 15, 19 and 22 belonged to cluster 2; and samples 8, 14, 16 and 20 belonged to cluster 3. The distribution of samples by clusters was the same as that of reported above. A drawback of the present work is the limited number of samples, which makes it difficult to draw conclusions; nevertheless, it is possible to find propolis samples in the same region having dissimilar phenol contents and enzyme inhibitory activities. In addition, great part of samples had relatively small amounts of phenols and consequently weak enzyme inhibitory activities. In this way, the activities correlated well with the phenol content, but in the same region, it was possible to find the samples with dissimilar properties.

3.4. Conclusion

Propolis samples from different places of Morocco had diverse amounts of total phenols, flavones, flavonols, flavanones and dihydroflavonols. Those samples with higher concentrations of these groups of compounds generally had the best capacity to inhibit the activities of acetylcholinesterase, α-amylase and α-glucosidase, lipoxygenase, tyrosinase, xanthine oxidase and hyaluronidase. A correlation between harvesting place of propolis samples and phenol content or inhibitory enzyme activities was not found in the present work, because in the same place, samples with dissimilar phenol contents and enzyme activities were

found. In addition, dissimilar activities and phenol contents were also found in the three propolis samples purchased from the market, which in turn reveals the importance of normalization of these natural products because propolis sold in the market shows distinct efficacious activities, not consistent with the requirements of quality, efficiency and safety that these products should have. Even in the absence of a deep chemical characterization of samples, it was possible to conclude the importance of polyphenols in inhibiting enzyme activities given the correlations found between polyphenol contents and activities. For the inhibitory activity of the enzymes studied in the present work, the results may suggest that a phenol normalization of the extracts may be enough, without the need for chemically characterizing them. Such results must be the target of further study for deeply evaluating the possibility to use these products in the combat of some diseases involving inflammation, hyperuricemia or gout, hyperglycemia, in those diseases characterized by a decline in cognitive function and mental atrophy, or as agents still for the treatment of hyperpigmentation of the human skin.

References

[1] M.G. Miguel, S. Nunes, S. A. Dandlen, A. M. Cavaco, M.D. Antunes, 'Phenols, flavonoids and antioxidant activity of aqueous and methanolic extracts of propolis (*Apis mellifera* L.) from Algarve, South Portugal', Food Sci. Technol (Campinas). 2014a, 34, 16-23.

[2] M.G. Miguel, O. Doughmi, S. Aazza, D. Antunes, B. Lyoussi, B, 'Antioxidant, anti-inflammatory and acetylcholinesterase inhibitory activities of propolis from different regions of Morocco', Food. Sci. Biotechnol. 2014b, 23, 313-322.

[3] A. Salatino,C. C. Fernandes-Silva, A. A. Righi, M. L. F. Salatino. 'Propolis research and the chemistry of plant products', Nat. Prod. Rep. 2011, 28, 925–936.

[4] S. Silici, S. Kutluca,'Chemical composition and antibacterial activity of propolis collected by three different races of honeybees in the same region', J. Ethnopharmacol. 2005, 99, 69–7

[5] Y.. Boufadi, J. Soubhye, A. Riazi, A. Rousseau, M. Vanhaeverbeek, J. Nève, Z. Boudjeltia, V. Antwerpen,'Characterization and Antioxidant Properties of Six Algerian Propolis Extracts: Ethyl Acetate Extracts Inhibit Myeloperoxidase Activity', Int. J. Mol. Sci. 2014, 15, 2327-2345.

[6] S. Huang, C. P. Zhang, K. Wang, G.Q. Li, F. L. Hu, ' Recent advances in the chemical composition of propolis', Molecules. 2014, 19, 19610–19632.

[7] R. Silva-Carvalho, F. Baltazar, C. Almeida-Aguiar, 'Propolis: a complex natural product with a plethora of biological activities that can be explored for drug development', Evid Based. Complement. Alternat. Med. 2015, 29, Article ID 206439.

[8] Y. Kang, Y. Lee, A. Koo, J.S. Yoon, S.W. Lim, Y.C. Kim, S.H. Sung 'ESP-102, a standardized combined extract of *Angelica gigas*, *Saururus chinensis* and *Schizandra chinensis*, significantly improved scopolamine-induced memory impairment in mice', Life. Sci. 2005, 76, 1691-1705.

[9] J. Chen, Y. Long, M. Han, T. Wang, Q. Chen, R.Wang,'Water-soluble derivative of propolis mitigates scopolamine-induced learning and memory impairment in mice', Pharmacol. Biochem. Behav. 2008, 90, 441-446.

[10] L. Scotti, T. Scotti, 'Computer aided drug design studies in the discovery of secondary metabolites targeted against age-related neurodegenerative diseases', Curr Top Med Chem. 2015, 15, 2239-2252.

[11] A. Murai, K. Iwamura, M. Takada, K. Ogawa, T. Usui, J. I. Okumura, 'Control of postprandial hyperglycaemia by galactosylmaltobionolactone and its novel anti-amylase effect in mice', Life. Sci. 2002, 71, 1405– 1415.

[12] H. Zhang, G.Wang, T. Beta, J. Dong, 'Inhibitory properties of aqueous ethanol extracts of propolis on alpha-glucosidase', *Evid* Based. Complement. Alternat. Med. http://dx.doi.org/10.1155/2015/587383. Accessed on 17 August 2015, Article ID 587383, 7p

[13] M. Popova, B. Lyoussi, S. Aazza, V. Bankova, G. Miguel,'Antioxidant and α-glucosidase inhibitory properties and chemical profiles of Moroccan propolis', Nat. Prod. Commun. 2015, 10, 1961-1964.

[14] Matsui, T., S. Ebuchi, T. Fujise, K. J. M. Abensudara, S. Doi, H. Yamada, K. Matsumoto, 'Strong antihyperglicemic effects of water-soluble fraction of Brazilian propolis and its bioactive constituent 3,4,5-tri-*O*-caffeoylquinic acid', Biol. Pharm. Bull. 2004, 27, 1797-1803.

[15] E. R. Sailer, L. R. Subramanian, B. Rall, R. F. Hoernhein, H. P. T. Ammon, H. Safayhi, 'Acetyl-11-keto-β-boswellic acid (AKBA): structure requirements for binding and 5-lipoxygenase inhibitory activity', Br. J. Pharmacol.1996, 117, 615-618.

[16] G. F. Sudina, O. K. Mirzoeva, M. A. Pushkareva, G.A. Korshunova, N. V. Sumbatyan, S. D. Varfolomeev, 'Caffeic acid phenethyl ester as a lipoxygenase inhibitor with antioxidant properties', FEBS Lett. 1993, 329, 21– 24.

[17] K. Mirzoeva, P. Calder, 'The effect of propolis and its components on eicosanoid production during the inflammatory response', Prostaglandins Leukot Essent Fat Acids. 1996, 55, 441-449.

[18] M. Touaibia, M. Guay, 'Natural product total synthesis in the organic laboratory: total synthesis of acffeic acid and phenethyl ester (CAPE), a potent 5-lipoxygenase inhibitor from honeybee hives', J. Chem. Soc. 2011, 88, 473-745.

[19] S. H. Moon, K. T. Kim, N. K. Lee, Y. S. Han, Y. S. Nah, S. G. Cho, Y. S. Park, H. D. Park, 'Inhibitory effects of naringenin and its novel derivatives on hyaluronidase', Food. Sci. Biotechnol. 2009, 18, 267-270.

[20] J. C. Silva, S. Rodrigues, X. Feás, L. M. tevinho, 'Antimicrobial activity, phenolic profile and role in the inflammation of propolis', Food. Chem. Toxicol. 2012, 50, 1790-1795.

[21] H. Miyataka, M. Nishiki, H. Matsumoto, T. Fujimoto, M. Matsuka, T. Satoh, 'Evaluation of propolis. 1. Evaluation of Brazilian and Chinese propolis by enzymatic and physico-chemical methods', Biol. Pharm. Bull. 1997. 20, 496-501.

[22] Y. K. Park, M. Ikegaki, 'Preparation of water and ethanolic extracts of propolis and evaluation of the preparations', Biosci. Biotechnol. Biochem. 1998, 62, 2230-2232.

[23] O. Nerya, J. Vaya, R. Musa, S. Izrael, R. Ben-Artie, S. Tamir, 'Glabrene and isoliquiritigenin as tyrosinase inhibitors from licorice roots' J. Agric. Food Chem. 2003, 51, 1201-1207.

[24] Y. Dong, M. Zhao, T. Zhao, M. Feng, H. Chen, M. Zhuang, L. Lin, 'Bioactive profiles, antioxidant activities, nitrite scavenging capacities and protective effects on H_2O_2-injured PC12 cells of *Glycyrrhiza glabra* L. leaf and root extracts', Molecules. 2014, 19, 9101-9113.

[25] S.bTazawa, T. Warashina, T. Noro, 'Tyrosinase inhibitors of Brazilian propolis. J. Nat. Med. 2001, 55, 111-118.

[26] P. Cos, L. Ying, M. Calomme, J. P. Hu, K. Cimanga, B. V. Poel, B., L. Pieters, A. J. Vlietinck, D.V. Berghe, 'Structure-activity relationship and classification of flavonoids as inhibitors of xanthine oxidase and superoxide scavengers', J. Nat. Prod. 1998, 61, 71-76.

[27] A. Ilhan, O. Akyol, A. Gurel, F. Armutcu, M. Iraz, E. Oztas, 'Protective effects of caffeic acid phenetyl ester against experimental allergic encephalomyelitis-induced oxidative stress in rats', Free. Radical. Bio. Med. 2004, 37, 386-394.

[28] K. Yoshizumi, N. Nishioka, T. Tsuji, 'The xanthine oxidase inhibitory activity and hypouricemia effect of the propolis on rats', Yakugaku Zasshi. 2005, 125, 315-321.

[29] F. Armutcu, S. Akyol, S. Ustunsoy, F. F. Turan, 'Therapeutical potencial of caffeic acid phenetyl ester and its anti-inflammatory and immunomodulatory effects (review)', Exp. Ther. Med. 2015, 9, 1582-1588.

[30] H. A. Mouse, M. Tilaoui, A. Jaafari, L. Ait M'barek, R. Aboufatima, A. Chait, A. Zyad, 'Evaluation of the *in vitro* and *in vivo* anticancer properties of Moroccan propolis extracts', Rev. Bras. Farmacogn. 2012, 22, 558– 567.

[31] I. Gülcin, S. Beydemir, I. G. Sat, O. I. Kufreviogl, 'Evaluation of antioxidant activity of cornelian cherry (*Cornus mas* L.)', Acta Aliment Hung. 2005, 34, 193-202.

[32] M. G. Miguel, S. Nunes, S. A. Dandlen, A. M. Cavaco, M. D. Antunes, 'Phenols and antioxidant activities of hydro-alcoholic extracts of propolis from Algarve, South of Portugal', Food. Chem. Toxicol. 2010, 48, 3418-3423.

[33] M. Popova, V. Bankova, D. Butovska, 'Validated methods for the quantification of biologically active constituents of poplar-type propolis', Phytochem. Anal. 2004, 15, 235–240.

[34] A. T. Mata, C. Proença, A. R. Ferreira, M. L. M. Serralheiro, J. M. F. Nogueira, M. E. M. Araújo, 'Antioxidant and antiacetylcholinesterase activities of five plants used as Portuguese food spices', Food. Chem. 2007, 103, 778-786.

[35] N. Uddin, M. R. Hasan, M. M. Hossain, A. Sarker, A. H. Hasan, A. F. Islam, M. M. Chowdhury, M. S. Rana, '*In vitro* α-amylase inhibitory activity and in vivo hypoglycemic effect of methanol extract of *Citrus macroptera* Montr. Fruit', Asian. Pac. J. Trop. Biomed. 2014, 4, 473-479.

[36] Y. Frum, A. M. Viljoen, '*In vitro* 5-lipoxygenase activity of three indigenous South African aromatic plants used in traditional healing and the stereospecific activity of limonene in the 5-lipoxygenase assay', J. Essent. Oil. Res. 2006, 18, 85-88.

[37] F. El-Hady, M.S. Abdel-Aziz, K.H. Shaker, Z. A. El-Shahid, 'Tyrosinase, acetylcholinesterase inhibitory potential, antioxidant and antimicrobial activities of sponge derived fungi with correlation to their GC/MS analysis', Int J Pharm Sci Rev Res. 2014, 26, 338– 345.

[38] M. Umamaheswari, A. Madeswaran, K. Asokkumar, 'Virtual Screening Analysis and *in-vitro* xanthine oxidase inhibitory activity of some commercially available flavonoids', Iran. J. Pharm. Res. 2013, 12, 317-323.

[39] A. Sahasrabudhe, M. Deodhar, 'Anti-hyaluronidase, anti-elastase activity of *Garcinia indica*', Int. J. Botany. 2010, 6, 299-303.

[40] K. Tadera, Y. Minami, K. Takamatsu, T. Matsuoka, 'Inhibition of α-glucosidase and α-amylase by flavonoids', J. Nutr. Sci. Vitaminol. 2006, 52, 149-152.

[41] K. Murata, K. Yatsunami, E. Fukuda, S. Onodera, O. Mizukami, G. Hoshino, T. Kamei, 'Antihyperglycemic effects of propolis mixed with mulberry leaf extract to patients with type 2 diabetes', Altern. Ther. 2004, 10, 78-79.

[42] M.G. Miguel, C. Cruz, M. L. Faleiro, M. T. F. Simões, A. C. Figueiredo, J. G. Barroso, L. G. Pedro, 'Salvia officinalis L. essential oils: effect of hydrodistillation, time on the chemical composition, antioxidant and antimicrobial activities. Nat. Prod. Res. 2011, 25, 526-541.

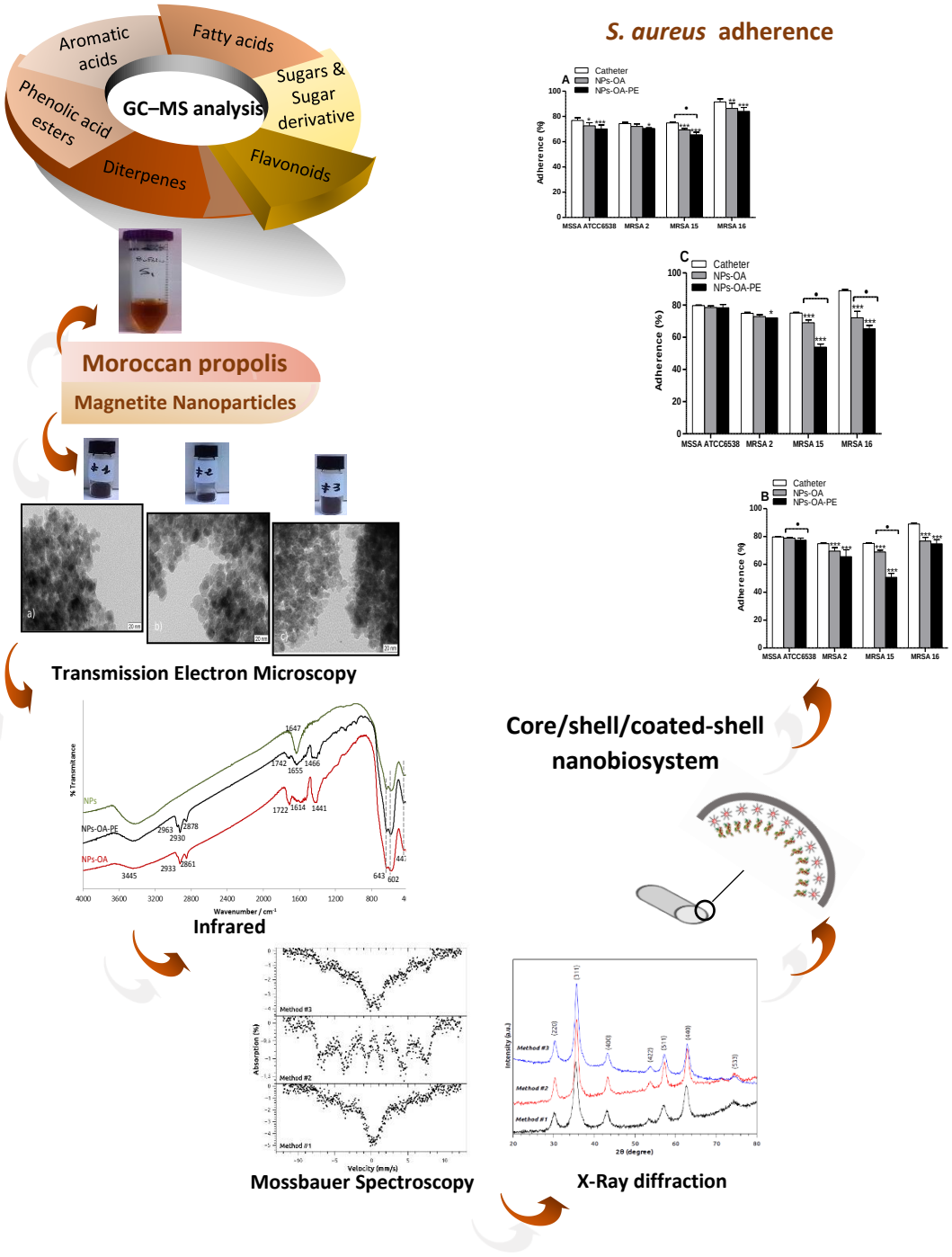
[43] M. A. R. Araújo, S. A. Libério, R. N. M. Guerra, M. N. S. Ribeiro, F. R. F. Nascimento, 'Mechanisms of action underlying the anti-inflammatory and immunomodulatory effects of propolis: a brief review', Rev. Bras. Farmacogn. 2012, 22, 208-219.

[44] B. Fu, H. Li, X. Wang, F. S. C. Lee, S. Cui, 'Isolation and identification of flavonoids in licorice and a study of their inhibitory effects on tyrosinase', J. Agric. Food Chem. 2005, 53, 7408-7414.

[45] J. Y. Lee, H. J. Choi, T. W. Chung, C. H. Kim, H. S. Jeong, K. T. Ha, 'Caffeic acid phenetyl ester inhibits alpha-melanocyte stimulating hormone-induced melanin synthesis through suppressing transactivation activity of microphthalmia-associated transcription factor', J. Nat. Prod. 2013, 76, 1399-1405

Chapter 4

Impact of Biohybrid Magnetite Nanoparticles and Moroccan Propolis on Adherence of Methicillin Resistant Strains of *Staphylococcus aureus*



Impact of Biohybrid Magnetite Nanoparticles and Moroccan Propolis on Adherence of Methicillin Resistant Strains of Staphylococcus aureus*

Abstract

Biofilm bacteria are more resistant to antibiotics than planktonic cells. Propolis possesses antimicrobial activity. Generally, nanoparticles containing heavy metals possess antimicrobial and antibiofilm properties. In this study, the ability of adherence of Methicillin Resistant Strains of *Staphylococcus aureus* (MRSA) to catheters treated with magnetite nanoparticles (MNPs), produced by three methods and functionalized with oleic acid and a hydro-alcoholic extract of propolis from Morocco, was evaluated. The chemical composition of propolis was established by gas chromatography mass spectrometry (GC-MS), and the fabricated nanostructures characterized by X-ray diffraction (XRD), transmission electron microscopy (TEM), Mossbauer spectroscopy and fourrier transform infrared spectroscopy (FTIR). The capacity for impairing biofilm formation was dependent on the strain, as well as on the mode of production of MNPs. The co-precipitation method of MNPs fabrication using Fe^{3+} and Na_2SO_3 solution and functionalized with oleic acid and propolis was the most effective in the impairment of adherence of all MRSA strains to catheters ($p < 0.001$). The adherence of the strain MRSA16 was also significantly lower ($p < 0.001$) when the catheters were treated with the hybrid MNPs with oleic acid produced by a hydrothermal method. The anti-MRSA observed can be attributed to the presence of benzyl caffeate, pinocembrin, galangin, and isocupressic acid in propolis extract, along with MNPs. However, for MRSA16, the impairment of its adherence on catheters may only be attributed to the hybrid MNPs with oleic acid, since very small amount, if any at all of propolis compounds were added to the MNPs.

*Adapted from 'Impact of Biohybrid Magnetite Nanoparticles and Moroccan Propolis on Adherence of Methicillin Resistant Strains of *Staphylococcus aureus*'. Soukaïna El-Guendouz, Smail Aazza, Badiaa Lyoussi, Vassya Bankova, João P. Lourenço, Ana M. Rosa Costa, José F. Mariano, Maria G. Miguel, and Maria L. Faleiro. Molecules. 2016 Sep; 21(9): 1208.

4.1. Introduction

Propolis is a natural resinous substance collected by honeybees from parts of plants. buds. and exudates. mixed with bees wax and salivary enzymes. Propolis is mainly constituted by resin (50%), wax (30%), essential oils (10%), pollen (5%), and other substances (5%), such as debris, minerals and organic compounds, despite the chemical variability that can be found. Such differences can be attributed to the different plants from where bees can collect the materials. as well as the different harvesting seasons of propolis. Polyphenols, terpenoids including steroids, naphthalene and stilbene derivatives, and fatty acids are some examples of organic compounds that can be found in propolis.[1,2] Different biological properties have been attributed to propolis, such as antimicrobial, anti-inflammatory, antioxidant, antidiabetic, spasmolytic, anaesthetic, anticancer, and immunomodulatory effects.[2,3]

Staphylococcus aureus is a Gram-positive bacterium that mainly colonizes the nares and is considered a human pathogen that shows increasing incidence in patients with prolonged hospitalization, submitted to intravenous drugs or under treatment with enteral feedings or dialysis, in postoperative surgical wounds in decubitus ulcers or in indwelling catheters.[4-6] Its incidence is increasing also in the community.[7] Over time, *Staphylococcus aureus* has developed resistance to several antibiotics and. presently. methicillin resistant strains (MRSA) are causing serious infections in hospitals or even in the community.[8,9] For these reasons, alternative approaches are required to fight this bacterial pathogen, and one of them is the use of natural products. Propolis comes as a natural candidate due to its multidirectional mechanism of action on bacterial cells, which renders the development of resistance unlikely.[10] The utilization of propolis extracts has already been assayed as anti-MRSA agents, as is shown in Table 4.1. which compiles the results of several authors who observed anti-MRSA activity in propolis of diverse origins.

Biofilm bacteria (adherent bacterial cells) are more resistant to antibiotics, cleaning and eradication than planktonic cells (cells in suspension). The percentage of infections associated with biofilm formation in medical devices is more than 25%. These infections cause great mortality and morbidity rates in patients with indwelling or implanted devices, with the consequent increasing of costs due to the long-term treatments.[11]

Different types of nanoparticles have been reported as possessing antimicrobial and antibiofilm properties, particularly those containing heavy metals.[12] Iron oxide magnetic nanoparticles were approved by FDA for biomedical uses, because they occur naturally in liver. spleen and human heart, consequently, they will be non-toxic and biocompatible at

physiological concentrations. In addition, the production of these nanoparticles is not difficult and they are chemically stable under physiological conditions. They are also easily functionalized. Functionalized iron oxide magnetic nanoparticles, such as magnetite nanoparticles (MNPs) have been evaluated for inhibiting microbial growth and biofilm formation of yeasts (*Candida albicans*, *C. tropicalis*, *C. krusei*, *C. glabrata*, and *Saccharomyces cerevisiae*), and bacteria (*Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Enterococcus faecalis*).[13]

The majority of the methods used for synthesizing stable, biocompatible and monodispersed MNPs include co-precipitation, thermal decomposition, hydrothermal synthesis, microemulsion, and sonochemical synthesis. However, other methods have been developed and reported: electrochemical synthesis, laser pyrolysis techniques, and microorganism or bacterial synthesis.[14] In order to prevent that MNPs form aggregates, and for raising its biocompatibility, stability and biological application, such NPs may be functionalized with some biomolecules.[14] Oleic acid has been used alone or along with natural products for preventing biofilm formation of pathogenic microorganisms (*C. albicans*. *C. tropicalis*. *S. aureus*. and *E. coli*) or for promoting its disruption.[12,15,16]

The main goal of the present work was to evaluate the ability of a hybrid nanosystem constituted by magnetite nanoparticles, produced by three methods based on two methods (co-precipitation and hydrothermal synthesis) and functionalized with oleic acid and a hydro-alcoholic extract of propolis from Morocco to prevent the adherence of MRSA strains on catheters, and therefore, impair the formation of biofilm.

Table 4.1. Utilization of propolis extracts as anti-Methicillin Resistant Strains of *Staphylococcus aureus* (anti-MRSA) agents.

Sample	Sample Extraction/Compounds	Strains	MIC *(mg/mL); MBC**(mg/mL)	Major Compounds	References
Twenty-four batches of propolis collected over two years (2010 and 2011) from different places in France	1. Methanol (MeOH)	Six human pathogenic bacterial strains collected by the Laboratory of Bacteriology at the University Hospital, Center of Angers, France)	0.090 - >0.100; -	Pinobanksin-3-acetate.pinocembrin. chrysin. galangin. prenyl caffeate	[17]
	2. Dichloromethane (DCM)	The same reported above	0.057 – 0.097; -	The same reported above	
	3. Mixture of DCM, MeOH, H_2O (31/19/4)	The same reported above	0.030; > 0.100	The same reported above	
	Pinobanksin-3-acetate	MRSA (0706C0025) MRSA (0702E0196)	>0.100; - >0.100; -		
	Pinocembrin	MRSA (0706C0025) MRSA (0702E0196)	>0.100; - >0.100; -		
	Chrysin	MRSA (0706C0025) MRSA (0702E0196)	>0.100; - >0.100; -		
	Galangin	MRSA (0706C0025) MRSA (0702E0196)	>0.100; - >0.100; -		
	Prenyl caffeate	MRSA (0706C0025) MRSA (0702E0196)	0.070; - 0.070; -		
Propolis. collected at Moravia. Czech Republic	Special propolis extract GH2002 (see the reference for the extraction method)	Ten strains	0.13–0.25; 0.5–1	-	[18]
Propolis samples from an apiary in Kamianna near Nowy Sącz in southern Poland	Hydro-alcoholic (70%) extract of propolis	Five strains from blood clinical origin MRSA ATCC 43300	0.39–0.78; 0.78–3.13 0.78; 3.13	Pinocembrin; kaempferol; galangin; chrysin; apigenin; quercetin; gallic acid; ferullic acid; caffeic acid; caffeic acid phenethyl ester; p-coumaric acid and cinnamic acid	[10]
The crude propolis and their respective ethanol extracts were sourced from the city of União da Vitória – State of Paraná, Brazil, provided by Novo Mel®.	Ethanol extracts	Strains (clinical isolate) were obtained from the Bacterial Library of the Microbiology Laboratory, Department of Pathology, Santa Casa de São Paulo -School of Medical Sciences.	1.42;	3-[4-Hydroxy-3-(oxobutyl)-phenylacrylic acid; 3-prenyl-3(*E*)-(4-hydroxy-3-methyl-2-butenol)-5-prenylcinnamic acid; 3-prenyl-4-(2-methylpropionyloxi) cinnamic acid; 3-prenyl-4-dihydrocynamoiloxi-cinnamic acid; dihydrokaemferide; 3-prenyl-4-hydroxycinnamic acid. caffeic acid; caffeoylquinic acid 1; caffeoylquinic acid 2; caffeoylquinic acid 3; caffeoylquinic acid 4; caffeoylquinic acid 5; cinnamic acid; *p*-coumaric acid; kaempferide; kaempferol; betuletol; 2.2-dimethyl-6-carboxyethenyl-2*H*-1-benzopirane; 2.2-dimethyl-8-prenyl-	[19]

				2*H*-1-benzopirano-6-propenoic acid; (*E*)-3-{4-hydroxy-3-[(*E*)-4-(2.3)-dihydrocynamoiloxi-3-methyl-2-butenyl]-5-renylphenyl-2-propenoic acid; 3.4-dihydroxy-5-prenyl-cinnamic acid; 3.5-diprenyl-4-hydroxy-cinnamic acid	
Two Jordanian propolis samples from two locations: University of Jordan (Type I). and Al-Hashmeah (Type II)	Type I crude aqueous methanol extracts Type II crude aqueous methanol extracts Pinobanksin-3-O-acetate Pinocembrin	MRSA isolated from hospitalized patients at theJordan University	4.69;	-	[20]
	Type II crude aqueous methanol extracts	The same reported above	18.75		
	Pinobanksin-3-O-acetate Pinocembrin		0.25; 0.25		
The propolis was from Guadalcanal Province (The Solomon Islands) for BeeVital & Herbal Apothecary (Withby. UK) 'Pacific propolis'	Propolis extracted with 95% ethyl alcohol (EEP). A portion of EEP suspended in water/ethanol (10/1) was partitioned between n-hexane (HEX). ethyl acetate (EA). n-BuOH (BUT) and water (WAT). EA (1-16) fractions obtained from EA fractionated by gel fi ltration using Sephadex® LH-20-100	One hundred and twenty clinical MRSA isolates were collected from the clinical laboratories of the New Royal Infirmary (Edinburgh. UK)	EEP: 0.064 - 0.128; - HEX: 0.512; - EA: 0.064 - 0.128 BUT: 0.128 - 0.256 WAT: >0.512 EA 9–EA 15: 0.016–0.064	Prenylflavanones: propolin H, propolin G, propolin D, propolin C	[21]
Purchased as ethanolic extract	Ethanolic extract of propolis (P8904, EEP, pH 7.3, Sigma, St, Louis, MO, USA)	MRSA (ATCC 33591)	1.024; - Propolis plus mupirocin for treating nares of the rabbits infected by MRSA resulted in more profound reduction in bacterial cell count and inflammatory response compared with the rest of the treatment modalities without this conjugation	-	[22]
Three samples of propolis were obtained from Croatia: sample 5587 (Zagreb) and samples 5582 and 5581 (Imotski)	Hydro-alcoholic extract (80%) of propolis (EEP). Galangin	Ten strains of MRSA	Sample 5587: 1.06; 2.00 Sample 5582: 4.98; 9.37 Sample 5581: 1.19; 2.37 Galangin: 0.16; 0.27	Flavones. flavonols. flavanones. galangin	[23]

* MIC: Minimal Inhibitory Concentration; ** MBC: Minimum Bactericidal Concentration; -: not referred by the authors.

4.2. Experimental

4.2.1. Chemicals

Hydrochloric acid (HCl) and L-(+)-Ascorbic acid were purchased from Merck (Darmstadt, Germany). Na_2CO_3 and Na_2SO_3 were purchased from Riedel de Haen (Seelze, Germany). Chloroform ($CHCl_3$) was purchased from LAB-SCAN (Dublin, Ireland). Oleic acid and Iron (II) sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$) were purchased from Sigma Aldrich (Steinheim, Germany). Iron (III) chloride anhydrous was purchased from Fluka Chemicals (Buchs, Switzerland). NaOH was from Pronalab (Madalena, Portugal). Ethanol was from Panreac Quimica (Barcelona, Spain). The medium brain-heart infusion (BHI) and Bacteriological agar type E were purchased from Biokar Diagnostics (Beauvais, France).

4.2.2. Propolis extraction

One gram of propolis from Morocco (region of Fez-Boulmane) was chopped into small pieces and extracted by maceration using 30 mL of 70% ethanol and maintained for one week at 37 °C under agitation (200 rpm). The resulting solution was filtered under vacuum. A clear solution. without further purification. was used for successive analyses.

4.2.3. GC-MS analysis of propolis extract

The analysis was performed with a Hewlett-Packard gas chromatograph 5890 series II Plus linked to a Hewlett-Packard 5972 mass spectrometer (Hewlett-Packard, Wilmington, DE, USA) system equipped with a 30 m long. 0.25 mm i.d.. and 0.5 µm film thickness HP5-MS capillary column. The work conditions were the same as previously reported.[24] Semi-quantification was carried out by internal normalization with the area of each compound. The addition of individual areas of the compounds corresponds to 100% area. Compound identification was performed using commercial libraries and comparison of mass spectra and retention times of reference compounds.

4.2.4. Preparation of magnetite nanoparticles

Magnetite nanoparticles were synthesized by three different methods.

4.2.4.1. Method #1

The alkaline co-precipitation of iron salts in aqueous solutions, as described by Kang *et al.*,[25] with some modifications, was as follows: 0.43 mL of HCl 12 M was diluted with 12.5 mL of distilled water, and then 2.6 g of $FeCl_3$ and 2.4 g of $FeSO_4 \cdot 7H_2O$ (2:1 molar ratio) were both added under continuous magnetic stirring. The resulting solution was poured into 125 mL

of NaOH (1.5 M) under vigorous stirring, which was maintained for 30 min. The last step generated an instant black precipitate. The magnetism was checked with a NdFeB permanent magnet. The precipitate was attracted by the magnetic field, and the supernatant was removed by decantation. The recovered magnetite was washed with distilled water for three times and then twice with ethanol. The product was air-dried overnight and grinded into powder.

4.2.4.2. Method #2

The co-precipitation, according to the procedure of Qu et al.[26] slightly modified, was as follows: 30 mL of a 2 M $FeCl_3$ stock solution, prepared by dissolving the necessary amount of iron salt in 2 M HCl, were diluted with an equal volume of deionized water. Afterwards, 20 mL of a 1 M Na_2SO_3 solution were added under stirring. Just after the mixing of Fe^{3+} and SO_3^{2-}, the color of the solution altered from light yellow to red, indicating the formation of a complex ion. Meanwhile, an ammonia solution was prepared by the dilution of 50.8 mL of concentrated ammonia to a final volume of 800 mL. The former solution was quickly poured into the diluted ammonia solution, under vigorous stirring, as soon as its color changed back from red to yellow again, as the complex decomposed to Fe^{2+} and SO_4^{2-}. A black precipitate formed immediately, but stirring was continued for 30 min. A permanent magnet was applied to the beaker containing the suspension, and a black powder could be seen to quickly settle on the bottom. The supernatant was discarded and fresh water was added to the beaker. After decantation, the powder was washed one time with an acidic solution (pH 3–4), then with distilled water until neutral pH, once with ethanol, and finally left to air dry overnight.

4.2.4.3. Method #3

Water-soluble magnetite nanoparticles were obtained by a hydrothermal method, according to Xuan et al.,[27] using a ferric salt as single iron precursor, and ascorbic acid as reducing agent. The so synthesized super paramagnetic Fe_3O_4 nanocrystals are capped with dehydroascorbic acid (the oxidized form of ascorbic acid). In a typical experiment, 0.33 g of $FeCl_3$ were dissolved in 25 mL of H_2O, under continuous stirring. Then, 10 mL of 0.6 M Na_2CO_3 (0.6 M) were added, drop by drop and, 10 min later, 0.12 g ascorbic acid were also added. After being stirred for another 15 min, the solution was transferred to a 40 mL Teflon sealed autoclave. The autoclave was kept at 160 °C for 3 h and then let cool down to room temperature. The final product was separated from the reaction medium by using a NdFeB permanent magnet. A rinsing process including three cycles of decantation / washing / decantation in deionized water and in alcohol was performed before air drying overnight.

4.2.5. Preparation of functionalized magnetite nanoparticles

Each magnetite type was functionalized with 6 mL of oleic acid (OA), the mixture being heated up to 80 °C on a water bath, under intense stirring for 60 min. Excess oleic acid was phase separated by dropwise addition of 4 mL of water, followed by OA-coated magnetite washing twice with ethanol and kept to dry overnight.[28]

4.2.6. Synthesis of the hybrid core/shell/coated shell nanomaterial

Functionalized magnetite was added into 10 mL of $CHCl_3$ (0.33% *w*/*v*) and was sonicated for 30 min. One mL of propolis sample at the MIC value (0.36 mg/mL) was added into the same volume of the magnetite suspension.

4.2.7. Characterization of the nanomaterial

Characterization of the nanomaterial was carried out by X-ray powder diffraction (XRD). transmission electron microscopy (TEM) (Hitachi High-Technologies Corporation, Tokyo, Japan). ^{57}Fe Mossbauer spectroscopy, and Fourier Transform Infrared (FTIR) spectroscopy (Bruker Tensor 27, Billerica, MA, USA).

4.2.7.1. X-Ray diffraction (XRD)

The structure type of all the samples was checked by powder X-ray diffraction on a Panalytical X′Pert Pro diffractometer (PANalitycal, Almelo, The Netherlands) using Cu Ka radiation filtered by Ni and an X′Celerator detector. The equipment was operated at 45 kV and 30 mA and the patterns were recorded in the range 20–80° 2θ, with a step size of 0.05° and 1500 seconds per step and compared with the ICDD PDF-2 database.

4.2.7.2. Transmission electron microscopy (TEM)

Samples were prepared by drop drying a diluted colloidal solution of NPs in ethanol onto 200 mesh Formvar-coated copper grids. Samples were observed using a Hitachi 8100 (Hitachi High-Technologies Corporation, Tokyo, Japan), 200 kV, LaB6 filament analytical transmission electron microscope.

4.2.7.3. Mössbauer spectroscopy

^{57}Fe Mossbauer spectroscopy was performed at room temperature on a Wissel constant acceleration transmission mode spectrometer (Wissenschaftliche Elektronik (WissEl) GmbH, Starnberg, Germany), using a proportional counter as detector and a ^{57}Co/Rh source. The resulting spectra were calibrated with an α-Fe foil and WMOSS software[29] was used for the

quantitative evaluation of the spectral parameters (least-squares fitting to Lorentzian peaks). All isomeric shifts are reported relative to the centroid of the α-Fe spectrum.

4.2.7.4. Fourrier Tranform Infrared Spectrsocopy (FTIR)

Infrared spectra were recorded in the wave numbers range 4000–400 cm^{-1}, in a Bruker Tensor 27 Fourier-transform infrared (FT-IR) spectrophotometer (Bruker Tensor 27, Billerica, MA, USA), using KBr wafers.

4.2.7.5. Impact of the nanoparticles on bacterial adherence

Catheter pieces treated with functionalized magnetite and propolis were filled with 100 μL of an overnight bacterial culture MSSA ATCC 6538, MRSA 2, MRSA 15, and MRSA 16 (Table 4.2). The bacterial strains were cultivated in Brain Heart Infusion (BHI). For solid media, agar (VWR) was added at 1.5%. w/v. Bacteria were maintained in BHI with 25% (v/v) glycerol at −80 °C and, when necessary were recovered in BHI. Prior to use. bacteria were transferred to fresh BHI agar plates and incubated at 37 °C.

Table 4.2. *Staphylococcus aureus* strains.

Bacteria	**Origin**	**Source**
Staphylococcus aureus ATCC 6538 (MSSA ATCC 6538)	Wound	American Type Culture Collection
Staphylococcus aureus methicillin-resistant 2 (MRSA 2)	Clinical	UAlg. CBMR. Portugal
Staphylococcus aureus methicillin-resistant 15 (MRSA 15)	Clinical	UAlg. CBMR. Portugal
Staphylococcus aureus methicillin-resistant 16 (MRSA 16)	Clinical	UAlg. CBMR. Portugal

The previously obtained suspension of the nanomaterial was used to fabricate a modified surface in a prosthetic device. This was achieved by submerging the catheter pieces into the fluid in order to create a coating film. The catheter pieces were then dried at room temperature. This process of submerging and drying was repeated for five times of 10 s each, and the catheter pieces were sterilized by ultraviolet radiation for 20 min.

Catheter pieces treated with functionalized magnetite were filled with 100 μL of an overnight bacterial culture and left to adhere for 30 min at room temperature in a flow cabinet (Faster BH-EN 2005, Milan, Italy). Following the catheter pieces were transferred to Falcon tubes with 10 mL of Brain Heart Infusion (BHI) (Oxoid). Each tube was sonicated in a bath sonicator (J.P, SELECTA. Barcelona. Spain) for 5 min. and the catheter was immediately removed. The bacterial suspension was serially diluted using phosphate buffer saline (PBS) and

the viability was determined according to the Miles and Misra technique.[30] Catheters treated with non-functionalized magnetite and catheters not treated with magnetite were used as control.[12]

4.2.8. Statistical analysis

For the statistical interpretation GraphPad Prism statistical software was used, version 5.03 (GraphPad Software, La Jolla California USA). The average of six independent experiments were analyzed and compared with one-way ANOVA using Tukey′s Multiple Comparison Test of each group, followed by two-way ANOVA test for revealing significant differences among the analyzed group. Significant differences were indicated by *p* values less than 0.05.

4.3. Results and discussion

4.3.1. Chemical composition of propolis extract

Table 4.3 depicts the chemical composition of the hydro-alcoholic extract of propolis. Flavonoids (31.9%), diterpenes (21.5%), and phenolic acid esters (16.5%) were the main groups of compounds found in this extract. Only four components were present at concentrations higher than 5%: the diterpene isocupressic acid (8.1%) (**1**), and the three flavonoids pinocembrin (7.4%) (**2**), pinocembrin chalcone (5.9%) (**3**), and galangin (5.3%) (**4**). The phenolic acid ester benzyl caffeate (4.7%) (**5**) was present in the extract at a concentration very close to 5% (Table 4.3).

Table 4.3. Chemical composition of hydro-alcoholic extract of Moroccan propolis.

Aromatic Acids	**%**	**Phenolic Acid Esters**	**%**	**Flavonoids**	**%**	**Diterpenes**	**%**	**Sugars and Sugar Derivatives**	**%**	**Fatty Acids**	**%**	
Benzoic acid	0.4	Pentenyl *p*-coumarate	0.7	Pinostrobin chalcone	2.7	Ferruginol	1.2	Monosaccharides	0.4	Hexadecanoic acid	-	
Hidroxybenzoic acid	0.1	Isopentenyl caffeate	1.8	Pinocembrin chalcone	5.9	Communic acid	2.7	Disaccharides	-	Octadecanoic acid	1.0	
Cinnamic acid	0.3	Pentenyl caffeate	0.9	Pinocembrin	7.4	Totarol	1.1	Glycerol	0.1	Octadecenoic acid	0.5	
p-Coumaric acid	0.3	Dimethylallyl caffeate	1.2	Pinobanksin	3.6	Imbricataloic acid	3.2	Inositol	Tr	Tetracosanoic acid	-	
Dimethoxycinnamic acid	0.6	Pentenyl ferulate	0.9	Pinobanksin 3-*O*-acetate	3.4	13-*epi*-Cupressic acid	2.2	Total	0.5	Total	1.5	
Ferulic acid	0.4	Benzyl ferulate	1.7	Galangin	5.3	Ferruginolon	1.2					
Isoferulic acid	0.4	Benzyl *p*-coumarate	1.3	Chrysin	3.6	Dehydroabietic acid	Tr					
Caffeic acid	0.8	Benzyl caffeate	4.7	Total	31.9	Isocupressic acid	8.1					
Total	3.3	Caffeic acid phenetyl ester	1.7			Junicedric acid	1.8					
		Cinnamyl ferulate	0.4			Total	21.5					
		Cinnamyl caffeate	1.2									
		Total	16.5									
Standard deviation does not succeed 6% for any of the constituents												

4.3.2. Characterization of the nanomaterial

4.3.2.1. XRD

Figure 4.1 displays the XRD patterns of the non-functionalized nanoparticles synthesized by each of the three methods. The diffraction peaks present in the patterns of all the samples indicate magnetite as the sole crystalline phase. The smaller width of the peaks in the pattern of Method #2 suggests a larger size of the nanoparticles in that sample, in agreement with transmission electron microscopy (TEM) observations. Figure 4.2 represents the diffractograms of both functionalized and nonfunctionalized nanoparticles, synthesized by Method #1, where it is evident that the functionalization procedure does not change the structure and composition of the nanoparticles.

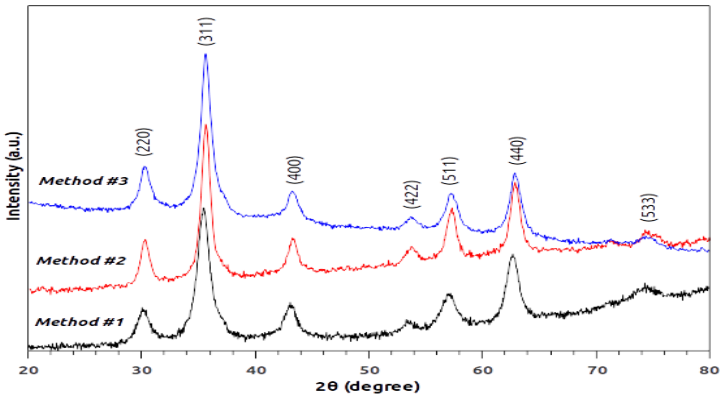


Figure 4.1. X-ray diffractograms of magnetite nanoparticles synthesized by the three methods. The labeled crystallographic planes refer to magnetite.

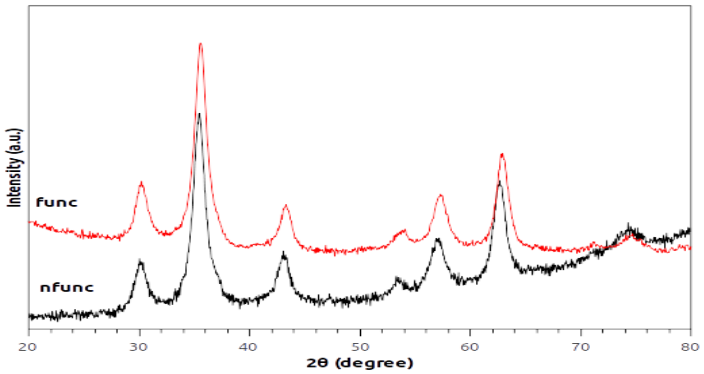


Figure 4.2. X-ray diffractograms of nonfunctionalized (unfunc) and functionalized with oleic acid (funct) magnetite nanoparticles, synthesized using Method #1.

4.3.2.2. TEM

This analysis was used to characterize the morphology of the nonfunctionalized nanoparticles and the results are presented in Figure 4.3 The images show that the three methods produced roughly spherical nanoparticles, although those produced by Method #2 are of more

regular shape, with diameters of about 15 nm, while Method #1 originates more angular shaped nanoparticles, with estimated sizes of 10 nm and Method #3 produces nanoparticles with approximately the same size but with a more regular spherical shape.

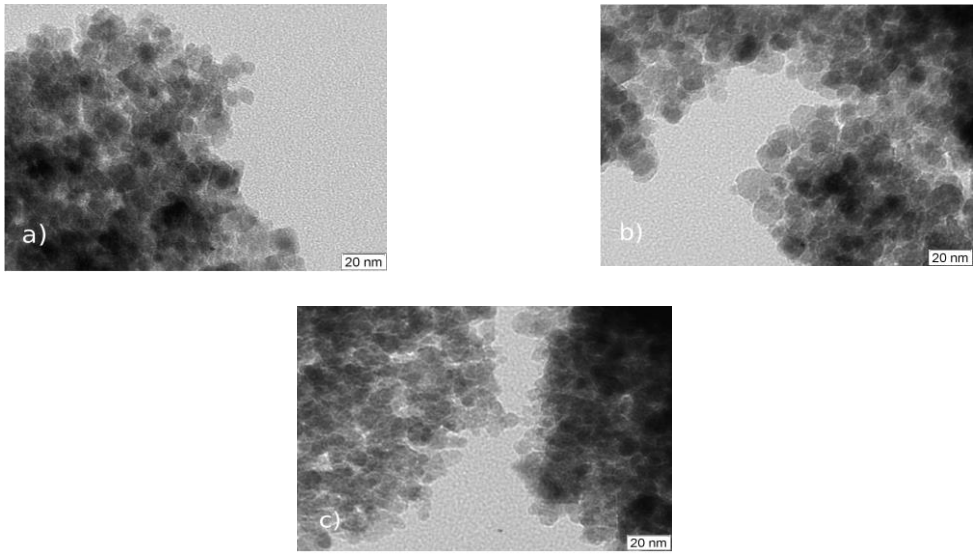


Figure 4.3. TEM micrograhphes of the nonfunctionalized nanoparticles sinthezised by Methods: #1 (**a**); #2 (**b**); and #3 (**c**).

4.3.2.3. Mössbauer Spectroscopy

In order to investigate the possible alteration of structure and composition by the functionalization procedure, two samples of magnetite synthesized by Method #1, with and without functionalization with oleic acid, were subject to Mössbauer analysis. Figure 4.4 shows the ^{57}Fe Mössbauer spectra of these samples. As can be seen, no apparent alteration on the spectra was introduced by the functionalization of the nanoparticles, which agrees with the XRD results.

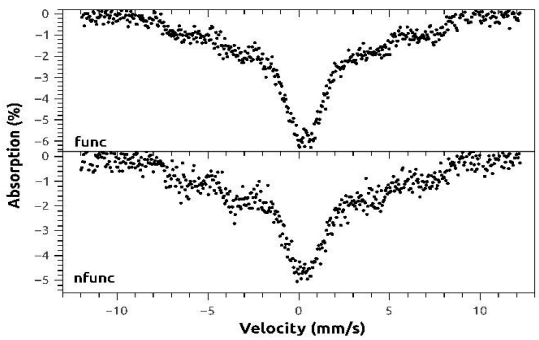


Figure 4.4. Room temperature ^{57}Fe Mössbauer spectra of magnetite nanoparticles. nonfunctionalized and functionalized with oleic acid. synthesized by Method #1.The acquisition time is different for the two spectra.

To further investigate the structure and composition of the as-synthesized MNPs, one sample of uncoated MNP synthesized by each of the three methods was analyzed by

transmission Mössbauer spectroscopy. The recorded spectra are shown in Figure 4.5. The room temperature spectra show the gradual passage from bulk-like to superparamagnetic (SPM) behavior, as the average size of the particles decrease. The Method #2 spectra displays a single sextet compatible with magnetite, with board lines indicating that these particles have a multidomain structure, although close to the single domain size limit. In the Method #3 sample, the central doublet evidences a predominant SPM size, with some contribution from the non-SPM particles sextet. correlating to a size distribution with smaller average particle size. Sample from Method #1 exhibits a similar behavior. Thus, it can be stated from the Mössbauer analysis that Method #2 produces Fe_3O_4 NPs that have on average a larger size than those produced by Methods #1 and #3, *which is in good agreement* with the results of both TEM and XRD.

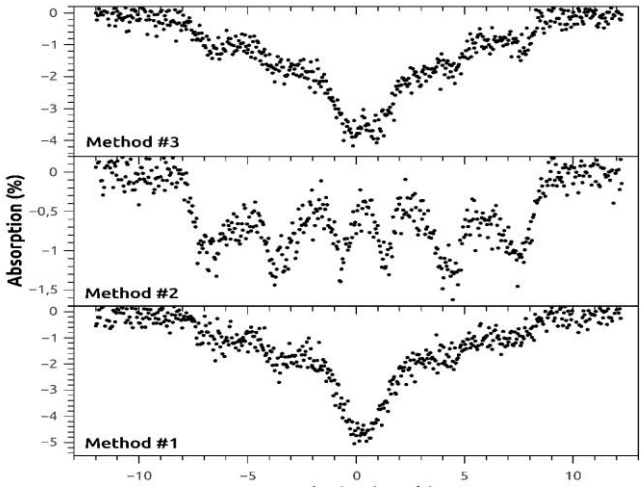


Figure 4.5. Room temperature ^{57}Fe Mössbauer spectra of the nonfunctionalized iron oxide nanoparticles synthesized by the tree different methods. The spectra were taken with different acquisition times.

4.3.2.4. FTIR

In the FTIR spectra of MNPs prepared by Methods #1 and 2 (Figure 4.6 a,b), an intense band near 600 cm^{-1} and the one centered at 447 cm^{-1} were observed. A splitting of the former into two bands (approximately 590 and 640 cm^{-1}) was observed. Besides these bands, two others are observed in these spectra: one centered near 3445 cm^{-1} and the other around 1640 cm^{-1}.

The FTIR spectrum of MNPs prepared by Method #3 (Figure 4.6c) does not differ much from the former, as the only significant absorption band of dehydroascorbic acid, the oxidation product of ascorbic acid, is from the carbonyl groups, at 1680 cm^{-1}. As a consequence, only a broadening of the 1640 cm^{-1} band relative to the other NPs samples is observed. Apart from that.

two inconspicuous bands, due to C-H asymmetric and symmetric stretching, may be denoted at nearly 2930 and 2850 cm^{-1}, respectively.

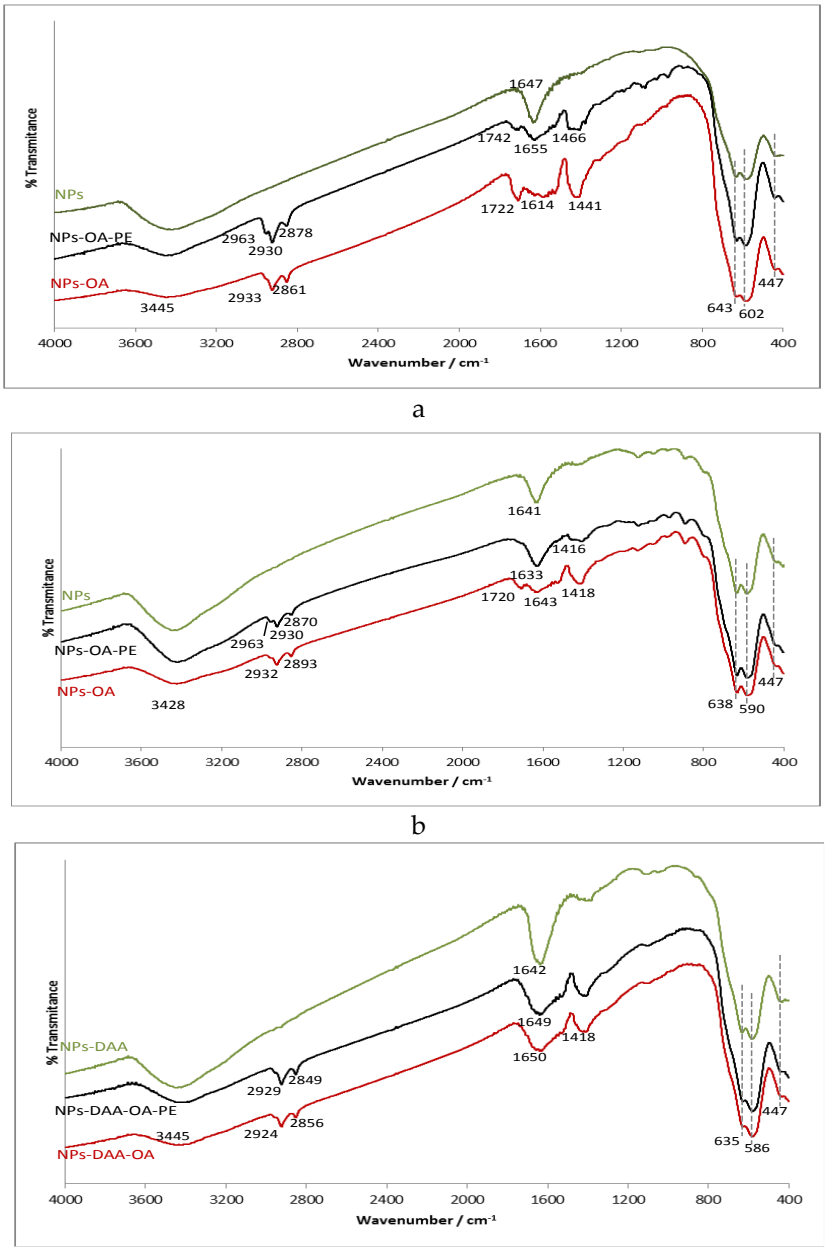


Figure 4.6. FTIR spectra of as obtained and functionalized magnetite NPs by: (**A**) Method #1; (**B**) Method #2; and (**C**) Method #3. OA—oleic acid. PE—propolis extract. DAA—dehydroascorbic acid.

Upon functionalization with oleic acid, the spectra of all MNPs show changes, indicating the presence of the fatty acid: the appearance of the C-H asymmetric and symmetric stretching bands, near 2930 and 2870 cm^{-1}, a broadening of the band at 1640 cm^{-1}, and a new band centered around 1430 cm^{-1}, due to the bending of the methylene groups in OA chain. The broadening of the 1640 cm^{-1} band is due to a shift in the C=O stretching band, originally near 1710 cm^{-1}, to lower wavenumbers, upon binding to the surface, that weakens the double bond character of the carbonyl. Nevertheless, in some samples (MNPs obtained by Methods 1 and 2), a band at

1720 cm^{-1} was still visible. After addition of propolis extract, this band remains in the first sample, although less intense, but disappears or at least fades away in the second one. In MNPs prepared by Method 3 this band is not present, meaning that the presence of dehydroascorbic acid somehow prevented the formation of an oleic acid bilayer, in the same manner as the compounds present in the propolis extract, that seem to decrease the amount of unbound oleic acid.

The effect of the addition of propolis extract on the FTIR spectra is mainly related to the aromatic compounds, since the aliphatic ones, acids, and esters absorb in the same regions as the compounds already present. Therefore, only a new band at 2963 cm^{-1}, attributable to the C-H stretching of unsaturated and aromatic hydrocarbons, as well as a broadening in the band centered near 1430 cm^{-1}, due to the C=C stretching absorption (in the region 1450–1470 cm^{-1}) are noteworthy in the spectra of the two first samples. In the last one, the spectra before and after addition of propolis extract seem practically superimposable, which means that only a very small amount, if any at all. of propolis compounds were added to the MNPs.

4.3.3. Impact of the nanoparticles on bacterial adherence

The effect of the nanoparticles on bacterial adherence was evaluated using catheters treated with different MNPs. The results are illustrated in Figure 4.7 a-c. The percentage of adherence of the cells of the MSSA strain was not affected by Methods #2 and #3 of nanoparticles production in comparison to the MRSA strains. However, the adherence of this strain was significantly lower ($p < 0.05$) by Method #1, particularly in the presence of propolis ($p < 0.001$).

The impact of the nanoparticles against the adherence ability of MRSA strains was dependent of the method of production of the nanoparticles and the strain, being the Method #2 the more efficient in inhibiting adherence of MRSA strains, particularly MRSA 15, which adherence was significantly lower ($p < 0.001$) when the catheters were treated with MNPs functionalized with oleic acid and propolis extract (50.67% ± 2.90 %). The adherence of MRSA 2 was also impaired by the nanoparticles produced by the Method #2 ($p < 0.001$). The adherence of the strain MRSA 16 was significantly lower ($p < 0.001$) when the catheters were treated with magnetite nanoparticles produced by Method #3 either just with oleic acid or nanoparticles with oleic acid and propolis extract.

Thus, in order to impair the adherence of MRSA strains, the use MNPs produced by the Method 2 and functionalized with oleic acid or oleic acid plus propolis extract are the more appropriate followed by the Method #3. To our best knowledge, this is the first study that shows

that catheters treated with magnetite nanoparticles functionalized with oleic acid and Moroccan propolis can impair the adherence of such problematic pathogenic bacteria, as MRSA strains.

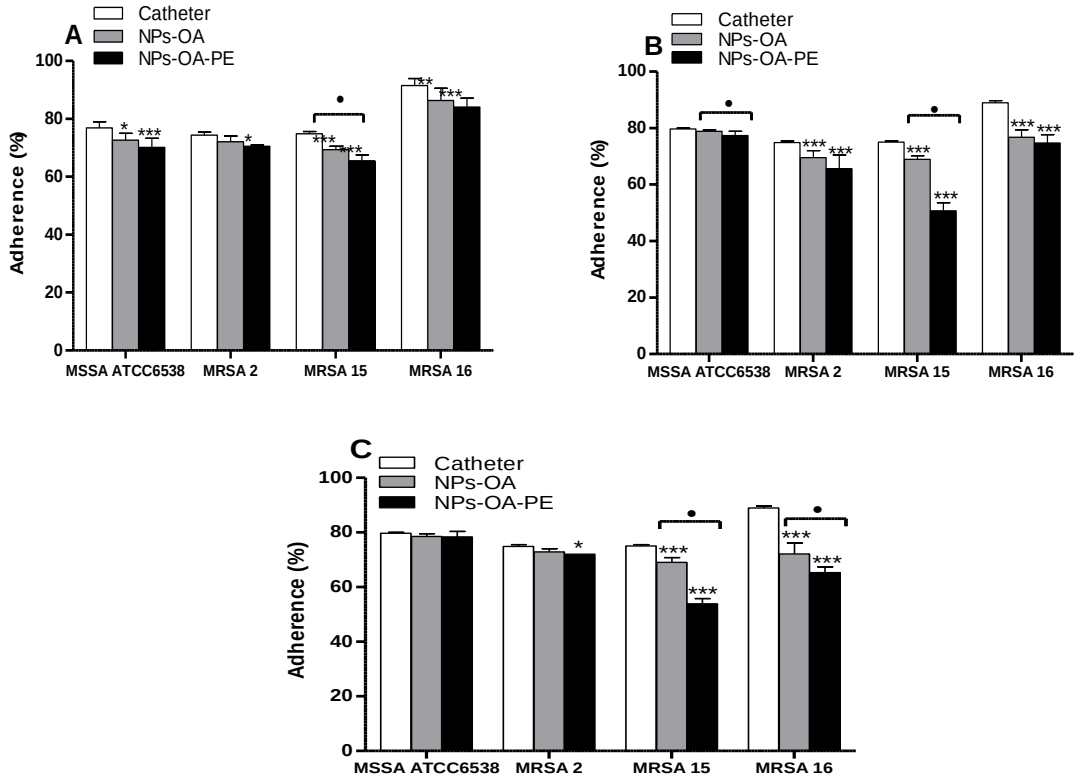


Figure 4.7. Percentage of adherence of microorganisms on catheter after contact with functionalized MNPs obtained by different methods: (**A**) Method #1; (**B**) Method #2; and (**C**) Method #3. OA—oleic acid. PE—propolis extract. Data represent the mean ± S.D from two separated experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ ($n = 6$). statistically significant when compared with catheter not submitted to any treatment. Square brackets indicate the use of One-Way ANOVA for each group. • $p < 0.05$ ($n = 6$).

The presence of diterpenes is common in propolis of Mediterranean origin.[31] This group of compounds was already reported in some Moroccan propolis.[24] Popova et al.[24] found at least three main types of propolis of Moroccan origin: flavonoid/phenolic acid esters-type, flavonoid-type, and diterpene-type. In the present work, flavonoid/diterpene-type, along with a relative high amount of phenolic acid esters, was found.

The characterization of the nanomaterials was followed by XRD, TEM, Mössbauer spectroscopy, and FTIR. The diffraction peaks present in the patterns of all the samples indicate magnetite as the sole crystalline phase[32] (ICDD PDF-2 card #01-071-6337). As reported by Mahdavi et al.[33] for functionalized MNPs with oleic acid. XRD data showed negligible effects of this fatty acid on the XRD spectra data. The morphology and particle size of nanoparticles were similar to those reported by Anghel et al.[15] for functionalized magnetite (Fe_3O_4/C_{18}), that is, the same type of functionalization (oleic acid) done in the present work. In the Mössbauer

spectroscopy, and at room temperature, spectra showed a gradual passage from bulk-like to superparamagnetic (SPM) behavior, as the average size of the particles decrease, a striking characteristic of the superparamagnetic nature of nanoparticles.[34]

FTIR spectroscopy contributes to provide a fast way of identification of MNPs. This technology is also useful when there is their functionalization, because it allows verifying the occurrence of such functionalization. In the identification of the nanoparticles, and for the Methods 1 and 2 there was confirmation of the presence of MNPs due to the intense band near 600 cm^{-1}, attributed to the stretching of the Fe-O bond of octahedral positions, and the one centered at 447 cm^{-1}, due to the stretching of tetrahedral Fe-O.[35] The splitting of the intense band near 600 cm^{-1} in two bands (approximately 590 and 640 cm^{-1}) is an effect of the finite size that causes the split of the vibrational energy levels in the nanoparticles.[36] Two other bands were observed: one centered near 3445 cm^{-1}, attributed to the O-H stretching both in hydroxyl groups on the MNPs surface and in adsorbed water molecules, and the other around 1640 cm^{-1} is attributed to the bending in these molecules.[36,37] Upon functionalization with oleic acid, the spectra of all MNPs show changes, indicating the presence of the fatty acid, nevertheless, in some samples (MNPs obtained by Methods #1 and #2), a band at 1720 cm^{-1} was still visible, probably due to the presence of unbound oleic acid molecules, forming a second layer.[36] The effect of the addition of propolis extract on the FTIR spectra was mainly related to the aromatic compounds.

The dependence of the method of production of MNPs as well as the strain on the impact of the adherence ability of microorganisms observed in the present work was also reported by some authors,[16,38] using other strains and methods of production of NPs. The different response of diverse strains to the same compound or extract is expectable since there are not universal antimicrobials. However, in the presence of functionalized nanoparticles with the same compound or extract, care must be taken in the production of such NPs, because unexpected activities may occur.

Recently, some works have demonstrated the capacity of propolis extract to prevent the adherence of several microorganisms (*Streptococcus mutans*, and several *Staphylococcus aureus* clinical strains) on abiotic (smooth glass or microplate) and biotic (HEp-2 (Human Epithelioma) and HeLa (cervical carcinoma)) surface.s[39-41] With the exception of Veloz et al.[34], in the remaining works, the authors did not describe the chemical composition of propolis extracts; only refer the importance of phenol content on the inhibition of biofilm formation,[39] or there is not ever any reference to the composition of the propolis sample.[41]

The anti-MRSA activity of propolis extracts from different origins and consequently with distinct chemical compositions has been reported, as can be seen in Table 4.1 nevertheless the anti-MRSA biofilm formation by propolis is very scarcely reported or even absent, as far as we know. The capacity for inhibiting the growth of MRSA by propolis extracts has been attributed to several compounds, including phenolic esters (benzyl caffeate) and flavonoids (pinocembrin. galangin and their derivatives), also detected in our sample, nevertheless it also presents isocupressic acid, a diterpene, in relative high amounts; which along with the other phenols may be responsible for the anti-adherence activity found.

It is noteworthy to refer some discrepancies on the anti-MRSA activities of some components present in propolis and found in different works developed by diverse authors. As examples: Boisard et al.[17] found that the flavonoids pinobanksin-3-acetate, pinocembrin, chrysin, and galangin did not show anti-MRSA activity, in contrast to the prenyl caffeate. Nevertheless, the activities of the extracts were higher than the isolated compounds, which evidence a synergetic activity among the compounds present in the extracts. These results disagree with those found by Darwish et al.[20] and Pepeljnjak and Kosalec[23]. These authors reported higher activity of pinobanksin-3-acetate, pinocembrin and galangin than crude extracts.

Functionalized Fe_3O_4/C_{18} nanoparticles without propolis extract present ability for inhibiting the adherence of MRSA strains, as already reported by Anghel et al.[15]. nevertheless, always inferior when compared to those samples also functionalized with propolis extract, which reveals the importance of this extract on the activity.

4.4. Conclusion

The capacity of functionalized MNPs for preventing the adherence of *S. aureus* to catheters was dependent on two factors: the bacterial strain and the mode of production of the NPs. The MSSA ATCC 6538 strain was less affected by the presence of the functionalized MNPs, independently on the type of production, in contrast to the MRSA strains. For MRSA strains, the MNPs produced using Fe^{3+} and Na_2SO_3 solution and functionalized with oleic acid and propolis extract was most effective in the prevention of adherence of almost all MRSA strains on catheters. The hybrid MNPs produced by a hydrothermal method also had an impact in the impaired of the adherence of the strain MRSA16 on catheters. The anti-MRSA activities reported for phenolic esters (benzyl caffeate) and flavonoids (pinocembrin. galangin and their derivatives), constituents of several types of propolis and also present in our sample can be the reason, along with MNPs, for the observed activities. However, the main compound,

isocupressic acid, may also have a role in the anti-adherence activities detected, although to the best of our knowledge no reference has been found upon its anti-MRSA potential. In addition, for MRSA16, the impairment of its adherence on catheters may only be attributed to the hybrid MNPs with oleic acid, since very small amount, if any at all, of propolis compounds were added to the MNPs, according to the FTIR spectra.

Nanotechnology may provide a new approach to prevent or disrupt the formation of biofilms on medical devices. The association of natural products, such as propolis, with nanotechnology may constitute an alternative to combat the formation of those biofilm in catheters by MRSA strains.

Reference

[1] G. A. Burdock, 'Review of the biological properties and toxicity of bee propolis (propolis)', Food Chem. Toxicol. 1998, *36*, 347–363.

[2] Y. Barlak, O. Deger, M. Çolak, S. C. Karatayh, A. M. Bozdayi, F. Yücesan, 'Effect of Turkish propolis extracts on proteome of prostate cancer cell line', Proteome Sci. 2011, 9. Doi:10.1186/1477-5956-9-74.

[3] J. M. Sforcin, 'Biological properties and therapeutic appications of propolis', Phytother. Res. 2016, 30, 894–905.

[4] H. F. L. Wertheim, M. C. Vos, A. Ott, A. Van Belkum, A. Voss, J. A. J. W. Kluytmans, P. H. J. Van Keulen, C. M. J. E. Vandenbroucke, M. H. M. Meester, H. A. Verbrugh, 'Risk and outcome of nosocomial *Staphylococcus aureus* bacteraemia in nasal carriers versus non-carriers', The Lancet. 2004, 364, 703–705.

[5] M. J. Rybak, K. L. LaPlante, 'Community-associated methicillin-resistant *Staphylococcus aureus*: A review', Pharmacotherapy. 2005, 25, 74–85.

[6] D. N. Frank, L. M. Feazel, M. T. Bessesen, C. S. Price, E. N. Janoff, N. R. Pace, 'The human nasal microbiota and *Staphylococcus aureus* carriage', PLoS One. 2014, 5, e10598. Doi:10.1371/journal.pone.0010598.

[7] H. F. Chambers, F. R. DeLeo, 'Waves of resistance: *Staphylococcus aureus* in the antibiotic era', Nat. Rev. Microbiol. 2009, 7, 629–641.

[8] C. M. Huang, C. H. Chen, D. Pornpattananangkul, L. Zhang, M. Chan, M. F. Hsieh, L. Zhang, 'Eradication of drug resistant *Staphylococcus aureus* by liposomal oleic acids', Biomaterials. 2011, 32, 214–221.

[9] M. Magana, A. Ioannidis, E. Magiorkinis, O. Ursu, C. G. Bologa, S. Chatzipanagiotou, M. R. Hamblin, G. P. Tegos, 'Therapeutic options and emerging alternatives for multidrug resistant *Staphylococcus* infections', Curr. Pharm. Des. 2015, 21, 2058–2072.

[10] R. D. Wojtyczka, A. Dziedzic, D. Idzik, M. Kepa, R. Kubina, A. Kabala-Dzik, J. Smolén-Dzirba, J. Stjko, M. Sajewicz, T. J. Wasik, 'Susceptibility of *Staphylococcus aureus* clinical isolates to propolis extract alone or in combination with antimicrobial drugs', Molecules. 2013, 18, 9623–9640.

[11] G. M. Cartelle, A. M. Holban, 'Advances in nanotechnology as an alternative against superbags', JSM Chem. 2014, 2, 1011.

[12] C. Chifiriuc, V. Grumezescu, A. M. Grumezescu, C. Saviuc, V. Lazăr, E. Andronescu, 'Hybrid magnetite nanoparticles/Rosmarinus. officinalis essential oil nanobiosystem with antibiofilm activity', Nanoscale Res. Lett. 2012, 7. Doi:10.1186/1556-276X-7-209.

[13] I. Liakos, A. M. Grumezescu, A. M. Holban, 'Magnetite nanostructures as novel strategies for anti-infections therapy', Molecules. 2014, 19, 12710–12726.

[14] W. Wu, Q. He, C. Jiang, 'Magnetic iron oxide nanoparticles: synthesis and surface functionalization strategies', Nanoscale Res. Lett. 2008, 3, 397–415.

[15] I. Anghel, A. M. Grumezescu, E. Andronescu, A. G. Anghel, A. Ficai, C. Saviuc, V. Grumezescu, B. S. Vasile, M. C. Chifiriuc, 'Magnetite nanoparticles for functionalized textile dressing to prevent fungal biofilms development'. Nanoscale Res. Lett. 2012, 7,501.

[16] A. Poiată, C. Tuchilus, D. Creangă, C. Stan, 'Magnetic nanoparticles influence on some bacterial cultures', Rom. J. Biophys. 2013, 23, 203–209.

[17] S. Boisard, A. M. Le Ray, A. Landreau, M. Kempf, V. Cassisa, C. Flurin, P. Richomme, 'Antifungal and antibacterial metabolites from a French poplar type propolis', Evid. Based. Complement. Alternat. Med. 2015. Doi:10.1155/2015/319240.

[18] A. Astani, S. Zimmermann, E. Hassan, J. Reichling, K. H. Sensch, P. Schnitzler, 'Antimicrobial activity of propolis special extract GH2002 against multidrug-resistant clinical isolates', Pharmazie 2013, 68, 695–701.

[19] L. C. Pamplona-Zomenhan, B. C. Pamplona, C. B. Silva, M. C. Marcucci, L. M. J. Mímica, 'Evaluation of the *in vitro* antimicrobial activity of an ethanol extract of Brazilian classified propolis on strains of *Staphylococcus aureus'*, Braz J. Microbiol. 2011, 42, 1259–1264.

[20] R. M. Darwish, R. J. A. Fares, M. H. A. Zarga, I. K. Nazer, 'Antibacterial effect of Jordanian propolis and isolated flavonoids against human pathogenic bacteria', Afr. J. Bacteriol. 2010, 9, 5966–5974.

[21] R. Raghukumar, L. Vali, D. Watson, J. Fearnley, V. Seidel, 'Antimethicillin-resistant *Staphlylococcus aureus* (MRSA) activity of 'Pacific propolis' and isolated prenylflavanones', Phytother Res. 2010, 24, 1181–1187.

[22] Y. Onlen, N. Duran, E. Atik, L. Savas, E. Altug, S. Yakan, O. Aslantas, 'Antibacterial activity of propolis against MRSA and synergism with topical mupirocin', J Altern. Complement Med. 2007, 13, 713–718.

[23] S. Pepeljnjak, I. Kosalec, 'Galangin expresses bactericidal activity against multiple-resistant bactéria: MRSA, Enterococcus spp. and Pseudomonas aeruginosa'. FEMS Microbiol. Lett. 2004, 240, 111–116.

[24] M. Popova, B. Lyoussi, S. Aazza, D. Antunes, V. Bankova, G. Miguel, 'Antioxidant and α-glucosidase inhibitory properties and chemical profiles of Moroccan propolis', Nat. Prod. Commun. 2015, 10, 1961–1964.

[25] Y. S. Kang, J. F. Rabolt, S. Risbud, P. Stroeve, 'Synthesis and characterization of nanometer-size Fe3O4 and □-Fe2O3 particles'. Chem. Mater. 1996, 8, 2209–2211.

[26] S. Qu, H. Yang, D. Ren, S. Kan, G. Zou, D. Li, 'Magnetite nanoparticles prepared by precipitation from partially reduced ferric chloride aqueous solutions', J. Colloid Interf. Sci. 1999, 215, 190–192.

[27] S. Xuan, L. Hao, W. Jiang, X. Gong, Y. Hu, Z. Chen, 'Preparation of water-soluble magnetite nanocrystals through hydrothermal approach', J. Magn. Magn. Mater. 2007, 308, 210–213.

[28] E. L. Foca-nici Ciurlica, C. Nadejde, D. E. Creanga, A. Carlescu, V. Badescu, 'Antibiotic coated magnetite nanoparticles for biological applications', 2016. http://www.jscimedcentral.com/Chemistry/chemistry-2–1011.pdf (Accessed on 3 April 2016).

[29] I. Prisecaru, 2016. 'WMOSS4 Mössbauer spectral analysis software', Available online: www.wmoss.org. (access on 5 February 2016).

[30] A. A. Miles, S. S. Misra, 'The estimation of the bactericidal power of blood', J. Hyg. (London) 1938. 38, 732–749.

[31] S. Huang, C. P. Zhang, K. Wang, G. Q. Li, F. L. Hu, 'Recent advances in the chemical composition of propolis', Molecules. 2014, 19, 19610–19632.

[32] M. L. Paramês, J. Mariano, Z. Viskadourakis, N. Popovici, M. S. Rogalski, J. Giapintzakis, O. Conde, 'PLD of Fe3O4 thin films: influence of background gas on surface morphology and magnetic properties', Appl. Surf. Sci. 2006, 252, 4610–4614.

[33] M. Mahdavi, M. B. Ahmad, M.nJ. Haron, F. Namvar, B. Nadi, M. Z. A3 Rahman, J. Amin, 'Synthesis, surface modification and characterization of biocompatible magnetic iron oxide nanoparticles for biomedical applications', Molecules. 2013, 18, 7533–7548.

[34] R. Lawaczeck, M. Menzel, H. Pietsch, 'Superparamagnetic iron oxide particles: contrast media for magnetic resonance imaging', Appl. Organomet. Chem. 2004, 18, 506–513.

[35] P. E. G. Casillas, C. A. R. Gonzalez, C. A. M. Pérez, 'Infrared spectroscopy of functionalized magnetic nanoparticles', In Infrared Spectroscopy—Materials Science, Engineering and Technology; Theophanides Theophile, Ed.; InTech: Rijeka, Croatia. 2012, 405–420.

[36] K. Yang, H. Peng, Y. Wen, N. Li, 'Re-examination of characteristic FTIR spectrum of secondary layer in bilayer oleic acid-coated Fe3O4 nanoparticles', Appl. Surf. Sci. 2010, 256, 3093–3097.

[37] M. Ma, Y. Zhang, W. Yu, H. Shen, H. Zhang, N. Gu, 'Preparation and characterization of magnetite nanoparticles coated by amino silane. Colloids Surf', A Physicochem. Eng. Asp. 2003, 212, 219–226.

[38] C. Chifiriuc, V. Lazăr, C. Bleotu, I. Călugărescu, A. M. Grumezescu, D. E. Mihaiescu, D. E. Mogosanu, A. S. Buteică, E. Buteică, 'Bacterial adherence to the cellular and inert substrate in the presence of CoFe2O4 and Fe3O4/oleic acid-core/shell', Dig. J. Nanomater. Biostruct. 2011, 6, 37–42.

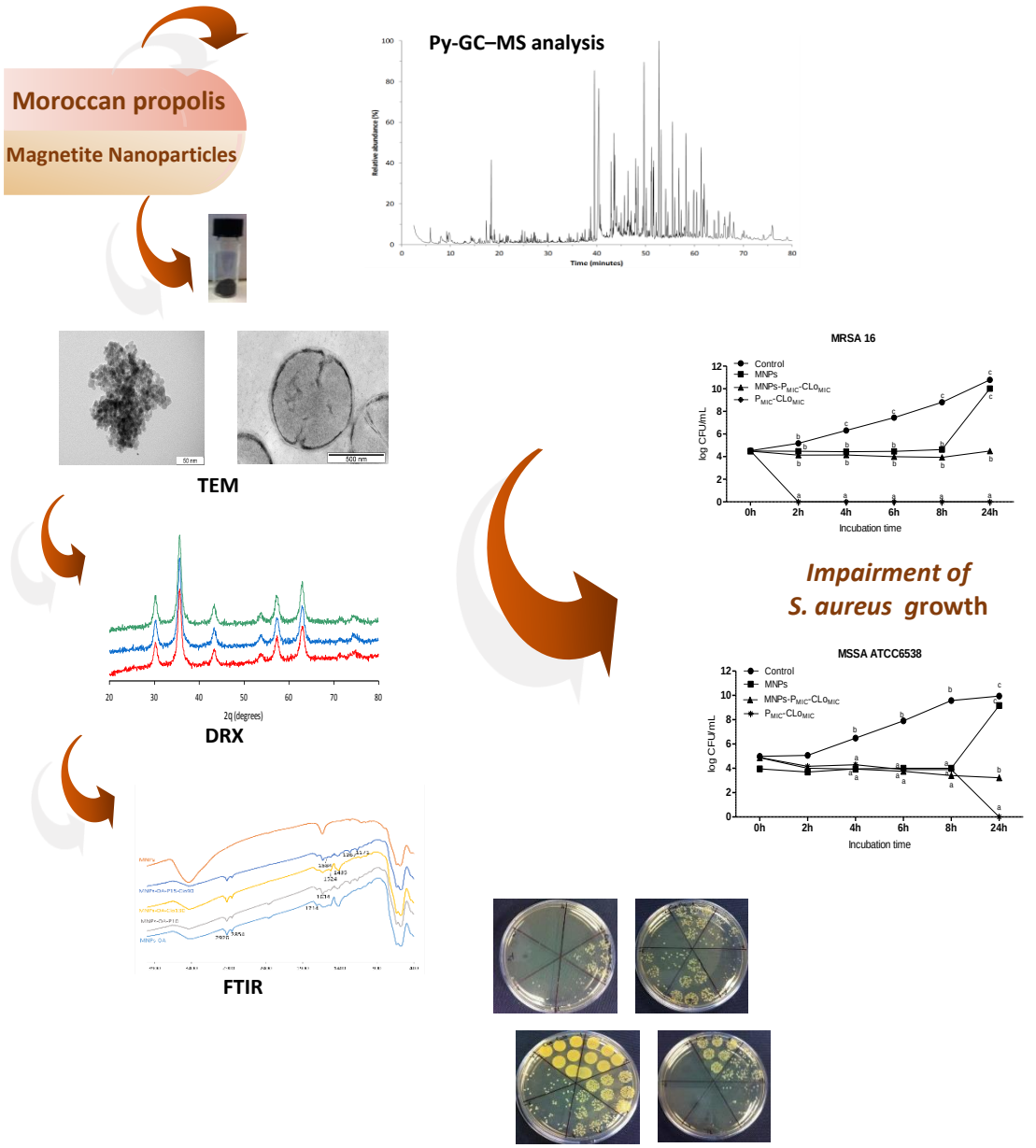
[39] C. Asawahame, K. Sutjarittangtham, S. Eitssayeam, Y. Tragoolpua, B. Sirithunyalug, J. Sirithunyalug, 'Antibacterial activity and inhibition of adherence of Streptococcus mutans by propolis electrospun fibers', AAPS Pharm. Sci. Tech. 2015, 16, 182–191.

[40] J. J. Veloz, N. Saavedra, A. Lillo, M. Alvear, L. Barrientos, L. A. Salazar, Antibiofilm activity of Chilean propolis on Streptococcus mutans is influenced by the year of collection', Bio. Med Res. Int. 2015, 2015, doi:10.1155/2015/291351.

[41] T. Stan, L. Mărutescu, M. C. Chifiriuc, V. Lazăr, 'Anti-pathogenic effect of propolis extracts from different Romanian regions on *Staphylococcus sp.* Clinical strains', Rom. Biotech. Lett. 2016, 21, 11166–11175.

Chapter 5

Magnetite nanoparticles functionalized with propolis against methicillin resistant strains of *Staphylococcus aureus*



Magnetite nanoparticles functionalized with propolis against methicillin resistant strains of *Staphylococcus aureus**♠

Abstract

Magnetite nanoparticles (MNPs) have been evaluated for inhibiting microbial growth and biofilm formation. In this study the effect of the nanocomposite Moroccan propolis extract / MNPs acting against methicillin resistant strains of *Staphylococcus aureus* (MRSA) was evaluated. Chemical composition of propolis was established by pyrolysis coupled to gas chromatography and mass spectrometry method (pyrolysis GC/MS). MNPs were obtained through the co-precipitation method. The fabricated nanostructure was characterized by X-ray Diffraction (DRX), Transmission Electron Microscopy (TEM), and Fourier Transform-Infrared Spectroscopy (FTIR). TEM of MNPs provided a particle average size of 15 nm, FTIR spectral analysis enabled a fast way of identification of MNPs, attesting the occurrence of the different combinations. The use of MNPs loaded with propolis and the antibiotic chloramphenicol at Minimum Inhibitory Concentration (MIC) value inhibited the bacterial growth of MSSA (methicillin susceptible strain of *S. aureus*) and MRSA strains. After the treatment with MNPs-OA-P-CLo nanocomposite (MNPs with oleic acid, propolis and chloramphenicol), the disruption of the bacterial cell was observed by TEM. The combination of propolis and chloramphenicol in free form at MIC value largely impaired both MSSA and MRSA strains as, after 2 h of treatment, no viable cells of MRSA 2 and MRSA 16 were recovered. Hence, the results elucidated a new antibacterial nanocomposite synthesis, for possible applications as prospective nanoantibacterial agents or drug carriers.

*Adapted from 'Magnetite nanoparticles functionalized with propolis against methicillin resistant strains of *Staphylococcus aureus*'. Soukaïna El-Guendouz. Badiaa Lyoussi. João P. Lourençoc. Ana M. Rosa Costa. Maria G. Miguel. Cristina B. Dias. Ana C. Manhita. Luisa Jordao. Isabel Nogueira. Maria L. Faleiro. Journal of the Taiwan Institute of Chemical Engineers, 102, 25-33.

5.1. Introduction

The biologically synthesized nanomaterials are an important aspect in biotechnology as well as in the field of nanomedicine. The capacity of nano-antimicrobial agents to penetrate bacteria and biofilm can make them as potential agents for controlling infectious diseases.[1] Metal nanoparticles, such as gold, silver, platinum, and zinc oxide have been largely used in different science fields, such as manufacturing and materials, environment, electronics, energy harvesting, mechanical industries,[2-4] and mainly in biomedicine.[5] Among all the metal nanoparticles, magnetite nanoparticles (MNPs) have always attracted researchers. In addition to the fact that the production of these nanoparticles is not complex, they are chemically stable under physiological conditions, non-toxic and biocompatible at physiological concentrations.[6] MNPs have been largely used for different biomedical applications, particularly as antimicrobial agents,[6,7] targeted drug delivery,[6] cellular labelling and separation,[8] and tissue repair.[9] In order to avoid aggregation of MNPs, enhance their biocompatibility, stability and biological application it is possible to functionalize this nanomaterial biomolecules.[6] For example, oleic acid alone or with natural products was used for preventing biofilm formation or promoting disruption of pathogenic microorganisms.[10-11]

One of the valuable natural products is propolis, produced by honey bees and has been used for different purposes medicinal purposes since ancient times.[12] Propolis, known as "bee glue" from the fact that it is used to cover surfaces, seal holes and close gaps in their hives, is a brawny sticky resinous substance, gathered from buds and the bark of trees. Bees use propolis not only to sterilize the hive environment from microbes, but also as an embalming substance, to mummify invader insects.[13] It is therefore considered a potent chemical weapon against pathogenic microorganisms. Raw propolis is typically composed of plant resins, waxes, essential and aromatic oils, pollens and other organic substances (debris, minerals and organic compounds). The chemical composition of propolis varies depending on its botanical origin and harvesting seasons.[6] Obviously, due to the high diversity in its chemical composition, propolis possesses a large number of biological and pharmaceutical properties, which has been scientifically demonstrated. Among those it can be highlighted the diuretic, antitumoral, antimicrobial, antioxidative, anti-inflammatory, anti-HIV, anti-neurodegenerative and antituberculosis activity.[6,14-17]

Staphylococcus aureus is a human pathogen and the most frequently opportunistic pathogen linked to healthcare associated infections (HAIs), specially targeting immunocompromised hosts.[18] *S. aureus* and particularly methicillin resistant strains (MRSA)

possess a high ability to cause diseases, which has been attributed to its large spectrum of virulence factors and its multiple resistances to antibiotics.[19] Therefore, an innovative concept based on the application of natural product and nanotechnology to obtain efficient biomaterials that provide an alternative therapeutically approach is needed.

We have previously reported that nanoparticle drug delivery system of Moroccan propolis exhibited inhibitory effect on the adherence ability of MRSA.[6] The aim of this study was to evaluate the effect of propolis extract combined with magnetite nanoparticles against MRSA strains, for possible use as pharmaceutical agent to face various bacterial infections.

5.2. Experimental

5.2.1. Chemicals

Hydrochloric acid (HCl), oleic acid and l-(+)-ascorbic acid were purchased from Merck (Darmstadt, Germany). Na_2CO_3 and Na_2SO_3 were purchased from Riedel de Haen (Seelze, Germany). Iron (III) chloride anhydrous was purchased from Fluka Chemicals (Buchs, Switzerland). The culture medium Brain Heart Infusion (BHI) and Bacteriological agar type E were purchased from Biokar Diagnostics (Beauvais, France). Tetramethylammonium hydroxide solution (TMAH, 25 wt% in methanol) was purchased from Sigma Aldrich (St Quentin Fallavier, France).

5.2.2. Propolis extraction

Hydro-alcoholic extracts of propolis from Morocco (region of Fez-Boulmane) were obtained, as described by El-Guendouz et al..[6,14]

5.2.3. Pyrolysis coupled to gas chromatography and mass spectrometry analysis of propolis extract

A system with a Frontier Lab PY-3030D double-shot pyrolyzer was used, with an interface temperature of 280°C. The pyrolyzer was coupled to a Shimadzu GC2010 gas chromatographer, also coupled to a Shimadzu GCMS-QP2010 Plus mass spectrometer. A capillary column Phenomenex Zebron ZB-5HT (30 m length, 0.25 mm internal diameter, 0.50 µm film thickness) was used for separation, with helium as carrier gas, adjusted to a flow rate of 2.2 mL min−1. The splitless injector operated at a temperature of 250°C. GC temperature programme was as follows: 40°C during 5 min, ramp until 300°C at 5°C min−1and then an isothermal period of 3 min. Source temperature was placed at 240°C, and the interface temperature was maintained at 280°C. Samples of approximately 300 µg of propolis powder were analysed by Py-GC/MS. Samples were derivatized with 3 µL of tetramethylammonium

hydroxide (25% in methanol) in a 50 µL Eco-cup capsule and placed in the double shot pyrolyzer using an Eco-stick. The sample was pyrolyzed using a single-shot method at 500°C for 12 s. The mass spectrometer was programmed to acquire data between 40 and 850 m/z. Compound identification was performed using AMDIS software integrated with NIST-Wiley database was used.

5.2.4. Preparation of magnetite nanoparticles (MNPs) functionalized magnetite nanoparticles (MNPs-OA)

MNPs and MNPs-OA were prepared as previously described by El-Guendouz et al..[6] MNPs were synthesized through co-precipitation method, afterwards they were functionalized with 6 mL of oleic acid (OA), heated up to 80°C under intense stirring for 60 min, then distilled water was added to separate oil phase and finally MNPs-OA were washed with ethanol and kept to dry at room temperature.

5.2.5. Characterization of the nanomaterial

Characterization of the nanomaterial was carried out by X-ray as previouselly descried by powder diffraction (XRD) (Panalytical X′Pert Pro diffractometer (PANalitycal, Almelo, Netherlands), transmission electron microscopy (TEM) (Hitachi High-Technologies Corporation, Tokyo, Japan), and Fourier Transform Infrared (FTIR) spectroscopy (Bruker Tensor 27, Billerica, MA, USA).

5.2.5.1. XRD

The structure type of all the samples was checked by powder X-ray diffraction on a Panalytical X′Pert Pro diffractometer (PANalitycal, Almelo, Netherlands) using Cu Ka radiation filtered by Ni and an X′Celerator detector as describe by El-Guendouz et al..[14]

5.2.5.2. FTIR

Infrared spectra were recorded as describe by El-Guendouz et al.,[6] where infrared spectra were recorded in the wavenumber range 4000–400 cm−1, in a Bruker Tensor 27 Fourier-transform infrared (FT-IR) spectrophotometer (Bruker Tensor 27, Billerica, MA, USA), using KBr wafers.

5.2.5.3. TEM

Samples were prepared by drop drying a diluted colloidal solution of NPs in ethanol onto 200 mesh Formvar/Carbon-coated copper grids. Samples were observed using a Hitachi H8100

(Hitachi High-Technologies Corporation, Tokyo, Japan), 200 kV, LaB6 filament analytical transmission electron microscope.

In order to evaluate the effect of nanoparticles on bacteria samples of cultures exposed to different treatments and untreated cultures (control) were analysed by TEM. Bacterial cultures (1 mL) were centrifuged for 10 min at 15,000 r.p.m and the pellets were resuspended in a mixture of fixatives composed by 2.5% (v/v) glutaraldehyde (EMS, Hatfield, PA, USA) and 4% formaldehyde (w/v) in PBS (Sigma, St. Louis, MO,USA) during 2 h at room temperature protected from light. Bacterial suspensions were centrifuged, resuspended in 1% (w/v) paraformaldehyde and kept at 4°C until further processing. The fixative was removed by washing with PBS. Bacterial pellets were resuspended in 10% gelatin (Sigma) in PBS and kept on ice until the solidification of gelatin and small cubs could be cut. The sample were post-fixed by incubation on ice for 2 h in the dark with 1% OsO_4 (EMS) in PBS, washed twice with PBS and twice with water before dehydration. Dehydration was performed by incubation for 10 min at room temperature with 50%, 70%, 96% and 100% ethanol (Merck). The sample was briefly washed with Epon 812 (EMS), and incubated over-night in the same resin at room temperature. Samples were mounted in molds (EMS) and the Epon812 was allowed to polymerise in an incubator set to 65 °C. Ultra-thin sections were obtained using an ultramicrotome (Leica ultracut R, Leica, Wetzlar, German) contrasted with saturated uranyl acetate (Merck) in water for 15 min followed by Reynolds lead citrate (Merck) for 3 min. Bacterial morphology assessed by TEM was performed with a Hitachi H8100 (Hitachi High-Technologies Corporation, Tokyo, Japan). Digital images were acquired with a bottom mounted CCD Keen-View camera (Olympus Soft Imaging Solutions GmbH, Munich, Germany).

5.2.6. Antimicrobial activity

5.2.6.1. Bacterial strains and growth conditions

The bacterial strains MSSA ATCC 6538, MRSA2, MRSA15, MRSA16 were cultivated in Brain Heart Infusion (Oxoid, Basingstoke, Hampshire, UK) (BHI). For solid media, agar (VWR) was added at 1.5%, w/v. The bacterial strains of *S. aureus* used in this study are indicated in Table 5.1. Bacteria were stored in BHI supplemented with 25% (v/v) glycerol at −80°C. Prior to use, bacteria were recovered in fresh BHI agar plates and incubated at 37°C.

Table 5.1. Minimum inhibitory (MIC) values of propolis extracts and chloramphenicol determined for the tested *S. aureus* strains.

Strains	Origin	Source	MIC (mg/mL)
Propolis			
S. aureus ATCC 6538	Wound	American Type Culture Collection	0.24
MRSA2	Clinical	UAlg, CBMR. Portugal	0.36
MRSA15	Clinical	UAlg, CBMR, Portugal	0.36
MRSA16	Clinical	UAlg, CBMR, Portugal	0.36
Chloramphenicol			
S. aureus ATCC 6538	Wound	American Type Culture Collection	0.025
MRSA2	Clinical	UAlg, CBMR. Portugal	0.09
MRSA15	Clinical	UAlg, CBMR, Portugal	0.13
MRSA16	Clinical	UAlg, CBMR, Portugal	0.09

5.2.6.2. Determination of the minimum inhibitory concentration

The MIC of propolis (P) samples and the antibiotic chloramphenicol (CLo) were determined using a microdilution method according El-Guendouz et al.[17] where the tested concentrations of propolis were 0.12; 0.24; 0.36; 0.49 and 0.73 mg/mL (based on preliminary assays), and for chloramphenicol the concentrations tested ranged between 0.015 - 0.140 mg/mL.

5.2.6.3. MNPs-OA-P-CLo microbial growth inhibition

To evaluate the effect of the MNPs-OA-P-CLo on the growth of different *S. aureus* strains, the viability of the bacterial cells was determined as previously described[20] with some modifications. Before treatment with the MNPs-OA-P-CLo, bacterial cultures were streaked for isolation on BHI agar. From this culture single isolated colonies were selected and used to inoculate 10 mL of BHI broth medium. The bacterial culture was grown on a shaking incubator at 37°C overnight. The resulting bacterial suspension was then adjusted to have an optical density measured at a wavelength of 600 nm, corresponding to a bacterial density of 10^8 CFU/mL. Then, the bacterial suspension was serially diluted to a bacterial density of 10^5 CFU/mL, using BHI broth.

Meanwhile, sterilize MNPs-OA (33%) were added to 400 µL of BHI and sonicated in bath sonicator (J.P.SELECTA, Barcelona, Spain) for 1 h Afterward a volume of 500 µL of the previously diluted bacterial suspension, 50 µL of propolis extract (0.24 or 0.36 mg/mL) and/or 50 µL chloramphenicol solution (0.025, 0.09 and 0.13 mg/mL) were added, the mixture was well vortexed for 60 s and allowed to incubate in a shaking water bath at 37°C. Propolis and

chloramphenicol were added at the MIC value previously determined for each bacterial strain. MNPs-OA, MNPs-OA-P and MNPs-OA-CLo at MIC values, as well as P-CLo at MIC values were also tested.

The number of CFU was determined on time 0, 2, 4, 6, 8 and 24 h using serial dilution in phosphate buffer saline (PBS) according to the Miles and Misra technique.[21] The experiment was repeated in triplicate. Bacterial culture without any treatment was used as control.

5.3. Results and discussion

5.3.1. Chemical composition of propolis extract

The Py-GC/MS technique was used to analyze the composition of propolis sample used in this work and Figure 5.1 shows a pyrogram of this analysis. The identification of the peaks was done using the NIST-Wiley reference database with a minimum match factor of 80, and Retention Indexes (RI) used for extra confirmation.

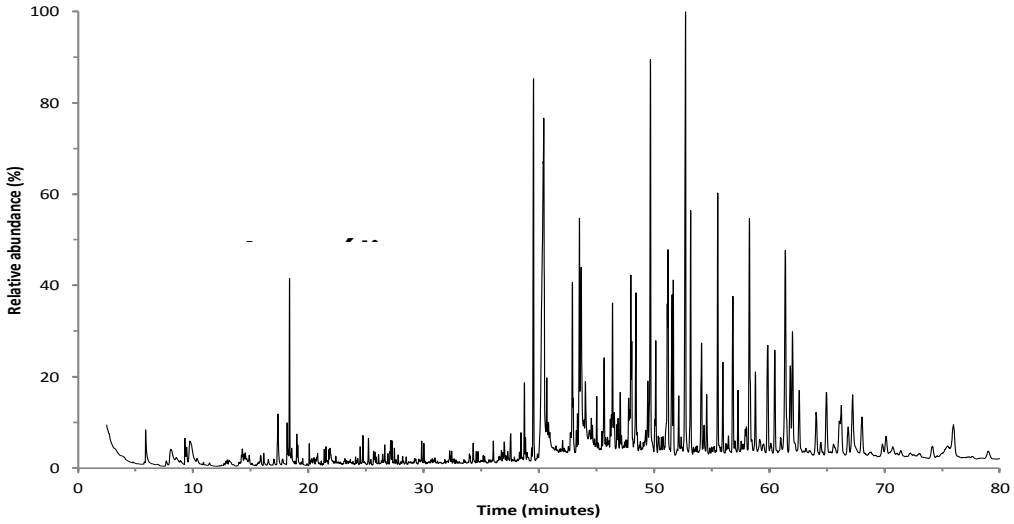


Figure 5.1. Pyrogram of analysis of the propolis sample using Py-GCMS.

The sample was pyrolized in the presence of TMAH, but the methylation was not complete and, for most cases, the fully derivatized and the free molecule are detected in the pyrogram. In the case of the identified chalcone, terpene and flavonoids, only a form of the molecule was detected, with the compounds presenting some OH groups methylated while others are free. For identification purposes, we assumed that these molecules had not been derivatized and considered their identification as tentative, because of the assumption made (Table 5.2).

Table 5.2. Possible bioactive compounds identified in the propolis sample.

Compound family	Retention time (min)	Compound name	Peak relative Area*
Benzoic acids methyl derivatives	18.361	Benzoic acid	+++
	25.894	*o*-Hydroxybenzoic acid	++
Cinnamic acids methyl derivatives	24.090	Hydrocinnamic acid	+
	27.135	Cinnamic acid	+
	34.297	Coumaric acid	+
	38.742	Caffeic acid	++
Phenyl ketones methyl derivatives	17.349	Acetophenone	++
	26.231	3- Hydroxyphenone	++
	26,438	Benzylideneacetone	++
Phenyl aldehydes	12.960	Benzaldehyde	+
Phenyl alcohols methyl derivatives	10.887	Phenol	+
	20.730	Catechol	+
	25.208	Vinyl guaiacol	++
	26.619	Benzene-4-ethenyl-1,2-dihydroxy	+
Chalcone**	48.422	Pinostrobin	+++
Flavonoids**	49.720	Dihydrochrysin	+
	51.566	5-Hydroxy-7-methoxy flavone (Tectochrysin)	+++
	52.788	5,7-Hydroxy flavone (Chrisin)	+
	54.337	5,7- di-Methoxy flavone	++
	56.446	4´,7 di-Methoxy-apigenin	++
Terpenoids*	33.968	β-Eudesmol	+
Others	35.168	Stilbene	+
	36.045	4-Hydroxycoumarin methyl derivat.	++

*Relative intensity of the chromatographic peak area among the possible bioactive compounds. Increasing number of signs denotes increased peak area. **Tentative identification assuming the compounds in these families were not derivatized.

The largest peaks in the pyrogram are compounds usually associated with beeswax, namely, alkanes and 1-alkenes, long chain alcohols, fatty acids from C16:0 to C34:0, unsaturated fatty acids such as C16:1 and C18:1, and small amounts of long chain esters. Because of the nature of the investigation being carried on, Table 5.2 presents only the list of the compounds which are usually associated with the bioactivity of propolis samples. The presence of terpenoid compounds in Greek propolis was associated with its bioactivity against bacteria and fungi.[22]

In the sample used for this study, a single sesquiterpene was identified in the sample, β-eudesmol, which had also been identified in the Greek samples.

Benzoic and cinnamic acids, phenyl alcohols, ketones and aldehydes were also identified in the propolis sample used in this study (Table 2), also reported in other propolis samples, generally possessing biological properties.[23,24]

The flavonoids are the family of compounds most commonly associated with the biological properties of the propolis. One flavanone and four flavones were identified in the

propolis sample (Table 5.2), with the 5-hydroxy-7-methoxy flavone (tectochrysin), being the most abundant compound of this family. Pinostrobin chalcone (2′,6′-Dihydroxy-4′-methoxychalcone) was also detected in relatively large amounts in this sample. The antimicrobial activity is associated with the inhibitory action that these compounds have against several microorganisms.[23]

5.3.2. Characterization of the nanomaterial

5.3.2.1. X-ray diffraction (XRD)

The characterization of the pristine and oleic acid functionalized nanoparticles was already done elsewhere.[6] The subsequent association with propolis and/or antibiotic did not change the crystalline structure of the nanoparticles. The XRD patterns (Figure 5.2) of these associations indicate magnetite as the sole crystalline phase.

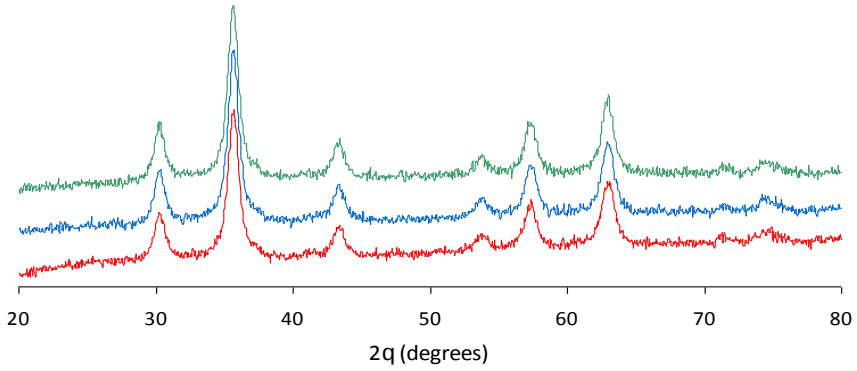


Figure 5.2. X-ray diffractograms of magnetite nanoparticles. (a): MNPs-OA-P_{MIC}-CLo_{MIC}; (b): MNPs-OA-P_{MIC}; (c): MNPs-OA-CLo_{MIC}.

5.3.2.2. TEM

TEM analysis was used to characterize the morphology of the non-functionalized nanoparticles (Figure 5.3) and the morphological alterations induced by treatment with nanoparticles on bacterial cells (Figure 5.4). In Figure 5.3, is shown that MNPs are roughly spherical nanoparticles with a size of 15 nm. The functionalization procedure does not change the structure and composition of the nanoparticles. Likewise, previous study demonstrates that functionalization of MNPs with OA or propolis did not affect MNPs properties.[6,25]

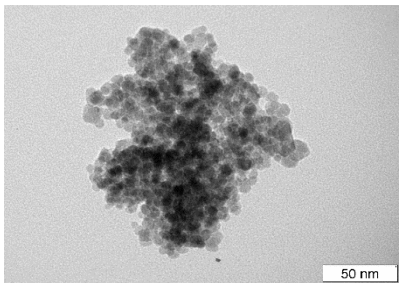


Figure 5.3. Transmission electron micrograph of the magnetite nanoparticles used in this work.

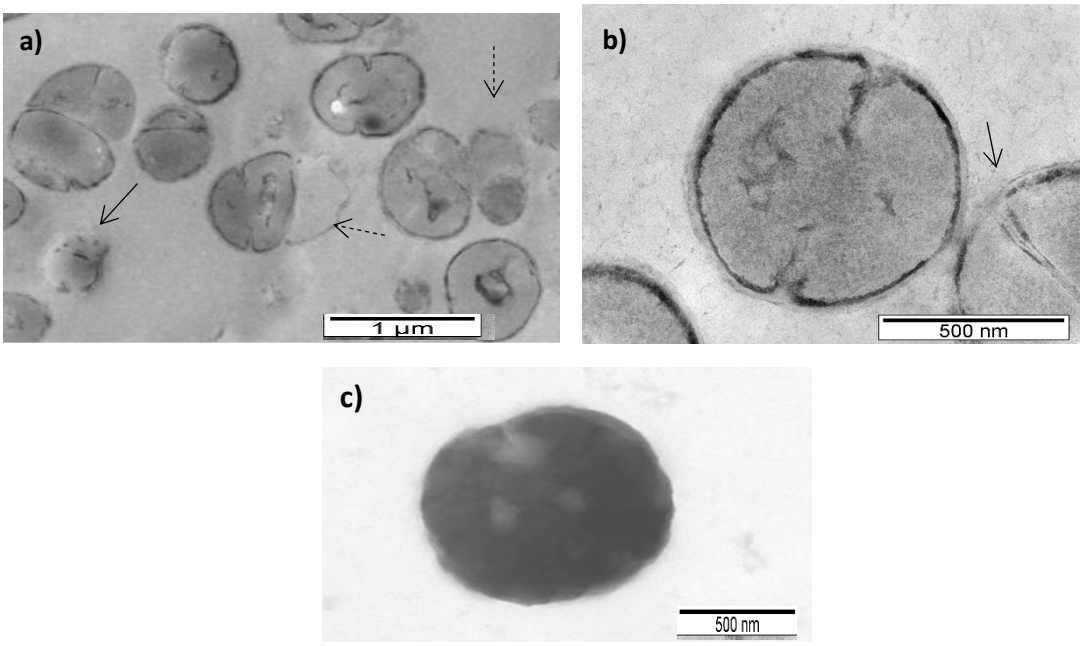


Figure 5.4. TEM micrographs of *S. aureus* bacterial cells treated with MNPs-OA-P_{MIC}-CLo_{MIC} (a, b) and control cells (c) in BHI for 24 h. The full arrows indicate disruptions on the cell wall and dashed arrows indicate loss of cell content.

The TEM images of the treated bacterial cells are illustrated in Figure 5.4. The bacterial cells treated with MNPs-OA-P_{MIC}-CLo_{MIC} showed marked morphological differences (Figure 5.4a, b) in comparison with the control bacterial cells, grown in BHI medium with no treatment (Figure 5.4c). The morphological changes observed in the bacterial cells treated with MNPs-OA-P_{MIC}-CLo_{MIC}include disruption and protrusions of the cell wall, in contrast to the control cells for which the bacterial surface was not compromised.

5.3.2.3. FTIR

Figure 5.5 presents the FTIR spectra of bare and functionalized MNPs. The spectra of MNPs and MNPs functionalized with oleic acid (MNPs-OA) are similar to those of NPs used by us in a former work.[6] The MNPs further functionalized with propolis extract (MNPs-OA-P_{MIC}) present a spectrum very similar to that of the corresponding NPs used in the above referred work, except for the two peaks at 1171 and 1267 cm^{-1}. These must correspond to the

stretching vibrations of C—O and C—O—C in alcohols, esters and sugars present in propolis. They should also be seen in the spectra of the former samples of propolis extract functionalized MNPs, so probably they were simply less resolved than in the current sample spectrum. The several samples of MNPs prepared with different concentrations of a functionalizing agent had identical spectra and, for that reason, each spectrum presented in Figure 5.5 is representative of the corresponding kind.

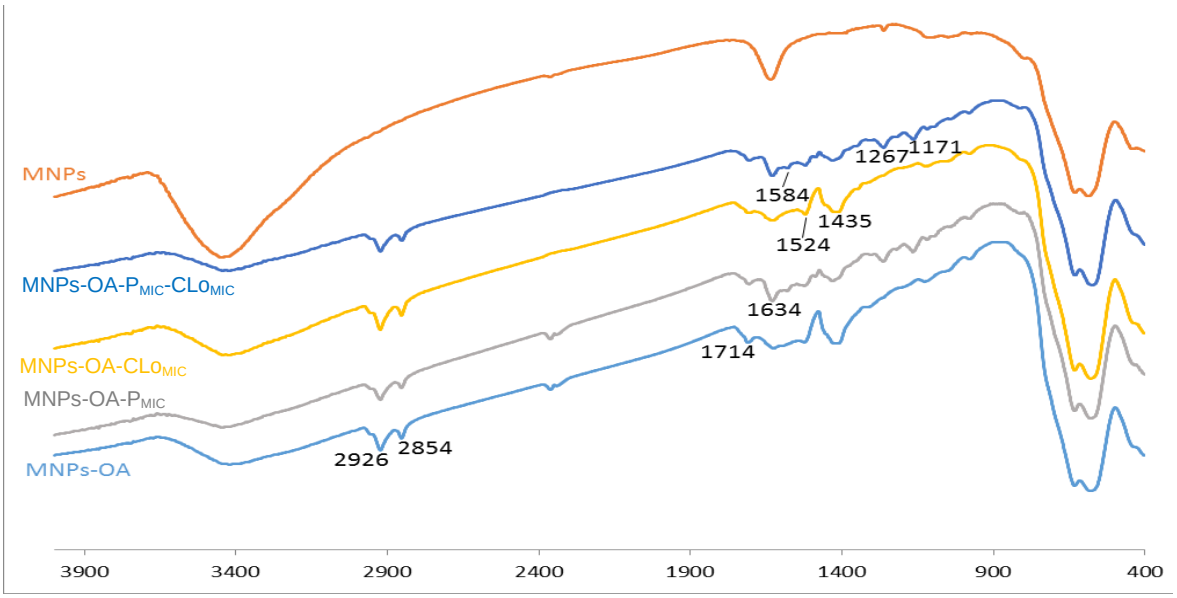


Figure 5.5. FTIR spectra of nude magnetite nanoparticles (MNPs); MNPs treated with oleic acid (MNPs-OA); MNPs treated with olecic acid and propolis extract at MIC value (MNPs-OA-P$_{MIC}$); MNPs treated with oleic acid and chloramphenicol at MIC Value (MNPs-OA-CLo$_{MIC}$) and MNPs treated with oleic acid, propolis extract at MIC value and chloramphenicol at MIC value (MNPs-OA-P$_{MIC}$-CLo$_{MIC}$).

All spectra of functionalized MNPs are very governed by the absorption bands of both magnetite and oleic acid. The presence of propolis components induces only subtle differences, as described previously[6] and stated above. The same goes for the presence of chloramphenicol (samples MNPs-OA-CLo$_{MIC}$ and MNPs-OA-P$_{MIC}$-CLo$_{MIC}$), whose major characteristic absorption bands[26] are desguized by those of magnetite and oleic acid: O—H and N—H stretching, at 3476, 3350, and 3258 cm^{-1} lay beneath the broad band centered near 3440 cm^{-1}; aromatic C—H and CH_2 asymmetric and symmetric streches at 3078, 2960, and 2905 cm^{-1}, fuse with the band centered at 2926 cm^{-1}; the only IR active C—Cl vibration is the C—Cl_2 asymmetric stretching at 778 cm^{-1}, which is masked by the very strong and broad band of magnetite.

Nevertheless, the broad band between 1475 and 1700 cm^{-1} presents indeed some differences in the spectrum of chloramphenicol functionalized NPs (MNPs-OA-CLo$_{MIC}$)

relative to that of MNPs-OA, which may be attributed to the C═O stretching, N—H bending, aromatic C═C stretching, and NO_2 asymmetric stretching, which occur between 1520 and 1686 cm^{-1}. Differences between these two spectra are also denoted in the band between 1300 and 1475 cm^{-1}. In this region occur absorptions due to aromatic ring and C—N stretching as well as NO_2 symmetric stretching. These observations support, at least to some extent, the adhesion of the drug to the oleic acid functionalized MNPs. However, when it is combined with propolis extract, that adhesion is less evident, as the spectrum of MNPs-OA-P_{MIC}-CLo_{MIC} very much resembles that of MNPs-OA-P_{MIC}. A small difference may be perceived at 1350 cm^{-1}, where the strong absorption band of NO_2 symmetric stretching is expected, which may be indicative of the presence of the drug.

5.3.3. Evaluation of antibacterial activity of propolis nanocomposite

The MIC of propolis sample and the antibiotic chloramphenicol were determined by the microdilution method for all *S. aureus* strains tested (Table 5.1). MIC values of propolis were 0.24 mg/mL for MSSA ATCC6538 and 0.36 mg/mL for MRSA2, MRSA15 and MRSA16. Whereas, the MIC values of CLo were 0.025 mg/ml for MSSA ATCC 6538, 0.09 mg/mL for MRSA2 and MRSA16, while MRSA 15 showed the highest MIC value, 0.13 mg/mL (Table 5.1). The assay using the combination of propolis and chloramphenicol at the MIC value with/without MNPs-OA ascertained an antimicrobial activity against the tested microorganisms (Figure 5.6). As shown in Figure 5.6 the growth of the bacterial cells of MRSA 15, MRSA16 and MSSA ATCC 6538 exposed only to MNPs-OA, just started to grow after 8 h of exposure, however, after 24 h reached similar CFU values to the control. Contrariwise, for MRSA 2 the growth started after six hours of incubation. The growth of the cells treated with free P_{MIC}-$CLo_{MIC}$was largely impaired especially for MRSA2 and MRSA16 for which no viable cells were recovered after 2 h exposure. For the strains MRSA15 and MSSA ATCC6538 the viability was maintained over initial values but after 24 h of exposure no viable cells were recovered. Moreover, MNPs-OA-P_{MIC} and MNPs-OA-CLo_{MIC}, have affected the growth of all tested strains, the bacterial cells were not able to recover during 8 h of exposure, but after 24 h is possible to observe a slight recovery for MNPs-OA-$P_{MIC.}$ On the other hands, assembling the P_{MIC} and CLo_{MIC} (MNPs-OA-P_{MIC}-CLo_{MIC}) have impaired the viability of all strains, in contrast with the MNPs-OA-P_{MIC} and MNPs-OA-CLo_{MIC} alone (Figure 5.6). This effect was more pronounced for MRSA15 for which no viable cells were recovered after 24 h.

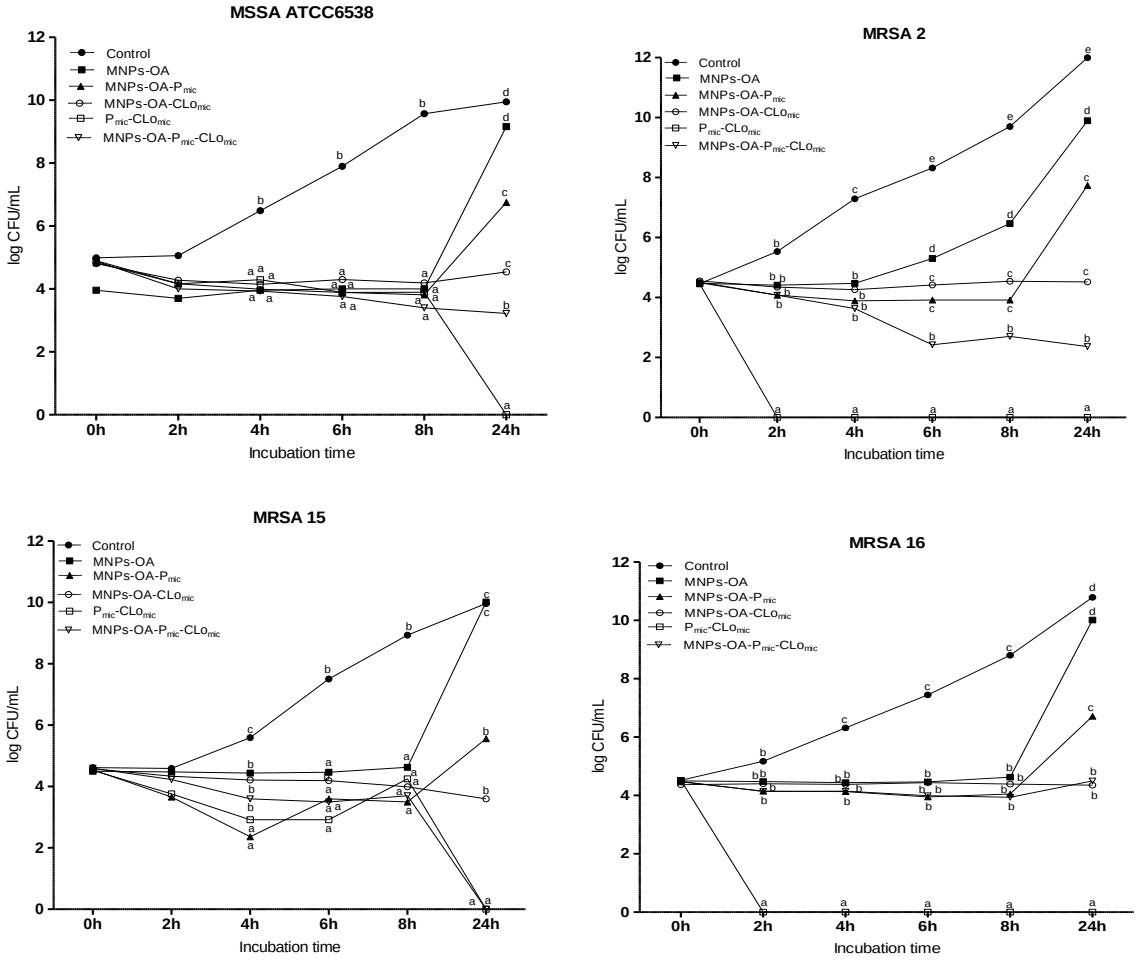


Figure 5.6. Effect of the MNPs-OA-P-CLo combination on the growth of the different strains of *S. aureus.* (propolis and chloramphenicol were tested at MIC values previously determined for each tested strain).

The fabricated nanostructure was characterized by DRX, TEM, and FTIR. The results (Figure 5.2 and 5.3) displayed the diffraction peaks present in the X-ray diffractograms and TEM functionalized MNPs (MNPs-OA-P_{MIC}-CLo_{MIC}; MNPs-OA-P_{MIC} and MNPs-OA-CLo_{MIC}). Besides indicating that magnetite showed a good crystallize structure and a roughly spherical shape, as describe by El-Guendouz et al.,[17] it makes on evident that the functionalization procedure does not change the structure and composition of the nanoparticles.

In the present study, the antimicrobial activity of propolis twined with synthesized MNPs-OA nanocomposite against important HAIs pathogens responsible for several difficult-to-threat infections in humans was determined. The produced MNPs only marginally affected the bacterial growth of the tested MSSA and MRSA strains, showing similar CFU numbers to the control cells. The different combinations tested in this study demonstrate that the MNPs-OA-P_{MIC}-CLo_{MIC} nanocomposite have antibacterial activity against MSSA ATCC6538, MRSA2, MRSA15 and MRSA16. In general, a differential growth inhibition pattern of tested strains

using the different treatments was observed. This has resulted in a significant decline in bacterial viability (Figure 5.5), which explain the results observed in transmission electron microscopy (Figure 5.4a–c) where obvious cellular damage among bacterial cells exposed to MNPs-OA-P_{MIC}-CLo_{MIC} (Figure 5.3a, b) in comparison with those not exposed to any treatment (Figure 5.4c). Furthermore, MNPs-OA-P_{MIC}-CLo_{MIC} showed a significant effect on imparing the bacterial recovering as compared to MNPs-OA-P_{MIC} or MNPs-OA-CLo_{MIC} alone, this effect could be considered as an additive effect of both components. Amongst the tested strains, MRSA2 and MRSA16 were the most susceptible for P_{MIC}-$CLo_{MIC,}$ whereas all strains used in this study were susceptible to MNPs-OA-P_{MIC}-CLo_{MIC} nanocomposite.

The utilization of propolis extracts has already been assayed as anti-MRSA agents, according the results of several authors who observed anti-MRSA activity in propolis of diverse origins.[24] The antimicrobial properties found can be attributed to several compounds that constitute the crude extract of propolis. For example, the cinnamic acids are usual components of the propolis and some antioxidant and antibacterial activities have been associated with some of these compounds.[23,24] However, this effect can also be related to the presence of flavonoids, as one of the most important compositions of crude propolis. This group of compounds was already reported in some Moroccan propolis.[6,27] Wojtyczka et al.[24] have shown that a hydro-alcoholic (70%) extract of propolis samples from Southern Poland with major components pinocembrin, kaempferol, galangin, chrysin, apigenin, quercetin had a high antibacterial effect on five MRSA strains isolated from blood clinical origin. However, the same propolis extract also had gallic acid, ferulic acid, caffeic acid phenethyl ester, *p*-coumaric acid and cinnamic acid. Boisard et al.[28] have shown that propolis samples from France impaired the growth of six MRSA strains, and the propolis samples contained pinobanksin-3-acetate, pinocembrin, chrysin, galangin, prenyl caffeate, as major compounds. Some of compounds cited by previous authors with antibacterial activity on MRSA strains were found also in our sample, such as pinostrobin, tectochrysin, 4′,7 dimethoxy-apigenin, chrysin and hydrocinnamic acid, which may be, at least, partially responsible for the antibacterial activity observed.

5.4. Conclusion

The importance of nanoparticles as drug delivery systems with optimized physicochemical and biological properties consists in the fact that they are taken up by the cells more easily than larger molecules, so they can be successfully used as delivery tools for currently available bioactive compounds. Overall, in this study, we report the synthesis of a

15 nm nanostructure, which when combined with propolis extract and the antibiotic CLo, exhibited antibacterial activity against different *S. aureus* strains, including MRSA. The action of the propolis nanocomposite targeted the bacterial cell wall evidencing their disruption and protrusions formation. The obtained results demonstrated that the proposed strategy prove to have an important benefit due to its antibacterial properties on demand for numerous medical applications.

References

[1] P.A. Araújo, F. Mergulhão, L. Melo, M. Simões, 'The ability of an antimicrobial agent to penetrate a biofilm is not correlated with its killing or removal efficiency', Biofouling. 2014, 29, 675–83.

[2] S.S. Mao, S. Shen, L. Guo, 'Nanomaterials for renewable hydrogen production, storage and utilization', Prog. Nat. Sci. Mater. Int. 2012, 22, 522–34.

[3] M. D. Moya-Ortega, C. Alvarez-Lorenzo, A. Concheiro, T. Loftsson, 'Cyclodextrin-based nanogels for pharmaceutical and biomedical applications', Int. J. Pharm. 2012, 428, 152–63.

[4] I. Khan, K. Saeed, I. Khan, 'Review: nanoparticles: properties, applications and toxicities', Arab. J. Chem. 2017. Doi:10.1016/j.arabjc.2017.05.011.

[5] A.N. Sahu., 'Nanotechnology in herbal medicines and cosmetics', Int. Res. Ayurveda. Pharma. 2013, 4, 472–4.

[6] S. El-Guendouz, S. Aazza, B. Lyoussi, V. Bankova, J.P. Lourenço, A.M. Rosa Costa, J.F. Mariano, M.G. Miguel, M.L. Faleiro, 'Impact of biohybrid magnetite nanoparticles and Moroccan propolis on adherence of methicillin resistant strains of *Staphylococcus aureus'*, Molecules. 2016, 21, 1208.

[7] I. Liakos, A. M. Grumezescu, A. M, 'Holban Magnetite nanostructures as novel strategies for anti-infections therapy', Molecules. 2014, 19, 12710–26.

[8] A.S. Arbab, L. A. Bashaw, B. R. Miller, E. K. Jordan, B. K. Lewis, H. Kalish, J. A. 'Frank Characterization of biophysical and metabolic properties of cells labeled with superparamagnetic iron oxide nanoparticles and transfection agent for cellular MR imaging', Radiology. 2003, 229, 838–46.

[9] X. H. Jing, L. Yang, X. J. Duan, B. Xie, W. Chen, Z. Li, H .B. Tan 'In vivo MR imaging tracking of magnetic iron oxide nanoparticle labeled, engineered, autologous bone marrow mesenchymal stem cells following intra-articular injection', Joint. Bone. Spine. 2008, 75, 432–8.

[10] C. Chifiriuc, V. Grumezescu, A. M. Grumezescu, C. Saviuc, V. Lazar, E. Andronescu 'Hybrid magnetite nanoparticles/Rosmarinus officinalis essential oil nanobiosystem with antibiofilm activity', Nanoscale. Res. Lett. 2012, 7, 209. Doi:10.1186/ 1556-276X-7-209.

[11] A. Poiata, C. Tuchilus, D. Creanga, C. Stan', Magnetic nanoparticles influence on some bacterial cultures', Rom. J. Biophys. 2013, 23, 203–9.

[12] G. A. Burdock, 'Review of the biological properties and toxicity of bee propolis (propolis)', Food. Chem. Toxicol. 1998, 36, 347–63.

[13] V. D.Wagh, 'Propolis: a wonder bee product and its pharmacological potentials', Adv. Pharmacol. Sci. 2013, 2013, 11.

[14] S. El-Guendouz, S. Aazza, B. Lyoussi, M. D. Antunes, M. L. Faleiro, M. G. Miguel, 'Anti-acetylcholinesterase, antidiabetic, anti-inflammatory, antityrosinase and antixanthine oxidase activities of Moroccan propolis', Int. J. Food. Sci. Technol. 2016, 51, 1762–73.

[15] J. M. Sforcin, 'Biological properties and therapeutic appications of propolis', Phytother. Res. 2016, 30, 894–905.

[16] S. El-Guendouz, N. Al-Waili, S. Aazza, Y. Elamine, S. Zizi, T. Al-Waili, A. Al-Waili, B. Lyoussi, 'Antioxidant and diuretic activity of co-administration of Capparis spinosa honey and propolis in comparison to furosemide', Asian. Pac. J. Trop. Biomed. 2017, 10, 974–980.

[17] S. El-Guendouz, S. Aazza, B. Lyoussi, V. Bankova, M. Popova, L. Neto, M. L. Faleiro, M. G. Miguel, 'Moroccan Propolis: a natural antioxidant, antibacterial, and antibiofilm against *Staphylococcus aureus* with no induction of resistance after continuous exposure', J. Evid. Based. Complement. Altern. Med. 2018, 19. Article ID 9759240, pages, https://doi.org/10.1155/2018/9759240 .

[18] European Centre for Disease Prevention and Control, Annual Epidemiological Report Stockholm: ECDC. https://ecdc.europa.eu/en/publications-data/ healthcare-associated-infections-acquired-intensive-care-units-annual, 2016.

[19] A. I. Cotar, M. C. Chifiriuc, S. Dinu, M. Bucur, C. Iordache, O. Banu, O. Dracea, C. Larion, V. Lazar, 'Screening of molecular virulence markers in *staphylococcus aureus* and Pseudomonas aeruginosa strains isolated from clinical infections', Int. J. Mol. Sci. 2010, 11, 5273–91.

[20] S. H. Hussein-Al-Ali, M. E. el Zowalaty, M. Z. Hussein, B. M. Geilich, T. J. Webster, 'Synthesis, characterization, and antimicrobial activity of an ampicillin-conjugated magnetic nanoantibiotic for medical applications', Int. J. Nanomedicine. 2014, 9, 3801–14.

[21] A. A. Miles, S. S. Misra. 'The estimation of the bactericidal power of blood', J. Hyg. 1938, 38, 732–49.

[22] E. Melliou, E. Stratis, I. Chinou, 'Volatile constituents of propolis from various regions of Greece–antimicrobial activity', Food. Chem. 2007, 103, 375–80.

[23] A. Kurek-Górecka, A. Rzepecka-Stojko, M. Górecki, J. Stojko, M. Sosada, G. Świerczek-Zięba, 'Structure and antioxidant activity of polyphenols derived from propolis', Molecules. 2014, 19, 78–101.

[24] R. D. Wojtyczka, A. Dziedzic, D. Idzik, M. Kepa, R. Kubina, A. Kabala-Dzik, J. Smolén-Dzirba, J. Stjko, M. Sajewicz, T. J. Wasik, 'Susceptibility of *Staphylococcus aureus* clinical isolates to propolis extract alone or in combination with antimicrobial drugs', Molecules. 2013, 18, 9623–40.

[25] M. Mahdavi, M. B. Ahmad, M. J. Haron, F. Namvar, B. Nadi, M. Z. A. Rahman, J. Amin, 'Synthesis, surface modification and characterisation of biocompatible magnetic iron oxide nanoparticles for biomedical applications', Molecules. 2013, 18, 7533–48.

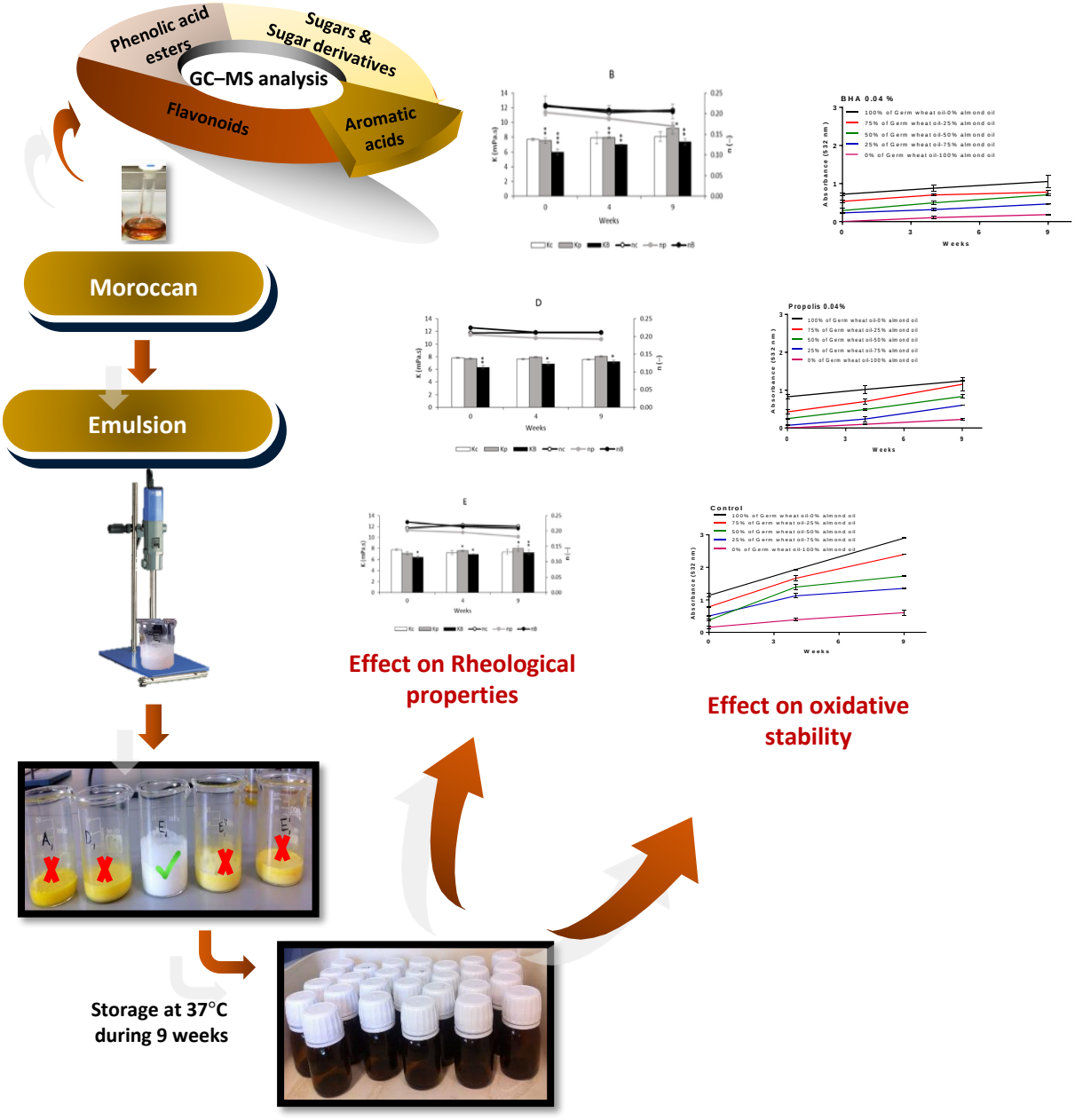
[26] D. Sajan, G. D. Sockalingum, M. Manfait, I. Hubert-Joe, V. S. Jayakumar, 'NIR-FT Raman, FT-IR and surface-enhanced Raman scattering spectra, with theoretical simulations on chloramphenicol', J. Raman. Spectrosc. 2008, 39, 1772–83.

[27] M. Popova, B. Lyoussi, S. Aazza, D. Antunes, V. Bankova, G. Miguel, 'Antioxidant and α-glucosidase inhibitory properties and chemical profiles of Moroccan propolis', Nat. Prod. Commun. 2015, 10, 1961–1964.

[28] S. Boisard, A. M. Le Ray, A. Landreau, M. Kempf, V. Cassisa, C. Flurin, P. Richomme, 'Antifungal and antibacterial metabolites from a French poplar type propolis', Evid. Based. Complement. Alternat. Med. 2015. Doi:10.1155/2015/319240

Chapter 6

Effect of poplar-type propolis on oxidative stability and rheological properties of O/W emulsions



Effect of poplar-type propolis on oxidative stability and rheological properties of O/W emulsions*♠

Abstract

Propolis is known to possess antioxidant activity. However, there is no information on this activity in emulsions O/W. The protective effect of propolis on the oxidation and rheological properties of emulsions O/W containing wheat germ and almond oils was evaluated. Emulsions O/W were prepared with different concentration of propolis extract, almond oil and wheat germ oil. All emulsions physically stable without phase separation were stored at 37 °C for 9 weeks. Chemical composition of propolis was established by Gas chromatography coupled to mass spectrometry. Rheological characterization of different emulsions was performed evaluating consistency index and flow behavior index. The oxidation was monitored by measuring the lipid hydroperoxides and thiobarbituric acid-reactive substances (TBARS) methods. Flavonoids, phenolic acid esters, and aromatic acids were the main groups of compounds found in propolis. The results showed that popolis was good antioxidant in the concentration of 0.02 and 0.04% when lipid phase was constituted by almond oil. The rheological behavior is typical of a non-Newtonian fluid, being almond oil more adequate for having a higher stable O/W emulsion.

*Adapted from 'Effect of poplar-type propolis on oxidative stability and rheological prop-erties of O/W emulsions'. Soukaina El-Guendouz, Smail Aazza, Badiaa Lyoussi, Nessrine, Majdoub, Vassya Bankova, Sara Raposo, Maria Dulce Antunes, Maria Graça Miguel. Saudi Pharmaceutical Journal. Volume 26, Issue 8, December 2018, Pages 1073-1082,

♠ The chemical characteristics of almond and wheat germe oils according to the specifications of the trade marks, the effect of adding propolis extract and BHA at different concentrations (0.01; 0.02 and 0.04 %) on TBARS hydroperoxide as well as on the apparent viscosity of O/W emulsions are described in appendix C

6.1. Introduction

Consumers have become more conscious and have a greater awareness of health and well-being. The demand for products with natural components instead synthetic ones has drastically risen, however, many of them still contain synthetic chemicals. Nowadays, one of the challenges of food and cosmetic industries is to formulate products not only with the adequate attributes as taste, color, fragrance, but also with functionality, safety and adequate stability, replacing some synthetic ingredients by natural ones.

Many formulated foods, pharmaceuticals and cosmetics contain a lipid phase dispersed in an aqueous medium, that is, they are oil-in-water (O/W) emulsions. The lipid phase may be constituted by vegetable oils (e.g. almond, and wheat germ oils). In cosmetic formulations, almond oil has been used in facial care, as moisturizer for excessively dry skin, sunburn and wind burn. It is used in lip balms, creams and in formulations for infants.[1] Wheat germ oil used in microemulsions, lotions and creams, has proved to be effective in treatment of sunburn and in some skin diseases such as psoriasis and eczema.[2]Although the antioxidant properties that have been attributed to almond and wheat germ oils, they may present susceptibility to oxidation, due to their triglycerides rich in polyunsaturated fatty acids (PUFAs).[2,3]

The oil phases of the emulsion may easily oxidize due to their large surface area that facilitates not only the interactions between the lipids and the water-soluble prooxidants but also favour the availability of the oil phase to oxygen dissolved in the aqueous phase which could be produced during the emulsification process, due to the overheating of the shear. In addition, formation of free radicals is also possible to occur in the emulsification process in the case of sonication, due to acoustic cavitation.[4] The oxidation of lipids can not only disturb the emulsion stability due to the formation of the new compounds but also weaken the quality of the product. Such compounds are unpleasant odorants and therefore responsible for the decline of the product quality, with the disadvantage of forming harmful radicals for human health.[4]

Several strategies have been developed to overcome the lipid oxidation and one of them is the utilization of antioxidants[4,5] Butylhydroxyanisole (BHA) and butylhydroxytoluene (BHT) are two synthetic compounds generally used as antioxidant in products containing fats or oils. However, several studies have reported that these compounds may be harmful for human health[6] although many other studies, as reviewed by[7] have claimed that the toxic effects may be attributed more to BHT metabolites than to the parent compound.

Beehive is an unparalleled source of natural products, such as propolis. It is a resinous substance collected by honeybees from parts of plants, buds, and exudates, mixed with bee wax and salivary enzymes. Bees use propolis to protect themselves from wind, rain, insects, microorganisms, employing it as a cement to seal cracks or open spaces in the hive, to sterilize the queen-bee posture site, and to mummify insect invaders.[8] The wide application of propolis in modern medicine and the increasing demand for it, due to its health benefits and use in cosmetic and food products, have drawn growing attention to its chemical composition which is complex and varies according to the floral biodiversity of the region. Polyphenols, terpenoids including steroids, naphthalene and stilbene derivatives, fatty acids, sugars, vitamins and minerals are some examples of compounds that can be found in propolis.[9,10] Propolis present a series of biological properties such as antimicrobial, anti-inflammatory, antioxidant, antidiabetic, spasmolytic, anaesthetic, anticancer, and immunomodulatory effects.[9-14] Propolis has been used in foods and beverages, chewing gums, cosmetics, creams, skin creams, among others, not only due to their preservative properties but also as their biological attributes for maintaining or improving health.[11-18] From the above, we can state that propolis can be a promising product that can replace the synthetic ones in the development of new GRAS (generally recognized as safe) products.

The main goal of the present work is to compare the ability of propolis (natural product) for preventing the lipid peroxidation of almond or wheat germ oils (natural products) used as lipid phase of the O/W emulsions with that of BHA (synthetic product) and at the same time evaluate its influence on some parameters of the emulsions' rheology.

6.2. Experimental

6.2.1. Chemicals

BHA was purchased from Sigma Aldrich Chemie, Steinheim, Germany. Almond oil and wheat germ oil were purchased from LBCHEM Labospirit, Lda Portugal. Tween 80, Span 80, Phenonip and Xanthan gum were purchased from Guinama S.L.U Spain. Thiobarbituric acid (TBA), ferrous chloride ($FeCl_2$) and ammonium thiocyanate (NH_4SCN) were purchased from Fluka, Biochemika, Sigma-Aldrich, Steinheim, Germany. Trichloroacetic acid was purchased from VWR, Leuven, Belgium. 1-Butanol, methanol and HCl was from Fisher Scientific UK Ltd, Loughborough, UK.

6.2.2. Propolis extraction

Extracts were obtained as reported by El-Guendouz et al..[13] Briefly, one gram of propolis from Morocco (region of Fez-Boulmane) was chopped into small pieces and extracted by maceration using 30 mL of 70% ethanol and maintained for 1 week at 37 °C under agitation (200 rpm). The resulting solution was filtered under vacuum. A clear solution, without further purification, was used to prepare the emulsions.

6.2.3. GC–MS analysis of propolis extract

The analysis was performed with a Hewlett–Packard gas chromatograph 5890 series II Plus linked to a Hewlett–Packard 5972 mass spectrometer (Hewlett-Packard, Wilmington, DE, USA) system equipped with a 30 m long, 0.25 mm i.d., and 0.5 µm film thickness HP5-MS capillary column. The work conditions were the same as previously reported.[13] Semi-quantification was carried out by internal normalization with the area of each compound. The addition of individual areas of the compounds corresponds to 100% area. Compound identification was performed using commercial libraries and comparison of mass spectra and retention times of reference compounds.

6.2.4. Preparation and storage of O/W emulsion

Oil-in-water (O/W) emulsions were prepared using Tween 80 and Span 80 as surfactants. The oil phases were constituted by the vegetable oils, wheat germ and almond. Phenonip (atimicorbial) and xanthan gum (stabilizer)[19] were also used. Two antioxidants, propolis and butylated hydroxyanisole BHA (positive control), were tested with three different concentrations 0.01% (w/w), 0.02% (w/w) and 0.04% (w/w) each. Briefly, O/W emulsions were prepared by mixing almond oil and wheat germ with different concentration each (A: 100% of wheat germ oil/0% almond oil; B: 75% of wheat germ oil/25% almond oil; C: 50% of wheat germ oil/50% almond oil; D: 25% of wheat germ oil/75% almond oil and E: 0% of wheat germ oil/100% almond oil). For each formulation (A, B, C, D and E) Tween 80 (1.1%, w/w), Span 80 (0.9%, w/w), Phenonip (0.8%, w/w), xanthan gum (0.9%, w/w), and propolis extract or BHA were added. Afterwards distilled water was added to 100% (w/w) and all the component were mixed with an ultra turrax homogenizer (IKA T18D Ultra Turrax, 50/60 HZ, 500 W, Germany) working at 16,000 rpm for 5 min. All emulsions physically stable were transferred to round glass amber bottles and stored in an incubator at 37 °C for 9 weeks. The assays were prepared in triplicate. A similar procedure was repeated to prepare a negative control (emulsions without

antioxidant). The concentrations of each ingredient used in this work to prepare the O/W emulsions were chosen after different assays (data not shown), since they produced the most stable emulsions.

6.2.5. Peroxide content determination in cosmetic emulsions

For the determination of primary oxidation products, lipid hydroperoxides were measured as describe by[20], with slight modification. One hundred (100 μL) of emulsion were mixed with 9.5 mL of methanol/butanol (2:1, v/v) by vortexing (10 s), then 0.1 mL of 30% ammonium thiocyanate and 0.1 mL of 0.02 M ferrous chloride in 3.5% hydrochloric acid were added. The absorbance of the solution was measured at 510 nm, 3 min after addition of the ferrous chloride against the control sample. Hydroperoxide content was expressed as absorbance (A_{510}). All emulsions were tested at weeks 0, 2, 4, 6 and 9.

6.2.6. Determination of lipid oxidation products (thiobarbituric acid-reactive substances)

Thiobarbituric acid-reactive substances (TBARS) were determined on the emulsions according to some authors,[21] with minor modifications. TBARS reagent was prepared by mixing 15% (w/v) trichloroacetic acid, 0.375% (w/v), 2-thiobarbituric acid in 1.76 mL of 12 M hydrochloric acid, then distilled water was added to 100 mL (w/v). Then one mL of emulsion sample and two mL the TBARS reagent were well vortexed for 30 s, placed in a boiling water bath for 15 min and then cooled down in room temperature for 30 min. 1-Butanol (2 mL) was added and mixture was centrifuged at 5000 rpm, for 10 min. The colorful supernatant was collected and the absorbance was measured at 532 nm.

6.2.7. Rheological studies

Rheological properties of the different samples were determined, using a Brookfield programmable rotational viscometer LVDV-II+Pro (Brookfield Engineering Laboratories Inc) equipped with the software Rheocalc 32 (version 2.4.). The experimental conditions were adjusted in preliminary tests and the studies were performed at 25 ± 1 °C and spindle LV-3C speeds ranging from 20 to 100 rpm, with an up-down rate ramp. The viscosity (η) was determined at shear rates between 2 and 21 s^{-1}. Each shear rate was imposed during 1 min to stabilized the viscosity. The assays were performed under the same conditions and identical container and were repeated at least two times and the experimental data fitted to the Power Law model (Eq. (1)) to establish the type of non-Newtonian emulsion behavior.

(1) $\tau=K\gamma^{n}$

where τ is the shear stress, γ is the shear rate, K is the consistency index, and n is index of fluid behavior.

The viscosity was related with the equation of Power-law model by the Eq. (2).

(2) $\eta=K\gamma^{n-1}$

From this model it can be inferred that if $n > 1$, the fluid is showing shear thickening behavior; if $n < 1$, it shows shear thinning behavior; if $n = 1$, it is exhibiting Newtonian behavior.

To eliminate the hypothesis of xanthan gum conditioned the rheological behavior of the emulsions, we proceed to the measure of viscosity in the same conditions of the assay. The viscosity of xanthan gum solution 0.9% (w/w) was 1240 ± 53 mPa·s, determined at a shear rate of 6.3 s^{-1} and present a shear thinning behavior.

6.2.8. Statistical analysis

TBARS Data were analyzed by two-way ANOVA using GraphPad Prism version 5.03 for Windows (GraphPad Software, La Jolla California, USA). Statistical significance was set as $p < 0.05$; when the analysis was statistically significant, the Duncan post hoc test was done. Rheological analyses were performed on duplicated samples. Statistical analysis was conducted with a SigmaPlot 12 software (Systat Software, version 12 for Windows), implementing the one-way ANOVA method. Significant differences between means (at the level of $P < 0.05$) were determined by Student–Newman–Keuls test.

6.3. Results and discussion

6.3.1. Chemical composition of propolis extract

Table 6.1 depicts the chemical composition of the hydro-alcoholic extract of propolis, and Figure C.1 (Appendix C) shows the chromatographic profile. Flavonoids (51.9%), phenolic acid esters (22.4%), sugars and sugar derivatives (9.2%) and aromatic acids (4.3%) were the main groups of compounds found in this extract. Flavonoids, the major group of phenol components, were mainly represented by galangin (8.7%), pinocembrin chalcone (6.6%), pinocembrin (6.6%), pinobanksin acetate (5.4%) and chrysin (5.4%), all of them with concentrations higher than 5%. The phenolic acid ester were the second abundant group in the propolis component and some of them were present also

at a concentrations higher than 5%: pentenyl caffeate isomers (7.1% and 5.2%) (Table 6.1). The phenolic composition of propolis extract is somehow similar to that of poplar origin.[22]

Table 6.1. Chemical composition of hydro-alcoholic extract of Moroccan propolis.

Aromatic Acids	**%**	**Phenolic Acid Esters**	**%**	**Flavonoids**	**%**	**Sugars and Sugar Derivatives**	**%**
E-caffeic acid	2.2	Pentenyl caffeate (isomere)	7.1	Galangin	8.7	Disaccharides	1.1
Dimethoxycinnamic acid	0.7	Pentenyl caffeate (isomere)	5.2	Pinocembrin chalcone	6.6	Glycerol	0.6
Z-Caffeic acid	0.6	Pentenyl caffeate (isomere)	3.6	Pinocembrin	6.6	Fructofuranose	2.0
Ferulic acid	0.5	CAPE	2.2	Pinobanksin acetate	5.4	Glucopyranose	1.8
p-Coumaric acid	0.3	Benzyl caffeate	1.3	Chrysin	5.4	Sugar	0.9
		Pentyl caffeate	1.3	Pinobanksin	4.8	Sugar	0.7
		Pentenyl caffeate (isomere)	0.7	Pinobanksin chalcone	2.7	Sugar	0.7
		Pentenyl caffeate	0.4	Tectochrysin	1.8	Sugar	0.5
		Pentenyl p-coumarate (isomere)	0.3	Pinobanksin acetate chalcone	1.6	Sugar	0.5
		Pentenyl p-coumarate (isomere)	0.2	Kaempferol	1.4	Sugar	0.2
		Pentenyl *p*-coumarate	0.1	Kaempferol methyl ether (isomere)	1.4	Sugar	0.2
				Quercetin methyl ether (isomere)	1.1		
				Pinostrobin chalcone	0.9		
				Quercetin	0.7		
				Quercetin methyl ether.	0.7		
				Kaempferol methyl ether	0.7		
				Alpinone chalcone	0.7		
				Quercetin	0.7		
Total	**4.3**	**Total**	**22.4**	**Total**	**51.9**	**Total**	**9.2**
Standard deviation does not succeed 6% for any of the constituents							

6.3.2. Protective effect of propolis extract on oxidative stability of W/O emulsion measured through peroxide content

Figure 6.1 showed that hydroperoxides increased as time increased to nine weeks for all emulsion comparing with the control (Blank). Propolis extracts and BHA at higher concentrations (0.02% and 0.04%) present the best protecting effect on the oxidation at the formulation D and E (Figure 6.1).

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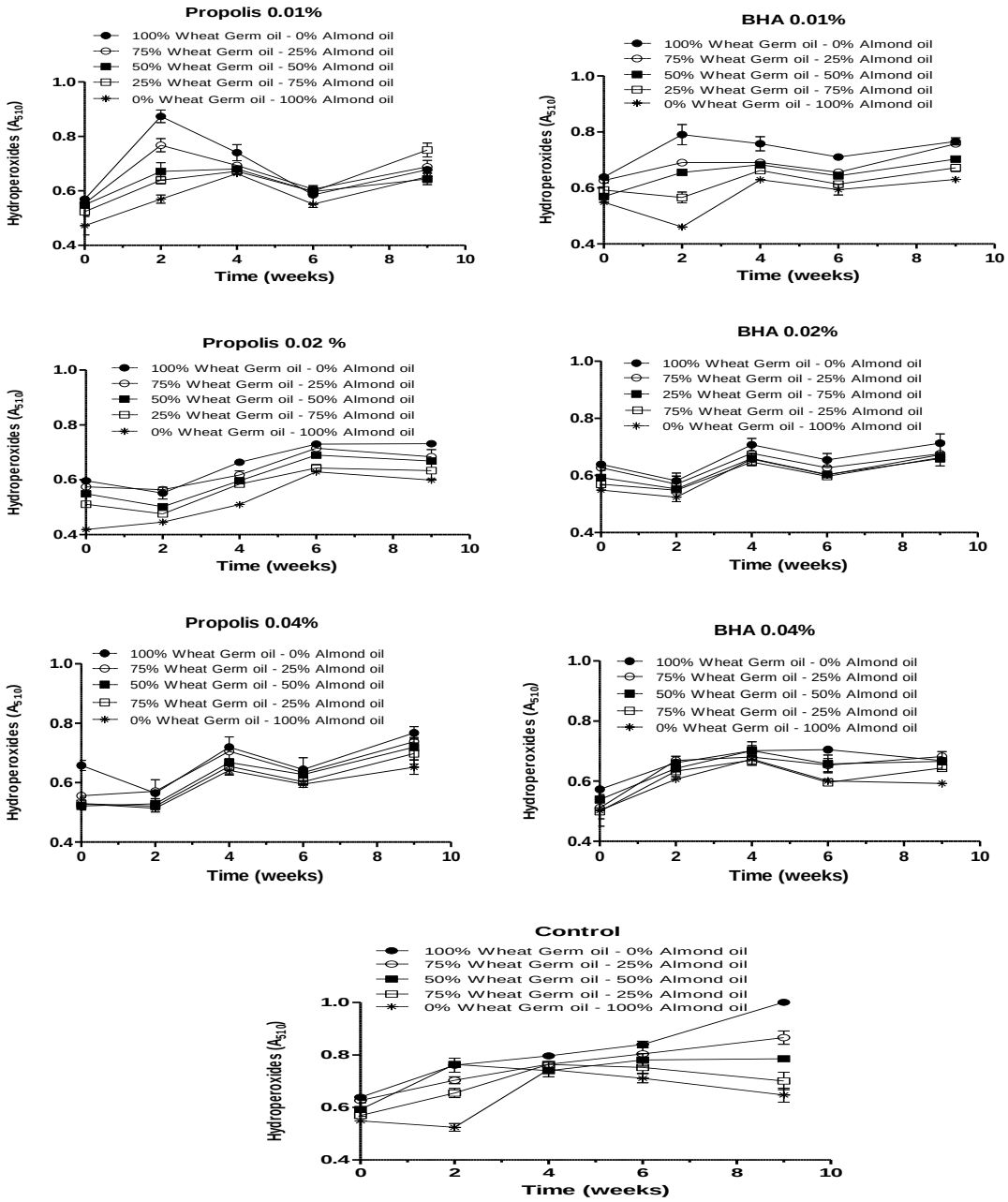


Figure 6.1. Effect of adding propolis extract and BHA at different concentrations (0.01%; 0.02% and 0.04%) on hydroperoxides (ABS_{510}) of O/W emulsions made with germ wheat oil and almond oil under storage conditions of 9 weeks at 37 °C. Values of mean (n = 6) ± standard deviation.

The results depicted in Appendix C, Table C.2 indicated that a significant ($p < 0.05$) increase of absorbance at $\lambda = 510$ nm, which means an evolution of primary oxidation products, was observed in all emulsions in comparison to the control before and after nine weeks of storage at 37 °C. It can also be seen from Appendix C, Table C.2, in the presence of propolis, or BHA that the higher concentration of almond oil is the best preventing lipid peroxidation (D: 75% almond oil and 25% wheat germ, E: 100% almond oil). Regarding the different

concentration of the propolis and BHA (0.01%; 0.02% and 0.04%), propolis revealed to have a similar effect as BHA in preventing oxidation.

Neither BHA nor propolis extract were so good for preventing lipid peroxidation of wheat germ as they were with almond oil. This may be explained by the predominance of polyunsaturated fatty acids constituting the triacylglycerols of wheat germ. Each additional double bond introduced into a fatty acid, at least doubles its rate of oxidation.[4] It might be concluded that the combination between propolis or BHA and almond oil has a protective effect against oxidation of O/W emulsions

6.3.3. Protective effect of propolis extract on oxidative stability of W/O emulsion measured through TBARS method

The reaction of reactive substances (degradation products of lipidic peroxidation, including malondialdehyde) with thiobarbituric acid, originates a pink pigment that is directly proportional to the amount of those reactive substances produced, which absorbance can be measured at $\lambda = 532$ nm. The increase of absorbance reveals, therefore, accumulation of reactive substances, including malondialdehyde, with thiobarbituric acid, which means oxidation. Figure 6.2 depicts the evolution of absorbance at $\lambda = 532$ nm of emulsions O/W with various proportions of almond and wheat germ oils in the presence of three concentrations of propolis extract or BHA at three concentrations (0.01, 0.02 and 0.04%), over time (9 weeks). A blank was performed without BHA or propolis extract. In all cases, the absorbance rose over time, being clearly evident that in the absence of any antioxidant (BHA or propolis extract), the accumulation of malondialdehyde after 9 weeks was higher when compared to the samples in which an antioxidant was added. regardless of the antioxidant used, the results also showed that they gradually lose their ability for preventing lipid peroxidation as the wheat germ concentration increases in emulsions (Figure 6.2). The Figure also shows that the raise of antioxidant ability is dependent on the concentration of BHA or propolis extract added to emulsions.

In Appendix C, Table C.3 it is possible to conclude that propolis extracts at higher concentrations (0.02 and 0.04%) work better as antioxidants than BHA, when the lipid phase was only constituted by almond oil (E), or at 0.04% when the oil phase was constituted by 75% almond oil and 25% wheat germ (0.04%). For all of the other samples, BHA revealed to be better than propolis extract as antioxidant.

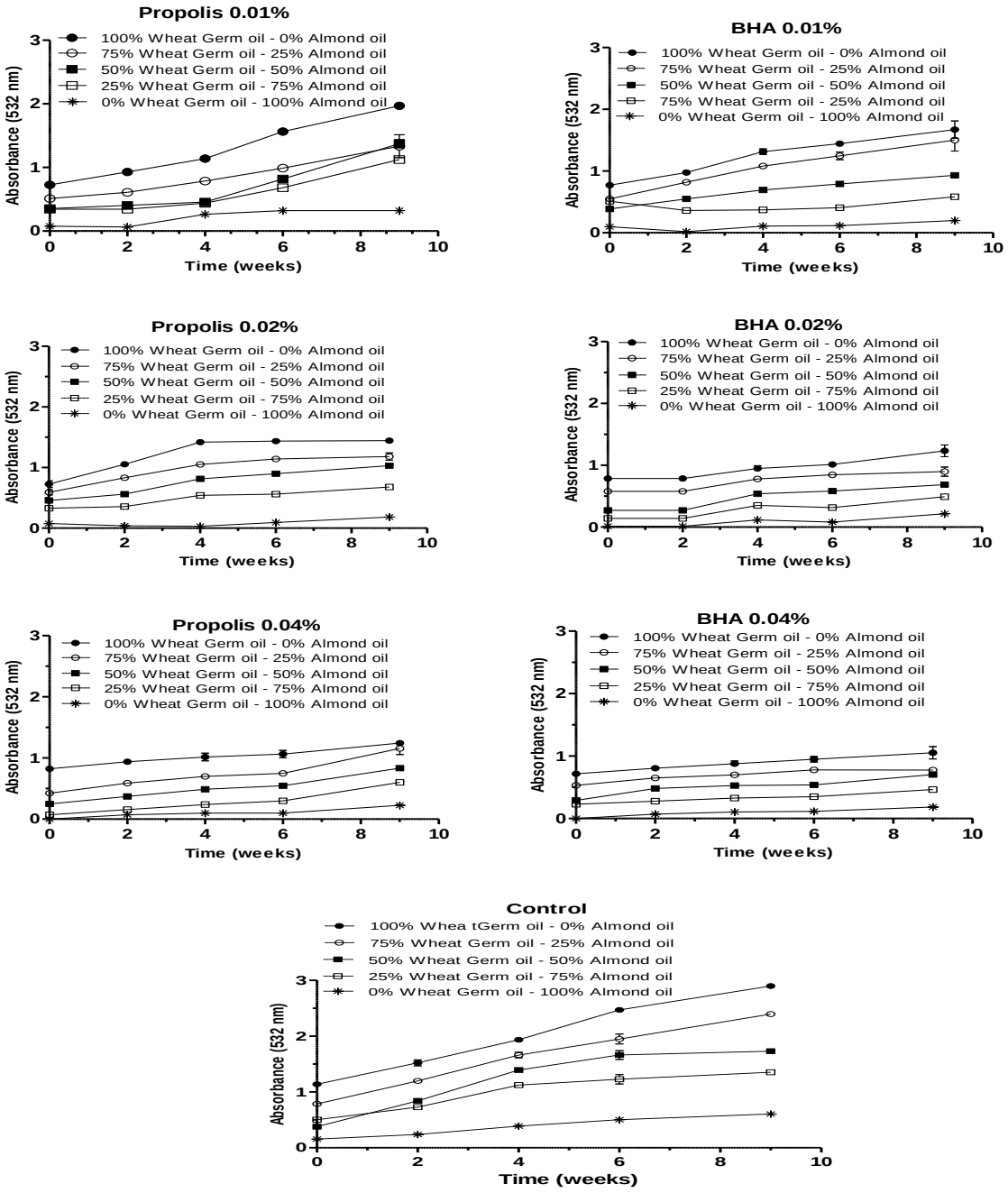


Figure 6.2. Effect of adding propolis extract and BHA at different concentrations (0.01%; 0.02% and 0.04%) on TBARS (ABS_{532}) of O/W emulsions made with germ wheat oil and almond oil under storage conditions of 9 weeks at 37 °C. Values of mean (n = 6) ± standard deviation.

The antioxidant capacity of propolis has been studied and in a review study made by Miguel et al.[23], the authors reported that the ability for preventing lipid peroxidation measured through the method of thiobarbituric acid, as done in the present work, was dependent on the flavonoids, caffeic acid and its derivatives caffeic acid phenethyl ester and 1,1-

dimethylallylcaffeate. In short, either flavonoids or phenolic acids and their esters need to have two ortho-hydroxyl groups in the aromatic ring to give high antioxidant ability to the compound.[23] The intermediate *o*-hydroxyl phenoxyl radical formed during the reaction of oxidation is stable owing to the unpaired electrons that can easily delocalize across the molecule.[24,25] In the present work, the chemical composition of propolis extract showed relative high amounts of caffeic acid derivatives with two ortho-hydroxyl groups, albeit also possessing a great percentage of flavonoids with two meta-hydroxyl groups (pinocembrin, pinobanksin, chrysin and their derivatives). In another study, Espinosa et al.[16] found that sinapic acid and rutin hydrate were the most efficient compounds, extracted from red propolis, to delay lipid oxidation of a functional emulsion, among 11 natural phenolic compounds (vanillic acid, caffeic acid, trans-cinnamic acid, 2,4-dihydroxycinnamic acid, *p*-coumaric acid, quercetin, trans-ferulic acid, trans,trans-farnesol, rutin, gallic acid or sinapic acid).

6.3.4. Rheological properties of W/O emulsion

For stabilizing an emulsion O/W, two categories of molecules can be used: those that adsorb at the oil-water interface and reduce interfacial tension (surfactants), and those that provide long-term emulsion stability by, for instance, modifying the viscosity of aqueous phase, that is, they are thickening agents (stabilizers).[19] In order to improve the stability of emulsions O/W, in some cases, surfactants or emulsifiers and stabilizers are used combined, due to the fact that they can act synergistically.[26] Xanthan gum has been used a stabilizer of emulsions not only to its high thickening attributes but also to its relative high solubility in cold water.[27] In the present work, the percentages of surfactants (Tween 80 and Span 80) and stabilizer (xanthan gum) were constant in all emulsions O/W.

Rheological characterization of the emulsions formulations, using different percentage of germ wheat oil and almond oil, with propolis extract and BHA as oxidants at different concentrations were performed. The rheological characteristics of the emulsion samples were measured applying a shear rate from 2.10 to 21.00 s^{-1}, with a 2.10 increment.

The thixotropic behavior was determined using the increment of the shear rate, as ascending scanning and decreasing the values of shear rate, as a descending scanning. It was found that for the same shear rate value, identical values of shear stress were obtained, which indicated that the emulsions do not present thixotropic behavior, as can be seen by Figure 6.3a, that shows the data for the fit to the power law model. The consistency index (K) and the flow behavior index (n) were obtained by fitting the viscosity vs shear rate data with the power-low

equation. The flow curves, shear stress versus shear rate, show that when shear rate increases, the values of viscosity decrease (Figure 6.3b), i.e. a shear thinning behavior (Figure 6.3a). The xanthan gum polymer has been characterized rheologically and exhibits, like the emulsion, a shear thinning behavior.

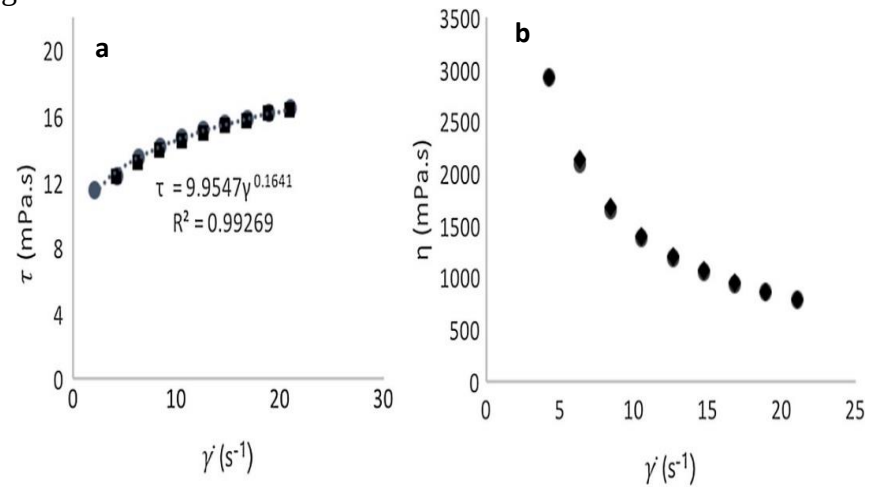


Figure 6.3. Flow behavior curve and Power law model (……) fitting (a) and relation between apparent viscosity and the shear rate (b), at 25 ± 1 °C with an up-down rate ramp.

Figures 6.4; 6.5 and 6.6, present the values of consistency index (K) and the fluid behavior index (n) for a control and for the various emulsions, with the antioxidants propolis extract and BHA, at different concentrations. All emulsions clearly show a fluid behavior index less than 1, more specifically $n < 0.25$, typical of shear thinning, which confirms the aforementioned results.

In Figure 6.4 the consistency index (K) for 0.01% emulsions with propolis extracts present a value slightly higher than the control and BHA emulsions, at same concentration. This index is a measure of the system consistency and is related with the viscosity. In this case, almost all of the viscosity values of emulsions with propolis extract are higher than those obtained with other samples, at the same concentration of 0.01% (Appendix C, Table C.4). For the emulsions with propolis extracts with 100% of germ wheat oil, the initial viscosity was 2312 ± 11 mPa·s, against 2020 ± 32 and 1760 ± 00 mPa·s, of control and BHA, respectively. After 9 weeks, propolis extract had an viscosity of 2184 ± 79 and control and BHA, 1834 ± 59 and 1936 ± 34 mPa·s, respectively.

Also, the flow behavior index (n) was tracked over time for the different concentrations of propolis extracts. The flow behavior index presented a more pronounced shear thinning behavior, as observed in the curves of Figure 6.3 (A, C, E), 5(C) and 6, the n values decrease

more sharply during the 9 weeks of storage. The n values of BHA had a similar behavior to the control, for the different concentrations and formulations.

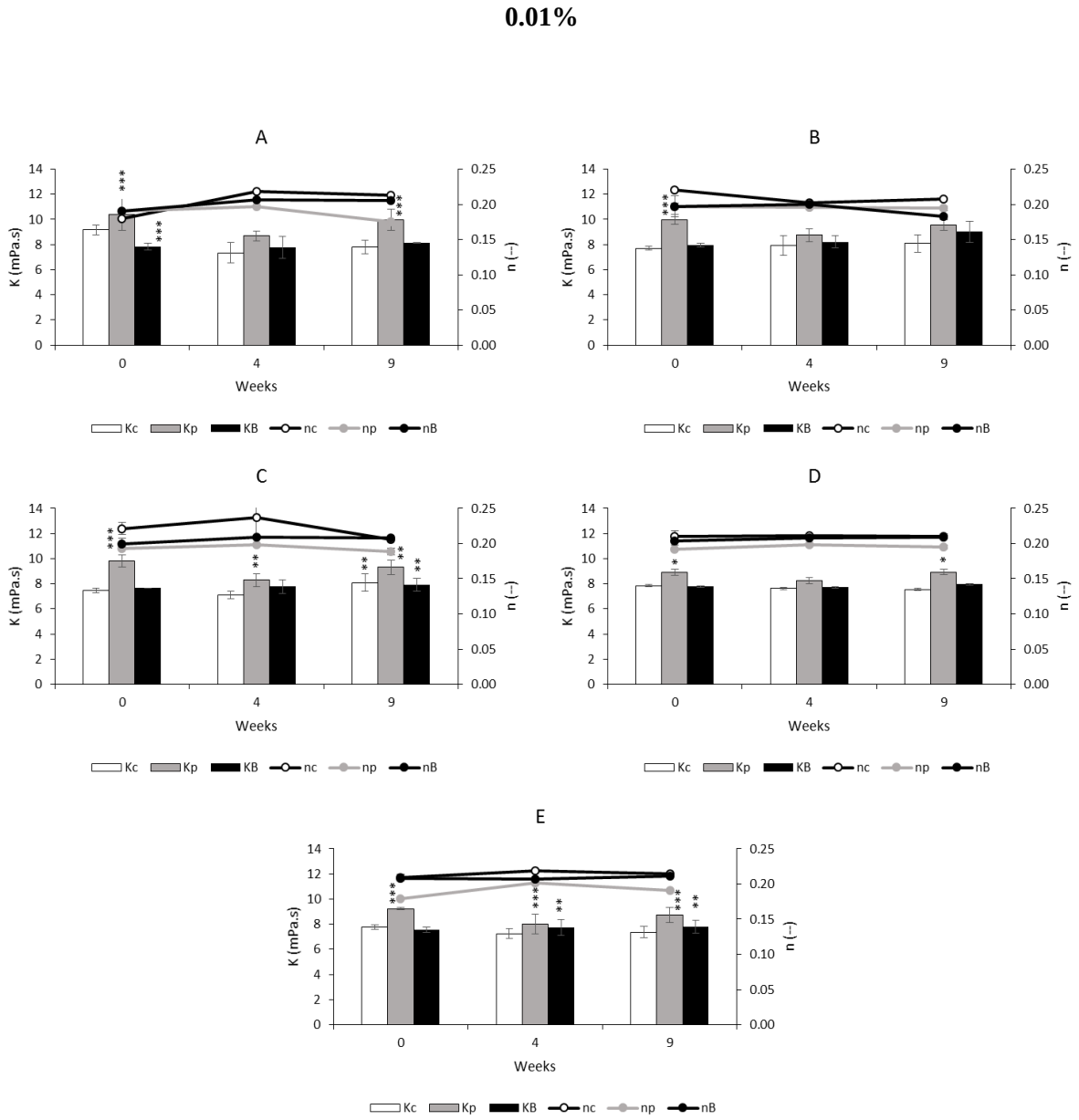


Figure 6.4. Effect of adding propolis extract (0.01%) and BHA on rheological characterization, with determination of consistency index (Kc, Kp, KB) and fluid behavior index (nc, np, nB), under storage conditions (37 °C for 9 weeks). Bars mean standard deviation (n = 4). Level of significance for the test ANOVA one-way: * P < 0.05; **P < 0.01; ***P < 0.001. A contains 100% of Germ wheat oil and 0% almond oil. B contains 75% of Germ wheat oil and 25% almond oil. C contains 50% of Germ wheat oil and 50% almond oil. D contains 25% of Germ wheat oil and 75% almond oil. E contains 0% of Germ wheat oil and 100% almond oil. Symbols: c, control; p, propolis; B, BHA.

0.02%

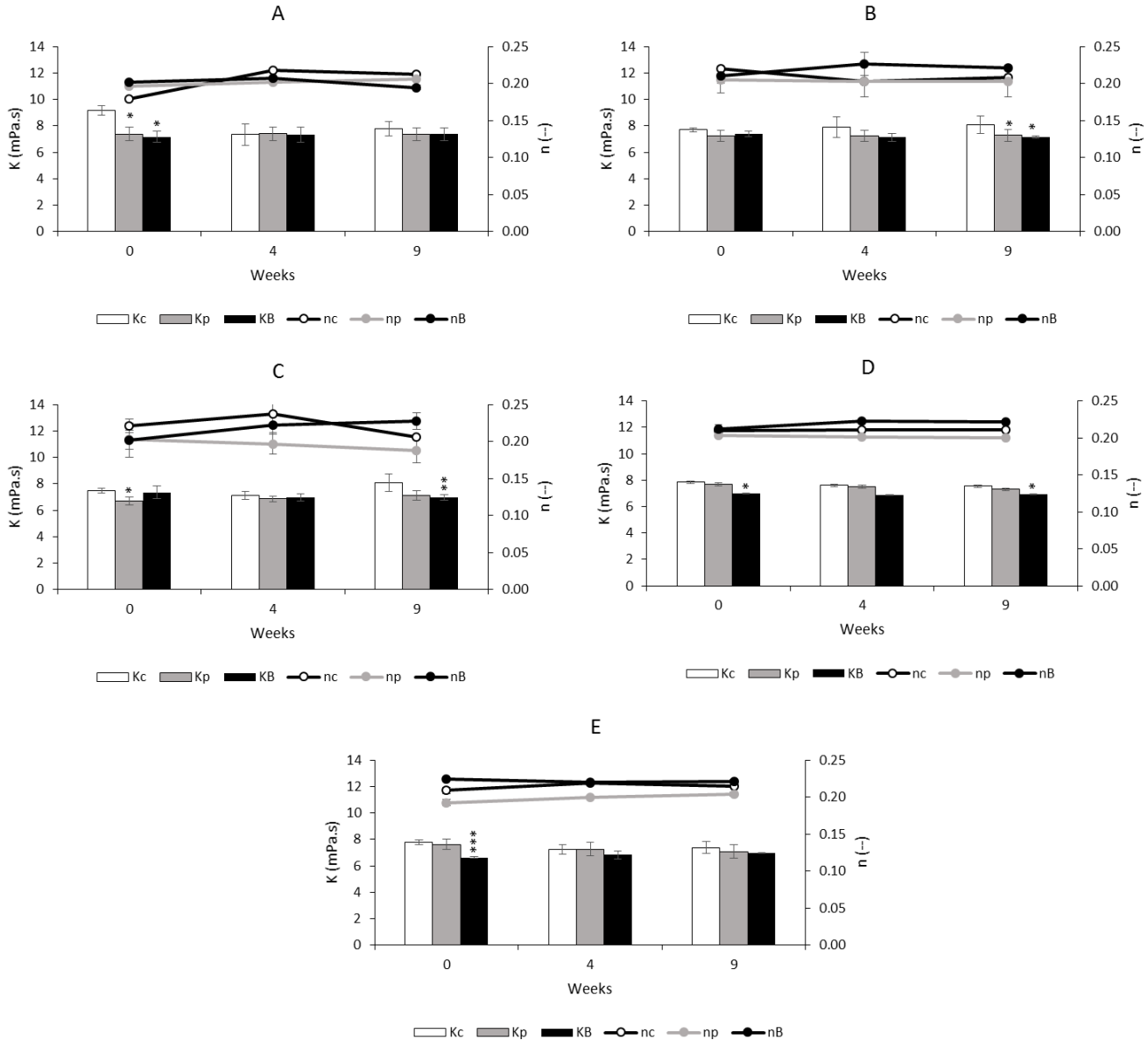


Figure 6.5. Effect of adding propolis extract (0.02%) and BHA on rheological characterization, with determination of consistency index (Kc, Kp, KB) and fluid behavior index (nc, np, nB), under storage conditions (37 °C for 9 weeks). Bars mean standard deviation (n = 4). Level of significance for the test ANOVA one way: * P < 0.05; **P < 0.01; ***P < 0.001. A contains 100% of Germ wheat oil and 0% almond oil. B contains 75% of Germ wheat oil and 25% almond oil. C contains 50% of Germ wheat oil and 50% almond oil. D contains 25% of Germ wheat oil and 75% almond oil. E contains 0% of Germ wheat oil and 100% almond oil. Symbols: c, control; p, propolis; B, BHA.

Depending on the composition of oils percent, wheat germ and almond oil, there is change in the viscosity tendency to increase or decrease, as the antioxidant added. Thus, in the case of propolis extract 0.01%, while the percentage of wheat germ oil decreases from 100% to 50% (A to C), the initial viscosity of the samples decreases, stabilizing the value when the percentage

of almond oil prevails (D and E) (Appendix C, Table C.4). The same happened with the storage period, where the values of viscosity of 0 weeks and 9 weeks decreased until the percentage of almond oil was 75% or higher, as can be seen in Appendix C, Table C.4. The decrease of viscosity, observed in 0.01% propolis extract emulsion and control, during the storage period may be occurred due to two possible reasons: a diffusion of water molecules from internal to the external aqueous phase or bursting of multiple globules due to osmotic pressure.[27] The same was observed by Mahmood and Akhtar,[28] in studies with multiple emulsions encapsulated with 5% green tea extract.

0.04%

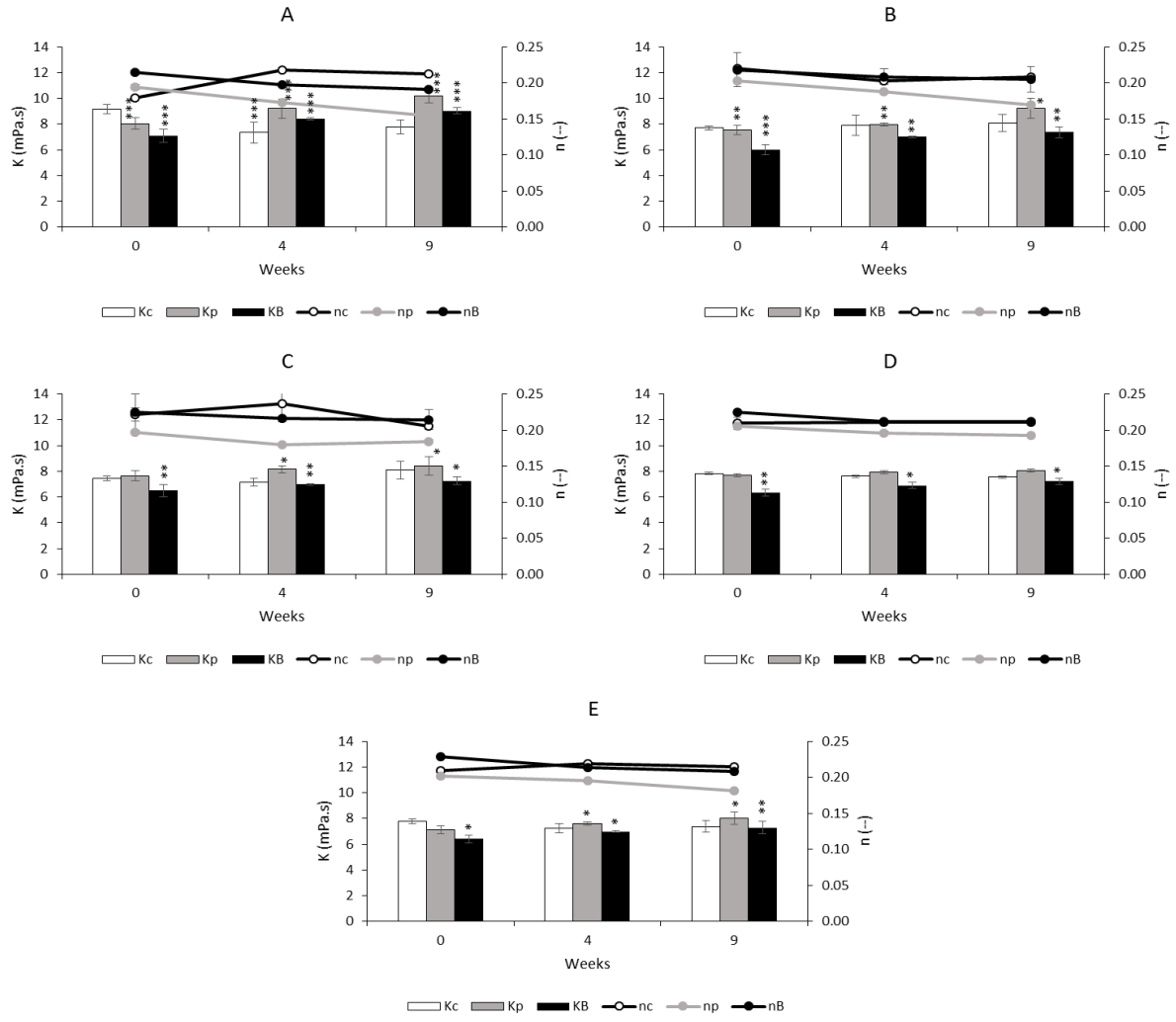


Figure 6.6. Effect of adding propolis extract (0.04%) and BHA on rheological characterization, with determination of consistency index (Kc, Kp, KB) and fluid behavior index (nc, np, nB), under storage conditions (37 °C for 9 weeks). Bars mean standard deviation (n = 4). Level of significance for the test ANOVA one-way: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. A contains 100% of Germ wheat oil and 0% almond oil. B contains 75% of Germ wheat oil and 25% almond oil. C contains 50% of Germ wheat oil and 50% almond oil. D contains 25% of Germ wheat oil and 75% almond oil. E contains 0% of Germ wheat oil and 100% almond oil. Symbols: c, control; p, propolis; B, BHA.

The different emulsions with 0.04% of antioxidant, propolis extract or BHA, showed an increase of viscosity with the storage period (9 weeks). In this case, this may be due to storage conditions where the temperature was 37 °C, enhancing the evaporation of the aqueous phase of the emulsion. Also, the antioxidants are prepared as a hydro-alcoholic solution, evaporation can be more severe and cause increased viscosity and accentuating the shear thinning character, visible in Figure 6.6, the curves representing the propolis extract behavior index.

6.4. Conclusion

In this study, we report that hydro-alcoholic extract of Moroccan propolis, mainly constituted by flavonoids (51.9%) and phenolic acid esters (22.4 %), was better antioxidant in the concentration of 0.02 and 0.04 % than BHA in the same concentrations in emulsions O/W when lipid phase was constituted by almond oil. While, a mix of almond oil and wheat germ was in various used percentages (75/25, 50/50, 25/75, respectively) BHA revealed to be more effective. Nevertheless, the ability of both antioxidants decreases with the increase in wheat germ percentage present in the O/W emulsion. Besides, the rheological behaviour is typical of a non-Newtonian fluid, more specifically a pseudoplastic fluid in which almond oil is more adequate for having a higher stable O/W emulsion. In this case, hydro-alcoholic extract of propolis at 0.02 % can replace BHA as antioxidant, since it reveals to be more effective than BHA.In conclusion, the characterization of the behavior of the culture under the storage conditions may allow evaluating the maintenance of the emulsion rheological properties and these results indicate that almond oil could have a stabilizing effect of the emulsion by minimizing flow variations with time aspect reflected in the measurement of viscosity after storage at 37 °C. The addition of antioxidant at a concentration of 0.01% seems also a beneficial effect in the emulsion.

Reference

[1] A. M. R. Alvarez, M. L. González Rodríguez, ‘Lipids in pharmaceutical and cosmetic preparations’, Grasas Aceites. 2000, 51, 74–96. Doi:10.3989/gya.2000.v51.i1-2.409

[2] Ajazuddin, A. Alexander, A. Khichariya, S. Gupta, R. J. Patel, T. K. Giri, D. K. Tripathi, ‘Recent expansions in an emergent novel drug delivery technology: Emulgel’, J. Control. Release. 2013, 171, 122–132. Doi:10.1016/j.jconrel.2013.06.030

[3] J. M. Roncero, M. Álvarez-Ortí, A. Pardo-Giménez, R. Gómez, A. Rabadán, J. E. Pardo, Virgin almond oil: Extraction methods and composition’, Grasas Aceites. 2016, 67. Doi:10.3989/gya.0993152

[4] C. C. Berton-Carabin, M. H. Ropers, C. Genot, Lipid oxidation in oil-in-water emulsions: involvement of the interfacial layer’, Compr. Rev. Food Sci. Food Saf. 2014, 13, 945–977. Doi:10.1111/1541-4337.12097.

[5] C. Poyato, I. Navarro-Blasco, M. I. Calvo, R. Y. Cavero, I. Astiasarán, D. Ansorena, Oxidative stability of O/W and W/O/W emulsions: Effect of lipid composition and antioxidant polarity', Food Res. Int. 2013, 51, 132–140. Doi:10.1016/j.foodres.2012.11.032.

[6] K. Yamaki, S. Taneda, R. Yanagisawa, K. Inoue, H. Takano, S. Yoshino. Enhancement of allergic responses in vivo and *in vitro* by butylated hydroxytoluene. Toxicol. Appl. Pharmacol. 2007, 223, 164–172. Doi:10.1016/j.taap.2007.05.007

[7] B. Nieva-Echevarría, M. J. Manzanos, E. Goicoechea, M. D. Guillén, '2,6-Di-tert-butyl-hydroxytoluene and its metabolites in foods. Compr. Rev. Food Sci. Food Saf. 2015, 14, 67–80. Doi:10.1111/1541-4337.12121.

[8] M. C. Marcucci, Propolis: chemical composition, biological properties and therapeutic activity. Apidologie. 1995, 26, 83–99. Doi:10.1051/apido:19950202

[9] Y. Barlak, O. Değer, M. Colak, S. C. Karataylı, A. M. Bozdayı, F. Yücesan, 'Effect of Turkish propolis extracts on proteome of prostate cancer cell line', Proteome Sci. 2011. 9, 74. Doi:10.1186/1477-5956-9-74

[10] G. A. Burdock, 'Review of the biological properties and toxicity of bee propolis (propolis)', Food Chem. Toxicol. 1998, *36*, 347–363.

[11] B. Clares, P. Gálvez, V. Gallardo, M. A. Ruiz, 'Elaboration, characterization, and stability study of a sunscreen emulsion for use as a towelette application in pediatric photoprotection', J. Cosmet. Sci. 2011. 62, 371–382.

[12] S. El-Guendouz, S. Aazza, B. Lyoussi, M. D. Antunes, M. L. Faleiro, M. G. Miguel, 'Anti-acetylcholinesterase, antidiabetic, anti-inflammatory, antityrosinase and antixanthine oxidase activities of Moroccan propolis', Int. J. Food. Sci. Technol. 2016a, 51, 1762–73.

[13] S. El-Guendouz, S. Aazza, B. Lyoussi, V. Bankova, J.P. Lourenço, A.M. Rosa Costa, J.F. Mariano, M.G. Miguel, M.L. Faleiro, 'Impact of biohybrid magnetite nanoparticles and Moroccan propolis on adherence of methicillin resistant strains of *Staphylococcus aureus'*, Molecules. 2016, 21, 1208.

[14] J. M. Sforcin, 'Biological properties and therapeutic appications of propolis', Phytother. Res. 2016, 30, 894–905.

[15] M. Duman, E. Ozpolat, 'Effects of water extract of propolis on fresh shibuta (Barbus grypus) fillets during chilled storage', Food. Chem. 2015, 189, 80–85. Doi:10.1016/j.foodchem.2014.08.091

[16] R. R. Espinosa, R. Inchingolo, S. M. Alencar, M. T. Rodriguez-Estrada, I. A Castro, 'Antioxidant activity of phenolic compounds added to a functional emulsion containing omega-3 fatty acids and plant sterol esters. Food. Chem. 2015, 182, 95–104. Doi:10.1016/j.foodchem.2015.02.130

[17] S. M. Osés, A. Pascual-Maté, M. A. Fernandez-Muiño, T. M. López-Diaz, M. T. Sancho, 'Bioactive properties of honey with propolis. Food. Chem. 2016, 196, 1215–1223. Doi:10.1016/j.foodchem.2015.10.050

[18] E. Sehn, L. Hernandes, S. L. Franco, C. C. M. Gonçalves, M. L. Baesso, 'Dynamics of reepithelialisation and penetration rate of a bee propolis formulation during cutaneous wounds healing', Anal. Chim. Acta. 2009, 635, 115–120. Doi:10.1016/j.aca.2009.01.019

[19] V. Krstonošić, L. Dokić, I. Nikolić, M. Milanović, 'Influence of xanthan gum on oil-in-water emulsion characteristics stabilized by OSA starch', Food. Hydrocoll. 2015. 45, 9–17. Doi:10.1016/j.foodhyd.2014.10.024

[20] P. Malinowska, A. Gliszczynska-swiglo, H. Szymusiak, 'Protective effect of commercial acerola, willow, and rose extracts against oxidation of cosmetic emulsions containing wheat germ oil. Eur. J. Lipid Sci. Technol. 2014, 116, 1553–1562. Doi:10.1002/ejlt.201300514.

[21] E. Hebishy, M. M. Buffa, B. Guamis, A. J. Trujillo, Stability of sub-micron oil-in-water emulsions produced by ultra high pressure homogenization and sodium caseinate as emulsifier, in: Chemical Engineering Transactions. 2013. 1813–1818. Doi:10.3303/CET1332303

[22] V. Bankova, M. Popova, S. Bogdanov, A. G. Sabatini, 'Chemical composition of European propolis: Expected and unexpected results', Zeitschrift Naturforsch. - Sect. C J. Biosci. 2002, 57, 530–533. Doi:10.1515/znc-2002-5-622

[23] M. G. Miguel, 'Chemical and biological properties of propolis from the western countries of the Mediterranean basin and Portugal', Int. J. Pharm. Pharm. Sci. 2013, 5, 403–409.

[24] E. Gregoris, R. Stevanato, 'Correlations between polyphenolic composition and antioxidant activity of Venetian propolis. Food Chem. Toxicol. 2010, 48, 76-82.

[25] W. M. Wu, L. Lu, Y. Long, T. Wang, L. Liu, Q. Chen, R. Wang, Free radical scavenging and antioxidative activities of caffeic acid phenethyl ester (CAPE) and its related compounds in solution and membranes: a structure-activity insight. Food Chem. 2007, 105, 107-115.

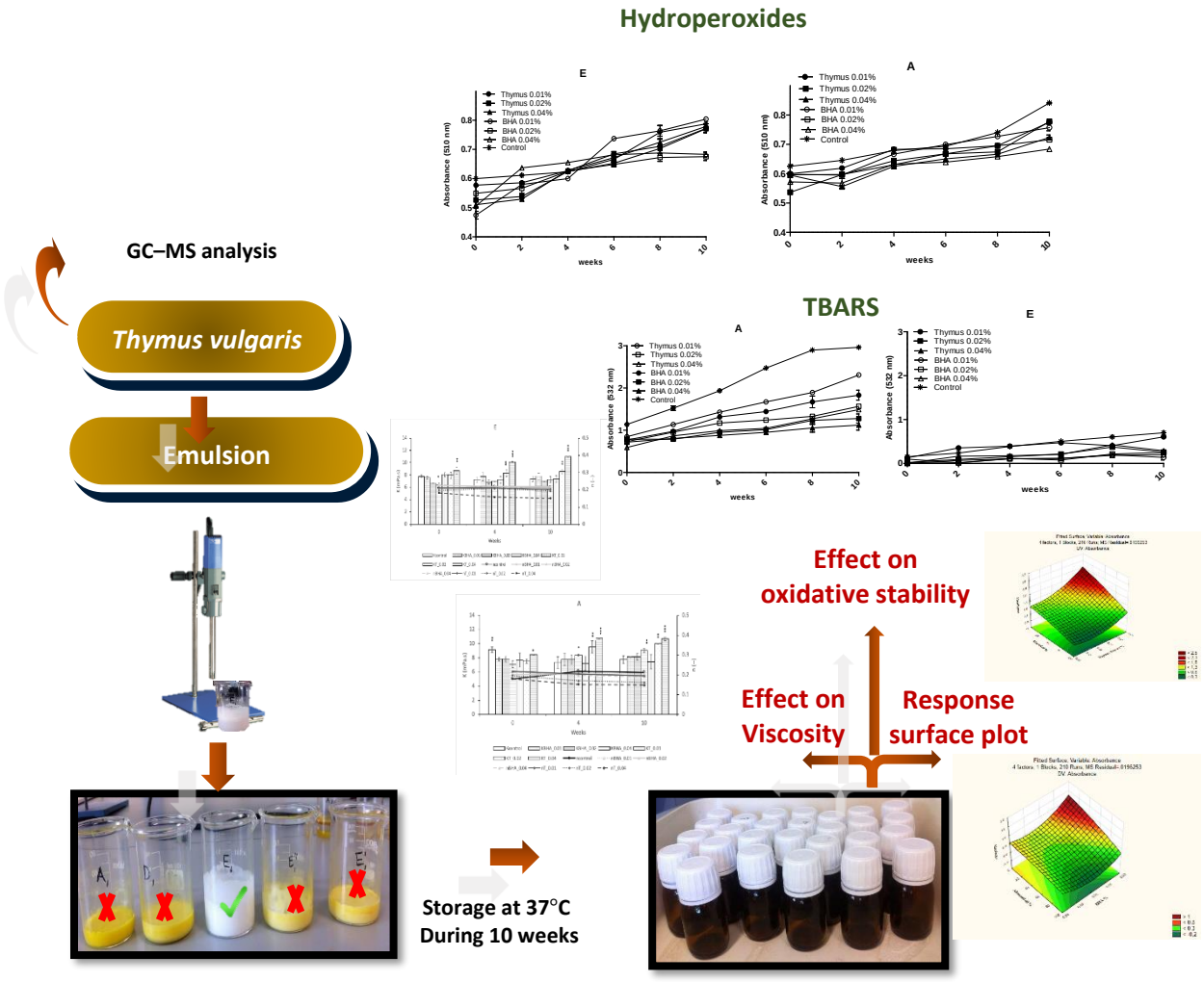
[26] S. Desplanques, F. Renou, M. Grisel, C. Malhiac, 'Impact of chemical composition of xanthan and accacia gums on the emulsification and stability of oil-in-water emulsions' Food. Hydrocol. 27, 401-410.

[27] F. Tirnaksiz, O. Kalsin 'A topical W/O/W multiple emulsions prepared with Tetronic 908 as a hydrophilic surfactant: formulation, characterization and release study. J. Pharm. Pharmaceut. Sci. 2005, 8, 299-315

[28] T. Mahmood, N. Akhtar, 'Stability of a cosmetic multiple emulsion loaded with green tea extract', Sci World J. 2013, Article ID 153695. http://dx.doi.org/10.1155/2013/153695.

Chapter 7

Antioxidant Activity of Thyme Waste Extract in O/W Emulsions



Antioxidant activity of thyme waste extract in O/W emulsions*♠

Abstract

Thymus vulgaris (thyme) is an aromatic plant and its essential oil has been applied as antimicrobial and antioxidant due to the presence of phenolic compounds. However, after steam distillation, the deodorized plant material is rejected, despite the possible presence of bioactive compounds. Ethanolic thyme waste extract revealed the presence of benzoic acid, 4-hydroxybenzoic acid, ferulic acid, caffeic acid, and sinapic acid. This waste thyme extract had the capacity for preventing the formation of primary and secondary lipid oxidation products in emulsions O/W (oil in water), constituted by diverse proportions of wheat and almond oils, without interfering with the viscosity parameters, for 10 weeks, at 37 °C. The increasing proportion of almond oil (≥50%) in the emulsion increases its resistance to oxidation, which is improved with the presence of an optimal concentration of tested thyme waste extract (0.02% and 0.04%). The waste thyme extract can, therefore, be used as an antioxidant either in food or pharmaceutical emulsions O/W, replacing the synthetic antioxidants.

*Adapted from 'Antioxidant activity of thyme waste extract in O/W emulsions'. Soukaïna El-Guendouz, Smail Aazza, Susana Anahi Dandlen, Nessrine Majdoub, Badiaa Lyoussi, Sara Raposo, Maria Dulce Antunes, Maria Graça Miguel. *Antioxidants* 2019, 8(8), 243.

♠ Information's about Apparent viscosity of the emulsions with thymus extract and BHA, at different concentrations under storage conditions are dispayed in appendix C

7.1. Introduction

In spite of emulsions O/W being largely used in food, pharmaceutical, cosmetic industries, and biomedical sciences,[1] they are thermodynamically unstable, tending to breakdown through coalescence and aggregation of the dispersed oil phase, over time.[2] Oxidation of the lipid phase of the emulsion O/W may occur, generating oxidative sub-products which interfere in the emulsion stability and unpleasant odorant compounds that impair the quality of the final product. The formation of free radicals can be avoided or decreased by adding antioxidants[3]. However, their use cannot be random and should be chosen taking into account their physicochemical properties, so that they preferentially remain at the interface O/W.[3]

Tocopherols, butylhydroxy toluene (BHT) or butylhydroxy anisole (BHA), gallates, and plant phenolics (quercetin or rosmarinic acid) have been used as antioxidants in emulsions O/W.[4,5] Thyme (*Thymus vulgaris* L.) is a source of a variety of natural bioactive materials, it contains many flavonoids, like lutein, apigenin, naringenin, luteolin, and thymonin,[6] beyond thymol and carvacrol in the volatile part of the plant.[7] These two phenolic compounds have been considered as good antioxidants.[7] The large production of essential oils originates waste that is usually poorly exploited. Such can be reversed and the deodorized plant that remains after the distillation may still contain bioactive compounds with antioxidant activity that must be valued.[8]

In the Algarve (Portugal), there is an essential oils' producer that struggles against the waste that remains after steam distillation. Despite the extraction of the volatile fraction, the remaining solid material still contains bioactive compounds. Some studies have shown that post-distillation waste material from thyme (*Thymus vulgaris*) still possess antioxidant and antimicrobial activities.[9,10] In spring and summer, there is production of essential oils obtained from the flowering aerial parts from diverse aromatic plants collected in organic agriculture farmers of Algarve, such as *Thymus vulgaris*. The goal of the present work was to evaluate the antioxidant capacity of an extract of deodorized *Thymus vulgaris* (thyme) by-product, obtained by a Portuguese producer of essential oils, in simple emulsions O/W in which the lipid phase was constituted by almond or wheat germ oils (natural products). The viscosity of the emulsion behavior type was also studied.

7.2. Experimental

7.2.1. Chemicals

Thiobarbituric acid (TCA), ferrous chloride ($FeCl_2$), ammonium thiocyanate (NH_4SCN), ferulic acid, DNP (2,4-dinitrophenylhydrazine), sulphuric acid (H_2SO_4), eryodictiol, quercetin, potassium hydroxide (KOH), *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide, NH_4I, 2-mercaptoethanol, and buthylated hydroxyanisole (BHA) were purchased from Fluka Biochemika, Sigma-Aldrich, Steinheim, Germany. 2,2-diphenyl-1-picrylhydrazyl (DPPH) was from Riedel-de Haën, Sigma-Aldrich, Seelze, Germany. Folin–Ciocalteu's phenol reagent and $AlCl_3$ were purchased from Panreac Quimica, Montcada i Reixac, Barcelona, Spain. Na_2CO_3 was purchased from Riedel de Haen (Seelze, Riedel-de-Haën Laboratory chemicals, Germany). Almond oil and wheat germ oil were purchased from LBCHEM Labospirit, Lda Portugal. Tween 80, Span 80, Phenonip and xanthan gum were purchased from Guinama, Valencia, S.L.U Spain. Trichloroacetic acid was purchased from VWR, Leuven, Belgium. 1-Butanol, methanol, and HCl were from Fisher Scientific UK Ltd., Loughborough, UK.

7.2.2. Plant extract

Solid material resulting from steam distillation of the flowering aerial parts of *Thymus vulgaris* L. from an organic agriculture in the Algarve region, Portugal, was a gift from a local producer of essential oils. That material was dried over 7 days at room temperature and then ground. Afterward, thyme was extracted with ethanol using Sohxhlet extraction method where 20 cycles were done before being concentrated using a rotavapor and kept until analysis at −4 °C in dark.

7.2.3. Chemical composition of thyme waste extract

Sample preparation: 100 µL of ethanolic sample (thyme waste extract) was evaporated with nitrogen stream and derivatized by the addition of 50 µL of the derivatization reagent. Afterwards, the sample was heated at 60 °C for 30 min. The derivatization agent was prepared as follows: an intermediate stock solution (Sol A) was prepared with 0.25 mL of *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) mixed with 10 mg NH_4I and 15 µL of 2-mercaptoethanol. Then, the Sol A was mixed with pure MSTFA (1/9). Compounds' identification was performed using retention times and spectra from a lab-made library, created with commercial standards also previously submitted to the same derivatization procedure.

The gas chromatography/mass spectrometry (GC) unit was a Bruker Scion 456-GC TQ with Phenomenex ZB5-MS capillary column (30 m × 0.25 mm ID, 0.25 µm df) at the following conditions: Sample volume: 1 µL, temperature. of injector: 280 °C; flow of He 1.0 mL/min;

Program temperature: 80 °C, hold: 1 min, 220 °C and increase 10 °C/min; 310 °C and increase 20 °C/final mine hold 7 min, in a total run time of 26.50 min. Source: 220 °C; temperature of transfer line: 260 °C; mass range: 50–350 (*m*/*z*). The software used was Bruker Daltonics MS Worksation version 8.2.1. and MS Search 2.0. (National Institute of Standards and Technology, Gaithersburg, MD, USA).

7.2.4. Total phenolics, flavones, flavonols, flavanones, and dihydroflavonols content determination in thyme extract

The total polyphenols content in thyme waste extract was determined using the El-Guendouz et al. method[11] with slight modification: 250 µL of extracts or standard (gallic acid at different concentrations) were mixed with 1 mL of 7.5% Na_2CO_3 and 1.25 mL of Folin-Ciocalteu reagent (0.2 N). The mixture was well vortexed and after 2 h of incubation at room temperature, the absorbance was measured at 760 nm. The amount of flavones and flavonols was determined according to the El-Guendouz et al. method[11]: 500 µL of thyme waste extract or standard (quercetin at different concentrations) were added to a volume of 500 µL of $AlCl_3$ (2%) and after one hour at room temperature the absorbance was measured at 420 nm. For the total amount of flavanones and dihydroflavonols compounds, the use of 2,4-dinitrophenylhydrazine (DNP) was determined, and the absorbance was measured at 486 nm as reported by El-Guendouz et al.[12] with some modifications. Briefly, 150 µL of sample or standard (eriodyctiol at different concentrations) and 100 µL of acidic DNP methanolic solution 1% were heated at 50 °C for 50 min in a water bath. After cooling at room temperature, the mixture was diluted to 10 mL with methanolic solution of KOH 10% (w/v). One mL of the resulting solution was added to 10 mL methanol and diluted to 50 mL with methanol.

Each experiment was carried out in triplicate. A calibration curve was used and the results were expressed as mg/g of gallic acid equivalents (GAE), mg/g of quercetin equivalents (QE) and mg/g of eriodyctiol equivalents (EE) for the quantification of phenols, flavones and flavonols, and dihydroflavonols, respectively.

7.2.5. Antioxidant activity determination of thyme waste extract

The antioxidant capacity of thyme was measured through DPPH assay according to the methods previously described.[13]

7.2.6. Preparation and storage of O/W emulsion

Oil-in-water (O/W) emulsions were prepared as described in our previous work,[14] but replacing propolis by thyme waste extract, where three different concentrations were used

(0.01, 0.02 and 0.04%), and five different formulations of oils' phases content (A: 100% of wheat germ oil/0% almond oil; B: 75% of wheat germ oil/25% almond oil; C: 50% of wheat germ oil/50% almond oil; D: 25% of wheat germ oil/75% almond oil and E: 0% of wheat germ oil/100% almond oil) for each concentration of thyme waste extract. BHA was used as positive control.

7.2.7. Viscosity studies

Viscosity studies of the different samples were determined using a Brookfield programmable rotational viscometer LVDV-II+Pro (Brookfield Engineering Laboratories Inc., Middleboro, MA, USA) equipped with the Rheocalc 32 (Brookfield Engineering Laboratories Inc., Middleboro, MA, USA) (version 2.4.) software as previously reported[14]. The shear data were analyzed according the power-law equation ($\tau = K.\ \gamma^n$) to obtain the consistency index (*K*) and the flow behavior index (*n*) for the different emulsions.

7.2.8. Peroxide content determination

Peroxide content determination was done at 0, 2, 4, 6, 8, and 10 weeks as described by El-Guendouz et al.[14] by measuring lipid hydroperoxide at the absorbance 510 nm. Three independent determinations for each sample were carried out.

7.2.9. Lipid oxidation products in formulated emulsions

Thiobarbituric acid-reactive substances (TBARS) were determined on the emulsions after 2, 4, 6, 8, and 10 weeks of incubation at 37 °C, according to El-Guendouz et al.[14] method. Three independent determinations for each sample were carried out.

7.2.10. Experimental design and statistical analysis

Viscosity analyses were performed on duplicated samples. Statistical analysis was conducted with a SigmaPlot 12 software (Systat Software, version 12 for Windows) (Systat, San Jose, CA, USA), implementing the one-way ANOVA method. Significant differences among means ($p < 0.05$) were determined by Student-Newman-Keuls test.

Central composite design (CCD) of the central response surface methodology (RSM) was used to study the effect of four independent variables in different combinations, X1 (BHA concentration %), X2 (thyme waste extract concentration %), X3 (almond oil/wheat germ oil ratio %), and X4 (storage time in weeks) on lipid peroxidation (dependent variable), using STATISTICA (Stat Soft. InC., Tulsa, OK, USA), version 10, Trial Version. All experiments were conducted in triplicate, data were analyzed by the analysis of variance (ANOVA), and *p*-

value lower than 0.05 was considered significant in surface response analysis. A total of 210 experiments were used to calculate the coefficient of the second-order polynomial regression of the four variables, and the model suitability was tested using the ANOVA test. A second-order polynomial equation was used to express the lipid peroxidation and secondary lipid oxidation (DO) (*Y*) as a function of the independent variables as

$$y = \beta_0 + \sum_{i=1}^{4} \beta_i x_i + \sum_{i=1}^{4} \beta_{ii} x_i^2 + \sum_{i<j}^{4} \beta_{ij} x_i x_j$$

where y represents the response variables; β_0 is a constant; β_i, β_{ii}, and β_{ij} are the linear, quadratic, and cross-product coefficients, respectively. x_i and x_j are the levels of the independent variables. Two-dimensional surface response contour plots were generated by varying two variables within the experimental range and holding the other two constants at the central point.

The regression model for the formation of peroxides in the formulated emulsions is presented in the following equation:

Peroxide content = $1.214 - 3.009\ TWE + 24.5\ TWE^2 - 5.9 \times 10^{-4}\ AO + 1.86 \times 10^{-8} \times AO^2 + 1.186 \times 10^{-2} \times BHA \times AO + 0.10 \times TWE \times AO + 0.015 \times W \times TE + 4.41 \times 10^{-5} \times W \times AO$

where TWE: Thyme waste extract (TWE); AO: Almond oil %; W: storage time (weeks); BHA: Butylated hydroxyanisole.

The regression model for secondary lipid oxidation products' content in the formulated emulsions is presented in the following equation:

Absorbance (TBARS) = $1.25 - 42.26\ TWE + 628.59\ TWE^2 - 0.0069\ AO - 3.38\ 10^{-4}\ AO^2 + 0.169 \times BHA \times TWE + 0.160\ TWE \times AO - 1.086 \times W \times TWE - 7.08 \times 10^{-4} \times W \times AO.$

where TWE: Thyme waste extract; AO: Almond oil %; W: storage time (weeks); BHA: Butylated hydroxyanisole.

7.3. Results and discussion

7.3.1. GC–MS analysis of thyme waste extract

The chemical composition of thyme waste extract, determined by GC/MS, is displayed in Table 7.1. Nine components representing 96.19% (i.e., relative %, rel. % of the total detected constituents) were identified in this extract. The main compound in the examined thyme waste extract was benzoic acid, which presents 40.88% (relative percentage). The amounts of 4-hydroxybenzoic acid, trans-cinnamic acid and p-coumaric acid found were 17.18%, 11.89%, and 10.34 rel%, respectively. Other identified phenolic acids were ferulic, caffeic, and sinapic

acids. Quercetin was also detected in this extract. Similar studies in Romania identified caffeic, chlorogenic, p-coumaric, and ferulic acids, luteolin, and apigenin as major compounds in the T. vulgaris extract.[15] In the deodorized leaves of thyme, rutin and apigenin were found as main phenols, along with quercetin, chlorogenic, ferulic, and gallic acid.[9]

Table 7.1 Chemical composition of thyme waste extract. Total polyphenols content and DPPH activity of thyme waste extract.

No.	Retention Time RT (time)	Relative Area Percent (%)	Compounds	*m/z* (I)
1	7.05	40.88	Benzoic acid	105(100); 179(93); 77(93); 135(82); 51(22); 180(13); 136(11); 73(8); 106(7); 75(7)
2	10.93	11.89	*trans*-Cinnamic acid (TMS)	131(100); 205(95); 103(95); 161(85); 77(74); 75(75); 145(45); 102(43); 135(25); 73(24)
3	11.83	17.18	4-Hydroxybenzoic acid (2TMS)	73(100); 267(72); 223(68); 193(46); 268(16); 224(15); 282(14); 75(12); 126(10); 91(10)
4	15.16	10.34	*p*-Coumaric acid (TMS)	73(100); 293(43); 219(40); 75(30); 249(30); 308(24); 179(11); 294 (10); 74(10); 220(8)
5	16.40	2.55	Ferulic acid (2TMS)	73(100); 338(45); 323(20); 75(34); 308(34); 249(30); 293(30); 59 (13); 219(13); 339(12)
6	16.70	8.74	Caffeic acid (TMS)	73(100); 219(68); 396(38); 191(16); 381(13); 397 (13); 220(12); 75(11); 74(8); 249(7)
7	17.37	3.71	Sinapic acid (2TMS)	73(100); 338(57); 368(45); 75(34); 353(32); 232(25) ; 279(15) ; 339(15) ; 59(14) ; 249(12)
8	21.91	0.90	Quercetin (5TMS)	73(100); 647(29); 648(16); 649(9); 559(8); 74(7) ; 75(6); 560(3); 147(3); 575(2)
Total*		96.19		
*Only the most abundant peaks were identified. I: intensity				
Phenols (mg EAG/g)		**Flavones (mg EQ/g)**	**Dihydroflavonols (mg EE/g)**	**DPPH (IC_{50} mg/mL)**
97.50±3.82		0.13±0.01	1.76±0.24	0.093±0.01

7.3.2. Polyphenol contents and antioxidant activity

Table 7.1 depicts the total phenolics and flavonoids contents of thyme extract. The sample exhibited 97.50 ± 3.82 mg GAE/g (total phenols), 1.76 ± 0.24 mg EE/g (flavanones and dihydroflavonols), and 0.13±0.01 mg QE/g (flavones and flavonols). The results agree with the literature, which reports the phenolics content of thyme to be relatively high in different extracts.[16,17]

Several studies have indicated that phenolic contents are strongly related to antioxidant activities. According to Gülçin et al.,[18] a high level of phenolic compounds indicates the elevated antioxidant capacity of thyme. Antioxidant activity was tested through DPPH test, and the IC_{50} was 93 µg/mL, similar to that of BHT (89 µg/mL), used as the control. Lower IC_{50} values (better activity) were found (13.4 µg/mL and 12.1 µg/mL) by other authors regarding DPPH scavenging activity of thyme extract.[16] Abdul et al.[19] reported IC_{50} values ranging from 48.1 µg/mL to 59.3 µg/mL, these values are higher than those found by Köksal et al.[16] but still lower than those found in our extract. The higher IC_{50} values (lower activity)

found in the present work cannot be compared to those reported by[16,19], since they studied the antioxidant activity of thyme extracts and in the present work, the antioxidant activity was determined in thyme waste extract in which bioactive compounds (thymol and/or carvacrol) were previously removed during the volatiles' extraction, by steam distillation. The transformations undergone by some compounds during distillation cannot also be excluded. Gavarić et al.[9] found lower antioxidant activity in deodorized leaf extracts of thyme than the extracts without previous treatment. According to Gavarić et al.[9] who studied the antioxidant activity of pure compounds and compared them with those of waste extracts, concluded that the antioxidant activity of these samples could be attributed to the relative high amounts of rosmarinic acid and rutin.

As our extract still possessed antioxidant activity, it was assayed as antioxidant in the emulsions.

7.3.3. Viscosity of O/W emulsion

Emulsions are thermodynamically unstable and they can be stabilized by surfactants or emulsifiers, or by thickening agents or stabilizers. Surfactants or emulsifiers and stabilizers can be combined and used for improving the stability of emulsions O/W.[20] In the present work, Tween 80 and Span 80, and xanthan gum were used as surfactants, and stabilizer, respectively.

The viscosity characterization was done for all emulsions O/W and was evaluated applying a shear rate from 2.10 to 21.00 s^{-1}, with a 2.10 increment. Figure 7.1 presents the data to fit the power law model, relating the viscosity vs. shear rate to the determination of the consistency index (K), while the flow behavior index (n) is represented in Figure 7.2. In Figure 7.1a, the emulsions do not present thixotropic behavior, these mixtures present identical values of shear stress for the same shear rate value. The emulsions present a shear thinning behavior, by the representation of flow curves, where shear stress is fitting with shear rate (Figure 7.1a). In Figure 7.1b, the values of viscosity diminishes, when shear rate rises, demonstrating this shear thinning behavior.

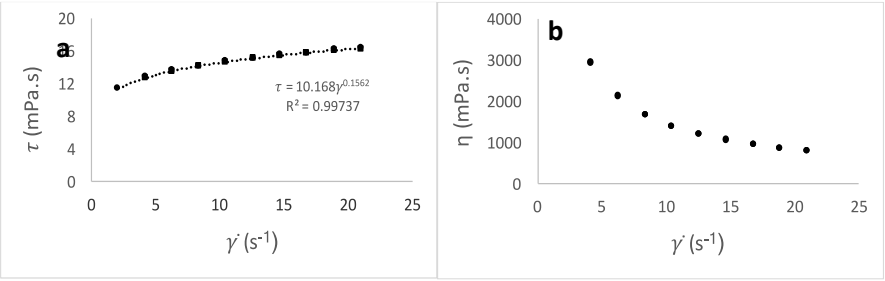


Figure 7.1. Flow behavior curve and power law model (⋯) fitting (**a**) and relation between apparent viscosity and the shear rate (**b**), at 25 ± 1 °C with an up-down rate ramp.

The control and the different emulsions, with the waste thyme extract and BHA, have the values of consistency index (K) and the fluid behavior index (n) typical of shear thinning performance, with the index less than 1 ($n < 0.22$), which confirms the aforementioned results (Figure 7.2).

As observed in Figure 7.2, the consistency index (K) for control remains constant over the 10 weeks of testing, presenting a slightly higher value in the formulation with 100% germ wheat oil, relative to BHA and waste thyme extract, with identical formulations (Figure 7.2A).

In general, for thyme formulations in all the storage weeks, the consistency index (K) is higher than the control and BHA formulations, having in week 0 a very similar value for different thyme waste extract concentrations. However, at week 4 and 10, the concentrations of 0.02 and 0.04 % presented a significant increase in the K, reaching the highest values for the concentration of 0.04%. This is reinforced with the viscosity values, since K index is a measure of the system consistency and is related to the emulsion viscosity. For the thyme waste extract (0.01%), the viscosity values are similar to those obtained with the other samples (results not shown). For the concentrations of 0.02% and 0.04% of thyme waste extract, after 10 weeks of storage, the higher viscosity values are 2136 ± 24 and 2451 ± 37 mPa s, respectively, against 1884 ± 57 mPa s for the control and 1722 ± 37 and 2046 ± 42 mPa s for BHA, respectively. These maximum values were obtained with 50% germ wheat oil and 50% almond oil.

The composition of the oil percentage (wheat germ and almond oils), as the antioxidant added, influences the viscosity trend. As shown in Figure 7.2A–C, the initial viscosity increases with the decrease of wheat germ oil percentage, from 100% to 50%, for all the thyme waste extract concentrations. However, for higher concentrations (Figure 7.2D,E), the initial viscosity decreases with an increase in the almond oil percentage (Appendix D, Table D.1). For the concentration of thyme extract 0.01%, a decrease of viscosity was observed with the 10 weeks of storage, as observed for the control. As reported in our previous work using propolis extract as an antioxidant,[14] this decrease in viscosity could occur by the diffusion of water molecules from the internal to the external aqueous phase or for another reason, the bursting of multiple globules due to osmotic pressure.[21] Mahmood et al.[22] observed an identical decrease in viscosity in studies with multiple emulsions encapsulated with 5% green tea extract.

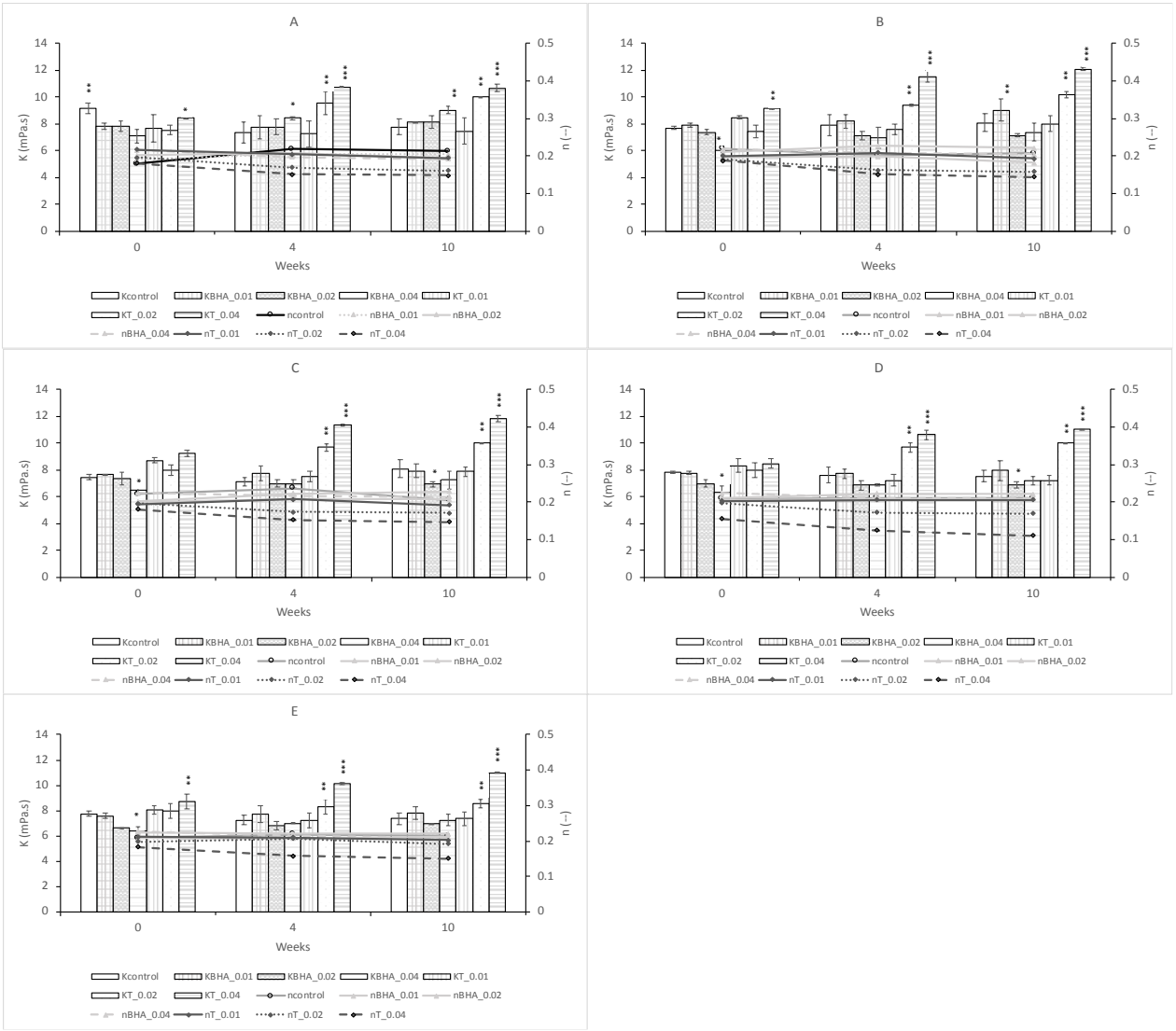


Figure 7.2. Effect of adding thyme extract and BHA, at different concentrations (0.01%, 0.02%, and 0.04%) on rheological characterization, with determination of consistency index (Kc, KBHA, KT) and fluid behavior index (nc, nBHA, nT), under storage conditions (37 °C for 10 weeks). Bars mean standard deviation (n = 4). Level of significance for the one-way ANOVA test: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. (**A**) 100% germ wheat oil and 0% almond oil. (**B**) 75% Germ wheat oil and 25% almond oil. (**C**) 50% Germ wheat oil and 50% almond oil. (**D**) 25% Germ wheat oil and 75% almond oil. (**E**) 0% Germ wheat oil and 100% almond oil.

An opposite behavior was observed when the concentrations of thyme waste extracts were 0.02 and 0.04%: the viscosity increased with the storage time. The increase of the viscosity is noteworthy at the two concentrations of thyme sample, with the highest values being reached at 0.04% in the system constituted by equal parts of germ wheat and oil almond oil (2451 ± 37 mPa s). The storage conditions could contribute to this situation, the temperature of 37 °C, could enhance the evaporation of the aqueous phase of the emulsion. In addition, the hydro-alcoholic extracts may undergo higher evaporation, for instance, than aqueous extracts, causing increased viscosity and accentuating the shear thinning character, as visible by the curves of the

thyme extract behavior index (Figure 7.1). A similar behavior was also observed for hydro-alcoholic extracts of propolis.[14]

Likewise, after 10 weeks, comparing the different emulsions, based on the concentration of thyme waste extracts, it was found that the viscosity increases in all formulations tested. For the 0.01% concentration, the viscosity values vary between 1696 ± 27 and 1780 ± 39 mPa s, depending on the composition of the emulsion, while for 0.04%, the viscosity values vary between 2451 ± 37 and 2144 ± 49 mPa s, always after 10 weeks of storage.

Also, the flow behavior index (n) for thyme waste extract had a more pronounced shear thinning behavior (Figure 7.2). For the different formulations, thyme 0.02 and 0.04% concentrations present a decrease in the flow behavior index with the storage time, contrary to what is observed with the control or BHA.

7.3.4. Peroxide content determination

Emulsion stability denotes the capacity to resist changes of physicochemical properties with the passage of time. It was reported that emulsion stability is strongly influenced by the concentration of the antioxidant agent and the types of oils used.[23] The optimization of all the main formulation factors is crucial for the successful preparation of stable emulsion products. Thus, in the present work, the effect of thyme waste extract on the oxidative properties of O/W emulsions formulated with two vegetable oils was examined. The emulsions were prepared using almond oil and wheat germ oil at different concentrations ranging from 0% to 100%, while thyme waste extract was chosen as a protector agent for its antioxidant properties, at concentrations ranging from 0.01% to 0.04%.

The results (Figure 7.3a) indicate that storage time, BHA concentration and thyme waste extract concentration in the emulsions had highly significant effects ($p = 0.000000$), and exerted great influence on the peroxide formation, whereas almond oil (L) and (Q) percentage in the emulsion did not produce a significant effect ($p > 0.05$). The cross-terms were statistically significant, except for 2 L by 4 L.

Three-dimensional surface plots were drawn to determine the interactive effect of the process variables on the formation of peroxides. The response surface plot of peroxide content in emulsions under different combinations is shown in Figure 7.3b–f. This set of dynamic graphs allowed us to evaluate the experimental interaction of different parameters on the formation of peroxides. Levels of peroxides in emulsions were found to increase highly with the storage time and slightly with the decrease of almond oil percentage (Figure 7.3b).

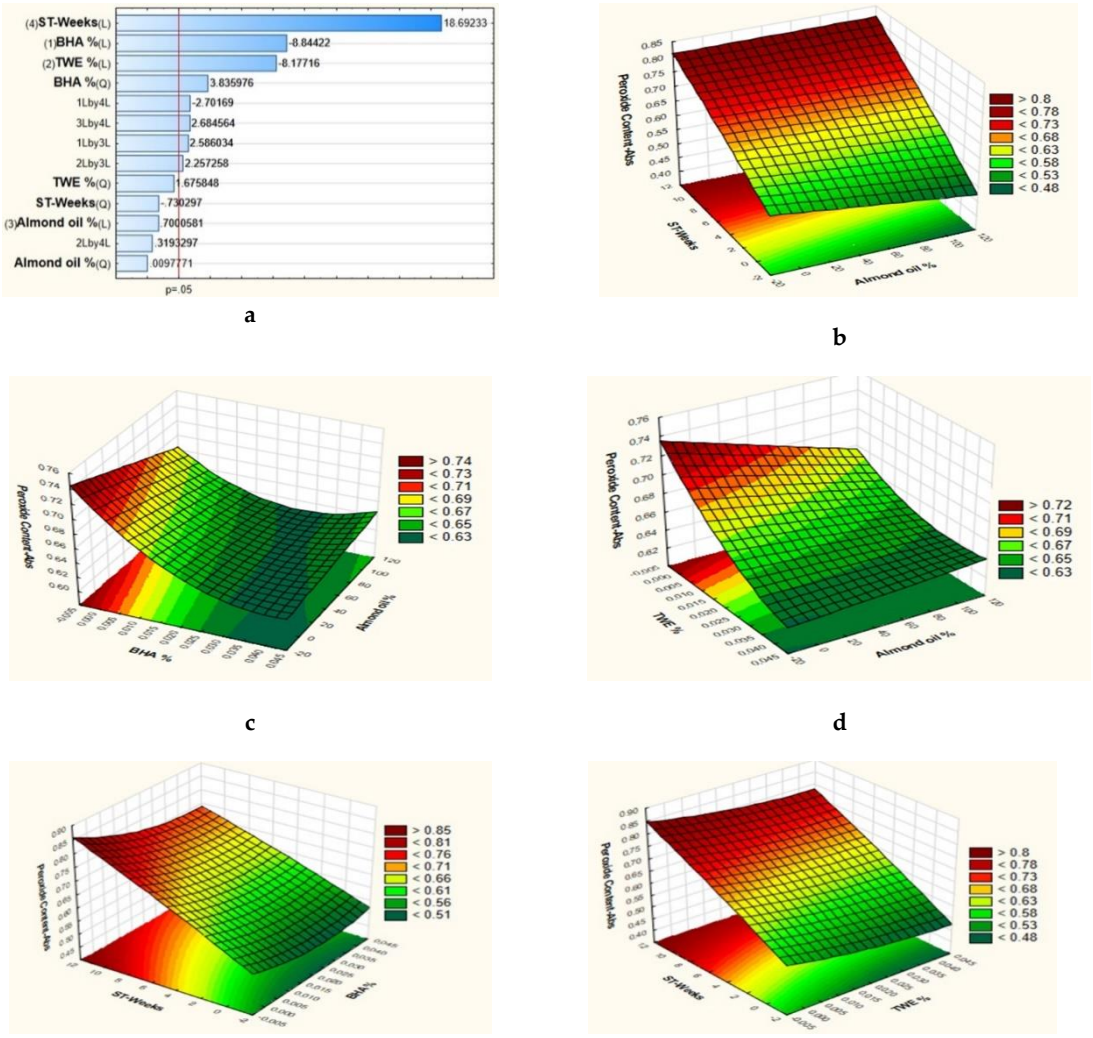


Figure 7.3. (**a**): Pareto chart showing the effects of the significant model coefficients. (**b**): Response surface plot showing the combined effect of storage time and emulsion almond oil %. (**c**): Response surface plot showing the combined effect of BHA concentration and emulsion almond oil %. (**d**): Response surface plot showing the combined effect of thyme extract concentration and emulsion almond oil %. (**e**): Response surface plot showing the combined effect of BHA concentration and storage time. (**f**): Response surface plot showing the combined effect of thyme extract concentration and storage time. TWE: Thyme waste extract; ST: storage time.

The 3D plots for the combined effects of BHA concentration and almond oil percentage in the emulsion are given in Figure 7.3c. BHA inhibited the formation of peroxides independently of the almond percentage used in emulsion. This inhibition increases with the increase of BHA concentration.

The presence of thyme waste extract strongly influences peroxide formation in all types of emulsions in the same way as BHA. The peroxide levels decreased with the increase in thyme waste extract concentration (Figure 7.3d), this is due to the same antioxidant effect exerted by this extract. Sinapic, ferulic, and caffeic acids, present in the thyme waste extract are considered

good antioxidants, having been some of these hydroxycinnamic acid derivatives used as natural antioxidants in food, beverages, and cosmetics.[24]

Figure 7.3e,f depict, respectively, the combined effect of BHA and thyme waste extract concentrations with the time storage on hydroperoxides formation. Thyme waste extract and BHA had the same behavior on peroxide formation during storage time. Hydroperoxides increased during time in all O/W emulsions. As compared to the BHA, thyme waste extract was more or less similarly efficient on controlling the evolution of primary oxidation products for almost all formulations. It is noteworthy that the presence of wheat germ oil at higher concentration mostly affects the emulsion stability being more prone to oxidation due to the predominance of unsaturated fatty acids.[3]

7.3.5. Secondary lipid oxidation products in formulated emulsions

Lipid and oil based formulations are susceptible to degradation through lipid peroxidation which represents a concerning chemical instability.[25] The aim of this part of the work is to evaluate the antioxidant effectiveness of thyme waste extract on MDA formation during oxidation of wheat and almond oil emulsion.

In order to examine the relative importance of the main effects and their interactions with statistical significance ($p < 0.05$), a standardized Pareto chart (Figure 7.4a) was employed. The results showed that the main factors (1) BHA concentration (L), (2) thyme waste extract concentration (L), (3) Almond oil % (L), (4) Storage time (L) and their interactions [BHA concentration (Q Thyme waste extract concentration (Q), Almond oil % (Q), 1 L by 4 L, 3 L by 4 L, 1 L by 3 L, 2 L by 3 L, 2 L by 4 L) that extend beyond the reference line were significant at the level of 0.05. BHA followed by thyme waste extract concentration presented the most significant factors, which affected oxidative stability of lipids in the investigated emulsion. All other factors and their interactions had less effect but were statistically significant at 95% confidence, except for the interaction of storage time (Q) expressed in weeks.

Initially (Figure 7.4b), interaction between factors like almond oil % and 10 weeks of storage time were examined to determine their impact on the secondary lipid oxidation expressed as absorbance. As it can be noticed in the plot, there is an increase in the absorbance with the increase of storage time and with the decrease of almond oil proportion in the emulsion. The increase of almond proportion in the emulsion enhances its resistance to oxidation. The lipid oxidation was shown to be low during the entire studied storage time when almond oil proportion in the emulsion was between 75 to 100%, whereas the inverse was observed between 0% to 25%.

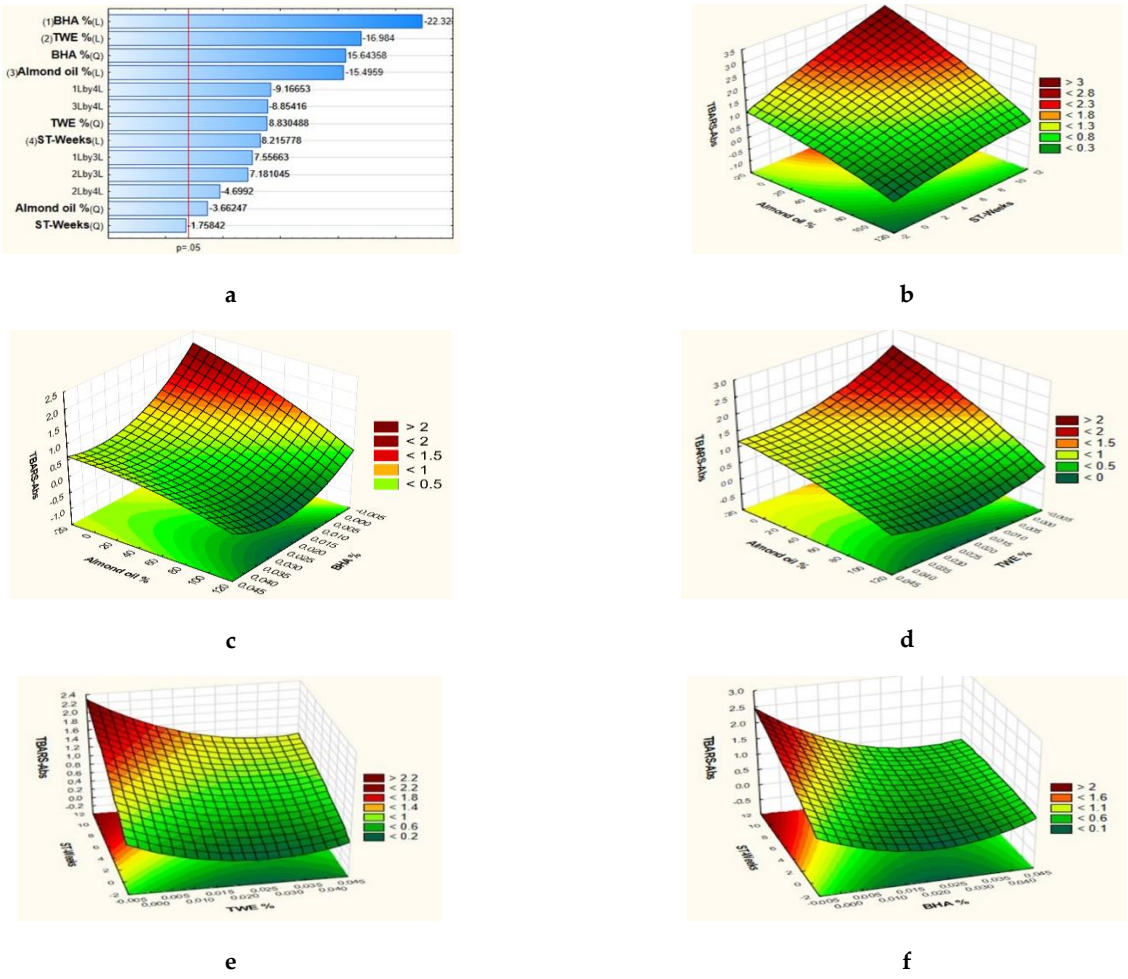


Figure 7.4. (**a**): Pareto chart showing the effects of the significant model coefficients. (**b**): Response surface plot showing the combined effect of storage time and emulsion almond oil %. (**c**): Response surface plot showing the combined effect of BHA concentration and emulsion almond oil %. (**d**): Response surface plot showing the combined effect of thyme extract concentration and emulsion almond oil %. (**e**): Response surface plot showing the combined effect of thyme extract concentration and storage time.(**f**): Response surface plot showing the combined effect of BHA concentration and storage time. TWE: Thyme waste extract; ST: storage time.

The response surfaces obtained for secondary lipid oxidation as a function of the percentage of almond oil and BHA concentration in emulsion are illustrated in Figure 4c. A significant increase of the lipid oxidation was observed along with the decrease of almond oil proportion for all BHA concentrations. This increase was extremely important in the absence of BHA. Figure 4d shows the response surface plot depicting the effect of thyme waste extract concentration and almond oil on secondary lipid oxidation. As seen for BHA concentration in emulsions, lipid oxidation decreases with the increase of thyme waste extracts until optimal concentration of 0.02% to 0.03% is reached, exhibiting the lowest absorbance, after which the phenomenon started to reverse. When compared to the BHA, thyme extract was more or less similarly efficient in controlling the evolution of MDA for almost all formulations.

Furthermore, whatever the antioxidant used; the values gradually increased during the storage period by raising the percentage of wheat germ oil.

According to Gallego et al.,[26] the emulsion stability increased with increasing concentration of natural extracts. Emulsions containing extract concentrations of 0.02% and 0.2% showed a higher stability than those prepared with the positive Trolox control (0.02%), and the 0.2% extract exhibited a similar antioxidant effect to that of BHA, at 0.004%. Besides, Poyato et al.[27] have shown that the type of antioxidant is a key factor in the control of oxidation process in W/O/W or O/W emulsions, which are formulated with highly polyunsaturated oils. The Banias et al.[28] study showed that thyme extracts have strong antioxidant effects in stabilizing lard.

Formulations with the combination of higher concentrations of thyme waste extract (0.02%, 0.04%) and almond oil (≥50%) were the best in protecting the oxidation of emulsion samples. According to Yun and Surh,[29] the ratio of oleic acid to linoleic acid could be used as a criterion for determining oil stability and fatty acid composition. Oleic acid (C18:1) has been estimated to be 10 to 40 times less susceptible to oxidation than linoleic acid (C18:2).

The effects of storage time with each of the two antioxidants (BHA and thyme extract) on emulsion lipid oxidation are displayed as 3D surface plot in Figure 7.4e,f, respectively. As shown, lipid oxidation increases with the storage time in the same way for both antioxidants. This oxidation was slower in the presence of any of the two antioxidants.

7.4. Conclusion

Thyme waste extract prevented the hydroperoxide formation and the accumulation of malondialdehyde in emulsions O/W, nevertheless this was dependent on its concentration as well as on the ratio of wheat germ and almond oil at the oil phase. The combination of higher concentrations of thyme waste extract (0.02%, 0.04%) and almond oil (≥50%) were the best in protecting the primary oxidation of emulsion samples (prevention of hydroperoxide formation). Thyme waste extract was only effective in the prevention of the accumulation of malondialdehyde when 100% almond oil was used; the presence of wheat germ hampered the antioxidant action of the thyme waste extract. Over the storage period (10 weeks), the antioxidant ability of thyme waste extract decreased, becoming more accentuated as the concentration of wheat germ oil increased. Depending on the ratio of oils (wheat germ and almond oil), there was also a change in the viscosity trend of emulsions O/W as the antioxidant was added: for all the thyme waste extract concentrations, the initial viscosity of the samples increased with the decrease of wheat germ oil percentage, from 100% to 50%; after this ratio,

the initial viscosity decreased by increasing the almond oil percentage. The results indicate that the extract of waste thyme obtained after steam distillation for producing essential oils can constitute a good antioxidant for stabilizing diverse types of materials such as emulsions O/W, provided that the oil phase is not predominantly constituted by polyunsaturated fats.

References

[1] M. Serdaroğlu, B. Öztürk, A. Kara, 'An overview of food emulsions of food emulsions: Description, classification and recent potential applications', Turk. J. Agric. Food Sci. Technol. 2015, 3, 430–438.

[2] C. Linke, S. Drusch, 'Pickering emulsions in foods–opportunities and limitations', Crit. Rev. Food Sci. Nutr.2018, 58, 1971–1985.

[3] C.C. Berton-Carabin, M. H. Ropers, C. Genot, 'Lipid oxidation in oil-in-water emulsions: Involvement of the interfacial layer', Compr. Rev. Food Sci. Food Saf. 2014, *13*, 945–977.

[4] E. A. Decker, D. J. McClements, C. Bourlieu-Lacanal, E. Durand, M. C. Figueroa-Espinoza, J. Lecomte, P. Villeneuve, 'Hurdles in predicting antioxidant efficacy in oil-in water emulsions', Trends Food Sci. Technol.2017, 67, 183–194.

[5] C. M. Seppanen, Q. Song, S. A. Csallany, 'The antioxidant functions of tocopherol and tocotrienol homologues in oils, fats, and food systems', J. Am. Oil Chem. Soc. 2010, 87, 469–481.

[6] A. B. Sharangi, S. Guha, 'Wonders of leafy spices: Medicinal properties ensuring human health', Sci. Int.2013, 1, 312–317.

[7] A. Komaki, F. Hoseini, S. Shahidi, N. Baharlouei, 'Study of the effect of extract of Thymus vulgaris on anxiety in male rats', J. Tradit. Complement. Med. 2016, *6*, 257–261.

[8] B. Pavlić, O. Bera, N. Teslić, S. Vidović, G. Parpinello, Z. Zeković, 'Chemical profile and antioxidant activity of sage herbal dust extracts obtained by supercritical fluid extraction', Ind. Crop. Prod. 2018, 120, 305–312.

[9] N. Gavarić, N. Kladar, A. Mišan, A. Nikolić, I. Samojlik, N. Mimica-Dulić, B. Božin, 'Postdistillation waste material of thyme (Thymus vulgaris L., Lamiaceae) as a potential source of biologically active compounds', Ind. Crop. Prod. 2015, 74, 457–464.

[10] M.Š. Pogačar, A. Klančnik, F. Bucar, T. Langerholc, S. S. Možina, Anti-adhesion activity of thyme (Thymus vulgaris L.) extract, thyme post-distillation waste, and olive (Olea europea L.) leaf extract against Campylobacter jejuni on polystyrene and intestine epithelial cells', *J*. Sci. Food Agric. 2016, 96, 2723–2730.

[11] S. El-Guendouz, S. Aazza, B. Lyoussi, M. D. Antunes, M. L. Faleiro, M. G. Miguel, 'Anti-acetylcholinesterase, antidiabetic, anti-inflammatory, antityrosinase and antixanthine oxidase activities of Moroccan propolis', Int. J. Food. Sci. Technol. 2016, 51, 1762–73.

[12] S. El-Guendouz, N. Al-Waili, S. Aazza, Y. Elamine, S. Zizi, T. Al-Waili, A. Al-Waili, B. Lyoussi, 'Antioxidant and diuretic activity of co-administration of Capparis spinosa honey and propolis in comparison to furosemide', Asian. Pac. J. Trop. Biomed. 2017, 10, 974–980.

[13] S. Aazza, B. Lyoussi, M. G. Miguel, 'Antioxidant activity of some Morrocan hydrosols', J. Med. Plants Res.2011, 5, 6688–6696.

[14] S. El-Guendouz, S. Aazza, B. Lyoussi, N. Majdoub, V. Bankova, M. Popova, S. Raposo, A. Dulce, M. G. Miguel, 'Effect of poplar-type propolis on oxidative stability and rheological properties of O/W emulsions', Saudi Pharm. J. 2018, *26*, 1073–1082.

[15] A. Mărculescu, L. Vlase, D. Hanganu, C. Drăgulescu, A. Iuliana, O. Neli-Kinga, 'Polyphenols analyses from Thymus species', J. Proc. Rom. Acad. Series B. 2007, 3, 117–121.

[16] E. Köksal, E. Bursal, İ. Gülçin, M. Korkmaz, C. Çağlayan, A. C. Gören, S. H. Alwasel, 'Antioxidant activity and polyphenol content of Turkish thyme (Thymus vulgaris) monitored by liquid chromatography and tandem mass spectrometry', Int. J. Food Prop. 2017, 20, 514–525.

[17] A. Sökmen, M. Gulluce, H. A. Akpulat, D. Daferera, B. Tepe, M. Polissiou, M. Sokmen, F. Sahin, 'The *in vitro* antimicrobial and antioxidant activities of the essential oils and methanol extracts of endemic Thymus spathulifolius, Food Control 2004, 15, 627–634.

[18] İ. Gülçin, E. Bursal, H. M. Şehitoğlu, M. Bilsel, A. C. Gören, 'Polyphenol contents and antioxidant activity of lyophilized aqueous extract of propolis from Erzurum, Turkey', Food Chem. Toxicol. 2010, 48, 2227–2238.

[19] M. Abdul Qadir, S. K. Shahzadi, A. Bashir, A. Munir, S. Shahzad, 'Evaluation of phenolic compounds and antioxidant and antimicrobial activities of some common herbs', Int. J. Anal. Chem. 2017, 2017, 6.

[20] S. Desplanques, F. Renou, M. Grisel, C. Malhiac, 'Impact of chemical composition of xanthan and acacia gums on the emulsification and stability of oil-in-water emulsions', Food Hydrocoll. 2012, 27, 401–410.

[21] F. Tirnaksiz, O. Kalsin, 'Topical W/O/W multiple emulsions prepared with Tetronic 908 as a hydrophilic surfactant: Formulation, characterization and release study', J. Pharm. Pharm. Sci. 2005, 8, 299–315.

[22] T. Mahmood, N. Akhtar, B. Khan, A. Rasul, S. Khan, 'Fabrication, physicochemical characterization and preliminary efficacy evaluation of a W/O/W multiple emulsion loaded with 5 % green tea extract', Braz. J. Pharm. Sci. 2013, 49, 341–349.

[23] J. Komaiko, D. J. McClements, 'Optimization of isothermal low-energy nanoemulsion formation: Hydrocarbon oil, non-ionic surfactant, and water systems', J. Colloid Interface Sci. 2014, 425, 59–66.

[24] N. Nićiforović, H. Abramović, 'Sinapic acid and its derivatives: Natural sources and bioactivity', Compr. Rev. Food Sci. Food Saf. 2014, 13, 34–51.

[25] H. Shrestha, R. Bala, S. Arora, 'Lipid-based drug delivery systems', J. Pharm. 2014, 1–10.

[26] M. Gallego, M. Skowyra, M. Gordon, N. Azman, M. Almajano, 'Effect of leaves of *Caesalpinia decapetala*on oxidative stability of oil-in-water emulsions', Antioxidants. 2017, *6*, 19.

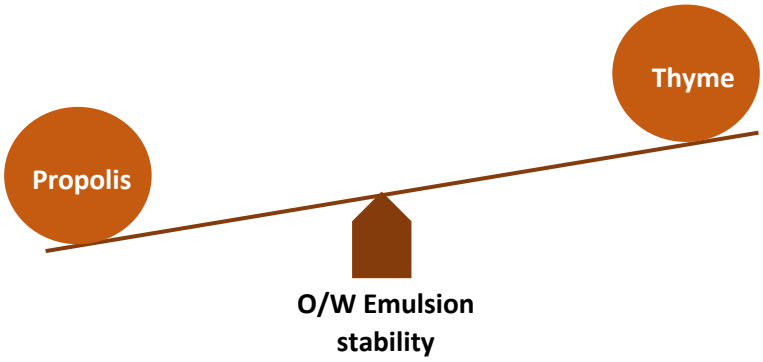
[27] C. Poyato, I. Navarro-Blasco, M. I. Calvo, R. Y. Cavero, I. Astiasarán, D. Ansorena, 'Oxidative stability of O/W and W/O/W emulsions: Effect of lipid composition and antioxidant polarity', Food Res. Int. 2013, 51, 132–140.

[28] C. Banias, V. Oreopoulou, C.D. Thomopoulos, 'The effect of primary antioxidants and synergists on the activity of plant extracts in lard', J. Am. Oil Chem. Soc. 1992, 69, 520–524.

[29] J. M. Yun, J. H. Surh, 'Fatty acid composition as a predictor for the oxidation stability of Korean vegetable oils with or without induced oxidative stress', Prev. Nutr. Food Sci. 2012, 17, 158–165.

Chapter 8

Natural antioxidants in emulsions O/W



Natural antioxidants in emulsions O/W*

Abstract

This study was aimed to compare the capacity of two natural products, propolis extract (PE) and thyme waste extract (TWE), to prevent the oxidation of oil in water (O/W) emulsion, as well as their impact on emulsion apparent viscosity (AV) behaviour in the presence of wheat germ and almond oils as lipid phase. For this, central composite design (CCD) and principal component analysis (PCA) were performed. Oxidation process was monitored by evaluating the formation of primary and secondary lipid oxidation products, at the same time the AV behaviour was determined through evaluating consistency index and flow behaviour index. The results revealed that the increase of PE % and TWE% decreases TBARS (Thiobarbituric Acid Reactive Substances) and hydroperoxides formation. Viscosity, increases with the rise of TWE% over (0.04%), whereas, lower concentrations of PE% decreases it. Those results have been confirmed in the PCA analysis. TWE showed higher resistance to oxidation, although, PE was more effective as antioxidant than TWE.

*Adapted from 'Natural antioxidants in emulsions O/W'. Soukaïna El-Guendouz, Smail Aazza, Susana Anahi Dandlen, Nessrine Majdoub, Badiaa Lyoussi, Sara Raposo, Maria Graça Miguel. *(Submitted).*

8.1. Introduction

Generally, emulsions are used in food, pharmaceutical, cosmetic industries among others, despite their thermodynamically instability over time, by breaking down or aggregating of the dispersed oil phase. Moreover, oxidation of the lipid phase may occur, giving rise to unwanted oxidative byproducts with impact on the emulsion stability and quality.[1] Among the strategies adopted to control this phenomena is the use of antioxidants mainly butylhydroxyanisole (BHA) and butylhydroxytoluene (BHT), that serve as synthetic antioxidants, however, these artificial compounds have been found to possess adverse effect on public health.[2,3]

Recently, our team has developed emulsions O/W in which almond and wheat germen oils were used as dispersed phase and propolis[4] and thyme waste extracts[5] were used as antioxidants. The present work deals to compare the antioxidant capacity of these extracts in the emulsions as well as their effects on the viscosity of the emulsions.

8.2. Experimental

8.2.1. Samples preparation

Propolis extract was performed as describe by El-Guendouz et al.,[4] and thyme waste was extracted as reported by El-Guendouz et al..[5]

8.2.2. Preparation and storage of emulsion O/W, antioxidant activity and viscosity of emulsions

Emulsions O/W were prepared as reported by El-Guendouz et al..[4,5]

For the determination of primary oxidation products, lipid hydroperoxides were measured as describe by El-Guendouz et al..[4] For the secondary oxidation products thiobarbituric acid-reactive substances (TBARS) were determined according to El-Guendouz et al..[4,5]

Rheological properties of the different samples were determined according to El-Guendouz et al..[4,5]

8.2.3. Statistical analysis

TBARS, hydroperoxides and apparent viscosity data were analyzed by two-way ANOVA using GraphPad Prism version 5.03 for Windows (GraphPad Software, La Jolla California, USA). Statistical significance was set as $p < 0.05$; when the analysis was statistically significant, the Duncan post hoc test was done.

Central composite design using the general second order polynomial models were used for the regression analysis of data and Principal component analysis (PCA) were performed using STATISTICA (Stat Soft. InC., Tulsa, OK, USA), version 10, Trial Version.

8.3. Results and discussion

Figure 8.1 allows to compare the antioxidant activities, measuring the primary and secondary oxidation products formation, and apparent viscosities of the emulsions O/W formulated with different concentrations of almond and wheat germ oils in the presence of propolis extract (PE) or thyme waste extracts (TWE) at 0.01 (Figure 8.1a), 0.02 (Figure 8.1b) and 0.04% (Figure 8.1c), after storage of 10 weeks, at 37 °C.

The results presented in Figures 1 a (0.01%), b (0.02%), and c (0.04%) and concerning the primary oxidation show that PE was more efficient in controlling and preventing lipid peroxidation. The statistical analysis reveals a significant difference ($p < 0.001$) of absorbance at $\lambda = 510$ nm between emulsion O/W containing PE and the ones with TWE regardless the type of formulations (A, B, C, D and E). Moreover, the higher concentration of PE and TWE, the lower absorbance recorded. Regarding the different formulations (A: 100% wheat germ oil, B: 75% wheat germ oil / 25% almond oil, C: 50% wheat germ oil/50% almond oil, D: 25 % wheat germ oil /75 % almond oil, E: 100% almond oil), it was verified that the presence of almond oil, even at low concentration, positively affect the emulsion stability (Figures 8.1, a, b, and c). Likewise, in the formation of the secondary peroxidation products, it was detected a very different behavior on their formation depending on the type of oil and the nature of antioxidant used. The presence of wheat germ oil, predominantly constituted by unsaturated fatty acids, made easier the oxidation (Figures 8.1a, b, and c).

Other factor determinant on the prevention of the stability of the formulated emulsions was the antioxidant used, in this regards, PE was more effective, this was concluded from the fact that a significant difference ($p < 0.001$) was found in the production of primary and secondary oxidation products in the emulsions O/W containing PE and those containing TWE after ten weeks of storage (Figures 8.1, a, b, and c).

In general, formulations with almond oil in an increased amount were more controlled regarding lipid peroxidation. Furthermore, PE especially when it was used at high concentration (0.04%) with the lipid phase almond oil, were the best form of O/W emulsion.

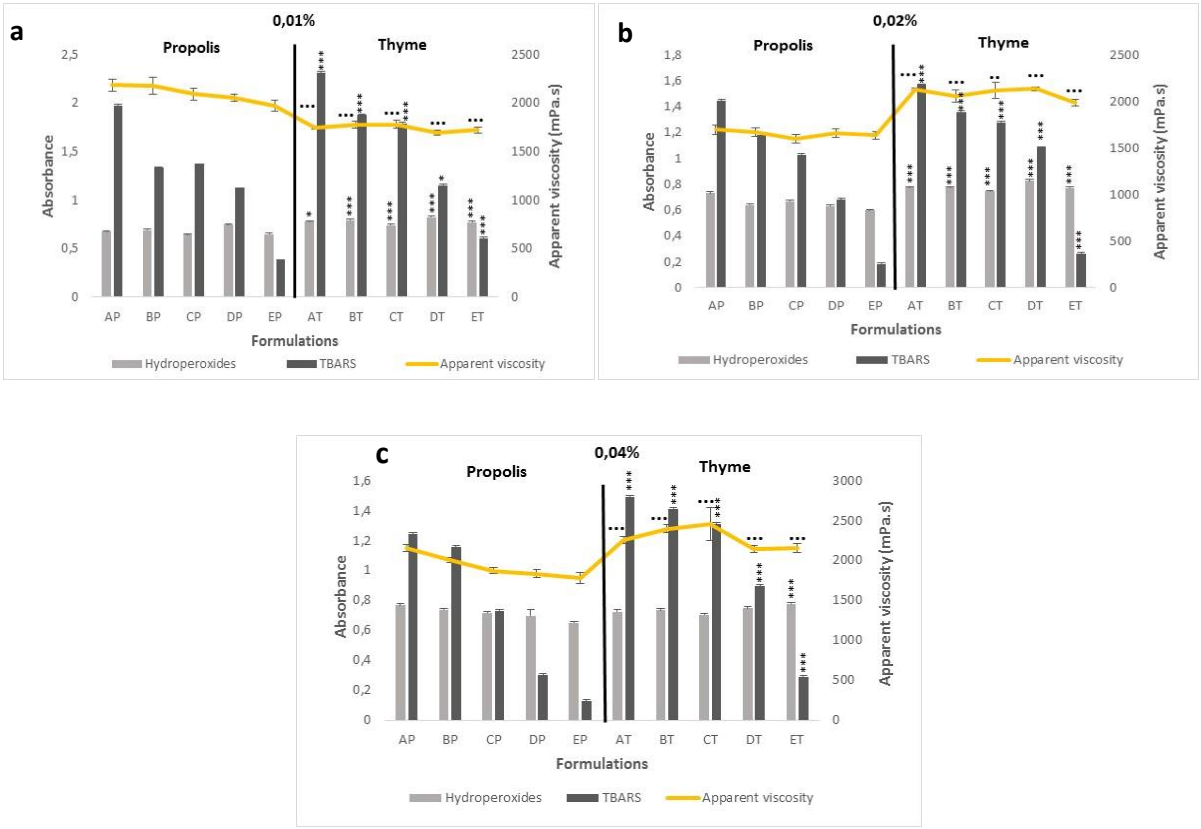


Figure 8.1. Effect of adding thyme extract and Propolis at different concentrations (0.01%; 0.02% and 0.04%) within O/W emulsions formulations (A, B, C, D and E) on TBARS (ABS_{532}), Hydroperoxide (ABS_{510}) and Apparent viscosity after storage of 10 weeks at 37 °C. Values of mean (n = 6) ± standard deviation. **P**: propolis extract, **T**: thyme extract, **A**: 100% wheat germ oil, **B**: 75% wheat germ oil / 25% almond oil, **C**: 50% wheat germ oil/50% almond oil, **D**: 25 % wheat germ oil /75 % almond oil, **E:** 100% almond oil. *** = P<0.001; **=P<0.01; *= P<0.05 and •••= P<0.001; ••=P<0.01; •= P<0.05 as compared to propolis.

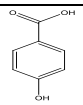
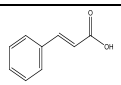
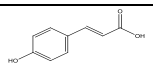
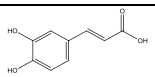
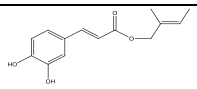
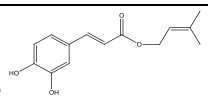
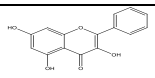
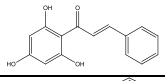
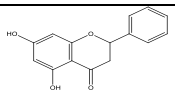
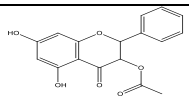
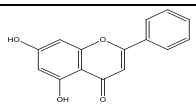
The formulated emulsions O/W were also tracked in viscosity stability terms, and the results are displayed in Figures 8.1, a, b, and c. Figure 8.1 shows the apparent viscosity (AV) (mPa.s) calculated from shear rate and shear stress, and reveals that many factors control the emulsion O/W viscosity, including percentage of almond oil or wheat germ oil, the nature of the antioxidant used as well as their concentration.

Regardless the type of the antioxidant added, the increase of almond oil affected largely the viscosity which was higher in formulation A (0% almond oil/ 100% wheat germ oil) as compared with that o formulation E (100% almond oil/ 0% wheat germ oil). As an example, this parameter varied from 2132 ± 11 (mPa.s) and 1700 ± 51 (mPa.s) to 1988 ± 34 (mPa.s) and 1638 ± 42 (mPa.s) for TWE and PE at 0.02% respectively. On the other hand, the concentration and type of the antioxidant (TWE or PE) appears with a high importance on controlling the viscosity. It is also apparent that the PE induced lower AV values than TWE, for the different formulations and concentrations, except for the concentration of 0.01%, where the opposite is true. For the concentration of 0.01%, PE presented viscosities superior to TWE. Thus, PE, in

all concentration tested (0.01%, 0.02%, 0.04%), was more efficient than TWE on controlling the AV, however when the concentration was higher the viscosity decreased. This finding was concluded from the statistical analysis, where, a significant difference was detected comparing all emulsion formulation (A, B, C, D and E) when PE or TWE were used ($p < 0.001$) (Figures 8.1, a b, and c).

The higher effectiveness of PE in emulsion O/W regarding oxidative stability and viscosity behavior as compared with TWE, may be attributed to the different major bioactive group of constituents present in PE[4] and absent in TWE[5] (Table 8.1), even if the latter also has important antioxidative capacity in the same matrix.

Table 8.1. Chemical composition of hydro-alcoholic extract of propolis and thyme waste extract (adapted from El-Guendouz et al.[4,5])

Components > 5%	**Thyme**	**Propolis**
Aromatic acids		
Benzoic acid	40.88	--
4-Hydroxybenzoic acid 	17.18	--
trans-Cinnamic acid 	11.89	--
p-Coumaric acid 	10.34	< 5
Caffeic acid 	8.74	--
Phenolic acid esters		
2-Methyl-2-butenyl caffeate 	--	7.1
3-Methyl-2-butenyl caffeate 	--	5.2
Flavonoids		
Galangin 	--	8.7
Pinocembrin chalcone 	--	6.6
Pinocembrin 	--	6.6
Pinobanksin acetate 	--	5.4
Chrysin 	--	5.4

Multiple regression analysis of variance (ANOVA) was used to check the adequacy and fitness of the developed models and results are given in Table 8.2. At a 95% confidence level, a model was considered significant, if the *p* value <0.05.

The model generated coefficients of regression based on analysis of variance (ANOVA) are given in Table 8.2. The statistical significance of the model was evaluated by the F-test (ANOVA). The higher F-values and the smaller P-values demonstrate that the applied model is significant. The precision of a model is judged by the regression coefficient (R2) which is always in between 0 and 1, and its order of magnitude suggests the aptness of the model ((Borugadda et al., 2015). The propriety and fitness of the models were evaluated by determination of the coefficient (R2). As shown in Table 8.2, in the formulated emulsions using PE as stabilizer, the determination of coefficient for TBARS content, hydroperoxyde content and apparent viscosity (AV) were 0.99, 0.93 and 0.88 respectively, demonstrating the significance of the model and that the accuracy and general ability of the polynomial model is good. Emulsions formulated using TWE presented a good statistical model, since the determination of the coefficient were 0.98, 0.93 and 0.95 for TBARS content, hydroperoxide content and AV, respectively. Thus, the regression model provides an excellent explanation of the relationship between the independent variables [almond oil (AO)%, PE% and TWE%] and the response variables.

PE and TWE had a significant linear and quadratic effect ($p < 0.05$) on all response variables. However, AO (%) had a significant effect ($p < 0.05$) on TBARS and hydroperoxide contents in TWE formulated emulsion, but was only significant for TBARS content when emulsions are formulated using PE.

Table 8.2. The results of analysis on variance (ANOVA) for the effects of independents variables.

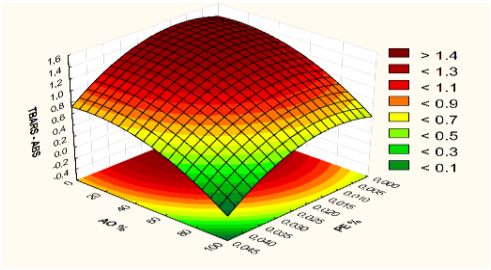
	TBARS		**Hydroperoxide**		**A. Viscosity**	
	F	**p**	**F**	**p**	**F**	**p**
Thyme Waste Extract (TWE)						
(1)TWE % L+Q	54.55	0.0044	23.76	0.0145	29.27	0.0108
(2)AO % L+Q	47.76	0.0053	28.58	0.0111	1.27	0.3985
1*2	1.38	0.3252	2.47	0.2142	1.38	0.3246
R^2	0.99		0.97		0.95	
R^2-adj	0.96		0.93		0.88	
Propolis Extract (PE)						
(1)PE % L+Q	58.88	0.0039	18.13	0.0211	142.55	0.0011
(2)AO % L+Q	37.16	0.0076	1.60	0.3367	4.42	0.1274
1*2	25.33	0.0151	0.22	0.6697	8.57	0.0611
R^2	0.98		0.93		0.95	
R^2-adj	0.96		0.81		0.88	

In order to study and optimize the composition of O/W emulsions, and to investigate the effects of process variables (TWE%, PE% and AO%) and their interactive effects on the viscosity, TBARS and hydroperoxide contents of the emulsions, the central composite design has been performed the three-dimensional plots were depicted in Figures 8.1 and 8.2, which can exhibit how the initial factors affected different responses, more clearly.

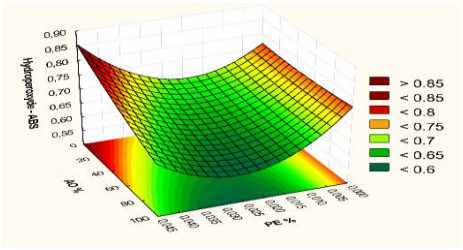
In what concerns TBARS content, Figures 8.2a and 8.3a illustrates the relationship between AO%, PE% and TWE%. As shown in Figure 8.2a, the increase in PE content (%) decreased the emulsion TBARS content. The same behaviour was found for almond oil. TBARS content (Figure 8.3a) decreased with the increase of TWE% in the emulsion till reaching an amount of 0.035% from which it starts to increase, demonstrating that high amount of this stabilizer could have an inverse effect. The lowest amount of TBARS produced during emulsions storage are found when using higher amount of almond oil (above 60%) and high amounts of propolis (0.04%) or moderate concentration of TWE (0.03%).

In what concerns hydroperoxide content, the 3D response surface plot shown in Figure 8.3a and 3b, illustrates the combined impact of AO%, PE% and TWE%. From this graph, it can be clearly observed that when the PE% proportion increased, hydroperoxide content decreased significantly ($p < 0.01$), but starts to increase when PE% overtakes 0.035%. We also noticed, that those amounts increase with the decrease of AO% in the emulsion. Emulsions enriched with PE between 0.020 to 0.030 produced lower amounts of hydroperoxide even with lower amount of AO%. The same phenomenon has been observed in Figure 8.3b, which illustrates the interaction between AO% and TWE%, showing that hydroperoxide amounts decreased with the rise of TWE%.

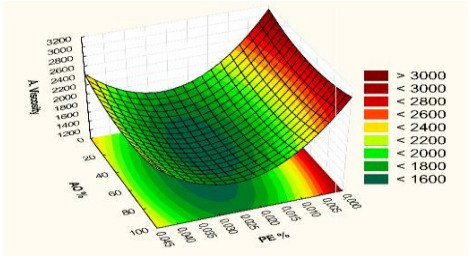
Regarding apparent viscosity (AV), emulsion viscosity (Table 8.2) was significantly influenced ($p < 0.05$) by the two antioxidants (PE% and TWE%) used in the emulsions, but AO% did not have any significant effect (Figures 8.2c and 8.3c). The rise of TWE% (Figure 8.3c), had an effect on enhancement of emulsion viscosity, whereas, it was decreased by the increase of PE% (Figure 8.2c) at lower concentration (<0.03%). When PE% overtakes this amount the emulsion viscosity starts to increase. The lowest viscosity was found in emulsion containing low amount of TWE or moderate amount of PE (between 0.02 and 0.03%).



a

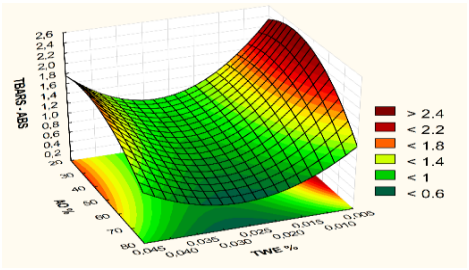


b

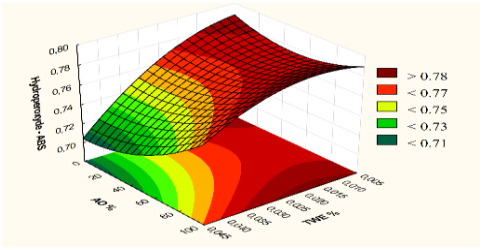


c

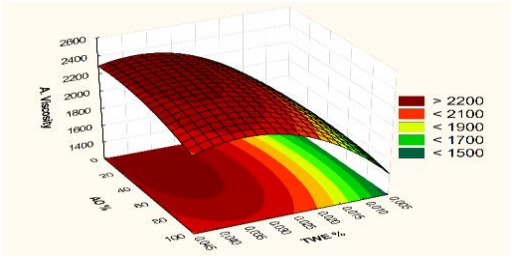
Figure 8.2. 3D surface plots representing the interaction effects of (E% and AO% on: (a) TRABRS, (b) Hydroperoxide (c) apparent viscosity (AV)



a



b



c

Figure 8.3. 3D surface plots representing the interaction effects of TWE% and(A)% on:(a) TRABRS, (b) Hydroperoxide (c) apparent viscosity (AV).

In order to assess the correlation between the different variables, data was subjected to PCA to provide an overview of the complete data set. The factor 1 (horizontal axis) explained 42.90% of the total variance, whereas the factor 2 (vertical axis) explained a further 31.02% (Figure 8.4). Combining PC1 and PC2 explained 73.92% of total variability which was high enough to represent all the variables.

It is clear that TBARS content was significantly positively correlated with wheat germ oil content (WGO%), and is also negatively correlated with AO%.

Thyme waste extract was correlated to emulsion viscosity and hydroperoxide content, the for parameter were found to be negatively correlated to PE%.

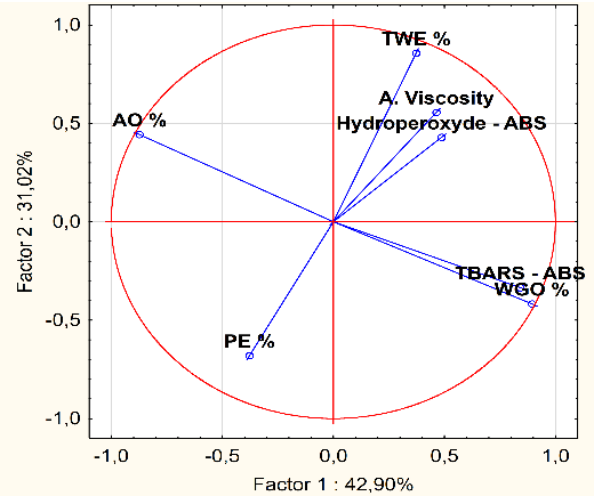


Figure 8.4. Principal component analysis (PCA) plot.

8.4. Conclusion

As a conclusion, we reported the antioxidant activity of PE and TWE. Besides, depending on the oils proportion the emulsions were more stable when PE was used at the concentration of 0.04%. This finding could be with great value for pharmaceutical and cosmetic industries, it presents an alternative way to formulate emulsion containing more safe and beneficial products from nature in the intention of replacing synthetic compounds. Moreover, the use of waste products and recycling them present a profit-making strategy from a lucrative view for cosmetic industries. Above all, the increase of the use of natural products in emulsion formulation is eco-friendly and could avoid several side effects resulted from synthetic products on human health.

References

[1] C. Linke, S. Drusch, 'Pickering emulsions in foods – opportunities and limitations' Crit Rev. Food. Sci. Nutr. 2018, 58, 1971-1985. doi: 10.1080/10408398.2017.1290578.

[2] K. Yamaki, S. Taneda, R. Yanagisawa, K. Inoue, H. Takano, S. Yoshino, 'Enhancement of allergic responses in vivo and *in vitro* by butylated hydroxytoluene', Toxicol. Appl. Pharmacol. 2007, 223, 164-172.

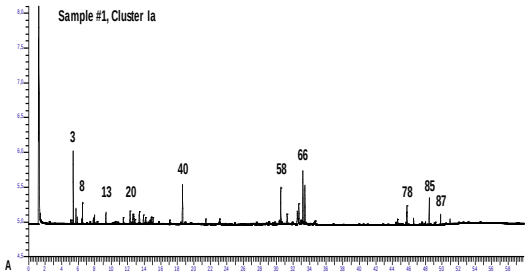
[3] B. Nieva-Echevarría, M. J. Manzanos, E.Goicoechea, M.D. Guillén2,6-Di-tert-butyl-hydroxytoluene and its metabolites in foods Compr. Rev. Food Sci. Food. Saf. 2015, 14, 67-80. 10.1111/1541-4337.12121.

[4] S. El-Guendouz, S. Aazza, B. Lyoussi, N. Majdoub, V. Bankova, M. Popova, S. Raposo, A. Dulce, M. G. Miguel, 'Effect of poplar-type propolis on oxidative stability and rheological properties of O/W emulsions', Saudi Pharmaceutical Journa,l. 2018, 26 8, 1073–1082. doi: https://doi.org/10.1016/j.jsps.2018.05.017

[5] S. El-Guendouz, S. Aazza, S. A. Dandlen, N. Majdoub, B. Lyoussi, S. Raposo, M. D. Antunes,V. Gomes, M. G. Miguel, 'Antioxidant Activity of Thyme Waste Extract in O/W Emulsions', Antioxidants **2019**, 8, 243; https://doi.org/10.3390/antiox8080243.

Chapter 9

Characterization of volatiles from Moroccan propolis samples



Characterization of volatiles from Moroccan propolis samples*

Abstract

The volatiles from twenty-four propolis samples collected from different geographical locations in Morocco were evaluated. Propolis volatiles isolated by hydrodistillation and analyzed by Gas Chromatography (GC) and Gas Chromatography-Mass Spectrometry (GC-MS). Cluster analysis based on propolis samples volatiles chemical composition defined two main uncorrelated clusters. Cluster I, with 23 out of the 24 samples analyzed, included three subgroups, Ia, Ib1 and Ib2, dominated by β-eudesmol (11-30%), cedrol (7-40%) and *n*-tricosane (6-34%), respectively. Cluster II included just one sample, with *ar*-curcumene (32%) as main component. The results obtained contributed to increase the knowledge on the chemical composition and variability of Moroccan propolis samples. The volatile profile of these samples was distinct from those of Algeria, Canary Islands and Ethiopia, the sole African propolis volatiles studied so far.

*Adapted from 'Characterization of volatiles from Moroccan propolis samples'. Soukaïna El-Guendouz, Badiaâ Lyoussi, Maria Graça Miguel & A. Cristina Figueiredo. Journal of Essential Oil Research, Pages 27-33, September 2018

9.1. Introduction

In addition to their role in the pollination of different plant species, bees produce a diverse array of products including honey, bee pollen, propolis and royal jelly. Since ancient times, bee products have a long medicinal history due to its broad applications and their beneficial effect on human health.[1]

Propolis, also called bee glue, is one of the most popular bee products, occurring usually as a sticky dark colored material made by honeybees (*Apis mellifera*) from buds, leaves, exudates and bark of various plants, mixed with beeswax and salivary enzymes.[2]

The large spectrum of biological properties attributed to propolis, and its potential use in food cosmetic and pharmaceutical industries, motivated a large number of studies, both on its chemical composition and validation of biological activity. [3-8]

Although several studies reported the phenolic composition of propolis, much less is known on its volatile composition.[4,9] despite its importance in propolis chemical characterization. Likewise, although some studies reported the phenolic composition,[10,11,8] as well as antioxidant, anti-inflammatory,[6,7,11-14] anticancer,[10] antimicrobial,[8] and enzymes inhibitory capacity (acetylcholinesterase, α-amylase, α-glucosidase, lipoxygenase, tyrosinase, and xanthine oxidase),[7,11,13] properties of Moroccan propolis, no previous report addressed its volatile composition.

Viewing to increase the knowledge on Moroccan propolis chemical composition and variability, this study evaluated the volatile composition of twenty-four propolis samples collected in different sites in Morocco.

9.2.Experimental

9.2.1. Propolis samples

In total, 21 raw propolis samples were collected, in spring-summer 2014, from *Apis mellifera* hives located at different sites in Morocco, two were purchased from the market and one was of the unknown origin (Figure 9.1 and Table.E.1, Appendix E). The samples were stored at −20°C until chemical analysis. Before extraction, the frozen samples were powdered using a mortar and a pestle.

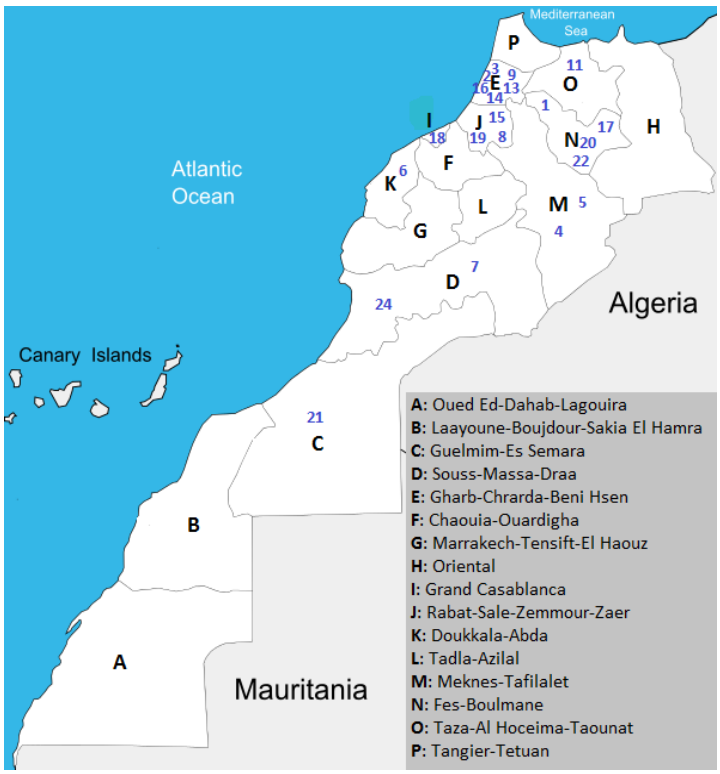


Figure 9.1: Map with the old regions of Morocco (1997–2015) in capital letters (map source Wikipedia) and the distribution of the provenance of the analyzed samples in Arabic numbers. Additional information in Appendix.E, Table.E.1.

9.2.2. Propolis volatile extraction

Propolis volatiles were isolated by hydrodistillation for 3 h using a Clevenger-type apparatus according to the European Pharmacopoeia method.[15] Given the low volatile yield, they were recovered from the graduated tube of the Clevenger apparatus after rinsing with distilled *n*-pentane (*n*-pentane ≥ 99% purity, HPLC grade, is in laboratory distilled prior to use, to remove stabilizers that may contaminate the sample, particularly low-volatile yield samples) when the distillation procedure was over and allowed to settle for about 10–15 min. For this procedure, the tap was opened anticlockwise, so that the water flowed out of the connecting tube until just below the filling funnel. Distilled *n*-pentane was introduced in the filling funnel followed by water, so that pentane evaporated, with the residual heat of the distillation flask and then condensed, and dissolved the volatiles, over the aqueous phase in the graduated tube. The tap was then opened clockwise, to recover the mixture of distilled *n*-pentane and volatiles in an appropriate vial. The mixture was then concentrated to a minimum volume of about

100 µl, at room temperature under nitrogen flux, using a blowdown evaporator system. After extraction and until analysis, the propolis volatile samples were stored at −20°C in the dark.

9.2.3. Propolis volatile composition analysis

9.2.3.1.Gas chromatography (GC)

Gas chromatographic analyses were performed using a Perkin Elmer Clarus 400 gas chromatograph equipped with two flame ionization detectors (FIDs), a data handling system and a vaporizing injector port into which two columns of different polarities were installed: a DB-1 fused-silica column (polydimethylsiloxane, 30 m × 0.25 mm i.d., film thickness 0.25 µm; J&W Scientific, Inc., Rancho Cordova, CA) and a DB-17HT fused-silica column ((50% phenyl)-methylpolysiloxane, 30 m × 0.25 mm i.d., film thickness 0.15 µm; J&W Scientific, Inc.). Oven temperature was programmed, 45–175°C, at 3°C/min, subsequently at 15°C/min up to 300°C and then held isothermal for 10 min; injector and detector temperatures, 280°C and 300°C, respectively; carrier gas, hydrogen, adjusted to a linear velocity of 30 cm/s. The samples were injected using split sampling technique, ratio 1:50. The volume of injection was 0.1 µL of a *n*-pentane essential oil solution (1:1). The percentage composition of the volatiles was computed, by the normalization method from the GC peak areas, calculated as mean values of two injections, from each sample, without using the response factors.

9.2.3.2.Gas chromatography–mass spectrometry (GC-MS)

The GC-MS unit consisted on a Perkin Elmer Clarus 600 gas chromatograph, equipped with DB-1 fused-silica column (30 m × 0.25 mm i.d., film thickness 0.25 µm; J&W Scientific, Inc.), and interfaced with a Perkin Elmer 600-T mass spectrometer (software version 5.4.2.1617; Perkin Elmer, Shelton, CT). Injector and oven temperatures were as above; transfer line temperature, 280°C; ion source temperature, 220°C; carrier gas, helium, adjusted to a linear velocity of 30 cm/s; split ratio, 1:40; ionization energy, 70 eV; scan range, 40–300 u; and scan time, 1 s. The identity of the components was assigned by comparison of their retention indices, relative to C_9–C_{30} *n*-alkane indices and GC-MS spectra from a laboratory-made library, created with reference essential oils, laboratory-synthesized components, laboratory-isolated compounds and commercially available standards.

9.2.4. Statistical analysis

The percentage composition of propolis volatiles was used to determine the relationship between the different samples by cluster analysis using Numerical Taxonomy Multivariate

Analysis System (NTSYS PC software, version 2.2, Exeter Software).[16] For cluster analysis, correlation coefficient was selected as a measure of similarity among all samples, and the unweighted pair group method with arithmetic averages (UPGMA) was used for cluster definition. The degree of correlation was evaluated according to Pestana and Gageiro.[17]

9.3. Results and discussion

Moroccan propolis volatiles were obtained in a yield <0.05% (v/w). Their chemical composition was a complex mixture in which 76 components were identified. The isolated propolis volatiles were fully chemically characterized, and the detailed relative amounts of all the identified components are listed in Appendix E, Table E.2. The main identified components (≥1%) are listed in Table 9.2, following their elution order on the DB-1 column, and arranged according to the lowest and the highest percentages found for each component in the three groups defined by agglomerative cluster analysis (Figures 9.2 and 9.3).

Despite this chemical diversity, the analyzed volatiles were predominantly sesquiterpene rich, or the main identified components were included in a chemical group designated other since the components grouped by their biosynthetic pathway were neither terpenes nor phenylpropanoids (Table 9.1). This 'others' group was mainly composed by compounds derived from the primary metabolism, namely aldehydes, hydrocarbons and fatty acids (Table 9.1). Several yet unidentified components attained relatively high percentages (≥5%) in some samples (Table 9.1). Data on the main mass spectra fragments of these components are provided in Table E.2 (Appendix E) and their fragmentation pattern in Figure E.1 in Appendix E

Cluster analysis showed two main uncorrelated clusters (S_{corr} < 0.2; Figures 9.2 and 9.3). Cluster I, with 23 of the 24 samples analyzed, included three subgroups (Ia, Ib1 and Ib2). Cluster Ia was formed by three of samples studied (1, 10 and 17, Scorr = 0.32; Figure 9.2), being dominated by β-eudesmol (11–30%). Cluster Ib1 included four samples (2, 9, 15 and 16, Scorr = 0.54), cedrol (7–40%) and *n*-tricosane (5–10%) being the main components. Cluster Ib2 comprised 16 samples (3–8, 11–14, 18–22 and 24), characterized by the dominance of *n*-tricosane (6–34%), *n*-heneicosane (2–28%) and *n*-pentacosane (2–17%). Clusters Ib1 and Ib2 showed some correlation (Scorr = 0.38) probably due to the relatively high content of *n*-tricosane in both groups of samples. Although not involved in the sampling as such, cluster II behaved as an outlier since it included just one sample, with *ar*-curcumene (32%) as the main component. To ascertain whether this extreme observation expressed a natural deviation, or a sample contamination with external elements, since it was obtained in a Morocco local market without information on provenance, requires further analysis. Although, in some cases, the

groups defined by agglomerative cluster analysis (Figure 9.2) showed some correlation with the data on dominant local flora (Appendix E,Table E.1), for example, with samples 1 and 17; samples 3, 4 and 5; or samples 15 and 16, other samples that shared similar local main flora were not included in the same cluster, such as samples 1 and 22. This indicates that other factors, in addition to the dominant local flora, may determine the final volatile composition of local propolis.

Table 9.1. Minimum and maximum percentage range of main components (≥1%) identified in the volatiles isolated by hydrodistillation from different propolis samples. For samples grouped on each of the clusters and subclusters, see Figure 9.1 and the full detailed composition in Table E.2. in Appendix E

Peak #	Components	RI	RI[a]	Propolis samples						
				Cluster I						Cluster II
				Ia		Ib1		Ib2		
				Min	Max	Min	Max	Min	Max	
3	α-Pinene	930	937	t	5.4	t	t	t	t	t
5	Camphene	938	938	t	1.2	t	t	t	t	t
8	β-Pinene	963	963	t	2.0	t	t	t	t	t
13	γ-Terpinene	1035	1035	t	1.2	t	t	t	t	t
17	*n*-Nonanal	1073	1073	t	1.0	t	1.9	t	11.3	t
20	*trans*-Pinocarveol	1106	1106	t	1.9		t	t	1.2	t
21	UI P2a	1107					6.0			
22	*cis*-Verbenol	1113	1122	t	0.1		1.2	t	2.1	t
23	UI P2b	1113					5.0			
24	*trans*-Verbenol	1114	1132	t	1.5	t	t	t	1.8	t
25	3-Methyl-1-indene	1114	1155	t	1.5	t	t	t	1.0	t
27	UI P2c	1122					3.6			
28	Borneol	1134	1137	t	1.2	t	0.3	t	0.6	t
29	Terpinen-4-ol	1148	1153	t	1.3	t	6.8	t	0.7	t
30	Octanoic acid	1149	1169	t	0.6	t	1.6	t	0.6	t
32	α-Terpineol	1159	1157	t	0.8	t	1.0	t	0.5	t
33	*n*-Decanal	1180	1183	t	2.7	t	4.7	t	13.0	t
37	2-*trans*-Decenal	1236	1266						5.4	
38	Nonanoic acid	1254	1279	t	0.6	t	t	t	7.0	t
39	*trans*-Anethole	1254	1288	t	t	t	3.4	t	1.8	t
40	Bornyl acetate	1265	1271	t	6.0	t	t	t	2.0	t
41	Carvacrol	1286	1299	t	t	t	0.9	t	2.2	t
42	2-*trans*-Undecenal	1323	1344						6.3	
43	α-Terpenyl acetate	1334	1349	t	0.9	t	2.1	t	t	t
45	β-Bourbonene	1379	1396	t	0.8	t	2.0	t	1.9	t
46	*n*-Dodecanal	1397	1410	t	t	t	4.8	t	5.1	t
48	Geranyl acetone	1434	1451	t	0.1	t	6.4	t	0.4	t
49	*ar*-Curcumene	1474	1473	t	0.9	t	7.2	t	16.6	31.8
50	UI A	1482		0.2	2.8	t	5.4	t	5.6	5.6
51	UI B	1483		0.2	2.5	t	2.1	t	4.7	5.5
52	UI C	1484		t	3.2	t	3.0	t	3.5	5.4
53	β-Bisabolene	1500	1506						t	10.5
54	*trans*-Calamenene	1505	1515	t	0.1	t	t	t	1.5	2.4
55	δ-Cadinene	1505	1519	t	0.1	t	t	t	1.4	t
57	*trans*-Nerolidol	1549	1550	t	0.8	t	0.8	t	4.1	t
58	Spathulenol	1551	1570	t	8.2	t	0.7	t	7.8	t
60	Cedrol	1574	1586	t	2.4	7.3	40.3	t	9.1	t
61	*epi*-Cedrol*	1579	1600				18.3			
62	UI P10	1605			14.8					
63	UI D	1606			6.4	t	3.5	t	6.4	3.7
64	γ-Eudesmol	1609	1626	4.4	14.4	1.1	5.8	t	3.5	5.1
65	T-Cadinol	1616	1630	t	2.0	t	t	t	1.5	t
66	β-Eudesmol	1620	1638	10.8	29.7	1.1	7.9	t	10.6	3.4
67	α-Eudesmol	1634	1643	2.0	20.9	t	4.5	t	9.1	5.7
68	α-Bisabolol	1656	1671	t	1.5	t	0.7	t	15.4	t
69	Bulnesol *	1656	1673				4.7			
70	*n*-Heptadecane	1700	1700	t	t	t	t	t	1.4	t
72	*n*-Nonadecane	1900	1900	t	0.2	t	2.4	t	13.5	t
73	Hexadecanoic acid	1908	1964	t	19.9	t	3.7	t	3.6	t

Peak #	Components	RI	RI[a]	Propolis samples						
				Cluster I						Cluster II
				Ia		Ib1		Ib2		
				Min	Max	Min	Max	Min	Max	
74	UI E	1908		t	1.9	t	2.1	t	1.8	t
75	UI F	1915		t	0.6	0.8	1.5	t	3.1	t
76	*n*-Eicosane	2000	2000	t	10.3	t	0.4	t	6.9	t
78	UI G	2050		t	2.3	4.1	17.5	t	11.8	t
79	UI H	2068							1.2	
80	UI I P13	2072							2.5	
81	*n*-Heneicosane	2100	2100	t	1.9	1.0	3.5	1.6	27.5	t
82	UI J P13	2112							13.9	
83	UI I Cl	2116	2116						1.4	
84	*n*-Docosane	2200	2200	t	0.8	t	1.7	t	8.8	t
85	*n*-Tricosane	2300	2300	t	3.2	4.5	10.4	6.1	33.5	t
86	*n*-Tetracosane	2400	2400	t	t	t	0.4	t	6.2	t
87	*n*-Pentacosane	2500	2500	t	1.4	t	3.3	2.1	17.4	t
88	*n*-Hexacosane	2600	2600	t	t	t	1.8	t	0.9	t
89	*n*-Heptacosane	2700	2700	t	0.7	t	1.3	t	10.3	t
90	*n*-Octacosane	2800	2800	t	t	t	t	t	1.0	t
91	*n*-Nonacosane	2900	2900	t	t	t	t	t	1.5	t

RI: In lab calculated Retention Index relative to C_9-C_{29} *n*-alkanes on the DB-1column. RI[a]: reported literature retention indices on DB-1 or similar phase column (100% Dimethylpolysiloxane) not from the authors lab[18]. t: trace (<0.05%). * Identification based on mass spectra only. UI: unidentified compound. Data on the main mass spectra fragments of these components is provided in the footnote of Supplementary Table E.2 and their fragmentation pattern in Supplementary Figure E.1.

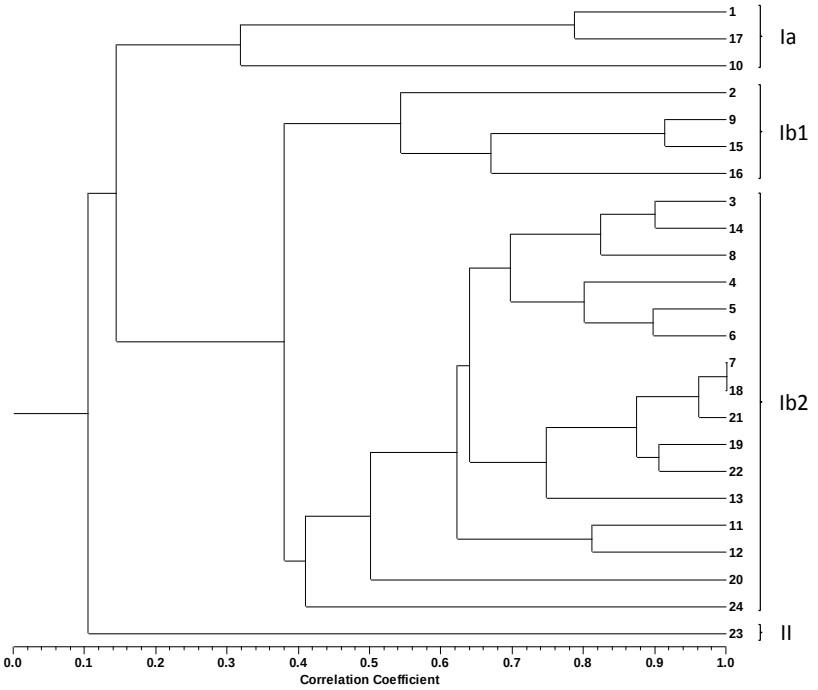


Figure 9.2: Dendrogram obtained by cluster analysis of the percentage composition of the volatiles isolated from the propolis samples evaluated, based on correlation and using unweighted pair group method with arithmetic average (UPGMA). For the origin of each propolis sample, see Figure 9.1 and Table E.1 in Appendix E.

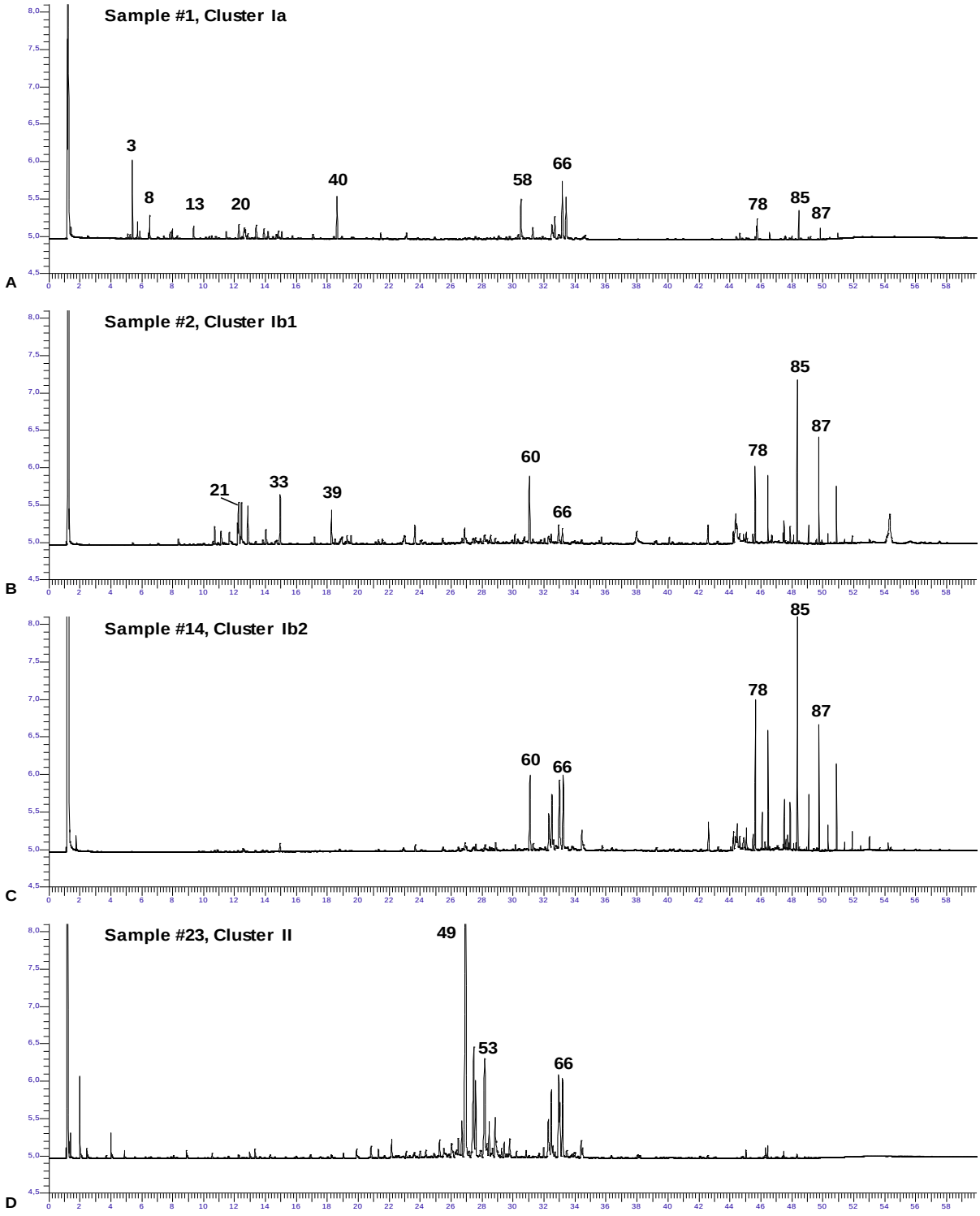


Figure 9.3: Representative gas chromatography profiles of the three groups defined by agglomerative cluster analysis based on the volatiles isolated from Moroccan propolis samples. Total run time is 61 min. For peak number correspondence, see Table 9.2 and Table E.2 in Appendix E..

The dominant flora in the Moroccan areas where the propolis samples were obtained shares some resemblances with that occurring in some countries from Southern Europe (Appendix E, Table E.1). Several of the most common constituents from European propolis

volatiles (references in 9), such as β-eudesmol, γ-eudesmol and α-bisabolol, among others, were also detected in the currently studied samples.

Studies regarding African propolis volatiles are scarce and limited to samples from Algeria, Canary Islands and Ethiopia (references in 9). Considering components with percentages ≥3%, only cedrol present in the volatile fraction of Algerian propolis was also detected in the volatile fraction of Moroccan propolis in this work. *trans*-Nerolidol, spathulenol, α-cadinene, isomers of cadinol or phellandrene, *trans*-calamenene and *cis*-verbenol were mono- and sesquiterpenes present either in some samples of this work or in those for Canarian Islands and Ethiopian origin (references in 9), in different percentages. In addition to other biotic and abiotic factors, these results confirm the importance of the flora surrounding the hives on the volatile composition of propolis.

9.4. Conclusion

The present work provides the first insights into Moroccan propolis concerning the chemical composition of its volatile fraction. Data showed that variations in propolis volatiles are mainly quantitative, suggesting that bees collected propolis from a similar botanical source. However, further research is needed to reveal the biological activity of the investigated volatiles, as well as their potential and contribution to the biological activity and medicinal application of propolis.

References

[1] S.U. Khan, S.I. Anjum, K. Rahman, M.J. Ansari, W.U. Khan, S. Kamal, B. Khattak, 'Muhamma da A and Khan HU, Honey: single food stuff comprises many drugs', Saudi J. Biol. Sci. 2018, 25, 320–325.

[2] A. Salatino, C.C. Fernandes-Silva, A.A. Righi, M.L.F. Salatino, *'Propolis research and the chemistry of plant products',* Nat. Prod. Rep. 2011, 28, 925–936.

[3] A.H. Banskota, Y. Tezuka, S.H. Kadoka, 'Recent progress in pharmacological research of propolis', Phytother. Res. 2001,15, 561–571.

[4] V. Bankova, M. Popova, B. Trusheva, 'Propolis volatile compounds: chemical diversity and biological activity: a review'. Chem. Cent. J. 2014, 8, 28.

[5] J.M. Sforcin, 'Biological properties and therapeutic applications of propolis', Phytother. Res. 2016, 30, 894–905.

[6] M.G. Miguel, S. Nunes, S.A. Dandlen, A.M. Cavaco, M.D. Antunes, 'Phenols, flavonoids and antioxidant activity of aqueous and methanolic extracts of propolis (*Apis mellifera* L.) from Algarve, South Portugal', Food. Sci.Technol. 2014a, 34, 16–23.

[7] M.G. Miguel, O. Doughmi, S. Aazza, D. Antunes, B. Lyoussi, 'Antioxidant, anti-inflammatory and acetylcholinesterase inhibitory activities of propolis from different regions of Morocco', Food. Sci. Biotechnol. 2014b, 23, 313–322.

[8] S. ElGuendouz, S. Aazza, B. Lyoussi, V. Bankova, J.P. Lourenço, A.M.R. Costa, J.F. Mariano, M.G. Miguel, M.L. Faleiro, 'Impact of biohybrid magnetite nanoparticles and Moroccan propolis

on adherence of methicillin resistant strains of *Staphylococcus Aureus'* Molecules. 2016a, 21, 1208.

[9] M.G. Miguel, A.C. Figueiredo, 'Propolis and geopropolis volatiles. In: Bee Products - Chemical and Biological Properties' J.M. Alvarez-Suarez, Edit., pp. 113–136, Springer International Publishing, Switzerland (2017).

[10] H.A. Mouse, M. Tilaoui, A. Jaafari, L.A. M'barek, R. Aboufatima, A. Chait, A. Zyad, 'Evaluatio n of the *in vitro* and in vivo anticancer properties of Moroccan propolis extracts', Rev. Bras. Farmacogn. 2012, 22, 558–567.

[11] M. Popova, B. Lyoussi, S. Aazza, D. Antunes, V. Bankova and G. Miguel, 'Antioxidant and α-glucosidase inhibitory properties and chemical profiles of Moroccan propolis' Nat. Prod. Comm. 2015, 10, 1961–1964.

[12] O. Abdelati, B. Bouchaib, N. Dari and H. Lahoucine, 'Antioxidant activities of propolis collected from different regions of Morocco', Int. J. Sci. Eng. Res. 2017, 8, 312–316.

[13] S. ElGuendouz, S. Aazz, B. Lyoussi, M.D. Antunes, M.L. Faleiro, M.G. Miguel, 'Anti-acetylcholinesterase, antidiabetic, anti-inflammatory, antityrosinase and antixanthine oxidase activities of Moroccan propolis', Int. J. Food. Sci. Technol. 2016b, 51, 1762–1773.

[14] S. El-Guendouz, N. Al-Waili, S. Aazza, Y. Elamine, S. Zizi, T. Al-Waili, A. Al-Waili, B. Lyoussi, 'Antioxidant and diuretic activity of co-administration of Capparis spinosa honey and propolis in comparison to furosemide', Asian. Pac. J. Trop. Med. 2017, 10, 974–980.

[15] Council of Europe, 'European Directorate for the quality of medicines' In: European Pharmacopoeia. 7th edn. pp. 241, European Directorate for the Quality of Medicines & HealthCare (EDQM) of the Council of Europe, Strasbourg, France (2010).

[16] J.F. Rohlf, *NTSYS-pc*, Numerical Taxonomy and Multivariate Analysis System, Version 2.1, User Guide. Applied Biostatistics, New York (2000).

[17] M.H. Pestana, J.N. Gageiro, 'Análise de dados para ciências sociais*'* A complementaridade do SPSS. Edições Sílabo, Lisboa (2000).

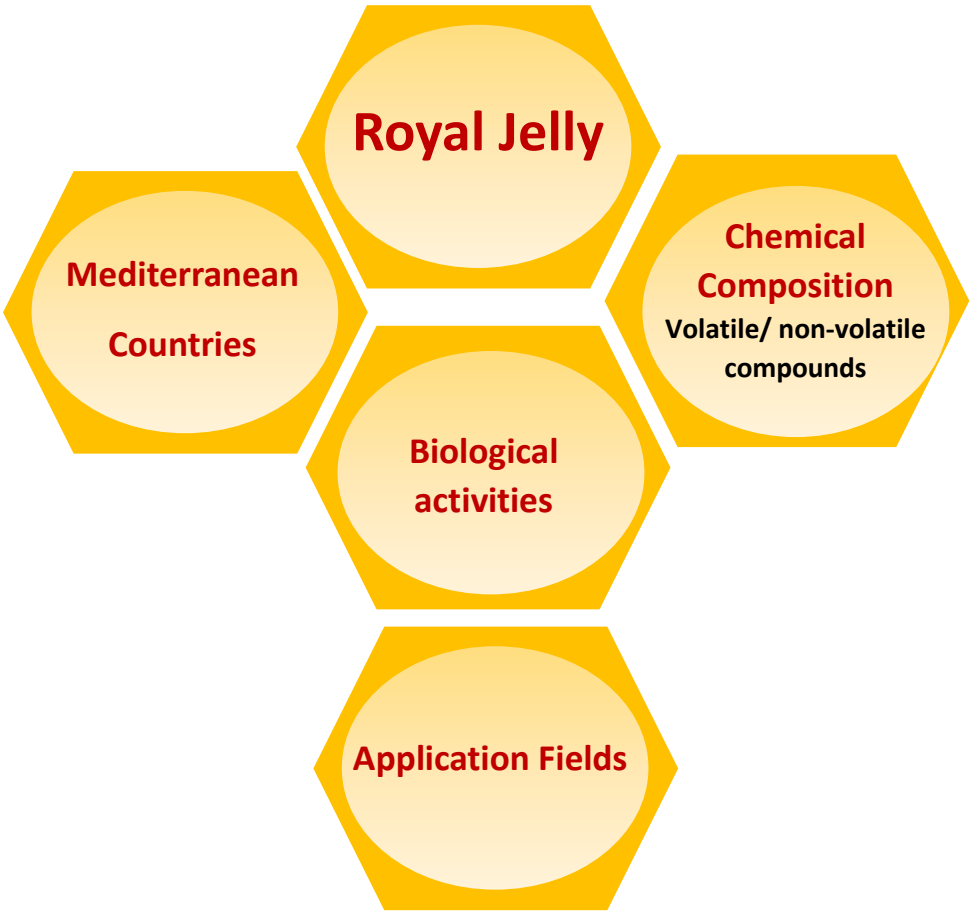
[18] P.J. Linstrom, W.G. Mallard, Eds., NIST Chemistry WebBook. NIST Standard Reference Database Number 69. National Institute of Standards and Technology, Gaithersburg MD. 20899, http://webbook.nist.gov (September 2018).

SECTION 2

Royal Jelly

Chapter 10

Insight in chemical composition and biological properties of Mediterranean Royal jelly



Insight in chemical composition and biological properties of Mediterranean Royal jelly*

Abstract

Royal jelly (RJ) is a secretion of the hypopharyngeal and mandibular glands of young *Apis mellifera* worker bees. It is privileged for the queen bee as exclusive nourishment for her entire life. This beehive product was used in traditional and folk medicine for human health care, this last has been subjected to several research highlighting their therapeutic properties and it was found that it exhibit a large spectrum of biological and pharmacological potential including antibacterial, antioxidant, anti-inflammatory, immunomodulatory and antitumor activities among others, which might be of high importance in modern medicine for the development of new drugs. Even if RJ is known since ancient times; research papers concerning its investigation are not this much developed as compared to the other beehive honey or propolis. In this review, we summarize our understanding so far, of data found in literature about the chemical composition and biological properties of RJ, in particular, that which originates from Mediterranean countries to stimulate future studies. Hence, more attention should be given to this natural product for better comprehension of its mechanism of action *in vitro* and *in vivo*, for possible standardization and achievement of new formulations.

*Adapted from 'Insight in chemical composition and biological properties of Mediterranean Royal jelly', Soukaïna El-Guendouz, Badiaa Lyoussi and Maria G. Miguel. Apicultural research. (*submitted*)

10.1. Introduction

Royal jelly (RJ) which is called 'bee milk' is a creamy-whitish fluid produced by the hypopharyngeal and mandibular glands, respectively, of *Apis mellifera* young newly emerged workers honeybees of 5–15 days old (called nurses). It is deposited in the bottom of each cell of the nest.[1] RJ is produced under partial digestion of essentially pollen and nectar.[1]

The hypopharyngeal gland is a pair of long glands coiled in the frontal part of the worker bee's head, dividing on oval saclike structure 'acini' attached to an axial collecting duct.[2] Mandibular glands consist of a pair of saclike glands, located on both sides of the head above the base of the mandible. Mandibular glands are present in queens, workers and also in drones but in very smaller size.[3] Both the hypopharyngeal and the mandibular glands of worker bees produce RJ but only the hypopharyngeal glands expresses proteins associated with major royal jelly proteins (MRJPs) and proteins such as carbohydrate metabolism and energy production. Hence, when the bees start flying out as foragers, those glands shrink and switch to the expression of enzymes involved in converting sucrose into its simple sugars such as a-glucosidase, glucose-oxidase, galactosidase, esterase, lipase, leucine aril amidase, and invertase; thus, the foragers are no longer able to produce RJ.[2-4]

Numerous studies have been dedicated to RJ in the last two decades by worldwide researchers. But few have been made with RJ from Mediterranean countries. The purpose of this review is to summarize available data of RJ from all countries surrounded by the Mediterranean Sea following the timescale of the research, compiling the chemical composition of the volatile and the nonvolatile part, biological properties and its different application,
in order to seek possible feature characterizing RJ from these areas. Further, showing the importance of the exploration of more researches by scientists to support its traditional uses,and possibly giving RJ a great industrial importance.

Its noteworthy that we will not provide a review covering the available literature about RJ from all over the world, instead, we are going to focus on the comparison of available data where the geographical origin of RJ is that of the Mediterranean countries, regarding its chemical composition of volatile and non-volatile part, biological properties and fields of application. To be noted also that papers reporting RJ with unknown geographic origin will not be included.

10.2. Royal jelly in history

The history of RJ dates back to ancient times; it has been known for centuries and has been used for numerous purposes in earlier civilizations. In ancient Egypt, RJ was mainly

consumed by the Pharaohs, it was considered as a symbol of nobility and power. Moreover, RJ was used for cosmetic intention, Cleopatra saw it as an important part of her beauty regimen, including both on her skin and as a consumable.[5] The first historical notes regarding royal jelly as a separate ingredient are from the ancient Greeks. Cassaignau[6] and Mraz[7] have reported, according to historians that RJ was consumed fresh together with inside honey of honeycombs, larvae, propolis and pollen. Moreover, Greeks believed that RJ was involved on the composition of the nectar "ambrosia" which granted immortality to the gods of Olympus. Aristotle, a Greek scientist, has studied the effect of RJ in queen bee, and reported its important function in the bees' society. He also associated RJ to the ability to an increase in physical strength as well as improvement of intellectual capacity.[8] It is noteworthy that honey and RJ were exclusively the breakfast of his school.[8,9] Traditional Chinese medicine sees RJ as a necessary ingredient. It was traditionally only found in sovereign gardens and was claimed to help ancient dynasties of China live longer and remain sexually potent even in old age.[11] Swammerdam, detailed on Biblia + Naturae the development cycle of the bee, declaring that the alleged "King" is actually be a queen, the drones are male and the workers are "neutral".[11] On 1672, it was discovered a kind of whitish 'porridge' in the bottom of the cells and states that the larvae are nourished by a substance other than honey without knowing their origin.[12] Later on, in 1734, Réaumur declares that this whitish 'porridge' comes from the secretion of bee workers, and coined the term "Royal Jelly" to name this substance, since it transforms the female bee larvae into a queen.[9,10] Levet[14] and Domerego[8] have reported that Langstroth, the oldest American beekeeper, was the first who did a chemical analysis of RJ but without scientific value.[14]

10.3. Organoleptic description and physical characteristics of RJ

The sensory characteristics are an important quality criteria for RJ. Naturally, it has a heterogenous texture due to the presence of undissolved granules of different sizes, composed mainly of pollen grains derived from the foraging activity of honeybees.[14] RJ is characterized with a whitish color which tends to yellow upon storage.[1] It has a distinguishable sharp odor and its taste being sour and sweet, partially soluble in water with a high buffering capacity ranging between (pH 3.4–4.5) and density of 1.1 g/mL.[14] RJ is also characterized by its gelatinous consistency; its viscosity is depends on its water content and freshness. When it is inappropriately stored, it slowly becomes more viscous, that it is why it should be kept in a frozen state for optimum quality. The increased viscosity appears when RJ is stored at room temperature or refrigerator at 5 °C. This, may be due to the increase in water insoluble nitrogenous compounds associated with a reduction in soluble nitrogen and free amino acids,

as a result of the continued enzymatic activities and interaction between the lipid and protein fractions.[3]

10.4. Mediterranean RJ

Numerous studies have been dedicated in the last two decades to RJ from worldwide researchers. But few studies were made with RJ from Mediterranean countries. In this part, similar to propolis part, we are summarizing available data of RJ from all countries surrounded by the Mediterranean Sea following the timescale of the researches, compiling the chemical composition of volatile and nonvolatile part, biological properties and its different application, in order to seek possible feature characterizing RJ from this areas, as well as showing the importance of the exploration of more researches by scientists to support its traditional uses, thing which can gives RJ a great industrial importance.

10.5. Chemistry of Mediterranean RJ

RJ is highly nutritious, rich in proteins, amino acids and various vitamins. Its composition is quite complex and rich in bioactive components. It is a product with a high-water content, it presents 60 to 70% of the total RJ composition, followed by carbohydrates which are present in RJ at the range of 11–23%, then proteins (9–18%), lipids (4–8%), minerals (0.8–3%), vitamins, phenols and amino acids.[15] The composition of RJ is dependent on several factors including seasonal and regional conditions of feeding, metabolites and changes in the physiology of nurse bees, larval age, the genetic of bees and their race.[12] It is noteworthy that quality and RJ composition is also highly dependent on the storage conditions postharvest.

Even if RJ is well known and has been used since ancient's time, its chemical composition remains less developed, especially in the Mediterranean countries where the scientific reports about the RJ composition are scarce or even absent. Thus, having a high potential of utilization, a detailed knowledge and exploration on the RJ chemical composition is of major importance.

On the basis of scientific publication output found on the search engines "web of science", "PubMed", "Scopus" and "Google Scholar", regarding the chemical composition of RJ originating from Mediterranean countries, only 62 scientific reports belonging to 8 countries have been found including France, Spain, Italy, Croatia, Greece, Turkey Egypt and Algeria. Figure 10.1 shows the contribution of each country cited above on the exploration of RJ. From the Figure 10.1, it is clear that very few researches have been done with RJ originating from Mediterranean countries, where Italy, France, Greece and Turkey published 35.7%, 21.4%, 21.4% and 17.8%, respectively of total research found, while Spain has only contributed with 3.6% (Figure 10.1).

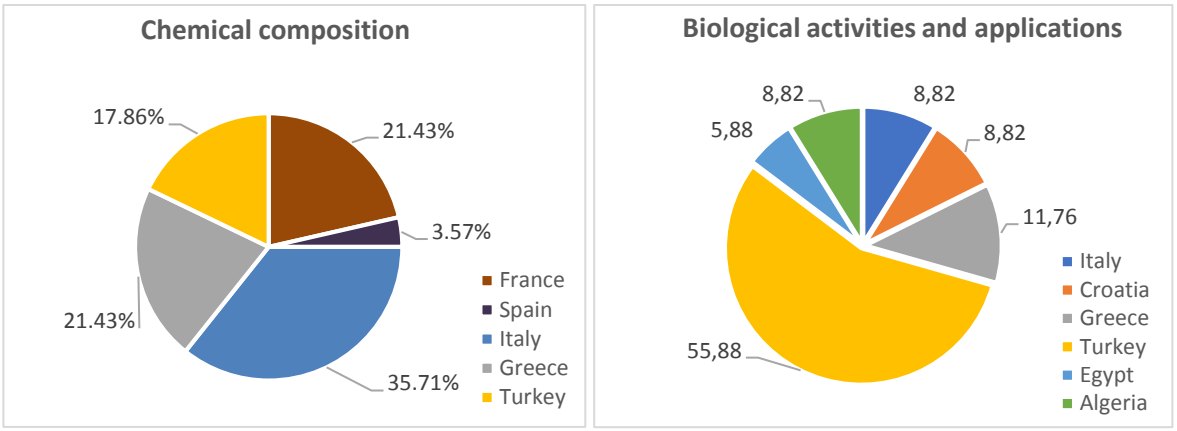


Figure 10.1. Graphical presentation of available scientific publication output on web of science, PubMed, Scopus and Google Scholar for RJ originating from Mediterranean countries in regards of its chemical composition, biological activities and application represented by their percentage share amongst 8 countries with a total 62 papers.

10.5.1. Non-volatile constituent of Mediterranean RJ

Even if RJ is well known and used since ancient's time, its chemical composition remains less developed, especially in the Mediterranean countries where the scientific reports about the RJ composition are scarce of even absent. Thus, having a high potential of utilization, a detailed knowledge and exploration on the RJ chemical composition is of major importance.

10.5.1.1. Water content

RJ reveals noticeable microbial stability in spite of the water content being generally higher than 60% and possessing an activity (a_w) above 0.92[13] The presence of a potent antibacterial protein (royalisin) in RJ may prevent microbial attacks, according to[14] More recently, Fontana et al.[15] have isolated short peptides from RJ (jelleines) possessing a high activity against Gram-positive, Gram-negative and yeasts.

In samples of RJ originating from France, the water content depended on the bee feeding: mean value of 65.3% in the absence of artificial bee feeding, and 66.3-66.7% in RJ obtained by feeding bees with honey or diluted sugars from sugarcane, starch hydrolysates or inverted beet sucrose[16] although in both cases they were within the range generally reported (60 to 70%)[16] However, some RJ samples showed lower water percentages (53.8% in Italian RJ, and < 50% in Greek RJ), but the majority of the reported samples in those countries[17,18] showed a water content values in concordance with the limits proposed by the International Honey Commission (IHC).[18] Likewise, in a study done by Karaali, Meydanoğlu, & Eke[19]; where they compared RJ samples from Italy and Turkey regarding moisture content; they found a water content of 61.60% and 65.32 %, respectively. Another study regarding moisture content of fresh royal

jelly samples origination from Anatolia, Turkey, revealed a mean water content of 66.8%, with 61.6% as a minimum and a maximum of 73.0%[20]

10.5.1.2. Sugar Composition

Carbohydrates represent about 30% of dry matter in RJ.[13] RJ carbohydrates consist essentially of the monosaccharides fructose and glucose, which are relatively constant and with a distribution that is quite similar to that of honey. In general, the amount of fructose and glucose represents 90% of the total sugars contained in RJ which make them the most abundant sugar in RJ.[21] In French RJ, some authors[16,22] stated that sugars determination could provide important information about the RJ's quality, including the detection of possible adulteration with honey or sugars. Indeed, they demonstrated that when bees were fed artificial food (not honey and pollen) maltose, maltotriose, sucrose and erlose contents in RJs increased up to 5.0%, 1.3 %, 4.0% and 1.3%, respectively, while in authentic samples those amounts should be <0.2%, not detectable, 0.2% 139 and 0.2%, respectively[16,22] However, in any case fructose and glucose were always the major carbohydrates. In RJ samples from Italy, France, Greece or Turkey, independent on the research team, glucose and fructose also dominated the carbohydrate fraction, although in diverse percentages. For example, Daniele & Casabianca[23] (2012) reported that fructose and glucose contents ranged between 2.3–7.8% and 3.4–7.7%, respectively, in Italian and French RJ samples, or 2.3%-145 6.9% and 3.7%-8.2%, respectively, in a specific region of Italy (Roma).[24] Maltose, maltotriose and gluconic acid, a product derived from glucose oxidation, were also presents in RJ samples from Italy and France.[23] Karaali et al.[19] compared RJ samples from Italy and Turkey and found a similarity in total sugar contents (11.20 % and 10.45%, respectively).

In Greece, RJ samples were analyzed for their sugar content and the authors[18] found that total sugar content ranged between 7.2% and 16.7%, where fructose (2.1%-7.3%) and glucose (2.4%-6.9%) dominated, followed by sucrose (0.0%-9.3%). Kolayli et al.[20] reported that in lyophilized RJ samples collected in Anatolia, Turkey, the most abundant monosaccharides were fructose and glucose with a mean of 6.9% and 4.95% respectively. Although disaccharides (sucrose, melezitose, maltose, lactose, trehalose) and trisaccarides (melibiose) have also been detected with an average of 4.3% and 0.5%, respectively. The other most abundant sugars found in Turkish RJ were melezitose and maltose but often in highly variable concentrations, while, lactose was detected in only three samples with a concentration ranging between 0.8% and 1.4%. Melibiose was detected in seven of the fifteen RJ samples, ranging from 0.1% to 1.0%.

It was argued that the presence of lactose, an important milk sugar, indicates possible contamination of the RJ samples by adulteration of RJ with milk or milk products.[20]

10.5.1.3. Protein, peptides and amino acids contents

Proteins represent more than 50% of the total RJ dry weight including minor royal jelly proteins and major RJ proteins (MRJPs). Eighty percent of RJ proteins are MRJPs, so called since it is, considered as playing specific physiological roles in queen honeybee development and include numerous essential amino acids, similar to ovalbumin and casein. MRJPs are the most of water-soluble proteins in RJ (31%). The MRJP family includes nine members which are encoded by nine different genes (MRJP1, MRJP2, MRJP3, MRJP4, MRJP5, MRJP6, MRJP7, MRJP8 and MRJP9) with molecular masses of 49–87 kDa.[1] It was reported that MRJPs are synthesized in the cephalic glands of the honey bee.[25] Later on, Xue et al.[21] characterized MRJP1 to MRJP8 by the cloning and sequencing of their respective cDNAs (complementary DNA). MRJPs represent the main intake of essential amino acids for the growth of the queen bee and larvae.[3] The most essential amino acids found in MRJPs are mainly arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine. Those amino acids are present in MRJP1 with a percentage of 48%, in MRJP2 with 47%, in MRJP3 with 39.3%. In addition, it was reported the predominance of arginine and lysine in MRJP4 with a percentage of 44.5% and in the MRJP5 with 51.4%. The arginine and methionine are represented as the predominant amino acids in the MRJP6 with a percentage of 42%, in the MRJP7 with 48.3%, in the MRJP8 with 49.5% and in the MRJP9 with 47.3%. While in the MRJP1, MRJP2 and MRJP4, leucine and valine are the predominant amino acids, leucine is the most abundant in the MRJP6, MRJP 7 and MRJP8.[3] It is noteworthy that peptides from RJ give it several multifunctional properties notably the antibacterial effect. The main peptides found in RJ are royalisin, jelleines, and royalactin.[26]

Only few studies were done concerning RJ composition in Mediterranean countries. In RJ samples from Bologna, and Campobasso, Italy, the protein content was in the range of 12.07% and 13.8 %.[27] In this study, the authors also intended to find a suitable marker for assessing the freshness of RJ and furosine (a product of Maillard's reaction) content was determined. The results showed that the concentration of this compound could increase significantly after 10 months of storage at room temperature or increased to a much lower level when the RJ was stored at 4 ºC. Other research regarding the protein identification of Italian RJ allowed the detection of two apalbumin 2 isoforms and several fragments of apalbumin 1 and

apalbumin 3. According to the authors[28] the presence of these fragments could be attributed to protease activity other than that of serine protease.

In the same work, Wytrychowski et al.[16] found that French RJ had a minimal and maximal protein content of 11.4 and 16.9%, respectively, with a mean protein content of 13.9%.[16] While, authentic Italian RJ showed a protein content of a minimal and maximal raging between 13.5% and 16.5% with an average of 15.0%. Lipophilic and hydrophilic fractions of each RJ originating from Italy was quantified.[29] found that all the RJs contained a greater amount of hydrosoluble proteins than liposoluble ones. The hydrosoluble protein content ranged between 0.831 and 0.967 µg/mg while the liposoluble protein content ranged between 0.207 and 0.396 µg/mg. Moreover, the hydrophilic protein profile of all the RJs was identified, in particular, MRJP1, MRJP2, MRJP3, their isoforms and their fragments. Gismondi et al. [29] identified the MRJP5 and its isoform and fragment in the lipophilic RJ portions. In addition, proteins in Italian RJ from three strains of honey bees were identified.[30] In this research, 57 most abundant proteins among a total of 157 proteins were detected in the studied RJs, of which most were assigned to the MRJPs with a particular heterogeneity of the MRJP3. Besides, glucose oxidase, 1-peroxiredoxin and 1-glutathione S-transferase (GST) S1 were also identified.[30] Regarding Greek RJ, Kanelis et al.[18] reported an average of 13.6% of protein content in 176 RJ samples from 34 major beekeeping areas of Greece. More recently, Pina et al.[31] did a targeted profiling of hydrophilic constituents of Greek RJ by hydrophilic interaction liquid chromatography–tandem mass spectrometry (LC-MS). This method of analysis leads to a detection of 64 out of 88 monitored analytes. In general, RJ hydrophilic bioactive components revealed the presence of lysine, ribose, proline, melezitose and glutamic acid to be in high abundance.[31] Moreover, Pina et al.[31] in their study, reported the presence of 18 hydrophilic nutrients, for the first time, as RJ constituents. The new reported RJ constituents included the biogenic amines trimethylamine, dimethylamine, trimethylamine-N-oxide, putrescine, cadaverine, spermine and spermidine, also choline, betaine and histamine, acetylcarnitine, pyroglutamic acid, creatine, creatinine and homoserine.

In Turkey, research done with Anatolian RJ samples revealed a minimal total protein value of 11.4% and a maximal value of 15.7% with a mean protein content of 14.1%.[20] Karaca et al.[32] demonstrated that a RJ sample from a local natural food store, Istanbul, Turkey, has lysine (62.4 mg/100 g) and proline (58.76 mg/100 g) as a main amino acid.

10.5.1.4. Lipid content

The lipid fraction of RJ is considered the most important components of RJ, even if it is present in a relatively low and variable concentrations that range between 8 and 19% of dry matter.[33] Fatty acids in RJ are known with a broad spectrum of medicinal and therapeutic properties associated with RJ including anticancer properties immunomodulatory, antimicrobial activities as well as the capacity to decrease cholesterol levels and the stimulation of glial cells and non-neuronal cells which produce the myelin insulation that coats healthy neurons.[26,34] This lipid fraction is predominated by organic acids (80-90%); most of them are free mono and dihydroxy acids and dicarboxylic acids containing 8–12 carbon atoms, with a rather unusual structure rarely encountered in nature unlike organic acids of most animal and plant materials which are mainly in the tri, di, and monoglyceride forms with minor amounts of free fatty acids.[13] RJ lipid fraction contains also phenols (4–10%), waxes (5–6%), steroids (3–4%) and phospholipids (0.4–0.8%).[35]

It was reported that RJ fatty acids consist mainly of monocarboxylic acids trans-10-hydroxy-2-decenoic acid (HDEA or 10-HDA) (32%), gluconic acid (24%) and 10-hydroxydecanoic acid (HDAA) (22%) representing more than 60% of the total amount of organic acids found in RJ.[21] Dicarboxylic acids (5%) besides several other acids are also present in RJ.[35] Likewise, MRJPs, 10-HDA is the major fatty acid in RJ being reported in RJ only, and to date never reported to be present in other bee products or even other natural products, thus, the 10-HDA content in RJ has been adopted as a criterion of the genuineness and quality of the RJ. RJs with 10-HDA content greater than 1.8% are considered as fresh and authentic samples.[33,36] 10-HDA has also been shown to have an important biological role in the development of the colony strategies.[12]

In the Mediterranean area, the lipid profile of RJ and its quantification have also been performed using various techniques. This approach had as ojective to find the best way to evaluate the lipid profile and quality of RJ samples. In general, when a chemical analysis of fatty acids content in RJ is required, only the quantification of 10-HDA in RJ samples is done, because it is the specific and major RJ fatty acid. Thus, Turkish researchers[37] who evaluated the 10-HDA content of Turkish RJ, reported that the levels of 10-HDA in Turkish RJ were 0.75 - 2.54%, while Kolayli et al.[20] described values of 10-HDA ranging from 1.0% to 3.9%.

In a study carried out with French RJ, Antinelli et al.[36] investigated the 10-HDA as a freshness parameter for RJ during storage time, and found that during 12 months storage, the 10-HDA loss rate was 0.1 and 0.2% at -18 and 4°C, respectively, while at room temperature storage, the loss rates were 0.4%. Wytrychowski et al. [16] assessed the 10-HDA content of

French RJ trough HPLC analysis and have reported a mean of 2.3%, (1.4%<10-HDA<3.7%). Ferioli, Marcazzan, Caboni,[38] investigated Italian RJs through Capillary Zone Electrophoresis (CZE) method for the determination of 10-HDA content and revealed that the 10-HDA content in RJ samples ranged between 0.8 and 3.2 g/100 g of RJ, and declared that CZE study gave comparable performances to HPLC in terms of analytical results, efficiency, sensitivity and analysis time.[38]

Gas chromatography analyses of the lipid fraction of Italian RJ, were performed to determine fatty acid profiles and quantify major phytosterols. Thus, lipid amounts ranged from 2.3 and 7.2 % of the fresh product in RJ, in which fourteen fatty acids and three phytosterols were identified. In addition, almost 70 % of the total fatty acids consisted of (E)-10-hydroxy-2-decenoic and 10-hydroxydecanoic acid thing which concord with the previous reported data.[39]

Melliou & Chinou[33] reported twenty-five compounds identified from the dichloromethane and methanol extracts of RJ from Greece, the most abundant were 10-hydroxy-2-decenoic acid, 10-hydroxydecanoic acid, and sebacic acid, while 7 of these were isolated for the first time as natural products: 10- acetoxydecanoic acid, trans-10-acetoxydec-2-enoic acid, 11-oxododecanoic acid, (11-S)- hydroxydodecanoic acid, (10R,11R)-dihydroxydodecanoic acid, 3,11-dihydroxydodecanoic acid, and (11-S),12-dihydroxydodecanoic acid.[33] Pyrzanowska et al.[40] reported that an analyzed sample of Greek RJ appeared very rich in fatty acids, mainly 10-hydroxy-2-decenoic acid with a percentage of 42.32%, followed by 10-hydroxydecanoic acid representing 19.56% of total fatty acids found and sebacic acid with 5%, besides 8- hydroxyoctanoic acid (6.16%), 3-hydroxydecanoic acid (2.17%) and 3,10-dihydroxydecanoic acid (4.59%). In another study, the same abundant fatty acids were found in Greek RJ as described by Pyrzanowska et al..[40] Lambrinoudaki et al.[41] noted that the chemical analysis of Greek-origin RJ disclosed a richness in medium chain fatty acids, compounds with hypolipidemic properties, which are presented particularly by 10-hydroxydecanoic acid and 10-hydroxy-2-decenoic acid with a value comprising 63% of the dry weight fatty content. 3,10-Dihydroxydecanoic acid (5.42%), sebacic acid (3.38%) and 8-hydroxyoctanoic acid (4.69%) were present in the studied sample, as well as some minor fatty acids.[40,42]

10.5.1.5. Minerals, vitamins, phenols and other components

Different other compounds with minor amounts and belonging to varied chemical classes, have been reported to be present in RJ such as trace and mineral elements, vitamins, phenolic compounds and nucleotides.[43] RJ composition reveals the presence of minerals, ash content

represents 0.8–3% of RJ fresh matter, while it represents 2-5 % of RJ dry matter.[14] The most abundant elements are, in descending order: potassium (K), calcium (Ca), sodium (Na), magnesium (Mg), zinc (Zn), iron (Fe), copper (Cu), manganese (Mn). Besides, it is possible to find a trace elements in RJ such as aluminium (Al), barium (Ba), strontium (Sr), bismuth (Bi), cadmium (Cd), mercury (Hg), plomb (Pb), tin (Sn), tellurium (Te), thallium (Tl), wolfram (W), antimony (Sb), chromium (Cr), nickel (Ni), titanium (Ti), titanium (V), cobalt (Co), molybdenum (Mo).[1] RJ trace and mineral elements are not quantitatively stable, depending not only on biological factors of bees, but also on factors outside the collony, the environment, procurement of food and production period.[34] It was disclosed that trace elements possess a key role in the therapeutic properties of RJ, since they exhibit a multitude of known and unknown biological functions.[44]

In France, concentrations of Bi, Cd, Hg, Pb, Sn, Te, Tl, W, Sb, Cr, Mn, Ni, Ti, V, Co and Mo in royal jelly were determined by double focusing magnetic sector field inductively coupled plasma mass spectrometry (SF-ICP-MS). The most abundant mineral elements in royal jelly samples were K, Na, Mg, Ca, P, and S. While, Zn, Fe, Cu, Al and Mn were the abundant trace elements.[45] Stocker et al.[45] conclude that the concentrations of trace and mineral elements were highly constant in the associated RJ samples. With regards to French RJ, other authors, have found that studied RJ samples showed Ca, P, Mg, Zn and Mn as the most abundant minerals.[46]

In addition, RJ also contains some vitamins, but only water-soluble ones, liposoluble vitamins such as vitamins A, D, E and K are absent.[12] Mainly, RJ is very rich in B group vitamins predominantly pantothenic acid (B5), whereas, thiamine (B1), riboflavin (B2), niacin (B3), pyridoxal (B6), biotin (B8), folic acid (B9) and cobalamin (B12) are present in lower quantities, and niacin (B3) and ascorbic acid (vitamin C) are present only in trace amounts.[43] As the variation in the concentration of oligo and mineral element in RJ, vitamin content is subjected to seasonal changes depending on the diversification of the source of pollen by worker bees, then the principale source of vitamins are pollen flowers.[12]

Other class of chemical compounds present in RJ are phenolic compounds or polyphenols, which are one of the most important group of compounds since they exhibit numerous biological and therapeutic properties.[47] The presence of phenols in RJ was reported only recently by several authors without providing a phenolic profile. However, biological properties of RJ are mainly attributed to bioactive fatty acids, proteins and phenolic compounds.[44] Previously, Guo, Kouzuma, &Yonekura[48] described the key role of tyrosyl residues of amino acids, which contain a phenolic group, in the antioxidant activity of RJ.

In Turkey, the total phenol contents of RJ were quantified through *in vitro* assay,[49] and the results showed that RJ contain a total phenol content of 59.16±1.94 mg GAE/100 g (gallic acid equivalent). In another study, nine Spanish RJ samples were subjected to fast analysis of polyphenols using automated TurboFlow™ liquid chromatography-Orbitrap high resolution mass spectrometry.[50] Only one ferulic acid was reported to be present in studied samples, with an amount ranging between 12,946 µg/kg and 18,936 µg/kg. While, Spanish RJ was reported to be flavonoids-rich according to the results of López-Gutiérrez et al.,[50] including acacetin (18-250 µg/kg), isorhamnetin-3-O-glucoside [not detected (nd)– 3,445 µg/kg], kaempferol-3-O-glucoside (nd- 2,329 µg/kg), formononetin (nd- 433 µg/kg), genistein (nd- 699 µg/kg) and apigenin (nd- 112 µg/kg). The results of this study were compared with those reported in the literature, and showed a difference in the detected compounds: the common flavonoids found were apigenin, acacetin and chrysin, while quercetin, kaempferol, galangin, pinocembrin or hesperidin were absent in the studied samples. Such results can be explained by the botanical origin of the RJ samples, the climate or the humidity which may affect the type and content of RJ polyphenols.[50] As a conclusion, it was mentioned that the results found showed a lack of homogeneity in this type of matrices and explains nonetheless the low numbers of studies about the composition of polyphenols in RJ.

In RJ it was also possible to find low quantitie of nucleotides as free bases (adenosine, guanosine, cytidine, iridin), phosphates (5'-monophosphate (AMP), adenosine 5'-diphosphate (ADP), and adenosine 5'-triphosphate (ATP)), acetylcholine and gluconic, benzoic, malic, citric, and lactic acids; and their origin is assumed to arise from the nurse bee.[12,22] Adenosine was frequently detected in RJ, it is known naturally as a purine nucleoside; composed of a molecule of adenine attached to a ribose sugar molecule resulting from the breakdown of adenosine triphosphate (ATP), which is the primary cellular energy source for transport systems and enzymatic activities of proteins *in vivo*; its real function and the role of the other minor components is still unclear.[12,22]

In general, and considering the review of the chemical composition of Mediterranean RJ above, we can state that the chemical composition of Mediterranean RJ showed a similarity to RJ originating from other countries.

10.5.2. Volatile constituent of Mediterranean RJ

In general, international investigation on RJ volatile is scarce. Even if they are found in very low concentrations, they bear precious information about RJ origin, quality and even freshness, although the RJ volatile fraction has not been investigated sufficiently studied up to now.[1] Similar to the other bee products, it was reported that, several factors can be responsible

for differences in the general composition of the volatile compounds of RJ, such us honeybee species, harvesting time, regions, storage conditions and extraction methods.[51] Predominantly, we can find on the volatile fractions of RJ different classes of compounds such us carboxylic acids (2- methylbutanoic acid, 3-methylbutanoic acid, pentanoic acid, 2-hexenoic acid…), phenols (hydroquinone), carbonyl compounds (particularly ketones), esters, aldehydes and alcohols.[52-54] In 2009, Nazzi et al.[54] analyzed diethyl ether extract of RJ originating from north eastern Italy, through chromatography coupled to mass spectrometry (GC-MS), to determine the volatile compounds. Twenty-six compounds were identified predominated with carboxylic acids, which are twenty-three aliphatic carboxylic acids and one aromatic carboxylic acid, the two other compounds were hydroquinone and methyl-4-hydroxybenzoate, while, two other compounds were not identified. Among the compounds identified in Italian RJ, seven had already reported as being present in RJ previously.[52] The new identified compounds were 2-hydroxyhexanoic acid, phenylacetic acid, pentanoic acid, 2-methylbutanoic acid, 2-hydroxy-4-methylpentanoic acid, 2- hexenoicacid, 3- methyl butanoic acid, hydroquinone and methyl-4-hydroxybenzoate.[55]

10.6. Royal Jelly: Biological properties, health benefits and fields of uses

RJ is receiving a special attention due to its high content of bioactive compounds which make it an attractive ingredient for several application. This fascinating bee product has been shown to exhibit numerous biological activities including antimicrobial,[56] antioxidant,[57] anti-inflammatory,[58] anti-tumoral,[59] vasodilative,[60] hypotensive,[61] hypocholesterolemiant, anti-allergic, antiaging and hepatoprotective,[62] among many others. This chapter will examine the current available literature on the biological properties, health benefits and fields of uses of RJ with known origin from countries boarding on the Mediterranean Sea. From the graphical presentation of available scientific publication output of RJ originating from Mediterranean countries in regards of its biological activities and applications (Figure 1), it is clear that there are some countries where the RJ research area is more developed than others, obviously from the number of papers in relation with RJ found, notably Turkey which is the leader in the exploration of the biological activities of RJ as a country boarding the Mediterranean, with more than 50% of the available research. The other countries (Italy, Croatia, Greece, Egypt and Algeria) shared the remaining percentage which ranged between 12.12% for Algeria and 413 6.06% for Egypt (Figure 10. 1).

Moreover, from Table 10.1, it is obvious that most RJ applications are in the field of 415 medicine and related bio-allied sciences. Thus, this part will be divided according to the 416 biological and therapeutic activity of RJ.

10.6.1. Antioxidant activity

The generation of cellular Reactive Oxygen Species (ROS) is a natural process occurring in human body, which is controlled through several mechanisms. In proper concentrations, ROS act as a defense against infectious agents, as well as the involvement in some cellular signaling systems. But, due to many environmental, lifestyle, and pathological situations, ROS could be produced in excess, leading to oxidative damage of the cell structure and biomolecules such as proteins and DNA, and consequently the development of chronic and degenerative illnesses including cancer, arteriosclerosis, arthritis, and neurodegenerative diseases, among others.[63] Therefore, scientific investigations has shown that foods containing natural antioxidant such as bee hive products are involved in the protection from the damage caused by the overabundance of ROS in the human organism.[64] Thereby, special attention has been given to the use of bee hive product such as honey, propolis, pollen and RJ as source of natural antioxidants, however scientific reports concerning RJ are less developed than other bee hive products.

With the exception of the work of Buratti, Benedetti, Cosio, [65] that evaluated the antioxidant activities of RJ from Italy comparing them with those from China, the works were from Turkish samples from different regions and evaluated by various research teams (Table 10.1). The *in vitro* antioxidant activity was evaluated using DPPH (2,2-Diphenyl-1-picrylhydrazyl radical scavenging activity, ferric reducing antioxidant capacity, total antioxidant activity, through the phosphomolybdenum method, and amperometric flow injection analysis (Table 10.1). Generally, the activities found were not different from other RJ samples from across the world.[20] In addition, the activities of RJ samples were generally lower than the remaining bee products, while the mixture of 'royal jelly– honey–pollen' had the greatest antioxidant power.[20] Such results may result from the relative total phenol content (TPC) and total flavonoid content (TFC) of RJ,[20,49,66] although other constituents also present in RJ contribute to the activities (vitamins, short peptides, short-chain hydroxyl and carboxylic fatty acids).[20]

The *in vivo* antioxidant activity of Turkish RJ samples was studied through the evaluation of their hepatoprotective activities against carbon tetrachloride (CCl_4) induced acute liver damage in rats.[66-68] The protective effect was achieved through the decreasing of the lipid peroxidation and improving endogenous antioxidant defense systems, along with reducing the

elevated levels of liver enzymes.[67] Moreover, Kanbur, Eraslan, Silici, & Karabacak[69] after submitting mice to high doses of fluoride, they observed adverse effects that could be reversed through the decrease of the erythrocyte and liver tissue malondialdeyde (MDA) levels, and increase of the erythrocyte and liver tissue superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) activities. According to the authors,[69] such results might demonstrate the beneficial antioxidant activity of RJ given to animals. Similar results were observed by Karadeniz et al.[70] and Silici, Ekmekcioglu, Kanbur, & Deniz,[71] when the oxidative stress was caused by cisplatin (CDDP) that damaged the kidneys and liver ofmice. Also, Cihan[72] studied the protective effect of RJ treatment at different concentrations on oxidative damage in liver and lung tissue after total body irradiation of adult male Sprague Dawley rats. This RJ treatment leads to a significant decrease in oxidative stress and biochemical parameters (decrease of MDA level, and increase of GSH-Px, CAT and SOD activities).[72] (Cihan, 2013). In a similar study focused on highlighting the antioxidative activity of RJ, Meltem et al,[73] showed that RJ treatment has a therapeutic effect on breast cancer in Sprague Dawley rats, when given together with a chemotherapeutic agent used usually in treatment, but it was inadequate and insufficient to reduce the oxidative stress caused by the breast cancer.

Additionally, but in rabbits, Aslan et al,[74] studied the potential neuroprotective effect of RJ against secondary injury after experimental SCI (spinal cord injury). A significant prevention of lipid peroxidation was observed as well as an augmentation of endogenous enzymatic or non-enzymatic antioxidative defense systems after treatment with RJ as compared with the control healthy animals.[74] The *in vivo* studies, either in mice, rats or rabbits showed that RJ samples were able to prevent lipid peroxidation, decreasing the MDA formation, or increasing the activity of some antioxidant enzymes (CAT, SOD, GSH-Px) after action of diverse types of pernicious factors (carbon tetrachloride, fluoride, cisplatin, N-methyl-Nnitrosourea or even irradiation).

10.6.2. Antimicrobial and anti-parasitic properties

The bacterial resistance against antibacterial agents is considered as one of the most urgent threats to the public health because the cure for bacterial infection is being increasingly complicated. This resistance can be a source of various illnesses that were once easily treatable with antimicrobial agents and have now become untreatable, generating dangerous infections, serious disability or even death. Thus, considering this evidence, a minor and more correct use

of antibacterials and antifungals is mandatory to improve infection control, as well as the research of new and natural source of therapeutic compounds.[75]

Numerous studies previously done on RJ showed that it possesses a wide range of medical activities particularly antimicrobial which has been demonstrated by several authors. Melliou & Chinou[33] investigated the antibacterial and antifungal capacity of Greek RJ and the isolated compounds against four Gram-positive bacteria, *Staphylococcus aureus* (ATCC 25923) and *Staphylococcus epidermidis* (ATCC 12228), *Streptococcus mutans, Streptococcus Viridans*; four Gram-negative bacteria, *Escherichia coli* (ATCC 25922), *Enterobacter cloacae* (ATCC 13047), *Klebsiella pneumoniae* (ATCC 13883), *Pseudomonas aeruginosa* (ATCC 227853); three human pathogen fungi, *Candida albicans* (ATCC 10231), *Candida tropicalis* (ATCC 13801) and *Candida glabrata* (ATCC 28838) through disc diffusion method and minimum inhibitory concentration determination (MIC). In the same work, the results of the antimicrobial activities were compared with those obtained from standard antibiotics (netilmicin, itraconazole and 5-flucytocine) in the case of the microorganisms from the American Type Culture Collection (ATCC), and the standard sanguinarine used especially for the oral pathogen. In this study, the authors reported RJ activities with a MICs values ranging from 0.67 to 1.3 mg/mL, nevertheless the new isolated compounds 11-oxododecanoic acid and (11S)-hydroxydodecanoic acid have been tested in the same experimentation and showed the highest and widest spectrum of antimicrobial activities, nevertheless always far from the values obtained with the standard antibiotics.

Koç et al.[76] honeybee products including RJ samples originating from Turkey for their ability to inhibit the growth of 40 yeast strains of . *albicans, C. glabrata, C. krusei, and Trichosporon spp.* The susceptibility to RJ was performed through broth microdilution method, which ranged with regard to all isolates between 0.06 and 1 µg/mL. nevertheless, propolis and pollen had better antifungal activities. According to the authors,[76] propolis and pollen (but not RJ) can even control some fluconazole resistant fungal strains. Although the authors have not correlated the antifungal activity of RJ samples with their chemical compositions, they consider that several compounds can be responsible for the properties found, including peptides.[76] In another study, Boukraâ, Niar, Benbarek, & Benhanifia,[77] analyzed the additive action of Algerian RJ and honey against *S.aureus* (ATCC 29523) and they reported a MIC value for four varieties of honey ranged between 20% and 21%, while, for RJ it was 2%. In this study, authors mixed honey and RJ and noticed that the honey MIC decreased more than 50% with 1% RJ. In this finding, the authors have shown that adding honey to RJ increases the antibacterial effect against *S. aureus*, and have considered that it is an effective combination of these natural

products mainly for clinical practice, and since they have no adverse effects on tissues, they can be safely used on wounds and inserted into cavities and sinuses to clear infection. Similarly, analysis of 18 Algerian RJ[78] was done on *P. aeruginosa*, and the results showed that honey MICs ranged between 12 and 18 % and RJ MIC was 4%, but after the combination each honey variety tested showed a greater than 90 % drop in MIC using 3% RJ, while the MIC of RJ dropped by 75% when used with the half concentration of honey that alone provides the MIC and by 50 % when used with one-third of the concentration of honey that alone provides the MIC.[77] Regarding the results of this investigation, Boukraâ et al.[77] affirmed that RJ may receive renewed recognition as wound healer. The same group of research studied the synergistic effect of starch and Algerian RJ against *S. aureus* and *E. coli*. According to the authors, this approach was done due to the fact that RJ possesses amylases, such as honey, which are able to hydrolyze starch originating dextrin and maltose that are responsible for the osmotic effect increase of honey, and consequently its antibacterial activity rise. The authors[79] determined that the MIC of a variety of freshly reaped RJ, then they tested a sub-MIC concentration with a set of concentrations of starch on the same bacteria strains to check the MIC variation. Boukraâ et al.[79] found a MIC value of RJ samples of 1.7% and 2% for *S. aureus* and *E. coli* respectively while the MIC with starch dropped with 61% and 30% against *S. aureus* and *E. coli*; according to their results, they concluded that the mixture of RJ and starch could be used to treat infections that are resistant to conventional drugs, at lower cost.[79]

The anti-parasitic activity of RJ was also evaluated by Nazzi et al.[55] whom studied the repellent properties of Italian RJ and its major volatile octanoic acid on Varroa destructor Anderson & Trueman, through a lab assay. The authors concluded that RJ and particularly, octanoic acid were able acting as repellent of mite. These results may partly explain the lower invasion rates of queen cells since the food provided to queen larvae is RJ, and field studies in bee colonies have confirmed that the compound may interfere with the process of cell invasion by mite.[55]

10.6.3. Anti-inflammatory activity

Inflammation is a bodily response following local injury caused by physical or chemical agents or microbial pathogens, which manifests in heat, redness, pain, swelling and disturbed physiological functions. In general, inflammation is a beneficial immune process, since it inactivates and/or destroys the invading organisms, removing the irritant and establishing the stage for tissue repair. Nevertheless, the inflammation process may become chronic, giving 556 rise to diverse inflammatory diseases. Usually, the drugs used to manage an inflammatory

condition are the nonsteroidal anti-inflammatory class, which have serious side effects mainly the gastric irritations which can evoke gastric ulcer.[80] So, a natural product with anti-inflammatory properties is of high importance. For instance, RJ has been proposed as an anti-inflammatory agent.

Karaca et al.[32] studied the effect of Turkish RJ on colitis induced by acetic acid and alteration of mast cell distribution (MC) in the colon of rats treated orally with RJ (150 mg/kg body weight) for 4 weeks. RJ was shown to protect the colonic mucosa against the injurious effects of acetic acid along with decreasing the number of MC and reducing the area of colonic erosion in the colon.[32] The same group of research verified the same protocol treatment with RJ on acetic acid-induced colitis by monitoring the distribution of $CD^{3+,}$ CD^{5+} , and CD^{45+} T-cell, and CD^{68+} cells in rats.[81] Karaca et al.[81] revealed that colitis induced by acetic acid increase the CD^{3+} , CD^{5+} and CD^{45+} Tcell, and CD^{68+} macrophage levels and RJ decreases the proliferative response of CD^{3+} and CD^{45+} T-cells while, no differences were observed for CD^{5+} T-cells and CD^{68+} macrophages which indicate that RJ has an anti-inflammatory effect.

Later on, a clinical experiment was done by Erdem & Güngörmüş[82] where they evaluated the effect of RJ on oral mucositis in patients undergoing radiotherapy and chemotherapy. In this experiment, patients received mouthwash therapy with benzydamine hydrochloride and nystatin rinses with or without RJ and the mean resolution time of oral mucositis was monitored. It was found that the addition of RJimproved the signs and symptoms of oral mucositis and markedly shortened its healing time as compared with the control.[82] In 2015, more investigation on Turkish RJ was carried out, where Aslan & Aksoy[83] screened its anti-inflammatory effects after inducing renal inflammation in rats with the use of ethylene glycol. The proinflammatory/anti-inflammatory cytokines, TNF-α, IL-1β and IL-18 levels in blood and renal tissue samples from the rats used in the application were measured and it was concluded that RJ exhibited an anti-inflammatory effect. The authors associated this effect to its antiradical and antioxidative properties.[83]

10.6.4. Immunomodulatory activity

Infectious diseases require efficient body defense mechanisms to control them through the process of immunomodulation. The immunomodulation process from a natural origin can afford an alternative to conventional chemotherapy for numerous diseases, mainly when host defense mechanisms have to be activated following conditions of impairment of the immune defense or when a selective immunosuppression is desired in situations like autoimmune disorders.[84] Šver et al.[85] showed that RJ exhibited immunomodulatory properties by

stimulating antibody production and immunocompetent cell proliferation in mice treated with fresh RJ or depressing humoral immune functions in rats. Later on, Erem, Deger, Ovali, & Barlak[86] investigated the effects of RJ on autoimmunity in graves' disease, where lymphocyte cells were obtained from the patients with Graves' disease and incubated with RJ, then MTT (methylthiazolyldiphenyl-tetrazolium bromide) test in lymphocyte cell culture, Th1 cytokines IFN-gamma, TNF-alpha, and IL-12, and Th2 cytokines IL-4 and IL-10 levels and TSHR antibody assays were assessed. The authors suggest, according to the results of this experiment, that RJ may exert an effect similar to an antithyroid drug to decrease thyroid-stimulating hormone receptor antibody levels (TSHR Ab).[86]

Egyptian RJ was also examined for its possible amelioration of peripheral blood leukocytes and lung inflammation in mouse conalbumin-induced asthma model. This was assessed by pretreating the experimental animals with RJ after sensitization with conalbumin for 18 days, Blood was collected for white blood cell differentiation, and the lungs were removed for histopathological studies. The sensitized and royal jelly-treated groups showed an increased incidence of asthma cascade events because the alleviation of inflammatory cells, which might be due to the immunostimulatory and vasodilatory effects of royal jelly being antagonistic to bronchial asthma cases.[87] Guendouz et al.[88] studied the preventive effects of RJ against the development of a systemic and intestinal immune response in mice allergic to cow's milk proteins by treatment with RJ and sensitization intraperitoneally with b-lactoglobulin (b-Lg), afterward, serum IgG and IgE anti-b-Lg were determined. The results showed a significant decrease of serum IgG, IgE and anti-b-Lg and reduction of plasma histamine level. Moreover, RJ significantly reduced intestinal dysfunction, by abolishing the secretory response induced by sensitization and prevention of length aberrations of jejunal villi. The authors speculate that using RJ may help prevent systemic and anaphylactic response in allergic mice.[88]

10.6.5. Antitumor Activity

Cancer is one of the most serious diseases worldwide; it represents the second-leading cause of death. Despite the fact that, survival rates are ameliorating due to the improvements and development of cancer treatment, there is a lack of anti-cancer drugs , which makes natural products with anticancer bioactive compounds with a high importance.[26] Orsolic & Bašić[89] investigated the effect of Croatian RJ on tumor development and metastasis in murine tumor models (mammary carcinoma and colon carcinoma) through intraperitoneal, subcutaneous and the intravenous injection of RJ before tumour-cell inoculation. It was found that the intravenous

application of tumor cells and RJ significantly inhibiteds the formation of metastases. Oršolić & Bašić,[89] according to the results found, RJ products given orally or systemically might control tumor growth and metastasis. On the other hand, Gismondi et al.[29] tested Italian RJ on the growth of three different mammalian cell lines: immortalized murine myoblasts (C_2C1_2), human prostate cancer (PC_3) and human neuroblastoma (SH-SY5Y) through treating cells with lipophilic, or hydrophilic RJs fraction, and observed an extraordinary antiproliferative activity of the lipophilic extract of all RJs against SH-SY5Y cells, which places in evidence the antitumoral feature against human neuroblastoma and thus making RJ as potential natural product for medical application to prevent the onset and slow down the growth of the individual compounds present in the lipophilic fraction, nevertheless they attributed the antiproliferative action to eicosane;dodecane, 2,6,11-trimethyl; octacosyl trifluoroacetate; phenantrenemetil-2 phenilcinnamate, and not to 10-HDA because all RJ samples had antiproliferative activity against SH-SY5Y cells and some of these RJ samples did not present this compound or its saturated form.

10.6.6. Anti-aging activities

Aging is a normal biological process that results from a progressive accumulation of damaged and defective cellular components and loss of cell or organ physiological function. Although the average lifespan in humans has extended over the last decades, more than often this does not mean good "quality of life", due to the degenerative diseases and dementia.[90] Mediterranean RJ has been the target of a study for finding ways to overcome the less cognitive ability common in the elderly as well as the deleterious effects of menopause, particularly at cardiovascular and osteoporosis levels. RJ from Mediterranean countries has been screened not only for its antimicrobial, antioxidant, antitumoral, immunomodulatory and anti-inflammatory activities, but also tested *in vivo* for its effect on the cognitive performance by orally treating naturally aged Wistar male rat's Greek RJ daily for 2 months.[40] The results showed an improvement of memory in rats as well as a depletion of dopamine and serotonin in the prefrontal cortex along with an increase in their metabolite concentration and turnover. Through this finding, Pyrzanowska et al.[40] validated the traditional use of RJ in providing a better quality of life in old age. Recently, the same group of research examined the effect of Greek RJ on neurotransmission in the central nervous system through its long-term administration, and they observed a decrease of gamma amino butyric acid (GABA) concentration in the striatum and hypothalamus of naturally aged Wistar male rats, with no change in liver and kidney functions. The authors did not attribute this property to any compound present in the RJ used,

although the chemical composition had been given (10- HDA and its saturated form constituted higher than 50% of the RJ sample.[42]

Mohamed et al.[91] studied the neuroprotective effect of Egyptian RJ administration on the changes in the function and structure of the brain following oral administration of tartrazine in rat pups. This artificial azo-dye derived from coal tar is used for coloring capsules of vitamins and antacids, in cosmetics and hair products, jam, jelly, chewing gums, biscuits, sauces and ice creams. However, allergic reactions, hyperactivity in children, and genotoxicity have been reported for tartrazine. The study performed by the authors led to the conclusion that tartrazine has several harmful effects on the neurotransmitters concentration which were counteracted by RJ (the level of GABA increased in group subjected to tartrazine and RJ when compared with the group treated with this dye, which had originated a decrease of that neurotransmitter), antioxidant and oxidative stress biomarkers were also positively affected (increase of the SOD and CAT activities and the levels of GSH, and decrease of the MDA levels) in the rat brain homogenate. Thus, RJ provides protection against the ruinous effects of tartrazine on rat pups' brain tissue function and structure.[91]

Other authors have checked the effect of Greek RJ on cardiovascular and bone turnover mark through clinical experiments in 36 postmenopausal healthy women, who received RJ, rich in medium chain fatty acids, daily for 3 months.[41] By the end of the experiment, it was noticed that RJ significantly raised the high-density lipoprotein – cholesterol (HDL-C 60.2 mg/dL versus 64.7 mg/dL, 7.7% increase), decreased in low density lipoprotein – cholesterol (LDL-C, 143.9 versus 136.2, 4.1% decrease) and in total cholesterol (224.4 to 216.1, 3.09% decrease). No significant statistical changes were observed in the remaining cardiovascular and bone turnover parameters. In general, the authors' conclusion was that RJ induced an improvement of the lipid profile of postmenopausal women and it can be efficient for controlling the menopause – associated dyslipidemia.[42] In a similar study, Kafadar et al.[92] studied the capacity of Turkish RJ to reduce bone loss due to osteoporosis in oophorectomized rat model. Oophorectomized rat models were treated with RJ, then blood samples were collected for biochemical analysis and bone mineral density (BMD) was measured, as well as weighing the uterine after scarifying the rats. Tissue samples were taken to determine bone calcium and phosphate level. According to the results of the experiment, treatment with RJ, Kafadar et al.[92] declared that the lumbar spine and proximal femur BMD results were statistically significantly higher in the royal jelly groups, as well as the bone tissue calcium and phosphate levels as compared to the non-treated contr

Table 10.1. Biological activities of RJ originating from countries of Mediterranean Sea

Geographical origin	Biological activity found	Type of evidence	Exploration method	References
Italy	Antiproliferative effect	Experimental (*in vitro*)	Investigation of RJs effect on the growth of three different mammalian cell lines: immortalized murine myoblasts (C_2C1_2), human prostate cancer (PC3) and human neuroblastoma (SH-SY5Y) through treating cells with lipophilic, or hydrophilic RJs fraction	[27]
	Anti-parasite effect varroa-repellent properties	Experimental (*in vivo* using bees)	Exploration of the semiochemicals affecting the process of host finding by the reproducing mites and identification of repellent compounds from royal jelly diverting the mites from queen cells.	[56]
	Antioxidant activity	Experimental (*in vitro*)	Antioxidant activity through electrochemical method and DPPH method	[66]
Croatia	Antitumoral effect	Experimental (*in vivo* in mice)	Investigation of the effect of RJ on tumor growth, metastasizing ability and induction of apoptosis and necrosis in murine tumor models (mammary carcinoma and colon carcinoma) through intraperitoneal, subcutaneous and intravenous injection of RJ before tumour-cell inoculation.	[88]
	Immunomodulatory potential	Experimental (*in vivo* in mice)	The CBA mice were pretreated with RJ, and received it once or twice a day at 7-day intervals. Enumeration of plaque forming cells, serum protein determination and blood parameters were assessed. RJ stimulate antibody production and immunocompetent cell proliferation in mice or depressing humoral immune functions in rats	[84]
Greece	Antimicrobial activity	Experimental (*in vitro*)	RJ samples were tested for their antimicrobial activity against Gram-positive and Gram-negative bacteria and fungi and exhibited interesting activities.	[33]
	Effects on neurotransmission in the central nervous system	Experimental (*in vivo* in wistar rats)	Old male Wistar rats were given RJ powder was daily for 2 months. levels of amino acids in selected brain structures were examined.	[41]
	Improvement of cognitive performance	Experimental (*in vivo* in old wistar rats)	Old male Wistar rats were given RJ powder was daily for 2 months. The spatial memory was assessed in the water maze and next the level of neurotransmitters, their metabolites and utilization in the selected brain regions were estimated. Greek Royal Jelly improves spatial memory and influences the concentration of brain neurotransmitters in naturally aged Wistar male rats	[39]
	Effect on cardiovascular and bone turnover	Clinical experience	36 postmenopausal healthy women received RJ for 3 months. Circulating cardiovascular risk markers and bone turnover parameters were compared between the baseline and the three-month visit	[40]

Turkey	Antifungal Activity against *Candida spp.* and *Trichosporon spp.*	Experimental (*in vitro*)	The broth microdilution method was used to assess the antifungal activity of RJ against yeasts	[76]
	Hepatoprotective effect	Experimental (*in vitro*)	Sprague–Dawley rats were treated with RJ and/or CCL_4 for 20 days. Blood samples were taken for biochemical analysis, Histopathological examination was assessed.	[68]
	Antioxidant activity	Experimental (*in vitro*)	Antioxidant activity through phosphomolybdenum, free radical scavenging activity tests	[50]
	Antioxidant activity	Experimental (*in vitro*)	Total phenolic contents, ferric reducing antioxidant capacity and 2,2-diphenyl-1-picryhydrazyl free radical scavenging activity were measured as antioxidant determinants.	[21]
	Hepatoprotective effect	Experimental (*in vivo* in Sprague Dawley rats)	Hepatoprotective activity against CCl_4 -induced hepatic damage in rats was evaluated. Animals were fed with RJ for 7 days following CCl_4 injection. Development of liver damage and oxidative stress were monitored.	[67]
	Antioxidant activity	Experimental (*in vitro*)	Antioxidant capacities of RJ were identified using FRAP, DPPH assays, phenolic and flavonoid contents measurement.	[67]
	Anti-inflammatory effect	Experimental (*in vivo* in Sprague Dawley rats)	Renal inflammation in rats was generated using ethylene glycol, RJ was given to animals and proinflammatory/anti-inflammatory cytokines, TNF-α, IL-1β and IL-18 levels in blood and renal tissue samples were measured	[59]
	Hepatoprotective effect	Experimental (*in vivo* in Sprague Dawley rats)	Rats received RJ and/or CCl_4 for 20 days, then blood samples were taken for biochemical analysis, as well as liver tissue for trace and major elements concentrations determination	[69]
	Neuroprotective effect	Experimental (*in vivo* in New Zealand male rabbits)	Neuroprotective effect of RJ on traumatic spinal cord injury was evaluated. Animals were given RJ after trauma then laminectomy was performed. Blood, cerebrospinal fluid and tissue sample from spinal cord were taken for measurements of antioxidant status or detection of apoptosis.	[73]
	Bone loss reduction in osteoporosis	Experimental (*in vivo* in Sprague Dawley rats)	Oophorectomized rats model treated with RJ, then blood samples were collected for biochemical analysis, bone mineral density (BMD) was measured, animals were sacrificed and uterine weights were measured, also tissue samples were taken to determine bone calcium and phosphate level	[90]
	Effect on mucositis in patients undergoing radiotherapy and chemotherapy	Clinical experience	Patients received mouthwash therapy with benzydamine hydrochloride and nystatin rinses in addition to royal jelly and the mean resolution time of oral mucositis was monitored	[81]

	Nephroprotective effect	Experimental (*in vivo* in Wistar albino rats)	Nephrotoxicity and oxidative stress in rats were induced using cisplatina and RJ was given to experimental animals. Blood was collected for determination of blood urea nitrogen, alanine aminotransferase, aspartate aminotransferase, triglyceride, total cholesterol, uric acid, total bilirubin, and total protein levels. In kidney samples malondialdehyde, glutathione peroxidase, superoxide dismutase and catalase activities analysis as well as for histopathological examinations	[72]
	Effect on colitis	Experimental (*in vivo* in Wistar albino rats)	Colitis in rats model was induced by acetic acid, then animals were treated orally with RJ. At the end of experiment colon samples were obtained for Mast cell and Histological analysis.	[32]
	Protective effect on oxidative stress parameters and apoptotic activity induced by Cisplatin.	Experimental (*in vivo* in Sprague Dawley rats)	Rats were treated with RJ for 15 consecutive days by gavage after injection of ciplatin The effect RJ against oxidative stress caused by Cisplatin injury of the kidneys and liver, was determined by measuring tissue biochemical and antioxidant parameters and investigating apoptosis immunohistochemically in rats treated with RJ	[71]
	Anti-inflammatory and cell regeneration	Experimental (*in vivo* in Sprague Dawley rats)	Effect of RJ on acetic acid-induced colitis on the distribution of CD^{3+}, CD^{5+}, CD^{45+} T-cell and CD^{68+} cells in rats was assessed by treating animals with RJ afterward colon samples were obtained for parameters determination	[80]
	Effect on autoimmunity in Graves' disease.	Experimental (*in vitro*)	Lymphocyte cells were obtained from the patients with Graves' disease. Were incubated with RJ, then MTT test in lymphocyte cell culture, Th1 cytokines IFN-gamma, TNF-alpha, and IL-12, and Th2 cytokines IL-4 and IL-10 levels and TSHR antibody assays were assessed. RJ may exert an effect similar to an antithyroid drug for decreasing thyroid-stimulating hormone receptor antibodies levels.	[85]
	Ameliorative effect damage caused by sodium fluoride exposure on biochemical parameters	Experimental (*in vivo* in mice)	Mice were given fluoride in drinking water, in association with royal jelly for 7 days, at the end of experimentation, blood samples and liver samples were taken. Erythrocyte and liver tissue malondialdehyde levels, superoxide dismutase, catalase and glutathione peroxidase activities, serum cholesterol, triglyceride, glucose, total protein and albumin levels, aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase activities were evaluated.	[70]
	Protective effect of RJ Against Radiation - induced oxidative stress in rats	Experimental (*in vivo* in Sprague Dawley rats)	Protective effect of RJ treatment at different concentration on oxidative damage in liver and lung tissue after total body irradiation was evaluated. Blood samples were collected and analyzed for alanine aminotransferase, aspartate aminotransferase, triglyceride, total cholesterol and gamma glutamyl transpeptidase levels. The lung and liver samples were kept for the measurement of malondialdehyde, glutathione peroxidase, superoxide dismutase and catalase activities.	[74]

	Antioxidant activity	Experimental (*in vivo* in Sprague Dawley rats)	Breast cancer was induced by the injection of N-methyl-Nnitrosourea to Sprague Dawley rats. Animals were treated with paclitaxel, RJ and paclitaxel + RJ during 30 days. Histopathological and biochemical parameters were evaluated in breast tissues.	[73]
Egypt	Ameliorative effects against asthma	Experimental (*in vitro* in mouse)	The modulatory effect of RJ on peripheral blood leukocytes and lung inflammation in a mouse conalbumin-induced asthma model was assessed by pretreating the experimental animals with RJ after sensitization with conalbumin for 18 days, then blood was collected for white blood cell differentiation, and the lungs were removed for histopathological studies	[86]
	Neuroprotective effect	Experimental (*in vivo* in Sprague Dawley rats pups)	Modulatory role of RJ against neurotoxic effect of tartrazine was investigated by introducing RJ with tartrazine to experimental animals for 30 days. Concentrations of different brain neurotransmitters, gamma amino butyric acid, dopamine, serotonin, the antioxidant and oxidative stress biomarkers were measured in the brain homogenates. An immunohistochemistry study was done to reveal the changes in brain structure.	[89]
Algeria	Immunomodulatory effect	Experimental (*in vitro* in mice)	Preventive effects of RJ against the development of a systemic and intestinal immune response in mice allergic to cow's milk proteins was assessed through treating sensitized animals, with β-lactoglobulin, with RJ for 7 days. IgG and IgE anti-β-Lg, plasma histamine levels, symptom scores, body temperature were determined. histological analysis of jejunums was done to determine local anaphylactic responses	[87]
	Antibacterial activity	Experimental (*in vitro*)	Additive Action of RJ and honey against *Staphylococcus aureus* and *Pseudomonas aeruginosa* aureus was assessed trough determination of minimum additive inhibitory concentration and determination of correlation between the antibacterial effect.	[76,77]
	Antibacterial activity	Experimental (*in vitro*)	A comparative method of adding RJ with and without starch to culture media was used. MIC of RJ and MIC of synergitism of RJ and starch were determined	[78]

10.7. Royal jelly allergy

Despite its high beneficial effect to human health, several allergic reactions induced by RJ are reported in literature around the world. In general, RJ consumption has been linked to contact dermatitis, acute asthma, anaphylaxis, hemorrhagic colitis and even death.[93-95] Diverse reports have signaled that RJ consumption leads to asthma and anaphylaxis in various case reports.[96-99] The authors have associated the appearance of allergic symptoms to the fact that those individuals had a history of asthma, allergic rhinitis, eczema, or atopic dermatitis,[99-101] thus RJ sensitization might be a more serious risk to atopic patients than those who are not atopic.[94] Moreover, contact dermatitis to RJ digestion was been also reported for the first time by Takahashi, Matsuo, & Ohkido,[102] where in a case report, a subject showed an exacerbation of dermatitis and it was presumed that this allergic reaction could be attributed to the amount of propolis components in the RJ consumed.

Besides Harper & Copeman[103] and Harada, Moriyama, & Tanaka[104] reports, other group of researchers reported cases where symptoms of allergy were observed at the time of their first intake of RJ. More recently, Paola et al.[94] describe a case with oral allergy syndrome (OAS) that appeared a few minutes after the ingestion of RJ and approved the previous allergic case reported about RJ. Testi et al.[105] demonstrated that severe anaphylaxis to RJ is attributed to the consumption of RJ jointed to the treatment with antibiotics cefonicid. Recent research has investigated RJ composition to reveal the compounds responsible for the allergic reaction in individuals. Katayama et al.[96] separated RJ extract by sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) and 2-dimensional electrophoresis (2-D), then immunoblotting of the SDS-PAGE and 2-D profiles were carried out to identify the allergenic spots, and concluded that the major allergens of RJ were MARJ1 and MARJ2 in our patients' population. While, Mizutani et al.[106] reported MARJP3 as a possible allergen in RJ-induced anaphylaxis. In accordance with these results, Okamoto et al.[107] affirmed that MRJP3 could suppress IL-4 production, showing potent antiallergic properties in a mouse model of immediate hypersensitivity, suggesting that MRJP3-derived antiallergic peptides by identifying the associated polypeptide regions may of clinical significance.

10.8. Conclusion

The chemical composition of Mediterranean RJ samples has been studied as well as their biological properties (antioxidant, anti-inflammatory, immunomodulatory, anticancer or anti-aging activities). Generally, there were not qualitative differences between the Mediterranean RJ samples and all remaining RJ worldwide, nevertheless exceptions could be found. For

example, in RJ samples from Greece were reported for the first time 16 new compounds as RJ constituents, along with 7 compounds isolated for the first time as natural products. In addition, it was also possible, for the first time, to know in a very detailed way the chemical composition of RJ samples, detecting a total of 348 and 276 different compounds in methanol and diethyl ether, respectively. In this study, the samples were coming from Italy. The biological properties found for RJ samples could constitute an alternative to some drugs used in conventional medicine that can present undesirable side effects, nevertheless in the majority of cases RJ samples have been less effective. Concerning honeybee products, in some cases propolis and pollen are more effective than RJ. Moreover, RJ is also more expensive than propolis or pollen, these two factors may partly explain lower number of studies upon RJ than propolis, pollen or honey. With very few exceptions, the study of biological properties of RJ samples are generally done using raw material and the chemical composition is not carried out or if done, there is not any clear attribution of the activities to the compounds identified. This was not a problem detected in the present review with Mediterranean RJ samples only, it is a common issue in the remaining RJ samples worldwide. Moreover, very few works have used *in vivo* assays and, in these cases, the bioavailability studies of active compounds of RJ, are absent, which limits the interest and importance of the natural product. Thus, this review intends to alert for the pressing necessity to cover aspects able to relate the biological activities found with the compounds present in RJ as well as their bioavailability. This, will allow better the comprehension of its mechanism of action *in vitro* and *in vivo*, also, a deep identification of their components is mandatory for its possible standardization and achievement of new formulations.

10.9. RJ in the Moroccan scenario

As reported above, RJ is widely investigated worldwide. However, in Morocco, there is still a serious lack of scientific data. As far as we know, research papers reporting RJ originated from Morocco are very scare or even inexistent, from where the necessity to focus on studying Moroccan RJ and exploring its health benefit.

References

[1] M. G. Miguel, S. El-Guendouz, 'Volatile compounds of royal jelly', Bee Products Chemical and Biological Properties. 2017. DOI: 10.1007/978-3-319-59689-1_9.

[2] Z. Y. Huang, G. W. Otis, 'Factors determining hypopharyngeal gland activity of worker honey bees (*Apis mellifera* L.)' Insect. Soc. 1989, 36, 264–276 https://doi.org/10.1007/BF02224880

[3] A. N. K. G. Ramanathan, A. J. Nair, V. S. Sugunan, 'A review on royal jelly proteins and peptides', J. Funct. Foods. 2018, 44, 255-264. https://doi.org/10.1016/j.jff.2018.03.008

[4] L. Jianke, F. Mao, D. Begna, F. Yu, Z. Aijuan, 'Proteome comparison of hypopharyngeal gland development between Italian and royal jelly producing worker honeybees (*Apis mellifera* L.)', J. Proteome Res. 2010. 9, 6578-94. https://doi.org/10.1021/pr100768t.

[5] H. Emonet, 'Étude de la médecine égyptienne antique et de sa pharmacopée: le papyrus Ebers', *Tours*. 2001, 210p.

[6] C. Cassaignau, 'L'Abeille et les produits de la ruche utilisés en nutrition et en thérapeutique' Th. Doct. ed. 1991.

[7] C. Mraz, 'Healt and the honeybee', Queen City Printers, New York.,. ed. New York. 1995.

[8] R. Domerego, 'Ces abeilles qui nous guérissent'. 2001.

[9] P. C. Molan, 'Bee World: Why honey is effective as a medicine', Amyris. ed. Bruxelles. 1999.

[10] T. Cherbuliez, R. Domerego, 'L'apithérapie : médecine des abeilles', Amyris. ed. Bruxelles. 2003.

[11] R. Chauvin, 'Traité de biologie de l'abeille', Tomes 1 et 2, ELSEVIER /. ed. 566p, ISBN : 2225828032. 1968.

[12] F. Fratini, G. Ciliaa, S. Mancini, A. Felicioli, 'Royal Jelly: An ancient remedy with remarkable antibacterial properties', Microbiol Res. 2016, 192, 130–141. https://doi.org/https://doi.org/10.1016/j.micres.2016.06.007.

[13] A. G. Sabatini, 'Quality and standardisation of Royal Jelly', J. ApiProd. ApiMed. Sci. 2009. https://doi.org/10.3896/IBRA.4.01.1.04.

[14] Fujiwara, S., Imai, J., Fujiwara, M., Yaeshima, T., Kawashima, T., & Kobayashi, K. (1990). A potent antibacterial protein in royal jelly. *The Journal of Biological Chemistry*, *265*, 849 11333-11337.

[15] Fontana, R., Mendes, M.A., de Souza, B.M., Konno, K., César, L.M.M., Malaspina, O., & 842 Palma, M.S. (2004). Jelleines: a family of antimicrobial peptides from the royal jelly of honeybees (*Apis mellifera*). *Peptides, 25*, 919-928. oi:10.1016/j.peptides.2004.03.016.

[16] M. Wytrychowski, S. Chenavas, G. Daniele, H. Casabianca, M. Batteau, S. Guibert, B. Brion, 'Physicochemical characterisation of French royal jelly: Comparison with commercial royal jellies and royal jellies produced through artificial bee-feeding', J. Food Compos. Anal. 2013. https://doi.org/10.1016/j.jfca.2012.12.002

[17] G. Sesta, L. Lusco, 'Refractometric determination of water content in royal jelly', Apidologie, 2008, 39, 225–232. https://doi.org/10.1051/apido:2007053.

[18] D. Kanelis, C. Tananaki, V. Liolios, M. Dimou, G. Goras, M. A. Rodopoulou, E. Karazafiris, A. Thrasyvoulou, 'A suggestion for royal jelly specifications Prijedlog globalnih standarda za matičnu mliječ,' Arh. Hig. Rada. Toksikol. 2015, 66, 275-284.

[19] A. Karaali, F. Meydanoğlu, D. Eke, 'Studies on composition, freeze-drying and storage of Turkish royal jelly', J. Apic. Res. 1988, 27, 182-187. https://doi.org/10.1080/00218839.1988.11100799.

[20] S. Kolayli, H. Sahin, Z. Can, O. Yildiz, M. Malkoc, A. Asadov, 'A Member of Complementary Medicinal Food: Anatolian Royal Jellies, Their Chemical Compositions, and Antioxidant Properties' Evid. Based. Complement. Alternat. Med. 2016, 21.

[21] X. Xue, L. Wu, K. Wang, 'Chemical composition of royal jelly. Bee Products - Chemical and Biological Properties'. 2017. https://doi.org/10.1007/978-3-319-59689-1_8.

[22] M. Wytrychowski, G. Daniele, H. Casabianca, 'Combination of sugar analysis and stable isotope ratio mass spectrometry to detect the use of artificial sugars in royal jelly production', Anal. Bioanal. Chem, 2012, 403, 451-456. https://doi.org/10.1007/s00216-012-5934-6.

[23] G. Daniele, H. Casabianca, 'Sugar composition of French royal jelly for comparison with commercial and artificial sugar samples', Evid. Based. Complement. Alternat. Med. 2012, 134, 1025- 1029. DOI: 10.1016/j.foodchem.2012.03.008.

[24] Sesta, G. (2006). Determination of sugars in royal jelly by HPLC. *Apidologie, 37*, 84–90 1038 https://doi.org/10.1051/apido:2005061.

[25] J. Hanes, J. Šimúth, 'Identification and partial characterization of the major royal jelly protein of the honey bee (*Apis mellifera* L.)', J. Apic. Res. 1992, 31, 22-26.

[26] M. Viuda-Martos, J. A. Pérez-Alvarez, J. Fernández-López, 'Royal jelly: health benefits and uses in medicine', Bee Products - Chemical and Biological Properties. 2017. https://doi.org/10.1007/978-3-319-59689-1_10.

[27] E. Marconi, M. F. Caboni, M. C. Messia, G. Panfili, 'Furosine: A suitable marker for assessing the freshness of royal jelly' J. Agri. Food. Chem. 2002, 50, 2825-2829. R.

[28] Scarselli, E. Donadio, M. G. Giuffrida, D. Fortunato, A. Conti, E. Balestreri, R. Felicioli, M. Pinzauti, A. G. Sabatini, A. Felicioli, 'Towards royal jelly proteome', Proteomics. 2005, 5, 769-76. https://doi.org/10.1002/pmic.200401149.

[29] A. Gismondi, E. Trionfera, L. Canuti, G. Di Marco, A. Canini, 'Royal jelly lipophilic fraction induces antiproliferative effects on SH-SY5Y human neuroblastoma cells', Oncol. Rep. 2017, 38, 1833-1844. https://doi.org/10.3892/or.2017.5851

[30] J. Li, T. Wang, Z. Zhang, Y. Pan, 'Proteomic analysis of royal jelly from three strains of western honeybees (*Apis mellifera*)', J. Agri. Food. Chem. 2007, 55, 8411-8422.

[31] A. Pina, O. Begou, D. Kanelis, H. Gika, S. Kalogiannis, C. Tananaki, G. Theodoridis, A. Zotou, 'Targeted profiling of hydrophilic constituents of royal jelly by hydrophilic interaction liquid chromatography–tandem mass spectrometry', J. Chromatogr. A. 2017 1531, 53-63.

[32] T. Karaca, F. Bayiroglu, M. Yoruk, M. S. Kaya, S. Uslu, B. Comba, L. Mis, ' Effect of royal jelly on experimental colitis induced by acetic acid and alteration of mast cell distribution in the colon of rats', Eur. J. Histochem. 2010, 54, e35. https://doi.org/10.4081/ejh.2010.e35.

[33] E. Melliou, I. Chinou, 'Chemistry and bioactivity of royal jelly from Greece', J. Agric. Food Chem. 2005, 53, 8987-8992. https://doi.org/10.1021/jf051550p.

[34] L. Ioana Bărnuţiu, L. Al Mărghitaş, D. S. Dezmirean, C. Manuela Mihai, O. Bobiş, 'Chemical composition and antimicrobial activity of royal jelly-review', J. Anim. Sci. Biotechno. 2011, 44, 67-72.

[35] Y. Terada, M. Narukawa, T. Watanabe, 'Specific hydroxy fatty acids in Royal Jelly activate TRPA1', J. Agri Food Chem. 2011, 59, 2627-35. https://doi.org/10.1021/jf10416460.

[36] J. F. Antinelli, S. Zeggane, R. Davico, C. Rognone, J. P. Faucon, L. Lizzani, 'Evaluation of (E)-10-hydroxydec-2-enoic acid as a freshness parameter for royal jelly' Food. Chem. 2003, 80, 85–89.

[37] M. Genç, A. Aslan,' Determination of trans-10-hydroxy-2-decenoic acid content in pure royal jelly and royal jelly products by column liquid chromatography', J. Chromatogr. A. 1999, 839, 265-8. https://doi.org/10.1016/S0021-9673(99)00151-X

[38] F. Ferioli, G. Marcazzan, M. Caboni, 'Determination of (E)-10-hydroxy-2- decenoic acid content in pure royal jelly: a comparison between a new CZE method and HPLC', J. Sep. Sci. 2007, 30, 1061–9.

[39] F. Ferioli, E. Armaforte, M. F. Caboni, 'Comparison of the lipid content, fatty acid profile and sterol composition in local Italian and commercial royal jelly samples', J. Am. Oil. Chem. Soc. 2014 91, 875-884. DOI:10.1007/s11746-014-2446-x

[40] J. Pyrzanowska, A. Piechal, K. Blecharz-Klin, I. Joniec-Maciejak, K. Graikou, I. Chinou, E. Widy-Tyszkiewicz, 'Long-term administration of Greek Royal Jelly improves spatial memory and influences the concentration of brain neurotransmitters in naturally aged Wistar male rats', J. Ethnopharmacol. 2014, 155, 343-351. https://doi.org/10.1016/j.jep.2014.05.032.

[41] I. Lambrinoudaki, A. Augoulea, D. Rizos, M. Politi, N. Tsoltos, M. Moros, I. Chinou, K. Graikou, E. Kouskouni, S. Kambani, K. Panoulis, P. Moutsatsou, 'Greekorigin royal jelly improves the lipid profile of postmenopausal women', Gynecol. Endocrinol. 2016, 32, 835-839.

[42] J. Pyrzanowska, A. Wawer, I. Joniec-Maciejak, A. Piechal, K. Blecharz-Klin, K. Graikou, I. Chinou, E. Widy-Tyszkiewicz, 'Long-term administration of Greek Royal Jelly decreases GABA concentration in the striatum and hypothalamus of naturally aged Wistar male rats', Neurosci. Lett. 2018, 675, 17-22. https://doi.org/10.1016/j.neulet.2018.03.034.

[43] S. Bogdanov, 'Royal jelly, bee brood: composition, health, medicine: A review', Bee Product Science. 2016. www.bee-hexagon.net.

[44] M. F. Ramadan, A. Al-Ghamdi, ' Bioactive compounds and health-promoting properties of royal jelly: A review', J. Funct. Foods. 2012, 4, 39-52. https://doi.org/10.1016/j.jff.2011.12.007.

[45] A. Stocker, P. Schramel, A. Kettrup, E. Bengsch, 'Trace and mineral elements in royal jelly and homeostatic effects', J. Trace. Elem. Med. Biol. 2005, 19, 183-9.

[46] J. B. Saunier, G. Losfeld, R. Freydier, C. Grison, 'Trace elements biomonitoring in a historical mining district (les Malines, France)', Chemosphere. 2013, 93, 2016-2023. https://doi.org/10.1016/j.chemosphere.2013.07.024.

[47] A. M. Gómez-Caravaca, M. Gómez-Romero, D. Arráez-Román, A. Segura-Carretero, A. Fernández-Gutiérrez,' Advances in the analysis of phenolic compounds in products derived from bees', J. Pharm. Biomed. Anal. 2006, 41, 1220-1234. https://doi.org/10.1016/j.jpba.2006.03.002.

[48] H. Guo, Y. Kouzuma, M. Yonekura, 'Structures and properties of antioxidative peptides derived from royal jelly protein', Food. Chem. 2009, 113, 238-245.

[49] D. Özkök, S. Silici, 'Antioxidant activities of honeybee products and their mixtures', Food. Sci. Biotechnol. 2017, 26, 201–206. https://doi.org/DOI 10.1007/s10068-017- 0027-0.

[50] N. López-Gutiérrez, M. Aguilera-Luiz, M. del, R. Romero-González, J. L. M. Vidal, A.G. Frenich, 'Fast analysis of polyphenols in royal jelly products using automated TurboFlow TM -liquid-chromatography–Orbitrap high resolution mass spectrometry', J. Chromatogr. A. 2014, 973, 17-28.

[51] V. A. Isidorov, S. Bakier, I. Grzech, 'Gas chromatographic-mass spectrometric investigation of volatile and extractable compounds of crude royal jelly', J. Chromatogr. B. Analyt. Technol. Biomed. Life. Sci. 2012, 15, 885-886. https://doi.org/10.1016/j.jchromb.2011.12.025

[52] F. P. Drijfhout, J. Kochansky, S. Lin, N. W. Calderone, 'Components of honeybee royal jelly as deterrents of the parasitic Varroa mite, Varroa destructor', J. Chem. Ecol. 2005, 31, 1747-64.

[53] R. Boch, D. A. Shearer, R. W. Shuel, Octanoic and Other Volatile Acids in the Mandibular Glands of the Honeybee and in Royal Jelly. J. Apic. Res. 1979, I8, 250-253.

[54] Y. Z. Zhao, Z. G. Li, W. L. Tian, X. M. Fang, S. K. Su, W. J. Peng, 'Differential volatile organic compounds in royal jelly associated with different nectar plants', J. Integr. Agric. 2016, 15, 1157-1165. https://doi.org/10.1016/S2095- 3119(15)61274-6.

[55] F. Nazzi, R. Bortolomeazzi, G. Della Vedova, F. Del Piccolo, D. Annoscia, N. Milani, 'Octanoic acid confers to royal jelly varroa-repellent properties', Naturwissenschaften. (2009). 96, 309-314. https://doi.org/10.1007/s00114-008-0470-0.

[56] K. Bílikova, S. C. Huang, I. P. Lin, J. Šimuth, C. C. Peng, 'Structure and antimicrobial activity relationship of royalisin, an antimicrobial peptide from royal jelly of *Apis mellifera*', Peptides. 2015, 68, 190-196. https://doi.org/10.1016/j.peptides.2015.03.001

[57] P. Jamnik, D. Goranovič, P. Raspor, 'Antioxidative action of royal jelly in the yeast cell', Exp. Gerontol. 2007, 42, 594-600. https://doi.org/10.1016/j.exger.2007.02.002

[58] Z. Aslan, L. Aksoy, 'Anti-inflammatory effects of royal jelly on ethylene glycol induced renal inflammation in rats', Int. Braz. J. Urol. 2015, 41, 1008–1013. Doi: 10.1590/S1677-5538.IBJU.2014.0470

[59] M. Nakaya, H. Onda, K. SasakiI, A. Yukiyoshi, T. Hirofumi, K. Yamada, 'Effect of royal jelly on bisphenol A-induced proliferation of human breast cancer cells', Biosci. Biotechnol. Biochem. 2007, 71, 253-255. DOI: 10.1271/bbb.60453.

[60] T. Oikawa, A. Kamogawa, Y. Akiyama, K. Sato, S. Nakajin, M. Shinoda, 'Biochemical studies on vasodilative factor in royal jelly', Yakugaku. Zasshi. 2017, 98, 139-145. https://doi.org/10.1248/yakushi1947.98.2_139.

[61] T. Matsui, A. Yukiyoshi, S. Doi, H. Sugimoto, H. Yamada, K. Matsumoto, 'Gastrointestinal enzyme production of bioactive peptides from royal jelly protein and their antihypertensive ability in SHR', J. Nutr. Biochem. 2002, 13, 80-86. https://doi.org/10.1016/S0955-2863(01)00198-X.

[62] C. Pavel, L. Mărghitaş, O. Bobiş, 'Biological activities of royal jelly-review', J. Anim. Sci. Biotechno. 2011, 44, 108-118.

[63] S. El-Guendouz, S. Aazza, B. Lyoussi, V. Bankova, M. Popova, L. Neto, M. L. Faleiro, M. Da Graça Miguel, 'Moroccan propolis: a natural antioxidant, antibacterial, and antibiofilm against *Staphylococcus aureus* with no induction of resistance after continuous exposure', Evid. Based. Complementary. Altern. Med. 2018, 4, 19p. DOI: 10.1155/2018/9759240.

[64] K. S. Kumar, D. Bhowmik, 'Medicinal uses and health benefits of Honey: An overview', J. Chem. Phar. Res. 2010, 2, 385-395.

[65] S. Buratti, S. Benedetti, M. S. Cosio, 'Evaluation of the antioxidant power of honey, propolis and royal jelly by amperometric flow injection analysis', Talanta. 2007. https://doi.org/10.1016/j.talanta.2006.07.006.

[66] Ö. Saral, O. Yildiz, R. Aliyazicioğlu, E. Yuluğ, S. Canpolat, F. Öztürk, S. Kolayli, Apitherapy products enhance the recovery of CCL4 -induced hepatic damages in rats', Turkish. J. Med. Sci. 2016, 46, 194–202. https://doi.org/10.3906/sag-1411-35.

[67] M. Cemek, F. Aymelek, M. E. Büyükokuroğlu, T. Karaca, A. Büyükben, F. Yilmaz, 'Protective potential of Royal Jelly against carbon tetrachloride induced-toxicity and changes in the serum sialic acid levels', Food Chem. Toxicol. 2010. https://doi.org/10.1016/j.fct.2010.07.013

[68] M. Cemek, F. Yılmaz, M. E. Büyükokuroğlu, A. Büyükben, F. Aymelek, A. Ayaz, 'Serum and liver tissue bio-element levels, and antioxidant enzyme activities in carbon tetrachloride-induced hepatotoxicity: protective effects of royal jelly', J. Med. Food. 2012. https://doi.org/10.1089/jmf.2012.0010.

[69] M. Kanbur, G. Eraslan, S. Silici, M. Karabacak, 'Effects of sodium fluoride exposure on some biochemical parameters in mice: Evaluation of the ameliorative effect of royal jelly applications on these parameters', Food Chem. Toxicol. 2009. https://doi.org/10.1016/j.fct.2009.02.008.

[70] A. Karadeniz, N. Simsek, E. Karakus, S. Yildirim, A. Kara, I. Can, F. Kisa, H. Emre, M. Turkeli, 'Royal jelly modulates oxidative stress and apoptosis in liver and kidneys of rats treated with cisplatin', Oxid. Med. Cell. Longev. 2011. https://doi.org/10.1155/2011/981793.

[71] S. Silici, O. Ekmekcioglu, M. Kanbur, K. Deniz, 'The protective effect of royal jelly against cisplatin-induced renal oxidative stress in rats', World J. Urol. 2011. https://doi.org/10.1007/s00345-010-0543-5.

[72] Y. Cihan, Protective Role of Royal Jelly Against Radiation-Induced Oxidative Stress in Rats. International Journal of Hematology and Oncology, 23. doi: 10.4999/uhod.11016

[73] Meltem, M., Diler, U.A., Ahmet, A., Şafak, E., Şen, T.M., Birgül, K.V., Hüseyin, A.U. (2017) The effects of royal jelly on the oxidant antioxidant system in rats with N- methyl- N-nitrosourea-induced breast cancer. *Turkish Journal of Biochemistry*. https://doi.org/https://doi.org/10.1515/tjb-2017-0140.

[74] A. Aslan, M. Cemek, M. E. Buyukokuroglu, K. Altunbas, O. Bas, Y. Yurumez, 'Royal jelly can diminish secondary neuronal damage after experimental spinal cord injury in rabbits', Food. Chem. Toxicol. 2012, 50, 2554-9. Doi: 10.1016/j.fct.2012.04.018.

[75] Centers for Disease Control and Prevention, 'Antibiotic resistance threats in the United Sates', Current. 2013. https://doi.org/CS239559-B.

[76] A. N. Koç, S. Silici, F. Kasap, H. T. Hörmet-Öz, H. Mavus-Buldu, B. D. Ercal, 'Antifungal Activity of the Honeybee Products Against Candida spp. and Trichosporon spp', J. Med. Food. 2011, 14, 128-134. https://doi.org/10.1089/jmf.2009.0296.

[77] L. Boukraâ, A. Niar, H. Benbarek, M. Benhanifia, 'Additive Action of Royal Jelly and Honey Against *Staphylococcus aureus'*, J. Med. Food. 2008, 11, 190- 192. https://doi.org/10.1089/jmf.2007.567.

[78] L. Boukraa, 'Additive activity of royal jelly and honey against *Pseudomonas aeruginosa',* Altern. Med. Rev. 2008.

[79] L. Boukraâ, A. Meslem, M. Benhanifia, S. M. Hammoudi, 'Synergistic effect of starch and royal jelly agaiCemeknst *Staphylococcus aureus* and *Escherichia coli'*, Evid. Based. Complementary. Altern. Med. 2009, 15, 755-757. https://doi.org/10.1089/acm.2008.0483.

[80] P. Chatterjee, S. Chandra, P. Dey, S. Bhattacharya, 'Evaluation of anti-inflammatory effects of green tea and black tea: A comparative *in vitro* study', J. Pharm. Adv. Res. 2012, 3, 136–138.

[81] T. Karaca, N. Şimşek, S. Uslu, Y. Kalkan, I. Can, A. Kara, M. Yörük, 'The effect of royal jelly on CD^{3+}, CD^{5+}, CD^{45+} T-cell and CD^{68+} cell distribution in the colon of rats with acetic acid-induced colitis', Allergol. Immunopathol. 2012, 40, 357-361. https://doi.org/10.1016/j.aller.2011.09.004.

[82] Ö. Erdem, Z. Güngörmüş, 'The effect of royal jelly on oral mucositis in patients undergoing radiotherapy and chemotherapy', Holist. Nurs. Pract. 2014.

[83] Z. Aslan, L. Aksoy, 'Anti-inflammatory effects of royal jelly on ethylene glycol induced renal inflammation in rats', Int. Braz J Urol. 2015. https://doi.org/10.1590/S1677-5538.IBJU.2014.0470.

[84] L. Ganju, D. Karan, S. Chanda, K. K. Srivastava, R. C. Sawhney, W. 'Selvamurthy, Immunomodulatory effects of agents of plant origin', Biomed. Pharmacother. 2003, 57, 296-300.

[85] L. Šver, N. Oršolić, Z. Tadić, B. Njari, I. Valpotić, I. Bašić, 'A royal jelly as a new potential immunomodulator in rats and mice', Comp. Immunol. Microbiol. Infect. Dis. 1996, 19, 31-38.

[86] C. Erem, O. Deger, E. Ovali, Y. Barlak, 'The effects of royal jelly on autoimmunity in Graves disease', Endocrine. 2006, 30, 175-183. https://doi.org/10.1385/ENDO:30:2:175.

[87] W. K. El-Aidy, A. A. Ebeid, A. E. R. M. Sallam, I. E. Muhammad, A. T. Abbas, M. A. Kamal, S. S. Sohrab 'Evaluation of propolis, honey, and royal jelly in amelioration of peripheral blood leukocytes and lung inflammation in mouse conalbumin-induced asthma model', Saudi J. Biol. Sci. 2015, 22, 780–788. https://doi.org/10.1016/j.sjbs.2014.11.005

[88] M. Guendouz, A. Haddi, H. Grar, O. Kheroua, D. Saidi, H. Kaddouri, 'Preventive effects of royal jelly against anaphylactic response in a murine model of cow's milk allergy', Pharm. Biol. 2017, 55, 2145-2152. https://doi.org/10.1080/13880209.2017.1383487.

[89] N. Oršolić, I. Bašić, 'Immunomodulation by water-soluble derivative of propolis: A factor of antitumor reactivity', J. Ethnopharmacol. 2003, 84, 265-273. https://doi.org/10.1016/S0378-8741(02)00329-X.

[90] Pan, M.-H.,Lai, C.-S., Tsai, M.-L., Wu, J.-C., & Ho, C.-T. (2012). Molecular mechanisms for anti-aging by natural dietary compounds. *Molecular Nutrition and Food Research, 56,* 998 88-115. DOI 10.1002/mnfr.201100509.

[91] A. A. R. Mohamed, A. A. A. Galal, Y. H. A. Elewa, 'Comparative protective effects of royal jelly and cod liver oil against neurotoxic impact of tartrazine on male rat pups brain', Acta Histochem. 2015. https://doi.org/10.1016/j.acthis.2015.07.002.

[92] I. H. Kafadar, A. Güney, C. Y. Türk, M. Öner, S. Silici, 'Royal jelly and bee pollen decrease bone loss due to osteoporosis in an oophorectomized rat model', Eklem. Hastalıkları. Cerrahisi. Dergisi. 2012, 23, 100-105.

[93] M. S. Abdullah, N. Abdullah, 'Skin test reactivity to bee hive products (honey bees, honey, royal jelly and pollen)', Jurnal. Teknologi. 2016, 72, 5–10. https://doi.org/10.11113/jt.v78.8827.

[94] F. Paola, D. D. Pantalea, C. Gianfranco, F. Antonio, V. Angelo, N. Eustachio, D. L. Elisabetta, 'Oral allergy syndrome in a child provoked by royal jelly', Case Reports in Medicine. 2014, 3p.

[95] Y. Yonei, K. Shibagaki, N. Tsukada, N. Nagasu, Y. Inagaki, K. Miyamoto, O. Suzuki, Y. Kiryu, 'Case report: Haemorrhagic colitis associated with royal jelly intake', J. Gastroenterol. Hepatol. 1997, 12, 495-499. https://doi.org/10.1111/j.1440- 1746.1997.tb00472.x

[96] M. Katayama, M. Aoki, S. Kawana,' Case of anaphylaxis caused by ingestion of royal jelly', J. Dermatol. 2008, 35, 222-4. https://doi.org/10.1111/j.1346- 8138.2008.00448.x

[97] R. Leung, F. C. K. Thien, B. Baldo, D. Czarny, 'Royal jelly-induced asthma and anaphylaxis: Clinical characteristics and immunologic correlations', J. Allergy. Clin. Immunol. 1995, 96, 1004-1007. https://doi.org/10.1016/S0091- 6749(95)70242-3.

[98] S. Peacock, V. Murray, C. Turton, 'Respiratory distress and royal jelly', Br. Med. J. 1995, 311, 1472. https://doi.org/10.1136/bmj.311.7018.1472a.

[99] F. C. K. Thien, R. Leung, B. A. Baldo, J. A. Weiner, R. Plomley, D. Czarny, 'Asthma and anaphylaxis induced by royal jelly', Clin. Exp. Allergy. 1996, 26, 216-222. https://doi.org/10.1111/j.1365-2222.1996.tb00082.x

[100] M., Harwood, S. Harding, R. Beasley, P. D. Frankish, 'Asthma following royal jelly', N. Z. Med. J. 1996, 109, 325.

[101] J. R. Laporte, L. Ibáñez, L. Vendrell, E. Ballarín, 'Bronchospasm induced by royal jelly', Allergy, 2008, 51, 440. https://doi.org/10.1111/j.1398-9995.1996.tb04645.x

[102] M. Takahashi, I. Matsuo, M. Ohkido, 'Contact dermatitis due to honeybee royal jelly', Contact. Dermatitis. 1983, 9, 452-5. https://doi.org/10.1111/j.1600-0536.1983.tb04462.x.

[103] J. I. Harper, P. W. M. Copeman, 'Beekeepers' dermatitis: contact allergy to beeswax, bees glue (propolis) and Royal Jelly', Br. J. Dermatol. 1983, 109, 97-97.

[104] S. Harada, T. Moriyama, A. Tanaka, 'Two cases of royal jelly allergy provoked the symptoms at the time of their first intake', Jap. J. Allergol. 2011, 60, 708-13.

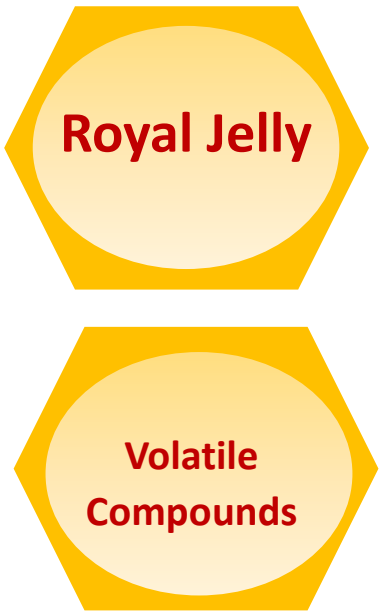
[105] S. Testi, L. Cecchi, M. Severino, M. Manfredi, G. Ermini, D. Macchia, S. Capretti, P. Campi, 'Severe anaphylaxis to royal jelly attributed to cefonicid', J. Investig. Allergol. Clin. Immunol. 2007, 17, 281.

[106] Y. Mizutani, Y. Shibuya, T. Takahashi, T. Tsunoda, T. Moriyama, M. Seishima, 'Major royal jelly protein 3 as a possible allergen in royal jelly-induced anaphylaxis', J. Dermatol. 2011, 38, 1079-1081. https://doi.org/10.1111/j.1346- 8138.2010.01179.x.

[107] I. Okamoto, Y. Taniguchi, T. Kunikata, K. Kohno, K.,Iwaki, M. Ikeda, M. Kurimoto, 'Major royal jelly protein 3 modulates immune responses *in vitro* and in vivo', Life. Sci. 2003, 73, 2029-2045.

Chapter 11

Volatile Compounds of Royal Jelly



Volatile Compounds of Royal Jelly*

Abstract

Royal jelly (RJ) has been used since ancient times in traditional medicine. It's a honeybee hypopharyngeal and mandibular glands secretion of young worker called nurse, yellowish, creamy, and acidic material with a slightly pungent odor and taste. RJ is primarily involved in the longevity of the queen bee and for feeding young larvae. The composition of RJ varies depending on seasonal and regional conditions, it may contain proteins, lipids, sugars, vitamins (A, B5, C, D, E) and essential amino acids particularly cystine, lysine and arginine. Investigation of Volatiles in RJ are scarce. In the few references found determining the volatile fractions of RJ, we can report that several factors can be responsible for differences in the general composition of the volatile's compounds of RJ such us honeybee species, harvesting time, regions, storage conditions and extraction methods. Predominantly, we can find on the volatile fractions of RJ different compounds such us carboxylic acids (2-methylbutanoic acid, 3-methylbutanoic acid, pentanoic acid, 2-hexenoic acid…), phenols (hydroquinone), carbonyl compounds (ketones), esters, aldehydes and alcohols.

In this chapter, based on the bibliographic investigations throughout the international literature, we detailed findings related to the chemical composition of volatile organic compounds of RJ determined by chromatography coupled to mass spectrometry (GC-MS).

*****Adapted from 'Volatile Compounds of Royal Jelly'. Maria G. Miguel and Soukaïna El-Guendouz. In Bee Products - Chemical and Biological Properties; Alvarez-Suarez, J.M. (Eds.). Springer International Publishing; 2017; pp. 191-198.

11.1. Introduction

Royal jelly (RJ) is a secretion rich in protein and lipids produced by the hypopharyngeal and mandibular glands, respectively, of *Appis mellifera* L. young worker honeybees (called nurses)[1] RJ is used by nurses to feed young larvae of workers and drones for the first 3 days, and is the exclusive food of the queen bee throughout its life. After this 3-day period, worker larvae are fed a mixture of RJ, pollen and honey.[2-4] Workers and queens are females developed from fertilized eggs, whereas unfertilized eggs become males, called drones.[4]

RJ is a viscous and creamy product. It is relatively acidic (pH 3.1–3.9) with a high buffering capacity ranging between 4 and 7, has a density of 1.1 g/mL, is partially soluble in water, ranges in colour from white to yellow, and has a slightly pungent odour and taste.[5-7] RJ is mainly composed of water (60-70%), proteins (9-18%), carbohydrates (7-18%), lipids (3-8%), minerals (0.8-3%), vitamins, phenols and amino acids.[8]

Major RJ proteins (MRJPs) are water soluble, constituting more than 80% of RJ proteins. The MRJP family has nine members, MRJP1-MRJP9, with molecular masses of 49-87 kDa.[4,8 and references therein] In some MRJPs there are repetitive pentapeptide regions concentrated with nitrogen-rich amino acids, and others with repetitive tripeptide regions concentrated with methionine (MRJP5).[10] Peptides are also constituents of RJ which possess antioxidant activity, mainly due to their capacity to scavenge hydroxyl free radicals, particularly those containing tyrosyl residues at their C-termini.[11] Free amino acids are also found in RJ, with proline, glycine, lysine and glutamic acid being the most abundant;[12] although Boselli et al. (2003)[2] also reported alanine, aspartic acid, homoserine, isoleucine, hydroxyproline, ornitine, phenylalanine, serine, threonine, tyrosine and valine as being present in considerable amounts.

Fructose and glucose are the main carbohydrates of RJ (over 90% of the total sugars), although fructose is the most predominant. Sucrose (in high variable concentrations) and oligosccharides trehalose, maltose, gentiobiose, isomaltose, raffinose, erlose, and melezitose (in very small concentrations) are also carbohydrates present in RJ.

The lipid fraction is predominantly composed of organic acids (80-90%), almost all in the free form (mono and dihydroxy acids and dicarboxylic acids with 8 and 10 carbon atoms) with characteristic arrangements. Phenols (4-10%), waxes (5-6%), steroids (3-4%) and phospholipids (0.4-0.8%) are also constituents of the lipid fraction. The fatty acid fraction predominantly consists of monocarboxylic acids *trans*-10-hydroxy-2-decenoic acid (HDEA or 10-HDA) (32%), gluconic acid (24%) and 10-hydroxydecanoic acid (HDAA) (22%). Other monocarboxylic acids have also been reported in RJ (7- and 8-hydroxyoctanoic, 3-

hydroxydecanoic, 9-hydroxydecanoic, 9-hydroxy-2-decenoic, 10-hydroxydecanoic, and 3,10-dihydroxydecanoic acids). Dicarboxylic acids are also present in RJ, but in lower amounts than hydroxycarboxylic acids (e.g. 2-octene-1,8-dioic and 2-decene-1,10-dioic acids). Mono- and di-esters of 10-hydroxy-2-*trans*-decenoic acid in which the hydroxyl group is esterified by another fatty acid were also reported in RJ, along with one hydroxyl-2-*trans*-decenoic acid 10-phosphate.[3,5 13-15]

In the lipid fraction of RJ, Lercker et al. (1982)[16] also detected sterols in the unsaponifiable extracts, such as 24-methylenecholesterol, the most abundant, along with sitosterol and Δ5-avenasterol in much lower amounts. More recently, other authors[3,17] also reported the presence of such phytosterols in Italian and Polish RJ samples. Isidorov et al. (2012)[16] reported the presence of 25-hydroxy-24-methylcholesterol in Polish RJ samples. Campesterol was also detected in Polish RJ by Isidorov et al. (2016).[18]

Only water-soluble vitamins have been reported in RJ. Pantothenic acid (B5) is the predominant vitamin in RJ, although others may also be present but in lower amounts: e.g. thiamine (B1), riboflavin (B2), niacin (B3), pantothenic acid (B5), pyridoxal (B6), biotin (B7), folic acid and ascorbic acid (vitamin C) (only in trace amounts).[19,20]

The most abundant elements in RJ include, in descending order: K, Ca, Na, Mg, Zn, Fe, Cu, Mn. Trace elements may also be found, such as Al, Ba, Sr, Bi, Cd, Hg, Pb, Sn, Te, Tl, W, Sb, Cr, Ni, Ti, V, Co, Mo.[6, 14]

Several authors[4,5, 21, 22] refer to the presence of phenols in RJ but do not provide a phenolic profile. In a review by Gómez-Caravaca et al. (2006)[23] focusing on the analysis of phenolic compounds in bee-derived products, the authors stated that no studies analysing the phenolic profile of royal jelly had been found. Generally, the antioxidant activity of samples is attributed to phenols. Some authors[10] reported that the antioxidant activity of royal jelly is attributed to the tyrosyl residues of amino acids, which contain a phenolic group. The quantification of total phenols in these studies could have therefore resulted from the presence of this amino acid in RJ. However, Isidorov et al. (2012)[16] identified several phenolic compounds either in the volatile fraction, obtained by head space-solid phase microextraction (HS-SPME), or in the ether extracts. The phenolic compounds are presented in Figure 11.1.

In general, the composition of RJ depends on seasonal and regional conditions.[4]

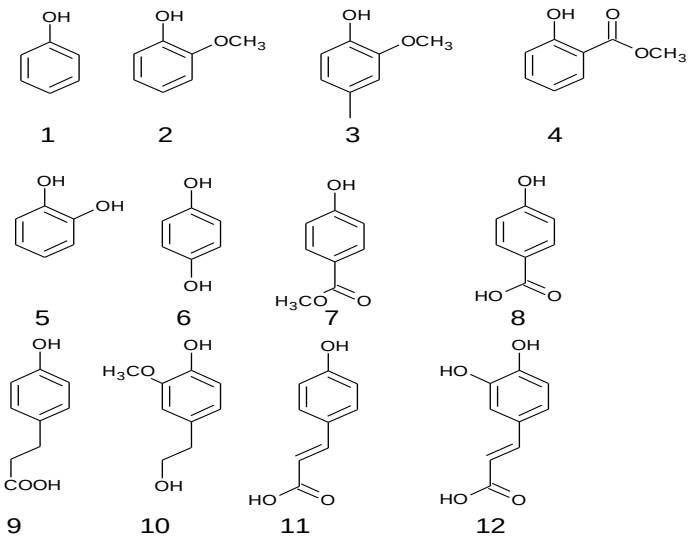


Figure 11.1. Phenols detected in Polish RJ. In volatile fraction: **1**: phenol; **2**: 2-methoxyphenol (o-guaiacol); **3**: 2-methoxy-p-cresol; **4**: methyl salicylate. In ether extract: **5**: pyrocatechol; **6**: hydroquinone; **7**: 4-hydroxybenzoic acid, methylester; **8**: 4-hydroxybenzoic acid; **9**: 4-hydroxyhydrocinnamic; **10**: 4-hydroxy-3-methoxyphenylethanol; **11**: *p*-coumaric acid; **12**: caffeic acid.

11.2. Volatiles in royal jelly

The volatiles in RJ have not been studied in detail; for this reason, very few references which included the identification of such compounds in RJ were found.[15, 16, 24,25,26, 27]

Royal jelly from colonies consisting of *Apis melliefera ligustica* and *A. mellifera carnica*, located in north eastern Italy, was extracted with diethyl ether and derivatized by silylation.[26] After this procedure, the authors identified 26 compounds by chromatography coupled to mass spectrometry (GC-MS). The compounds were predominantly carboxylic acids (23 aliphatic carboxylic acids and 1 aromatic carboxylic acid). The remaining two compounds identified were hydroquinone and methyl-4-hydroxybenzoate. Two other compounds were not identified. Seventeen compounds found by Nazzi et al. (2009)[26] had already been reported by other authors.[15,24,25] The new compounds found in RJ by Nazzi et al. (2009)[26] were 2-methylbutanoic acid, 3-methylbutanoic acid, pentanoic acid, 2-hexenoic acid, 2-hydroxy-4-methylpentanoic acid, 2-hydroxyhexanoic acid, phenylacetic acid, hydroquinone and methyl-4-hydroxybenzoate.

None of the compounds reported above were detected three years later by Isidorov et al. (2012)[16] in volatile fractions of RJ from two colonies of *A. mellifera carnica* situated in two distinct regions of Poland (north eastern and south western), obtained after extraction by HS-SPME and analysed by GC-MS. According to the authors, the volatile fraction predominantly consisted of carbonyl compounds (37.2-43.3%), particularly ketones, with 2-heptanone alone

accounting for 20%. The other carbonyl compounds identified were, in descending order of quantity: acetone > 2-nonanone > benzaldehyde > 2-butanone > 2-pentanone, hexanal (all of them in significantly lower percentages than 2-heptanone). The second major component was the alkene 1-pentadecene (17-21%). Phenols were the third major group of compounds (7.2-12%), in descending order of quantity: phenol > methyl salicylate > 2-methoxyphenol > 2-methoxy-*p*-cresol.

In the same work, the authors[16] evaluated the effect of storage temperature (-18 ºC, 4 ºC and room temperature) on the volatile profile of RJ. They verified that samples kept for 10 months at room temperature showed the greatest quantitative modifications, particularly the phenol content which decreased twofold (phenol from 12 to 7% and methyl salicylate from 7 to 3%), as well as the volatile C2 and C4 acid content (acetic acid from traces to 7% and butyric acid from not detected to 5%) and C5 aldehyde content (2-methylbutanal, 3-methylbutanal and 2-methyl-2-butenal were absent or in trace amounts in the fresh material while after 10 months their percentages were about 1%). The authors suggested that the increase in C2 and C4 carboxylic acids in RJ kept at room temperature may have been due to rancidity.[16]

More recently, Zhao et al. (2016)[27] also used GC-MS to analyse the volatile fraction of 30 RJ samples collected from 30 apiaries during flowering seasons of different nectar plants (ten types of RJ: *Schisandra chinensis*, *Medicago sativa*, *Vitex negundo*, *Citrullus lanatus*, *Sophora alopecuroides*, *Brassica campestris*, *Robinia pseudoacacia*, *Helianthus annus*, *Litchi chinensis*, *Tilia tuan*) in China after extraction by HS-SPME. A total of 89 compounds were identified by the authors, distributed amongst the following compound classes: esters and aldehydes (25 and 17.5%, respectively), ketones (15%), acids (10%), and alcohols (10%). Although 89 compounds were identified, the authors prudently decided that only 40 of these could be constitutive volatile components of RJ samples because they were present in relatively high amounts and in at least 2 types of RJ.

Zhao et al. (2016)[27] found that 2-nonanone and acetic acid were common to all RJ samples, whereas octanoic acid, benzaldehyde and phenol were present in 9 RJ types, toluene and octanoic acid were present in 8 RJ types, and 2-pentanone and (benzoic acid, methyl ester) were present in 7 RJ types. On the other hand, there were 14 compounds that were only present in 2-4 RJ types, thus making them unsuitable for classification purposes: (1-propen-2-ol, formate), ethanol, (butanoic acid, ethyl ester), (acetic acid, butyl ester), hexanal, ethylbenzene, octanal, (benzene, 1,2,3,5-tetramethyl-), (2-furancarboxaldehyde, 5-methyl-), benzoxazole, 2-furanmethanol, methyl salicylate, (4H-pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl-), and 2-furan-carboxaldehyde, 5-(hydroxymethyl)-). Ethanol and furfural, which are not constitutive

compounds of RJ, were present in some samples possibly due to the fermentation of carbohydrates and heating during the extraction process, respectively.[27]

The results obtained by Zhao et al. (2016)[27] allowed the classification of all samples into three main groups; nevertheless, there was one group which only contained RJ from the flowering season of *Tilia tuan*, which was considerably different from the samples belonging to the other two groups. This sample mainly consisted of alcohols, whereas acids, esters, aldehydes and ketones predominated in diverse proportions in the other samples, allowing them to be grouped in the remaining classes.

In short, there are differences in the chemical composition of the volatile fractions of RJ samples studied by several authors.[16,26,27] Several factors were pointed out by Zhao et al. (2016)[27] as being responsible for these differences: honeybee species, harvesting time, region, storage method and processing technology. In fact, when comparing the results obtained by these three groups it is possible to verify differences, even within the same group when working with different RJ of varying origin. Nevertheless, when only comparing the results of Zhao et al. (2016)[27] and Isidorov et al. (2012),[16] who extracted the volatile fraction of RJ samples by the same procedure (HS-SPME) although using different fibers, a common compound is found in all samples: 2-nonanone. This seems to be a general characteristic of the volatile fraction of RJ, independent of the nectar plant, fiber, region, honeybee species, storage method and processing technology. Other components were also common to both works: ethanol, acetic acid, ethylacetate, 2-pentanone, 2-methylbutanal, toluene, hexanal, benzaldehyde, phenol, octanoic acid, and methylsalicilate; however, Zhao et al. (2016)[27] found that these compounds could also be absent in at least one sample in the study. Such absences in RJ of different nectar origin reveal that the presence of these compounds may be dependent on the nectar plant. This is reinforced by the fact that octanoic acid was also identified in the study of Nazzi et al. (2009),[26] despite the use of a different extraction method to that of Zhao et al. (2016)[27]. However, the group also verified that octanoic acid was absent in the flowering season of *Sophora alopecuroides*.

The presence of carbonyl and volatile phenolic compounds along with octanoic acid in RJ may give rise to repellent effects against the mite *Varroa destructor* and antibacterial activity.[16]

11.3. Conclusion

From above, we can conclude that carboxylic acids; as 2-methylbutanoic acid, 3-methylbutanoic acid and pentanoic acid, 2-hexenoic acid among others, phenols; carbonyl

compounds, aldehydes and alcohols are the commun groups of compounds found in RJ Volatiles In sum, several factors are presented as responsible for differences in the general composition of the volatiles compounds of RJ including honeybee species, harvesting time, regions, storage conditions as well as extraction methods.

References

[1] T. Fujita, H. Kozuka-Hata, H. Ao-Kondo, T. Kunieda, M. Oyama, T. Kubo, 'Proteomic analysis of the royal jelly and characterization of the functions of its derivation glands in the honeybee', J. Proteome. Res. 2013,12, 404-411.

[2] E. Boselli, M. F. Caboni, A. G. Sabatini, G.L. Marcazzan, G. Lercker, 'Determination and changes of free amino acids in royal jelly during storage', Apidologie. 2003, 34, 129-137.

[3] F. Ferioli, E. Armaforte, M. F. Caboni, 'Comparison of the lipid content, fatty acid profile and sterol composition in local Italian and commercial royal jelly samples', J. Am. Oil. Chem. Soc. 2014, 91, 875-884.

[4] Y. Wang, L. Ma, W. Zhang, X. Cui, H. Wang, B. Xu, 'Comparison of the nutrient composition of royal jelly and worker jelly of honey bees (*Apis mellifera*)', Apidologie. 2016, 47, 48-56.

[5] M. F. Ramadan, A. Al-Ghamdi, 'Bioactive compounds and health-promoting properties of royal jelly: a review', J. Funct. Foods. 2012, 4, 39-52.

[6] Z. Nabas, M. S. Y. Haddadin, J. Haddadin, I. K. Nazer, 'Chemical composition of royal jelly and effects on symbiotic with two different locally isolated probiotic strains on antioxidant activities', Pol. J. Food. Nutr. Sci. 2014, 64, 171-180.

[7] C. I. Mureşan, L. A. Mărghitaş, D. S. Dezmirean, O. Bobiş, V. Bonta, I. Zacharias, R. Mărgãoan, C. Paşca, 'Quality parameters for commercialized royal jelly', Bulletin. UASVM. Animal. Science. and. Biotechnologies. 2016, 73. Doi: 10.15835/buasvmcn-asb:11630.

[8] R. Balkanska, I. Karadjova, M. Ignatova, 'Comparative analyses of chemical composition of royal jelly and drone brood', Bulg. Chem. Commun. 2014, 46, 412-416.

[9] L. I. Bărnuțiu, L. A. Mărghitas, D. S. Dezmirean, C. M. Mihai, O. Bobiş, 'Chemical composition and antimicrobial activity of royal jelly – review', J. Anim. Sci. Biotechnol. 2011, 44, 67-72.

[10] M. D. Drapeau, S. Albert, R. Kucharski, C. Prusko, R. Maleszka, 'Evolution of the yellow/major royal jelly protein family and the emergence of social behaviour in honey bees', Genome. Res. 2006, 16, 1385-1394.

[11] H. Guo, Y. Kouzuma, M. Yonekura, 'Structure and properties of antioxidative peptides derived from royal jelly proteína', Food. Chem. 2009, 113, 238-245.

[12] W, Liming, Z, Jinhui, X, Xiaofeng, L, Yi, Z, 'Jing Fast determination of 26 amino acids and their content changes in royal jelly during storage using ultra-performance liquid chromatography', J. Food. Compos. Anal. 2009, 22, 242–249.

[13] N, Noda, K, Umebayashi, T, Nakatani, K, Miyahara, K, 'Ishiyama Isolation and characterization of some hydroxy fatty and phosphoric acid and esters of 10-hydroxy-2-decenoic acid from the royal jelly of honey bees (*Apis mellifera*)', Lipids. 2005, 40,833-838.

[14] A. G. Sabatini, G. L. Marcazzan, M. F. Caboni, S. Bogdanov, L. B. de Almeida-Muradian, 'Quality and standardisation of royal jelly', JAAS. 2009, 1,1-6.

[15] Y. Terada, M. Narukava, M. Watanabe, 'Specific hydroxyl fatty acids in royal jelly activate TRPA1', J. Agric. Food. Chem. 2011, 59, 2627-2635.

[16] G. Lercker, P. Capella, L. S. Conte, F. Ruini, G. Giordani, 'Components of royal jelly II: the lipid fraction, hydrocarbons and sterols', J. Apic. Res. 1982, 21,178–184.

[17] V. A. Isidorov, S. Bakier, I. Grzech, 'Gas-chromatographic-mass spectrometric investigation of volatile and extractable compounds of crude royal jelly', J. Chromatogr. B. 2012, 885-886: 109-116.

[18] V. A. Isidorov, S. Bakier, M. Stocki, 'GC-MS investigation of the chemical composition of honeybee drone and queen larva homogenate', J. Apic. Sci. 2016, 60. Doi: 10.1515/JAS-2016-0011.

[19] M. Ciulu, N. Spano, M. I. Pilo, G. Sanna, 'Recent advances in the analysis of phenolic compounds in unifloral honeys', Molecules. 2016, 21,451–482.

[20] S. Bogdanov, 'Royal jelly, 'bee brood: composition, health, medicine: A review', Bee Product Science. from www.bee-hexagon.net. April 2016.

[21] T. Nagai, R. Inoue, 'Preparation and the functional properties of water extract and alkaline extract of royal jelly', Food. Chem. 2004, 84, 181-186.

[22] J-R Liu, Y-C Yang, L-S Shi, C-C Peng, 'Antioxidant properties of royal jelly associated with larval age and time of harvest', J. Agric. Food. Chem. 2008, 56, 11447-11452.

[23] A. M. Gómez-Caravaca, M. Gómez-Romero, D. Arráez-Román, A. Segura-Carretero, A. Fernández-Gutíerrez, 'Advances in the analysis of phenolic compounds in products derived from bees', J. Pharm. Biomed. Anal. 2006, 41, 1220-1234.

[24] R, Boch, D. A. Shearer, R. W. 'Shuel Octanoic and other volatile acids in the mandibular glands of the honeybee and in royal jelly', J. Apicult. Res. 1979, 18, 250–252.

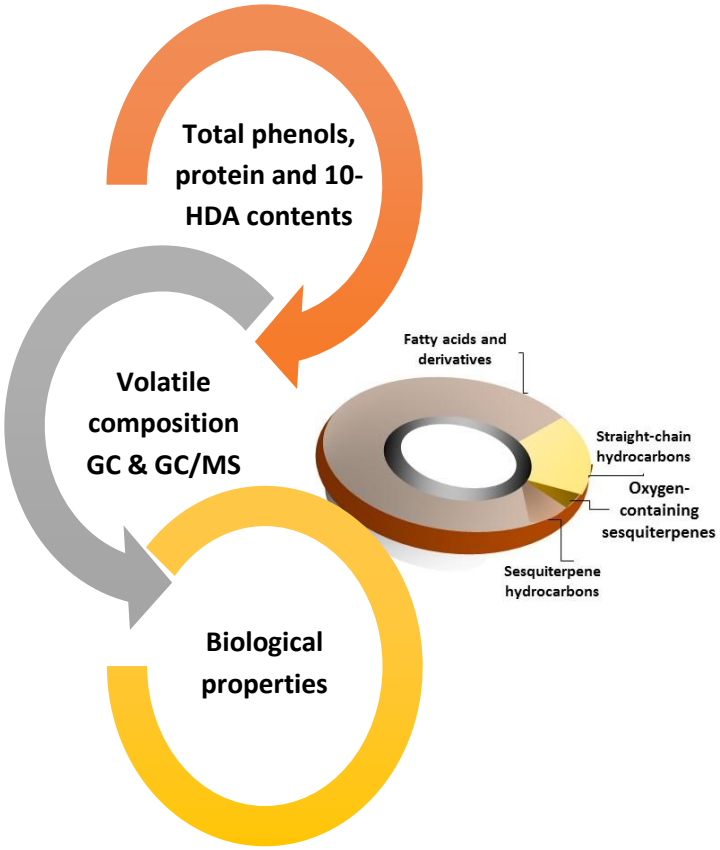
[25] F. P. Drijfhout, J. Kochansky, S. Lin, N. W. Calderone, 'Components of honeybee royal jelly as deterrents of the parasitic varroa mite, Varroa destructor', J. Chem. Ecol. 2005, 31, 1747–1764.

[26] F. Nazzi, R. Bortolomeazzi, G. della Vedova, F. del Piccolo, D. Annoscia, N. Milani 'Octanoic acid confers to royal jelly varroa - repellent properties', Naturwissenschaften. 2009, 96, 309-314.

[27] Y-z. Zhao, Z-g. Li, W-l. Tian, X-m. Fang, S-k. Su, W-j, 'Peng Differential volatile organic compounds in royal jelly associated with different nectar plants', J. Integr. Agric. 2016, 15,1157–1165. Doi:10.1016/S2095–3119(15)61274-6

Chapter 12

Chemical characterization and biological properties of royal jelly samples from the Mediterranean area



Chemical characterization and biological properties of royal jelly samples from the Mediterranean area*

Abstract

Royal jelly (RJ) is a bee product that has high nutritional value and is beneficial for the human health, earning importance as a functional food. Thus, the characterization of its main biological properties is with high importance. In this work, six RJ samples obtained in Morocco, Portugal and Spain were evaluated in terms of total phenols and flavone/flavonol contents, total protein, 10-hydroxy-2-decenoic acid (10-HDA), volatiles composition, and antioxidant, anti-inflammatory properties, and inhibition of tyrosinase, xanthine oxidase and acetylcholinesterase activities. Total phenolic content ranged from 3-9 mg Gallic Acid Equivalent/g RJ, and flavone/flavonol content between 0.1-0.5 mg Quercetin Equivalent/g RJ. 10-HDA content varied between 0.88-1.24 % and total protein from 5.47-29.72 %. Gas chromatography and gas chromatography-mass spectrometry analysis (GC-MS) showed RJ volatiles dominated by linolenic acid, 2-decenoic acid and octanoic acid in variable amounts. The antioxidant activity was monitored through nitric oxide scavenging activity (NO) and hydrogen peroxide scavenging capacity (H_2O_2), where the IC_{50} ranged from 2.270-3.356 mg/mL and from 0.238-1.453 mg/mL, respectively. Anti-acetylcholinesterase activity IC_{50} ranged from 0.705-4.645 mg/mL, while, xanthine oxidase inhibition IC_{50} ranged between 3.348-11.860 mg/mL. The results showed that phenols and flavonoids highly contributed to the RJ biological properties, in contrast to 10-HDA and proteins.

*Adapted from 'Chemical characterization and biological properties of royal jelly samples from the Mediterranean area'. Soukaïna El-Guendouz, Alexandra M. Machado, Smail Aazza, Badiaâ Lyoussi, M. Graça Miguel, M. Conceição Mateus and A. Cristina Figueiredo. Natural product communications (accepted).

12.1. Introduction

Honeybees, *Apis mellifera*, are a source of a variety of precious and valuable natural products with health-promoting bioactive compounds including honey, bee bread, bee venom, bee pollen, propolis and royal jelly (RJ).[1] RJ is a milky-white and strongly acidic (pH 3.1–3.9) fluid produced by the hypopharyngeal and mandibular glands of *A. mellifera* L. young worker honeybees (called nurses).[2] RJ serves as the most important part of honeybee larvae diet, playing a major role in caste differentiation. It is the food given to all young larvae of workers and drones in their maturation process; whereas the queen honeybee is fed RJ for her entire life cycle, nurse honeybees are fed RJ for only 3 days, then after this 3 day period, worker larvae are fed a mixture of RJ, pollen and honey.[3]

RJ is mainly composed of water (50–70%), proteins (9–18%), carbohydrates (7–18%), lipids (3-8%), mineral salts (1.5%), vitamins, polyphenols, enzymes and hormones.[4] A unique and chemically interesting feature in RJ is its lipid fraction, where 80–85% are uncommon short chain (8-12 carbon atoms) hydroxy and dicarboxylic free fatty acids.[2] The principal compound is (*E*)-10-hydroxy-2-decenoic acid (10-HDA) which represents more than 50% of the free fatty acids content and whose presence has not been reported in any other natural product or even in any other bee related product. In contrast to the protein, lipid, carbohydrate and phenolic characterization of RJ which has been deeply studied, its volatile fraction is scarcely discussed, according to the review from Miguel and El-Guendouz.[2]

Multitude pharmacological proprieties have been attributed to RJ, among these antioxidant,[4] anti-inflammatory,[1,5] anti-aging,[1] neuroprotective,[1] antimicrobial,[1,6] anti-allergic[1] and antitumoral properties.[1] Due to these attributes, RJ has been used in foods, cosmetics and pharmaceutical industry.[7]

The present work aims at unravelling the volatile fraction, total phenols, flavonoids and proteins of RJ from different geographical origins (Morocco, Portugal and Spain), as well their *in vitro* biological properties including, antioxidant, anti-inflammatory anti-acetylcholinesterase and anti-xanthine oxidase.

12.2. Experimental

12.2.1. Chemicals

ABTS (2,2'-azino bis (3thylbenzothiazoline-6-sulphonic acid), potassium dihydrogen phosphate (KH_2PO_4) and dipotassium hydrogen phosphate (K_2HPO_4), were purchased from VWR, Leuven, Belgium. Phenazine methosulfate (PMS), nicotinamide adenine dinucleotide disodium salt hydrate (NADH) and gallic acid (GA) were purchased from Acros organics, New

Jersey, USA. Nitrotetrazolium blue chloride was purchased from Sigma Aldrich Chemie, Steinheim, Germany. Phosphate buffer saline (PBS) was from Fisher Scientific, New Jersey, USA. Sodium nitroprussiate dehydrate and 2,2'-diphenyl-1-picrylhydrazyl (DPPH) were from Riedel-de Haën, Sigma-Aldrich, Seelze, Germany. Griess reagent system was purchased from Promega Corporation, Madison, USA. Folin Ciocalteu's phenol reagent, $AlCl_3$, and hydrogen peroxide (H_2O_2) were purchased from Panreac Quimica, Montcada i Reixac, Barcelona, Spain. Na_2CO_3 were purchased from Riedel de Haen (Seelze, Germany, Riedel-de-Haën Laboratory Chemicals, Germany). Quercetin was perchased Alfa Aesar GmbH & CoKG, Carsluhe, Germany. Acetylthiocholine iodide (ATCI), AChE (Type VI-S) from the electric gel Electrophorus electricus, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), sodium tetraborate, 5-lipoxygenase from soya bean, linoleic acid, mushroom tyrosinase, L-DOPA, xanthine oxidase, xanthine and methanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Boric acid and Tris–HCl were purchased from Merck, Darmstadt, Germany. Bio-Rad reagent, Bio-Rad laboratories GmbH, Heidemannstrabe, Munchen, Germany. 10-hydroxy-2-decenoic acid (10-HDA) from TCI, USA, and *n*-pentane from Carlo Erba reagents, Barcelona, Spain.

12.2.2. Royal jelly samples

In total six RJ samples from Morocco (MA), Portugal (PT) and Spain (ES) were studied. Samples from Morocco were purchased from beekeepers in Fès-Boulemane region (RJ1_MA, RJ3_MA, RJ4_MA) and Rabat-Salé-Kénitra region (RJ2_MA). Samples from Portugal (RJ5_PT) and Spain (RJ6_ES) were purchased from a commercial store. RJ3_MA and RJ4_MA were in powder form, whereas the remaining had a buttery-like appearance.

Except for volatile fraction, for proteins (Bio-Rad assay) and 10-HDA evaluations, all RJ samples were diluted in distilled water (50 mg/mL) and ultrasonicated for 60 min. After sonication, RJ samples solution were centrifuged for 10 min at 5,000 rpm and the supernatants were used as the sample solution for the following tests. The samples were stored at -20 °C until analysis.

12.2.3. Estimation of total phenolic content

The total polyphenol content in RJ samples solution was determined as previously described[8] with a slight modification. RJ sample solution (25 µL) was mixed with 125 µL of Folin-Ciocalteu reagent (0.2 N) for 5 min, then 100 µL of 7.5% Na_2CO_3 were added. All samples were incubated at room temperature for 2 h, and their absorbance was read at 760 nm. The blank solution contained distilled water instead of RJ. The total polyphenol content was

expressed as mg of Gallic Acid Equivalents per gram of RJ (mg GAE/g RJ) using a calibration curve. The concentration range of gallic acid was 0-1 mg/mL.

12.2.4. Estimation of total flavones and flavonol content

The method described by El-Guendouz et al.[8] was used for total flavonoid determination. Briefly, 100 µL of $AlCl_3$ (20%) was added to 100 µL of RJ samples solution. After 1 h of incubation at room temperature, the absorbance was measured at 420 nm. The total flavonoid contents were expressed as mg of Quercetin Equivalents per gram of RJ (mg QE/g RJ) using a calibration curve of 0-1 mg/mL.

12.2.5. Determination of total protein content in royal jelly

Bradford method (Bio-Rad assay) was used to protein content determination with minor modifications.[9] Each RJ samples (250 mg) was suspended in 10 mL methanol/water (50/50; v/v) and sonicated for 60 min. Afterwards, the pH was adjusted to 2.5 with phosphoric acid and the sample solutions were diluted 10 times. Five mL of Bio-Rad reagent diluted to 1:5 was added into 200 µL of RJ solutions and the mixture was well vortexed, then the absorbance was measured at the 595 nm after 5 min incubation. The total protein content was expressed as mg of Bovine Serum Albumin Equivalents per gram of RJ (mg BSAE/g RJ) using the BSA standard curve where the initial concentration was 0.3-1 mg/mL.

12.2.6. Determination of 10-HDA in RJ

The 10-HDA content of RJ was determined by high-performance liquid chromatography (HPLC) using an Agilent Technologies 1220 infinity LC liquid chromatography equipped with a column used was LichroCART 250-4 (Lichrospher 100 RP-18e (5 µm).[10] Ten mL of an aqueous methanol solution (50:50 v/v with Milli Q water) was added on 250 mg of RJ samples, sonicated for 60 min and adjusted at pH 2.5 with phosphoric acid. Afterwards, the sample solution was diluted 10 times and filtered through a membrane (0.45 µm). Twenty µL were injected into the HPLC to quantify 10-HDA. The mobile phase was constituted by methanol : water Milli Q (60:40) at pH 2.5 adjusted with phosphoric acid. The flow rate was adjusted to 1.0 mL/min, and detection was performed at 225 nm. The concentration of 10-HDA in RJ samples solution was determined using calibration curve of standard ranging from 0-80 µg/mL.

12.2.7. Royal jelly volatiles extraction

RJ volatiles were isolated by hydrodistillation for 1 h using a Clevenger-type apparatus

according to the European Pharmacopoeia method.[11] Given the volatiles low yield, they were recovered from the graduated tube of the Clevenger apparatus after rinsing with distilled *n*-pentane (*n*-pentane ≥99% purity, HPLC grade, is in lab distilled prior to use, to remove stabilizers that may contaminate the sample, particularly low volatiles yield samples) when the distillation procedure was over, and allowed to settle for about 10-15 min. For this procedure, the tap was opened anti-clockwise, so that the water flowed out of the connecting tube until just below the filling funnel. Distilled *n*-pentane was introduced in the filling funnel followed by water, so that pentane evaporated, with the residual heat of the distillation flask, and then condensed, and dissolved the volatiles, over the aqueous phase in the graduated tube. The tap was then open clockwise, to recover the mixture of distilled *n*-pentane and volatiles in an appropriate vial. The mixture was then concentrated to a minimum volume of about 100 µL, at room temperature under nitrogen flux, using a blow-down evaporator system. After extraction and until analysis, the RJ volatiles samples were stored at -20 °C in the dark.

12.2.8. Royal jelly volatiles composition analysis

12.2.8.1. Gas Chromatography (GC)

Gas chromatographic analyses were performed using a Perkin Elmer Clarus 400 gas chromatograph equipped with two flame ionization detectors (FIDs), a data handling system and a vaporizing injector port into which two columns of different polarities were installed: a DB-1 fused-silica column (polydimethylsiloxane, 30 m x 0.25 mm i.d., film thickness 0.25 µm; J & W Scientific Inc., Rancho Cordova, CA, USA) and a DB-17HT fused-silica column [(50% phenyl)-methylpolysiloxane, 30 m x 0.25 mm i.d., film thickness 0.15 µm; J & W Scientific Inc.]. Oven temperature was programmed, 45-175 °C, at 3 °C/min, subsequently at 15 °C/min up to 300 °C, and then held isothermal for 10 min; injector and detector temperatures, 280 °C and 300 °C, respectively; carrier gas, hydrogen, adjusted to a linear velocity of 30 cm/s. The samples were injected using split sampling technique, ratio 1:50. The volume of injection was 0.1 µL of a *n*-pentane-essential oil solution (1:1). The percentage composition of the volatiles was computed, by the normalization method from the GC peak areas, calculated as mean values of two injections, from each sample, without using the response factors.

12.2.8.2. Gas Chromatography-Mass Spectrometry (GC-MS)

The GC-MS unit consisted on a Perkin Elmer Clarus 600 gas chromatograph, equipped with DB-1 fused-silica column (30 m x 0.25 mm i.d., film thickness 0.25 µm; J & W Scientific, Inc.), and interfaced with a Perkin-Elmer 600T mass spectrometer (software version 5.4.2.1617, Perkin Elmer, Shelton, CT, USA). Injector and oven temperatures were as above; transfer line

temperature, 280 °C; ion source temperature, 220 °C; carrier gas, helium, adjusted to a linear velocity of 30 cm/s; split ratio, 1:40; ionization energy, 70 eV; scan range, 40-300 u; scan time, 1 s. The identity of the components was assigned by comparison of their retention indices, relative to C_8-C_{30} *n*-alkane indices and GC-MS spectra from a lab-made library, created with reference essential oils, laboratory-synthesized components, laboratory isolated compounds and commercially available standards.

12.2.9. Antioxidant activity

12.2.9.1. DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging activity

Scavenging of the DPPH radical was assayed following the method previously described,[12] with some modifications. RJ samples solution (5-50 mg/mL) with different concentrations were prepared and 350 µL of each solution were added to 650 µL of DPPH solution (60 µM).

The absorbance of sample (Abs_{sample}) was read after 30 min of incubation time at room temperature and compared with the absorbance of the control (Abs_{cont}) in which the sample was replaced by distilled water. The scavenging effect percentage was calculated from the formula $[(Abs_{cont} - Abs_{sample}) / Abs_{cont}] \times 100$. Analyses were carried out in triplicate. The percentage was plotted against the extract concentration, and IC_{50} values were determined (concentration of sample able to scavenger 50% of the DPPH free radical). Butylated hydroxytolu-ene (BHT) was used as positive control at a concentration ranging between 0.03 and 1 mg/mL.

12.2.9.2. ABTS (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) free radical-scavenging activity

Determination of the ABTS radical scavenging activity was carried out as reported by El-Guendouz et al..[13] Briefly, the ABTS radical was generated by reaction of a 7 mM ABTS aqueous solution with $K_2S_2O_8$ (2.45 mM) in the dark, for 16 h, and adjusting the absorbance at 734 nm to 0.7, at room temperature. RJ samples solution (5-50 mg/mL) (350 µL) were added to 650 µL of ABTS and the absorbance at 734 nm was read after 6 min. The capability to scavenge the $ABTS^+$ was calculated using the formula: ABTS scavenging activity (%) = $[(Abs_{cont} - Abs_{sample}) / Abs_{cont}] \times 100$. Analyses were carried out in triplicate. The sample concentration providing 50% inhibition (IC_{50}) was obtained by plotting the inhibition percentage against RJ concentrations. BHT was used as positive control at a concentration ranging between 0.03 and 1 mg/mL.

12.2.9.3. Scavenging ability of superoxide anion radical

Scavenging ability of superoxide anion radical was evaluated as previously reported.[12] Superoxide anions were generated in a non-enzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS-NADH) system by oxidation of NADH and assayed by reduction of nitrotetrazolium blue chloride, (NBT). The superoxide anion was generated in 200 µL of phosphate buffer (19 mM, pH = 7.4) containing 25 µL of NBT (514 µM) solution, 25 µL of NADH (1989 µM) solution and 25 µL of different concentrations of RJ (5-50 mg/mL). The reaction was started with the addition of 25 µL of PMS solution (816 µM) to the mixture.

The reaction mixture was incubated at room temperature for 10 min and the absorbance reading was performed at 560 nm. The percentage of inhibition was calculated using the following equation: Inhibition = $[(Abs_{cont} - Abs_{sample}) / Abs_{cont}] \times 100$. Tests were carried out in triplicate. The sample concentration providing 50% inhibition (IC_{50}) was obtained by plotting the inhibition percentage against RJ concentrations. Ascorbic acid was used as positive control at a concentration ranging between 0.03 and 1 mg/mL.

12.2.9.4. Nitric oxide (NO) scavenging activity

The nitric oxide (NO) scavenging activity was measured as in Majdoub et al..[14] In this method, 50 µL of different concentrations of each sample (5-50 mg/mL) was added to 50 µL of 10 mM sodium nitroprusside in phosphate-buffered saline (PBS) into a 96-well plate and the plate was incubated at room temperature, for 90 min. Finally, an equal volume (100 µL) of Griess reagents was added to each well and the absorbance was immediately read at 532 nm. Several concentrations of samples were made and the percentage of inhibition was calculated from the formula: $[1 - (Abs_{sample} - Abs_{sample}\ blank) / (Abs_{control} - Abs_{control}\ blank)] \times 100$, where $(Abs_{sample} - Abs_{sample}\ blank)$ is the difference in the absorbance of a sample, with or without 10 mM sodium nitroprusside, and $(Abs_{control} - Abs_{control}\ blank)$ is the difference in the absorbance of the PBS control, with or without 10 mM sodium nitroprusside. Tests were carried out in triplicate. The sample concentration providing 50% inhibition (IC_{50}) was obtained by plotting the inhibition percentage against extracts concentrations.

12.2.9.5. Hydrogen peroxide scavenging capacity (H_2O_2)

The ability of the RJ extracts to scavenge hydrogen peroxide was determined according to the method of Ruch et al.,[15] with slight modifications. A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (50 mM, pH = 7.4). The volume (150 µL) of each RJ concentration (5-50 mg/mL) were added to 600 µL of hydrogen peroxide solution (40 mM),

1 mL of phosphate buffer (50 mM, pH = 7.4) and 1650 µL of distilled water. Absorbance of hydrogen peroxide at 230 nm was determined 20 min later against a blank solution containing the phosphate buffer without hydrogen peroxide. Tests were carried out in triplicate and the values of IC_{50} were determined as reported above. Ascorbic acid was used as positive control at a concentration ranging between 0.03 and 1 mg/mL.

12.2.10. Enzymatic activities

12.2.10.1. Inhibition of lipoxygenase

The inhibition of lipoxygenase was carried out as previously reported.[8] The reaction was initiated by the addition of 100 µL of samples, 50 µL linoleic acid (0.001 M), 937 µL borate buffer and 5 µL 5-lipoxygenase solution (0.054 g in 1 mL borate buffer 0.005%, Tween 0.1 M, pH 9), then the absorbance was read at 234 nm after 2-3 min incubation. The enzymatic reactions were performed in absence or in presence of RJ samples solution (5-50 mg/mL) and their kinetics were compared. The inhibition percentage of the enzyme was calculated and the IC_{50} values were compared. Analyses were carried out in triplicate. Nordihydroguaiaretic acid was used as positive control at a concentration ranging from 1 to 0.03 mg/mL.

12.2.10.2. Inhibition of tyrosinase

The evaluation of the inhibitory action of RJ on tyrosinase was performed according to El-Guendouz et al.[8] with slight modifications. Briefly, 420 µL phosphate buffer (50 mM, pH 6.5), 200 µL of sample (5-50 mg/mL), 180 µL of mushroom tyrosinase solution (100 U/ml) were mixed. The assay mixture was incubated at 25 °C, for 40 min. Following incubation, 180 µL of 5 mM L-DOPA solution was added and the amount of dopachrome produced in the reaction mixture was determined spectrophotometrically at 492 nm after 3 min. The percentage of inhibition of tyrosinase activity was calculated and the IC_{50} was determined. Kojic acid was used as positive control at a concentration ranged between 1 and 0.03 mg/mL.

12.2.10.3. Inhibition of acetylcholinesterase

The acetylcholinesterase inhibition assay was performed following Miguel et al.[16] with minor modification. Into 180 µL of Tris-HCl buffer (0.1 M, pH 8), 200 µL of different RJ sample solution (5-50 mg/mL) and 150 µL of acetylcholinesterase enzyme (0.28 U/mL) were added, then the mixture was left to stand for 15 min at room temperature. Afterwards, 180 µL of iodine acetylcholine (0.005 g in 10 mL of Tris-HCl buffer) and 375 µL of 5,5' dithiobis-2-nitrobenzoic acid (DTNB) (0.059 g in 50 mL of buffer) were added and the mixture was incubated for 30 min at room temperature. The inhibition percentage of enzyme was calculated

as follows: Inhibition = [(Abs_{cont} − Abs_{sample}) / Abs_{cont}] x 100. The analyses were carried out in triplicate. Samples concentration providing 50% inhibition (IC_{50}) was obtained plotting the inhibition percentage against sample concentrations. Galantamine was used as positive control (0.03-1 mg/mL).

12.2.10.4. Inhibition of xanthine oxidase

Xanthine oxidase inhibiting was performed as El-Guendouz et al.[8] An amount of 200 µL of RJ sample solution (5-50 mg/mL), 350 µL of phosphate buffer (pH 7.5, 50 mM) and 50 µL of xanthine oxidase enzyme solution freshly prepared (0.4 units/mL in phosphate buffer, pH 7.5) were mixed and incubated at room temperature for 15 min. Then 330 µL of 0.150 mM xanthine solution were added and the mixture was reincubated for 30 min under the same condition. The absorbance was measured at 290 nm after adding 100 µL of 1 M HCl. The assay was done in triplicate and IC_{50} values were calculated from the percentage of inhibition. Allopurinol was used as positive control at the concentration of 1mg/mL.

12.2.11. Statistical analysis

Statistical analysis was performed with the Statistical Package for the Social Sciences (SPSS) 23.0 software (SPSS Inc., Chicago, IL). Statistical comparisons were made with one-way analysis of variance followed by Tukey multiple comparisons. The level of significance was set at $P < 0.05$. Correlations between phenol and flavonoid content and enzyme inhibitory activities were achieved by Pearson correlation coefficient (r) at a significance level of 95%.

The percentage composition of RJ volatiles was used to determine the relationship between the different samples by cluster analysis using Numerical Taxonomy Multivariate Analysis System (NTSYS PC software, version 2.2, Exeter Software).[17] For cluster analysis, correlation coefficient was selected as a measure of similarity among all samples, and the Unweighted Pair Group Method with Arithmetical Averages (UPGMA) was used for cluster definition. The degree of correlation was evaluated according to Pestana and Gageiro[18] in very high (0.90-1.0), high (0.70-0.89), moderate (0.40-0.69), low (0.20-0.39) and very low (<0.20).

12.3. Results and discussion

12.3.1. Phenols and flavonoids contents

Total phenols, flavones and flavonols found in RJ samples solution are displayed in Table 12.1 as means ± standard deviation, with each determination performed in triplicate. The comparison of the values between the different RJ samples revealed a significant difference between powder form of RJ samples from Morocco RJ3_MA and RJ4_MA and those from

Portugal (RJ5_PT) Spain (RJ6_ES) and Morocco (RJ1_MA and RJ2_MA). Total polyphenol content varied from 3.1 mg GAE/g (RJ1_MA) to 9.0 mg GAE/g (RJ3_MA) in RJ samples, while flavonoid content ranged from 0.1 mg QE/g (RJ1_MA) to 0.5 mg QE/g (RJ3_MA) (Table 12.1).

The values of phenols in RJ samples were higher than those reported by Özkö and Silici[19] for RJ samples originating from Turkey (59.2 mg GAE/100 g). Other authors[20,21] reported higher amounts of total phenols in local and commercial RJ samples from Romania (14.6-39.9 mg GAE/g RJ and 15.4-32.5 mg GAE/g RJ, respectively) than those found in the present samples. Although the amounts of total flavonoids have been calculated with different standard compound (rutin) than that used in the present work, Nabas et al.[22] reported an amount of 1.3±0.1 μg rutin/mg RJ in Jordanian RJ samples, much lower than those found in samples of the present work (Table 12.1).

Table 12.1. Phenols, flavonoids, protein and 10-HDA content of RJ samples represented as mean ± SD

Samples code	Phenols (mg GAE/g RJ)	Flavonoids (mg QE/g RJ)	Protein content (%)	10-HDA (%)
RJ1_MA	3.1 ± 0.1^{b}	0.1 ± 0.0^{d}	11.68 ± 0.40^{c}	1.20 ± 0.02^{b}
RJ2_MA	3.0 ± 0.1^{b}	0.1 ± 0.1^{d}	29.72 ± 0.89^{e}	1.02 ± 0.01^{d}
RJ3_MA*	9.0 ± 0.8^{a}	0.5 ± 0.0^{a}	7.96 ± 0.31^{b}	0.88 ± 0.01^{f}
RJ4_MA*	8.9 ± 0.5^{a}	0.4 ± 0.1^{b}	5.47 ± 0.50^{a}	0.95 ± 0.01^{e}
RJ5_PT	3.1 ± 0.1^{b}	0.2 ± 01^{d}	6.62 ± 0.50^{ab}	1.13 ± 0.02^{c}
RJ6_ES	3.1 ± 0.0^{b}	0.3 ± 0.0^{c}	25.56 ± 0.22^{d}	1.24 ± 0.01^{a}

* RJ powder form. Values followed by different letters in the same column are significantly different by Duncan test. ($p < 0.05$).

12.3.2. Proteins content

Like for phenols and flavonoids contents, there was a significant difference on protein content in RJ samples, Table 12.1. Samples from Morocco and Spain (RJ2_MA and RJ6_ES) presented the highest amount of protein content (29.72 ± 0.89 and 25.56 ± 0.22 %, respectively), while samples RJ4_MA showed the lowest amount (5.47± 0.50 %).

Kanelis et al.[23] found 10.5 to 21.0% of protein in RJ samples from Greece. In the present study, there were two samples (RJ2_MA and RJ6_ES) in which the protein content was higher than 20%, the higher percentage reported by Kanelis et al.[23] for Greek RJ. Pavel et al.[24] reported that fresh and commercial samples of RJ from Romania presented protein values ranging from 7.1 to 17.7%. Samples from different regions of Bulgaria were tested and similar results to those reported by Pavel et al.[24] were found. Balkanska et al.[25] reported mean protein values of 16.1 and 13.2 %. In the present study, the lowest protein content was observed in powder samples. There are two methods considered adequate for determining the total protein

content:[9] the Bradford and Lowry methods. According to the authors, both methods are equally sensitive, although the Lowry method being more time consuming. In the present study both methods were used and the results were different (data not shown in Table 12.1). For example, in the Lowry method, samples RJ3_MA and RJ4_MA had the highest concentrations of proteins (> 60%) remarkably high for RJ samples. Therefore, some interferences could have happened between Lowry reagent and some components of the RJ, such as phenolic compounds.[26] The same samples had the highest concentrations of phenol and flavonoid compounds (Table 12.1).

12.3.3. 10-Hydroxy-2-decenoic acid (10-HDA) content

10-HDA content values were significantly different in RJ samples varying from 0.88 to 12.4 %, Table 12.1. The overall comparison showed variation between all samples. In this case, the sample from Spain (RJ6_ES) presented the higher amount of 10-HDA followed by that from Morocco (RJ6_ES: 1.24±0.01 %, RJ1_MA: 1.20±0.02 %), while samples from Morocco in the powder form (RJ3_MA and RJ4_MA) had the lowest values (0.88±0.01 and 0.95±0.01 % respectively).

The values found in the present work were within the range of values reported by several authors. Ferioli et al.[27] reported that 10-HDA content in fourteen samples originating from Italy, Australia, China and South America ranged between 0.8 and 3.2 g/100 g of RJ. Kanelis et al.[22] described that the content of 10-HDA in the RJ from different countries ranged from 0.8 to 3.4%.

Several factors could be considered as indicators of freshness and quality during the storage (10-HAD, proteins, moisture, viscosity, coloration), nevertheless none of them consider the natural variability among RJ samples,[28] as observed in the present work.

12.3.4. Royal jelly volatiles

Royal jelly volatiles were obtained in a yield <0.05% (v/w). Their chemical composition was a complex mixture in which up to sixty-six components were identified. The isolated royal jelly volatiles are listed in Table 12.2, following their elution order on the DB-1 column, and arranged according to the two groups defined by agglomerative cluster analysis (Figure 14.1).

Cluster analysis showed two moderately correlated clusters ($S_{corr} > 0.54$) (Figure 14.1). Cluster I, comprised sample 1 from Morocco and the sample from Portugal, which were dominated by linolenic acid (RJ_1_MA 35%, RJ_5_PT 50%), octanoic acid (RJ_1_MA 17%, RJ_5_PT 18%) and trans-2-decenoic acid (RJ_1_MA 24%, RJ_5_PT 9%). Cluster II included the four remaining samples, whose volatiles main compounds were trans-2-decenoic acid (30-47%), octanoic acid (21-38%) and linolenic acid (2-17%).

It is still noteworthy the marked difference in the linolenic acid and trans-2-decenoic acid between RJ3_MA (2% and 47%, respectively) and RJ5_PT (50% and 9%, respectively) (Table 12.2). Other remarkable difference was the n-heneicosane (C_{21}) content, between RJ6_ES and the remaining RJ samples. The percentage of this straight chain saturated hydrocarbon in RJ6_ES was 19%, whereas in the remaining RJ samples the percentages did not exceed 0.3% (Table 12.2). The amounts of n-tricosane (2%), n-pentacosane (1%) and n-heptacosane (1%) were higher in RJ2_MA than in the remaining RJ samples (traces-1%) (Table 12.2).

The presence of the synthetic antioxidant butylated hydroxytoluene (BHT) in RJ4_MA denotes its addition, maybe for preventing oxidation. 2-Furfural (4.6%) only present in RJ3_MA, which is not a constitutive metabolite of RJ samples, may derive from a prolonged storage of this sample or heat exposure before extraction.[29] The plasticizer dibutyl sebacate was detected, despite in relative amount ≤1%, in samples RJ3_MA, RJ4_MA and RJ6_ES, which may indicate a prolonged storage in plastic containers that should be avoided.

The presence of fatty acids in RJ was also reported by Nazzi et al.[30] nevertheless in the present work hydroxyl-derivatives of fatty acids were not detected in the volatile fraction, such as 10-HDA, maybe because of the different type of extraction used in both cases. In the present work, the hydroxylated-fatty acids may have remained in the hydrolate not being recovered by pentane. Other factors, such as harvesting time, region of origin, storage method and processing technology may determine the differences found between the volatile composition of the current studied samples and those previously reported.[29-32]

Table 12.2. Percentage composition of the volatiles isolated by hydrodistillation from different RJ samples. For samples grouped on each of the clusters and subclusters see Figure 12.1.

Components	RI	Royal jelly samples					
		Cluster I		Cluster II			
		RJ 1_MA	RJ 5_PT	RJ 2_MA	RJ 3_MA	RJ 4_MA	RJ 6_ES
n-Octane	800	t	0.1	t	t	t	t
2-Furfural	825	t	0.1	t	4.6	t	t
2-Heptanone	886	t		t	t		t
n-Nonane	900						t
trans-1,3-Nonadiene *	916						1.0
Benzaldehyde	927	t	t	t	t	t	t
α-Pinene	930	t	t	t	t	t	t
Hexanoic acid (= Caproic acid)	968	t	0.2	t	t	t	t
β-Myrcene	975	t	t	t	t		
2,4-Heptadienal	975	t		t	t		t
Benzene acetaldehyde	1002					t	t
p-Cymene	1003					t	t
Limonene	1009						t
2-Methyl decane	1046					t	t
Fenchone	1050					t	
2-Nonanone	1058	t		t	t	t	t
n-Nonanal	1073	t		t	t	t	t
Linalool	1074					t	
Octanoic acid (= Caprylic acid)	1149	16.8	17.9	37.8	35.6	30.4	21.1
Ethyl octanoate	1177					0.6	
n-Decanal	1180	t		t	t	0.8	t
Cuminaldehyde	1200					t	t
trans-Anethole	1254		t			t	t
Nonanoic acid (= Pelargonic acid)	1263	t		t	t	0.8	t
2-Undecanone	1275					t	
Carvacrol	1286	t	t	t	t	t	t
trans-2-Nonenoic acid	1291	t		t	t	t	1.0
Carvacrol acetate	1348						t
Decanoic acid (= Capric acid)	1356	0.5	0.3	t	1.8	3.7	3.4
trans-2-Decenoic acid	1383	23.6	8.5	29.8	46.7	44.3	38.8
n-Dodecanol	1468	t		t	t		t
ar-Curcumene	1474					0.9	
α-Zingiberene	1492					t	
Butylated hydroxytoluene (BHT)	1492					1.0	
β-Bisabolene	1500					0.5	
n-Pentadecane	1500	t	0.2	t	t	0.1	t
Dodecanoic acid (= Lauric acid)	1550	t		t	t	t	t
Cedrol	1574					t	
n-Hexadecane	1600	t		t	t		
T-Cadinol (= *epi*-α-cadinol)	1616					0.8	1.2
α-Cadinol	1626					t	t
δ-Dodecalactone	1640					t	t
n-Heptadecane	1700	t		t	t	t	t
Tetradecanoic acid (= Myristic acid)	1723						t
n-Hexadecanal	1776						t
Hexadecanoic acid (= Palmitic acid)	1908	2.5	2.6	2.1	t	2.2	1.9
n-Octadecanal	2008	t	0.6	t	t	t	t
n-Octadecanol	2095	t	1.2	t	t	t	t
Methyl oleate	2096		t				
n-Heneicosane	2100	0.2	0.3	t	t	t	19.1
Linolenic acid	2249	35.2	50.3	17.1	2.2	7.2	6.4
Oleic acid	2250	11.7	7.3	5.7	t	2.4	3.2
Dibutyl sebacate	2250				t	1.2	t
Ethyl linolenate	2251	t	t	t	t	t	t

Components	RI	Royal jelly samples					
		Cluster I		Cluster II			
		RJ 1_MA	RJ 5_PT	RJ 2_MA	RJ 3_MA	RJ 4_MA	RJ 6_ES
n-Tricosane	2300	0.6	0.6	1.9	t	t	t
n-Pentacosane	2500	0.6	0.6	1.4	t	t	t
n-Heptacosane	2700	0.7	0.7	1.3	t	t	t
n-Nonacosane	2900	0.2	0.3	t	t	t	t
% identification		97.6	95.6	97.1	90.9	96.9	97.1
Grouped components							
Monoterpene hydrocarbons		t	t	t	t	t	t
Oxygen-containing monoterpenes		t	t	t	t	t	t
Sesquiterpene hydrocarbons						1.4	
Oxygen-containing sesquiterpenes						0.8	1.2
Phenylpropanoids			t			t	t
Fatty acids and derivatives		95.3	90.9	92.5	86.3	91.6	75.8
Straight-chain hydrocarbons		2.3	2.8	4.6	t	0.1	19.1
Others		t	1.9	t	4.6	3.0	1.0

RI: In lab calculated Retention Index relative to C_8-C_{30} *n*-alkanes on the DB-1column. RJ: royal jelly, 1-4: sample number, MA, PT and ES: two letters code for countries names, Morocco, Portugal and Spain, respectively. * Identification based on mass spectra only. t: trace (<0.05%).

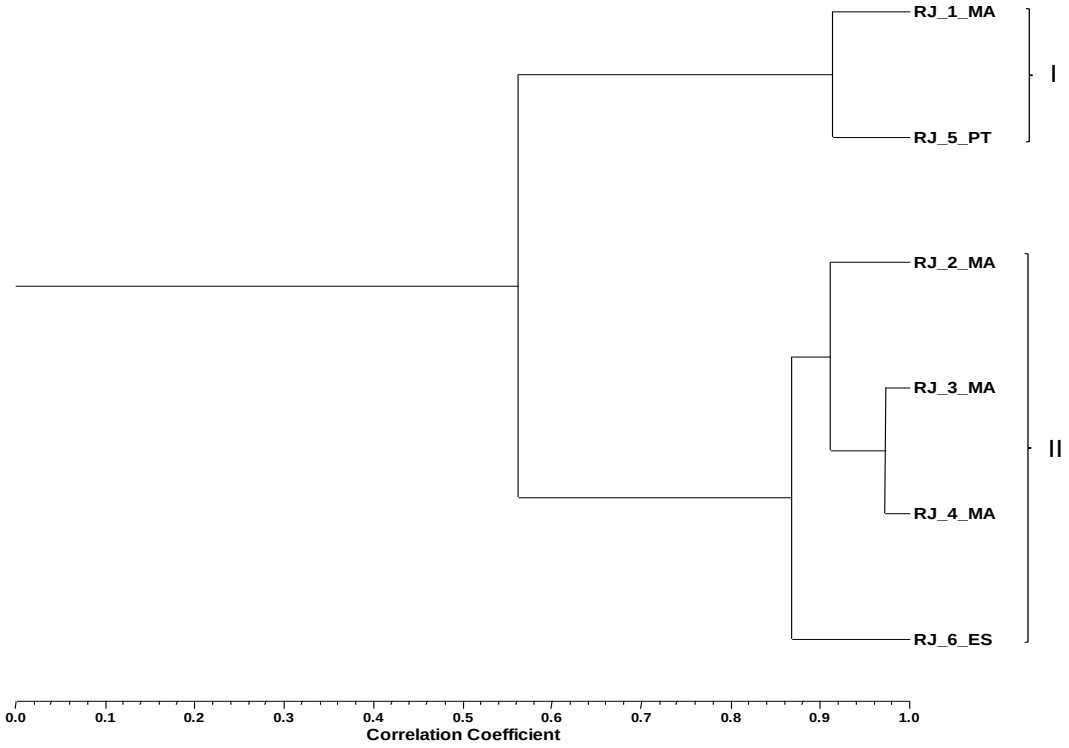


Figure 12.1. Dendrogram obtained by cluster analysis of the percentage composition of the volatiles isolated from the royal jelly samples evaluated, based on correlation and using unweighted pair-group method with arithmetic average (UPGMA). RJ: royal jelly, 1-4: sample number, MA, PT and ES: two letters code for countries names, Morocco, Portugal and Spain, respectively.

12.3.5. Antioxidant activities

The antioxidant activity was checked by evaluating the capacity for scavenging diverse free radicals (DPPH, ABTS, superoxide, NO) and H_2O_2. A significant variability ($p < 0.05$) of

antioxidant properties was found among samples, Table 12.3. Among all RJ samples, RJ3_MA and RJ4_MA (sample in powder form from Morocco) were in the forefront regarding their antioxidant capacities, with the lower IC_{50} values: 2.007 and 2.842 mg/mL; 2.018 and 2.076 mg/mL; 0.018 and 0.019 mg/mL; 2.270 and 2.270 mg/mL; 0.506 and 0.895 mg/mL for their ability of scavenging ABTS and DPPH, superoxide, NO free radicals, and H_2O_2, respectively. The sample from Portugal (RJ5_PT) possessed the higher IC_{50} in almost all antioxidant assays (lower activity). Samples from Morocco RJ2_MA and the one from Spain (RJ6_ES) presented intermediate IC_{50} values.

Nevertheless, such activities were significantly lower ($p < 0.05$) to those found for the positive controls (BHT, ascorbic acid and curcumin) (Table 12.3). The relative weak antioxidant activity evaluated in *in vivo* system was already previously observed,[33] because the increase of the superoxide dismutase, catalase, and gluthatione peroxidase activities as well as the gluthatione levels, in rats with N-methyl-N-nitrosourea (MNU) induced breast cancer, was not enough to reduce the oxidative stress.

The antioxidant activities increased depending on the concentration of the total phenolic content in the sample, which was proved by the inverse correlation found between the IC_{50} values found and the concentration of total phenols (Table 12.4).

Table 12.3. Antioxidant activity of RJ, measured by distinct methods, and expressed in IC_{50} (mg/mL), (mean ± SD).

Samples code	DPPH (mg/ mL)	ABTS (mg/ mL)	Superoxide (mg/ mL)	NO (mg/ mL)	H_2O_2 (mg/ mL)
RJ1_MA	5.724±0.099^{b}	12.851±0.114^{b}	0.076±0.001^{c}	3.356±0.039^{a}	1.028±0.002^{b}
RJ2_MA	6.383±0.619^{b}	9.548±1.239^{c}	0.092±0.001^{b}	2.869±0.009^{b}	0.238±0.015^{f}
RJ3_MA*	2.018±0.007^{c}	2.007±0.007^{d}	0.018±0.000^{d}	2.270±0.169^{c}	0.506±0.001^{e}
RJ4_MA*	2.076±0.033^{c}	2.842±0.067^{d}	0.019±0.000^{d}	2.270±0.169^{c}	0.895±0.009^{c}
RJ5_PT	11.720±0.093^{a}	15.736±0.000^{a}	0.101±0.001^{a}	2.926±0.144^{b}	1.453±0.008^{a}
RJ6_ES	0.215±0.014^{d}	12.724±0.344^{b}	0.075±0.000^{c}	3.252±0.116^{a}	0.775±0.004^{d}
BHT	0.022±0.001^{d}	ND	ND	ND	ND
BHT	ND	0.004±0.000^{e}	ND	ND	ND
Ascorbic acid	ND	ND	0.013±0.001^{e}	ND	ND
Curcumin	ND	ND	ND	0.040±0.002^{d}	ND
Ascorbic acid	ND	ND	ND	ND	0.098±0.005^{g}

RJ powder form. Values followed by different letters in the same column are significantly different by Duncan test. (p <0.05).

The observed correlation between antioxidant activities and the amounts of the group of compounds (phenols, flavonoids) in samples showed the importance of these groups of compounds on the activity, suggesting that the observed antioxidant activity and scavenging ability against active oxygen species and free radicals can be assigned to phenolic fractions in RJ. On the other hand, and on opposite to Balkanska et al.,[34] who reported 10-HDA to be

responsible for 74% of the antioxidant capacity of the RJ samples, in the present work a positive correlation was detected between the antioxidant activities, expressed as IC_{50} values, and the total 10-HDA content in the studied RJ samples (Table 12.4). As such, 10-HDA content influenced negatively the antioxidant activity, since the higher the 10-HDA concentration, the greater the amount of RJ required to provide 50% inhibition.

Some authors[19,35-36] reported that protein and phenolic fractions of RJ have high antioxidant and free radical scavenging activities against reactive oxygen species. The antioxidant impacts were attributed to some biomolecules and compounds of RJ such as albumin proteins as well as the polyphenolic compounds.[10,37] In this study, only phenolic compounds contributed to the antioxidant activity, due to the negative correlation observed between total phenol content in samples and IC_{50} values (Table 12.4), except for hydrogen peroxide scavenging activity. In this case, proteins revealed to have a role in the capacity for scavenging hydrogen peroxide (negative correlation between IC_{50} values and total amount in samples), despite the small number of samples used in this assay.

Table 12.4. Pearson correlation coefficients among total phenols flavonoids, proteins content, 10-HDA and antioxidant activities (IC_{50}).

	Total phenol	**Flavo-noids**	**Proteins content**	**10-HDA content**	**DPPH**	**ABTS**	**Superoxide scavenging**	**Nitric oxide**	**Hydrogen peroxide**
Total phenol	1	0.890**	-0.581*	-0.826**	-0.486*	-0.923**	-0.958**	-0.890**	-0.198
Flavo-noids	0.890**	1	-0.354	-0.722**	-0.591**	-0.837**	-0.862**	-0.822**	-0.297
Protein content	-0.581*	-0.354	1	0.357	-0.186	0.292	0.488*	0.466	-0.614**
10-HDA content	-0.826*	-0.722**	0.357	1	0.169	0.875**	0.708**	0.926**	0.446

* Correlation is significant at the $p < 0.05$ level. ** Correlation is significant at the $p < 0.01$ level.

12.3.6. Effects on inhibitory activity of enzymes

Inhibitory propriety of RJ on lipoxygenase (LOX), tyrosinase, acetylcholinesterase (AChE) and xanthine oxidase were investigated for all samples and results are displayed in Table 12.5. The screening of the LOX inhibitory activity in RJ samples revealed an important activity, Table 12.5, with a significant difference between all tested samples ($p < 0.05$). Samples RJ3_MA and RJ4_MA (Morocco/ powder form) exhibited the higher capacity to inhibit the 5-LOX enzyme with the lower IC_{50} values (1.115 and 1.108 mg/mL respectively), followed by sample from Spain (RJ6_ES: 2.796 mg/ mL) and the ones from Morocco (RJ1 and RJ2) and Portugal (RJ5), Table 12.5. However, the exhibited activities were much lower when compared to the positive control Nordihydroguaiaretic acid (Table 12.4). Moreover, person correlation

coefficients among total phenols, and flavonoids content and 5-LOX enzyme inhibitory activity demonstrated a high inverse correlation which means that 5-LOX inhibitory activity can be attributed to those groups of compounds.

Diverse studies have demonstrated the anti-inflammatory activity of RJ by suppressing the production of proinflammatory cytokines due to phenolic compounds.[38,39] The present work permitted to demonstrate that RJ is also able to inhibit LOX, which has been considered both an indicator of antioxidant and anti-inflammatory activities.[8]

Table 12.5. Enzyme inhibitory activities (IC_{50}= mg/g RJ) of RJ represented as mean ± standard error.

Samples code	Lipoxygenase	Tyrosinase	Acetylcholinesterase	Xanthine oxidase
RJ1	3.077±0.062^{a}	9.344±0.473^{a}	3.568±0.019^{b}	10.992±0.226^{ab}
RJ2	3.088±0.009^{a}	4.729±0.114^{c}	4.645±0.027^{a}	11.860±0.283^{a}
RJ3*	1.115±0.085^{c}	3.544±0.335^{d}	0.705±0.017^{c}	3.348±0.019^{d}
RJ4*	1.108±0.007^{c}	3.610±0.075^{d}	1.198±0.170^{c}	3.768±0.161^{d}
RJ5	3.072±0.032^{a}	5.863±0.108^{b}	3.140±0.210^{b}	9.968±0.291^{b}
RJ6	2.769±0.027^{b}	4.827±0.389^{bc}	3.063±0.063^{b}	8.115±0.112^{c}
Nordihydroguaiaretic acid	0.003±0.000^{e}	ND	ND	ND
Kojic Acid	ND	0.006±0.000^{e}	ND	ND
Galantamine	ND	ND	0.006±0.000^{d}	ND
Allopurinol	ND	ND	ND	0.015±0.001

* RJ powder form. Values followed by different letters in the same column are significantly different by Duncan test. (p <0.05).

As tyrosinase inhibitors, Table 12.5, RJ samples from Morocco (RJ3_MA and RJ4_MA/ powder form) were the most efficient (3.544 and 3.610 mg/mL, respectively), followed by another sample from Morocco (RJ2_MA: Rabat-Salé-Kénitra region) with an IC_{50} value of 4.729 mg/mL, sample from Spain (RJ6_ES) with an IC_{50} value of 4.827 mg/mL, sample from Portugal (RJ5_PT) with an IC_{50} value of 5.863 mg/mL; and sample from Morocco (RJ1_MA: Fès-Bouleman). Similarly, to that observed for lipoxygenase activity, the kojic acid (positive control) was more active in the inhibition of the tyrosinase enzyme. An inverse correlation was observed between the inhibition activity presented as IC_{50} values and the concentrations of total phenols, and flavonoids content, demonstrating that these compounds have an important role tyrosinase inhibition, Table 12.6.

For melanin overproduction disorders, several treatments are based on tyrosinase inhibitor substances, since tyrosinase is the key enzyme in melanogenesis.[40] Han et al.[41] investigated RJ samples from Inje County, Gangwon Province, Korea and have shown that RJ reduces melanin synthesis by down-regulation of tyrosinase mRNA transcription. Peng et al.[42] in Taiwan RJ attributed the suppression of skin pigmentation to 10-HDA. In the present work, the inhibition of tyrosinase (IC_{50} values) is well negatively correlated with the content of phenols and flavonoids; but positively correlated with 10-HDA content, Table 12.6. The

presence of 10-HDA seems to promote the action of tyrosinase since a positive correlation between IC_{50} values and amounts of 10-HDA was observed, in contrast to that observed by those authors.[42]

Table 12.6. Pearson correlation coefficients among total phenols flavonoids, proteins content, 10-HDA and enzyme inhibitory activities.

	Total phenol	Flavonoids	Proteins content	10-HDA content	LOX	Tyrosinase	AChE	Xanthine Oxidase
Total phenol	1	0.890**	-0.581*	-0.826**	-0.987**	-0.609**	-0.911**	-0.930**
Flavonoids	0.890**	1	-0.354	-0.722**	-0.912**	-0.788**	-0.875**	-0.937**
Proteins content	-0.581*	-0.354	1	0.357	0.532*	-0.24	0.689**	0.541*
10-HDA content	-0.826**	-0.722**	0.357	1	0.786**	0.656**	0.616**	0.652**

* Correlation is significant at the $p < 0.05$ level. ** Correlation is significant at the $p < 0.01$ level.

All RJ samples investigated during this study exhibited anti-AChE effect, Table 12.5. Samples from Morocco RJ3 and RJ4 (powder form), exhibited higher AChE inhibitory activity than the remaining samples, with IC_{50} values of 0.705 and 1.198 mg/mL, respectively. Samples from RJ1_MA (Rabat-Salé-Kénitra region), Portugal (RJ5_PT) and Spain (RJ6_ES) showed an intermediary IC_{50} values (3.568; 3.140 and 3.063 mg/mL, respectively), while the lowest ability to inhibit AChE activity was for sample RJ2_MA (Fès-Boulemane region). Among the six samples investigated as shown in Table 12.5, all samples presented a significantly ability for inhibiting AchE, but lower to that of the galantamine. Furthermore, a strong negative correlation was also found between the amounts of amounts of phenol and flavonoids and IC_{50} values, Table 12.6.

Alzheimer's disease (AD) is mainly associated with aging and characterized by a cognitive impairment and irreversible and progressive mental atrophy.[19] Up to date, there is no conclusive treatment for AD, for the treatments just delay the progression of the disease and manage some of the symptoms.[14] A common pharmacological therapy for this disease is based on drugs containing acetylcholinesterase inhibitors having the capacity to restore the level of acetylcholine in the brain. Thus, the search for sources of AChE inhibitors compounds is highly required. Recently, Pan et al.[43] demonstrated that RJ was able to decrease the levels of oxidative markers such as malondialdehyde (MDA) and also to diminish the levels of AChE in the brain of ovariectomized cholesterol-fed rabbits, which are more susceptible to AD. In other *in vivo* study, the authors[44] observed that RJ was also able improving the cognitive ability of rats submitted to trimethylin, and stimulate the regenerating process of the injured hippocampal dentate gyrus. In the present work, total phenol and flavonoid contents negatively correlated

with IC_{50} values, which reveal to be a role in the activity found in opposite to the amounts of proteins and 10-HDA which positive correlation between the IC_{50} values and their amounts in RJ has a negative role on the inhibition of AChE, Table 12.6.

All RJ samples investigated exhibited an XO inhibitory activity, Table 12.5. Overall, significant differences between activities of tested samples were observed. As expected, samples from Morocco RJ3_MA and RJ4_MA (powder form), had the lower IC_{50} (values of 3.348 and 3.768 mg/mL), which means a good XO inhibitory activity. While, for this test, sample from Spain was in second rank with an IC_{50} value of 8.115 mg/mL, followed by samples from Portugal RJ5 and those from Morocco RJ1_MA then RJ2_MA (9.968; 10.992 and 11.860 mg/mL, respectively). As reported for the other inhibitory enzyme activities, there was a negative correlation between the IC_{50} and the total phenol, flavonoids, while for 10-HDA and proteins contents a positive correlation was observed, therefore an unfavourable action on the biological activity.

Xanthine oxidase (XO) is an important enzyme with the function of catalyzing the hydroxylation of hypoxanthine to xanthine and xanthine to uric acid, which is excreted by kidneys.[41] When in excess in the body uric acid may lead to the gout. XO plays an important role in various forms of ischemic and other types of tissue and vascular injuries, inflammatory diseases, and chronic heart failure. Indeed, finding new XO-inhibitors is health promising to face much human pathology, especially inhibitors deriving from natural products. Reports on the action of RJ on XO are scarce, as far as we know, Nagai et al.[46] showed that RJ from Japan was able to inhibit XO and therefore, to diminish the production of superoxide anion radicals, though dark honeys presented better activities. The anti-xanthine oxidase IC_{50} values observed in the present work for Moroccan, Spanish and Portuguese RJ, as observed for the remaining enzyme activities was also negatively correlated with the amounts of phenol and flavonoids, but positively with the levels of proteins and 10-HDA, Table 12.6. The results of the present work show the importance of phenols on the inhibition of XO, AChE, LOX and tyrosinase. Nevertheless, 10-HDA and proteins contents presented a negative role on the same enzymes activity.

12.4. Conclusions

The studied RJ samples from Portugal, Spain Morocco showed antioxidant, anti-inflammatory and AChE, LOX, XO and tyrosinase inhibitor properties. The negative correlation between IC50 values for antioxidant and enzyme inhibitor activities found in RJ and polyphenolic contents, and the positive correlations between the same IC50 values and HDA

or proteins contents, suggest that the properties can be attributed mainly to polyphenolic compounds and not to 10-HDA or proteins contents. On the other hand, the high content of fatty acid present in the RJ samples (mainly linolenic acid, 2-decenoic acid and octanoic acid) may also be contributing to their biological activities.

From this finding, it can be argued, that the modification of RJ samples nature to powder form affect the 10-HDA and other compounds' content which can be reflected on its biological properties. Moreover, and independent on its geographic origin, and of its natural variability, RJ biological properties may support its use as healthy food as well as in the prevention and treatment of some diseases and mild ailments.

In addition, the presence of the non-constitutive 2-furfural; BHT; and plasticizer dibutyl sebacate in some samples may indicate a prolonged storage or heat exposure of RJ; addition as antioxidant; and storage of RJ in plastic containers over a relative prolonged storage in plastic containers.

References

[1] Pasupuleti VR, Sammugam L, Ramesh N, Gan SH. Honey, propolis, and royal jelly: A comprehensive review of their biological actions and health benefits. *Oxid. Med. Cell Longev*. 2017, 2017:1259510. doi: 10.1155/2017/1259510. Epub 2017 Jul 26.

[2] Miguel MG, El-Guendouz S. Volatile compounds of royal jelly. In Bee Products - Chemical and Biological Properties; Alvarez-Suarez JM (Eds.); Springer International Publishing; 2017; pp. 191-198.

[3] Wang Y, Ma L, Zhang W, Cui X, Wang H, Xu B. Comparison of the nutrient composition of royal jelly and worker jelly of honey bees (*Apis mellifera*). *Apidologie* 2016, 47, 48–56.

[4] Kocot J, Kiełczykowska M, Luchowska-Kocot D, Kurzepa K, Musik I. Antioxidant potential of propolis, bee pollen, and royal jelly: Possible medical application. *Oxid. Med. Cell Longev*. 2018, 2018:7074209. doi: 10.1155/2018/7074209. eCollection 2018.

[5] Teixeira RR, de Souza AV, Peixoto LG, Machado HL, Caixeta DC, Vilela DD, Baptista NB, Franci CR, Espindola FS. Royal jelly decreases corticosterone levels and improves the brain antioxidant system in restraint and cold stressed rats. *Neurosci. Lett*. 2017, 655, 179–185.

[6] Pavel CI, Mărghitaş Lal, Bobiş O, Dezmirean DS, Şapcaliu A, Radoi I, Mădaş MN. Biological activities of royal jelly – *Review. Anim. Sci. Biotechnol*. 2011, 44, 108-118.

[7] Ramadan MF, Al-Ghamdi A. Bioactive compounds and health promoting properties of royal jelly: A review. *J. Funct. Foods* 2012, 4, 39−52.

[8] El-Guendouz S, Aazza S, Lyoussi B, Antunes MD, Faleiro ML, Miguel MG. Anti-acetylcholinesterase, antidiabetic, anti-inflammatory, antityrosinase and antixanthine oxidase activities of Moroccan propolis. *Int. J. Food Sci. Technol*. 2016, 51, 1762-1773.

[9] Hartfelder K, Bitondi MMG, Brent CS, Guidugli-Lazzarini KRG, Simões, ZLP, Stabentheiner A, Tanaka ÉD, Wang Y, Standard methods for physiology and biochemistry research in *Apis mellifera*. *J. Apic. Res*. 2013, 52,1-48.

[10] Liu J-R, Yang Y-C, Shi L-S, Peng C-C. Antioxidant properties of royal jelly associated with larval age and time of harvest. *J. Agric. Food Chem*. 2008, 56, 11447–11452.

[11] Council of Europe. European Directorate for the Quality of Medicines, in European

Pharmacopoeia, 7th Edition, Strasbourg, France, 2010, pp 241.
[12] Aazza S, Lyoussi B, Miguel MG. Antioxidant and antiacetylcholinesterase activities of some commercial essential oils and their major compounds. *Molecules*, 2011, 16, 7672-7690.
[13] El-Guendouz S, Aazza S, Lyoussi L, Bankova V, Popova M, Neto L, Faleiro ML, Miguel MG. Moroccan propolis: a natural antioxidant, antibacterial, and antibiofilm against *Staphylococcus aureus* with no induction of resistance after continuous exposure. *Evid. Based Complement. Alternat. Med*. 2018, 19, DOI: 10.1155/2018/9759240.
[14] Majdoub N, El-Guendouz S, Rezgui M, Carlier J, Clara Costa C, Kaab LBB, Miguel MG. Growth, photosynthetic pigments, phenolic content and biological activities of *Foeniculum vulgare* Mill., *Anethum graveolens* L. and *Pimpinella anisum* L. (Apiaceae) in response to zinc. *Ind. Crops Prod*. 2017, 109, 627–636.
[15] Ruch RJ, Cheng SJ, Klaunig JE. Prevention of cytotoxicity and inhibition of intracellular communication by antioxidant catechins isolated from Chinese green tea. *Carcinogenesis* 1998, 10, 1003-1008.
[16] Miguel MG, Doughmi O, Aazza S, Antunes D, Lyoussi B. Antioxidant, anti-inflammatory and acetylcholinesterase inhibitory activities of propolis from different regions of Morocco. *Food Sci. Biotechnol*. 2014, 23, 313-322.
[17] Rohlf JF. NTSYS-pc, Numerical Taxonomy and Multivariate Analysis System, version 2.1, user guide, Applied Biostatistics, New York, 2000.
[18] Pestana MH, Gageiro J.N. Análise de dados para ciências sociais. A complementaridade do SPSS. Edições Sílabo, Lisboa, 2000.
[19] Özkö D, Silici S. Antioxidant activities of honeybee products and their mixtures. *Food Sci. Biotechnol*. 2017, 26, 201-206.
[20] Pavel CI, Mărghitaş LA, Dezmirean DS, Tomoş LI, Bonta V, Şapcaliu A, Buttstedt A. Comparison between local and commercial royal jelly - use of antioxidant activity and 10-hydroxy-2-decenoic acid as quality parameter. *J. Apic. Res*. 2014, 53, 116-123.
[21] Čeksterytė V, Kurtinaitienė B, Venskutonis PR, Pukalskas A, Čiūtė RK, Balžekas J. Evaluation of antioxidant activity and flavonoid composition in differently preserved bee products. *Czech J. Food Sci*., 2016, 34, 133–142.
[22] Nabas Z, Haddadin MSY, Haddadin J, Nazer IK. Chemical composition of royal jelly and effects of symbiotic with two different locally isolated probiotic strains on antioxidant activities. *Pol. J. Food Nutr. Sci*. 2014, 64, 171-180.
[23] Kanelis D, Tananaki C, Liolios V, Dimou M, Goras G, Rodopoulou MA, Karazafiris E, Thrasyvoulou A. A suggestion for royal jelly specifications. *Arh. Hig. Rada. Toksikol*. 2015, 66, 275-284.
[24] Pave CI, Mărghitaş LA, Dezmirean DS, Tomoş LI, Bonta V, Şapcaliu A, Buttstedt A. Comparison between local and commercial royal jelly - use of antioxidant activity and 10-hydroxy-2-decenoic acid as quality parameter. *J. Apic. Res*. 2014, 53, 116-123.
[25] Balkanska R, Zhelyazkova I. Determination of amino acids and protein content in fresh and commercial royal jelly from Bulgaria. *Bull. Chem. Soc. Ethiop*. 2015, 29, 485-490.
[26] Niamke S, Kouame LP, Kouadio JP, Koffi D, Faulet BM, Dabonne S. Effect of some chemicals on the accuracy of protein estimation by the Lowry method. *Biokemistri* 2005, 17, 73-81.
[27] Ferioli F, Marcazzan GL, Caboni MF. Determination of (E)-10-hydroxy-2-decenoic acid content in pure royal jelly: A comparison between a new CZE method and HPLC. *J. Sep. Sci*. 2007, 30, 1061-1069.
[28] Antinelli JF, Zeggane S, Davico R, Rognone C, Faucon JP, Lizzani L. Evaluation of (E)-10-hydroxydec-2-enoic acid as a freshness parameter for royal jelly. *Food Chem*. 2003, 80, 85-89.
[29] Vázquez L.C, Díaz-Maroto MC, Guchu E, Pérz-Coello MS. Analysis of volatile compounds of

eucalyptus honey by solid phase extraction followed by gas chromatography coupled to mass spectrometry. *Eur. Food Res. Technol*. 2006, 224, 27-31.

[30] Nazzi F, Bortolomeazzi R, della Vedova G, del Piccolo F, Annoscia D, Milani N. Octanoic acid confers to royal jelly varroa - repellent properties. *Naturwissenschaften* 2009, 96, 309–314.

[31] Isidorov VA, Bakier S, Grzech I. Gas-chromatographic-mass spectrometric investigation of volatile and extractable compounds of crude royal jelly. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 2012, 885–886, 109–116.

[32] Zhao Y-Z, Li Z-G, Tian W-l, Fang X-M, Su S-K, Peng W-J. Differential volatile organic compounds in royal jelly associated with different nectar plants. *J. Integr. Agric*. 2016, 15, 1157–1165.

[33] Malkoç M, Altay DU, Alver A, Ersöz Ş, Ş TM, Kural BV, Uydu HA. The effects of royal jelly on the oxidant-antioxidant system in rats with N-methyl-N-nitrosourea-induced breast cancer. *Turk. J. Biochem*. 2017, 43, 176-183.

[34] Balkanska R. Correlations of physicochemical parameters, antioxidant activity and total polyphenol content of fresh royal jelly samples. *Int. J. Curr. Microbiol. Appl. Sci*. 2018, 7, 3744-3750.

[35] Nagai T, Inoue R. Preparation and the function properties of water extract and alkaline extract of royal jelly. *Food Chem*. 2004, 84, 181–186.

[36] Silici S, Ekmekcioglu O, Eraslan G, Demirtas A. Antioxidative effect of royal jelly in cisplatin-induced testes damage. *Urology* 2009, 74, 545-551.

[37] Guo H, Kozuma Y, Yonekura M. Isolation and properties of antioxidative peptides from water-soluble royal jelly protein hydrolysate. *Food Sci. Technol. Res*. 2005, 11, 222–230.

[38] Kohno K, Okamoto I, Sano O, Arai N, Iwaki K, Ikeda M, Kurimoto M. Royal jelly inhibits the production of proinflammatory cytokines by activated macrophages. *Biosci. Biotechnol. Biochem*. 2004, 68, 138–145.

[39] Susilowati H, Murakami K, Yumoto H, Amoh T, Hirao K, Hirota K, Matsuo T, Miyake Y. Royal jelly inhibits *Pseudomonas aeruginosa* adherence and reduces excessive inflammatory responses in human epithelial cells. *Biomed Res Int.* 2017, doi: 10.1155/2017/3191752. Epub 2017.

[40] Petrillo A, González-Paramás AM, Era B, Medda R, Pintus F, Santos-Buelga C, Fais A. Tyrosinase inhibition and antioxidant properties of *Asphodelus microcarpus* extracts. *BMC Complement. Altern. Med*. 2016, 16, 453. doi: 10.1186/s12906-016-1442-0.

[41] Han SM, Yeo JH, Cho YH, Pak SC. Royal jelly reduces melanin synthesis through down-regulation of tyrosinase expression. *Am. J. Chin. Med*. 2011, 39, 1253-1260.

[42] Peng C-C, Sun H-T, Lin I-L, Kuo P-C, Li J-C. The functional property of royal jelly 10-hydroxy-2-decenoic acid as a melanogenesis inhibitor. *BMC Complement Altern Med*. 2017, 17, 392, doi: 10.1186/s12906-017-1888-8.

[43] Pan Y, Xu J, Jin P, Yang Q, Zhu K, You M, Hu F, Chen M. Royal jelly ameliorates behavioral deficits, cholinergic system deficiency, and autonomic nervous dysfunction in ovariectomized cholesterol-fed rabbits. *Molecules* 2009, 24, 1149; doi:10.3390/molecules24061149.

[44] Hattori N, Ohta S, Sakamoto T, Mishima S, Furukawa S. Royal jelly facilitates restoration of the cognitive ability in trimethyltin-intoxicated mice. *Evid. Based Complement. Alternat. Med*. 2011, 1-6, doi:10.1093/ecam/nep029.

[45] Battelli MG, Bortolotti M, Polito L, Bolognesi A. The role of xanthine oxidoreductase and uric acid in metabolic syndrome. *Biochim. Biophys. Acta* 2018, 1864, 2557-2565.

[46] Nagai T, Sakai M, Inoue R, Inoue H, Suzuki N. Antioxidative activities of some commercially honeys, royal jelly, and propolis. *Food Chem*. 2001, 75, 237-240.

SECTION 3

General Conclusions and Future Perspectives

General Conclusions

In the present work, it has been performed an exhaustive evaluation of two Moroccan bee products, propolis and royal jelly (RJ) from various regions, highlighting their biological activities criteria, chemical composition as well as the exploration of their possible applications in the medicine, pharmaceutical and food industries. From the results obtained, the following conclusions were drawn.

The none-volatile part of Mediterranean propolis is characterized by the presence of phenolic acids and their esters as well as flavonoids. However, propolis samples originating from Sicily (Italy), Croatia, Malta, Creta (Greece), Tukey, Cyprus, Egypt, Lybia, Algeria and Morocco revealed to contain diterpenes as a part of their bioactive components. Regarding the volatile fraction, it was mainly presented by benzoic acid and its esters, mono- and sesquiterpenes, being the oxygenated sesquiterpene ß-eudesmol characteristic of poplar propolis, whereas the hydrocarbon monoterpene α-pinene has been related with the presence of conifers. Regardless the chemical composition, Mediterranean propolis showed a large spectrum of biological activity, which was exploited mainly in medicine and bio-allied sciences.

The evaluation of the antioxidant activities of Moroccan propolis samples from various geographical origins, through several methods, revealed significative differences. These differences can be linked to the bioactive compounds that each propolis sample contains. Moreover, the most active propolis sample have shown a good antibacterial effect against *Staphylococcus aureus* strains without inducing any development of resistance to antibiotics. Also, it has antibiofilm and anti-quorum sensing potential, as well as the capacity to reduce the virulence of the *S. aureus* investigated strains.

Furthermore, the studied propolis samples revealed to contain a high amount of total phenols, flavones, flavonols, flavanones and dihydroflavonols with various distribution depending on the propolis geographical origin. Besides, we have illustrated through this study, the capacity of the investigated samples to inhibit the activities of acetylcholinesterase, α-amylase and α-glucosidase, lipoxygenase, tyrosinase, xanthine oxidase, and hyaluronidase enzymes. The cited activities could be a result of the action of polyphenols given the positive correlations found between polyphenol contents and those activities.

Regarding the geographical origins, there was no specification of one region on the behalf of the others, the results showed an heterogeneity, but we can limit the good propolis samples in the ones coming from the north of Morocco, especially from Fez-Boulemane, Gharb-

Chrarda-Beni-Hsen and Rabat-Sale-Zemmour-Zaer regions considering the best activity in the majority of the assays.

In order to involve most active propolis samples in some applications, an association between propolis extract, being a natural product, and nanotechnology to prevent or disrupt the formation of biofilms on medical devices was performed. The magnetite nanoparticles (MNPs)/ propolis extract nanocomposite revealed a good capacity to prevent adherence of *S. aureus* strains on an experimental catheter, regardless the bacterial strain and the mode of production of the NPs. This capacity was attributed to the chemical composition of the propolis extract used to perform this work, being rich in flavonoids, diterpenes, and phenolic acid esters according to the GC-MS analysis data. In addition, 15 nm nanostructures were synthetized and combined with propolis extract and the antibiotic chloramphenicol, this nanostructure was tested for the purpose of drug delivery systems against different *S. aureus* strains, and the results have shown that MNPs functionalized with propolis and the antibiotic chloramphenicol at MIC value inhibited the bacterial growth of MSSA and MRSA strains. The TEM analysis proved that the bacterial cell wall evidencing their disruption and protrusions formation after exposure to the nano-antibacterial agent MNPs/propolis. Similar to the results aforementioned, the antimicrobial property is attributed to the several compounds that constituted the crude extract of propolis used detected trough pyrolysis GC/MS, including tectochrysin, which was the most abundant compound in the tested sample, pinostrobin chalcone as well as benzoic and cinnamic acids.

On the other hand, sample from the region of Fez-Boulmane showed that it is mainly constituted by flavonoids and phenolic acid esters (according to GC–MS analysis) and possessing a good anti-oxidant activity, have been introduced in the formulation of O/W emulsion in the intention to substitute the synthetic antioxidant. Propolis at the concentration 0.02 and 0.04% was more effective on prevention oxidation of O/W emulsion during the storage, especially when the lipid phase was almond oil, thing which was supported by the rheological results. While, when the lipid phase, was constituted by different proportion of almond oil and wheat germ oil, propolis was less potent as compared to the BHA. Thus, we conclude that hydro-alcoholic extract of propolis at 0.02 % and 0.04% coupled with almond oil is more adequate for having a higher stable O/W emulsion and can replace BHA as antioxidant.

Likewise, thyme being well known with its antioxidant activity and used in several emulsions for various applications, its waste extract has been tested for the same purpose as propolis in O/W emulsion and displayed a potential to prevent the hydroperoxide formation and the accumulation of malondialdehyde in emulsions O/W, at the concentrations of 0.02%, 0.04%

and almond oil as the oil phase, without interfering with the viscosity parameters, for 10 weeks, at 37 °C as compared to BHA. The GC/MS analysis revealed that benzoic acid, 4-hydroxybenzoic acid, *trans*-cinnamic acid, *p*-coumaric acid, ferulic, caffeic, and sinapic acids were present in this extract. Thus, thyme waste extract can be considered as a good antioxidant for stabilizing O/W emulsions and this could be attributed to the bioactive compounds it contains. It is noteworthy that the presence of wheat germ hampered the antioxidant action of the propolis and thyme waste extracts. In sum, our results indicate that the extract of propolis and thyme waste obtained after steam distillation for producing essential oils can be used as a good antioxidant for stabilizing diverse types of materials such as emulsions O/W, with the oil phase poor in polyunsaturated fats.

Through this work, we reported the high antioxidant activity of propolis and thyme waste extracts in O/W emulsions with the presence of almond oil the results were compared to check the efficiency of propolis. It has been found that the emulsion stability depends on various parameters. Regardless the percentage of oil phase in the emulsion, a significant difference between emulsion containing propolis and thyme waste extracts in term of the prevention of the stability over storage time has been found. Moreover, propolis extract, in all concentration tested (0.01%, 0.02%, 0.04%) was more efficient than thyme on controlling the evolution of apparent viscosity.

A first insight into the volatile fraction of Moroccan propolis was done in this work using GC and GC/MS analysis. Cluster analysis of volatile isolated by hydro-distillation from the propolis samples defined two main uncorrelated clusters. The first one was dominated by β-eudesmol, cedrol, and *n*-tricosane, while cluster II contains only one sample that showed the *ar*-curcumene as major constituent. Moroccan propolis showed a major chemical variability in the volatile profile, distinct from those of Algeria, Canary Islands, and Ethiopia. The variations in propolis volatiles were mainly quantitative, suggesting that bees collected propolis from similar botanical sources. Furthermore, no full correlation has been recorded between samples and local flora, for the reason that some samples have some correlation with data on dominant local flora, but others not, thing which can be explained by the fact that other factors contribute in the final volatile composition of local propolis besides dominant local flora. However, dominant flora in the Moroccan areas where the propolis samples were collected has some similarity with that occurring in some countries from Southern Europe like Croatia, Greece, and Portugal.

In general, RJ investigation was limited in few countries from the Mediterranean, mainly France, Italy, Greece, and Turkey. Available data have shown that its composition is quite

complex and are in concordance to RJ originating from other countries. RJ has a higher-water content between 46.8%-73.2%, an important sugar amount including monosaccharides (fructose, glucose), disaccharides and trisaccharides with different distribution depending the geographical origin but not exceeding 17.9% which was reported in French RJ samples. The protein content in RJ was in interval of 11.4 and 15.7% for sample from Turkey, Italy France, and Greece. For lipid content, only quantification of 10-HDA in RJ samples was effective, being the specific and major RJ fatty acids. Thus, its content was limited between 0.75 and 19.56%, with the high content belonging to Greek RJ samples. Vitamins, minerals, and phenols have also been detected in RJ samples from the Mediterranean countries. Concerning volatile constituents, their composition is related to several factors including honeybee species, harvesting time, regions, storage conditions and extraction methods. only samples from Italy were reported, they were dominated by carboxylic acids and some newly identified compounds such as 2-hydroxyhexanoic acid, phenylacetic acid, pentanoic acid, and 2-methylbutanoic acid. For RJ fields of uses, their biological properties were mainly exploited in medicine, through various procedures, highlighting their health benefit.

In the present thesis, it was also reported the chemical composition of RJ volatiles from Morocco, Spain, and Portugal, which was a complex mixture in which up to sixty-six components were identified and gathered in two moderately correlated clusters. Cluster I included sample 1 from Morocco and the sample from Portugal, mainly constituted by linolenic acid, octanoic acid, and *trans*-2-decenoic acid. While the second cluster comprised samples from Morocco and Spain containing *trans*-2-decenoic acid, octanoic acid, and linolenic acid as main compounds. The antioxidant and the enzyme inhibitory activities showed a significant difference. Samples in powder form originating from Morocco exhibited the best activities, in the opposite to the other samples from Morocco which were practically in the last range concerning their enzyme inhibitory activity, as compared to the ones from Portugal and Spain. Overall, except the powder form, RJ from Morocco was less effective as compared to the ones from Portugal and Spain, this could be related to the harvesting method which is mainly traditional and storage condition in Morocco, besides the geographic origin.

Future perspectives

Hive products stole the scientist attention worldwide, especially propolis and RJ, which are considered one of the most important beehive products playing a critical role inside the hive and possessing a large spectrum of biological activities.

Unfortunately, the potential of the propolis from Mediterranean countries to be used in the field of the food industries, animal husbandry, cosmetic, etc, was less developed. The combined efforts done by the scientific researchers on propolis from the Mediterranean countries should be boosted in order to make better its use. Appropriate methods should be developed for the standardization of Mediterranean-propolis-type which could make challenge for serious competition to the well-known Brazilian and Chinese ones. As aforementioned, the investigation upon propolis, the approach of propolis typification could be performed according to the plant source.

Regarding propolis from Morocco, it is clearly concluded that can be used in different field, as antioxidant source that can be highly involved in the ROS related human diseases. Being a natural product, future research in natural products from Morocco should be orientated to propolis for its biomedical application to combat nosocomial infections and the biofilm formation on medical device caused by *S. aureus.*

The results showed that Moroccan propolis possesses a high capacity to inhibit various key enzymes involved in several diseases. This finding must be the target of further study for deeply evaluating the possibility to use this product in the combat of some diseases involving inflammation, hyperuricaemia or gout, hyperglycaemia, in those diseases characterized by a decline in cognitive function and mental atrophy, or as agents still for the treatment of hyperpigmentation of the human skin.

The action of Moroccan propolis/nanocomposite elucidated in the present thesis demonstrated that the proposed strategy proves to have an important benefit due to its antibacterial properties on demand for numerous medical applications. Nanotechnology may provide a new approach to prevent or disrupt the formation of biofilms on medical devices, the association of natural products, such as propolis, with nanotechnology may constitute an alternative to combat the formation of those biofilms in catheters by MRSA strains. This axe of research is with high importance and future research are imposed for isolation and identification of the bioactive components responsible for this activity of Moroccan propolis, as well as determination of inner mechanisms of action.

Regarding propolis volatiles, we illustrate the diversity of propolis and we present it as an important source of exogenous antioxidant. However, further research is needed to reveal the biological capacity of the investigated volatiles, as well as their potential and contribution to the biological activity and medicinal application of propolis.

As reviewed previously, many *in vitro* assays and preclinical investigation have been performed making in evidence important biological properties of RJ. Nevertheless, *in vitro* and *in vivo* investigation of RJ originating from Mediterranean countries is still very limited, and more scientific research and exploration is imposed. Scientists are recommended to give more intention, using the facilities and available tools in research nowadays, for exploiting RJ, identify its bioactive components, their isolation and purification, as well as determination of inner mechanisms of action, which could lead to the formulation of new products, originated from natural source, presenting no side effects, for pharmacological and therapeutic uses.

The results found, regarding the investigated RJ samples may be compared to well-known RJs worldwide, but serious attention should be given to the development of this bee-product, researchers have to focus on exploring this fascinating 'boon' of nature.

In general, this thesis represents a strong background and opens a series of future research aspects to explore Moroccan propolis and RJ. Overall, Morocco is well known for its biodiversity and the scientific report is very developed in the field of exploration of medicinal plants. Nevertheless, scientific research upon hive products is very limited in some scientific papers and still confined to honey. Although, many researches remain to be carried out by scientists for investigating propolis and RJ, to determinate their chemical composition for its standardization and reliability. Exploiting the available high developed methods, standardized quality controls, along with suitable clinical experiments are highly recommended in order to ensure the effectiveness, quality and safety necessary for, why not, its commercialization not only as food supplement but also as medicine.

Appendix A

Table A.1. Chemical constituents of the non-volatile part of propolis from different Mediterranean Sea countries and their related biological properties

Geographical origin	Extract	Major constituents	Plant source	Bioactivity	Method	References
Southern European Coast						
France						
Not reported	NI	**Phenolic acids esteres**: benzyl and prenyl caffeate caffeate (22 mg/g) **Phenolic acids:** caffeic acid (5-76.9 mg/g), *p*-coumaric acid (10.2-61.4 mg/g), ferulic acid (3.9-20.8 mg/g), isoferulic acid (4.1-16.2 mg/g) **Flavonoids** pinobanksin-3 acetate (38.1-42.8 mg/g), pinocembrin (33-49.6 mg/g), chrysin (23.1-36.5 mg/g), galangin (20.4-31.1 mg/g)	*Populus* spp. section Aigeiros and especially *P. nigra* L.	Antioxidant, Anti-AGEs (advanced glycation end-products), Antifungal, Antibacterial effects	HPLC-DAD HPLC-MS GC/MS	[40] [41] [165]
Different geographical places	Ethyl acetate extracts	**Phenolic acids:** caffeic acid, *p*-coumaric acid, **Flavonoids:** chrysin, pinobanksin, pinobanksin3-acetate, galangin, kaempferol, tectochrysin and pinocembrin	Poplar type of propolis (blue and orange types)	NE	HPTLC-MS	[42]
Spain						
East Andalusia	Ethanolic extracts	**Phenolic acids:** caffeic acid (2.7-7.9 mg/g EEP), *p*-coumaric acid (2.3-12.7 mg/g EEP), ferulic acid (1.8-12.5 mg/g EEP), Cinnamyldenacetic acid (0.8-22.9 mg/g EEP **Flavonoids:** pinobanksin (1.5-14.6 mg/g EEP),), chrysin (29.7-43.9 mg/g EEP), pinocembrin (43.2-85.3 mg/g EEP), galangin (12.6-45.9 mg/g EEP), pinobanksin 3-acetate (42.6-108.1 mg/g EEP), Caffeic Acid Phenethyl Ester ester with small quantity.	*Castanea sativa*, *Quercus* sp., *Populus* sp., *Eucalyptus* sp., *Salix* sp., *Pinus* sp.	Antioxidant	LC-PDA-MS	[35]
Not reported	Ethanolic extracts	**Flavonoids:** pinocembrin (134 µg/ml), galangin (83 µg/ml), naringenin (48 µg/ml), acacetin (31 µg/ml), chrysin (12 µg/ml).	NI	NE	HPLC–ESI/MS	[38]
Galicia Castilla y Leon	Meyhanolic extract	**Phenolic acids:** caffeic acid (5.6-25.0 mg caffeic acid /g); *p*-coumaric acid (0.0-48.6 mg caffeic acid /g), cinnamic acid (2.9–32.0 mg caffeic acid /g), ferulic acid (0.00–2.2 mg caffeic acid /g), isoferulic acid (3.5-35.6 mg caffeic acid /g), and dimethylcaffeic acid (DMCA) (7.1-35.8 mg caffeic acid /g), **Phenolic acids esters**: caffeic acid benzyl ester (18.9-92.6 mg caffeic acid /g), *p*-coumaric cinnamyl ester (0.00–12.5 mg caffeic acid /g), *p*-methoxy cinnamic acid cinnamyl ester (0.00–12.6 mg caffeic acid /g), caffeic acid phenethyl ester (0.00–33.2 mg caffeic acid /g),	NI	NE	HPLC–DAD NIR	[37]

La Alcarria and Nerpio	Ethanolic extracts	**Phenolic acids:** caffeic acid, isoferulic acid **Phenolic acids esters**: 3-methyl-2-butenyl-*trans*, - caffeate (0.9-13%), 2-Methyl-2-butenyl mz/w-isoferulate (<0.1-0.9%) **Flavonoids:** pinocembrin (0.5-5.5%), pinobanksin (1.6-11.1%) and their derivatives, **Hydrocabons:** heptacosane (2.1-11.8%), nanocosane (0.7-5.7%), henitriacontane (0.3-3%) pentacosane (0.5-4.1%) **Long chain fatty acids:** hexadecanoic acid (0.2-3.2%), Tetracosanoic acid (0.8-8.4%), hexacosanoic acid (0.2-1.2%)	*Populus nigra*	NE	GC-MS	[39]
Not reported	Ethanolic extracts	**Flavonoids**: dihydroxyflavone, chrysin, pinocembrin, apigenin, galangin, pinobanksin, pinobanksin acetate. **Phenolic acids esters**: caffeic acid phenethyl ester.	*Populus* resin	NE	EASI-MS	[36]
Italy						
Not reported	Ethanolic extracts	**Flavonoids:** pinocembrin (166µg/ml); galangin (40 µg/ml); naringenin (57 µg/ml); Acacetin (31 µg/ml); Chrysin (26 µg/ml)	NI	NE	HPLC–ESI/MS	[38]
Piedmont, Emilia Romagna, and Sicily	Ethanolic extracts	**Phenolic acids esteres:** Benzyl caffeate (0.9%), Phenethyl caffeate (0.2%) **Flavonoids:** pinocembrin (0.2%), pinobanksin 3-*O*-acetate, chrysin (0.4%), galangin (0.2%), **Rich on diterpenic acids (53%).**	*P. nigra*	NE	GC-MS	[29]
Not reported	Ethanolic extracts	**Flavonoids:** rich in pinocembrin and galangin	NI	Antibacterial	HPLC	[44]
Not reported	Ethanolic extracts	**Phenolic acids esters**: caffeic acid phenethyl ester (≥20 mg/g), **Phenolic acids:** caffeic acid (≥10 mg/g), **Flavonoids:** chrysin (≥40 mg/g); pinocembrin (≥40 mg/g); galangin (≥15 mg/g)	NI	Antioxidant activity	HPLC-MS/MS	[45]
Bologna	Ethanol acetone chloroform	**Phenolic acids esters**: CAFFEIC ACID PHENETHYL ESTER (2.36-4.27 mg/g); caffeic acid cinnamyl ester (2.6-4.03 mg/g); and pinobanksin 3-*O*-acetate (12.31-23.31 mg/g). **Phenolic acids:** *p*-coumaric acid (6.51-11.01 mg/g); ferulic acid (1.39-2.17 mg/g); caffeic acid (5.26-7.85 mg/g); **Flavonoids:** Apigenin (4.39-5.97 mg/g); chrysin (4.7-8.23 mg/g); galangin (10.63-18.52 mg/g); kaempferol (3.65-7.10 mg/g); quercetin (6.14-10.33 mg/g); naringenin (7.27-11 mg/g); pinocembrin (16.8-24.89 mg/g); pinostrobin (1.97-3.65 mg/g); pinobanksin (4.47-7.45 mg/g)	*Populus* spp	Antioxidant activity	HPLC-MS/MS	[47]
Not reported	Ethanolic extracts	**Phenolic acid esters**: 3,4-dimethyl caffeic acid (0.3-12.1 mg/g), caffeic acid prenyl ester (0.3-10.3 mg/g), caffeic acid benzyl ester (0.7-22.3 mg/g); caffeic acid phenylethyl ester (0.4-13.1 mg/g); caffeic acid cinnamyl esters (1.3-24.8 mg/g) **Phenolic acids:** caffeic acid (0.3-6.7 mg/g); *p*-coumaric acid (0.3-10.2 mg/g) ferulic acid (0.8-8.7mg/g); **Flavonoids:** chrysin (0.9-33 mg/g); pinocembrin (0.7-25 mg/g); galangin (1.1-16.3 mg/g); pinobanskin-3-*O*-acetate (0.6-31.9 mg/g).	NI	NE	HPLC-DAD	[48]

Different locations of central Italy	Ethanolic extracts	**Phenolic acids:** caffeic acid (2600-25000 µg/g), *p*-coumaric acid (1900-5400 µg/g) ferulic acid (3100-5200 µg/g). **Flavonoids:** quercetin (600-8500 µg/g), apigenin (1100-5000 µg/g), kaempferol (1400-8500 µg/g), chrysin (6000-39000 µg/g), galangin (4700-30000 µg/g), benzyl salicylate benzyl cinnamate	poplar origin.	NE	GC-MS	[43]
All over Italy, islands included	Ethanolic extracts	**Phenolic acids:** Ferulic acid (2.61-52.59 mg/g), *m*-coumaric acid (2.96-11.94 mg/g), isoferulic acid (2.69 -101.04 mg/g), (2.96-11.94 mg/g), caffeic acid (1.78 -4.66 mg/g), *trans*-cinnamic acid (3.24-4.05 mg/g), **Flavonoids**: Chrysin (18.19-27.07 mg/g), Galangin (11.32- 15.89 mg/g), Pinocembrin (11.94 -12.09 mg/g), Pinostrobin (5.17 -6.56 mg/g),	poplar origin.	Antioxidant activity	HPLC-ESI-TQ MS	[46]
Not reported	Ethanol, distilled water, olive oil cyclodextrin extract and their mixture	**Phenolic acids:** Ferulic acid (7.06-87.64µg/g), boropinic acid (21.71-213.10µg/g), 4-geranyloxyferulic acid (0.50-107.12µg/g), **Coumarin derivatives:** umbelliferone (4.54-145.37µg/g), 7-isopentenyloxycoumarin (15.21-295.84µg/g), auraptene (0.95-162.68µg/g),	NI	NE	HPLC	[49]
Not reported	Not mentioned	**Phenolic acids:** Two new cinnamic acid 4-(3´-Hydroxymethyl-3´-methylallyloxy), cinnamic acid and 4-(3´-hydroxymethyl-3´-methylallyloxy)-3-methoxycinnamic	NI	NE	HPLC	[50]
Slovenia						
Dolenjska	Ethanolic extracts Eth 70% and Eth 95%	**Phenolic acids:** *p*-coumarin acid (351-465.26 µg/ml); ferulic acid (199.-225.56 µg/ml); caffeic acid (333.39-465.26 µg/ml); **Phenolic Acid Esters:** caffeic acid benzyl ester (148.12-166.82 µg/ml); caffeic acid cinnamyl ester (342.13-355.3 µg/ml); **Flavonoids:** pinobanksin (292.28-294.45 µg/ml); apigenin (151.61-160.56 µg/ml); kaempferol (161.15-179.62 µg/ml); pinobanksin-3-o-acetate (292.19-301.13 µg/ml); kaempferide (104-65-110.46 µg/ml); chrysin (233.37-242.7 µg/ml); pinocembrin (226.57-253.05 µg/ml); galangin (188.17-207.38 µg/ml);	NI	Antioxidant and Antimicrobial activities	LC/MS	[51]
Not reported	Dichloromethane Extract	Slovenian test solutions mainly reflected patterns characteristic for blue-type propolis rich in flavonoids and with low quantities of phenolic acids	Poplar type	NE	HPTLC	[53]
Savinjska Valley	Ethanolic extracts	**Phenolic Acid Esters:** caffeic acid isoprenyl ester, caffeic acid benezyl ester, caffeic acid phenyethyl, caffeic acid cinnamyl ester, *p*-coumaric benzyl ester, **Flavonoids**: chrysin, pinocembrin, apigenin, kaempferol, rhamnetin, pinobanksin derivates (pinobanksin-5-methyl-ether pinobanksin-3-O-acetate, pinobanksin-3-O-propionate, pinobanksin-3-O-butyrate, pinobanksin-3-O-pentanoate) **Phenolic acid derivates:** caffeic acid derivate, coumaric acid derivate, ferulic acid derivate	NI	Antifungal activity	LC/MS LC-DAD	[52]
Croatia						

(Kopacki rit, Lonjsko polje, island Krk, Virovitica, GorskiKotar, Istra north coast, island Rab)	Ethanolic extracts	**Phenolic acids:** vanillin (43±4 -140±8 g/l); *p*-coumaric acid (71±1 -879.39 g/l); ferulic acid (151±8 -1046±42 g/l); **Flavonoid:**.chrysin (940±52 -1355±66 g/l); galangin (1107±50 -1627±78 g/l); **Phenolic Acid Esters:** caffeic acid phenethyl ester (263±18 -486±24 g/l)	Populus tremula *Pinus spp.*	Antioxidant Properties	HPLC-DAD	[65]
Lokrum Island and Trogir	Ethanolic extracts	**Phenolic acid esters:** (15.2-16.2%) **Phenolic acids:** (4-4.5%) **Flavonoids:** (35.2-35.5%) mainly pinocembrin, pinobanksin, pinobanksin-3-O-acetate, chrysin and galangin **Diterpenes: (4.8-8.6%)** mainly isocupressic acid, pimaric, imbricatoloic acid, agathadiol, totarol, 13epi-torulosal, communic acid, 13-epicupressic acid, abietic acid and ferruginolon.	cypress trees *Cupressus sempervirens*	Antibacterial and antioxidant	GC-MS	[61]
Several locations throughout the central, continental Croatia	Ethanol-H_2O in two ratios 70:30 and 80:20 (v/v)	**Phenolic acid:** p-coumaric acid (2.34-4.4%), 3,4-dymethoxycinnamic acid (2.26 -1.28%), ferulic acid (2.01-3.71 %), caffeic acid (1.32-3.30 %), **Phenolic acid esters:** benzyl caffeate (7.56- 9.41 %), phenylethyl caffeate (2.59- 3.34%), **Flavonoids:** pinocembrin (2.26 -1.28%), pinobanksin 3 acetate (4.20 -5.94%), galangin (2.66-3.64 %), chrysin (3.25 -5.42 %),	Poplar propolis type	Antioxidative activity Antibacterial Activity	GC-MS	[66]
Not reported	80% ethanol	Planar chromatographic fingerprint was marked as blue-type propolis which caracterised wih *p*-coumaric acid and ellagic acid, caffeic acid, quercetin, and apigenin	blue-type propolis	ND	HPTLC	[59]
Continental Croatia	80% ethanol	**Flavonoids:** chrysin (2478.5 µg/g propolis), pinocembrin (2069.1 µg/g propolis), galangin (1441.6 µg/g propolis), isorhamnetin (32.2 µg/g propolis)	NI	Antioxidative activity	HPLC	[67]
Different place in Croatia	80% ethanol	**Phenolic acids:** o-coumaric acid, p-coumaric acid, caffeic acid, **Flavonoids:** 6'-hydroxyflavone, chrysin, quercetin, galangin, apigenin	NI	ND	Thin-Layer Chromatography	[56]
Several places	Ethanolic extracts	**Phenolic acids:** ferulic acid (0.0017-0.959 mg/g); pcoumaric acid (0.0023-0.1464 mg/g); **Flavonoids:** tectochrysin (0.248-1.2004 mg/g); (0.3706- 47.4879 mg/g); pinocembrin (0.2487-7.6651 mg/g); chrysin (1.0249-4.1356 mg/g)	NI	Antiproliferative and cytotoxic effect on the HeLa cells	HPLC	[68]
Different regions of Croatian	80% ethanol	**Phenolic acids:** caffeic acid (0.1204-0.4196 mg/mL), *p*-coumaric acid (nd-0.2968 mg/mL), isoferulic acid (nd-0.5068 mg/mL), **Flavonoids:** pinocembrin (nd-0.5616 mg/ml), tectochrysin (nd-1.1816 mg/ml), pinocembrin-7-methyl ether (nd-1.1621mg/ml), chrysin (nd-0.5616 mg/ml),	NI	ND	Thin-Layer Chromatography	[57]
Different geographic origin	80% ethanol	**Phenolic acids:** caffeic acid (0.09-0.39 mg/mL), ferulic acid (nd-0.82 mg/mL), **Flavonoids:** galangin (0.25-0.73 mg/mL), pinocembrin (0.43-1.12 mg/ml), chrysin (0.73-1.44 mg/ml), kaempferol (0.24-0.41 mg/ml),	NI	ND	Two-Dimensional Thin-Layer	[58]

					Chromatography	
Continental and Adriatic regions	80% ethanol	Continental region: **Phenolic acids:** Caffeic acid (0.27-2.67%) **Flavonoids:** naringenin (0.32-2.41 %), chrysin (0.22-5.32 %), pinocembrin (nd-3.98 %), galangin (nd-2.12 %) Adriatic regions: **Phenolic acids:** Caffeic acid (nd-2.91%) **Flavonoids**: naringenin (nd-1.14 %), chrysin (0.03- 3.64 %), pinocembrin (0.06- 5.46 %), galangin (0.02- 3.07 %)	NI	Antibacterial	HPLC Thin-Layer Chromatography	[54]
Samples from Continental and Mediterranean Regions	Methanolic extract	**Phenolic acids:** ferrulic acid (nd-1.020 mg/ml), *p*-coumaric acid (nd-1.031 mg/ml), **Flavonoids:** chrysin (nd- 8.020 mg/ml), apigenin (nd- 1.230 mg/ml), kaempferol (nd-0.672 mg/ml), tectochrysin (nd- 16.07 mg/ml), galangin (nd- 8.710 mg/ml), pinocembrin (nd- 6.390 mg/ml), pinocembrin-7-metylether (nd- 0.423 mg/ml),	NI	Antioxidant and Antimicrobial Activities	HPLC	[62]
Different regions of Croatia	Methanolic extract	**Phenolic acids:** vanillic acid, ferulic acid, *p*-coumaric acid, caffeic acid, 3,4-dihydroxybenzoic acid and, 2-5- 3,4-dihydroxybenzoic acid, trans-cinnamic acid, **Phenolic acid esters:** verbascoside **Flavonoids:** pinobanksin, apigenin, galangin, diosmetin, tectochrysin, rhamnetin, quercetin, kaempferol, sakuranetin, apigenin-7-O-glucoside, apigenin-7-O-glucoside	NI	NE	LC-QQQ ULC-QTOF	[63]
Not reported	Different preparation including Diethyl ether	**Phenolic acid esters:** cinnamic acid esters (28.3%), 2-phenylethyl (*E*)-caffeate (2.7%), benzyl (E)-caffeate (2.5%) **Phenolic acids:** cinnamic acids (10.3 %), (*E*)-isoferulic (5.1%), (*E*)-caffeic (3.2%), **Flavonoids:** 5-dimethoxyflavanone -4',7-dimethoxyflavanone (8.9%), pinocembrin (8.8%), chrysin (7.7%), pinobanksin (5.3%), pinobanksin-3-acetate (8.6%), galangin (2.1%)	Poplar	NE	GC-MS	[64]
Bosnia and Herzegovina						
Sjetovac	Methanol extract	**Phenolic acids:** *p*-coumaric acid, ferulic acid, benzoic acid **Flavonoids:** chrysin, galangin, pinobanksin, pinocembrin, pinobanksin 3-*O*-acetate, kaempheride, apigenin, naringenin **Phenolic acid esters:** CAFFEIC ACID PHENETHYL ESTER, caffeic acid benzyl caffeate, cinnamyl caffeate, conyferyl benzoate, conyferyl *p*-coumarate, benzyl *p*-coumarate, benzyl ferulate, 1,3-diferulyl-2-acetyl-glycerol, 1-*p*-coumaryl-2-acetyl-3-ferulyl-glycerol	*Populus* species	NE	NMR, IR, and UV spectroscopy followed by OPLS	[60]
Several places	Ethanolic extracts	**Phenolic acids:** ferulic acid (0.0017-0.959 mg/g); p-coumaric acid (0.0023-0.1464 mg/g); **Flavonoids:** tectochrysin (0.248-1.2004 mg/g); (0.3706-47.4879 mg/g); pinocembrin (0.2487-7.6651 mg/g); chrysin (1.0249-4.1356 mg/g)	NI	Antiproliferative and cytotoxic effect on the HeLa cells	HPLC	[68]
Albania						

Near Tirana	Ethanolic extracts	**Phenolic acid esters: (53%)** **Flavonoids: (38%)**	NI	Antibacterial, antifungal and antiviral activity	GC-MS	[69]
Malta						
Not reported	Ethanolic extracts	**Phenolic acids:** (0.1-2.7%) **Diterpene:** (18.7-92.5%) mainly isocupressic acid, communic acid, pimaric acid and imbricatoloic acid, totarol and 13-epitorulosal **Triterpene:** (0.1-4.9%) **Sesquiterpenyl esters:** 2-Acetoxy-6-*p*-methoxybenzoyl jaeschkeanadiol, 2-acetoxy-6-*p*-hydroxybenzoyl-jaeschkeanadiol, ferutinin and teferin	Genus Ferula, *Populus spp*. and *Ferula spp.*	Antibacterial *Staphylococcus aureus*	GC–MS	[70]
Not reported	NI	**Diterpenic profile**	NI	NE	GC–MS	[28]
North and Central Malta	Methanol	The diterpenoid totarol was the predominant constituent	*Pinus halepensis* Mill., *Eucalyptus gomphocephala* DC., *Cupressus sempervirens* L., *Ceratonia siliqua* L., *Lycopersicum esculentum* Miller, *Cucurbita pepo* L., *Prunus persica* (L.) Batsch and *Prunus armenaica* L.	Cytotoxicity test on human cancer cell lines	GC–MS	[71]
Greece						
Not reported	Ethanolic extracts	**Phenolic acid esters: (0.9-28.9%)** **Phenolic acids: (0.1-5.4%)** **Flavonoids: (0.9-46.7%):** mainly pinocembrin, pinobanksin, pinobanksin-3-*O*-acetate, chrysin and galangin	cypress trees *Cupressus sempervirens*	Antibacterial and antioxidant	GC-MS	[61]

		Diterpenes: (4.7-81.9%) mainly iso- cupressic acid, pimaric, imbricatoloic acid, agathadiol, totarol, 13epi-torulosal, communic acid, 13-epi-cupressic acid, abietic acid and ferruginolon.				
six different regions of Greece	Ethanolic extracts	**Phenolic acids: (0.1-1.2%)** **Flavonoids: (0.1-8.7%)** **Diterpenes:** 14,15-dinor-13-oxo-8(17)-labden-19-oic acid (1.5-4.9%); pimaric acid (4.3-10.8%); isoagatholal (9.2-29.1%); agathadiol (2.7-8.3%); isocupressic acid (9.2-29.1%); communic acid (1.8-6.4%); totarol (3.1-6.5%), **Triterpenes: (0.7-4.1%)**	*Pinus* sp. and *Cupressus sempervirens*	NE	GC-MS	[75]
Larnaka,	Ethanolic extracts	**Phenolic acids:** *p*-Coumaric acid (0.25-2.18 mg/g EEP); cinnamic acid (0.04-4 mg/g EEP), caffeic acid (0.14-3.87 mg/g); ferulic acid (0.11-1.81mg/g). **Flavonoids:** chrysin (0.26-145.7mg/g EEP); apigenin (0.25-15.85mg/g EEP); pinocembrin (0.32-33.34 mg/g EEP), pinobanksin (0.32-33.74mg/g EEP), pinobanksin-3-O-acetate (0.38-55.78 mg/g EEP). **Anthraquinones** mainly emodin (2.87-53.974 mg/g EEP), and chrysophanol (0.3-36.61 mg/g EEP), **Diterpenes:** totarol (0.32-33.74mg/g EEP), abietic acid (0.25-345.1 mg/g EEP); isopimaric acid (1.94-259.6 mg/g EEP)	Coniferae trees especially *Pinus* sp.	Antioxidant, antibacterial, and antifungal activities,	GC–MS	[31]
Not reported	Ethanolic extracts	**Phenolic acids ester:** Cinnamic acid (0.12-1.44%), **Flavonoids:** pinostrobin chalcone (1.12-16.93%), chrysin (0.94-2.55%); techtochrysin (0.58 – 6.45%); pinocembrin (0.24-7.94%), **Terpenes:** totarol (0.33-25.75%) (diterpene), ß-elemene (sesquiterpene)(13.28-13.79%)	*Pinus* sp	NE	GC-MS	[73]
West Macedonia and Rhodes	Methanolic extract	**Phenolic acids:** caffeic acid (0.64 -4.17 %), coumaric acid (0.83-3.00 %), ferulic acid (0.53 -1.41 %) **Phenolic acids ester:** caffeic acid phenethyl ester (0.36 -2.04 %), **Flavonoids:** apigenin (0.48 -2.74 %), galangin (1.32 -8.55 %), quercetin (0.42 -2.09 %), luteolin (0.29 -1.12 %),	NI	Antioxidant activity	HPLC	[77]
Nafplio, Amorgos, Crete, Kos, Lakonia, Imathia, Arkadia and Corfu island	Hydroalcoholic	**Phenolic acids:** Gallic acid (61.0 - 263.2 µg/g), *p*-coumaric acid (nd - 846 µg/g), caffeic acid (nd - 228.3 µg/g), (0.48 -2.74 µg/g), (0.48 -2.74 µg/g), **Phenolic acids ester:** caffeic acid phenethyl ester (nd - 402.4 µg/g), (0.48 -2.74 µg/g), (0.48 -2.74 µg/g), (0.48 -2.74 µg/g), (0.48 -2.74 µg/g), chlorogenic acid (nd - 301.6 µg/g) **Flavonoids**: pinocembrin (361.2 – 13992 µg/g), apigenin (nd - 1989.8 µg/g), chrysin (169.7 - 9940.3 µg/g), galangin (nd - 2529.1 µg/g), Pinobanksin (nd -326.9 µg/g), quercetin (nd -596.5 µg/g), kaempferol (nd -1343 µg/g), pinobanksin-3o-acetate (nd – 2809 µg/g), (+)-catechin (nd – 2717µg/g),	NI	Antioxidant activity	HPLC-PDA-ESI/MS	[76]
Nigrita,	Ethanolic extracts	**Phenolic acids:** caffeic acid (1.7%) **Phenolic acids Esters**: Phenethyl caffeate (4.8%), benzyl caffeate (4.3%), 3,3-dimethylallyl caffeate	Buds of poplars	Antimicrobial activity	GC-MS	[74]

		Flavonoids: chrysin (4.9%), galangin (5.8%), Pinostrobin (3.2%), Pinocembrin (10.3%), pinobanksin (1.5%), pinobanksin 3-o-acetate (6.4%)				
Agrinio, Arta, Preveza and Andros,Chalkidiki,	*N*-butanol extract	Diterpenic and phenolic compounds	NI	Antimicrobial activity	GC and GC-MS	[72]
Turkey						
Kazan and Marmaris regions	Ethanolic extracts	**Phenolic acid ester:** caffeic acid pentenyl ester, 3,3-dimethylallyl caffeate, and isopent-3-enyl caffeate	Populus sp	Antibacterial activity	^{1}H and ^{13}C NMR spectra	[98]
Turkey	Ethyl alcohol extracts	**Flavonoids** (0.16-33.36%), mainly quercetin, chrysin, 7 3' 4'-trihydroxyflavone, 4' 5'-dihydroxy-7-methoxyflavone **Aromatic acid esters (**0.46-61.05%), **Aromatic alcohols** (0.46-2.32%) **Aromatic acids** (0.2-3.48%), **Aliphatic carboxylic acids** (0.66-3.04%), **Terpenes:** (0.15-0.41%), **Aliphatic carboxylic acid esters** (0.27-2.17%)	NI	Antibacterial activity	GC–MS	[78]
Yozgat, Izmir, Kayseri, Adana, Erzurum, Artvin	Alcohol extract	**Flavonoids:** pinocembrin, pinobanksin, pinobanksin-3*O*-acetate, chrysin, galangin, **Phenolic acids:** *p*-coumaric acid, ferulic acid, **Phenolic Acid Esters:** benzyl *p*-coumarate, benzyl ferulate, phenylethyl caffeate, cinnamyl cinnamate. **Diterpenes (4-5%)**: dihydroabietic acid, abietic acid, isopimaric acid	*Populus* spp *Populus euphratica*	Antibacterial activity	GC–MS	[79]
Denizli Denizli-Sarayköy, AydIn, Denizli-BaskarcI, AydIn, Konya and Tekirdag	Alcohol extract	**Flavonoids:** chrysin (12.62- 18.57%), apigenin (7.29- 14.02%), naringenin (1.19- 8.4%), **Phenolic Acid Esters:** 3-4-dimethoxy-cinnamic acid (0.52- 1.29%),	NI	Antioxidant, Antibacterial, activities,	GC–MS	[80]
Bursa, Erzurum, Gumushane and Trabzon	Ethanolic extracts	**Phenolic acids:** (1.32- 18.15%) **Phenolic Acid Esters:** (3.10- 31.86%) **Flavonoids:** (4.72- 50.55%) **Terpenes:**(1.92- 7.7%)	One sample from Castanea sativa,	NE	GC–MS	[108]
Ankara-Kazan and Mugla-Marmaris	Ethanolic extracts	**Phenolic acids:** benzoic acid (0.2- 0.34%), ferulic acid (0.09- 0.29%) **Diterpenes**: isopimaric acid (11.17- 26.88%), dehydroabietic acid (10.61%), abietic acid (11.39%)	*Pinus brutia* L	NE	GC–MS	[97]
Bartin Province	Hexane, Ethanol and Ethyl acetate extract	**Phenolic acids:** gentisic acid (199.56-6897.8 µg/g), vanillic acid (204.6-6534.2 µg/g), caffeic acid (3522.8-19100 µg/g), syringic acid (523.64-1337.3 µg/g), *p*-coumaric acid (3356-7568.4 µg/g), ferulic acid (12878-136800 µg/g), homogentisic acid (309-1268 µg/g), 3,4-dihydroxybenzoic acid (253.22-1339.8 µg/g),	NI	Antibiofilm Activity	UPLC-ESI-MS/MS	[81]

		Flavonoids: rutin (126.73-3565.1 µg/g), myricetin (55.87-1206.70 µg/g), quercetin (47.93-1006.1 µg/g), naringenin (245.43-1148.2 µg/g), genistein (364. 76-2008.2 µg/g), kaempferol (121.77-1355.7 µg/g),				
Asian and European part of Marmara region, and Aegean region,	Ethanolic extracts	**Flavonoid:** Pinostrobin chalcone (7.10-11.82%), pinocembrin (4.24-24.61%), **Terpenes.** Totarol (7.27-24.47%); 1R-α-Pinene (monoterpene) (3.48%)	*Cupressus sempervirens*	NE	GC-MS	[73]
Anatolia	Metthanolic extracts	**Phenolic acids:** vanillic acid (3.21- 33.38 mg/100 g), proto-catechuic acid (2.57-27.80 mg/100 g), abscisic acid (10.73-466.6 mg/100 g), ferulic acid (22.33-712.69 mg/100 g), benzoic acid (24.27-726.12 mg/100 g), caffeic acid (125.94-465.81 mg/100 g), p-coumaric acid (57.11-457.98 mg/100 g), **Phenolic acid esteres**: chlorogenic acid (1.88- 53.46 mg/100 g) **Flavonoids:** rutin (3.29-5504 mg/100 g), quercetin (77.62-1651.68 mg/100 g), epicatechin (3.16-10.62 mg/100 g),	NI	Antioxidant, antibacterial, and antifungal activities,	HPLC	[242]
Not reported	Ethanolic extracts	**Phenolic acids:** *p*-coumaric acid (4.1-6.4 mg/g); ferulic acid (7.1-15.6 mg/g); caffeic acid (4.3-4.5 mg/g)	NI	Antioxidant,	HPLC	[99]
Anatolia	Ethanolic extracts	**Phenolic acids:** 4-pentenoic acid, 5-phenyl (2.34%); caffeic acid (0.14-2.36%), **Flavonoid:** naringenin (0.4-7.42%); chrysin (1.61-8.05%); pinobanksin and its derivatives (0.54-6.7%); quercetin pinobanksin and its derivatives (1.8-3.6%); galangine.	NI	Antimicrobial activity	GC–MS	[83]
Mersin (South Anatolia) and Castanea Bursa (West Anatolia)	Methanol extracts	***Eucalyptus*-type** propolis main component are aromatic acids mainly cinnamic acid and its esters ***Castanea*-type** propolis contained only trace amount of aromatic acids, it consisted mainly of sugar and glycosides.	*Populus* sp., *Eucalyptus* sp. and *Castanea sativa*	Antimicrobial activity	GC-MS	[189]
Kayseri	Ethanolic extracts	**Flavonoids:** Chrysin, **Phenolic acid ester:** Cinnamyl cinnamate **Fatty and aromatic acids:** 9-Octadecenoic acid , 2-Propenoic acid , Caffeic acid **Ketone and terpene** including 2-Propen-1-one, 4H-1-Benzopyran-4-one, Benzofuran-3-one,	NI	Antifungal activity	GC-MS	[94] [95]
Different localities	Ethanol-water Extract	1-*Populus nigra* and *Populus tremula* L. types **Flavonoids:** apigenin, chrysin, caffeic acid, naringenin, pinobanksin, galangin and pinocembrin, **Phenolic Acid Esters:** caffeic acid phenethyl ester, **Phenolic acids:** caffeic acid, **2 nonphenolic** type was rich in terpenes	1-*Populus nigra L.* and *Populus tremula L.* types -2 nonphenolic type (*Fabaceae, Lamiaceae, Rosaceae, Castanea sativa* Mill., *Lotus*	Antioxidant activity	HPTLC	[89]

			corniculatus L., *Salix* spp.)			
Erzurum (East Anatolia)	Ethanolic extracts	**Flavonoids:** Naringenin (nd-2.51 %), **Aromatic acids:** Benzoic acid (1.04-1.25%), **Phenolic Acid Esters:** Benzyl cinnamate (nd-2.91 %), **Anthraquinone:** Chrysophanol (nd-20.82 %)	*Populus alba, Populus tremuloides and Salix alba.*	Antibacterial	GC–MS	[95]
Hakkari	Ethanol extracts	**Falvonoids:** apigenin, chrysin, galangin, gengkwanin, kaempferol, naringenin, pinocembrin, pinostrobin chalcone, sulfuretin, tectochrysin. **26 Coumarins including** angelicin, bergapten, bergaptol, columbianetin, decursin, isogeijerin, jatamansin, lomatin, methoxsalen, oroselone, osthole, oxypeucedanin, prangenin, psoralen, seselin	Poplar type	NE	GC–MC	[90]
Hatay	Alcohol extracts	**Phenolic acids:** caffeic acid (4.37 %), cinnamyl cinnamate (6.95 %) **Phenolic Acid Esters:** Benzyl cinnamate (4.43 %), methyl cinnamate (3.55 %) **Terpenoids:** α-Elemene (3.51%), β-Eudesmol (7.61%), α-Eudesmol (4.51%) **Other components:** Indolin, 2-methylen (2.31%), Cyercene (4.45%)	NI	Preservative effect on egg quality during storage	GC–MC	[100]
Kayseri	80% ethanol	**Phenolic acids:** Caffeic acid (3.45%) **Flavonoids:** chrysin (10.62%) **Fatty acids:** 9-Ocadecenoic acid (1.46%), 2- **Aliphatic acids**: Propenoic acid (4.88%) **Phenolic Acid Esters:** Cinnamyl cinnamate (3.21%)	*Populus* spp.	Antifungal activity	GC–MC	[91] [92]
Anatilia	Ethanol (96%)	**Aromatic alcohols**: 2-napthalenemethanol (0.3-2.18%) **Phenolic acids:** **Flavonoids**: isalpinin (4.97-6.17%), pinocembrin (7.01-16.26%), pinostropin (2.26-13.06%), naringenin (0.9-6.2%), chrysin (1.45-9.86%), pinobanksin and its derivatives (4.3-11.5%), quercetin and its derivatives (1.1-9.1%) apigenin and its derivatives (0.2-3.8%) **Phenolic Acid Esters:** Cinnamyl cinnamate (0.23-5.28%).	NI	Antimicrobial activity	GC–MC	[93]
Elazig Province	70% ethanol	**Flavonoids:** Chrysin (5.33%), Acacetin (3.02%), Naringenin (2.67%), **Terpenes:** Farnesol (20.64%), **Aromatic acids** and **Aromatic acids ester** with low concentration	NI	Application in poultery farming	GC-MS	[102] [103] [101]
Karabuk	Water extract	**Aromatic acid**: Benzoic acid (1.12%), cinnamic acid (1.04%) **Aromatic acid ester:** dimethyl caffeic acid (1.79%), cinnamyl- cinnamate (1.86%). **Faty acids:** oleic acid (1.95%), **Flavonoids:** chrysin (18.1%), pinocembrin (16.8%), pinostrobin chalcone (7.12%), galangin (7.88%), Tectochrysin (9.5%), sakuranetin (1.05) **Terpenes** in low concertation: abietic acid, α eudesmol, dehydroabietic acid β-selinene	NI	Application in poultery farming	GC-MS	[274]

Hatay and Bursa province	Ethanolic extracts	**Phenolic** Acid Esters: 1,2-Benzenedicarboxylic acid, bis(8-methyl nonyl) ester (nd-7.01%), Benzyl cinnamate (nd-1.23%), **Phenolic** acids: benzoic acid (nd– 4.73 %), (3-phenyl-2-propenoic acid (3.23-0.91 %) **Flavonoids**: tectochrysin (3-4.31%), pinocembrin (5.43-10.63%), pinobaksin (1.61-3.77%), Chrysin (2.8-7.04%)	NI	Antimicrobial activity	GC- MS	[107]
Different regions in Turkey	Ethanolic extracts	**Phenolic acids**: caffeic acid (0.88 mg/g), p-coumaric acid (0.49 mg/g), ferulic acid (0.56 mg/g), *trans*-cinnamic acid (0.51mg/g), **Flavonoids:** pinocembrin (18.71 mg/g), chrysin (16.65 mg/g), galangin (13.66 mg/g), pinobanksin (3.01 mg/g), pinostrobin (2.6 mg/g),	NI	Antioxidant and antiproliferative	HPLC-PDA	[106]
Kayseri	Ethanolic extracts	**Phenolic Acid Esters:** Cinnamic acid, 3,4 dimethoxy (21.62%), Cinnamic acid 4 methoxy (22.61%), 3,4 *bis* cinnamate (23.76%) **Phenolic acids:** 2-phenylacetic acid (12.11%), *p*-cinnamic acid (19.96%) **Flavonoids:** Pinobanksin (33.38%), Flavone 3',5,6-triethoxy-3,4' flavone (35.02%), flavon acetic acid (35.6%), Other components: 2,4-di-*tert*-butyl-6-nitrophenol (26.36%)	Poplar	Protective effect against Propetamphos-induced changes in haematological and biochemical parameters	GC- MS	[109]
Euro-Siberia, Irano-Turanian region	Ethanolic extracts	**Phenolic Acid Esters:** (nd-2.18 mg/g propolis) **Aromatic alcohol:** (3.65-11-45 mg/g propolis) **Phenolic acids:** (nd-8.55 mg/g propolis) **Flavonoids:** (1.34-64.3 mg/g propolis)	NI	Antibacterial and protective effect against damage caused by TCDD	GC- MS	[104] [105]
Different regions of Turkey	NI	**Phenolic Acid Esters:** (nd-2.18 mg/g propolis) **Phenolic acids:** *p*-Hydroxybenzoic acid (0.46-2.24 mg/mL), caffeic acid (3.96-34.78 mg/mL), *p*-coumaric acid (0.19-4.91 mg/mL), cinnamic acid (3-5.28 mg/mL), ferulic acid (1-19.42 mg/mL), **Flavonoids:** Quercetin (1.11-4.33 mg/mL), chrysin (1.24-2.22 mg/mL), pinocembrin (0.94-2.81 mg/mL), galangin (0.96-1.70 mg/mL),	Samples of orange, blue and the third subtype propolis	Antibacterial and antioxidant activities	UHPLC–LTQ/orbitrap /MS/MS	[85]

Anatolia	Dried propolis ethanolic extract (DEEP)	**Phenolic Acid Esters:** Chlorogenic acid (25.09-582.38 mg phenolic/kg DEEP), **Phenolic acids:** caffeic acid (284.43-546.66 mg phenolic/kg DEEP), gallic acid (13.18 - 35.86 mg phenolic/kg DEEP), (284.43 mg phenolic/kg DEEP) **Flavonoids**: gallocatechine (687.77-2106.09 mg phenolic/kg DEEP), epigallocatechin (102.3-351.12 mg phenolic/kg DEEP) myricetin (140.27-158.28 mg phenolic/kg DEEP), DEEP: dried propolis ethanolic extract	NI	Antioxidant activity	HPLC	[96]
Elazig province	Ethanolic extract	**Flavonoids:** chrysin (5.3%), acacetin (3.0%), naringenin (2.7%) **Terpene:** Farnesol (20.6%)	NI	Effect on performance, nutrient utilization and carcass charac-teristics in broilers exposed to lead	GC- MS	[84]
Region of Bingol,	Ethanol extract	**Phenolic Acid Esters:** caffeic acid phenethyl ester (2574.4 µg/g) **Flavonoids:** chrysin (23.9 µg/g), acacetin (41.1 µg/g) , apigenin (126.7 µg/g), pinostrobin (548.8 µg/g), naringenin (143.3 µg/g), quercetin (35.8 µg/g), galangin (38.3 µg/g),	NI	Effects of propolis on apoptosis in cancer cells.	HPLC–MS/MS	[88]
Bursa	95% ethanol	**Phenolic Acid Esters** (13.1%) **Flavonoids:**(31.8%) **Terpenoids:** (4.50%) **Aliphatic acids**:(2.22%) **Alcohols**: (1.71%) **Aromatic acids: (**1.52%)	*Castanea.sativa*	Protective effects against alcohol induced oxidative stress in male rats.	GC- MS	[252]
Kayseri province	Ethanolic extracts	**Flavonoides:** pinocembrin (14.75%), chrysin (7.67%), galangin (4.9%) Presence of aromatic acids, aromatic acid ester and some terpens in low concetration	NI	Protective effect	GC–MS	[87]
Bursa	Water extract	**Fatty acids:** 25.36% **Aromatic acids** 6.34 % : benzoic acid, caffeic acid, , **Phenolic Acid Esters** 5.73%: 4,3 acetyloxycaffeate, 2-propenoic acid methyl ester, caffeic acid methyl ester **Flavonoids** 17.37% : chrysin and pinobanksin	NI	Anticarcinogenic effect	GC/MS	[86]
Levantine Coast						
Cyprus						
Not reported	Ethanolic extracts`	**Flavonoids:** chrysin (0.24-5.94 mg/g EEP); pinocembrin (3.7-20.26 mg/g EEP); pinobanksin (0.55-1.59 mg/g EEP); pinobanksin-3-O-acetate (4.29-5.67 mg/g EEP).	*Pinus* sp	Antioxidant, antibacterial, and	GC–MS	[31]

		Anthraquinones: emodin (1.8-4.08 mg/g EEP), **Diterpenes:** dehydroabietic acid (17.75-46.97 mg/g EEP), abietic acid (16.57-23.44 mg/g EEP), isopimaric acid (5.5-17.44 mg/g EEP),		antifungal activities,		
South West, south and central Cyprus	Ethanolic extracts	**Phenolic acid ester (1.1-17%)** **Phenolic acids (0.1-4.5%)** **Flavonoids (0.7-38.5%):** mainly pinocembrin, pinobanksin, pinobanksin-3-O-acetate, chrysin, galangin **Diterpenes (0.7-32.6%):** mainly isocupressic acid, pimaric, imbricatoloic acid, agathadiol, totarol, 13epi-torulosal,	cypress trees *Cupressus sempervirens*	Antibacterial and antioxidant effects	GC-MS	[61]
Lebanon						
South of Lebanon	Ethanolic extracts	**Phenolic acids:** caffeic acid (nd- 0.142 mg/g), ferulic acid (nd- 1.184 mg/g), **Flavonoids** : rutin (0.072- 0.206 mg/g), quercetin (0.055- 0. 0.474 mg/g), genistein (nd-0.015 mg/g), apigenin (nd- 0.176 mg/g), keampferol (0.571 - 0.714 mg/g), chrysin (3.480-5.596 mg/g), pinocembrin (2.688 – 3.112 mg/g), galangin (3.080 – 4.064 mg/g)	NI	Cytotoxic activity	LC-MS/MS	[110]
Northern African Coast						
Egypte						
Upper	Ethanolic extracts	**Aliphatic acid:** lactic acid (0.31-18.5%), succinic acid (0.16-8.00%) and, hydroxyacetic acid (0.04-4.3%) **Phenolic acids:** 4-methoxy-cinnamic acid (1.81%) **Di and Triterpenes:** cycloartenol (7.10%); ß-amyrin (0.17-4.7%), triterpene of ß-amyrin type (0.08-4.8%), **Flavonoids:** pinocembrin chalcone (0.04-3.67%), pinocembrin (1.1-1.35%), pinobankasin-3-acetate (0.42-1.1%)	Populus nigra *Populus* spp	Antimicrobial Activity	GC-MS	[196]
East Nil Delta	Ethanolic extracts	**Fatty acid:** stearic acid (1.9%-6.4), oleic acid (3.2%-12.3%), palmitic acid (1.4%-13.3%) **Phenolic acids:** Benzoic acid (2.7%), ferulic acid (2.4%), caffeic acid (4.4%), 3,4-dimethoxy-cinnamic acid (2.90%), cinnamic acid (2.80%), trans- *p*-coumaric acid (2.30%) **Flavonoids:** pinocembrin (6.06%), pinobankasin-3-acetate (1.16%) **Esters**: Phthalate ester (0.3%-3.8%)	*Populus* spp	Antiviral and Antimicrobial Activities	GC/MS	[111]
Not reported	Ethanolic extracts	**Esters:** octadecatrienyl ferulate (1.11%,) tridecyl hydroferulate (isomer) – (11.24%) **Di and Triterpenes**: cycloartenol (2.05%), cycloartenol (2.12%) isomer, α-amyrin (2.53%). B-amyrin (2.44%), triterpene of α-amyrin type (2.00%-3.61%). **Phenolic acids and Aliphatic acid:** low amounts	NI	Antioxidant and Antimicrobial Activities	GC/MS	[112]
Middle delta region	Ethanolic extracts	**Phenolic acids:** Vanillic acid (3.05±0.53 µg/g), benzoic acid (2.31±0.34 µg/g), caffeic acid (7.435±0.36 µg/g), chlorogenic acid (4.22±0.28 µg/g), gallic acid (6.35±0.18 µg/g), tannic acid (10.64±0.81 µg/g), ferulic acid (5.14±0.54 µg/g), **Flavonoids:** Catechol (8.12±0.25 µg/g), catechin (4.22±0.19 µg/g), Esculetin (6.39±0.36 µg/g)	NI	Antioxidant and Antimicrobial	HPLC	[113]
Libya						

Different localities	NI	Mainly diterpenes	NI	Anti-parasitic and Antimicrobial Assays	LC-HRMS	[114]
Tarhona	Ethanolic extracts	Compounds belonged to phenolic, flavonoids, terpenoids, phytosterol and coumarine.	NI	Antioxidant and antimicrobial activities	Phytochemical Screening through chemical tests were using standard procedures to identify the constituents as described by literature	[115]
North East	Ethanolic extracts	**Diterpenes:** 13-epitorulosal, acetyl-13-epi-cupressic acid and 13-epi-cupressic acid	NI	Antiprotozoal activity	HPLC–ELSD, ^{1}H and ^{13}C NMR	[116]
Tunisia						
Menzel mhiri	Methanolic extracts	**Flavonoids:** myricentin 3,7,4',5'-tetramethyl ether, quercetin 3,7,3'-trimethyl ether, chrysin, galangin, tectochrysin, pinocembrin, pinobanksin, dimethyallyl caffeate, phenylethyl caffeate	NI	NE	HPLC	[117]
Algeria						
Stetif region	Ethanolic extracts	**Phenolic acid:** cinnamic acid (0.9%-1.31%), 2,3,4-trihydroxybenzoic acid (10.74 %), cinnamicacid, 3,4-dimethoxy- (1.39%), cinnamicacid, 4-methoxy-3-(2.32%) **Phenolic acid esters:** trimethyl cinnamate (1.24%-20.03%), benzoicacid, 2-methyl ester (4.96%), trimethyl 3,4-bis(trimethyloxy) cinnamate (60.27%) **Flavonoids:** chrysin (8.76%) **Diterpenes:** pimaric acid (1.09%-4.69%), dehydroabietic acid (2.50 %), abietic acid 4.65%) **Triterpenes:** lanostan-3-one, 11. β,18-epoxy (5.36%), Pregn-4-en-3-one, 17-hydroxy-, (17. α.) (6.07 %),	NI	Bactericidal and immunomodulators activities	GC/MS	[118]
Different region of algeria	Ethanolic extracts	**Caffeic acid derivatives**: 3-methyl-3-butenyl-(*E*)-caffeate, 2-methyl-2-butenyl-(*E*)-caffeate and phenethyl-(*E*)-caffeate **Flavonoids:** chrysin, apigenin, kaempferol, and galangin, pinobanksin, pinobanksin-3-acetate, and pinobanksin-3-(*E*)- caffeate	*Populus* sp	Antioxidant Activity	HPLC-DAD	[119]

		Diterpenes: cupressic acid, isocupressic acid, imbricatoloic acid, torulosal, isoagathotal ,torulosol , agathadiol , cistadiol ,18- hydroxy-*cis*-clerodan-3-ene-15-oic acid				
M'Sila	Ethanolic extracts	**Phenolic acid esters:** 3,3-dimethylallyl caffeate (0.6%-1.7%), 3,3-dimethylallyl caffeate (1.4%-3.0%), Benzyl caffeate (4.5%), Phenethyl caffeate (1.7%-4.1%) **Flavonoids:** chrysin (0.4%-5.6%), galangin (5.3%-5.9%), pinobanksin (9.3%-7.4%), pinobanksin 3-o-acetate (5.3%-8.9%) **Diterpenes**: diterpenic hydroxyacid M=322 (12.4%-21.1%), pimaric acid (0.8%-1.2%)	Buds of poplars	Antimicrobial Activity	GC-MS	[74]
North Algeria		**Phenolic acid esters:** 3-methyl-3butenylcaffeate (1.9%), 2-methyl-2-butenylcaffeate (1.1%), 3-methyl-2- butenylcaffeate (2.6%), benzyl caffeate (2.6%), phenylethylcaffeate (2.4%) **Flavonoids**: pinostrobin chalcone (1.3%), pinocembrin chalcone (5%), pinocembrin (8.7%) sakuranetin (1.7%), pinobanksin (6.8%), dihydroxymonomethoxy flavanone m=430 (1.1%), pinobanksin 3-*O*-acetate (4.8%), chrysin (8.4%), galangin (6.7%) **Diterpenes**: communic acid (1.7%), pimaric acid and imbricataloic acid (2.4%), dehydroabietic acid (1.8%), isocupressic acid (2.1%), junicedric acid (3.7%)	Poplar	Antioxidant and microbicidal avtivities	GC-MS	[61]
Morocco						
Beni Mellal region	Alcoholic and ethyl acetate extracts	**Flavonoids:** wogonoside, quercetin-arabinose glucoside, apigenin dihexoside, rhamnetin hexoside, baicalin or wogonin glucoside, rhamnetin or isorhamnetin, saphnin or daphnitin, afzelechin-catechin dimmer	*Ceratonia siliqua*, *Olea europeae*	Anticancer	HPLC/ESI-MS	[120]
Morocco	Ethanolic extracts	**Phenolic acid esters:** (pentenyl caffeates and phenylethyl caffeate caffeic acid phenethyl ester **Flavonoids:** pinocembrin, galangin (1.2%), Chrysin (1.1%), pinobanksin-3-*O*-acetate **Diterpenes:** isocupressic acid (3.2%-19.5%), agathadiol, (11.3%), totarol (1.5-%10.1%), 13-*epi*-cupressic acid (9.5%), communic acid (7.9%) and imbricataloic acid (2.7%-4.8%) **Triterpenes:** lupeol, α-amyrin, lupeol acetate, and α-amyrin acetate, lanosterol, cycloartenol and 24-methylene cycloartenol,	Poplar type *Cupressus sempervirens*	Antidiabetic and Antioxidant activities	GC/MS	[121]
Morocco	Ethanolic extracts	**Flavonoids**: caffeine, chrysin, galangin and pinocembrin	NI	Antioxydant, Antimicrobial	HPLC	[122]

HPLC-DAD: High-Performance Liquid Chromatography- Diode-Array Detector; HPLC-MS: High-Performance Liquid Chromatography- Mass Spectrometry; GC-MS: Gas Chromatography-Mass Spectrometry; HPTLC-MS: High Performance Thin Layer Chromatography- Mass Spectrometry; LC-PDA-MS: Liquid Chromatography-Photodiode-Array-Mass Spectrometry; HPLC–ESI/MS: High-Performance Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry; NIR: Near Infrared Spectroscopy ; EASI-MS: Easy Ambient Sonic Spray-Ionization Mass Spectrometry; HPLC-MS/MS: High Performance Liquid Chromatography- Mass Spectrometry/Mass Spectrometry; HPLC-ESI-TQ MS: High Performance Liquid Chromatography-Electro Spray Ionization-Tandem Triple Quadrupole-Mass Spectrometry; LC-MS: Liquid Chromatography-Mass Spectrometry; LC-DAD: Liquid Chromatography-Diode-Array Detector; LC-QQQ: Liquid Chromatography-Triple Quadrupole; ULC-QTOF: Untargeted Liquid Chromatography-Quadrupole Time-Of-flight; NMR: Nuclear Magnetic Resonance; IR: Infrared; OPLS: Orthogonal Projections To Latent Structures; HPLC/PDA-ESI/MS: High-Performance Liquid Chromatography/Photodiode-Array-Electrospray; Ionization/Mass Spectrometry; UPLC-ESI-MS/MS: Ultrahigh-Performance Liquid Chromatography- Electrospray Ionization/ Mass Spectrometry System ; HPLC-PDA : HPLC- Photodiode Array Detection; UHPLC LTQ/ORBITRAP/MS/MS: Ultrahigh Performance Liquid Chromatography-Linear Ion Trap-High Resolution Orbitrap Mass Spectrometry System ; DEEP: Dried Propolis Ethanolic Extract; LC-HRMS: Liquid Chromatography -High Resolution Mass Spectrometry; HPLC–ELSD: Evaporative Light Scattering Detector; EEP: Ethanolic Extract of Propolis; NI: Not Indicated; NE : Not Evaluated

Appendix A

Table A.2. Chemical constituents of the volatile part of propolis from different Mediterranean Sea countries and their related biological properties

Propolis samples and its Geographical origin	Volatile extraction method	Main abundant components	Plant source	Bioactivity	Volatiles analysis method	References
Southern European Coast						
France						
Berry and Lyonnais	Hydrodistillation	ß-Eudesmol, guaiol, ß-caryophyllene, ß-selinene, α-guaiene, naphtalene, benzyl, benzoate, benzyl cinnamate, benzyl acetate, trimethylbenzene, methylethylbenzene	NI	NE	GC and GC-MS	[125]
Italy						
Pise and Livourne	Distillation	Anethole, α-pinene and ß-pinene, vanillin, eugenol and borneol	NI	NE	GC and GC-MS	[126]
Not reported	Headspace solid-phase microextraction	Benzoic acid (3.1- 30.1%) and its esters, Vanillin (0.07-5.44%), δ-cadinene (1.29-13.31%), γ -cadinene (1.36-8.85%), α-muurolene (0.78-6.59%), β-eudesmol (2.33-12.83%), T-cadinol (2.73-9.95%) α-cadinol (4.84-9.74%)	NI	NE	HS-SPME-GC–MS	[127]
Croatia						
Slavonia Dalmatia	Hydrodistillation	Benzoic acid (0.7-27%), benzyl alcohol (3.1 and 18.2%), naphthalene (17.8%), limonene (6.4 and 10.5%), β-pinene (0.6 and 6.5%), γ-terpinene (0.6-5.6%),	NI	NE	GC-MS	[128]
Eight different areas	Headspace solid-phase microextraction /simultaneous distillation extraction	α-Eudesmol (up to 19.9%), β-eudesmol (up to 12.6%), γ-eudesmol (up to 10.5%), benzyl benzoate (up to 28.5%), 4-vinyl-2-methoxyphenol (up to 18.1%), γ-cadinene (up to 21.4%), *cis*-calamenene (up to 11.4%), α-bisabolol (up to23%), benzyl cinnamate (up to11.8%);	NI	Antioxidant	GC and GC-MS	[65]
Albania						
Tirana region	Distillation	Cadinene (10.5%), methoxyacetophenone, (9.0%), sesquiterpene alcohol M = 222 (18.5%), heptacosane (6.6%). tricosane (4.8%), nonacosane (5.4), heneicosane (3.6%), pentacosane (4.1%), hentriacontane (4%)	NI	NE	GC-M S	[129]
Greece						
Agrinio, Arta, Preveza and Andros Chalkidiki,	Hydrodistillation	α-Pinene (7.9-45.8%), α-eudesmol (3-12.1%), junipene (1.5-11.7%), *n*-decanal (2.8-10.3%), δ-cadinene (0.3-8.4%), %), β-eudesmol (0.7-7.4%), manoyl oxide (0.1-7.1%), cedrol (4.3-6.3%), manool (0.6-5.2%), γ-eudesmol (1.8-4%), *trans*-β-Terpineol (2.2-6.6%), *n*-nonanal (2.5-5.4%), α-muurolene (1.5-5%); *trans*-totarol (0.6-3.2%);	NI	Antibacterial	GC and GC-MS	[130]
Turkey						
Malatya	Headspace solid-phase microextraction	α-Terpinene (21.8%) , β–pinene (6.4%), ß-cariophyllene (8.1%), junipene (9.1%), α –terpineol (12.3%), cinnamyl alcohol (8.7%), Cinnamaldehyde (6.1%).	NI	Inhibitory effect on the development	GC-MS	[131]

				of experimental tuberculosis infection		
North East North esat Anatolia)	Headspace solid-phase microextraction	Cedrol (4.1-15.6%), α-bisabolol (14.3%), phenyl ethyl alcohol (1.1-7.7%), decanal (0.6-6.7%), ethyl benzoate (0.1-6.5%), benzyl alcohol (0.7-7.4%), δ-cadinene (0.2-5.6%), nonanal (0.3-5%), ß-eudesmol (1-3%), γ-cadinene, (0.3-2.9%), calamenene (0.7-2.8%),	NI	Antimicrobial, Antioxidant	HS-SPME-GC–MS	[132]
Northern African Coast						
Tunisia						
Kef, Nabeul, Bizerte	Solid-phase microextraction	α-Pinene (45-90%), *cis*-sabinol (4.2%), α-terpineol (2.1%), *p*-mentha-1(7) ,2-dien-8-ol (3%), β-fenchyl alcohol (3.6%), β-copaene (0.04-3.2%), junipene (2.8%), γ-cadinene (2.08%), cedrol (0.1-8.2%),	NI	Antifungal Activity	Headspace Coupled with GC-MS	[133]
Algeria						
Benibelaîd, Kaous) (El-malha,	Hydrodistillation	2-Hexenal (1.14-11.5%), myristic acid (1.65-5.66%), thymol (4.47%), linoleic acid (1.86-5.16%), isooctane (0.23-3.89%), α-cedrol (0.15-2.57%), cyclotetradecane (0.3-6%).	NI	NE	GC-MS	[134]

Table A.3. Biological properties of propolis from different Mediterranean Sea countries

Geographical origin	Propolis type plant source	Type of extract isolated	Biological activity detected	Reference
Southern European Coast				
Spain				
Basque Country (Bizkaia, Gipuzkoa and Araba)	*Populus* sp.*; Fraxinus* sp.*; Ulmus* sp.*; Salix* sp.*; Castanea sativa; Rubus ulmifolius; Quercus* sp.*; Betula* sp.	Ethanolic and propylene glycol extracts	Antioxidant activity through ABTS and DPPH scavenging tests	[138]
Basque Country (Bizkaia, Gipuzkoa and Araba)	*Populus* sp.*; Fraxinus* sp.*; Ulmus* sp.*; Salix* sp.*; Castanea sativa; Rubus ulmifolius; Quercus* sp.*; Betula* sp.	Ethanolic and propylene glycol extracts	Antimicrobial effects against *Streptococcus mutans CECT 479T, Staphylococcus aureus CECT 435, Streptococcus pyogenes CECT 191; H. pylori CIP 103995, Salmonella enterica CIP 6062, Escherichia coli CECT 101; Candida albicans CECT 1394, Saccharomyces cerevisae CECT 1383.*	[137]
East Andalusia (southern Spain)	*Castanea sativa; Quercus* sp. *Populus* sp. *; Eucalyptus* sp. *Salix* sp.*; Pinus* sp.	Ethanolic extract	Antioxidant activity through ABTS and DPPH scavenging tests	[35]
Galicia, Castilla y León	NI	Methanolic extract	- Antioxidant activity through DPPH and total antioxidant capacity TEAC assays - Biocide properties using Bacillus subtilis ATCC 6633 (CECT 356).	[140]
Barcelona	NI	70% extract	Fungistatic and fungicidal effects on fungi isolated from Venezuelan patients. *Candida albicans, C. krusei, C. guilliermondii* and *C. tropicalis*	[164]
Galicia, Castilla y León	*Populus spp*., is the main plant	Methanolic extract	-Antioxidant Activity, Inhibiting Capacity of the ABTS Radical	[136]
France				
Monbliahue	NI	70% ethanol	Antibacterial assay*: Staphylococcus aureus, Escherichia coli* and Antifungal assay: *Candida albicans*	[163]
Rennes	NI	80% ethanol	Antiviral effect: Herpes simplex virus 1	[164]
Different regions of France from 'Ballot-Flurin Apiculteurs' company	NI	Water, 95% EtOH, 70% EtOH, and MeOH extracts.	Antioxidant activity: DPPH and ORAC assays Anti-AGEs Activity	[40]
Different regions of France from 'Ballot-Flurin Apiculteurs' company	NI	Water, 95% EtOH, 70% EtOH, and MeOH extracts.	Antifungal Activity: *Candida albicans*, *C. glabrata* and *Aspergillus fumigatus.* Antibacterial Activity: 36 human pathogenic bacterial strains including, *Acinetobacter baumannii strains, Escherichia coli, Pseudomonas aeruginosa,* and *Staphylococcus aureus*	[41]
Italy				
Laboratory C. Sessa (Milano)	NI	Ethanolic extract	Antioxidant activity: Quenching of DPPH, Scavenger effect on superoxide anion, Xanthine oxidase activity inhibition, TBARS assay	[143]
D. Ulrich Spa Laboratory (Torino)	NI	Dry propolis dissolved in sterile broth containing	Antimicrobial activity: *S. aureus, C. albicans, S pyogenes, K. pneumoniae, S. pneumoniae, M. catarrhalis, H. influenzae, E. coli, P. mirabilis and P. aeruginosa, H. influenzae*	[161]

		dimethylsulphoxide and Tween 80		
Piedmont, a region situated in north-west Italy	NI	95% EtOH	Antibacterial effect against *Streptococcus pyogenes*	[44]
Piedmont region hilly zone and a mountain valley	hybrids of poplar, alders, poplars, hazels and pine trees.	Ethanolic extract	Sensitivity to honey bee propolis 44 *Staphylococcus aureus* strains from different source	[165]
Specchiasol S.p.A. Verona Italy	NI	ethanol 70%	Antimicrobial activity: 263 bacterial clinical isolates were tested, mainly *Staphylococcus spp* strains and *Streptococcus spp.* strains	[167]
Venetian propolis	NI	different alcohol concentrations, in the range 60–100% v/v	Antioxidant activity: Inhibition of lipid peroxidation, Folin–Ciocalteu, enzymatic, DPPH scavenging and EEAC assays	[143]
Roma	NI	Ethanolic extract	Antibacterial activity: *Escherichia coli* and *Staphylococcus aureus*	[168]
Roma	NI	70% extract	Fungistatic and fungicidal effects on fungi isolated from Venezuelan patients. *Candida albicans, C. krusei, C. guilliermondii* and *C. tropicalis; C. tropicalis.*	[162]
ABOCA S.p.A.	NI	85% ethanol	Antibiofilm activity: *P. aeruginosa* Antioxidant capacity: chemiluminescence and DPPH assays Anti-inflammatory: Cytokine production assay	[142]
Apulia	*Quercus ilex, eucalyptus spp., rosmarunis officinalis, , quercus spp., spartium, iunceum, acacia spp., juglansregia, prenus ceraus*		Antifungal activity	[161]
All over Italy, islands included	Poplar origin	Ethanolic extracts	Antioxidant activity: DPPH, reducing power, chelating activity, deoxyribose assay	[46]
Slovenia				
Savinjska Valley	NI	96% EtOH	Antioxidative activity of Propolis extract in yeast cells of *S. cerevisiae ZIM 2155*, through determination of intracellular oxidation and mitochondrial Proteome analysis	[145] [52]
Dolenjska,	NI	70% EtOH 96% EtOH	Antioxidant activity: reducing power test, DPPH Free Radical-Scavenging, determination of the $O^{\cdot}{}_2^-$ scavenging activity, β-Carotene–Linoleic Acid System determination, Metal-Chelating Activity Antibacterial activity against different gram -/+ bacterial strains Antifungal activity: yeast and moulds including *C. albicans, Penicillium sp.*	[51]
Croatia				
Continental Croatia	NI	80% EtOH	Antioxidative activity through FRAP assay.	[67]
Metković, Čisla (Omiš), Vis island, Pelješac and Kutina	NI	80% ethanol	Antioxidative Activity using ABTS+ and DPPH	[146]
Croatian propolis	NI	80% ethanol	Radioprotective ɣ-irradiated human white blood cells *in vitro*.	[227]
Lokrum Island and Trogir	NI	70% ethanol	Antimicrobial Activity against: *Staphylococcus aureus*, and *Streptococcus*	[61]

Kopački Rit, Lonjsko Polje, island Krk, Virovitica, Gorski Kotar, Istra, north coast, island Rab	NI	EtOH extract	Antioxidant activity: DPPH assay, FRAP assay,	[65]
Samples from Continental and Mediterranean Regions	NI	Methanolic extract	Antioxidant activity of propolis samples through DPPH and the FRAP tests Antimicrobial Activity: bacteria (*Escherichia coli* and *Staphylococcus aureus*) and yeasts (*Candida albicans* and *Aspergillus niger*)	[62]
Several locations throughout the central, continental Croatia	Poplar propolis type	EtOH-H2O in two ratios 70:30 and 80:20 (v/v)	Antioxidative activity through DPPH radical scavenging assay, Ferric Reducing antioxidant power. Antibacterial Activity *: E.coli, S. aureus, M. catarrhalis, E. faecalis*	[66]
Zagreb	Poplar	Water and 40 or 80% (v/v) ethanol	Antioxidant activity using β-Caroteneelinoleic acid assay, Fe^{2+} chelating activity, Antimicrobial activity	[148]
Continental and Adriatic regions	NI	80% ethanol	Antibacterial activity against Gram-positive bacterial strain *Bacillus subtilis* NCTC 8236,	[54]
Dalmatia	NI	Not mentioned	Antioxidant activity using change of peroxide number (PN), TBARS and of DPPH-radical activity	[147]
Malta				
Different location	NI	70% ethanol	Antibacterial activity: *Staphylococcus aureus, E. coli WF + and Candida albicans*	[70]
Greece				
Nigrita	NI	70% EtOH	Antimicrobial Activity: *Staphylococcus aureus* and *Escherichia coli*	[74]
North-west greece (preveza region)	NI	*n*-butanol extract	Antibacterial activity: *Staphylococcus aureus, S. epidermidis, Enterobacter cloacae,* Klebsiella *pneumoniae, Pseudomonas aeruginosa* Antifungal activity: *candida albicans, C. tropicalis, C. glabrata,*	[72]
Agrinio, Arta, Preveza and Andros and Chalkidiki	NI	Volatile extract of propolis	Antibacterial activity: *Staphylococcus aureus, S. epidermidis, Escherichia coli, Enterobacter cloacae, Klebsiella* pneumoniae and *Pseudomonas aeruginosa* Antifungal activity: human pathogen fungi Candida *albicans, C. tropicalis and C. glabrata.*	[130]
West Macedonia and Rhodes	NI	methanol, 80% methanol, and aqueous	Antioxidant activity through DPPH and FRAP assays	[77]
Central and southern Greece, Aegean Sea islands, and Cyprus (Larnaka, SE Cyprus),	NI	70% ethanol	Antibacterial Activity: 18 pathogenic and non-pathogenic bacterial strains (target strains) Antioxidant activity: DPPH and FRAP assays, Antifungal activity: pathogenic fungi	[31]
Kerkyra, N. Manolada, Veroia, Elassona, Chios, N. Moudania, Didimoticho, Tichero, Alexandroupoli	NI	Ethanol (96 %) w	Antibacterial Activity: propolis against *Paenibacillus* larvae	[169]

central, southern and northern Greece	NI	70% ethanol	Antibacterial bioassay: *Staphylococcus aureus, S. epidermidis, Escherichia coli, Enterobacter cloacae, Klebsiella pneumoniae, Pseudomonas aeruginosa* Antifungal activity: *candida albicans, C. tropicalis, C. glabrata*, Antioxidant activity: Rancimat method	[61]
Nafplio, Amorgos, Crete, Kos, Lakonia, Imathia, Arkadia and Corfu island	NI	Hydroalcoholic	Antioxidant activity: DPPH Radical Scavenging Assay	[76]
Turkey				
Bursa	NI	70% EtOH	Antimicrobial Activity: *Staphylococcus aureus* and *Escherichia coli* Antifungal activity: c*andida albicans*	[74]
Kazan-Ankara, Marmaris-Mugla	NI	30, 50, 70, and 96% ethanol	Antimicrobial Activity: against different Gram^{+} and^{-} bacteria Antifungal activity: *candida albicans*	[98]
Kayseri Province	*Populus nigra* L.	Ethanol extracts	Antioxidant activity through total antioxidant activity using the phosphomolybdenum method and DPPH	[154]
Balikesir and Istanbul	NI	96% ethyl alcohol	Antibacterial Activity against *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *beta-hemolytic Streptococci*	[170]
Mugla, Antakya, Alanya, Beysehir, Hadim, and Tas¸kent	NI	95% ethanol 100% methanol.	Antifungal and antibacterial activity	[171] [173]
Alanya, Antakya, Beys¸ehir, Hadim and Tas¸kent	NI	100% methanol.	Antifungal activity *alternaria alternata* and *Fusarium oxysporium f. sp. melonis*	[172]
Mamak, Kemaliye 1 and Kemaliye 2 regions	NI	Ethanol exctract	Antimicrobial activity of propolis against methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium*	[174]
Yozgat, Izmir, Kayseri, Adana, Erzurum and Artvin.	*P. nigra* and *P. euphratica*	70% ethanol	Antibacterial effects against *Staphylococcus aureus*, *Escherichia coli* and their clinical isolates	[79]
Izmir province (west of Anatolia)	Pinus spp.	70% ethyl alcohol and propylene glycol	potential antibacterial and antifungal activities of propolis on oral pathogen microorganisms	[180]
Mersin (South Anatolia) and Castanea Bursa (West Anatolia)	*Populus* sp., *Eucalyptus* sp. and *Castanea sativa*	Methanol extracts	Antimicrobial activity against a panel of microorganisms (barteria and yeast)	[95] [93] [188]
Anatolia	NI	80% ethanol	Antifungal activity against *Candida* spp., *Trichosporon* spp., *C. glabrata*, and *Rhodotorula*.	[189] [184]
Denizli, Denizli-Sarayköy, AydIn, Denizli-Bas, karcI, AydIn, Konya and Tekirdag cities	NI	96% ethanol	Antimicrobial activity against different Gram+ and Gram- bacteria Antioxidant effects: DPPH assay, heat-induced oxidation of an aqueous emulsion system of β-carotene and linoleic acid	[80]

Region of Yomra, Trabzon,	*Picea orientalis, Fagus orientalis, Castanes sativa, Rhodddendron ponticum, Rhododendron luteum, Rubus caucasicus*	70% ethanol	Antibacterial effect of propolis in mouthrinse against oral microorganisms and human gingival fibroblasts	[182]
Trabzon	NI	99% ethyl alcohol	Amoebicidal, as well as cysticidal, properties for Acanthamoeba trophozoites and cysts.	[186]
Anatolia	NI	Ethanol extracts	Antimicrobial activity against certain oral pathogens	[83] [181]
Hatay province	NI	Methanol extracts	Antimicrobial effect against spoilage and pathogenic food-related microorganisms	[183]
Kayseri	NI	70% ethanol	Antileishmanial effect of propolis against the *Leishmania tropica* parasite	[191]
Erzurum	NI	Lyophilize aqueous extract	Antioxidant activity: ferric thiocyanate method, Fe^{3+} reducing power, cupric ions (Cu_2^{+}) reducing assay, chelating activity on ferrous ions (Fe^{2+}), H_2O_2 scavenging activity, DPPH, $ABTS^{+}$, O_2 scavenging activity and $DMPD^{+}$	[135]
Euro-Siberia, Irano-Turanian regions	NI	Ethanol extracts	Antibacterial activity against anaerobic bacteria causing oral cavity infections	[104]
Different region of turkey mainly Bursa, Ankara, Muðla, Konya,	NI	96% ethanol	Antibacterial activity against two foodborne pathogens, *Salmonella enteritidis* and *Listeria monocytogenes*	[78]
Kayseri (Central Anatolia) in Turkey	Poplar propolis sample	70% ethanol	antimicrobial activity of propolis on *Enterococcus faecalis* and *Candida albicans*	[179]
Honey Agricultural Cooperative	NI	methanol, ethanol, hexane, and water extracts	potent inhibitory activity against the zinc metalloenzyme carbonic anhydrase	[193]
Bingol, Rize, Tekirdag and Van	NI	Ethanol:Ethylacetate:Water:HCl, Methanol:HCl, Ethanol:Water:HCl, Methanol:Water:HCl, Ethanol:Acetone:Water:HCl, Water:HCl	Antioxidant: DPPS, ABTS	[96]
Adana Hatay province Bursa province	NI	Ethanolic extract	Antiparasital propreties : *Leishmania tropica*	[107] [192]
Anatolia	NI	100% methanol.	Antioxidant Capacity: FRAP assay Antifungal Assay and Antibacterial Assay: six bacteria and two yeasts, *S. aureus, E. coli, S. typhimurium, B. cereus, L. monocytogenes, P. aeruginosa, A. niger* and *C. albicans*.	[82]

Bartýn, Artvin, Tekirdað, Edirne, Kýrklareli, Ýzmir, Muðla, Çanakkale and Ankara	NI	ethanol (96 %)	Antibacterial Activity: propolis against *Paenibacillus larvae*	[169]
Black Sea region of northern Turkey	*Chestnut sativa*	Double distilled water extract	Sedative effect by the Monoamine oxidase inhibition was measured using peroxidase-linked spectrophotometric assay in enzyme isolated from rat liver microsomes antioxidant activity: ferric reducing power	[150]
Kayseri, Central Anatolia	NI	80% ethanol	Antifungal activity of propolis against yeasts isolated from blood culture.	[177]
Kazan, Ankara	NI	80% ethanol	Antimicrobial and anti-biofilm activity against planktonic species microorganisms.	[178]
Different areas of Turkey	NI	70% ethanol	Inhibition effects of propolis on the crucial enzymes, urease, xanthine oxidase and acetylcholinesterase	[192]
Bartýn Province, which is located in Northern Turkey	NI	96% ethanol Ethyl acetate Hexane	Antibiofilm activity of propolis against clinical Methicillin-Susceptible Staphylococcus aureus strains	[81]
East Anatolia Region of Turkey	Fabaceae (38.4%), Asteraceae (20.2%), and Fagaceae (11.2%) families	absolute ethanol	Antioxidant activities: the hydrogen peroxide scavenging activity, FRAP, DPPH radical scavenging activity and metal-chelating activity	[153]
Corlu, Kirklareli and one location in Ordu in the Black sea region in the north of Turkey	NI	100% methanol.	Microbiological propreties against *Staphylococcus aureus* isolated from instant soups	[176]
Central Anatolia Region	NI	70% ethanol	Antibacterial activity test: *Escherichia coli, Salmonella, Staphylococcus aureus* and *Listeria monocytogenes* Antioxidant activity: by ELISA using Total Antioxidant Status assay kit	[149]
Hatay province	NI	Methanol extracts	Antibacterial activity against 13 different species of agricultural bacterial pathogens	[187]
Hatay province	NI	Methanol extracts	Antibacterial activity against 20 species of bacteria.	[172]
Trabzon and its surrounding	NI	Ethanolic extracts	Antioxidant activity trough reducing power, radical scavenging activity, metal chelating activity and the analysis of intracellular oxidative stress.	[155]
Black Sea region	NI	Methanol extracts	Antioxidant capacities of propolis were identified through FRAP and DPPH assays,	[152]
Hydroalcoholic extract	*Populus nigra* L., *Populus tremula* L., *Fabaceae, Lamiaceae, Rosaceae, Castanea sativa* Mill., *Lotus corniculatus* L., *Salix* spp.	Ethanol-water extract	Antioxidant activity through HPTLC-DPPH	[89]

Trabzon	NI	Dimethylsulfoxide and Water extract	Antioxidant actifity trough Ferric Reducing Antioxidant Potential and Total antioxidant capacities	[23]
Kayseri	*Populus* spp.	80% ethanol	Antifungal activity trough disc diffusion method, agar dilution method and National Committee for Clinical Laboratory Standards (NCCLS, M27A) broth microdilution method against yeasts isolated from patients with superficial mycoses	[91]
Yomra, Trabzon,.	*Picea orientalis, Fagus orientalis,Castanes sativa, Rhodddendron ponticum, Rhododendron luteum, Rubus caucasicus*	ethanolic extract	Antibacterial property of glass-ionomer cement (GIC) containing propolis against *Streptococcus mutans* as well as on *S. mutans* biofilm formation.	[185]
Different regions in Turkey	NI	ethanolic extract	Antioxidant activity using DPPH, ABTS, cupric Reducing Antioxidant Capacity and FRAP assays	[106] [151]
Anatolia	NI	Methanol extract	Antifungal and Antibacterial activities and antioxidant activity	[82]
Different regions of Turkey	Samples of orange, blue and the third subtype propolis	Ethanolic extracts	Antioxidant activity trough DPPH and antibacterial activity against oral cavity bacteria	[85]
Levantine Coast				
Cyprus				
Not reported	NI	70% ethanol	Antibacterial bioassay: *Staphylococcus aureus*, *S. epidermidis*, *Escherichia coli*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* Antifungal activity: *Candida albicans*, *C. tropicalis*, *C. glabrata*, Antioxidant activity: Rancimat method	[61]
East Cyprus	NI	70% ethanol	Antibacterial Activity: 18 pathogenic and non-pathogenic bacterial strains (target strains) Antioxidant activity: DPPH , FRAP assays, Antifungal activity: pathogenic fungi	[31]
Northen African Coast				
Egypt				
-Upper (Baniswief, Fayoum, Assiut and Souhag) -East Nile Delta (Dakahlia, Ismailia and Sharkia provinces) -Reclaimed land at El-Saff - Not reported	NI NI	Ethanolic extract 70% ethanol	Antifungal activity: effect on growth, conidia germination and aflatoxin production of two isolates of aflatoxigenic fungi. Antibacterial assay: Staphylococcus aureus, Escherichia coli, Streptococcus mutans and lactobacilli in saliva Antifungal assay: Candida albicans The antiviral: Infectious Bursal Disease Virus and Reo-Virus Antioxidant assay: DPPH scavenging activity	[194] [195] [107] [108] [196] [197] [120] [161]

Not reported	NI	ethanolic extract	Anti-protozoal activity on blastocystis species.	[201]
Libya				
Tukra, Al`Aquriyah	NI	Absolute ethanol	Antiprotozoal activity against *Trypanosoma brucei*	[112]
Tukra, Qaminis, Bayda, Kufra, Ghadames, Tripoli, Khaser Khiar and Khumas	*Citrus* spp, *Peganum harmala*, *Pina christi*, *Thymus capitatus* L., *Tamarix africana*, *Ceratonia siliqua*, *Arbutus pavarii*	Absolute ethanol	Anti-parasitic activity: Anti-trypanosomal, Anti-leishmanial assay, Anti-*Mycobacterium marinum*, Anti-Plasmodium falciparum and Anti-*Crithidia fasciculata* assays	[110]
Tunisia				
Monastir	NI	Ethanol extract	Anti-cariogenic and anti-biofilms activity	[199]
Algeria				
M 'Sila	NI	70% EtOH	Antimicrobial Activity: *Staphylococcus aureus* and *Escherichia coli* Antifungal activity: *Candida albicans*	[72]
Beni belaïd area	*Eucalyptus globulus*, *Ficus carica*, *Punica granatum*, *Rubus fructicosus* and *Opuntia ficus-indica*	MeOH/CH2Cl2 and MeOH/H2O	Stromelysin-1 inhibitor	[202]
-North Algeria Tigzirt , Ouled ali, Ain El Arba, Yennarou, Ain ouassara, Ksar el hirane	NI	Methanol extracts Ethanol extracts	Antioxidant activity: DPPH assay, Scavenging Lipid Peroxidation,	[115] [156]
Sétif region: Babor, Setif, Ain-Abbassa and Boutaleb Beni Belaid, Jijel Bejaia and Oued ghir	NI NI NI	70% ethanol CH_2Cl_2-MeOH 4. -70% ethanol - water, 50% ethanol, 85% ethanol, and 50% methanol	Bactericidal and immunomodulators activities Antioxidant activity using DPPH and ABTS assays, FRAP, and cupric reducing antioxidant power. Antioxidant activity: Carotenoid content, Determination of reducing power, Phosphomolybdenum Assay, DPPH and by electrochemical techniques	[114] [157] [156]
Tiaret, Tlemcen , Sidi bel Abbes and Mascara	-*Populus nigra*, *Ziziphus lotus*, *Ceratonia siliqua*, *Juniperus phoenicia* and *Citrus* among others	Ethanolic extract	Antimicrobial capacity: six reference strains and two clinical isolated strains	[200] [201]

ABTS: 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid; DPPH: 2,2-diphenyl-1-picrylhydrazyl; FRAP: Ferric Reducing Antioxidant Power; TEAC: Trolox Equivalent Antioxidant Capacity; NI: Not Indicated; NE: Not Evaluated; ORAC: oxygen radical absorbance capacity; TBARS: of tiobarbituric acid reactive species; EEAC: catechin equivalent antioxidant capacity; DMPD+: N, N-dimethyl-p-phenylenediamine radicals

Table A.4. Different field of application of propolis from Mediterranean Sea countries

Country	Field of application	Screening objectives	Treatment strategy	Main finding	References
Southern European Coast					
Spain					
Granada	Medicine/pharmacy and bio-allied sciences	The assessment of the effect of oral supplementation with antioxidant substances capable of diminishing oxidative aggression and free radical's generation associated to aging	Healthy Wistar rats feeds a diet supplemented with 2 % of propolis in over 3 months. Blood sampling was done for measuring hematological and biochemical parameters and liver were conserved for further analysis.	Synergic effect of overall propolis compounds reducing the oxidative stress and glucose and cholesterol plasma levels associated with aging.	[205]
Zaragoza	Food industry	Investigation of the incorporation of propolis-based dietary supplement into apple juice to decrease the intensity of the heat treatment required to inactivate 5 $\log_{10}$ cycles of Escherichia coli O157:H7.	The minimum inhibitory and minimum bactericide concentrations of propolis-based dietary supplement were determined. A thermocouple was used, to monitor the temperature, where were the treatment media, commercial apple juice, propolis and tested bacterial culture Samples were taken, and the survivors were enumerated. Sensory tests were also done	In apple juice, the controlled incorporation of propolis reduced the thermal treatment needed to reach the goal inactivation level, thing which makes the possibility of obtaining a sensorially appealing, low-pasteurized apple juice with the functional properties provided by propolis.	[260]
France					
Plantex, Sainte-Geneviève-des-Bois	Medicine/pharmacy and bio-allied sciences	Effect of Propolis and cranberry in reducing the motility and the biofilm formation of uropathogenic Escherichia coli responsible on urinary tract infections	assays were performed with strains isolated from patients with cystitis, pyelonephritis or asymptomatic urinary tract infections (colonisation) Motility was evaluated through the swarming and swimming capacity of the isolates in presence/absence of cranberry ± propolis. Early and late biofilm formation was observed with the Biofilm Ring test and the crystal violet method	Propolis potentiates the effect of cranberry on (uropathogenic Escherichia coli) UPEC, representing a new strategy to prevent recurrent urinary tract infections	[206]
West France	Medicine/pharmacy and bio-allied sciences	Photoprotective properties of propolis	In Oil/Water emulsion containing propolis, physical determination of the reduction of the energy in the UV range was performed (SPF determination).	According these results, propolis appears to have interest effect in photoprotection, because its properties are similar to thus of homosalate (positive control).	[207]
Italy					
Laboratory Carlo Sessa, Milan	Medicine/pharmacy and	Preclinical investigation of associating synthetic and natural (resveratrol/propolis) anticancer	Androgen-resistant prostate cancer cell line DU145 was treated for 48h with vinorelbine bitartrate, resveratrol and propolis at different concentrations	Propolis and resveratrol exercise their cytotoxicity through two different types of cell death: a necrosis is triggered in	[208]

	bio-allied sciences	treatment in the therapy of prostate cancer.	MTT assay, Lactate Dehydrogenase activity, cell membrane integrity, cell redox status, and oxygen species production, reduced glutathione levels, genomic DNA fragmentation (COMET assay) with special attention on the presence of apoptotic DNA damage (TUNEL test), and possible mitochondrial transmembrane potential alteration (deltapsi) tests were performed. Results were compared with vinorelbine bitartrate (semi-synthetic drug)	DU145 by treatment with resveratrol, while apoptotic cell demise was induced by propolis. Possible use of these propolis/resveratrol in alternative to classic chemotherapy, and in combination with very low concertation of vinorelbine	
Laboratory Carlo Sessa, Milan	Medicine/pharmacy and bio-allied sciences	Effect of propolis and its main active ingredient caffeic acid phenethyl ester (caffeic acid phenethyl ester) on formation of aberrant crypt foci (ACF) and tumors in the rat colon *in vivo*	Azoxymethane (AOM) used as carcinogenic agent and different group of rats were treated with propolis extracts and caffeic acid phenethyl ester, for aberrant crypt foci determination, the colons were rapidly removed for tumor evaluation	Caffeic acid phenethyl ester (50 mg/kg i.p.) reduced the formation of aberrant crypt foci and tumor induced by azoxymethane, while propolis ethanolic extract was without effect.	[209]
Laboratory Carlo Sessa, Milan	Medicine/pharmacy and bio-allied sciences	Evaluation the effect of propolis during chronic inflammatory events through cultures of human cartilaginous tissues and chondrocytes	The inflammatory events were stimulated with interleukin-1 β (IL-1 β) and treated with indomethacin, propolis and caffeic acid phenethyl ester. The determination of nitric oxide and glycosaminoglycans in cultures of human cartilaginous tissues and chondrocytes were performed.	Propolis and caffeic acid phenethyl ester: - Were able to reduce nitric oxid production and glycosaminoglycans. contrast the harmful effects of interleukin-1 β - prevent the inhibition of glycosaminoglycans synthesis in chondrocytes Propolis possess a protective action of propolis in cartilage alteration	[210]
Laboratory Carlo Sessa, Milan	Medicine/pharmacy and bio-allied sciences	Effect of propolis extract, with and without caffeic acid phenethyl ester, and some of its components (caffeic, ferulic, cinnamic and chlorogenic acids, galangin and pinocembrin) on cyclooxygenase (COX) activity in rats model	Animals were treated with a Bacterial lipopolysaccharide (LPS), lungs were removed and homogenised in protease inhibitor buffer and the protein concentration in the homogenates was measured. Homogenates were pre-incubated with/without test compounds then were incubated in the presence of excess arachidonic acid. The amount of prostaglandine E and 6-keto-prostaglandine F was measured by radioimmunoassay and Inhibition of prostanoid generation by test compounds was calculated	Caffeic, ferulic, cinnamic and chlorogenic acids and pinocembrin, has no activity on cyclooxygenase. caffeic acid phenethyl ester and propolis were equipotent inhibitors, while galangin was less potent. The effect of propolis extract without caffeic acid phenethyl ester was 10 times less potent than the extract containing caffeic acid phenethyl ester. Caffeic acid phenethyl ester and galangin contribute to the overall activity of propolis	[212]
Laboratory Carlo Sessa, Milan	Medicine/pharmacy and bio-allied sciences	The effect of an ethanolic extract of propolis, with and without caffeic acid phenethyl ester, and some of its components on	Murine monocyte/macrophage J774 cell line was used and the amount of prostaglandine E2 was measured radioimmunoassay after stimulation for 24h, with	Pinocembrin and caffeic, ferulic, cinnamic and chlorogenic acids did not affect the activity of cyclooxygenase isoforms, while caffeic acid phenethyl ester and galangin were more effective	[211]

		cyclooxygenase (COX-1 and COX-2) activity in J774 macrophages has been investigated.	lipopolysaccharide to cyclooxygenase-2 or arachidonic acid to induce cyclooxygenase-1	Ethanolic extract of propolis deprived of caffeic acid phenethyl ester, was about ten times less potent than the extract containing caffeic acid phenethyl ester in the inhibition of both cyclooxygenase-1 and cyclooxygenase -2. cyclooxygenase 1 and cyclooxygenase -2 activity was concentration-dependently inhibited by propolis.	
Propolis samples from local beekeepers	Medicine/pharmacy and bio-allied sciences	Use of propolis as lipid bioactive nano-carrier to prepare solid lipid nanoparticles (SLNs) intended for topical nasal drug delivery.	Propolis was used as a lipid material to prepare solid lipid nanoparticles (SLNs). *In vitro* and ex vivo permeation tests of diclofenac sodium, the model drug, and polyphenols were carried out.	*In vitro* and ex vivo studies: - the tested dug and polyphenols do not cross the membranes - solid lipid nanoparticles dispersion based on propolis can be used as a release system for diclofenac and flavonoids for the local treatment of diseases of the nasal cavity, due to the presence of flavonoids.	[213]
Laboratory Carlo Sessa, Milan	Stomatological field	Trial to develop a new topical mucoadhesive formulation containing propolis for possible application in the stomatological field	A gel formulation was developed using propolis extract and its effectiveness was checked *in vitro* through permeation study Only caffeic acid phenethyl ester permeation flux was considered being the component responsible of the activity of propolis An *in vivo* test was done to test the mucoadhesion behaviour and the comfort *in vivo.*	-*In vitro* study showed that caffeic acid phenethyl ester and other propolis components were able to permeate the *in vitro* porcine buccal mucosa. -*In vivo* evaluation of mucoadhesive gel revealed adequate comfort and non-irritancy during the period of study and it was well accepted by the volunteers. Propolis can be involved in the stomatological field being an antimicrobic, anti-inflammatory and analgesic agent.	[214]
Veneto region	Medicine/pharmacy and bio-allied sciences	The investigation of the efficacy of propolis as broad spectrum UVB and UVA photoprotection sunscreens for cosmeceutical use	Sun protection factor; the UVA/UVB ratio and the critical wavelength were measured for propolis,	Propolis gives good broad spectrum UVB and UVA photoprotection. Possibility to use propolis in natural sunscreens formulation.	[215]
Campania, southern Italy	Improvement of livestock	An evaluation of the effect of propolis on mortality of fish eggs due to mycosis through the study of its efficacy on the fish growth rate and the histochemical and ultrastructural characteristics of muscle fibres.	Fish (*Oncorhynchus mykiss* and *Salmo truttain*) was fed two different experimental conditions: traditional feeding and feeding with addition of propolis. The growth rate was determined. The muscles were stained for myosin ATPase and succinic dehydrogenase to evaluate fibre type.	Fish fed propolis showed: - Reduction of mortality of fish eggs - A more rapid muscular growth Possibility of the use of propolis as an antifungal agent and a natural product that can improve fish farming.	[269]
Croatia					
Zagreb	Medicine/pharmacy and bio-allied sciences	Effect of propolis and its polyphenolic derivative compounds (caffeic acid and caffeic acid phenethyl ester) on the growth, metastatic capacity,	Mice were injected with tumor cells to generate metastasis in the lung, the tested components were given to animals *per os* or intraperitoneally before or after tumor cell inoculation.	Propolis and its polyphenolic derivative compounds act as immunomodulatory agent trough: - inducing apoptosis and necrosis, - decreasing the number of lung colonies,	[216]

		immune reaction, apoptosis and necrosis of a transplantable mammary carcinoma (MCa) of CBA mouse.	Several trial for treated and untreated animals were performed regarding: Weight and cellularity of spleen and femur, leukocyte count for blood samples, response of spleen lymphocyte to polyclonal mitogens and apoptosis/necrosis analysis	- inhibiting tumor growth and prolong survival of treated mice	
Zagreb	Medicine/pharmacy and bio-allied sciences	Evaluation of antimetastatic effect of a water-soluble derivative of propolis (WSDP) on CBA mouse	Tumor were generated as describe by.[208] Spleen were removed and lymphocyte phenotyping, macrophage cultures and lymphocyte activating factor activity assay, *in vitro* colony assay of target cell destruction, weight and cellularity of spleen, lymph nodes and femur, and leukocyte count, plaque-forming cells assay, plaque-forming cells assay and Apoptosis and necrosis analysis were done.	The use of water-soluble derivative of propolis leads many changes in several immunological parameters such as: - The production of lymphocyte activating factor by peritoneal macrophages and the efficacy of those macrophages to kill tumor cell - Responses of lymphocytes to mitogen, - Weight and cellularity of spleen, Propolis exerts immunomodulatory activity	[217]
Zagreb	Medicine/pharmacy and bio-allied sciences	Evaluation of the effect of propolis and its polyphenolic on the metastatic potential and immune responses of a transplantable mammary carcinoma (MCa) of CBA mouse	Metastases in the lung were generated as describe by[208] and tested samples (water-soluble derivative of propolis, caffeic acid, caffeic acid phenethyl ester and quercetin were given to mice before tumor cells inoculation. Weight and cellularity of spleen, femur, and leukocyte count, inhibition of 3H-methyl thymidine, NO_2^- and NO_3^- determination, cytotoxicity to tumor cells response of spleen lymphocytes to polyclonal mitogens and apoptosis analysis were performed	Tested compounds were: - Cytotoxic to tumor cells, - Apt to induce apoptosis and necrosis. Propolis acts as immunomodulatory agents Propolis and its phenolic derivatives compounds could be used for the control of tumor growth	[218]
Zagreb	Medicine/pharmacy and bio-allied sciences	Investigation the effect of propolis and its polyphenolic compounds on the growth and metastatic potential of a transplantable mammary carcinoma (MCa) of the mouse	Mouce were given per os the tested compounds (propolis, caffeic acid and caffeic acid phenethyl ester) before or after to the tumor cell inoculation. Total cells number, differential cell count in the peritoneal cavity, and determination of functional activity of macrophages were performed	Tested compounds: - Reduced the number of metastases in the lung. - Were cytotoxicity to tumour cells - Were able to induce apoptosis and/or necrosis. Propolis and its phenolic compounds activity was related to the immunomodulatory properties of the compounds	[219]
Zagreb	Medicine/pharmacy and bio-allied sciences	Investigation the effect of water-soluble propolis derivatives and some of its polyphenolic compounds (caffeic acid, quercetin, chrysin and naringenin) on the development of Ehrlich ascites tumor (EAT).	Animals were treated per os with tested compounds 2h prior the of Ehrlich ascites tumor inoculation. Total number and differential count of cells present in the peritoneal cavity, determination of functional activity of macrophages and survival analysis were effectuated.	Propolis treated animals shows: - A reduction of the volume of ascitic fluid induced by of Ehrlich ascites tumor cells and total number of cells present in the peritoneal cavity - An increase of the number of polymorphonuclear (PMN) cells - A decrease of the number of macrophages in the peritoneal cavity in of Ehrlich ascites tumor -bearing mice treated with test components.	[222]

				All tested compounds affected the functional state of macrophages increasing their tumoricidal activity	
Not reported	Medicine/pharmacy and bio-allied sciences	Effect of the combination of the anticancer drug irinotecan with propolis on the growth of Ehrlich ascites tumor (EAT) and the life span of tumor-bearing Swiss albino mice were studied.	Test components were given to mice intraperitoneally 3 days before the injection of EAT cells then Survival and tumor growth analysis were performed	Results demonstrate the synergistic action of irinotecan and thing which increase survival time and tumor regression	[223]
Zagreb	Medicine/pharmacy and bio-allied sciences	Screening of the efficacity of propolis and its derivative flavonoids on raising irinotecan-induced cytotoxicity to tumor cells in mice displaying Ehrlich ascites tumors (EAT) at the same time cushion normal blood, liver, and kidney cells.	Propolis and its flavonoids (quercetin, naringin) were given to animals before tumor cell inoculation then the solution containing peritoneal cells was removed for further evaluation (the total number and differential count of the cells present in the peritoneal cavity, functional activity of macrophages, hematological and biochemical parameters, and micronucleus assay)	In treated animals with a combination of test compounds and irinotecan is was observed a substantial inhibition of the growth of of Ehrlich ascites tumor cells. Pretreatment with test components prior to irinotecan expanded number of micronucleated cells Propolis and its related flavonoids showed a great immunomodulatory efficacity by deteriorating toxic and genotoxic effects leaded by irinotecan to normal cells without influencing the irinotecan cytotoxicity in tumor cells.	[221]
Not reported	Medicine/pharmacy and bio-allied sciences	Examination of propolis regarding its antitumor, genotoxic, chemopreventive, and immunostimulative effects combined to an anticancer drug of local chemoimmunotherapy and hyperthermal intraperitoneal chemotherapy (HIPEC) in a mouse-bearing Ehrlich ascites tumor (EAT)	Mice were treated with propolis/Cisplatin respectively before/after implantation of Ehrlich ascites tumor cells in the peritoneal cavity of mice were harvested and cells then total number of cells, differential count of the cells present in the peritoneal cavity, functional activity of macrophages, comet assay, and micronucleus assays were performed	- Combination of propolis and cisplatin affect the tumor growth and enhance the survival. - Propolis increases macrophage activity and sensitivity of tumor cells to hyperthermal intraperitoneal chemotherapy and reduces cisplatin toxicity to normal cells, without affecting cisplatin cytotoxicity on of Ehrlich ascites tumor cells.	[220]
Not reported	Medicine/pharmacy and bio-allied sciences	Investigation of the effect of water-soluble derivative of propolis (WSDP) on cytopenias caused by radiation and/or chemotherapy and its antimetastatic efficiency, alone or in combination with chemotherapeutic agents, on the blood leukocytes count and hematopoiesis	Metastases in the lung were generated as describe by[208] and mice were treated with water-soluble derivative of propolis and/or Epirubicin before and after tumor cells inoculation. Blood cell counts, Macrophage cultures and IL-1 activity assay and glutathione content assay were performed.	WSDP combined with Epirubicin inhibited metastasis formation; modulate the hematological parameters (number of red and white blood cells in peripheral blood) Propolis alone: present an antimetastasis activity, it: -Increased number of exogenous CFUs, increased cellularity of hematopoietic tissue and the number of leucocytes in peripheral blood; increased myeloid and megakaryocytic types of CFUs	[224]

Zagreb	Medicine/pharmacy and bio-allied sciences	Screening of the radioprotective effects of propolis and its polyphenolic compounds on the mortality of mice exposed to gamma-irradiation.	Three days before irradiation mice were pretreated with test components (caffeic acid, naringin, quercetin and Chrysin) daily then were inspected twice a day, at least 6 h apart for morbidity and mortality.	Propolis and its polyphenolic compounds: - Offered a protection against hematopoietic death - Postponed the onset of mortality - Reduced the symptoms of radiation sickness. Propolis and its polyphenolic compounds protect mice from the lethal effects of irradiation.	[225]
Zagreb	Medicine/pharmacy and bio-allied sciences	Study of the possible protection of propolis against ionizing radiation in the whole-body mice	Animals were treated intraperitoneally with test components before and/or after irradiation with γ-rays and alkaline comet assay was performed on whole blood samples. Also, genotoxic effects of all test components were assessed.	Propolis and related flavonoids given to mice before irradiation protected mice from lethal effects of whole-body irradiation and diminish primary DNA damage in their white blood cells.	[226]
Zagreb	Medicine/pharmacy and bio-allied sciences	Inspection of antitumor capacity propolis with/without an anticancer drug in mice injected with 4T1 mammary carcinoma	Mice were pretreated with propolis 14 days before tumor injection of tumor cells in gluteal region. Then tumor growth, dihydropyrimidine dehydrogenase (DPD) transcriptional and translational level were determined.	Animals pretreated with propolis combined with 5FU(5-Fluorouracil): -prolonge the suppressive effect of 5FU on tumor growth, - reduced the number of metastasis and decreased dihydropyrimidine dehydrogenase protein level.	[227]
Not reported	Medicine/pharmacy and bio-allied sciences	Assessment of the radioprotective effects of quercetin and the propolis in CBA mice exposed to a single radiation dose 4 Gy.	Mice were treated before/after gamma-irradiation (therapy) with quercetin or propolis. Leukocyte count was determined and DNA damage in leukocytes was assessed using the alkaline comet assay. Genotoxic effects of all test components were also evaluated in non-irradiated mice.	Pretreated animals were less sensitive to irradiation. Propolis and quercetin: - protect against primary leukocyte DNA damage in mice. - Appear not genotoxic to non-irradiated mice. Propolis and quercetin could be used in supportive therapy in humans, notably in the treatment of cancer patients exposed to radiotherapy.	[231]
Zagreb	Medicine/pharmacy and bio-allied sciences	The evaluation of the possible radioprotective effects of propolis on γ-irradiated human white blood cells	Irradiation of blood samples was performed with gamma rays and the alkaline comet assay; the analysis of structural chromosome aberration and the cytokinesis-block micronucleus assays were effectuated	Propolis and its polyphenolic components were able to reduce the number of necrotic cells Tested samples diminish the levels of primary and more complex cytogenetic DNA damage in white blood cell	[232]
Zagreb	Medicine/pharmacy and bio-allied sciences	Investigation of possible synergistic action of anticancer drug Irinotecan (IRI) propolis on Swiss albino mice injected with Ehrlich ascites tumor (EAT)	mice were administered Irinotecan and/or propolis before injection of Ehrlich ascites tumor peripheral white blood cell count, cell types washed from the peritoneal cavity, functional activity of macrophages from peritoneal cavity, and the level of primary DNA damage in leukocytes, kidney, and liver cells using the alkaline comet assay were done	The addition of propolis to Irinotecan treatment enhanced antitumor activity of Irinotecan and prolongs survival in Ehrlich ascites tumor-bearing mice.	[233]
Not reported	Medicine/pharmacy and bio-allied sciences	Assessment of the positive possible effect of topical treatment with propolis before the usability of thermography in psoriatic lesion regression	inflammation was induced using irritants and was followed through histopathological assessment of the skin, thermographic scanning, total number of inflammatory cells in the peritoneal cavity, differential analysis of cells in the peritoneal cavity, macrophage	Topically applied propolis with irritants reduced the lipid peroxidation, total number of inflammatory cells in the skin, peritoneal cavity, functional activity of macrophages, number of micronuclei in mouse peripheral blood reticulocytes and enzymatic activity of	[230]

			spreading index, hematological and biochemical parameters, frequencies of micronucleated reticulocytes, lipid peroxidation and glutathione assay in the skin.	alkaline phosphatase and aspartate transaminase Propolis improve psoriatic-like skin lesions by suppressing functional activity of macrophages and reactive oxygen species production	
Zagreb	Medicine/pharmacy and bio-allied sciences	Effect of propolis on renal and liver function in alloxan-induced diabetic mice.	Animals were treated with a daily dose of propolis after inducing diabetes was with alloxan. Survival analysis, body weights, hematological and biochemical parameters were measured, also, renal and liver oxidative stress marker malonaldehyde levels and histopathological changes were observed.	Diabetic mice treated with propolis showed a: - raise of body weight, haematological and immunological parameters of blood - 100% survival of diabetic mice. - Dropped the number of vacuolized cells and degree of vacuolization; - enhancement of the ruination of fatty acid metabolism in diabetes. Test components did not improve renal histopathology in diabetic mice.	[228]
Not reported	Medicine/pharmacy and bio-allied sciences	Exploration of antidiabetic activity of propolis in alloxan-induced diabetes in mice.	Diabetes was induced in mice through the injection of alloxan monohydrate, then were treated with propolis or epigallocatechin gallate (EGCG) for 7 days. Body weight, determination of glucose level in serum, Survival analysis, haematological analysis were done. Intraperiotoneal liquid samples and total number of cells present in the peritoneal cavity, total number of cells present in the peritoneal cavity were measured, also protein measurement in liver and kidney tissue, lipid peroxidation, alkaline comet (SCGE) assay and pperipheral blood micronucleus (MN) assays were performed	Administration of epigallocatechin or propolis: - raise the body weight to diabetic mice - raise the haematological/immunological blood parameters, - showed 100% survival of the mice. - diminution of lipid peroxidation in liver, kidney and brain tissue - reduction of DNA damage in peripheral lymphocytes -Propolis exbibit an oxidative and anti-inflammatory potential and could be used in strategies for the prevention and treatment of diabetes mellitus	[229]
19 samples from continental and costal part	Medicine/pharmacy and bio-allied sciences	Assessment of the antiaggregatory activity of propolis on platelet aggregation on the whole blood samples of healthy volunteers	Platelet aggregation was determined by impedance aggregometry on the whole blood samples of the healthy volunteers	In low micromolar concentrations propolis showed an important antiaggregatory potential. suggesting that propolis supplementation may influence platelet aggregation and consequently thrombus formation.	[234]
Bosnia and Hercegovina and Croatia					
Not reported	Medicine/pharmacy and bio-allied sciences	Study of cytotoxic effects of propolis on cervical tumor cell line (HeLa)	Cervix adenocarcinoma cell line was treated with propolis and cytotoxicity was evaluated trough MTT assay	Propolis exhibited an antiproliferative and cytotoxic effect on the HeLa without correlation with concentration of particular components	[68]
Albania					

Not reported	Stomatological field	Examination of the possible positive effect of propolis from Albania on the inflamed pulpal tissue after pulpotomy in piglets.	In piglets, two teeth each were infected using special pathogenic microbial flora, the teeth were treated after vital amputation with propolis radiography then histopathology analyses were performed to survey the infected pulp tissue treated with propolis up to 3 months.	Application of Albanian propolis reveled great anti-inflammatory and regenerative effect after 3 month creating dentin barrier. Propolis could be clinical used.	[235]
Greece					
Preveza region	Medicine/pharmacy and bio-allied sciences	Examination of propolis chemical constituents (diterpenes and flavonoids) for their cytostatic activities against human malignant and normal cell strains.	The MTT assay using propolis was checked against two human solid tumor cell lines—HT-1080 fibrosarcoma cells and HT-29 colorectal adenocarcinoma cells and on the HL-60 human promyelocytic leukemia cell line (ECACC), human skin fibroblast strain AG01523c, two other fibroblast strains (DSF9 and DSF60),	Propolis extract and the isolated diterpenes were cytostatic activity, especially against human colon cancer cells Manol isolated from Greek propolis exhibited a promising profile as an antiproliferative agent. Propolis is an antitumor natural product.	[236]
Kalavrita	Medicine/pharmacy and bio-allied sciences	The encapsulation of propolis extract in β -cyclodextrin (β-CD) cavity seeking to raise the solubility of its components in water for medical application	Propolis extract was sonicated in aqueous solutions of β -cyclodextrin in order to form a complex propolis / β -cyclodextrin, the preparation was filtered and dried. Aqueous solubility of propolis in this complex was monitored by drawing a solubility diagrams and by measuring the fraction of propolis extract constituents that was dissolved in water.	Smaller molecules were more efficient in encapsulation but more difficult to release from the β -cyclodextrin cavity as compared with 'bigger' molecules such as flavonoids, anthraquinones and terpenes. The release of compounds from encapsulated propolis depends on their chemical properties and their relative abundance in the matrix.	[237]
Arcadia region	Food industry	Development of a protocol for the addition propolis into milk and the evaluation if its presence could confer anti-listerial activity all along the storage of milk under optimal or improper refrigeration conditions	Propolis extract with/without glycerol, was added into extended shelf-life milk, artificially contaminated with *Listeria monocytogenes*. *L. monocytogenes* growth and survival was monitored and sensory evaluation was performed	Adding propolis in milk, contaminated with L. monocytogenes, leads to a pronounced and dose-dependent anti-listerial effect during storage. Extended shelf-life milk with added propolis received average consumer acceptability scores.	[261]
Turkey					
Bursa	Medicine/pharmacy and bio-allied sciences	Study of the anticarcinogenic effect of propolis in human lymphocytes culture	Blood samples of healthy volunteers were incubated and exposed to different concentrations of propolis. Micronucleus Mitotic index (MI) was determined	Mitotic index decreases in the peripheral blood lymphocytes added to the different concentrations of propolis propolis at high concentrations could become carcinogenic	[86]
Trabzon	Medicine/pharmacy and bio-allied sciences	Exploration of the influence of propolis on respiratory burst within a cancer cell lines	K-562 cell cultures and mononuclear cell (MNC) cultures from a peripheral blood sample were incubated with extracts for 24h. intracellular dichlorofluorescein (DCFH) test through flow-cytometric fluorescence analysis was done.	Fluorescence which was higher in the control group ty decreased by the increasing of propolis extract K-562 cell culture, no effect on mononuclear cell culture. Propolis extracts inhibit respiratory burst within cancer cell lines probably by their antioxidant potentials.	[261]
Bursa province	Medicine/pharmacy and	Investigation of the anticarcinogenic and antimitotic	Tissue samples were obtained from patients with bladder carcinomas and exposed to	Propolis decrease cell division	[239]

	bio-allied sciences	effects of propolis on transitional carcinoma cell cultures.	propolis	Propolis could be used as an antimitotic and anticarcinogenic agent.	
Sakarya, Kemaliye, C-anakkale, Van, Yalova and Hacettepe	Medicine/pharmacy and bio-allied sciences	Investigation of the anti-apoptotic efficacity of propolis on the caspase pathway in the human breast carcinoma cell lines.	Propolis extratcts were incubated with MCF-7 cells during 2 days culture. Cell growth and cytotoxicity were measured colorimetrically by MTT assay. Apoptotic cell death was determined by the TUNEL method and caspase activity was investigated by immunocytochemistry.	Propolis induced apoptosis and increase number of TUNEL positive cells. Immunpositivity for all caspases was detected after treatment with propolis extracts Propolis act as anti-tumour by increasing apoptosis through the caspase pathway.	[240]
Trabzon	Medicine/pharmacy and bio-allied sciences	Evaluation of the effect of propolis on protein expression profile in PC-3 prostate cancer cell lines.	PC-3 prostate cancer cell lines were incubated with propolis extract MTT assays, Lysis of PC-3 cancer cell lines after incubation and Expression Difference Mapping (Proteomic profiling) using SELDI-TOF-MS assays were assessed	Propolis reduced the cell viability propolis may have antiproliferative activity through differentiating protein expression profile in PC-3 prostate cancer cell lines	**[**23]
Trabzon region	Medicine/pharmacy and bio-allied sciences	Study of the cytotoxic activity of propolis on Liver, Colon, Breast, Cervix and Prostate Cancer Cell Lines	Cytotoxic activity of propolis was determined through MTT assays against the five human cancer cell lines: prostate adenocarcinoma (PC-3), hepatocellular carcinoma (HepG2), colon adenocarcinoma (WiDr), cervix adenocarcinoma (HeLa) and mammary adenocarcinoma (MCF-7)	Propolis exhibited a powerful anti-proliferative activity against the five human cancer cell lines tested reducing their proliferation. The activity found may be due to its phenolic content	[245]
Sivas	Medicine/pharmacy and bio-allied sciences	Examination of the cytotoxicity and nitric oxide synthase (iNOS) effect of propolis (EEP) against several cancer cell lines and a non-tumor cell line along with encapsulation	Microencapsulation by complex coacervation of propolis was done cytotoxicity of propolis alone or with encapsulation was screened against PC-3, U87MG, A549, mPANC96, CaCo-2, MCF-7, HeLa, MDA-MB-231 and a non-tumor cell line HEK293, with MTT assay and inhibition levels of inducible nitric oxide synthase (iNOS) were also determined.	Propolis free and loaded microparticles exhibited similar cytotoxic and nitric oxide inhibition activities. The results found revel the high potential of propolis as an anti-inflammatory agent, from its possible use the industry as a natural supplement	[242]
Trabzon	Medicine/pharmacy and bio-allied sciences	Investigation of the apoptosis-inducing capacity of dimethyl sulfoxide extracts of propolis in HL-60 Myeloid Cancer Cell Lines.	Propolis extract was incubated with HL-60 cells, and caspase-3 activity evaluated. The cells were analyzed using flow cytometry for the determination of the cell cycle characteristics of HL-60 cells with and without extract	Propolis increased the apoptosis from undetectable levels to 60.1 %, while it maintains cell viability. propolis increase apoptosis and the activity of caspase-3 in HL-60 cells. Propolis may have beneficial effects in the treatment of cancer	[244]
Trabzon	Medicine/pharmacy and	Investigation of the effect of dimethyl sulfoxide and water	propolis extracts were incubated for 24 h with PC-3 cells and total RNA was extracted	propolis decreased the expression of Voltage-Gated Sodium Channel isoforms to varying extents.	[243]

	bio-allied sciences	extracts of Turkish propolis on mRNA expression of Nav 1.5 and 1.7 a isoforms of Voltage-Gated Sodium Channel (VGSC) proteins in PC-3 human prostate cancer cells	Real-time polymerase chain reaction (RTPCR) assay was used	propolis may have antimetastatic activity in PC-3 cells due to down-regulation of expressions mRNA of Voltage-Gated Sodium Channel α-isoforms.	
Trabzon	Medicine/pharmacy and bio-allied sciences	Study of the antiproliferative and proapoptotic effect of propolis on human lung cancer cell line.	A549 cells was used and the cytotoxic activity of propolis was tested using MTT assay. Apoptosis, mitochondrial membrane potential and cell cycle using flow cytometry, endoplasmic reticulum stress using RT-PCR, and caspase activity using luminometric analysis were investigated	Propolis showed selective toxicity against A549 cells. It arrested the cell cycle of A549 cells at the G phase, induced endoplasmic reticulum stress, caspase activity, and apoptosis and reduced mitochondrial membrane potential. Propolis reduce cancer cell proliferation.	[246]
Region of Bingol,	Medicine/pharmacy and bio-allied sciences	Study of the effects of propolis on apoptosis in cancer cells.	MCF-7 cells were incubated with propolis for 24 h after treatment with homocysteine (Hcy) Cell proliferation, lipid peroxidation, intracellular reactive oxygen species, TUNEL, cell migration and expression levels of apoptotic genes among other assays was performed	Propolis inhibited caspase- 3 and -9 induced by Hcy in MCF-7 cells. Propolis at low doses, has protective effects against homocysteine-induced toxicity.	[88]
Different regions in Turkey	Medicine/pharmacy and bio-allied sciences	Examination of the antiproliferative effect on several human breast cancer cell lines and proliferative effect on fibroblasts and mouse mesenchymal stem cell line	Two different breast cancer cell lines; MDA-MB-231, UACC-3199 and on two normal cell lines; fibroblasts and mouse mesenchymal stem cell lines was used and cell viability was tested through XTT assay	Propolis sample showed significant anti-proliferative effect on MDA-MB-231 and UACC breast cancer cell lines. As well as a proliferative effect on both fibroblasts and mouse mesenchymal stem cells.	[106]
Bursa	Medicine/pharmacy and bio-allied sciences	Study of genotoxic potential of propolis in peripheral blood lymphocytes	Blood samples were incubated and exposed to increasing concentrations of propolis and sister chromatid exchange (SCE) rates was determined	Propolis increased SCE rates and have genotoxic effects in high concentrations. This activity could be related to the complex content of propolis through a synergistic effect.	[239]
Kayseri	Medicine/pharmacy and bio-allied sciences	Protective effect of propolis on the damage in biochemical and haematological parameters induced by propetamphos	Propolis was done to animals in drinking water during 28 days with/without propetamphos Blood samples were taken for biochemical and haematological analysis.	Propolis alleviated the harmful effects of propetamphos (increase of the levels of glucose, triglyceride, activities of aspartate aminotransferase, alkaline phosphatase, and alanine aminotransferase, Lymphocyte percentage, decrease of total protein, neutrophil percentage and total leukocyte)	[109]
Turkish propolis	Medicine/pharmacy and	Protective effect of propolis	DNA damage was induced using hydrogen peroxide Cells were treated with propolis at different concentration	Propolis decrease H_2O_2 -induced DNA damage in cultures. Propolis showed a chemopreventive activity	[248]

	bio-allied sciences	on foreskin fibroblast cells against hydrogen peroxide induced oxidative DNA damage.	Anti-genotoxicity was assessed before, simultaneously, and after treatment of propolis extract.		
Erzurum	Medicine/pharmacy and bio-allied sciences	Investigation of the protective effect of propolis on histological changes, oxidative stress and DNA damage in rat liver induced by 2,3,7,8 TCDD (2,3,7,8-Tetrachlorodibenzo-P-dioxin)	Rats were intraperitoneally administered with propolis and / or Tétrachlorodibenzo-p-dioxine daily for 21 days. the liver samples were obtained by the end of the experiment for the determination of antioxidant enzyme activities, level of glutathione, rate of hepatocyte micronucleus analysis and histological examinations.	Presence of propolis with Tétrachlorodibenzo-p-dioxine: - alleviated the pathological effects in hepatic tissue -Prevent the suppression of antioxidant enzymes in the livers of animals - showed a strong protective effect against hepatocyte micronucleus. It can be concluded that propolis has beneficial influences and was able to antagonize Tetrachlorodibenzo-P-dioxin toxicity in the liver.	[104]
Kayseri province	Medicine/pharmacy and bio-allied sciences	Evaluation the protective effect of propolis on lipopolysaccharide (LPS)-induced experimental model of liver injury in rats.	Propolis was administered to rats animals after LPS administration. After six hours DNA methylation analysis in the liver tissue samples were determined, Genomic Template Stability (GTS, %) was evaluated in the blood tissue samples	Propolis showed a protective effect against changes of both genomic stability values and methylation profiles, It alleviates tissue damage and malondialdehyde level changes The use of propolis before lipopolysaccharide protect the hepatic damage	[87]
Istanbul	Medicine/pharmacy and bio-allied sciences	Exploration of the effects of propolis against cyclosporine (CsA) induced hepatotoxicity and nephrotoxicity in rats	propolis alone or with cyclosporine was given by gavage to animals every day. Feed intake and body weight were determined and blood sample biochemical analysis. The kidney and liver tissues were used for biochemical analysis	Propolis improve reduction in body weight and ameliorate the toxicity of cyclosporine by scavenging the free radicals and increasing the antioxidant activities.	[151]
Not reported	Medicine/pharmacy and bio-allied sciences	Investigation the effects of propolis on the expression of DNA repair enzymes.	Fibroblast cells were incubated with propolis then cell viability was determined using MTT. DNA damage was determined using comet assay and mRNA expression of target enzymes was detected using RT-PCR.	Antigenotoxic effect of propolis is through preventing oxidative damage by radical scavenging, metal chelating, or modulation of antioxidant response, rather than increasing expression of DNA repair enzymes.	[241]
Black Sea Region	Medicine/pharmacy and bio-allied sciences	Evaluation of the hepatoprotective effect of propolis	Animals were fed with propolis for 7 days after the injection of CCl4 injection. Activities of the alanine transaminase, aspartate transaminase, malondialdehyde, superoxide dismutase, and catalase were determined. Antioxidant capacities of propolis were identified through FRAP and DPPH assays,	Propolis enhanced the healing of CCl4 induced liver damage, due to their antioxidant properties and bioavailability.	[151]
Trabzon	Medicine/pharmacy and	Evaluation of the radioprotective effect of ethanolic against ɣ-ray-	Fibroblast cells were pretreated with propolis at different concentrations then were exposed to ɣ-rays.	Propolis decreased DNA damage on cells caused by the ɣ-rays.	[249]

	bio-allied sciences	induced DNA damage on fibroblast cells	Comet assy was done	propolis prevent ɣ-ray-induced DNA damage in fibroblast cells and showed a radioprotective activity	
Region of Bingol,	Medicine/pharmacy and bio-allied sciences	Evaluation of the cytoprotective role of propolis on Hyper homocysteinemia induced vascular endothelial cell injury.	Human vascular endothelial cells were incubated with Hyper homocysteinemia and propolis in complete media for 24h. Cell proliferation, lipid peroxidation, intracellular reactive oxygen species, TUNEL and active caspase-3 positive cells assays was performed.	Hyper homocysteinemia causes oxidative stress increasing the reactive oxygen species generation, Bax/Bcl-2 ratio and the expression of caspase3 protein. The co-treatment with propolis protects human vascular endothelial cells against damage induced by Hyper homocysteinemia	[250]
Bursa	Medicine/pharmacy and bio-allied sciences	Exploration of the protective effects of propolis on serum lipid profile and liver enzymes against alcohol induced oxidative stress in male rats.	The ethanol-propolis extract with or not alcohol was given to rats for 15 days. At the end of the treatment, serum lipid levels, activities of liver enzymes and biochemical parameters were measured.	Propolis treatment group showed a decrease in cholesterol and triglyceride levels, as well as in alkaline phosphatase and aspartate transaminase enzyme activities, increased lactate dehydrogenase enzyme activity caused an increase in HDL level and a decrease in LDL level. Propolis has a protective against degenerative diseases and against alcohol-induced oxidative stress	[251]
Bingöl region	Medicine/pharmacy and bio-allied sciences	Evaluation of the effect of propolis on rat brain astrocytes	Astrocytes were exposed to propolis at doses for 24 h, and then the cell viability was monitored by MTT assay. Levels of glial fibrillary acidic protein (GFAP), transcriptional nuclear factor-κB, poly (ADP-ribose) polymerase (PARP) and angiostatins were measured to determine the molecular mechanisms underlying cell responses to propolis	Propolis has dose-dependent cytotoxic effect through modulation of cytoskeleton rearrangements and pro-apoptotic signaling pathways. Propolis could be used in the prevention and treatment of neuronal impairments, including malignant tumors and neurodegenerative disorders associated with excessive astrocytic activation.	[252]
Erzurum	Medicine/pharmacy and bio-allied sciences	Evaluation of propolis effects on some biochemical parameters in rats treated with sodium fluoride	Rats received propolis with/without fluoride in drinking water and at 21st day, blood samples were collected from the heart Glucose, triglyceride, cholesterol, total protein, and uric acid levels, and aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) activities, malondialdehyde (MDA) levels, glutathione Peroxidase (GSH-Px) In the plasma, erythrocyte superoxide dismutase (SOD) and catalase (CAT) activities were measured.	Propolis improved some oxidative stress parameters and certain other biochemical parameters. propolis not was not able to completely eliminate the free radicals and the other adverse effects generated by fluoride	[253]
Marmara, Sakarya region	Medicine/pharmacy and bio-allied sciences	Study of the effects of propolis on endotoxin-induced uveitis on Male Wistar albino rats	Animals received intraperitoneal lipopolysaccharide endotoxin followed by aqueous extract of propolis or vehicle, 24h later, aqueous humor (AH) was collected from eyes of tumor necrosis factor-α (TNF- α) and hypoxia-inducible factor-1α (HIF-1a) for immunohistochemical and ultrastructural analysis.	The animals treated with propolis it was observed: - A reduction of ciliary body NF-kB/p65 immunoreactivity and aqueous humor levels of hypoxia-inducible factor-1α and tumor necrosis factor-α. - A fewer vacuoles and reduced mitochondrial degeneration in the retinal pigment epithelium,	[254]

				The anti-inflammatory capacity of propolis against uveitis demonstrated, suggest a its possible potential application for the treatment of inflammatory ophthalmic diseases.	
Zongul-dak, Bursa and Artvin	Medicine/pharmacy and bio-allied sciences	Exploration of the immunomodulatory effects propolis using blood mononuclear cells (PBMC).	BMC cells were preincubated for 30 min with propolis at different concentration and stimulated or not with phytohaemagglutinin Concentrations of neopterin, tryptophan, kynurenine and pro-inflammatory cytokines, tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-g) were determined as well as the viability of the cells was performed	Propolis with dose-dependently suppressed the enhancement of neopterin production and tryptophan degradation by enzyme indoleamine 2,3-dioxygenase (IDO) as well as release of cytokines caused by mitogen phytohaemagglutinin Propolis show an immunomodulatory property and could be used for patients showing symptoms of chronic inflammation.	[255]
Elazig province	Medicine/pharmacy and bio-allied sciences	Investigation of the effects of propolis on biochemical parameters, MDA levels and some antioxidant enzyme activities in broilers under oxidative stress originating from lead.	Propolis was supplemented to basal ration of broiler chicks during 42 days after inducing oxidative stress using lead and ambient temperature was gradually decreased from 32° C on day 1 to 22°C at the end of the experiment. Newcastle disease vaccination was performed on day 10, and the Gumboro vaccination took place on day 18. Blood samples were collected from brachial vein before cervical dislocation. Plasma, liver and heart samples were taken for analysis	It was concluded that propolis alleviate the damage and the biochemical changes resulted from the oxidative stress induced by lead Propolis showed similar antioxidant effects as vitamin C	[256]
Elazig province	Medicine/pharmacy and bio-allied sciences	Investigation of the effects of propolis on performance, nutrient utilization and carcass characteristics in broilers exposed to stress caused by lead.	Propolis with/without lead was supplemented to basal Blood samples were collected from brachial vein before cervical dislocation. Plasma, liver and heart samples were taken for analysis	Lead inclusion in diet of birds had negative effects on the performance and propolis supplements improved the growth, nutrient utilization and carcass yield	[84]
Arious regions of Turkey	Medicine/pharmacy and bio-allied sciences	Study of the effect of propolis on respiratory burst status and the secretion of PMN elastase by neutrophils	The Flow Cytometric Cell Probe Oxidative Burst Test was used to determinate if respiratory burst occurred or not and what its severity was after a 4 h incubation with propolis at different concentration PMN elastase activity was determined by Flow Cytometric Cell Probe Elastase Test	Turkish propolis may exhibit anti-inflammatory activities by reducing respiratory burst and secreting Polymorphonuclear leukocytes elastase.	[257]
Bursa	Medicine/pharmacy and bio-allied sciences	Exploration of propolis cream as compared to silver sulfadiazine (SSD) for healing of burned wounds in rats.	Wistar rats were and burned model was constituted on the back of all rats and the burned area were covered daily with propolis or ulfadiazine skin cream by the end of the experiment burned skin tissue samples were collected from rats for histopathological examinations.	Propolis was significantly effective in healing of burned skin wounds in rat model.	[258]

Rize	Stomatological field	Evaluation of the effect of propolis on bond strength dentin of a self-etch adhesive when used as final irrigant.	Human third molars were used dentin surfaces were treated with irrigation solution of propolis then were bonded using Clearfil SE Bond with Tygon.	20% propolis solution as a final irrigant was found to have a favorable effect on the dentin bond strength of the tested self-etch adhesive to coronal denti when compared with other irrigation solutions.	[259]
Hatay province	Food industry	Exploration of the positive effect of propolis Storage of Sweet Cherry	Fruits were dipped in extracted propolis immediately after harvest and then kept at 0 ºC, 85-90 % relative humidity for 4 weeks. fungal decay and fruit quality (weight loss, total soluble solids, titratable acidity, skin color, stem browning, surface pitting, appearance and taste) were assessed	Propolis revealed to be effective in preventing fungal decay in cherries for 4 weeks, but adversely affected sensory quality and stem color of cherries.	[262]
Hatay province	Food industry	Exploration of the efficacity pf propolis controlling fungal decay in grapefruits the effect of propolis on the storage life of Star Ruby grapefruit, as well as the capacity to preserve the fruit quality during storage.	After harvesting, fruits were soaked in propolis extract and then stored at 8 °C and 90% relative humidity for 6 months. The change of physiological, fungal decay, and some fruit quality characteristics (weight loss, fruit juice content, total soluble solids, titratable acidity, juice pH, percent of fruits with green calyx, and skin color) were assessed monthly.	In the opposite to fruits treated with propolis extract at 10%, those treated with propolis 5% were: - Effective in preventing fungal decay. - Successfully protected during experimental storage without losing important qualities after the harvest.	[263]
Fanus Natural Company, Trabzon,	Food industry	Exploration of the effect of propolis on the chemical, microbiological and sensory quality in vacuum-packed fresh shibuta (*Barbus grypus*) fillets through storage	Fish fillets were treated with propolis extract, vacuum packed in nylon/LDPE pouches and stored in a refrigerator (2 ± 1 C) and analyzed at 3-day intervals to determine the shelf life. The total crude protein, pH values, total volatile basic nitrogen (TVB-N), value of thiobarbituric acid (TBA) and microbiological analysis and sensory evaluation were determined	Treatement with propolis before storage of vacuum-packed shibuta fillets, affect positivelly the sensory quality, total volatile basic nitrogen value and microbiological growth which resulted a prolongation of the product's shelf-life. Propolis could be used as natural preservative for fresh fish products	[190]
Kayseri	Food industry	Examination of the antifungal activity of Turkish propolis in unpasteurized fruit juices against four mould isolates from spoiled juices	The minimum inhibitory concentration of three isolates of *Penicillium spp.* and one isolate of *Zygomycetes spp* was determined in juices of apple, orange, white grape and mandarin containing propolis	Propolis appears to have an antifungal activity against mould isolated from spoiled fruit juices. Possibility to use propolis as natural preservative for foods susceptible to fungal spoilage.	[169]
Kayseri	Food industry	Antifungal activity of Turkish propolis in fruit juices	Determination of the minimum inhibitory concentration of propolis dilluted with different fruit juices and tested on *Candida kefyr, C. parapsi losis, C. famata, C. glabrata, C. pelliculosa and Pichia ohmeri*	Propolis showed a significant antimicrobial activity against the yeast isolates from spoiled fruit juices. Possibility to use propolis as a natural preservative for foods prone to fungal spoilage	[94]
Kayseri region	Food industry	Examination of the influence of propolis in apple juice against *Escherichia coli* and *E. coli*	Inhibitory effect of the propolis extract in sterilized apple juice inoculated with bacteria then incubated at 25 and 4 days was determined trough and the number of cells in the tubes was counted	Propolis extract showed significant antimicrobial activity against *E. Coli* and *E. coli* O157:H7, - propolis could be used as a natural preservative for the foods prone to microbial spoilage.	[265]

		O157:H7 strains of the spoilage and pathogenic bacteria.			
Kayseri region	Food industry	Determination the effect of propolis application on the reduction of patulin content of apple juice	Different concentrations of propolis were added into apple juice after *P. expansum* inoculation Patulin in juice was determined with High-performance liquid chromatography equipped with diode array detector	Propolis reduced the patulin and it can be used as a natural antifungal agent for P. expansum inhibition instead of chemical preservatives.	[92]
Konya region	Food industry	Screening of the antibacterial effectiveness of chitosan–propolis coated polypropylene films against foodborne pathogens	Antibacterial activity of chitosan-coated polypropylene films incorporating propolis extract were evaluated against six foodborne pathogens using the ISO 22196 method designed for the antibacterial treated plastic products.	Incorporation of propolis to coating increased the antibacterial activity against all pathogens tested. Propolis is a promising antimicrobial agent for the food packaging applications.	[266]
Hatay province	Food industry	Establishment of the effect of propolis on egg weight loss, hatchability, chick performance, and to screen its antimicrobial activity on egg-shells.	Eggs were sprayed with different treatment including propolis at 3 doses and incubated in a commercial incubator. Eggs were placed on sterile stomacher bags then washed through whole-egg waching technique to recover the shell-associated microorganisms. hatching and chick performance procedure were performed	Eggs sprayed with propolis had: - A lower egg weight loss - A reduced bacterial activity - No change on hatchability, embryonic mortality, body weight gain, and relative growth Instead chemical disinfectant, propolis as a natural product may be used as hatching egg disinfectant	[267]
Hatay province	Food industry	Study of the effect of propolis egg shell coatings on interior egg quality	Eggs were immersed in propolis and dried and were stored for 4 weeks. Albumen index, Haugh unit, Yolk Index and pH measurement were monitored during the experience	Coating of eggs with propolis extract improved interior egg quality during storage.	[268] [100]
Elazig province	Improvement of livestock field	Evaluation of the effect of dietary propolis as alternative to antibiotic on performance and digestibility in broilers exposed to heat	Chicks were reared a thermo neutral chamber and fed a basal diet and supplemented with 5 g/kg diet the ethanol extracts of propolis.	Propolis supplementation improved the influence on the body weight gain, feed intake, feed conversion and digestibility of caused by heats stress	[103]
Elazig Province	Improvement of livestock field	Study of the effect of propolis supplementation on basal diet of chicks on feed intake, body weight, body weight gain, feed conversion rate, digestibility ,egg production and qualities in laying hens exposed to heat stress.	Chicks were fed a basal diet supplemented or not with propolis and kept in cages in temperature-controlled rooms during two months Feed intake, body weight, body weight gain, feed conversion rate, digestibility, egg production and qualities were monitored	Propolis supplementation increased growth performance and digestibility, improved the egg shell thickness and egg weight in comparison to non-supplemented birds. Supplementation of laying hens with propolis attenuated heat stress-induced oxidative damage.	[101]
Elazig Province	Improvement of livestock field	Investigation of the antioxidant activity of propolis on biochemical indicators and	Bird were fed a basal diet supplemented with propolis at different concentration and were exposed to heat stress	Propolis decreased lipid peroxidation and regulated antioxidant enzyme activities in the broilers caused by heat stress.	[270]

		antioxidant enzyme activities of broilers exposed to heat stress (at 34 °C)	On day 41, birds were sacrificed blood samples were collected from the brachial vein and plasma, liver, muscle, kidney and heart samples were taken for biochemical analysis	Propolis may be used in the prevention of oxidative stress in broilers exposed to heat stress.	
Kayseri province	Improvement of livestock field	Screening of the effect of dietary propolis supplementation on performance and eggs quality in laying hens	Laying hens fed dietary propolis supplementation during 32 weeks, Live weight, feed conversation ratio, egg production, egg weight, mortality, Haugh unit and shell thickness were determined 28 days intervals throughout the experiment	Propolis supplementation increased live weight without affecting the other studied parameters Propolis did not induce any adverse effect on performance, egg quality or even survival rate	[271]
Kayseri province	Improvement of livestock field	Examination of the effects of dietary propolis as alternative to antibiotic on growth and laying performances, nutrient digestibility and egg quality in laying hens under heat stress	Birds subjected to prolonged heat stress supplemented or not by propolis or an antibiotic (flavomycin) were compared with those obtained in chickens subjected to a normal temperature.	The heat stress causes an increase in mortality, live weight and weight gain, food intake and feed efficiency, egg production as well as nutrient digestibility, an alteration in shell qualities, while propolis was comparable to the antibiotic in its effect, it significantly reduced the negative effects of heat stress on performance, nutrient digestibility and shell characteristics	[102]
Not reported	Improvement of livestock field	Study of the the dietary addition of propolis on growth performance, carcass characteristics, internal organ weights and some serum variables in quail (Coturnix coturnix japonica) birds.	Propolis was added to the daily diet of the quails during 35 days, then the body weight gain, feed consumption and feed efficiency were determined weekly. Carcass characteristics, internal organ weights and serum variables were determined by the end of the experimentation	Propolis supplementation improve the weight gain, feed efficiency and carcass weight Birds feed propolis supplementation showed a higher serum HDL and lower level Propolis could be used as a natural substitute for antibiotics in poultry diets.	[272]
Karabuk	Improvement of livestock field	Study of the dietary supplementation on performance, egg characteristics, lipid peroxidation, antioxidant enzyme activity and mineral levels in Japanese quail	Japanese quail received propolis in the basal diet during 74 days and eggs were daily collected and weighed. Feed intake and feed conversion ratio of the groups, egg external and internal characteristics were determined. By the end of the experimentation liver tissue specimens and blood samples were taken for biochemical analysis	Propolis supplementation - Improved the feed conversion ratio, egg characteristics such as shell weight, shell thickness and shell rate -Increased the liver superoxide dismutase and catalase enzyme activities and the concentration of Ca in the serum, - Reduced glutathione and glutathione peroxidase activity was reduced in the propolis group ($P<0.05$). Propolis supplementation improved feed conversion ratio, serum Ca level and antioxidant status	[273]
Malatya,	Improvement of livestock field	Exploration of the effect of water extract of propolis on tuberculosis infection in guinea-pigs	Animals received propolis after subcutaneous inoculation of tubercle bacilli during 30 days, then were sacrificed for histological analysis	Formation of necrosis was less prominent in the group treated without statistical significance. The granuloma was more prominent Propolis showed a limited effect on the development of tuberculosis infection in this guinea-pig model.	[32]

Kayseri province	Improvement of livestock field	Investigate the effectiveness of propolis and Whitfield's ointment, used separately or in combination, for the treatment of dermatophytosis in cattle.	cattle showing clinical signs of dermatophytosis, then propolis ointment with / without Whitfield's ointment were applied topically once a day for two weeks. The shape, size, position, distribution and appearance of skin lesions and the general condition of the animals were observed clinical examinations were recorded Hair and scale samples were collected for mycological examination	Application of propolis and Whitfield's ointment was a highly effective treatment for dermatophytosis, and was better than the use of either substance alone.	[271]
Balikesir	Improvement of livestock field	Study of the propolis influence on microbiologic and biochemical parameters of Rainbow trout (Oncorhynchus mykiss) after exposure to the pesticide (cypermethrin)	Propolis at different concertation was treated to the animals for 96 h and not fed for 12 h before. pH, Lactic acid, TVB-N (volatile base-nitrogen), Malondialdehyde measurement and Microbiological analyses were assessed	Levels of malondialdehyde, lactic acid, total volatile base-nitrogen, total counts of psychrophilic, and mesophilic bacteria increased in cypermethrin groups while in propolis group decreased. Propolis changed positively the fillet quality and some biochemical and microbiologic functions of fishes that exposed to cypermethrin.	[275]
Elazig province	Improvement of livestock field	Study of the effects of dietary propolis growth performance and antioxidant status in blood of juvenile Rainbow trout	Propolis was given to the fish, subjected to two different flow rates with or without flow stress, as a supplementation in diets on final weight, condition factor, feed conservation ratio, protein efficiency ratio and vitamin A, C and E concentrations and malondialdehyde levels in serum as well as plasma superoxide dismutase activities were determined	Long-term low flow rate caused oxidative stress in juvenile rainbow trout leading to a decrease in final weight, condition factor, feed conservation ratio, protein efficiency ratio as well as A, C and E vitamin concentrations in serum, also malondialdehyde level increased in serum and plasma superoxide dismutase enzyme activity decreased in plasma. Propolis supplementation decreased the negative effects of flow stress on fish trout	[274]
Levantine Coast					
Lebanon					
South of Lebanon	Medicine/pharmacy and bio-allied sciences	Study of the anti-proliferative and proapoptotic activity and the effect of propolis on cell cycle distribution were evaluated	Jurkat leukemic T-cells, glioblastoma U251 cells, and breast adenocarcinoma MDA-MB-231 cells was used and MTT cytotoxic assays, flow cytometry as well as western blot analysis were established	Propolis reduced the cell viability tested cells, increase in p53 protein expression levels and PARP cleavage Cell cycle distribution showed an increase in the SubG0 fraction. Lebanese propolis showed an important cytotoxicity and anti-proliferative activity	[110]
Cyprus					
Bağlıköy region	Food industry	Exploration of the effect of different agent including propolis on the post-harvest quality of pomegranate with and without modified atmosphere packaging (MAP)	Treated fruits with different samples were stored in MAP bags or uncovered for 150 days under known condition. weight loss, visual quality, chilling injury and gray mold were monitored and antioxidant activity, ascorbic acid, anthocyanin and titratable acidity were determined.	Propolis combined with MAP: - Contribute positively for the maintenance of fruit, weight, juice content and visual quality and marketability -Control gray mold development and slowed the occurrence of chilling injury	[277]
Northern African Coast					

Egypt					
Not reported	Medicine/pharmacy and bio-allied sciences	Study the effect of crude Egyptian propolis on tumor in mice induced by Ehrlich ascitis carcinoma (EAC) cell line	Mice were orally administrated by propolis for 7 days through a gastric intubation 2h before intraperitoneal injection of Ehrlich ascitis carcinoma. Ehrlich ascitis carcinoma cells were collected d from the peritoneal of mice and the homogenates was used for the determination of enzyme activities and cell cycle analysis	Mice propolis treated shows: - Inhibition of tumor growth and the proliferation of Ehrlich ascitis carcinoma. - Decrease of the lipid peroxide level - Increase of glutathione and glutathione S-transferase concentrations - Inhibition of cell cycle progression and inducted apoptosis. - Arrested Ehrlich ascitis carcinoma cells in G0/G1 which leads to a decrease in the viability, DNA, total RNA and protein level of tumor cells Anti-tumor mechanism may be effectuated through induction of apoptosis and preventing oxidative damage.	[278]
Not reported	Medicine/pharmacy and bio-allied sciences	Evaluation of the capacity of propolis on the inhibition of proliferation and growth of Ehrlich ascitis carcinoma (EAC) cell line in mice	Propolis was administered through gastric intubations 2 h prior to injection of EAC in mice, then dosed for a period of 11 more days until spontaneous death of animals. Survival analysis were monitored and counting of Ehrlich ascites carcinoma cells, were done using ascitic fluid from peritoneal cavity, also blood samples were collected for immunological, hematological, biochemical studies.	Propolis treated group revealed: - A reduction in the volume, total cell count, viable percentage - An increase in the percentage of dead cells, mean survival time, life span percentage and treated vs positive control (T/C) percentage. - An increase in the lymphocyte transformation rate, phagocytic activity and killing power - Presence of leucocytosis in cancer-bearing mice and propolis-treated groups with granulo- cytosis and monocytosis. - An improvement in total protein and albumin level, globulin level and creatinine level.	[279]
Beni-Suef	Medicine/pharmacy and bio-allied sciences	Investigation of the potency of propolis/pollen on antimutagenic, anti-histopathologic and antioxidant status against cisplatin induced chromosomal abnormalities in bone marrow cells, histological alterations and oxidative stress in liver, kidney and testis tissues of mice.	Mice were treated with propolis/pollen/cisplatin (according treatment of each group) for 14 days then mice were sacrificed and liver, kidneys and testes tissues. Hepatic, renal and testicular disorder were monitored histologically, genotoxicity and cytotoxicity were done through the bone marrow chromosomal aberration assay and mitotic index and oxidative stress was assessed via lipid peroxidation, catalase activity and the concentration of the reduced form of glutathione	Cisplatin caused: - Toxic histological and genetical manifestations - Increase of peroxidized lipid content - Decrease catalase activity and glutathione concentration in the homogenate of liver, kidney and testis tissues, Propolis and bee pollen defeat this toxic effect.	[280]
Not reported	Medicine/pharmacy and bio-allied sciences	Exploration of the protective effect of propolis extract on Doxorubicin induced testicular injury	Rats received propolis and/or doxorubicin for 3 weeks. And the effect of the treatment of on the growth of solid Ehrlich carcinoma in mice were investigated.	Doxorubicin: - Reduce sperm count, markers of testicular function, steroidogenesis and gene expression of testicular 3b-hydroxysteroid dehydrogenase (3b-HSD), 17b-	[281]

			Blood samples and testicular samples were collected for determination of testosterone, luteinizing hormone, and follicle-stimulating hormone. The testes and the epididymis were removed for tissue analysis.	hydroxysteroid dehydrogenase (17b-HSD) and steroidogenic acute regulatory protein (StAR). - Increased testicular oxidative stress, inflammatory and apoptotic markers. Treatment with propolis extract avoid those modifications without affecting its antitumor activity.	
Not reported	Medicine/pharmacy and bio-allied sciences	Synthesis and evaluation of anticancer activity of the new formulated propolis-loaded nano-in-microparticles (NIMs) against human liver cancer (HepG2) and human colorectal cancer (HCT 116) cells.	The prepared Solid dispersions, Solid dispersion and propolis-loaded nano-in-microparticles were characterized using SEM, TEM, dynamic light scattering, FTIR, differential scanning colorimetry and UV-Visible spectrophotometry. The therapeutic efficiency of formulated propolis was bio-assessed via cytotoxicity measurements, mitochondrial dysfunction, apoptosis induced cell death, cell cycle arrest.	-great increase in propolis solubility with a controlled release profile in different gastrointestinal tract environments. Propolis-loaded NIMs: - Induce more cytotoxic effect on HepG2 cells than HCT-116 cells - Present three-fold higher therapeutic efficiency than free propolis. - Apoptosis of HepG2 cells	[282]
El Aliubiya region	Nanotechnology for medical applications	Trial for the synthesis of a sensitive electrochemical sensor for microRNAlet-7a detection in normal serum samples, hepatocellular carcinoma patients and human liver cancer cells	Carbon paste amended with silver nanoparticles and propolis were used for sensor creation (probe/CP/AgNPs/propolis) Characterization was done through: surface characteristics were achieved using scanning, Fourier-transform infrared spectroscopy, spectrophotometer, transmission electron microscope, energy dispersive X-ray analysis and elemental mapping techniques. Prepared biosensor was assayed for detection of miRNAlet7a in extracted miRNA from human serum samples using square wave voltammetry and electrochemical impedance spectroscopy technics	Providing optimal condition, the composed sensor showed good rapid and sensible response reaching a very low detection limit. A remarkable result was obtained for miRNA-let 7a detection in hepatocellular carcinoma patients and/or in hepatic cancerous cultured cell lines Huh7 and HepG2.	[283]
Not reported	Medicine/pharmacy and bio-allied sciences	Exploration of the modulatory effect some hive products including propolis on peripheral blood leukocytes and lung inflammation in a mouse conalbumin-induced asthma model	Animals were sensitized and challenged with the antigen conalbumin, then 24h later received dexamethasone/honey/ royal jelly/propolis once per day for 18 consecutive days. Blood was collected for white blood cell differentiation, and the lungs were removed for histopathological analysis.	Animals propolis-treated with propolis extract: - Displayed appreciable curative effect against asthma, - Important diminution in inflammatory scores This useful effect could be a result of the bioactive compounds present in propolis such as the flavonoids and phenols	[284]
Not reported	Medicine/pharmacy and bio-allied sciences	Evaluation of the healthful effect of propolis alone or in combination with praziquantel on biochemical, immunological, parasitological, and histological	Schistosoma mansoni-infected mice were treated with propolis/praziquantel/day (according each treatments) for 4 weeks starting 49 days after infection Blood sample was collected for immunological and histopathological studies	Treatment with propolis: - Failed to stamp out the worm - Reduce the hepatic granuloma number, lymphocytic infiltration and aggregation, hepatic and splenic	[285]

		disorder caused by experimental schistosomiasis in mice		myeloperoxidase activity and plasma and liver and thymus nitric oxide levels together - Normalize plasma proteins and - Mitigation of oxidative stress in the examined Treatment with propolis and praziquantel: -anti-schistosomal activity of praziquantel was potentialized - Amelioration of the histological and biochemical alteration associated with schistosomiasis.	
Not reported	Medicine/pharmacy and bio-allied sciences	Exploration of the effect of propolis on cryptosporidiosis in experimentally infected dexamethasone-immunosuppressed rats.	Rats were immunosuppressed with dexamethasone with/without infection with Cryptosporidium oocysts then given orally extract of propolis and/or nitazoxanide (standard anti-cryptosporidial drug) - Faecal pellets were tacked to monitor oocysts shedding. -Leukogram and serum proteins were monitored using blood samples -Histopathological studies were done with Ileum specimens	Rats infected and tertaed with propolis revealed: - the highest diminution of oocysts shedding in faecal samples - Obvious increase in neutrophils count and α2- and β-globulins levels Histopathological modifications appear in the ileum of animals infected with cryptosporidium Propolis modulate the immunity dexamethasone-immunosuppressed rats	[286]
Not reported	Stomatological field	Screening of the capacity of propolis to block oral candida cells adhesion and therefore preventing their colonization.	The isolate obtained from a patient diagnosed with acute pseudo-membrane candidiasis were exposed to propolis growth profile, surface bound proteins, polymorphism for candida albicans surface were checked	Low concentration of propolis and less time were enough to cause a decrease in the adhesion and germ tube formation, Propolis acts mainly on the surface bound proteins and, to a lesser extent, on EPS thus preventing adhesion	[287]
Not reported	Textile material for medical application	Use of propolis being a natural product, for the production of cotton textile proceeding antibacterial activity and many effective characteristics.	Cotton fabric samples were treated with propolis different crosslinking agents. The efficiency of the interaction of propolis with cotton cellulose was monitored type, nature and concentration of the crosslinking agent, concentration of propolis, and conditions of curing were explored. Antibacterial activity, SEM, FTIR, durability to washing, UV protection and water repellency of propolis-modified cotton fabrics were performed.	Use of propolis generate a cotton textile with superior antibacterial activity, water repellent, ease of care characteristics and UV protection. The antibacterial activity, fabric properties, and performance properties depend on propolis concentration, condition of treatment and type, nature and concentration of the crosslinking agent.	[288]
Cairo	Food industry	Exploration of the efficiency of the use of propolis on the shelf-life and different quality criteria of fresh oriental sausage.	Fresh oriental sausage was dealt with propolis, kept in a refrigerator at 5 °C and examined every 3 days until occurrence of signs of spoilage. Sensory, chemical and microbiological deteriorative criteria were assessed.	Propolis treated samples, as compared with control revealed a longer shelf-life as they decomposed after 21 days. - A decrease of the thiobarbituric acid value and total volatile bases nitrogen. - Proteolytic, lipolytic and total mould and yeast counts were somewhat lower Propolis may eb used as natural preservative in fresh oriental sausage processing instead synthetic one.	[289]

Giza province	Improvement of livestock field	Examination of the advantageous effects of propolis on growth performance and immune function of turkey poults exposed to oxidative stress induced by Paraquat injection	Turkey poults received basal diet supplemented or not with propolis and daily subcutaneous Paraquat injection for 7 days. Productive performance was determined Blood samples were collected for the determination of biochemical and immunological parameters.	Propolis supplementation in poults group: - Greatly improve the Paraquat effects on weight gain, feed intake, and diminished feed conversion ratio. - Decrease the Malondialdehyde and corticosterone, - Raise the T3 levels - Significantly limited the Paraquat -suppressive effects on cell- and humoral-mediated immunity and lymphocyte proliferation of turkey poults - Reversion the Paraquat -induced DNA fragmentation and heat shock protein 70 (Hsp70) over-expression in blood cells	[299]
Not reported	Veterinary Medicine	Examination of the effect of propolis given alone or in combination with inactivated *Pasteurella multocida* vaccine on rabbits challenged with a virulent strain of *Pasteurella multocida*.	Rabbits non-vaccinated against pasteurellosis were treated with propolis and/or vaccine for six weeks. Clinical signs, body weight, and mortality rate were followed, Leukogram, serum biochemistry, and immune response were evaluated At the end of experiment, animals were subjected to challenge with a virulent strain of *P. multocida* and histopathological investigation were done on tissue specimens two weeks later.	Groups treated with propolis/vaccine: - Improvement of the general health conditions, liver and kidney functions - Reduction of the severity of adverse clinical signs, mortality rates, and histopathological changes associated with challenge of rabbits with P. multocida strain.	[298]
Not reported	Veterinary Medicine	The evaluation of the protective effect of propolis with/without inactivated P. multocida vaccine in rabbits challenged with virulent P. multocida strain.	Rabbits non-vaccinated against pasteurellosis was injected subcutaneously (S/C) with propolis and/or vaccine, for six weeks during which clinical signs, body weight, and mortality rate was monitored erythrogram and biochemical parameters were done after blood samples collection histopathological investigation was done on tissue specimens two weeks later after being challenged with a virulent strain of P. multocida at the end of experiment.	Propolis improved general health conditions, liver and kidney functions in addition to reduction of the severity of adverse clinical signs, mortality rates, and histopathological changes associated with challenge of rabbits with P. multocida strain.	[299]
Not reported	Improvement of livestock field	Evaluation of the efficacy of propolis on Aeromonas hydrophila fish growth performance, immunostimulation and resistance to Aeromonas hydrophila	Oreochromis niloticus fish were fed basal diet containing propolis and were intraperitoneally challenged by A. hydrophila at the end of the feeding period for 15 more days. Growth performance and organ index were checked and blood samples were collected for analysis	Animals fed propolis as supplementation showed: - A best growth rate and feed conversion ratio, - An increase in the average daily gain, specific growth rate and feed efficiency ratio, Hematocrit-level, - A significant increase in serum lysozyme and serum bactericidal activities - A high relative level of protection against A. hydrophila Propolis enhanced the growth, immunity and resistance of O. niloticus against A. hydrophila	[300]

Not reported	Improvement of livestock field	Exploration of the effectiveness of dietary bee-pollen or propolis to crush the genotoxic and endocrine disturbing effects of malathion polluted water in *Oreochromis niloticus* (*O. niloticus*).	Acute toxicity experiment: fish were kept on a die with/without propolis/pollen and exposed daily to malathion in water for 96h. Chromosomal aberrations, micronucleus and DNA-fragmentation was monitored to determine protective efficiency.	For the group treated with propolis/ bee pollen and exposed to an acute toxicity it was observed: -A decrease of in the frequency of chromosomal aberrations, micronucleus and DNA-fragmentation. - An increase of survival rate	[301]
Tunisia					
Mahares, a south suburb of Sfax	Medicine/pharmacy and bio-allied sciences	Exploration of the effect of propolis administered to cobalt-treated rats on the biochemical changes and renal damages.	Pregnant Wistar rats were treated with CoCl2 with/without propolis-supplemented diet. At the end of the experimental period blood samples were collected for biochemical assays. Urinary samples were collected. After the sacrifice, the kidneys kept for oxidative stress markers analysis, various biochemical assays and histological studies.	Treatment of animals with propolis leads to: -A lower malondialdehyde levels, - A higher antioxidant activities - Cobalt-related histopathological changes in the kidneys were at lower severity. - Propolis is presented as brilliant agent for the controlling of cobalt-induced nephrotoxicity	[289]
Algeria					
Not reporetd	Medicine	Study of the protective effect of propolis on peroxidative damage in rat heart mitochondria which was induced by the administration of an acute dose of doxorubicin	Rats were pretreated with propolis during four days before administration of Doxorubicin and scarified after 24h. Mitochondria were extracted from a homogenate of rat heart for the evaluation of the peroxidative lesions biochemically and biophysically,	propolis pretreatment: - Decrease of the peroxidative damage in the heart mitochondria. - Decrease of both mitochondrial malondialdehyde formation and production of superoxide anion, - Restoration of respiratory chain ratio and P/O - Decrease in the rate and the amplitude of mitochondrial swelling. Antioxidants from natural sources may be useful in the protection of cardiotoxicity in patients who receive doxorubicin.	[290]
Eastern Algeria	Medicine	Exploration of the protective effect of propolis on rat kidney against acute oxidative stress induced by doxorubicin.	Wistar rats were pre-treated with propolis for seven days, on day 7 then doxorubicin was intravenously administered Serum creatinine concentration, scavenging effect of flavonoids, lipid peroxidation and glutathione concentration were measured.	Animals pretreated with propolis showed: -A restoration of the renal functions - A reduction of the toxic effect of doxorubicin.	[291]
Tigzirt	Medicine	Study of the precautionary and remedial efficiency of propolis during α-Tropomyosin induced uveitis, over the regulation of	Animals were injected daily with propolis for 5 days prior to immunization or for 9 days commencing 5 days post immunization with α-Tropomyosin extract with α-Tropomyosin extract, and sacrificed at day 14. Histological examination,	Treatment with propolis: decreased the retinal histological damages induced by α-Tropomyosin, reduce the nitric oxide and tumor necrosis factor, -α levels and curtail nitric oxide synthase and nuclear factor-κB expression in retina, rise the urea and interleukin-4 levels arginase-1 expression.	[292]

		inducible nitric oxide synthase (NOS_2) and arginase-1	NOS_2, arginase-1, and nuclear factor-κB (NF-κB) expression were monitored plasmatic production of nitric oxide, urea, IL-4, and TNF-α was performed	Propolis attenuated retinal damages through the induction of Th2 response and the inhibition of and nuclear factor -κB/ nitric oxide synthase pathway	
Morocco					
Beni Mellal region	Medicine	Screening of the antitumor potential of a Moroccan propolis extracts	Mice were subcutaneously grafted with tumor P815 tumor-bearing mice (DBA2/P815) fragments and after 5 days were treated in oral route with propolis every four days, five times The tumor volume of each mouse was evaluated	Daily treatment of animals with propolis reduced the tumor volume as compared with the control	[120]
Oulad Ayyad near Beni-Mellal	Medicine	Investigation of a possible analgesic effect of black propolis from morocco in rats and mice	Propolis extracts was administered before the antinociceptive test, which was performed trough writhing, formalin and hot plate tests	Propolis extract: - Decreased the pain induced by injection of acetic acid. - Showed a significant effect in the hot plat test - Decreased the painful stimulus in formalin test propolis activated both central and peripheral mechanisms to elicit the analgesic effect	[293]
region of Salé	Medicine	Screening of protective effect of propolis in Proteinuria, Crystaluria, nephrotoxicity and hepatotoxicity induced by ethylene glycol ingestion.	Rats received ethylene-glycol in drinking along with/without propolis daily, for 30 days. Urine and blood samples were collected Urinalyses for pH, crystals, protein, creatinine, uric acid and electrolytes, and renal and liver function tests were performed.	Ethylene-glycol: - Raise urinary volume, pH, calcium, phosphorus, uric acid and protein excretion. - Reduce creatinine clearance, and magnesium - Leads to a crystaluria, Propolis moderate this disorder and ameliorates the deterioration of liver and kidney function	[294]
Outat el Haj	Medicine	Evaluation of possible protective effect of hydro-ethanolic extract of propolis against paracetamol-induced liver and kidney damage in rats models	Animals were pretreated with propolis for eight days before treatment with paracetamol+ propolis for more seven days. blood samples were collected for measurement of the liver enzymes, serum albumin, protein and creatinine, blood urea nitrogen, hematological parameters, and urine volume, protein and albumin.	The treatment with propolis allowed the prevention of biochemical changes in blood rats samples, it increase hemoglobin, serum total protein, albumin, and uric acid, decreased blood creatinine, blood urea nitrogen, alanine aminotransferase, aspartate aminotransferase and lactate dehydrogenase activities, white blood cells, and platelet count as compared to the control. - Propolis act with a dose dependent manner on prevention of proteinuria, low hemoglobin and body weight loss.	[295]

TUNEL assay: Terminal deoxynucleotidyl transferase dUTP nick end labeling assay; SELDI-TOF-MS: Surface-enhanced laser desorption/ionization -ime-of-flight - mass spectrometry; LDPE pouches: Low Density; Polyethylene Pouches. PARP: Poly (ADP-ribose) polymerase; SEM: scanning electron microscope; TEM: Transmission Electron Microscopy FTIR: Fourier Transform InfraRed spectroscopy

Appendix B

Table B.1. Chemical composition of hydro-alcoholic extract of Moroccan propolis.

	Aromatic Acids	**%**	**Diterpenes**	**%**	**Triterpenes**	**%**	**Sugars and Sugar Derivatives**	**%**	**Fatty Acids**	**%**
Sample 7	Benzoic acid	1.2	Totarol	1.2	Lanosterol (3-α-OH)	1,2	Monosaccharides	52,6	Hexadecanoic acid	2,8
			Imbricataloic acid	1.3	α-amyrin	0,4	Disaccharides	9,3	Octadecanoic acid	1,9
			Dehydroabietic acid	0.3	Lanosterol (3-β-OH)	2,9	Glycerol	1,6	Octadecenoic acid	0,5
					Lupeol	3,5	Inositol	5,0	Tetracosanoic acid	0,8
	Total	**1.2**	**Total**	**2.8**	**Total**	**9**	**Total**	**68.5**	**Total**	**6**
Sample 1	(El-Guendouz et al., 2016a)									
Standard deviation does not succeed 6% for any of the constituents										

Appendix C

Table C.1: Chemical characteristics of almond and wheat germ oils

	Almond Oil	Wheat germ oil
Chemical characteristics		
Peroxide value	Max 3.0 meqO_2/kg	Max 15 meqO_2/kg
Saponification value	190-200 mg KOH/g	-
Faty acide content		
Palmitic acid	4.0-9.0 %	14.0-19.0 %
Palmitoleic acid	Max 0.8 %	-
Stearic acid	Max 3.0 %	<2 %
Oleic acid	62.0-86.0 %	10.0-23.0%
Linoleic acid	20.0-30.0 %	52.0-65.0%
Linolenic acid	Max 0.4 %	3.0-10.0%
Arachidonic acid	-	<3 %

-: not reported

Table C.2. Effect of adding Propolis and BHA at different concentrations (0.01; 0.02 and 0.04 %) on hydroperoxides (ABS_{510}) of O/W emulsions made with germ wheat oil and almond oil under storage conditions of 9 weeks at 37 °C. Values of mean (n=6) ± standard deviation.

		A: 100% of Germ wheat oil / 0% almond oil		B: 75% of Germ wheat oil / 25% almond oil		C: 50% of Germ wheat oil / 50% almond oil		D: 25% of Germ wheat oil / 75% almond oil		E: 0% of Germ wheat oil / 100% almond oil	
		Week 0	**Week 9**	**Week 0**	**Week 9**	**Week 0**	**Week 9**	**Week 0**	**Week 9**	**Week 0**	**Week 9**
0.01 %	**Control**	0.638±0.020^{d}	1.001±0.001^{a}	0.627±0.015^{c}	0.856±0.044^{a}	0.592±0.044^{b}	0. 785±0.010^{a}	0.569±0.015^{bc}	0.700±0.057^{a}	0.549±0.005^{b}	0.647±0.045^{a}
	Propolis	0.569±0.005^{e}	0.677±0.013^{c}	0.554±0.006^{d}	0.688±0.040b^{b}	0.549±0.015^{c}	0.643±0.036^{b}	0.524±0.022^{cd}	0.749±0.044^{a}	0.472±0.059^{bc}	0.651±0.036^{a}
	BHA	0.628±0.020^{d}	0.766±0.022^{b}	0.627±0.15^{c}	0.757 ±0.005^{ab}	0.569±0.015^{c}	0.702±0.006^{ab}	10.749±0.044^{b}	0.671±0.022^{a}	0.539±0.005^{b}	0.630±0.018^{a}
0.02 %	**Control**	0.638±0.020^{d}	1.001±0.001^{a}	0.627±0.015^{c}	0.856±0.044^{a}	0.592±0.010^{b}	0.0.785±0.010^{a}	0.569±0.015^{bc}	0.700±0.057^{a}	0.549±0.005^{b}	0.647±0.045^{a}
	Propolis	0.596±0.002^{de}	0.731±0.011^{bc}	0.574±0.013^{cd}	0.638±0.045^{b}	0.549±0.015^{c}	0.668±0.017^{b}	0.510±0.012^{cd}	0.633±0.053^{a}	0.419±0.010^{c}	0.598±0.017^{a}
	BHA	0.648±0.020^{ed}	0.713±0.055^{bc}	0.627±0.015^{c}	0.676±0.074^{b}	0.538±0.044^{c}	0.659±0.019^{b}	0.569±0.015^{bc}	0.662±0.024^{a}	0.529±0.005^{b}	0.672±0.013^{a}
0.04 %	**Control**	0.638±0.020^{d}	1.001±0.001^{a}	0.627±0.015^{c}	0.856±0.044^{a}	0.592±0.010^{a}	0.785±0.010^{a}	0.569±0.015^{bc}	0.700±0.057^{a}	0.549±0.005^{b}	0.647±0.045^{a}
	Propolis	0.657±0.029^{cd}	0.767±0.036^{b}	0.555±0.008^{d}	0.737±0.024^{b}	0.521±0.013^{c}	0.719±0.074^{ab}	0.529±0.020^{bcd}	0.697±0.06^{a}	0.529±0.036^{bc}	0.651±0.041^{a}
	BHA	0.572±0.012^{cd}	0.668±0.051^{d}	0.511±0.063^{d}	0.682±0.007^{b}	0.538±0.018^{c}	0.665±0.014^{b}	0.500±0.019^{d}	0.643±0.016^{a}	0.6502±0.091^{bc}	0.592±0.011^{a}

For each percentage of oils (A, B, C, D and E), different letters denote significant differences according to Duncan multiple range test ($p<0.05$)

Table C.3. Effect of adding propolis extract and BHA at different concentrations (0.01; 0.02 and 0.04 %) on TBARS (ABS_{532}) of O/W emulsions made with germ wheat oil and almond oil under storage conditions of 9 weeks at 37 °C. Values of mean (n=6) ± standard deviation.

		A: 100% of Germ wheat oil / 0% almond oil		B: 75% of Germ wheat oil / 25% almond oil		C: 50% of Germ wheat oil / 50% almond oil		D: 25% of Germ wheat oil / 75% almond oil		E: 0% of Germ wheat oil / 100% almond oil	
		Week 0	Week 9	Week 0	Week 9	Week 0	Week 9	Week 0	Week 9	Week 0	Week 9
0.01 %	Control	1.138±0.005^{e}	2.899±0.016^{a}	0.998±0.005^{d}	2.499±0.016^{a}	0.849±0.005^{c}	1.999±0.016^{a}	0.505±0.005^{b}	1.499±0.016^{a}	0.238±0.005^{b}	0.625±0.016^{a}
	Propolis	0.072±0.095^{f}	1.969±0.032^{b}	0.591±0.078^{f}	1.334±0.312b^{c}	0.456±0.03^{ij}	1.373±0.110^{b}	0.343±0.083^{f}	1.125±0.067^{b}	0.076±0.033^{gh}	0.388±0.041^{c}
	BHA	0.771±0.017^{f}	1.672±0.042^{c}	0.549±0.055^{f}	1.501±0.038^{b}	0.487±0.013^{h}	0.932±0.026^{e}	0.510±0.015^{d}	0.985±0.004^{c}	0.099±0.001^{g}	0.099±0.018^{de}
0.02 %	Control	1.138±0.005^{e}	2.899±0.016^{a}	0.998±0.005^{d}	2.499±0.016^{a}	0.849±0.005^{c}	1.999±0.016^{a}	0.505±0.005^{b}	1.499±0.016^{a}	0.238±0.005^{b}	0.625±0.016^{a}
	Propolis	0.725±0.095^{f}	1.444±0.036^{d}	0.591±0.078^{f}	1.180±0.104^{cd}	0.457±0.06^{hi}	1.030±0.043^{d}	0.377±0.124^{f}	0.679±0.025^{d}	0.076±0.033^{gh}	0.183±0.023^{f}
	BHA	0.787±0.046e^{f}	1.234±0.164^{e}	0.598±0.036^{f}	0.898±0.129^{e}	0.272±0.012^{k}	0.685±0.013^{g}	0.140±0.038^{gh}	0.493±0.002^{e}	0.013±0.006^{hi}	0.216±0.006d^{e}
0.04 %	Control	1.138±0.005^{e}	2.899±0.016^{a}	0.998±0.005^{d}	2.499±0.016^{a}	0.849±0.005^{c}	1.999±0.016^{a}	0.505±0.005^{b}	1.499±0.016^{a}	0.238±0.005^{b}	0.625±0.016^{a}
	Propolis	0.824±0.057^{f}	1.243±0.018^{e}	0.425±0.054^{g}	1.156±0.171^{cd}	0.248±0.014^{k}	0.732±0.044^{f}	0.070±0.009^{h}	0.302±0.008^{e}	0.000±0.000^{i}	0.127±0.019^{f}
	BHA	0.717±0.024^{f}	1.054±0.170^{f}	0.532±0.027^{e}	0.772±0.04^{e}	0.292±0.069^{jk}	0.704±0.026^{g}	0.230±0.015^{g}	0.464±0.019^{d}	0.000±0.00^{i}	0.185±0.013^{d}

For each percentage of oils (A, B, C, D and E), different letters denote significant differences according to Duncan multiple range test ($p<0.05$)

Table C.4. Apparent viscosity of the emulsions with propolis extract and BHA, at different concentrations (0.01, 0.02 and 0.04 %), under storage conditions of 9 weeks at 37 °C. Apparent viscosity (mPa.s) was determined at a shear rate of 6.3 s^{-1}, with a LV-3C spindle. Values of mean (n=4) ± standard deviation.

		A 100% of Germ wheat oil / 0% almond oil		B 75% of Germ wheat oil / 25% almond oil		C 50% of Germ wheat oil / 50% almond oil		D 25% of Germ wheat oil / 75% almond oil		E 0% of Germ wheat oil / 100% almond oil	
		Week 0	Week 9	Week 0	Week 9	Week 0	Week 9	Week 0	Week 9	Week 0	Week 9
0.01 %	Control	2020±34^{l}	1834±59^{hji}	1852±23^{gl}	1884±57^{l}	1796±06 ^{jml}	1872±57 ^{nj}	1844±28^{mn}	1776±51^{hnm}	1826±37^{mnj}	1736±34^{hl}
	Propolis	2312±11^{a}	2184±79^{o}	2288±17^{a}	2176±85^{o}	2222±20 ^{a}	2090±59 ^{o}	2018±20^{a}	2052±57^{a}	2040±11^{a}	1972±57^{oa}
	BHA	1760±00^{gi}	1936±34^{jl}	1822±14^{ilj}	2004±85^{m}	1754±25 ^{ij}	1844±45 ^{ljm}	1820±34^{jnm}	1858±65^{m}	1784±34^{jnml}	1832±45^{n}
0.02 %	Control	2020±34^{l}	1834±59^{hji}	1852±23^{gl}	1884±57^{l}	1796±06 ^{jml}	1872±57 ^{nj}	1844±28^{mn}	1776±51^{hnm}	1826±37^{mnj}	1736±34^{hl}
	Propolis	1680±40^{c}	1700±51^{e}	1688±62^{dhg}	1670±48^{chgd}	1548±34 ^{b}	1596±40 ^{db}	1782±37^{inm}	1662±48^{e}	1736±68^{ilj}	1638±42^{f}
	BHA	1640±51^{b}	1716±85^{fh}	1716±23^{fgh}	1722±37^{gh}	1684±57 ^{fih}	1676±28 ^{eihf}	1634±14^{ce}	1642±31^{dc}	1594±08^{c}	1620±23^{dc}
0.04 %	Control	2020±34^{l}	1834±59^{hji}	1852±23^{gl}	1884±57^{l}	1796±06 ^{jml}	1872±57 ^{nl}	1844±28^{mn}	1776±51^{hnm}	1826±37^{mnj}	1736±34^{hl}
	Propolis	1840±51^{ij}	2158±54no	1730±20^{h}	2004±68^{nm}	1740±34 ^{hji}	1872±57 ^{mj}	1774±31^{gnm}	1836±68^{lnm}	1622±20^{e}	1784±57^{lnm}
	BHA	1680±68^{d}	2046±42^{ml}	1418±42^{b}	1708±62^{ehg}	1558±54 ^{cd}	1700±40 ^{gih}	1526±65^{b}	1685±37^{f}	1546±31^{bd}	1692±74^{g}

For each percentage of oils, different letters indicate statistically significant differences according to Student-Newman-Keuls ($p < 0.05$)

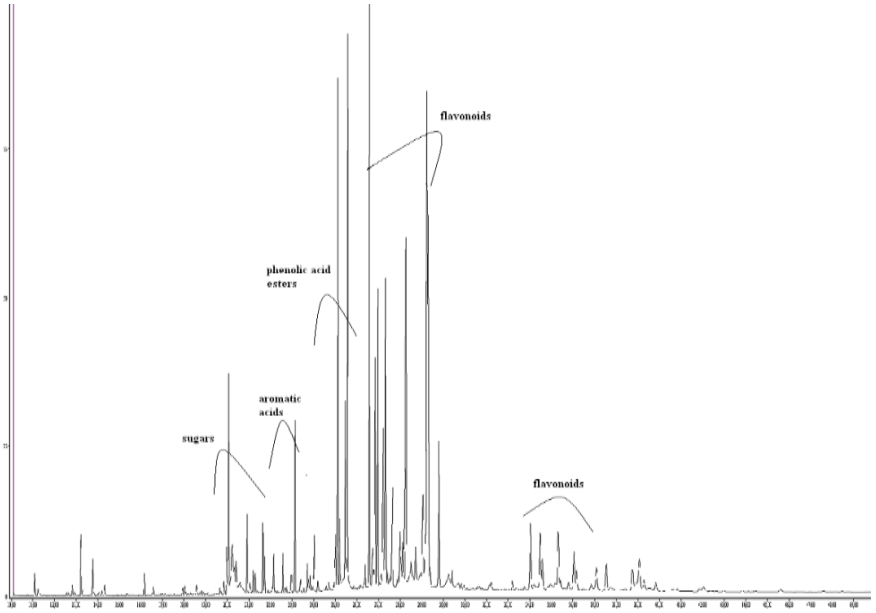


Figure C.1. GC Chromatogram of hydro-alcoholic propolis extract

Appendix D

Table D.1. Apparent viscosity of the emulsions with thymus extract and BHA, at different concentrations (0.01, 0.02 and 0.04 %), under storage conditions of 9 weeks at 37 °C. Apparent viscosity (mPa.s) was determined at a shear rate of 6.3 s^{-1}, with a LV-3C spindle. Values of mean (n=4) ± standard deviation.

		A 100% of Germ wheat oil / 0% almond oil		**B 75% of Germ wheat oil / 25% almond oil**		**C 50% of Germ wheat oil / 50% almond oil**		**D 25% of Germ wheat oil / 75% almond oil**		**E 0% of Germ wheat oil / 100% almond oil**	
		Week 0	**Week 9**	**Week 0**	**Week 9**	**Week 0**	**Week 9**	**Week 0**	**Week 9**	**Week 0**	**Week 9**
0.01 %	Control	2020±34	1834±59	1852±23	1884±57	1796±06	1872±57	1844±28	1776±51	1826±37	1736±34
	Thymus	1823±10	1744±15	1984±24	1776±37	2028±21	1780±39	1920±18	1696±27	1904±41	1720±27
	BHA	1760±00	1936±34	1822±14	2004±85	1754±25	1844±45	1820±34	1858±65	1784±34	1832±45
0.02 %	Control	2020±34	1834±59	1852±23	1884±57	1796±06	1872±57	1844±28	1776±51	1826±37	1736±34
	Thymus	1720±42	2132±11	1723±15	2058±69	1860±14	2116±87	1848±57	2136±24	1848±34	1988±34
	BHA	1640±51	1716±85	1716±23	1722±37	1684±57	1676±28	1634±14	1642±31	1594±08	1620±23
0.04 %	Control	2020±34	1834±59	1852±23	1884±57	1796±06	1872±57	1844±28	1776±51	1826±37	1736±34
	Thymus	1884±35	2256±47	2060±84	2399±34	2080±97	2451±37	1824±54	2144±49	2008±23	2156±66
	BHA	1680±68	2046±42	1418±42	1708±62	1558±54	1700±40	1526±65	1685±37	1546±31	1692±74

For each percentage of oils, different letters indicate statistically significant differences according to Student-Newman-Keuls ($p < 0.05$)

Appendix E

Table E.1. Data on collection place and dominant local flora

Sample	Region*	Dominant local flora
1	Fez-Boulemane	*Cistus*, *Juniperus*, *Lavandula*, *Olea*, *Pinus*, *Pistacia*, *Quercus* and *Rosmarinus*
2	Gharb-Chrarda-Beni-Hsen	*Vaccinium*
3	Gharb-Ghrada-Beni-Hsen	*Ceratonia*, *Citrus*, *Silybum* and *Ziziphus*
4	Meknes-Tafilalet	*Ceratonia*
5	Meknes-Tafilalet	*Ceratonia*
6	Doukkala-Abda	*Eucalyptus*
7	Souss-Massa-Draa	*Citrus*, *Euphorbia* and *Thymus*
8	Rabat-Sale-Zemmour-Zaer	*Eucalyptus*, *Juniperus* and *Quercus*
9	Gharb-Ghrada-Beni-Hsen	*Vaccinium*
10	Morocco local market	
11	Taza-Al Hoceima-Taounate	*Cistus*, *Juniperus*, *Lavandula*, *Olea*, *Pinus*, *Pistacia*, *Quercus* and *Rosmarinus*
12	Unknown provenance	
13	Gharb-Chrada-Beni-Hsen	*Ceratonia*, *Chamaerops*, *Eucalyptus*, *Euphorbia*, *Juniperus*, *Olea*, *Pinus*, *Pistacia*, *Quercus* and *Thuja*
14	Gharb-Chrada-Beni-Hsen	*Eucalyptus*
15	Rabat-Sale-Zemmour-Zaer	*Ceratonia*, *Chamaerops*, *Eucalyptus*, *Euphorbia*, *Juniperus*, *Olea*, *Pinus*, *Pistacia*, *Quercus* and *Thuja*
16	Gharb-Chrada-Beni-Hsen	*Ceratonia*, *Chamaerops*, *Eucalyptus*, *Euphorbia*, *Juniperus*, *Olea*, *Pinus*, *Pistacia*, *Quercus* and *Thuja*
17	Fez Boulemane	*Cistus*, *Juniperus*, *Lavandula*, *Olea*, *Pinus*, *Pistacia*, *Quercus* and *Rosmarinus*
18	Grand-Casablanca	*Citrus*, *Euphorbia* and *Thymus*
19	Rabat-Sale-Zemmour-Zaer	*Eucalyptus*, *Marrubium*, *Mentha*, *Ricinus*, thistles and *Vaccinium*
20	Fez -Boulmane	*Bupleurum* and *Thymus*
21	Guelmim-Es Semara	*Acacia*, *Argania* and *Euphorbia*
22	Fez -Boulmane	*Cistus*, *Juniperus*, *Lavandula*, *Olea*, *Pinus*, *Pistacia*, *Quercus* and *Rosmarinus*
23	Morocco local market	
24	Souss-Massa-Draa	*Acacia*, *Argania*, *Citrus* and *Euphorbia*

Table E.2. Percentage composition of the volatiles isolated by hydrodistillation from different propolis samples. Samples ordered by appearance on each cluster. For samples grouped on each of the clusters and subclusters see Figure 9.2.

Peak #	Components	RI	RI[a]	Propolis samples																								
				Cluster I																								Cluster II
				Ia			Ib1				Ib2																	
				1	17	10	2	9	15	16	3	14	8	4	5	6	7	18	21	19	22	13	11	12	20	24	23
1	Tricyclene	921	927	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t
2	α-Thujene	924	929	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t
3	α-Pinene	930	937	5.4	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t
4	Benzaldehyde	930	926	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t
5	Camphene	938	938	1.2	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t
6	Thuja-2,4(10)-diene	940	947	0.4	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t
7	Sabinene	958	961	0.5	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t
8	β-Pinene	963	963	2.0	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t
9	α-Terpinene	1002	1005	0.6	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t
10	*p*-Cymene	1003	1008	0.9	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t
11	β-Phellandrene	1005	1009	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t
12	Limonene	1009	1016	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t
13	γ-Terpinene	1035	1035	1.2	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t
14	2-Methyl decane*	1046	1058	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t
15	2,5-Dimethyl styrene	1059	1076	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t
16	Terpinolene	1064	1064	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t
17	*n*-Nonanal	1073	1073	t	1.0	t	1.9	t	t	t	t	t	t	t	0.2	1.0	t	t	t	t	t	t	t	t	t	11.3	t	t
18	Linalool	1074	1082	t	0.8	t	t	t	t	t	t	t	t	t	0.1	0.6	t	t	t	t	t	t	t	t	t	t	t	t
19	α-Campholenal	1092	1127	0.9	t	t	t	t	t	t	t	t	t	t	t	0.7	t	t	t	t	t	t	t	t	t	t	t	t
20	*trans*-Pinocarveol	1106	1106	1.9	t	t		t	t	t	t	t	t	1.2	t	0.9	t	t	t	t	t	t	t	t	t	t	t	t
21	UI P2a	1107					6.0																					
22	*cis*-Verbenol	1113	1122	0.1	t	t		1.2	t	t	t	t	t	2.1	t	t	t	t	t	t	t	t	t	t	t	1.0	t	t
23	UI P2b	1113					5.0																					
24	*trans*-Verbenol	1114	1132	1.5	t	t	t	t	t	t	t	t	t	t	0.8	0.3	t	t	t	t	t	t	t	t	1.8	t	t
25	3-Methyl-1-indene	1114	1155	1.5	t	t	t	t	t	t	t	t	1.0	t	0.8	1.0	t	t	t	t	t	t	t	t	t	t	t
26	Pinocarvone	1121	1121	0.7	t	t		t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t
27	UI P2c	1122					3.6																					
28	Borneol	1134	1137	1.2	t	t	t	0.3	t	t	t	t	t	0.6	t	0.5	t	t	t	t	t	t	t	t	t	t	t	t
29	Terpinen-4-ol	1148	1153	1.3	t	t	t	0.4	6.8	t	t	t	0.7	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t
30	Octanoic acid	1149	1169	0.6	t	t	1.6	0.3	t	t	t	t	t	t	t	0.6	t	t	t	t	t	0.4	t	t	t	t	t
31	Myrtenal	1153	1153	0.6	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t
32	α-Terpineol	1159	1157	0.8	t	t	1.0	t	t	t	t	t	t	t	0.3	0.5	t	t	t	t	t	t	t	t	t	t	t	t

Peak #	Components	RI	RI[a]	Propolis samples																								
				Cluster I																								Cluster II
				Ia			Ib1				Ib2																	
				1	17	10	2	9	15	16	3	14	8	4	5	6	7	18	21	19	22	13	11	12	20	24	23
33	*n*-Decanal	1180	1183	1.3	2.7	t	4.7	2.3	t	0.8	0.8	t	2.0	6.3	7.9	9.4	t	t	t	t	t	0.6	t	t	3.2	13.0	t
34	*trans*-Carveol	1189	1199	0.2	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t
35	2-Phenyl ethyl acetate	1228	1257	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t
36	*trans*-Cinnamaldehyde	1228	1266	0.8	t	t	0.8	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t
37	2-*trans*-Decenal	1236	1266																							5.4	
38	Nonanoic acid	1254	1279	t	0.6	t	t	t	t	t	0.7	t	0.2	t	0.6	0.7	t	t	t	t	t	t	t	t	t	7.0	t
39	*trans*-Anethole	1254	1288	t	t	t	3.4	t	t	t	t	t	0.4	1.8	0.6	0.3	t	t	t	t	t	t	t	t	t	t	t
40	Bornyl acetate	1265	1271	6.0	t	t	t	t	t	t	t	t	t	0.6	2.0	t	t	t	t	t	t	t	t	t	t	t	t
41	Carvacrol	1286	1299	t	t	t	0.9	t	t	t	t	t	t	t	t	0.6	t	t	t	t	t	2.2	t	t	t	t	t
42	2-*trans*-Undecenal	1323	1344																							6.3	
43	α-Terpenyl acetate	1334	1349	0.9	t	t	t	0.9	2.1	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t
44	Decanoic acid	1356	1366	t	t	t	t	0.3	t	t	0.5	t	t	t	t	t	t	t	t	t	t	0.7	t	t	t	t	t
45	β-Bourbonene	1379	1396	0.8	t	t	2.0	t	t	t	1.6	t	0.8	t	1.9	1.5	t	t	t	t	t	0.4	t	t	t	t	t
46	*n*-Dodecanal	1397	1410	t	t	t	2.2	1.8	4.8	t	2.3	t	1.6	3.8	4.5	3.5	t	t	t	t	t	0.4	t	t	t	5.1	t
47	Longifolene	1399	1412	t	t	t	t	t	t	t	t	t	0.3	t	0.3	0.3	t	t	t	t	t	t	t	t	t	t	t
48	Geranyl acetone	1434	1451	0.1	t	t	t	t	t	6.4	0.4	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t
49	*ar*-Curcumene	1474	1473	t	t	0.9	2.8	7.2	t	3.5	t	t	3.2	2.8	3.4	2.9	t	t	1.4	t	2.0	0.7	12.3	16.6	t	6.3	31.8
50	UI A	1482		0.2	1.0	2.8	1.3	5.4	t	t	t	t	1.6	2.0	1.3	1.0	t	t	t	0.8	t	0.4	5.6	3.3	t	t	5.6
51	UI B	1483		0.2	0.5	2.5	1.2	2.1	t	t	t	t	1.4	2.1	1.0	0.7	t	t	t	0.8	t	0.3	4.7	2.5	t	t	5.5
52	UI C	1484		t	3.2	2.0	1.3	3.0	t	t	0.5	t	1.6	1.8	t	1.4	t	t	t	t	t	t	3.5	1.9	t	t	5.4
53	β-Bisabolene	1500	1506																							t	10.5
54	*trans*-Calamenene	1505	1515	0.1	t	t	t	t	t	t	t	t	t	t	t	1.1	t	t	t	t	t	t	t	t	1.5	t	2.4
55	δ-Cadinene	1505	1519	0.1	t	t	t	t	t	t	t	t	1.4	t	t	t	t	t	t	t	t	t	t	t	1.0	t	t
56	1,4-Dimethylazulene	1507	1523	0.2																		0.6					
57	*trans*-Nerolidol	1549	1550	0.8	t	t	0.8	t	t	t	4.1	t	t	3.7	3.8	0.5	t	t	t	t	t	t	t	t	t	t	t
58	Spathulenol	1551	1570	8.2	t	t	t	0.7	t	t	t	t	0.2	t	0.3	2.5	t	t	t	t	t	t	7.8	t	t	t	t
59	β-Caryophyllene oxide	1561	1575		t					0.8								t	t	t	t				t	t	t
60	Cedrol	1574	1586	2.4	t	0.4	7.3	24.2	40.3	19.5	4.8	9.1	7.8	7.3	4.2	3.7	t	t	t	1.3	8.3	t	5.1	t	8.4	t	t
61	*epi*-Cedrol*	1579	1600							18.3																	
62	UI P10	1605				14.8																					
63	UI D	1606		4.1	6.4		1.2	2.5	t	3.5	3.1	6.4	2.1	3.3	1.7	2.6	t	t	t	t	3.0	t		3.5	t	t	3.7
64	γ-Eudesmol	1609	1626	4.4	14.4	12.1	1.1	1.1	1.1	5.8	3.3	3.5	3.0	t	t	2.1	t	t	t	t	t	t	t	t	t	t	5.1
65	T-Cadinol	1616	1630	2.0	t	t	t	t	t	t	t	t	1.5	t	t	0.5	t	t	t	t	t	t	t	t	t	t	t
66	β-Eudesmol	1620	1638	12.8	29.7	10.8	2.3	3.2	1.1	7.9	9.0	10.6	5.5	t	5.5	5.1	t	t	t	4.7	4.5		3.7	1.8	t	t	3.4
67	α-Eudesmol	1634	1643	9.1	20.9	2.0	1.7	2.5	t	4.5	6.6	9.1	5.5	t	3.7	3.8	t	t	t	8.2	t		3.0	1.4	t	t	5.7
68	α-Bisabolol	1656	1671	0.9	1.5	t	0.7	0.4	t	t	0.7	1.9	8.5	t	0.4	0.6	t	t	t	t	t	t	t	t	15.4	t	t

Peak #	Components	RI	RI[a]	Propolis samples																							
				Cluster I																							Cluster II
				Ia			Ib1				Ib2																
				1	17	10	2	9	15	16	3	14	8	4	5	6	7	18	21	19	22	13	11	12	20	24	23
69	Bulnesol *	1656	1673							4.7																	
70	*n*-Heptadecane	1700	1700	t	t	t	t	t	t	t	t	t	0.7	t	1.4	0.8	t	t	t	t	t	t	t	t	t	t	t
71	*n*-Octadecane	1800	1800	t	t	t	t	t	t	t	t	t	0.2	t	0.5	t	t	t	t	t	t	t	t	t	t	t	t
72	*n*-Nonadecane	1900	1900	0.2	t	t	2.4	1.7	t	t	5.9	1.5	3.6	3.4	5.1	3.1	12.3	11.5	13.5	1.6	4.3	6.5	t	t	4.2	t	t
73	Hexadecanoic acid	1908	1964	0.2	t	19.9	1.5	1.6	3.7	t	0.5	t	1.6	1.5	0.6	1.7	t	t	t	t	2.0	3.6	t	t	t	t	t
74	UI E	1908		0.2	t	1.9	1.5	0.3	2.1	t	0.4	t	1.0	t	0.5	t	t	1.8	t	t	1.6	1.5	1.0	t	t	t	t
75	UI F	1915		0.6	t	t	0.8	1.0	1.5	0.9	3.1	1.7	t	t	1.4	1.6	t	t	t	t	t	t	1.0	t	2.9	t	t
76	*n*-Eicosane	2000	2000	0.1	t	10.3	0.4	0.2	t	t	2.3	t	1.6	1.4	1.9	0.6	4.9	1.0	6.9	1.9	4.1	6.1	t	t	t	t	t
77	Abietatriene	2027	2078								0.5																
78	UI G	2050		2.3	t	t	4.1	7.0	17.5	6.8	3.9	11.6	4.9	3.3	1.0	1.5	t	t	t	6.2	5.8	t	11.8	5.4	3.0	t	t
79	UI H	2068										1.2															
80	UI I P13	2072																				2.5					
81	*n*-Heneicosane	2100	2100	0.9	1.9	t	2.8	2.8	3.5	1.0	9.3	7.3	6.0	4.9	6.7	4.1	24.9	21.7	27.5	12.8	12.0	12.7	1.6	12.1	7.3	5.5	t
82	UI J P13	2112																				13.9					
83	UI I Cl	2116										1.4															
84	*n*-Docosane	2200	2200	0.8	t	t	1.0	1.7	t	0.8	3.7	1.3	t	1.3	1.5	1.8	t	1.5	8.0	5.2	6.2	8.8	3.9	1.9	t	0.8	t
85	*n*-Tricosane	2300	2300	3.2	2.7	t	7.4	7.6	10.4	4.5	14.0	19.5	10.6	8.1	10.1	6.1	28.7	33.5	24.0	26.8	18.1	13.1	16.2	24.2	8.5	9.8	t
86	*n*-Tetracosane	2400	2400	t	t	t	0.4	t	t	t	0.7	1.2	1.2	0.5	1.5	0.8	2.5	1.0	1.5	6.2	2.6	5.2	t	t	t	0.9	t
87	*n*-Pentacosane	2500	2500	1.0	1.4	t	3.3	2.5	t	t	4.9	7.9	2.1	3.3	3.0	3.5	14.6	17.4	9.5	13.3	7.6	6.7	5.2	14.1	3.6	6.5	t
88	*n*-Hexacosane	2600	2600	t	t	t	1.8	t	t	t	t	t	t	t	0.4	0.7	t	t	t	t	t	0.9	t	t	t	t	t
89	*n*-Heptacosane	2700	2700	0.2	0.7	t	t	1.3	t	t	1.9	5.3	2.6	2.0	2.8	3.4	10.3	7.9	5.3	6.6	4.2	2.7	3.3	1.6	t	1.0	t
90	*n*-Octacosane	2800	2800	t	t	t	t	t	t	t	t	t	t	t	t	0.6	t	t	t	t	t	0.3	t	t	t	1.0	t
91	*n*-Nonacosane	2900	2900	t	t	t	t	t	t	t	t	t	0.3	t	t	1.4	1.5	t	t	t	t	0.3	t	t	t	t	t

RI: In lab calculated Retention Index relative to C_9-C_{29} *n*-alkanes on the DB-1column. RI[a]: reported literature retention indices on DB-1 or similar phase column (100% Dimethylpolysiloxane) not from the authors lab [18]. t: trace (<0.05%). * Identification based on mass spectra only. UI: unidentified compound. Unidentified compounds MS (EI, 70 eV) *m/z* (Intensity ≥20%): *UIP2a*: 41 (65), 43 (37), 55 (71), 56 (35), 57 (32), 69 (100), 70 (37), 84 (20), 91 (20), 98 (24). *UIP2b*: 41 (82), 42 (24), 43 (87), 55 (54), 56 (100), 57 (44), 69 (99), 70 (59), 84 (35). *UIA*: 41 (36), 55 (23), 77 (39), 79 (45), 81 (22), 91 (67), 93 (55), 94 (35), 105 (79), 106 (22), 107 (41), 117 (21), 119 (100), 121 (37), 131 (41), 147 (23), 159 (41), 187 (44), 202 (49). *UIP10*: 41 (56), 53 (20), 55 (32), 67 (48), 69 (28), 77 (37), 79 (49), 81 (40), 84 (24), 91 (62), 93 (55), 95 (45), 105 (54), 107 (42), 108 (28), 109 (82), 119 (33), 121 (36), 131 (40), 137 (37), 145 (38), 159 (37), 161 (23), 183 (23), 187 (100), 202 (42). *UID*: 41 (29), 43 (25), 59 (63), 79 (29), 81 (25), 91 (44), 93 (39), 95 (22), 105 (42), 107 (37), 119 (41), 133 (24), 135 (35), 147 (27), 161 (100), 162 (28), 189 (31), 204 (35). *UIG*: 41 (64), 43 (38), 53 (21), 55 (66), 67 (47), 69 (68), 71 (29), 77 (26), 79 (59), 81 (100), 91 (50), 93 (72), 94 (29), 95 (97), 105 (42), 107 (53), 109 (53), 119 (35), 121 (43), 123 (40), 133 (30), 134 (24), 135 (31), 136 (28), 137 (83), 148 (20), 257 (49). *UIJP13*: 41 (67), 43 (66), 55 (25), 67 (30), 69 (100), 79 (21), 81 (24), 91 (22), 93 (42), 105 (29), 107 (40), 109 (61), 119 (24), 133 (21).

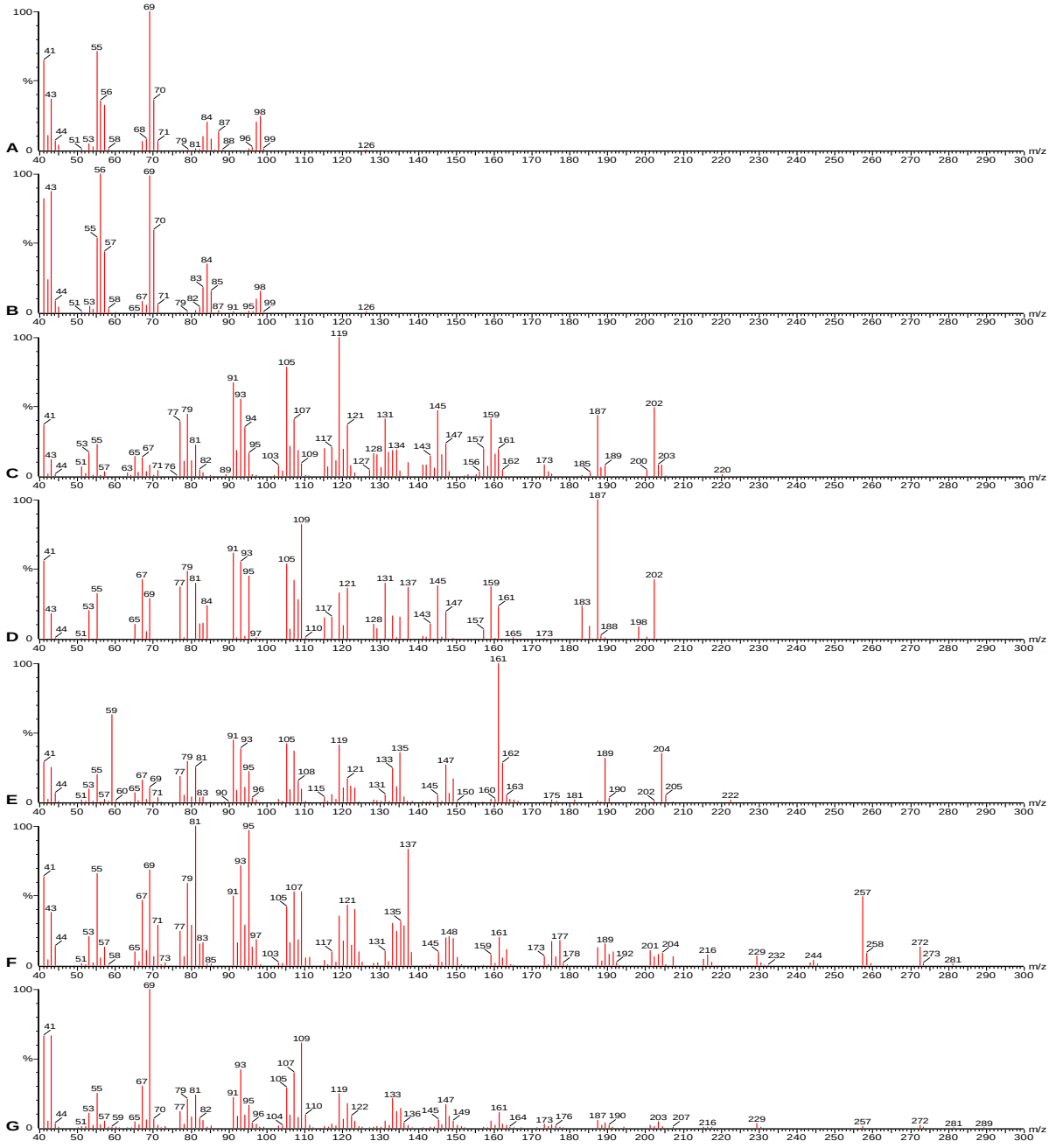


Figure E.1: Mass spectra of the main unidentified (≥5%) compounds UIP2a (A), UIP2b (B), UIA (C), UIP10 (D), UID (E), UIG (F) and UIJP13 (G) from the evaluated propolis samples.

Appendix F

Antioxidant and diuretic activity of co-administration of Capparis spinosa honey and propolis in comparison to furosemide*

*Adapted from 'Antioxidant and diuretic activity of co-administration of Capparis spinosa honey and propolis in comparison to furosemide'. Soukaïna El-Guendouz, Noori Al-Waili, Smail Aazza, Youssef Elamine, Soumia Zizi, ThiaAl-Waili. Ali Al-Waili, Badiaa Lyoussi. Asian Pacific Journal of Tropical Medicine Volume 10. Issue 10. October 2017. Pages 974-980.

Abstract

To study the antioxidant properties of *Capparis spinosa* (*C. spinosa*) honey and propolis and the effect of combined honey and propolis administration on urine volume and electrolytes in rats. *C. spinosa* honey [1 000 mg/kg body weight (b.wt)], propolis (100 mg/kg b.wt), honey/propolis mixture (*C. spinosa* honey 1 000 mg/kg b.wt/ propolis extract 100 mg/kg b.wt), distilled water (1 mL/kg b.wt) and furosemide (10 mg/kg b.wt) were orally administered to five groups of rats for 21 d. Urine volume. blood and urine sodium, potassium and chloride were measured. The antioxidant activity of propolis and honey was assessed and their total phenols and flavonoids were determined. Propolis and *C. spinosa* honey contain polyphenols including flavonoids and propolis demonstrated higher antioxidant activities than honey. Honey significantly increased urine volume and urine electrolyte excretion. Propolis had no significant effect on urine volume, but co-administration of propolis and honey caused significant diuresis. No major changes were observed in plasma electrolytes with the use of honey, propolis or their combination. Honey and propolis have antioxidant activity and contain polyphenols including flavonoids that are more pronounced in propolis. Honey has a significant diuretic activity alone or in combination with propolis. This is the first study comparing the diuretic effect of co-administration of propolis and *C. spinosa* honey with furosemide.

F.1. Introduction

It was found that honey has potential therapeutic effects. In modern medicine, honey has been introduced, in particular, as part of wound and ulcer management. Many studies including ours have found that honey has various biological activities including anti-inflammatory effect by modulating levels of cytokines and prostaglandins, antimicrobial activity against a wide range of pathogenic microbes, and antioxidant properties by upgrading antioxidant system.[1-6]

Regarding the kidney function, we have found that honey has beneficial effects on renal function in normal volunteers such as increasing urine output and creatinine clearance. It also increases urinary nitric oxide and decreases urinary prostaglandins level in human.[6] In addition, our studies demonstrated that honey could protect liver and kidney during acute blood loss and after carbon tetrachloride intoxication by normalization of liver enzymes and kidney function.[7,8] In the earlier observation, we found that honey increases urine output and creatinine clearance and has protection against lead-induced toxicity.[9] Other studies showed that honey has a favorable effect in hypertension, diabetic nephropathy and in cisplatin and cyclophosphamide induced nephro-toxicity.[10-12] Recently, it was found that carob honey collected from Morocco has diuretic, natriuretic and kaliuretic activity without side effects of hypokalemia that was observed with the use of widely prescribed diuretic, furosemide.[13]

Honey has been mentioned in Holy books, the Talmud, the Bible, and the Quran as a healer of human diseases. In the Surat Al-Nahel (the Bee chapter) it says (translating the meaning): and thy LORD taught the bee to build its cells in mountains, on tree and in men's habitations, then to eat of all the fruits of the earth and find with skill the spacious paths of its LORD, there issues from within their bodies a drink of varying colors, wherein is healing for men, verily in this is a sign for those who give thought.

Propolis is another bee product that bees collect from different plant sources and use it as part of the defense mechanism in their hives. Large amount of data demonstrated that propolis has vast majority of biological activities such as antioxidant and free radical scavenger activity with high content of antioxidant ingredients, antimicrobial activity against wide range of pathogens, immune stimulating and anti-malignant effect, and anti-inflammatory activities by modulating various pro-inflammatory cytokines.[14-18] Propolis has ameliorating effect on kidney function in diabetic nephropathy as well as a reno-protective effect in paracetamol, carbon tetrachloride and doxorubicin induced nephro-toxicity.[19-23] Recently, it was found that Moroccan propolis extract decreases urinary protein excretion and ameliorates the deterioration of liver and kidney function caused by ethylene glycol ingestion as well as it has a potential to be used in the treatment and prevention of urinary tract calculus, crystaluria. and proteinuria.[24]

In Morocco, honey and propolis are widely used in traditional medicine as part of the management of various diseases. However, scientific investigations regarding their biological activities are limited, and the diuretic effect of *Capparis spinosa* (*C. spinosa*) honey has not been studied.

The present study aimed to evaluate the effect of combined administration of *C. spinosa* honey and propolis collected in Morocco on urine volume and urinary and plasma electrolyte including sodium, potassium, and chloride. The different phenolic contents of propolis and honey were assessed, and the antioxidant capacities of propolis and honey were studied. This is the first study investigating the diuretic effect of co-administration of propolis and honey and their effect on plasma and urine electrolytes in comparison to furosemide, a widely prescribed diuretic in clinical practice.

F.2. Experimental

F.2.1. Chemicals

Ethanol was from Panreac and gallic acid gallic acid was purchased from Acros Organics, New Jersey, USA. Rutin, 2.4-dinitrophenylhydrazine, Butylated hydroxytoluene and azino-bis3-ethylbenzothiazoline-6-sulphonic acid (ABTS) were purchased from Fluka Biochemika, Sigma-Aldrich, Steinheim, Germany. Sodium dihydrogen phosphate and di-sodium hydrogen phosphate was from Riedel-de-Haën Laboratory Chemicals**,** Germany. Potassium ferricyanide and ferric chloride were purchased from Fluka Chemicals Buchs, Switzerland, while ascorbic acid was purchased from Merck, Darmstadt, Germany.

F.2.2. Preparation of samples

Moroccan propolis samples were obtained from beekeeper's association, Fez, Boulemane, Morocco. The collected propolis was crushed to fine powder. and was extracted with the use of ethanol 70%. The alcoholic extract solution was then filtered through a filter paper and the alcohol content was evaporated with the use of a rotary evaporator under reduced pressure. The residue was dissolved in a minimal volume of ethanol and stored at a low temperature (−20 °C) until it was used in the experimentation. *C. spinosa* honey was also obtained from beekeeper's association, Fez, Boulemane, Morocco, and was used in the experiment. A loop diuretic, furosemide, was used as a reference drug (control) for the comparison.

F.2.3. Determination of total phenolic, total flavonic and flavonolic content

Each experiment was repeated three times on *C. spinosa* honey or propolis and the results were expressed as mean ± SEM. The total polyphenol content in both propolis and *C. spinosa* honey samples was determined using the method of Slinkard and Sinleton.[25] Using a calibration curve, the total polyphenol content was expressed as mg/g of gallic acid equivalents (GAE). The concentration range of gallic acid was 1 mg/mL. The amount

of flavones and flavonols in the propolis extract and *C. spinosa* honey samples was determined according to the method of Miguel *et al.*.[26] The total flavones and the flavonols contents were calculated as rutin equivalents (mg/g) using a calibration curve. The concentration range of rutin was 1 mg/mL. Furthermore, the total amount of flavanones and dihydroflavonols compounds was determined with the use of 2. 4-dinitrophenylhydrazine, and the absorbance was measured at 700 nm.[27] UV/visible spectrophotometer, Ultrospec 1100 pro, Amersham Biosciences, Texas, USA was used.

F.2.4. Antioxidant activity assays

The antioxidant capacity of the propolis and *C. spinosa* honey was determined by evaluating their ability for scavenging free radicals, azino-bis3-ethylbenzothiazoline-6-sulphonic acid (ABTS) and nitric oxide (NO), and by their reducing power. The latter was determined according to the method described by Laskar *et al.*[28]. *C. spinosa* honey (50 µL) or propolis (50 µL) were mixed with 500 µL of 0.2 M sodium phosphate buffer (pH = 6) and 500 µL of 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min and then was centrifuged for 10 min at 3 000 rpm. The supernatant liquid (500 µL) was mixed with 500 µL of distilled water and 100 µL of 0.1% ferric chloride. The absorbance of the mixture was measured at 700 nm. Ascorbic acid was used as a positive control.

$ABTS^{+\bullet}$ radical scavenging activity was determined as described by Aazza *et al.*.[29] Briefly. the ABTS radical was generated by reaction of a 7 mM $ABTS^{+\bullet}$ aqueous solution with $K_2S_2O_8$ (2.45 mM) in the dark for 16 h and adjusting the absorbance at 734 nm to 0.7 at room temperature. Twenty-five microliter of *C. spinosa* honey or propolis extract were added to 275 µL of $ABTS^{+\bullet}$ and absorbance at 734 nm was read after 6 min. The capability to scavenge the $ABTS^{+\bullet}$ was calculated using the formula: ABTS scavenging activity (%) = $[(A_0 - A_1)/A_0] \times 100$ (%), where A_0 is the absorbance of the control (without sample) and A_1 is the absorbance in the presence of honey or propolis sample. The sample concentration providing 50% inhibition (IC_{50}) was obtained by plotting the inhibition percentage against honey or propolis concentrations. Butylated hydroxytoluene (BHT) was used as a positive control. NO scavenging activity was measured with the use of Griess reagent and ascorbic acid was used as a positive control.[30]

F.2.5. Diuretic effects of propolis and C. spinosa honey

Thirty male Wistar rats [(190 ± 40) g] were obtained from the animal house-breeding center, Faculty of Science, Dhar Mehrez, Fez, and were housed under standard environmental conditions. The animals had a free access to tap water and standard laboratory rat food. The

protocol was approved by the institutional committee on animal care following the French Technical Specifications for the Production, Care and Use of the Laboratory Animals, University Sidi Mohamed Benabdelah, Faculty of Science, Dhar Mehrez, Fez.

The animals were divided into five groups six rats each. Group 1 were treated with oral honey 1 000 mg/kg body. weight (b.wt); group 2 were treated with oral propolis extract 100 mg/kg b.wt; group 3 were treated with a mixture of propolis extract 100 mg/kg b.wt and honey 1 000 mg/kg b.wt; group 4 were treated with oral furosemide 10 mg/kg b.wt; group 5 were treated with oral distilled water (1 mL/kg b.wt). The experiment continued for a total of 21 d. Each rat was individually kept in a metabolic cage and the urine output was collected, measured, and filtered for testing. Blood samples were collected in capillary tubes containing ethylene diaminetetraacetic acid by retro-orbital puncture under light diethyl ether anesthesia. Blood and urine sodium. chloride and potassium were measured with the use of flame spectrophotometer.

F.2.6. Statistical analysis

All the data were analyzed using graph pad prism (version 5) and the results were expressed as mean ± SEM. ANOVA test and student's *t*-test were used for analysis. Data with $P < 0.05$ was considered significant.

F.3. Results and discussion

F.3.1. Total phenolic, flavones and flavonols contents

Propolis showed a high amount of phenolic compounds [(327.039 ± 0.020) mg/g of GAE], which is significantly higher than that found in the honey [(2.538 ± 0.020) mg/g of GAE]. The amount of flavones and flavonols content in *C. spinosa* honey was (0.190 ± 0.100) mg eq rutin/g, which is significantly less than the level of flavones and flavonols in propolis [(159.500 ± 0.090) mg eq rutin/g. $P < 0.05$]. The propolis extract showed a higher amount of total flavanones and dihydroflavonols content [(132.235 ± 0.060) mg eq naringin/g] than that found in *C. spinosa* honey [(0.140 ± 0.060) mg eq naringin/g. $P < 0.05$].

F.3.2. Antioxidant activity assays

As the absorbance increases, the antioxidant activity increases. The propolis extract and honey have high total antioxidant activity measured by the ferric reducing antioxidant power assay. The effect was dose-dependent in all samples (Table F.1). However, ascorbic acid and

propolis had higher antioxidants activity than honey, which was evident by higher absorbance with lower concentration of ascorbic acid or propolis.

Table F.1. Reducing power of honey, propolis and ascorbic acid solutions at different concentrations (mean ± SEM).

Honey		Propolis		Ascorbic Acid	
Concentration (mg/ml)	Absorbance (700nm)	Concentration (mg/ml)	Absorbance (700nm)	Concentration (mg/ml)	Absorbance (700nm)
3.91	0.09±0.01*	0.08	0.12±0.01*	0.03	0.22±0.01
7.81	0.09±0.01*	0.16	0.15±0.01*	0.06	0.31±0.01
15.63	0.11±0.01*	0.31	0.20±0.01*	0.13	0.44±0.01
31.25	0.14±0.01*†	0.63	0.29±0.01*	0.25	0.67±0.01
62.50	0.18±0.01*†	1.25	0.61±0.01*	0.51	0.93±0.04
125.00	0.26±0.01*†	2.50	0.65±0.08*	1.01	1.60±0.02
250.00	0.43±0.01*†	5.00	0.91±0.08*	2.02	2.87±0.07
500.00	0.66±0.02*†	10.00	1.61±0.08*	4.04	2.99±0.01

As compared to ascorbic acid*P<0.05; as compared to propolis† P<0.05.

Propolis showed a high antioxidant capacity towards the ABTS radical [IC_{50} = (0.016 ± 0.01) mg/mL] which was not significantly different from BHT [(0.014 ± 0.016) mg/mL]; whereas *C. spinosa* honey showed a lower scavenging activity towards the $ABTS^{+\bullet}$ radical [IC_{50} = (11.460 ± 0.02) mg/mL] as compared to the propolis extract ($P < 0.05$) or BHT, Propolis had a greater NO scavenging activity [IC_{50} = (0.45 ± 0.01) mg/mL] than *C. spinosa* honey [IC_{50} = (20.69 ± 0.04) mg/mL] but its activity was lower than ascorbic acid [IC_{50} = (0.05 ± 0.01) mg/mL. $P < 0.05$].

F.3.3. Effect on urine volume

C. spinosa honey increased urine volume and caused significant diuresis, which was obviously starting on day 1, and became significant at day 5 of the experiment. The urine volume remained significantly higher for the honey-treated rats than the control rats at all-time intervals (Table F.2). The propolis extracts decreased urine volume, which was insignificant when it was compared to the urine volume before propolis administration. However, the mixture of propolis and *C. spinosa* honey caused a significant increase in the urine volume on days 19 and 21 (Table F.2).

Table F.2. Urine volume (mL) with daily oral administration of propolis. *C. spinosa*honey. honey and propolis mixture. furosemide. and distilled water(mean ± SEM).

	Day 0 (basline)	Day 5	Day 7	Day 15	Day 19	Day 21
Control	5.30±0.75	5.25±0.60	5.37±0.20	5.85±0.20	4.75±0.40	5.62±0.40
Honey	4.25±0.20	7.00±0.60*+#	8.70±0.70*+#^	10.5±0.50*+x#^	11.25±0.40*+x#^	11.5±0.40*+x#^
Propolis	6.10±0.50	5.20±0.50x^	4.30±0.22x^	4.30±0.60x^	4.50±0.20x^	4.95±0.20x^
Honey/ Propolis	6.00±0.40	6.60±0.70+#	6.30±0.20x#	6.60±0.60+x#	7.60±0.20*+x #	8.00±0.40*+x#
Furosemide	4.25±0.40	7.50±0.58+	9.00±0.30*+	12.75±0.20*+	13.10±0.40*+	13.25±0.40*+

As compared to day 0 (baseline). $^{*}P < 0.05$; as compared to the control. $^{+}P < 0.05$; as compared to furosemide. $^{x}P < 0.05$; as compared to propolis. $^{\#}P < 0.05$; as compared to honey/propolis mixture. $^{\wedge}P < 0.05$.

F.3.4. Effect on urinary electrolyte excretion

C. spinosa honey significantly increased excretion of sodium, potassium and chloride as compared to baseline, while furosemide significantly increased the excretion of sodium and potassium without significant changes in the chloride urinary excretion. In contrast, propolis extracts significantly decreased sodium and potassium excretion ($P < 0.05$). However, the mixture of propolis and honey increased urine sodium and chloride excretion and decreased potassium excretion (Table F.3).

Table F.3: Effect of oral administration of propolis, *C. spinosa* honey, honey and propolis mixture, furosemide and distilled water on urine electrolytes level in normal rats (mean ± SEM).

Groups	Dose (mg/kg b.wt)	Urine sodium (meq/L)		Urine potassium (meq/L)		Urine chloride (meq/L)	
		Baseline	Day 21	Baseline	Day 21	Baseline	Day 21
Honey	1 000	74.0±1.0	83.0 ±1.0*	22.40 ±0.50	44.60±0.50*	105.05 ±2.00	123.02± 1.00*
Propolis	100	79.0± 1.0	71.0±1.0*	16.10±1.00	11.25 ±1.00*	101.45± 3.00	99.50± 3.00
Honey/Propolis	1 000/100	74.0±1.0	80.0±1.0*	17.70±1.00	14. 20±1.00*	90.02± 1.00	97.00± 1.00*
Furosemide	10	49.0 ±0.9	88.0±0.1*	16.10± 2.00	24.25 ±1.00*	99.00 ± 1.00	101.00 ±2.00

$^{**}P < 0.05$ compared to the control and to baseline.

F.3.5. Effect on plasma electrolyte levels

There was no significant effect of *C. spinosa* honey, propolis, or propolis/honey mixture on plasma levels of sodium and potassium while honey and furosemide significantly decreased plasma chloride level ($P < 0.05$) (Table F.4).

Table F.4: Effect of oral administration of propolis, *C. spinosa* honey, honey and propolis mixture, furosemide, and distilled water on plasma electrolyte levels in normal rats (mean ± SEM) (meq/L).

Treatment	Basline			Day 21		
	Sodium	Potassium	Chloride	Sodium	Potassium	Chloride
Water, (control)	142.5 ±5.50	2.79± 0.40	109.50±4.00	141.00±3.50	3.00 ± 0.70	112.00±2.00
Furosemide	142.5±5.50	2.88 ±0.40	110.75 ±5.00	140.00± 6.50	2.80±0.90	99.75 ±4.00
Honey	143.25±5.40	2.83±0.40	118.00 ±2.00	143.00 ±2.70	3.04±0.50	108.00 ±3.00*
Propolis	141.25±3.50	2.95± 0.50	106.00±5.00	140.00 ± 5.00	3.05±0.50	104.00 ±2.300*
Honey/ Propolis	140.5±5.50	2.70 ±0.50	113.50±9.00	141.00 ± 3.50	3.00±0.60	104.00± 5.00

$^{*}P < 0.05$ compared to the control and to baseline.

F.3.6. Effect on osmolarity and clearance of free water

C. spinosa honey, propolis, or the mixture of honey/propolis showed no significant effect on the plasma osmolarity (Table F.5). However, *C. spinosa*honey or the mixture of honey/propolis significantly increased urine osmolality and osmolar clearance while the

propolis extracts significantly decreased urine osmolality and osmolar clearance ($P < 0.05$).

Table F.5: Effects of oral administration of propolis, *C. spinosa* honey, honey and propolis mixture, furosemide, and distilled water on various parameters measured (mean ± SEM).

	Uosm ($mOsm/kgH_2O$)	**Posm ($mosm/kgH_2O$)**	**COsm (µl/min)**	**CH_2O (µl/min)**
Control	1 580±9	286±6	27.62±2.0	-22.62±2.5
Honey	1 660±9*	286±6.9	46.4±3.5^{*+}	-38.5±4.3*
Propolis	1 421±8*	280±8.8	12.68±9.0^{*+}	-10.68±1.3^{*+}
Honey/Propolis	1 600±9*	282±7.5	34.04±1.2^{*+}	-28.04±1.9^{+}

As compared to the control $^{*}P < 0.05$; as compared to *C. spinosa* honey, $^{+}P < 0.05$.

The results presented showed that propolis collected from Morocco contains high amount of total phenols. Recently, it was shown that propolis collected from different regions of Morocco contains phenols, flavonoids, and has antioxidant and anti-inflammatory activities.[31] High total phenols contents were found in Portuguese propolis samples (329.00 mg/g of GAE), and in Chinese propolis samples [(302.0 ± 4.3) mg/g of GAE]. However, lesser amount of total phenols was found in Portuguese (151 mg/g of GAE) and in Brazilian propolis [(120.0 ± 3.5) mg/g of GAE].[27,32,33] In the present study, Moroccan propolis showed a higher amount of phenolic compounds [(327.039 ± 0.020) mg/g of GAE] than that reported in Chinese, Portugal and Brazilian propolis.

The results showed that *C. spinosa* honey contains phenolic compounds, but less than propolis. The amount of phenolic compounds in *C. spinosa* honey was lesser than that previously reported for Tualang honey [(251.7 ± 7.9) mg gallic acid/kg] and for Manuka honey [(52.63 ± 1.21) mg gallic acid/100 g].[34,35] However, the mean flavones and flavonols content in *C. spinosa* honey was higher than that reported for Croatian acacia honey (43.66 mg/kg), and for Burkina Faso acacia honey (61.4 mg/kg).[36,37] This could be due to the different floral and geographical origins of honey sources since the compositions of bee honey depend on its geographical floral origin, season, environmental factors and treatment of beekeepers.[38]

The antioxidant activities of chestnut (*Castania sativa* Mill.) honeys and propolis in Turkey were evaluated and found to be high and related to the sample concentrations. Furthermore, the ethanolic propolis extracts showed the highest antioxidant activity, which is similar to our findings that propolis extracts explored higher antioxidant activity than honey.[39] Recent study conducted on 32 different chestnut honeys collected from different areas in the

Black Sea region of Turkey showed that the levels of phenolic materials ranged from 70 mg GAE/100 g to 105 mg GAE/100 g which was lower than *C. spinosa* honey [(2.538 ± 0.020) mg GA/g], and the levels of total flavonoid between 4.04 mg QE/100 g and 7.01 mg QE/100 g. However, the concentrations of compounds from all three polyphenol classes varied depending on the unifloral character of the chestnut honey. Ferric reducing/antioxidant power was used to measure antioxidant capacities of chestnut honey and its values ranged from 330 μmol $FeSO_4$/100 g to 470 μmol $FeSO_4$/100 g, the antioxidant capacity of honey depends on its unifloral character.[40]

$ABTS^{+\bullet}$ method was used for evaluating the ability of propolis and *C. spinosa* honey samples for scavenging free radicals that gives an indication of the total antioxidant capacity of the samples. HBT and $ABTS^{+\bullet}$ assay demonstrated a better activity than *C. spinosa* honey samples. Nevertheless, the activity of propolis was not significantly different from the activity of HBT. The propolis extract has a higher ferric reducing power than *C. spinosa* honey.

NO is an important bio-regulatory molecule generated from the amino acid *L*-arginine. It has important effects on various biological systems. However, during infections and inflammations, its concentrations become higher. It was demonstrated that chronic exposure to inducible NO radical is associated with various carcinomas and inflammatory conditions.[41,42] In the present study, propolis and honey showed NO scavenging activity in which propolis was more pronounced. It is well known that phenolic compounds and flavonoids have NO scavenging activity. Therefore, flavonoids and phenolic compounds present in propolis or honey might be responsible for the observed NO scavenging activity. We have found that honey increases NO end products in various human biological fluids such as blood and urine.[43,44] However, it was found that gelam honey inhibited inducible NO and PGE_2 in rat inflammatory model.[45] This makes honey having a dual function: it can increase NO when there is a demand for it to facilitate normal physiological functions or to recover abnormalities related to low availability of NO, and honey can scavenge extra amount of NO generated in inflammatory and pathological conditions.

The administration of honey causes a significant increase in the urine volume and urinary excretion of sodium and chloride. Furthermore, there was no effect of honey on plasma electrolytes. This is similar to our previous study in which carob honey collected from Morocco showed a diuretic activity without causing hypokalaemia.[13] Propolis did not reveal significant effects on urine volume, though it caused a mild reduction in urine volume. However, the mixture of *C. spinosa* honey and propolis caused a moderate diuretic effect.

Several studies have shown that flavonoids exhibit diuretic effects.[46,47] This effect could explain, partially, the diuretic activity of *C. spinosa* honey. The glucose and fructose in honey sample might cause diuresis. However, we found that the use of artificial honey did not result in a significant increase in urine volume in normal individuals.[6] In spite of higher amount of flavonoids and phenols found in propolis as compared to *C. spinosa* honey, propolis did not show a diuretic activity in the present study. More studies should be conducted to verify the effect of propolis on urine volume and urine electrolytes. Different doses or different ways of collection and preparation might result in different propolis activity. In this regard, we have found that propolis extract caused a significant increase in urine output in rat exposed to ethylene glycol toxicity and in restored renal function test, it increased creatinine clearance.[24] Studies have shown that NO causes natriuresis and diuresis and inhibits fluid reabsorption in renal tubules.[48,49] In renal physiology, PGE_2 was found to inhibit sodium chloride transport in the collecting ducts.[50] We have found that oral honey increases urinary NO and decreases urinary prostaglandins level in normal individuals.[51] These findings might explain, partially, the diuretic effect of honey observed in this study.

F.4. Conclusion

Aberrantly, the results are interesting and have potential clinical application. The present study is the first to investigate the effect of combined administration of propolis and honey on urine output and plasma and urine electrolytes compared with furosemide. More studies including the effect of such combination on histopathology of the kidney will be useful. Although *C. spinosa* honey has a similar diuretic activity to the Moroccan carob honey and honey collected from United Arab Emirates was better than artificial honey,[6,13] comparison of this type of honey with other types of honey such as Manuka honey or artificial honey will help to explore whether different honey samples have a different diuretic activity. The result will pave the way for further studies, including clinical trial, to investigate the diuretic effect of honey and the use of honey and propolis in acute or chronic kidney pathological entities where oxidative process plays a major role in the pathogenesis

References

[1] A. Zaghloul, H. El-Shattawy, A. Kassem, 'Honey, a prospective antibiotic: Extraction, formulation, and stability', Pharmazie. 2001, 56, 643-647.

[2] N. Al-Waili, A. Al Ghamdi, M. J. Ansari, Y. Al-Attal, A. Al-Mubarak, K. Salom, 'Differences in composition of honey samples and their impact on the antimicrobial activities against drug multiresistant bacteria and pathogenic fungi', Arch. Med. Res. 2013, 44, 307-316.

[3] K. Jaafar, J. Haidar, S. Kuraydiyyah, T. Ghaddar, K. Knio, B. Ismail, et al. 'Physicochemical, melissopalynological and antioxidant properties of artisanal honeys from Lebanon', J. Food. Sci. Technol. 2017, 54, 2296-2305.

[4] S. B. Almasaudi, A. T. Abbas, R. R. Al-Hindi, N. A. El-Shitany, U. A. Abdel-Dayem, S. S. Ali, et al. 'Manuka honey exerts antioxidant and anti-inflammatory activities that promote healing of acetic acid-induced gastric ulcer in rats', Evid. Based. Complement. Alternat. Med. 2017, 2017: 1-12.

[5] W. S. Ghazali, A. C. Romli, M. Mohamed, 'Effects of honey supplementation on inflammatory markers among chronic smokers: a randomized controlled trial', BMC. Complement. Altern. Med. 2017, 17, 175-184.

[6] N.S. Al-Waili, 'Effects of honey on the urinary total nitrite and prostaglandins concentration', Int. Urol. Nephrol. 2005, 37, 107-111.

[7] N.S. Al-Waili, K.Y. Saloom, M. Akmal, F. Al-Waili, T. N. Al-Waili, A. N. Al-Waili, et al. Honey ameliorates influence of hemorrhage and food restriction on renal and hepatic functions, and hematological and biochemical variables. Int. J. Food. Sci. Nutr. 2006, 57, 353-362.

[8] N. S. Al-Waili, K. Y. Saloom, T. N. Al-Waili, A. N. Al-Waili, M. Akmal, F. S. Al-Waili, et al., 'Influence of various diet regimens on deterioration of hepatic function and hematological parameters following carbon tetrachloride: a potential protective role of natural honey', Nat. Prod. Res. 2006, 20, 1258-1264.

[9] A. F. Fihri, N. S. Al-Waili, R. El-Haskoury, M. Bakour, A. Amarti, M. J. Ansari, et al., 'Protective effect of Morocco carob honey against lead-induced anemia and hepato-renal toxicity', Cell. Physiol. Biochem. 2016, 39, 115-122.

[10] O. O. Erejuwa, S. A. Sulaiman, M. S. Wahab, K. N. Sirajudeen, M. S. Salleh, S. Gurtu, 'Differential responses to blood pressure and oxidative stress in streptozotocin-induced diabetic Wistar-Kyoto rats and spontaneously hypertensive rats: effects of antioxidant (honey) treatment', Int. J. Mol. Sci. 2011, 12, 1888-1907.

[11] R. Hamad, C. Jayakumar, P. Ranganathan, R. Mohamed, M. M. El-Hamamy, A. A. Dessouki et al., 'Honey feeding protects kidney against cisplatin nephrotoxicity through suppression of inflammation', Clin. Exp. Pharmacol. Physiol. 2015, 42, 843-848.

[12] K. M. Zoheir, G. I. Harisa, O. M. Abo-Salem, S. F. Ahmad, 'Honey bee is a potential antioxidant againstcyclophosphamide-induced genotoxicity in albino male mice', Pak. J. Pharm. Sci. 2015, 28, 973-981.

[13] R. El-Haskoury, S. Zizi, S. Touzani, N. Al-Waili, A. Al-Ghamdi, B. Lyoussi, 'Diuretic activity of carob (Ceratonia siliqua L.) honey: comparison with furosemide', Afr. J. Tradit. Complement. Altern. Med. 2016, 12, 128-133.

[14] K. Ozgur, T. Fulya, A. Cengiz, '*In vitro* antimicrobial activity of propolis samples from different geographical origins against certain oral pathogens', Anaerobe. 2007, 13, 140-145.

[15] J. Sforcin, 'Propolis and the immune system: a review', J. Ethnopharmacol. 2007, 113, 1-5.

[16] N. Al-Waili, A. Al-Ghamdi, M. Ansari, Y. Al-Attal, K. Salom, 'Synergistic effects of honey and propolistoward drug multi resistant Staphylococcus aureus, Escherichia coli and Candida albicans isolates in single and polymicrobial cultures', Int. J. Med. Sci. 2012, 9, 793-800.

[17] J. M. Sforcin, 'Biological properties and therapeutic appications of propolis', Phytother. Res. 2016, 30, 894–905.

[18] V. Pasupuleti, L. Sammugam, N. Ramesh, S. H. Gan, 'Honey, propolis, and royal jelly: A comprehensive review of their biological actions and health benefits', Oxid. Med. Cell. Longev. 2017, 2017. Doi: 10.1155/2017/1259510.

[19] S. K. Nirala, M. Bhadauria, 'Propolis reverses acetaminophen induced acute hepatorenal alterations: a biochemical and histopathological approach', Arch. Pharm. Res. 2008, 31, 451-461.

[20] O. M. Abo-Salem, R. H. El-Edel, G. E. Harisa, N. El-Halawany, M. M. Ghonaim, 'Experimental diabetic nephropathy can be prevented by propolis: Effect on metabolic disturbances and renal oxidative parameters', Pak. J. Pharm. Sci. 2009, 22, 205-210.

[21] W. Zhu, Y. H. Li, M. L Chen, F. L. Hu, 'Protective effects of Chinese and Brazilian propolis treatment against hepatorenal lesion in diabetic rats', Hum. Exp. Toxicol. 2011, 30, 1246-1255.

[22] M. Bhadauria, 'Propolis prevents hepatorenal injury induced by chronic exposure to carbon tetrachloride', Evid. Based. Complement. Alternat. Med. 2012, 2012, 1-12.

[23] K. Boutabet, W. Kebsa, M. Alyane, M. Lahouel, 'Polyphenolic fraction of Algerian propolis protects rat kidney against acute oxidative stress induced by doxorubicin', Indian. J. Nephrol. 2011, 21, 101-106.

[24] N. El Menyiy, N. Al Waili, M. Bakour, H. Al-Waili, B. Lyoussi, 'Protective effect of propolis in proteinuria, crystaluria, nephrotoxicity and hepatotoxicity induced by ethylene glycol ingestion', Arch. Med. Res. 2016, 47, 526-534.

[25] K. Slinkard, V. Singleton, 'Total phenol analysis: Automation and comparison with manual methods', Am. J. Enol. Vitic. 1977, 28, 49-55.

[26] M. G. Miguel, S. Nunes, S. A. Dandlen, A. M. Cavaco, M. D. Antunes, 'Phenols and antioxidant activities of hydro-alcoholic extracts of propolis from Algarve, South of Portugal', Food. Chem. Toxicol. 2010, 48, 3418-3423.

[27] M. Popova, V. Bankova, D. Butovska, 'Validated methods for the quantification of biologically active constituents of poplar-type propolis', Phytochem. Anal. 2004, 15, 235-240.

[28] R. Laskar, I. Sk, N. Roy, N. Begum, 'Antioxidant activity of Indian propolis and its chemical constituents', Food. Chem. 2010, 122, 233-237.

[29] S. Aazza, B. Lyoussi, C. Megías, I. Cortés-Giraldo, J. Vioque, A. C. Figueiredo, et al., 'Anti-oxidant, anti-inflammatory and anti-proliferative activities of Moroccan commercial essential oils', Nat. Prod. Comm. 2014, 9, 587-594.

[30] C. Ho, L. Tang, S. M. Lin, F. Liew, 'Evaluation of peroxynitrite scavenging capacities of several commonly used fresh spices', Food. Chem. 2010, 119, 1102-1107.

[31] M. Miguel, O. Doughmi, S. Aazza, D. Antunes, B. Lyoussi, 'Antioxidant, anti-inflammatory and acetyl cholinesterase inhibitory activities of propolis from different regions of Morocco', Food. Sci. Biotechnol. 2014, 23, 1-10.

[32] M. Ahn, S. Kumazawa, Y. Usui, J. Nakamura, M. Matsuka, F. Zhu, et al. 'Antioxidant activity and constituents of propolis collected in various areas of China', Food. Chem. 2007, 102, 1383-1392.

[33] L. Moreira, L. Dias, J. Pereira, L. Estevinho, 'Antioxidant properties, total phenols and pollen analysis of propolis samples from Portugal', Food. Chem. Toxicol. 2008, 46, 3482-3485.

[34] M. Mohamed, K. Sirajudeen, M. Swamy, N. Yaacob, S. Sulaiman, 'Studies on the antioxidant properties of Tualang honey of Malaysia', Afr. J. Tradit. Complement. Altern. Med. 2010, 7, 59-65.

[35] M. Khalil, N. Alam, M. Moniruzzaman, S. Sulaiman, S. Gan, 'Phenolic acid composition and antioxidant properties of Malaysian honeys', J. Food. Sci. 2011, 76, C921-C928.

[36] P. J. Taormina, B. A. Niemira, L. R. Beuchat, 'Inhibitory activity of honey against foodborne pathogens as influenced by the presence of hydrogen peroxide and level of antioxidant power', Int. J. Food. Microbiol. 2001, 69, 217-225.

[37] A. Meda, C. E. Lamien, M. Romito, J. Millogo, O. G. Nacoulma, 'Determination of the total phenolic, flavonoid and proline contents in Burkina Fasan honey, as well as their radical scavenging activiity', Food. Chem. 2005, 91, 571-577.

[38] J. Da Costa Leite, L. Trugo, L. Costa, L. Quinteiro, O. Barth, V. Dutra, 'Determination of oligosaccharides in Brazilian honeys of different botanical origin', Food. Chem. 2000, 70, 93-98.

[39] A. Sarikaya, E. Ulusoy, N. Özturk, M. Tuncel, S. Kolayli, 'Antioxidant activity and phenolic acid constituents of chestnut (Castalinia sativa Mill) honey and propolis', J. food. Bioch. 2009, 33, 470- 484.

[40] S. Kolayli, Z. Can, O. Yildiz, H. Sahin, S. Karaoglu, 'A comparative study of the antihyaluronidase, antiurease, antioxidant, antimicrobial and physicochemical properties of different unifloral degrees of chestnut (Castanea sativa Mill.) honeys', J. Enz. Inhibit. Med. Chemist. 2016, 31, 210-218.

[41] R. Huie, S.Padmaja, 'The reaction of no with superoxide', Free. Rad. Res. Communit. 1993, 18, 195-199.

[42] N. S. Al-Waili, 'Oxidants and antioxidants in breast cancer: A possible tumor marker', FASEB. J. 2003, 17, 861.

[43] N. Al-Waili, 'Identification of nitric oxide metabolites in various honeys: effects of intravenous honey on plasma and urinary nitric oxide metabolites concentrations', J. Med. Food. 2003, 64, 359- 364.

[44] N. Al-Waili, N. Boni, 'Honey increased saliva, plasma, and urine content of total nitrite concentrations in normal individuals', J. Med. Food. 2004, 7, 377-380.

[45] M. Kassim, M. Achoui, M. Mansor, K. M. Yusoff, 'The inhibitory effects of Gelam honey and its extracts on nitric oxide and prostaglandin E(2) in inflammatory tissues', Fitoterapia. 2010, 81, 1196-1201.

[46] H. Jouad, M. Lacaille-Dubois, B. Lyoussi, M. Eddouks, 'Effects of the flavonoids extracted from Spergularia purpurea Pers. on arterial blood pressure and renal function in normal and hypertensive rats', J. Ethnopharmacol. 2001. 76, 159-163.

[47] J. Wu, A. Muir, 'Isoflavone content and its potential contribution to the antihypertensive activity in soy bean angiotensin I converting enzyme inhibitory peptides', J. Agricul. Food. Chem. 2008, 56, 9899-9904.

[48] H. Garcia, I. Pomposiella, L. Govin,' Nitric oxide inhibits ADH-stimulation osmotic water permeability in cortical collecting ducts', Am. J. Physiol. 1996, 270, F206-F210.

[49] T. Noonan, O. Banks, 'The role of nitric oxide in saline-induced natriuresis in rats', Proceed. Soc. Exp. Biol. Med. 1999, 221, 376-381.

[50] J. Chen, M. Zhao, W. He, G. L. Milne, J. Howard, J. Morrow, et al., 'Increased dietary NaCl induces renal medullary PGE_2 production and natriuresis via the EP_2 receptor', Am. J. Physiol. Renal. Physiol. 2008, 295, F818-F825.

[51] N. S. Al-Waili, N. S. Boni, 'Natural honey lowers plasma prostaglandin concentrations in normal individuals', J. Med. Food. 2003, 6, 129-133.

Appendix G

Preliminary screening of the possible protective effect of Moroccan propolis against chromium-induced nephrotoxicity in animal model

*Adapted from 'Preliminary screening of the possible protective effect of propolis against chromium-induced nephrotoxicity in animal model'. Soukaïna El-Guendouz, Soumia Zizi,Youssef Elamine, Badiaa Lyoussi. Veterinary world. (accepted)

Abstract

Hexavalent chromium (Cr (VI)) compounds has been shown to induce nephrotoxicity associated with oxidative stress in humans and animals. Thus, the aim of the present study, was to investigate the nephroprotective effect of bee propolis, as highly antioxidant natural product, *in vivo* using animal model. First of all, total phenols and flavonoids contents of propolis sample were estimated *in vitro*. Afterwards, to study the protective effect of propolis on renal damages caused by an injection of a single dose of potassium dichromate (15 mg / kg.b.wt), twenty-four male Wister rats were divided into test and control groups. Propolis treatment was performed by oral gavage of 100 mg /kg.b.wt/day, while control groups received water instead. The 24 hours urine was collected and blood samples were withdrawn before and after each treatment for further analysis. Propolis revealed to be rich in polyphenols and flavonoids. Chromate provoked a nephrotoxic effect expressed by drastic decrease in glomerular filtration assessed by creatinine clearance. However, the administration of propolis attenuated the renal damages induced by the chromate. This attenuation can be seen by the increase of creatinine clearance when comparing propolis treated group to the non-treated group. Propolis showed a protective potential against chromate induced nephrotoxicity through the amelioration of chromate's toxic effects. It might be concluded that propolis could be an effective as chemo-protectant in the management of potassium dichromate-induced nephrotoxicity.

G.1. Introduction

The biotoxic effects of many heavy metals such as arsenic, barium, cadmium, chromium, lead, and selenium on human health are of great concern. Those metals originate from various sources comprising mining, natural weathering of the earth's crust, sewage discharge, industrial effluents and many others. In general, exposure to those toxicants can be by ingestion, inhalation or skin contact. Chromium (Cr), a plentiful metal in the Earth's crust, this dangerous element exists in the environment at different degrees of oxidation ranging from (+2) to (+6), however, the most common oxidation states of chromium are trivalent Cr^{+3} and hexavalent Cr^{+6}. [1] It was reported that both states (Cr^{+3} and Cr^{+6}) are toxic to humans, animals and plants.[2-4]

Naturally occurring chromium compounds are generally in the trivalent state, while hexavalent chromium compounds (the most toxic form) are mostly derived from human activities.[5] According to Jaishankar et al.[6] Cr^{+3} has low membrane permeability, which make it generally innocuous comparing to Cr^{+6}. The toxicity of this last form is more hazardous because it penetrates the cells much more easily than does the Cr^{+3}.[6] While, Cr^{+3}, in trace concentrations, is essential for regular operation of human vascular and metabolic systems as well as combating diabetes in trace. Contrariwise Cr^{+6} may cause health problems including: allergic reactions, skin rash, nose irritations and nosebleed, ulcers, immune system deterioration, genetic material alteration, kidney and liver damage, and may even go as far as death of the individual.[7,8].

Moreover, chromium exposure has been associated with kidney diseases, according to the urinary data, these kidney toxicants seem to target mainly the proximal tubular cells leading to an oxidative stress, endoplasmic reticulum stress and mitochondrial damages resulting an apoptosis and necrosis. [7,8] On the other hand, nowadays, natural products provide an excellent strategy toward identifying new bioactive compounds for combating a large number of human diseases especially those induced by oxidative stress. Propolis is a resinous substance collected by honeybees from parts of plants, buds, and exudates and mixed with bees wax and salivary enzymes. It is mostly constituted by resin, wax, essential oils and pollen. Its richness in bioactive polyphenols makes in a suitable natural product for the present contribution.

The chemical variability found in propolis can be attributed to the geographical location, collection site, botanical origin, bee species and the climate.[9,10] Different studies have shown that propolis have a broad spectrum of biological properties, such as antimicrobial, anti-inflammatory, antioxidant, antidiabetic, spasmolytic, anaesthetic, anticancer and immunomodulatory effects.[9-15]

To the best of our knowledge, the protective effect of propolis against K2Cr2O7-induced nephrotoxicity has not been explored. The aim of this work was to investigate whether using propolis as a natural product, can prevent or improve the effects of a chromium induced nephrotoxicity in animal model. G.2. Experimental

G.2. Experimental

G.2.1. Chemicals

Ferulic Acid, DNP (2,4-Dinitrophenylhydrazine), Quercetine, Sulphuric acid (H_2SO_4), Potassium Hidroxyde (KOH), and Eryodictiol were purchased from Fluka Biochemika. Folin Ciocalteu's phenol reagent was from Panreac Quimica, Montcada i Reixac, Barcelona, Spain. Na_2CO_3 and Al_2Cl_3 were from Riedel de Haen (Seelze, Germany, Riedel-de-Haën Laboratory chemicals, Germany. Potassium dichromate ($K_2Cr_2O_7$) was purchased from Sigma-Aldrich and

propolis sample was purchased from beekeeper association, in the region of Fez, Boulemane, Morocco and was stored in dark at room temperature.

G.2.2. Ethical approval

The ethical approval was obtained from Sidi Mohamed Ben Abdallah University in Fez, the Animal Facility and the Laboratory of Physiology-Pharmacology and Environmental Health, Faculty of Science Dhar EL Mahraz of Fez, Morocco (USMBA-PPSE 2016-05). The care and handling of the animals were in accordance with the internationally accepted standard guidelines for the Care and Use of Laboratory Animals and the protocol was approved by our institutional committee on animal care, University Sidi Mohamed ben Abdallah Faculty of science Dhar EL Mehraz Fez, Morocco. All efforts were made to minimize animal suffering and the number of animals used.

G.2.3. Sample preparation

Propolis sample was obtained from a beekeeper's association in Fez, Morocco. Ten milliliters of distilled water were added to one hundred milligrams of propolis powder for total phenols and flavonoids determination. Regarding the *in vivo* experiment, distilled water was added to obtain the required propolis concentration which was orally administered to the animals for 10 days.

G.2.4. Determination of phenolic groups

G.2.4.1. Total phenol content

The total polyphenol content in propolis sample was determined using the method of El-Guendouz et al.[10] Propolis (25 µL) were mixed with 125 µL of Folin-Ciocalteu reagent (0.2 N) and 100 µL of 7.5% Na_2CO_3, and the absorbance was measured at 760 nm after two hours of incubation at room temperature. The total polyphenols content was expressed as milligrams of Ferulic acid equivalents per milliliter (mg FAE mL^{-1}) using a calibration curve.

G.2.4.2. Total flavones/flavonols content

The amount of flavones and flavonols in the propolis was determined according to the method of El- Guendouz et al..[10] Briefly, 100 µL of Al_2Cl_3 (20%) were added to 100 µL of extract, and after one hour of incubation at room temperature the absorbance was recorded at 420 nm. Total flavones and the flavonols content were calculated as Quercetin equivalents per milliliter (mg QE mL^{-1}) using a calibration curve.

G.2.4.3. Total flavanones and dihydroflavonol contents

The quantification of total flavanone and dihydroflavonol compounds was performed according to El- Guendouz et al.[10] where 75 µL of sample or standard (eriodictyol) and 2 mL of DNP (2,4-dinitrophenylhydrazine) solution (1g DNP in 2 mL 96% sulphuric acid, diluted to 100 mL

with methanol) were mixed and heated at 50°C for 50 min. After cooling at room temperature, the mixture was diluted to 10 mL with methanolic KOH solution (10%, w/v), then, one mL of the resulting solution was diluted to 50 mL with methanol and the absorbance was measured at 486 nm.

G.2.5. Experimental animals'

Adult male Wistar rats weighing 190±20 g were obtained from our Animal House Breeding Center, Department of Biology, Faculty of Science Dhar El Mehrez Fez, Morocco. The animals, were housed under standard environmental conditions (25±2 °C, 55±5% humidity and 12h/12h light/dark cycle) and allowed free access to tap water and standard laboratory rat chow.

G.2.5.1. Experimental design

The rats were divided into four groups of six rats each, as follow:

Group I: served as healthy control and received distilled water (10 mL / kg.b.wt/ day);

Group II: served as healthy group treated with propolis (100 mg / kg b.wt/day);

Group III: rats of this group received distilled water (10mL / kg.b.wt/ day) and were injected with potassium dichromate (15 mg / kg b.wt/single dose).

Group IV: received propolis (100 mg /kg.b.wt/day) and were injected with 15 mg / kg b.wt of potassium dichromate.

The animals were kept in metabolic cages, experimentation was continued for 10 days and the animals received the treatments daily. Potassium dichromate aqueous solution was administered by subcutaneous injection of single dose at day 4 of the experiment and propolis was given by oral gavage daily.

G.2.6. Biological mediums sampling and analysis

The 24 hours urine samples were collected in graduated cylinders; to determine the urine volume for each rat; filtered, centrifuged and stored at -20°C until day of analysis. Blood samples were withdrawn at the beginning, before starting the experimental protocol, and at the (after 10 days), centrifuged at 4000 r.p.m for 10 minutes, and then the plasma was collected and stored at -20 °C until analysis.

Plasma and urine electrolytes concentration, glucose, tiglycerides and cholesterol profiles were measured by flame spectrophotometry. Concentration of creatinine in plasma and urine was determined by the Jaffe alkaline picrate method, and creatinine concentration was measured by spectrophotometer at 500 nm. Creatinine clearance was used to evaluate the renal function, or more exactly, the glomerular filtration rate, and was calculated from plasma and urinary creatinine levels. following the formula:

(Creatinine $_{Urine}$ / Creatinine $_{Serum}$) X (Volume $_{Urine}$ (ml) / Time (hours) X 60)

G.2.7. Statistical analysis

All data were analyzed using GraphPad Prism 5. and the results were expressed as Mean±SD. Within group, means comparison was performed through the analysis of variance (ANOVA test). Significant difference between control and experimental groups were assessed using student's t-test. *p*- values < 0.05 were considered to be significant.

G.3. Results and discussion

G.3.1. Phenols

The concentrations of total phenols, flavones and flavonols, and flavanones and dihydroflavonols found in propolis sample used in this work are displayed in Figure-1. Propolis showed a high amount of phenolic compounds with a value of 6.21±0.02 mg FAE/mL. Regarding the flavones and flavonols content it was high as 3.91± 0.20 mg QE /mL, while the amount of flavanones and dihydroflavonols amount was 2.31± 0.01 mg QE /mL in the studied propolis sample (figure G.1).

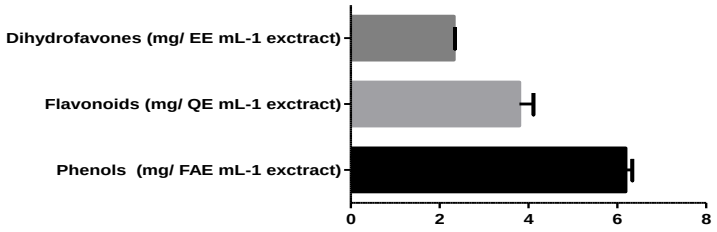


Figure G.1. Phenol content found in propolis sample (Mean ± SD).

G.3.2. Nephrotoxicity induction and chemoprotective effect of propolis

G.3.2.1. Urinary volume

In the present work, we evaluate the effect of propolis on alleviating the chromate induced nephrotoxicity in Wister rats after single subcutaneous dose injection. The results displayed in Figure G.2, shows that the urinary volume increase significantly (P<0.001) in the fifth day (8.01±0.12 mL; 7.76±0.15 mL) and sixth day (7.92±0.12 mL; 7.85±0.15 mL) for the none treated nephrotoxic group and for that injected with dichromate and treated with propolis respectively as compared to the healthy control group. Afterward, for the none treated nephrotoxic animals, a significant decrease (P<0.001) of the urine volume was observed starting from day seven (2.22±0.25 mL) until the end of the experiment (0.12±0.10 mL). Regarding the nephrotoxic group treated with propolis, a significant decrease (P<0.001) of the diuresis on day seven (1.05±0.38mL) was observed, however, this diuresis starts recovering the normal volume

on day eight. It worth mentioning that the administration of propolis to healthy rats did not affect their 24h urinary volumes. (Figure G.2).

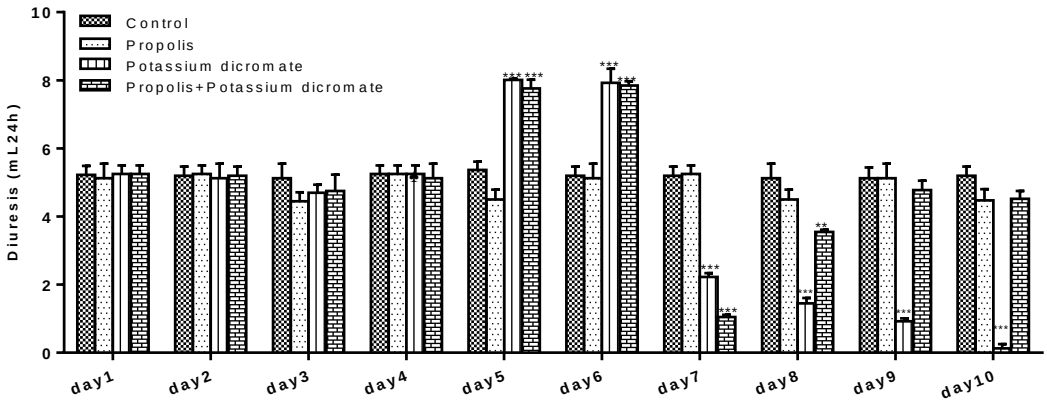


Figure G.2. Average of urinary volume excretion (UV) of control and treated rats during 10 days of treatment. The volume of 24 h urine was measured and reported as mean ±S.E.M for six rats in each group. ♠ chromium injection day.

G.3.2.2. Effect on electrolyte levels

The effects of the interventions with propolis along with/without chromium on urinary and plasma sodium and potassium are summarized in Table G.1. The results showed that a significant changes can be seen in some electrolytes following chromium injection. While, no significant changes in unary Na^+ and K^+ were induced for the healthy group treated with propolis, as compared with control made of healthy animals. Chromium injection in the absence of any treatment induced a significant decrease ($P<0.001$) of both parameters (Table G.1). However, propolis supplementation attenuated the decrease observed following chromium injection.

Table 1. Concentrations of Urinary and Plasma electrolyte in control and treated rats after 10 days of treatment.

Groups	Urinary electrolyte concentration		Plasma electrolyte concentration	
	Na^+ (mmol/L)	K^+ (mmol/L)	Na^+ (mmol/L)	K^+ (mmol/L)
Control	75.25±1.47	54,6±0,75	141.00±0,70	3.1±0.81
Propolis	76.30±0.47	49,25 ±0,24	139.00±0,70	3.08±0.11
Potassium dichromate	22.02±1.01***	7.4±0,18***	136.00±1.41*	7.00±0.5***
Propolis/ Potassium dichromate	58.80±0.15*	35.56±0,18*	127.00±1.58*	4.17±0.14*

Data are presented as Mean ± SD, **P < 0.05 compared to control, **P<0.01 compared to control, ***P <0.001 compared to control.

Regarding the plasma electrolyte Na^+, both nephrotoxic groups revealed a significant difference ($P<0.05$) as compared to the control healthy group. While, the plasmatic K^+ has significantly increased ($P<0.001$) in the none treated nephrotoxic group (7.00±0.5 mmol/L) group as compared to the control (3.1±0.81 mmol/L) (Table G.1). However, propolis treatment maintained this parameter around the normal values (4.17±0.14 mmol/L).

On the other hand, according to Figure G.3, the administration of propolis to healthy rats did not affect the creatinine clearance during the experiment comparing with the control. In contrast, the injection of chromate caused a drastic reduction of renal function. This reduction can be seen by the significant decrease (P<0.001) of the creatinine clearance by the end of the experiment. Regarding the group supplemented with propolis, a significant alleviation against the adverse effect of chromium injection was observed (Figure G.3).

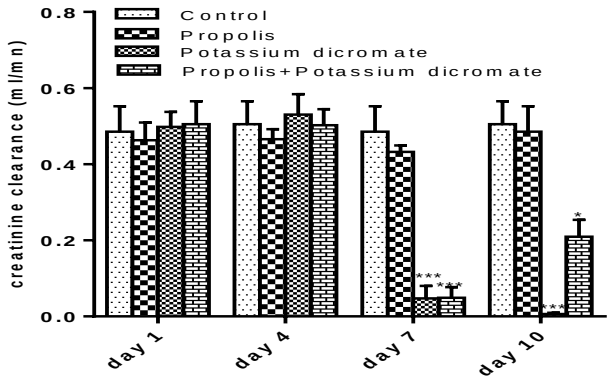


Figure G.3. Effect of propolis and potassium dichromate on creatinine clearance in normal Wistar rats treated and control. Data are presented as Mean ± SD, **P < 0.05 compared to control, **P<0.01 compared to control, ***P <0.001 compared to control.

G.3.2.3. Osmolality and the clearance of free water

All groups showed no significant change in plasma osmolality as compared to the control. In contrast, the injection of dichromate alone decreased significantly free water clearance which provides more evidence about the occurrence of renal function disorder as compared to control group. As for the other parameters propolis administration restored significantly (P<0.01) the renal function via maintaining these parameters in normal values (Table G.2).

Table G.2. Concentrations of biochemical parameters in control and treated rats after 10 days of treatment

	U_{osm} (mosm/kgH2O)	P_{osm} (mosm/kgH2O)	C_{Osm} (µl/min)	C_{H20} (µl/min)
Control	1580 ±9	286 ±6	27.62 ±2	-22.6 ±2
Propolis	1526 ±9	278 ±6	24.7±2.2	-20.20 ±
Potassium dichromate	440 ±9***	227 ±8	3.5 ±0.5***	-1.6±0.1 ***
Propolis/Potassium dichromate	1880 ±9	254 ±6	14.4± 1.2**	-11.30 ± **

Data are presented as Mean ± SD, ***P* < 0.05 compared to control, ****P*<0.01 compared to control, *****P*<0.001 compared to control.

G.3.2.4. Blood glucose, triglycerides, cholesterol levels and creatinine clearance:

The results of biochemical parameters showed that the dichromate lead to a significant increase in blood glucose concentrations, triglycerides and cholesterol levels (P<0.001; P<0.001; P<0.01

respectively). The values of those parameters increased from 1.15±0.14 g/L, 0.95±0.22 g/L and 0.47±0.08 g/L in normal rats to 2.78±0.10 g/L, 1.34±0.03 g/L and 1.23±0.01 g/L respectively in nephrotoxic individuals. The supplementation with propolis moderated the acute effect of chromate on the mentioned parameters and the values were 1.81±0.16 g/L, 1.02±0.02 g/L and 0.61±0.12 g/L, respectively (Table G.3).

Table G.3: Effects of oral administration of propolis, potassium dichromate, Propolis/Potassium dichromate and distilled water on various parameters measured (mean ± SD)

	Glucose (g/L)	**Triglycerides (g/L)**	**Cholesterol (g/L)**
Control	1.15±0.14	0.95±0.22	0.47±0.08
Propolis	0.85±0.12**	0.75±0.11	0.51±0.05
Potassium dichromate	2.78±0.10***	1.34±0.03***	1.23±0.01**
Propolis/ Potassium dichromate	1.81±0.16*	1.02±0.02**	0.61±0.12*

**P < 0.05 compared to control, **P<0.01 compared to control, ***P <0.001 compared to control.

Heavy metals toxicity in organism, including that of chromium, was associated to the generation of reactive oxygen species (ROS) leading to an oxidative stress.[16] Chromium is well-known as oxidizing agent able to induce directly a tissue damages. The humans and animals' exposure to Cr^{6+} compounds induces carcinogenic, mutagenic, and teratogenic effects. [6,17] Many studies showed that oral feeding of chromium leads to a significant increase in organo-somatic indexes of liver, kidneys, heart and spleen.[18] Balakrishnan et al.[7] reported that administration of chromium to rats significantly reduced the antioxidant markers of kidney such as superoxide dismutase and glutathione (GSH). Moreover, it increased the peroxidation markers such as malondialdehyde (MDA). Furthermore, Velma and Tchounwou[16] found that the kidney appears to be more vulnerable and sensitive to Cr-induced toxicity than the liver.
In this context, some authors found that chromium chelating agents or antioxidants such as a-tocopherol, ascorbic acid, and glutathione can ameliorate chromium-induced oxidative damage by scavenging the ROS and/or preventing biological macromolecules from oxidative injury. [19-21] Kandhare et al.[22] and Chetyrkin et al.[23] reports that L-arginine and pyridoxamine, which have antioxidant properties, significantly restored alteration in serum and urine biochemical parameters. Several authors have shown that propolis exhibited a strong antioxidant property.[9,12,24-27] This activity was attributed to some of its bioactive compounds. In general, phenolic compounds and flavonoids has been found to be the major components of propolis, and they were reported to be the responsible of its antiradical activity. [9,12,26-28] In concordance with those authors, El-Guendouz et al.[10] and Miguel et al.[12] have found that propolis samples

with higher concentrations of phenolic compounds exhibit a higher antioxidant power and anti-inflammatory properties.

In this study, total phenols contents found in the propolis sample are within the range of those generally detected in methanolic extract of Portuguese propolis, 2.93-8.76 mg pinocembrin equivalent /mL and 1.28-2.76 mg quercetin equivalent /mL, for total phenols and flavonoids contents respectively.[29] Our results are in concordance with those reported for Moroccan ones, which were ranging between 0.039 and 8.864 mg FAE/ mL for phenols, 0.020 and 4.320 mg QE/mL extract for the flavonoids, and 0.031 and 0.532 mg EE/mL extract for dihydroflavones.[10] Even in the absence of a deep histological study to evaluate the kidney damage caused by $K_2Cr_2O_7$, it was evident to state the occurrence of renal function disorder given the severe decrease of the urinary volume and an acute increase of urinary and plasma electrolyte Na^+ and K^+, blood glucose, triglycerides and cholesterol levels following dichromate intervention. Furthermore, a significant change in creatinine clearance, osmolality and clearance of free water were recorded as a result of this chromium toxicity. While, after the pretreatment of rats with propolis before the chromium injection, it was observed a significative acceleration in the recovery of the previously mentioned affected parameters. In parallel, we found that chronic administration of propolis did not affect urinary volume of rats.

In sum, injection of chromate decreased urinary output, glomerular filtration rate assessed by creatinine clearance as compared with the control rats. This effect can be explained by the fact that the glomerular filtration rate decreased due to the obstruction of urinary outflow. Besides, the obstruction of the urinary outflow leads to an accumulation of waste products in the blood including sodium and potassium. Those observations were proved by our results, and explain the death of nephrotoxic animals by the end of the experiment. The levels of the electrolytes Na^+ and K^+ decreased in urine and increased in plasma for chromate injected group, while, the group of animals pretreated with propolis showed a lower increment of the electrolyte levels in urine. This finding suggests that propolis exerted a protective effect on renal damage as it alleviates the chromium effect. In this regard, we have found that propolis extract increased creatinine clearance until restoration of renal function. Many studies focused on the protective role of the antioxidant effect of propolis against the toxicity of several heavy metals. Wen et al.[30] reported a possible protective effect of propolis against Aluminum-induced toxicity, while El Masry et al.[31] demonstrated the same effect but against lead-induced neurotoxicity. Other researchers, [32] showed the positive influence of the administration of propolis on reducing the concentration of mercury in the muscles, kidneys and liver in animal models. In a previous study, [33] our group of research have reported that this propolis sample exhibit a great potential

as antioxidant agent after the *in vitro* evaluation of its ability for scavenging free radicals, azino-bis3-ethylbenzothiazoline-6-sulphonic acid (ABTS) and nitric oxide (NO), and by their reducing power. Hence, we can attribute the found effect in this work to this bioactive property of propolis.

On the whole, our current results, showed that propolis have been useful in preventing kidney damage caused by dichromate, suggesting that this nephroprotective effect was through its antioxidant and ROS scavenging properties via the action of flavonoids and phenolic compounds it contains.

G.4. Conclusion

In summary, chromium injection in rats caused kidney failure and it was alleviated by propolis intervention. Propolis protected against functional alterations of the kidney. The phenolic compounds were possibly involved in its beneficial effect.

From above, we speculate that propolis by virtue of its renoprotective property, ameliorates potassium dichromate-induced loss of functional integrity in kidney tissues. On the basis of this study, it should be taken into consideration that the supplementation of natural antioxidants such as propolis may act as a protective agent against the toxicity of chromates and, therefore, could be a significant intervention for future testing in kidney failure. Those results will be with a high interest and can be with a potential clinical application. However, there is a clear need to characterize the quality and quantity of the constituents of propolis and to identify the mechanism of action to confirm the present results.

References

[1] Haq, Z., Jain, R.K., Khan, N., Dar, M.Y., Ali, S., Gupta, M., Varun, T.K. (2016) Recent advances in role of chromium and its antioxidant combinations in poultry nutrition: A review. *Vet. World.*, 9(12): 1392-1399.

[2] Hamidi, O., Mohammad, C., Hasan, G., Ali, A. Hassan, M. (2016) Effects of chromium (III) picolinate and chromium (III) picolinate nanoparticles supplementation on growth performance, organs weight and immune function in cyclic heat stressed broiler chickens. *Vet. Dergi. Kafkas.*, 1: 10-16.

[3] C. S. Liu, H. W. Kuo, J. S. Lai, T. I. Lin, 'Urinary. N-acetyl-betaglucosaminidase as an indicator of renal dysfunction in electroplating workers', Int. Arch. Occup. Environ. Health. 1998, 71, 348-52. Doi: 10.1007/s004200050291.

[4] M. Mohanty, H. Kumar Patra, 'Effect of ionic and chelate assisted hexavalent chromium on mung bean seedlings (Vigna Radiata l. Wilczek. Var k-851) during seedling growth', J. Stress. Physiol. Biochem. 2013, 9, 232–241.

[5] Agency for Toxic Substances and Disease Registry (ATSDR), 'Chromium Toxicological Profile', US Department of Health and Human Services, Agency for Toxic Substances and Disease Registry; Atlanta, GA. 2012.

[6] Jaishankar, M., Mathew, B.B., Shah, M.S., Gowda, K.R.S. (2014) Biosorption of Few Heavy Metal Ions Using Agricultural Wastes. *Int. J. Environ. Res. Public. Health.*, 2: 1–6.

[7] R. Balakrishnan, C. S. Satish Kumar, M. U. Rani, M. K. Srikanth, G. Boobalan, A. G. Reddy, 'An evaluation of the protective role of α-tocopherol on free radical induced hepatotoxicity and nephrotoxicity due to chromium in rats', Indian. J. Pharmacol. 2013, 45, 490-5.

[8] Hong, S., Jason, B., Max, C., (2015) Oral Chromium Exposure and Toxicity. *Curr Environ. Health. Rep*., 2(3): 295–303.

[9] M. G. Miguel, S. Nunes, S. A. Dandlen, A. M. Cavaco, M. D. Antunes, 'Phenols and antioxidant activities of hydro-alcoholic extracts of propolis from Algarve, South of Portugal', Food. Chem. Toxicol. 2010, 48, 3418-3423.

[10] El-Guendouz, S., Aazza, S., Lyoussi, B., Antunes, M.D., Faleiro, M.L., Miguel, M.G. (2016) Anti-acetylcholinesterase, antidiabetic, anti-inflammatory, antityrosinase and antixanthine oxidase activities of Moroccan propolis., *Int. J. Food. Sci. Tech.,* 51: 1762–1773.

[11] El-Guendouz. S, Lyoussi. B, Miguel. M.G.C. (2019). Insight on Propolis from Mediterranean Countries: Chemical Composition, Biological Activities and Application Fields. *Chem Biodivers.* 16(7).

[12] Miguel, M.G., Doughmi, O., Aazza, S., Antunes, D., Lyoussi, B. (2014) Antioxidant, anti-inflammatory and acetylcholinesterase inhibitory activities of propolis from different regions of Morocco. *Food. Sci. Biotechnol.,* 23: 313-322.

[13] El-Guendouz, S., Aazza, S., Lyoussi, B., Bankova, V., Lourenço, J.P., Costa, A.M.R., Mariano, J.F., Miguel, M.G., Faleiro, M.L. (2016) Impact of biohybrid magnetite nanoparticles and Moroccan propolis on adherence of Methicillin resistant strains of *Staphylococcus aureus*. *Molecules.,* 21: 1208.

[14] Sforcin, J.M. (2016) Biological properties and therapeutic applications of propolis. *Phytother. Res.,* 30: 894–905.

[15] El-Guendouz, S., Aazza, S., Lyoussi, B., Majdoub, N., Bankova, V., Popova, M., Raposo, S., Antunes, D., Miguel, M.G. (2018) Effect of poplar-type propolis on oxidative stability and rheological properties of O/W emulsions. *Saudi. Pharm. J.* 26: 1073-1082.

[16] V. Velma, P. B. Tchounwou, 'Oxidative stress and DNA damage induced by chromium in liver and kidney of goldfish, *carassius auratus*', Biomark. Insights. 2013, 25, 43-51. Doi: 10.4137/BMI.S11456.

[17] Thomas, L.D.M., Max, C. (2019) Mechanisms of chromium-induced toxicity. *Curr. Opin. Toxicol.,* 14:1-7.

[18] S. Geetha, M. Sai Ram, S. S. Mongia, V. Singh, G. Ilavazhagan, R. C. G. Sawhney Evaluation of antioxidant activity of leaf extract of Seabuckthorn (Hippophae rhamnoides L.) on chromium (VI) induced oxidative stress in albino rats', J. Ethnopharmacol. 2003, 87, 247-51.

[19] L. Arreola-Mendoza, J.L. Reyes, E. Melendez, D. Martin, M. C. Namorado, E. Sanchez, L. M. Del Razo, 'Alpha-tocopherol protects against the renal damage caused by potassium dichromate', Toxicol, 2006, 218, 237–246.

[20] H. K. Park, B. C. Jeong, M. Sung, M. Y. Park, E. Y. Choi, B. S. Kim, H. H. Kim, I. I. Kim, 'Reduction of oxidative stress in cultured renal tubular cells and preventive effects on renal stone formation by the bioflavonoid quercetin', J. Urol. 2007, 179, 1620-1626.

[21] R. K. Bijarnia, T. Kaur, K. Aggarwal, C. 'Tandon Modulatory effects of N-acetylcysteine on hyperoxaluric manifestations in rat kidney', Food. Chem. Toxicol. 2008, 46, 2274-2278.

[22] A. D. Kandhare, M. V. Patil, S. L. Bodhankar, 'L-Arginine attenuates the ethylene glycol induced urolithiasis in nephrectomized hypertensive rats: role of KIM-1, NGAL, and Nos', Ren. Fail. 2015, 37, 709-721.

[23] S. V. Chetyrkin, D. Kim, J. M. Belmont, et al, 'Pyridoxamine lowers kidney crystals in experimental hyperoxaluria: a potential therapy for primary hyperoxaluria', Kidney. Int. 2005, 67, 53-60.

[24] M. C. Marcucci, 'Propolis: chemical composition, biological properties and therapeutic activity', Apidologie. 1995, 26, 83-99.

[25] A. Mavri, H. Abramovic, T. Polak, J. Bertoncelj, P. Jamnik, S. S. Mozina, B. Jersek, 'Chemical properties and antioxidant and antimicrobial activities of Slovenian propolis' Chem. Biodivers. 2012, 9, 1545-1558.

[26] N. I. Potkonjak, D. S. Veselinovic, M. M. Novakovic, S. Z. Gorjanovic, L. L. Pezo, D. Z. 'Suznjevic Antioxidant activity of propolis extracts from Serbia: A polarographic approach', Food. Chem. Toxico.l 2012, 50, 3614–3618.

[27] A. L. Piccinelli, T. Mencherini, R. Celano, Z. Mouhoubi, A. Tamendjari, R. Aquino, L. Rastrelli, 'Chemical composition and antioxidant activity of Algerian propolis', J. Agric. Food. Chem. 2013, 61, 5080-5088.

[28] R. Christov, B. Trusheva, M. Popova, V. Bankova, M. Bertrand, 'Chemical composition of propolis from Canada, its antiradical activity and plant origin', Nat. Prod. Res. 2006, 19, 673-8.

[29] M. G. Miguel, S. Nunes, S. A. Dandlen, A. M. Cavaco, M. D. Antunes, 'Phenols, flavonoids and antioxidant activity of aqueous and methanolic extracts of propolis (*Apis mellifera* L.) from Algarve, South Portugal', Food. Sci. Technol. 2014, 34**.** http://dx.doi.org/10.1590/S0101-20612014000100002.

[30] Wen, Y.F., Zhao, J.Q., Nirala, S., Bhadauria, M. (2012) Aluminum-Induced Toxicity and Its Response to Combined Treatment of HEDTA and Propolis in Rats. *Pol. J. Environ. Stud.*, 21: 1437-1444.

[31] Masry, T.A,, Emara, A.M,, Shitany, N.A. (2011) Possible protective effect of propolis against lead induced neurotoxicity in animal model. *J. Evol. Biol. Res.*, 3: 4-11.

[32] Jose, L., Valverde, P., Agata, Z., Hussein B.K., Sylwia, E.S., Ewa, R. (2018) Influence of the administration of propolis and bee pollen preparation on the concentration of mercury in the muscles, kidneys and liver of white storks. *Med. Weter.* 74 (3), 171-174 171.

[33] El-Guendouz, S., Al-Waili, N., Aazza, S., Elamine, Y., Zizi, S., Al-Waili, T., Al-Waili, A., Lyoussi, B. (2017) Antioxidant and diuretic activity of co-administration of Capparis spinosa honey and propolis in comparison to furosemide. Asian. *Pac. J. Trop. Biomed.*, 10(10).