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MASTER'S THESIS

MASTER « **MEDICALE BIOTECHNOLOGY**»

OPTION: « **BIOMEDICAL**»

Saffron (*Crocus sativus* L.) secondary metabolites : antibacterial and antioxidant activities & *insilico* screening for biomarkers

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Foreword:

This end-of-study master's degree project is part of a **Safranval** research project funded by the science and technology academy Hassan II for the 2016-2020 period entitled:

Contribution to the knowledge of Moroccan saffron using biotechnological tools (physio-morphological study, chemical and molecular identification and selection of performant cultivars.

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Abstract

Crocus sativus. L, an aromatic and medicinal plant whose stigma is used as the saffron spice, the red gold. Known for its aromatic and therapeutic properties, the saffron spice harbours many biomedical and biotechnological potentials. The goal of this study is to bring forward the antioxidant and antibacterial properties of the Moroccan saffron. The samples used for the study are cultivated in the Talouine region and were found to have a phenolic content of 6.33 mg GAE/g DW (TPC using Folin-ciocalteu reagent) and an antioxidant potential whose IC50 is of 2.21 mg (DPPH method). No antibacterial activity was recorded during this study against 5 bacterial strains. From 6767 *crocus sativus* ESTs, available in NCBI database, 1,890 gene ontology terms were recovered, and 28 putative proteins related to secondary metabolism were constructed using GOs, EggNOG and Mercator predictions. Six sequences, were retrieved, having the highest Max BLAST score against *crocus sativus*, to propose, for the first time, biomarkers that will be used for the authenticate and study the traceability of pure Moroccan *crocus sativus*, to valorise the spice and make it more competitive in international markets.

Key words: *crocus sativus, secondary metabolites, antibacterial, antioxidant, ESTs, Gene Ontology, EggNOG, Mercator.*

Résumé

Crocus sativus. L, une plante aromatique et médicinale dont les stigmates sont utilisés comme l'épice du safran, surnommé l'or rouge. Connue pour ses propriétés aromatiques et thérapeutiques, le safran recèle de nombreux potentiels biomédicaux et biotechnologiques. Le but de cette étude est de caractérisé les propriétés antioxydantes et antibactériennes du safran marocain. Les échantillons utilisés pour l'étude proviennent de la région de Talouine, il présente une teneur en phénols de 6,33 mg GAE/g DW (TPC utilisant le réactif de Folin-ciocalteu) et un potentiel antioxydant dont l'IC50 est de 2,21 mg (méthode DPPH). Aucune activité antibactérienne n'a été perçu dans les tests menés contre 5 souches bactériennes. À partir de 6767 EST de *crocus sativus,* disponibles dans la base de données NCBI, 1 890 termes d'ontologie génique ont été générés et 28 protéines putatives liées au métabolisme secondaire ont été construites à l'aide des prédictions GO, EggNOG et Mercator. Six séquences, ont été récupérées, ayant le BLAST Max score contre *crocus sativus le* plus élevé, pour proposer, pour la première fois, des biomarqueurs qui seront utilisés pour l'authentification et l'étude de la traçabilité du *crocus sativus* marocain pur, pour valoriser l'épice et la rendre plus compétitive sur les marchés internationaux.

ملخص

الزعفران، نبتة عطرية وطبية تستخدم مياسم الزهور في الأساس كتوابل، يشتهر الزعفران بالذهب الأحمر ويعرف بخصائصه العطرية والعلاجية، كما يزخر بالعديد من الخصائص الطبية الحيوية والمقومات التكنولوجيا الحيوية.

أجريت هذه الدراسة بهدف إبراز الخصائص المضادة للأكسدة وللبكتيريات التي يتميز بها الزعفران المغربي. العينات المستخدمة في الدراسة من منطقة تالوين ووجد أنها تحتوي على كمية فينول بمقدار 6.33 ميليغرام، أيضا ضمت نسبة مشجعة من مضادات الاكسدة بمعدل تركيز أدنى مانع وفعال 2.21 ميليغرام. فيما يخص نشاط مضاد للجراثيم، لم يتم رصد أي نشاط خلال هذه الدراسة ضد 5 سلالات بكتيرية.

من أصل 6767 من علامات التسلسل، تم استرداد 1،890 (ESTs) المتاحة في قاعدة البيانات NCBI مصطلحًا في علم الأنطولوجيا الجينية، وتم إنشاء 28 بروتينًا مفترضًا متعلقًا بعملية التمثيل الغذائي و الثانوي (ميتابوليزم ثانوي) باستخدام EggNOG Mercator وGOs

تم عزل ستة متواليات، حاصلة على أعلى نقاط ماكس بلاست بالتوازي مع الزعفران، لاقتراح، لأول مرة، المؤشرات الحيوية التي سيتم استخدامها لمصادقة ودراسة إمكانية تتبع الزعفران المغربي الأصلي ، .من أجل تثمين التوابل المشتقة منه وجعلها أكثر تنافسية في الأسواق الدولية.

List of abbreviations

ABC: ATP-binding cassette

AD: Alzheimer's disease

AR: Annotation Rule

AS: Annotation Score

ATP: Adenosine triphosphate

BBB: The Blood-Brain Barrier

BCH: carotene hydroxylase

BDNF: Brain-Derived Neurotrophic Factor

BHC: β-carotene hydroxylase enzyme

BLAST: The Basic Local Alignment Search Tool

BP: Biological Process

CATH-Gene3D: Protein Structure Classification – Gene3D database

CC: Cellular Component

CCD: Carotenoid Cleavage Dioxygenase

CD4/CD8: Cluster of Differentiation 4/ Cluster of Differentiation 8

CDD: Conserved Domains Database

cDNA: Complementary DNA

COG: Clusters of Orthologous Groups of proteins

CREB: Cyclic-AMP Response Element Binding Protein

CVD: Cardiovascular Diseases

DB: Database

DMAPP: Dimethylallyl Diphosphate

DNA: Deoxyribonucleic acid

DOXP: 1-deoxy-D-xylulose 5-phosphate pathway

DPPH: 2,2-Diphenyl-1-picrylhydrazyl

DT: Direct Term

DXR: 1-deoxy-d-xylulose 5-phosphate reductoisomerase

DXS1: 1-deoxy-D-xylulose-5-phosphate synthase 1

EST : Expressed Sequence Tag

FOX: Cefoxitin

gDNA: Genomic DNA

GGPP: Geranylgeranyl Diphosphate

GI: GeneBank identifiers

GO: Gene Ontology

GSS: Genome Survey Sequence

HAMAP: High-quality Automated and Manual Annotation of microbial Proteomes

HDF: Human Dermal Fibroblasts

HIV: Human Immunodeficiency Virus

HMM: Hidden Markov Model

IL-1β: interleukin-1β

IL-6: Interleukin 6

IPP: Isopentyl Diphosphate

ITM: Islamic Traditional Medicine

IZ: Inhibition Zone

KAAS: KEGG Automatic Annotation Server

Keap1: Kelch-like ECH-associated protein 1

KEGG: Kyoto Encyclopedia of Genes and Genomes

KO: KEGG Orthology

KOG: EuKaryotic Orthologous Groups

LCY: Lycopene cyclase

LMBM: Laboratory of Microbiology and Molecular Biology

MCAO: Middle Cerebral Artery Occlusion

MDR: Multidrug Resistance

MDR1: Multi-Drug Resistance 1 (P-glycoprotein)

MEP : 2-C-methyl-D-erythritol 4-phosphate

MF: Molecular Function

mRNA: Messenger RNA

NCBI: National Center for Biotechnology Information

NF-κB: Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells

NHEK: Human Epidermal Keratinocytes

NK-cell: Natural Killer Cell

NO: Nitric Oxide

NOGs: Non-Supervised Orthologous Groups

NR: Non-redundant

Nrf-2: Nuclear factor-erythroid factor 2-related factor 2

OD: Optical Densities

ORFs: Open Reading Frames

p65NF-κB: Nuclear factor NF-kappa-B p65 subunit

PANTHER: Protein Analysis Through Evolutionary Relationships

PCR: Polymerase chain reaction

p-CREB: phospho-CREB

PD: Parkinson's disease

Pfam: Protein Families Database

PIR : Non-redundant Reference Protein Database

PIRSF: The Protein Information Resource Super Family system

PRINTS: The Protein Fingerprint Database

PROSITE: Database of protein domains, families, and functional sites

PUFA: Polyunsaturated Fatty Acids

RNA: Ribonucleic Acid

ROS: Reactive Oxygen Species

SFLD: Structure–Function Linkage Database

SMART: Simple Modular Architecture Research Tool

TIGRFAMs: The Institute for Genomic Research (TIGR) protein families

TNF-α: Tumor Necrosis Factor Alpha

TPC: Total phenolic Content

tRNA: Transfer Ribonucleic acid

TZP: Piperacillin + Tazobacteam

UVA: Ultraviolet A

UV–Vis : Ultraviolet–visible spectroscopy

VGF: Nerve Growth Factor Inducible

ZCD: Zeaxanthin 7,8(7',8')-cleavage dioxygenase

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I. Introduction:

The *Crocus sativus* stigma, referred to as saffron, is a well-known spice produced mainly in the Mediterranean area. Described as the red gold, it is the costliest spice in the world. While many countries have climates that support the growth of the saffron flower, the varieties in the environment, such as altitude, soil type and so, impact the secondary metabolites produced in the flowers. Incidentally, molecular characteristics of the stigma vary considerably as the geographical location and harvest and preservation methods change.

Saffron has been studied for its unique secondary metabolites: crocins, picrocrocin and safranal. Not only do they harbour a high therapeutic potential against known dangerous disease, but they were also reported as being excellent protectors against oxidative stress. Since the latter has been associated to cancer, neurodegenerative and cardiovascular diseases, ailments that take millions of lives daily; researchers around the planet are investigating natural substances that show antioxidant potential and a high content in beneficial metabolites such as carotenoids, flavonoids and phenols. Saffron happens to be one of the spices that is attracting increasing interest, especially in countries with high saffron production such as Iran, India, Morocco and Italy.

For the Moroccan saffron, few studies discuss its particular characteristics. While quality reports showcase the superior quality of Moroccan saffron, very few studies have been published debating the specifics of its quality and therapeutic potential.

The initial purposes of this work are to determine total phenolic content in the methanolic extract of saffron stigma using Folin-ciocalteu reagent and to measure the antioxidant activity of the extract through the 1,1-diphenyl-2-picryl hydrazyl (DPPH) assay. Evidence of a high content in phenols as a high antioxidant potential will attest to the special quality of the Morocco-grown saffron. Moreover, we are going to study is the antibacterial potential of the methanolic extract of the stigma. As the search for new drugs and phytochemicals that are effective against pathogenic bacteria continues, many papers reported antibacterial activity of saffron stigma and plant by-products.

Given the high value of the saffron spice, and since it is highly demanded, not only as a spice for seasonings, but as a natural source of health beneficial and therapeutic components, the number of fraudulent products rises continuously. For the saffron spice, many other fibres such as corn etamines, flowers or parts of flowers are dyed and fragranced into resembling saffron.

As a second axis of study, we are going to perform an in-silico molecular screening of the *crocus sativus* ESTs database, available in NCBI. The goal is to first provide a functional analysis of this database and mine for enough relevant data to design primers that can be used as biomarkers to provide a molecular tool, to verify the authenticity of any sample by a simple PCR run. Using primers molecularly unique to *crocus sativus,* we will be able to trace and authenticate marketed saffron for the Moroccan consumer.

Our work will help characterize and valorise the Moroccan saffron and provide new data in a growing research area, where information about the saffron grown in our region is lacking.

II. *Crocus sativus*: the plant

II.1 Taxonomy:

The taxonomic classification of C. sativus series is as follows:

- Division: Spermatophyta
- Sub-division: Angiospermae
- Class: Monocotyledonae
- Sub-class: Liliidae
- Order: Liliales
- Family: Iridaceae
- Genus: Crocus

a.Sub-genus: Anthers with extrose dehiscence

b.Section Crocus: Scape subtended by a membranous prophyll

c.Series Crocus: Corm tunics finely fibrous, usually reticulate; flowers autumnal; leaves rather numerous, usually 5–30, appearing with the flowers or shortly after; bracts flaccid, usually not closely sheathing the perianth-tube, membranous, white or transparent with no marking; anther yellow; style branches 3, usually red and often expended at the apex, entire or at most fimbriate; seed coats covered with dense mat of papillae. 2n= 12, 14, 16, 26 (Saxena, 2010)

II.2 Botanical aspects:

Crocus Sativus is classified under the genus crocus (Figure 1).

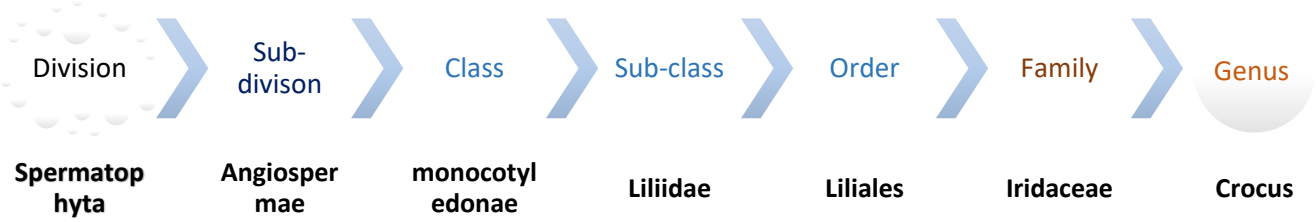


***Figure 1**: Taxonomy of crocus sativus*

The perennial plant belongs to the Iridaceae family of the Asparayales order. This plant has been cultivated for more than four thousand years in different regions of the world. (Saxena, 2010). It was reported that saffron was documented for the first time in the 7th BC in an Assyrian botanical reference. (Ahmed et al., 2016). *Crocus sativus* is well adapted with a specific kind of climate: cool to cold winter with little rainfall, autumn-winter-summer precipitation with warm summers with little rainfall. The agroclimatic conditions influence the quality of saffron greatly.(Ahmed et al., 2016; Saxena, 2010)

The plant aestivates during spring protects itself from summer drought thanks to a compact corm (Figure 2).

Figure 2: *crocus sativus plant: from bulb to flower*
Pictures taken during harvest season of 2020 by Pr. Sbabou

The saffron plant starts to grow actively from autumn until late spring. It produces 5 to 11 slender, almost vertical leaves about 40 cm of length. Then, purple buds appear and give flower during winter. The plant only flowers during October. The flower has a large variety of vivid colours, from a dark mauve to a pale pastel shade of lilac (figure 2). Each plant can carry up to four flowers each one has 3 dark red to reddish brown stigmas (25-30 cm long).

Since the flowers of saffron derive from the sterile triploid variety, they are unable to produce fertile seeds for reproduction. Therefore, the plant depends on humans to dig the corms up, break them apart and replant them. Each one of the divisions (usually 10 cormlets) gives rise to new plants. Harvesting and drying processes are considered the strongest factors that affect the spice's taste and flavor. (Ahmed et al., 2016)

II.3 Cytogenetics of saffron

Saffron is a triploid monocot sterile specie with a 3n = 24 (basic chromosome number x = 8) number of chromosomes (Chichiriccò, 1984). The karyotype of saffron is 2n = 3x = 24 without any noteworthy variations in karyology between different geographical origins. Thus, the acknowledged karyotype consists of 8 triplets:

1. subarcocentric (1, 2)
2. metacentric (3, 4, and 8)
3. submetacentric (6, 7)

Although pollen grains of saffron are mostly alive, they have a low germination rate that is due to the cytological abnormalities. The pollen tube cannot penetrate the ovule in C. sativus, suggesting that the flower probably originated from a self-incompatible species. This self-incompatibility lessens the likelihood of seed production in saffron close to impossible.

Saffron being a perennial sterile plant reproduces only vegetative by means of the corms.(A. Mir Shafat et al., 2020)

II.4 History of saffron cultivation

Inscriptions and decoration on objects found by archeologists indicate that saffron was first used and made famous by the Indians and the Egyptians. It is the oldest spice whose history could be traced back to more than 5000 years in the high valleys of Kashmir and the high plains of Iran. Used by the Egyptians and the Hebrews, saffron was later on passed on to Greeks and Romans. the Arab-Islamic show that in the 10th century, saffron was already cultivated in Syria, Iran, Afghanistan, India and the Maghreb. According to some historic references, saffron cultivation in Morocco is evidenced starting from the XVI century. Nevertheless, according to Bellakhdar (1997), it would actually be prior to this date. However, Hadiqat al-azhar, Al-Wazir Al Ghassani, a Moroccan writer of the XVI century, proclaimed an important production of saffron in the region of Marrakech and in the gardens of Fes.

The Book of Agriculture “Kitab al-filaha” by Ibn Al Awwam (end of the XII century), devoted a long chapter to agricultural techniques of saffron in Andalusia . The exact time of its introduction in Morocco remains unknown. The sole apparent information is that it was cultivated a long time ago in the region of Taliouine, on lands of the Amazigh Souktana tribes.

In view of that, Bellakhdar (1997), theorized that the Romans have probably transmitted to the Amazigh tribes the saffron agricultural techniques learned from Greeks. The same tribes continued to practice its cultivation on local level.(Zaazaa et al., 2017)

II.5 Saffron cultivation in Morocco

II.5.1 The agricultural practices

Crocus sativus L. or "Zaâfaran" in Morocco, is mainly grown in Taliouine, located at the junction of the Low Atlas in the South and the High Atlas mountains in the North, at an altitude of about 1000m, a latitude of 30° 36'N, and longitude of 8°25'W (Figure 3). The exact locations at where saffron is grown are located at about 1200 to 1400m over an area of 565 ha; these locations are cooler than Taliouine but still are considered a warm microclimate. More recently, a saffron cultivation has started in the Taznakht area (Ouarzazate province) over an area of 105 ha.(El Aymani et al., 2019; Negbi, 1999)

Figure 3: *Cultivation zone of saffron in Morocco. (Dubois, 2010)*

Although Morocco is ranked fourth producer of Saffron after Iran, India and Greece, with only 1.5% of total world production (3 tons).(El Aymani et al., 2019)

During August and September, the onion-shaped corms, are dug up; they are covered with fibrous tunics, the daughter corms are separated. They undergo a selection process where the rotten, bruised or damaged corms are eliminated. The external 2 to 3 tunics are removed from the remaining corms, leaving only the interior one. The selection process goes on and only the

corms with a diameter greater than 2.5 cm are used in propagation. The rest are used as animal food. Corms can be stored for several weeks in a cool, dry environment. However, for better sprouting, it is preferable if they are used shortly after having been dug up.(Ahmed et al., 2016; Negbi, 1999)

II.5.2 Saffron processing: from flower to stigma

Flower picking takes place early in the morning, while the flower is still closed. Flowers are picked at the base of the segments, and put into baskets in thin layers to avoid excess pressure and deformation of the stigmas. Picking goes on for the first two to three morning hours.

The flowers are brought indoors for separation straightaway after harvest. During the process, the stigmas plus the uppermost 2 mm of the style are separated from the rest of the flower organs. If the style portion is longer than 2 mm, saffron is considered to be of inferior quality.

The fresh red stigmas are dried directly after harvest, to ensure optimum conditions for quality product. The Saffron is handled very gently and cautiously to avoid stigma breakage. The stigmas are placed in thin layers on a cloth and dried in the sun for a 2 h period or in the shade after 7 to 10 days. Drying is complete before the stigmas break or crumble. Air-dried saffron retains its purplish red colour, its fragrance and its aroma, and is the priciest type of saffron. (Negbi, 1999)

II.5.3 Saffron production in Morocco: an update

Saffron cultivation has gained in importance in Morocco over the past few years. According to data from the Ministry of Agriculture, the area reserved for this crop increased from 610 ha in 2008 to 1,826 hectares in 2018. Thanks to the “Maroc Vert” Plan, the area of saffron farmed has more than tripled in just 10 years, exceeding the objective set by the agricultural strategy by 35% that was in fact, 1,350 hectares at the start.

The production of saffron in Morocco increased from 1,500 kg in 2008 to 6,860 kg in 2018, generating in the process, a rather remarkable revenue: from 16 million dirham in 2008, to 139 million dirhams in 2018 (AgriMaroc.ma, n.d.).

III. The *Crocus sativus* stigma:

The different uses of the *crocus sativus* stigma, especially as a spice, has been well documented throughout history in different cultures. The recorded history says that the dried stigma was

utilised as a drug, dye, perfume and condiment in ancient Egypt, Greece, India, Persia and Rome.

III.1 The ethnomedicinal uses of *crocus sativus* in different cultures:

In Islamic Traditional Medicine (ITM), saffron was widely recommended as a nerve tonic, stimulator of the menstrual flow. It was also used for treating dysmenorrhea, gastric ulcers and premature ejaculation. In Iran, the stigma known as "Kal mas" or "Zaferan" is consumed to strengthen the heart.

In Indian Traditional Medicine, the stigma, generally known as "Kesar", is used as an immunity booster and has been recognized as a nerve sedative, appetizer, stimulant and aphrodisiac. In Traditional Chinese Medicine, the use of stigma is recommended for treating disorders of nervous system, relieving asthma, pertussis and inflammations. In Iraq, the stigma and style, known as "Zeferan" have been used to treat insomnia and migraine in addition to stimulatung metabolism . In Spain, the spice, popularly known as "Azafran´ " is used for relieving toothache. Saffron extract known as "Zafferano" in Italy, is recommended as a digestive and sedative concoction and the infusion is used as a mouth rinse. (Mohtashami et al., 2021)

III.2 The saffron Secondary metabolites:

The *Crocus sativus* plant is mainly composed of more than 150 substances (Table 1) comprising water, nitrogenous matter, anthocyanins, glycosides, monoterpenes, aldehydes, flavonoids, vitamins, volatile oil, proteins, amino acids, carbohydrates, minerals, raw fibers and gums(Bathaie & Mousavi, 2010), (Ghaffari & Roshanravan, 2019) .

Table 1 *chemical composition of saffron extract. (A. Mir Shafat et al., 2020)*

Component	Mass (%)
Carbohydrates	12.0 to 15.0
H_2O	19.0 to 14.0
Polypeptides	11.0 to 14.0
Cellulose	4.0-7.0
Lipids	3.0-8.0
Minerals	1.0-1.5
Miscellaneous	40.0

The famous spice is made only of the stigmas of the *crocus sativus* flower. The stigma contains the highest concentration of bio-active molecules (Gómez-Gómez et al., 2017):

- Crocin and Crocetin, the two molecules classified as carotenoids, primarily responsible for the characteristic red color of the stigma, as well as the intense golden colour that saffron provides to aqueous solutions.
- Picrocrocin, responsible for the bitter taste of the spice.
- Safranal, along with approximately 40 other compounds, create the unique saffron aroma.(José Bagur et al., 2017)

III.3 The biosynthesis of the saffron's main secondary metabolites:

By definition, secondary metabolites are compounds whose presence isn't mandatory for the organism's growth and normal development, they are however necessary for the plant's interaction with its environment. These molecules can be divided into 5 major classes; terpenoids, alkanoids, cyanogenic glucosinolates and phenolic compounds.

The *crocus sativus* stigma contains different volatile and non-volatile bioactive molecules. The most abundant molecular species identified in literature are terpenoids and polyphenols.

Staring with the phenolic compounds, who are important constituents of the human diet, mainly due to their physiological properties as anti-allergenic, anti-artherogenic, anti-inflammatory, anti-microbial, antioxidant, anti-thrombotic, cardioprotective, and vasodilatory effects(Bastola et al., 2017). In the stigmas, phenols like kaempferols glycosides have been identified, as well as flavonols, a sub-category of flavonoids , whose basic structure comprises two phenyl groups joined by a three-carbon bridge. The glycosides are compounds produced during the secondary metabolism of plants that are distinguished by having two distinct parts in their molecule: a sugar and an organic molecule, both linked by alpha (α) or beta (β) glycosidic bonds. Saffron possesses other bioactive compounds, such as kaempferols and its glycosides. Kaempferols, the main flavonoids in the stigma, are gaining a rising interest for their antioxidant activity as a food supplement, in functional foods, in pharmaceutical formulations.(Moratalla-López et al., 2019).

Terpenoids, mostly known as terpenes, are composed of

- ⇒ Five-carbon isopentane units,
- ⇒ Isopentyl Diphosphate (IPP),
- ⇒ Dimethylallyl diphosphate DMAPP

All of which are isomers typically synthesised in plastids, by the MEP/DOXP pathway in plants. (Seifi & Shayesteh, 2020).

Terpenoids are present as tetraterpenoids (C40), commonly called carotenoids. Their biosynthesis starts with product of the MEP pathway: geranylgeranyl diphosphate (GGPP). The condensation of two GGPP molecules by phytoene synthase (PSY) generating C40 linear 15-cis-phytoene.

After a series of enzymatic reactions, the first coloured pigment is synthesized: Lycopene. (Figure 4)

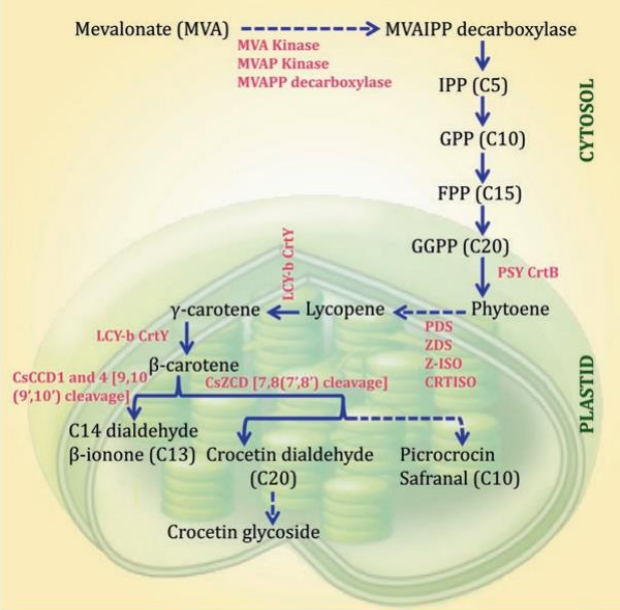


***Figure 4**: The biosynthetic pathway of apocarotenoids. (Vahedi et al., 2019)*

The red pigment is then cyclized, producing several types of carotene molecules, including the orange pigment β-carotene.

The lipophilic pigments are responsible for different colours in several parts of the plants (flower, fruit, leaves…). They can be sorted into carotenes, xanthophylls and lycopene, producing, respectively, orange, yellow and red colouring (Ghaffari & Roshanravan, 2019).

The hydroxylation of β-carotene by the β-carotene hydroxylase enzyme (BHC) generates zeaxanthin C40H26O4.

To form the unique and unmatched bioactive molecules characteristic of the saffron spice, zeaxanthin and β-carotene act as the main substrates to the key enzyme: carotenoid cleavage dioxygenase (CDD). The two precursors are converted by different CCDs leading to different apocarotenoid compounds (Liu et al., 2020). The reaction involves the incorporation of the two oxygen atoms from molecular oxygen into their substrates across a double bond, leading to the production of two ketone- or aldehyde-containing cleavage products. These cleavage products

are categorised as **apocarotenoids.** (Baba & Ashraf, 2016; Seifi & Shayesteh, 2020; Vahedi et al., 2019)

III.4 Properties of Saffron apocarotenoids

The common precursor for the characteristic stigma molecules is zeaxanthin as mentioned before. These molecules include crocin and crocetin (the red colour), picrocrocin (bitter taste) and safranal (saffron fragrance) (Figure 5). In this section, we will discuss in further detail the specific properties of each one of these compounds.

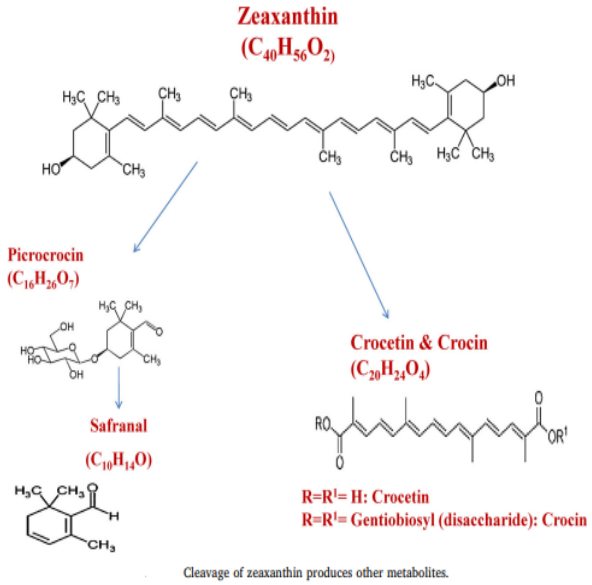


Figure 5*: the products of the enzymatic cleavage of zeaxanthin in the saffron stigma. (Ghaffari & Roshanravan, 2019)*

III.4.1 Crocin and crocetin:

Crocin (C44H64O24), derived from the word crocos meaning saffron in German (José Bagur et al., 2017), is a unique member of the carotenoids class, since it is water-soluble. This unusual attribute is owed to the terminal glycosyl units, added through the process of glycosylation, to the crocetin molecule during the biosynthesis in the cytosol (Dhiman & Kharkwal, 2020; Razak et al., 2017)

About 30% of the dry matter of saffron is composed of crocins (combination of C-1 or a-C, C-2 , C-3, C-4, and C-5)(Table 2). Crocin content of the saffron crop is influenced by the geographical area where it is grown and the method of metabolites extraction carried out (A. Mir Shafat et al., 2020; Dhiman & Kharkwal, 2020).

Table 2 some of the crocins identified in the saffron stigma extract. (A. Mir Shafat et al., 2020)

Compound	Sugar moieties	Chemical formula	Isomer occurrence in saffron
Crocetin	R1 = R2 = OH	$C_{20}H_{24}O_4$	cis − trans
Crocin 1	R1 = β-D-glucosyl, R2 = H	$C_{26}H_{34}O_9$	Trans
Crocin 2	R1 = β-D-gentiobiosyl, R2 = H	$C_{32}H_{44}O_{14}$	cis − trans
Crocin 2^0	R1 = R2 = β-D-glucosyl	$C_{32}H_{44}O_{14}$	cis − trans
Crocin 3	R1 = β-D-gentiobiosyl, R2 = β-D-glucosy	$C_{38}H_{54}O_{19}$	cis − trans
Crocin 4	R1 = R2 = β-D-gentiobiosyl	$C_{44}H_{64}O_2$	cis − trans
Crocin 5	RI = 3β-D-glucosyl, R2 = β-D- gentiobiosy	$C_{50}H_{24}O_{29}$	cis − trans

Crocins present in C. sativus are powerful free radical quenchers and have been used in traditional medicine since thousands of years. They display a variety of health benefi ts and have gained attention due to their sedative, analgesic, and anticancer properties (Baba & Ashraf, 2016)

Combined with other phytoconstituants, crocetin is also responsible for the crimson colour. Crocetin is in reality a polyene chain with a carboxyl group at both ends and is lipophilic in nature. In its anionic form, it is highly soluble in water. The resultant products of the crocetin are hydrophilic when esterified with hydrophilic gentiobiose(s) or any other sugar precursors (Finley & Gao, 2017). Enzymatic or acid hydrolysis of crocin yields crocetin as a product(A. Mir Shafat et al., 2020)

III.4.2 Picrocrocin

Picrocrocin (C16H26O7) , the compound of bitterness, is a colourless monoterpene glycoside, it constitutes up to 26% of the dry matter of stigma (Ghaffari & Roshanravan, 2019). Its precursor is zeaxanthin, and it itself is a precursor to safranal. The drying process of saffron is directly responsible for the taste-aroma ratio since the more zeaxanthin is metabolized, the bitterer the taste will be. (Dhiman & Kharkwal, 2020)

III.4.3 Safranal

The compound of aroma, monoterpene aldehyde ($C_{10}H_{14}O_4$), also known as safranal, is an unstable volatile component, formed by the hydrolysis and dehydration of Picrocrocin, during the drying and storage of the saffron. It constitutes 30–70 % of essential oil and about 0.001–0.006 % of dry matter(Baba & Ashraf, 2016)

Safranal can form up to 65% of the total aroma components, estimated to be around 40 substances. Safranal is an indispensable component of saffron aroma, the mechanism by which it is produced from picrocrocin is yet to be fully elucidated. Whether it is produced non-enzymatically, enzymatically, or by both the processes, more studies are needed to unravel the mechanism.

(Bathaie & Mousavi, 2010; Dhiman & Kharkwal, 2020; José Bagur et al., 2017)

III.4.4 Other pigments

The colour compounds of saffron are carotenoids and glycosidic, alpha-carotene, beta-carotene, lycopene, zeanthingentiobioside, glycoside, gentio-glycoside, beta-crocetin di-glycoside and gama-crocetin. .(Dhiman & Kharkwal, 2020)

III.5 Regulation of the apocarotenoid levels in the cell:

Apocarotenoid biosynthesis pathway is highly regulated in C. sativus. It involves the synchronisation of many individual pathways especially since it is a tissue and developmental stage-specific process. Moreover, the stable levels of carotenoids depends on the storage capacity, as well as plastid biogenesis, flowering, and fruit development.

The main genes that govern the production of carotenoids are PSY, LCY, CCD, BCH and ZCD, together with the two key genes of the MEP pathway DXS1 and DXR (Akhtar & Swamy, 2019; Gómez-Gómez et al., 2017)

It was reported that expression of main carotenoid and apocarotenoid biosynthesis genes was considerably higher in stigma compared to the rest of the flower, which provided the molecular basis for the stigma-specific accumulation of saffron apocarotenoids (Figure 6).(Akhtar & Swamy, 2019; Baba et al., 2015; Gómez-Gómez et al., 2017)

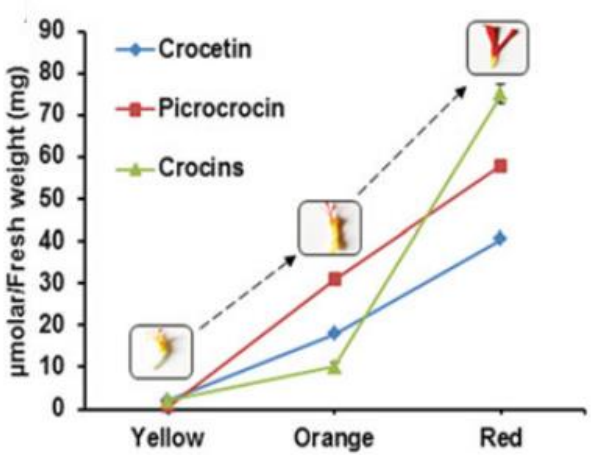


Figure 6: *the contents of crocins, picrocrocin and crocetin in different stages of the maturation of the stigma. (Baba & Ashraf, 2016)*

Most of these secondary metabolites are stored in a glycosylated form, which chemically stabilizes them and renders them suitable for storage in different organelles.

The accumulation of these carotenoids, seems to be controlled by the transcript levels of PSY, BCH, and β-LCY2a

Coordinated transcriptional regulation of biosynthetic genes is one of the most important mechanisms responsible for the final levels of secondary metabolites in plant cells. Then again, only a few of those transcription factors have been identified and characterised.

The main apocarotenoids that accumulate during the stigma development are crocetin, its glucoside derivatives, crocins, and picrocrocin. Their accumulation starts early in the development of the stigma. Electron micrographs of the stigma revealed that carotenoid-derived metabolites were accumulated in vacuoles.(Figure 7) They uninterruptedly increased in size, reaching a maximum diameter of up to 1.5 nm in the late red stigmas. Another fundamental participant in carotenoid accumulation is the Or gene. This gene is not directly involved in carotenoid biosynthesis at transcript level, but can regulate PSY abundance at post-transcriptional level. Within saffron proteome, several Or homologs were identified(Baba & Ashraf, 2016; Gómez-Gómez et al., 2017)

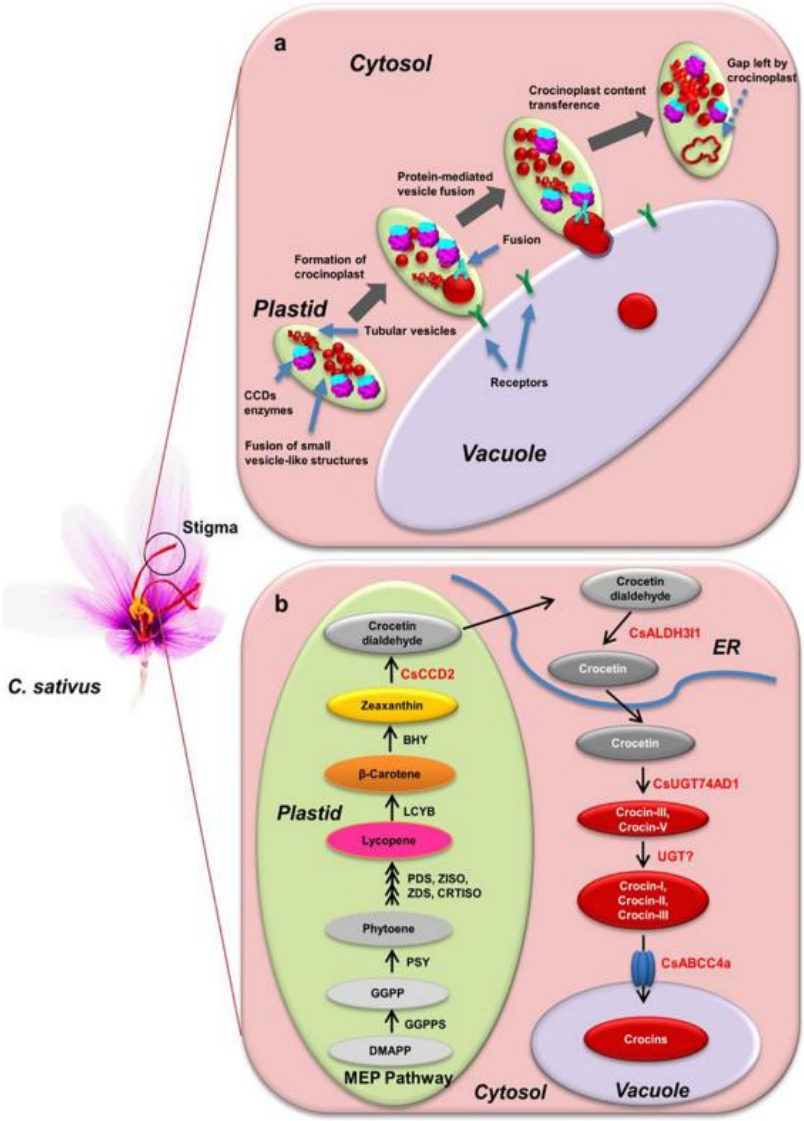


***Figure 7**: the route of apocarotenoids accumulation in the Saffron stigma. (Liu et al., 2020)*

IV. Pharmacological properties of the *Crocus sativus* stigma:

The saffron spice has been considered as the costliest spice for over 3000 years. Not only has it been used in different cuisine for its crimson colour and particular taste, especially in Persian, Indian, European, Arab, and Turkish cuisines. It is also widely used in traditional medicine to treat some illnesses (Finley & Gao, 2017).

Modern science has established in its turn the many pharmacological and biomedical attributes of the unique molecular assortment present exclusively in the *crocus sativus* stigma. Since saffron is a source of a variety of pigments carotenoids a, flavonoids and polyphenols, it is safe to say that the red spice is a rich source of antioxidant compounds.

IV.1 Antioxidant activity

IV.1.1 An overview:

Oxygen is indispensable for the life, in particular cell respiration. However, the metabolism of oxygen can generate reactive elements called free radicals, in particular the superoxide ion (O2–) and the hydroxyl ion (OH–).

A free radical is defined as "an atom or group of atoms with one or more unpaired electrons which exist in a free form." Free radicals play a significant role in the development of tissue damage and pathological events in living organisms. Free radicals damage DNA, vital cellular proteins and membrane lipids (lipid peroxidation), which might lead to cell death.

Oxidative stress is mediated by reactive oxygen species (ROS), molecules that are produced during the normal and aberrant cellular metabolism that utilizes molecular oxygen. The imbalance between production of as example: O2, H2O2, OH, ROO and the ability of the normal detoxification systems in favour of the oxidants leads to oxidative stress, which itself leads to cellular damage. The damage is caused by the interaction of ROS with cellular constituents.

The way to limit the harm of these ROS is by way of antioxidants. An antioxidant is defined as a chemical substance that inhibits, stops, or controls the oxidation of an oxidizable substrate. It can be an organic or inorganic compound.

The balance between antioxidations and oxidations is believed to be critical in maintaining a healthy biological system.(Angaji et al., 2012; Bolhassani et al., 2014; Gulcin, 2020)

IV.1.2Antioxidants of the stigma:

Saffron stigma contains high levels of molecules considered as powerful antioxidants and can potentially prevent and treat some diseases. (Table 3)

***Table 3**: compounds of the crocus sativus stigma with antioxidant capacity. (Ghaffari & Roshanravan, 2019)*

Family	Name	Note
Carotenoids	Crocetin	
	Crocin	The unique water soluble carotenoid and the major component of saffron
	α- carotene	
	β- carotene	
	zeaxanthin	
Monoterpene aldehydes	picocrocin	
	Safranal	60% of the volatile components
Flavonoids		

As the production of excess free radicals is associated with cancer, aging, cardiovascular disease, diabetes and other diseases, experimental data showed that saffron and its active ingredient crocin have the potential to prevent oxidative damage of the brain, liver, and kidney induced by chronic restraint stress in rats.

The methanolic extract of saffron shows high antioxidant activity, and crocin, picrocrocin and safranal in saffron exhibit radical scavenging activity (Figure 8) (Xing et al., 2021). The antioxidant effect of saffron extracts is possible because the presence of sugar moieties attached to the terminal COOH groups of the crocetin skeleton (crocin structure) plays a role in the penetration of cell membranes. Carotenoids also influence the strength and fluidity of membranes, consequently affecting its permeability to oxygen and other molecules(Bolhassani et al., 2014).

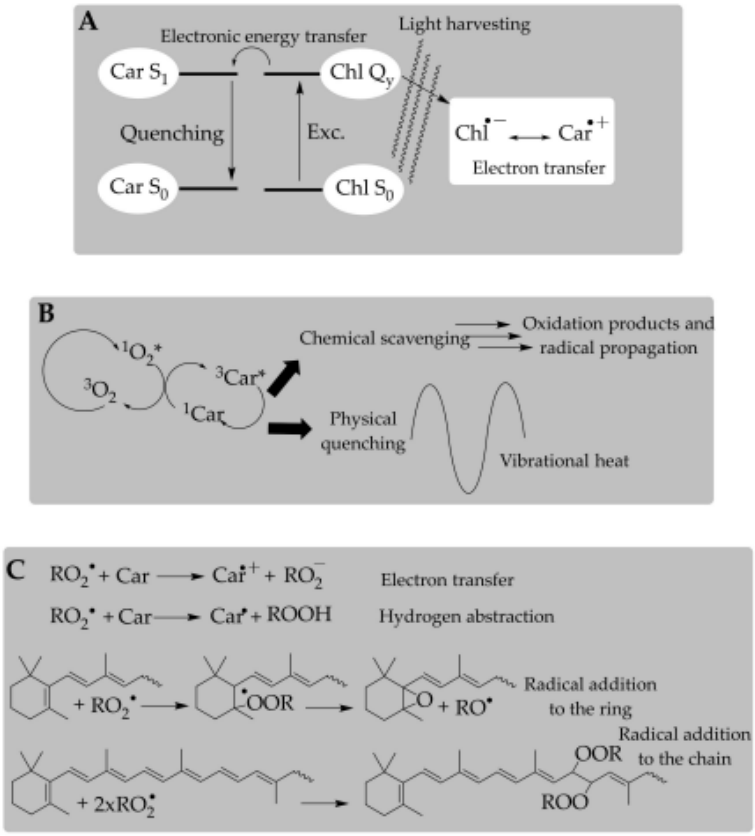


Figure 8*: Mechanisms of the antioxidant action shown by carotenoid pigments in different processes. (A) The electron transfer from the characteristic conjugated polyenic chain of carotenoids to chlorophylls. (B)Physical quenching of sing. (C)Electron transfer, hydrogen abstraction, and radical addition in the antioxidant activity against peroxyl radicals. (Pérez-Gálvez et al., 2020)*

A process like lipid peroxidation generates peroxyl radicals. These peroxyl radicals damage the lipids in the cell membranes. Among many other ROS, peroxyl radicals are efficiently scavenged by carotenoids(Mitra, 2020). Safranal and crocin can capture free radicals, and crocetin can effectively remove free radicals and inhibit lipid peroxidation thanks to the presence of hydroxyl in carboxylic group. Thus, they may potentially be used for cancer prevention, as well as the treatment of cardiovascular and psychiatric disorders (Xing et al., 2021)

The antioxidant activity of saffron compounds can protect DNA and tRNA from damaging chemical reaction in ligand–polynucleotide complexes (Kanakis et al., 2009).

Another study showed that the aqueous extract of saffron has, on top of antioxidant activity, an ability to block the reactive oxygen species and intracellular signals activation to downregulate the apoptotic pathway, thereby improving cell viability(Rodriguez-Ruiz et al., 2016). The aqueous extract also decreased the arterial blood pressure in a dose-dependent manner. Researchers revealed that safranal is the major contributor to the hypotensive activity of saffron (Ghaffari & Roshanravan, 2019)

Alongside the carotenoids, the polyphenols of the stigma also exhibit high antioxidant potential, and it is largely due to their redox properties, which make them act as reducing agents, hydrogen donors, and singlet oxygen quenchers(Angaji et al., 2012). Flavonols with varied hydroxyl substitution can act as strong antioxidants as they are considered as polyphenolic compounds(Gulcin, 2020).

The synergistic effect of all the bioactive constituents gives to saffron spice a significant antioxidant activity.

IV.2 Other medicinal benefits of the *Crocus sativus* stigma

Most research papers attribute the potential therapeutic value of saffron to its three principal components: safranal, picrocrocin and crocins (Table 4).

***Table 4**: Biomedical activity of saffron compounds. (Moratalla-López et al., 2019)*

Bioactive Compound	Bioactivity [Reference]	Model	Dose
trans-crocetin	Cross the blood–brain barrier and reach the central nervous system [102,103]	Rats	Oral administration (100 mg/kg)
Crocetin	Neuroprotection [83]	Hemi-Parkinson rats	Peripheral administration (25, 50 and 75 µg/kg body weight)
	Improved post-shock survival and reduced apoptosis [127]	Rats	Bolus injection (2 mg/kg body weight)
	Cardioprotective effects (after myocardial ischemia reperfusion injury) [121]	Adult male Wistar rats	Intragastric administration (50 mg/kg/day)
Crocins	Hepatoprotective effects [158]	Rats	Intraperitoneally (25 mg/ kg body weight/day for 4 weeks)
Safranal	Antidepressant [110]	Rats	Peripheral administration (15.5 mg/kg body weight.)
	Anticonvulsant [111]	Mice	Injected (0.15 and 0.35 mg/kg)
Picrocrocin	Antitumor effects [100]	Human colon adenocarcinoma (Caco-2-cell model)	8–24 µM
Saffron extracts	Satiating [113]	Human (randomized, double-blind, placebo-controlled, parallel-group)	Oral administration (capsule: 176.5 mg extract/day for 8 weeks)
	Reduce cognitive deterioration (Alzheimer's disease) [134]	Patients (randomized double-blind parallel-group)	Oral administration (capsule: 30 mg/day for 12 months)
Saffron	Premenstrual syndrome [115]	Women (double-blind, randomized and placebo-controlled trial)	Oral administration (capsule: 30 mg/day for 6 months)
	Neuroprotection (macular degeneration) [130]	Albino rats with light-induced photoreceptors degenerations	Oral administration (1 mg/kg/day for 6 weeks)
	Improve the symptoms of children with deficit hyperactivity disorder [131]	Children	Oral administration (20–30 mg/ day for 6 weeks)
	Effective treatment in depression and anxiety [140]	Patients (double-blind controlled clinical trial)	Oral administration (30 mg/day per 6 weeks)

Carotenoids have shown two beneficial effects to human health: a reinforcement of the immune response as well as a reduction of the risk of degenerative diseases such as cancer, cardiovascular diseases, cataract and muscular degeneration. The modulation of immune responses using carotenoids covers increasing natural killer cell (NK-cell) activity in the senior population, increasing the lymphocyte response to mitogens, protection of immune cells from their own bactericidal production of reactive species, increasing total white blood cells and

CD4/CD8 ratio in HIV infected patients. C. sativus has also been used to treat several medical conditions such as gastrointestinal disorders, urological infections as well as in treating malignancies (Bolhassani et al., 2014).

IV.2.1Anti-carcinogenic effect:

Cancer remains the largest cause of mortality in the world almost 10.0 million cancer deaths occurred in 2020(Sung et al., 2021). Large varieties of natural substances are being studied for their anti-carcinogenic ability.

Amongst these natural substances, Saffron extracts inhibit tumour formation and cancer cell growth. Many studies have shown that saffron has the potential to treat a range of cancers, such as gastric cancer, colorectal cancer, hepatic cancer, pancreatic cancer, prostate cancer, cervical cancer, ovarian cancer, breast cancer, skin cancer, lung cancer and leukaemia (Table 5).

Table 5: *Some of the anti-tumor and cytotoxic effects of saffron's crocetin. (Hashemi & Hosseinzadeh, 2019*

Some examples of Anti-tumor and cytotoxicity effects of crocetin.

Type of tumor	Cell lines or animal models	Concentration/Dose,	Mechanism of action
Breast	MDA-MB 231 cells	1 and 10 μM	Downregulation of MMPs expression
	MCF-7 cells	50 μM	Modulation of the expression of ATG1 and Beclin-1
	Wistar rats	100 mg/kg (oral),	Decreased tumor size, latency period, and tumor number
Lung	A549 cells	IC50 about 0.41 mM	Inhibited proliferation and enhanced apoptosis
	A549 and VA13 cells	l-100 μg/ml	Inhibition of nucleic acid and protein synthesis
	Mice	50 mg/kg (i.p),	Decrease glycoproteins and polyamines levels, Scavenge free radicals
	Mice	20 mg/kg (i.p),	
Cervical and Ovarian	HeLa and SKOV3 cells	IC50 from 100 to 120 μM	Induction of p53 as the regulation the G1 checkpoint
	Mice bearing cervical tumor	40 mg/kg (oral)	Downregulation of the proinflammatory cytokine
Gastric	BGC-823 cells	IC50 about 200 μM	Reduction of MMP, caspase 3 activation and cytochrome *c* translocation into the cytosol
	Rat	50, 75, and 100 mg/kg	Changes in serum antioxidant activity and lactate dehydrogenase
Colon	SW480 cells	0.2, 0.4, 0.8 mM	Induction cell cycle arrest through P21induction
	HCT116 (p53+/+), HCT116 (p53−/−), HT29 (p53mt) cells	100 μM	Cytotoxic effect by p53-dependent and p53-independent manner
Liver	Hep G2	IC50 about 0.6 μM	Increasing of Nrf2 and decreasing of LDHA levels
Pancreatic	MIAPaCa-2, BxPC3, Capan-1, and ASPC-1	200 μM	Changes in cell cycle proteins, Cdc-2, Cdc-25C, Cyclin-B1, and EGFR
	Nude mice	4 mg/kg (oral)	Induction of apoptosis as well as inhibition of proliferation

The antitumor effects of saffron and its components may be due to their inhibitory effects on cellular DNA or RNA synthesis, free radical chain reactions, and interaction of carotenoids with topoisomerase II(Xing et al., 2021)

MDR (multidrug resistance) is a major cause of chemotherapy failure. Cancer cells often develop cross-resistance to structurally and functionally unrelated cytostatic drugs, a phenomenon that has been termed MDR. The particular activity of ATP-dependent multidrug transporters, who belong to the superfamily of ATP-binding cassette (ABC) proteins, is significant in this process.

P-glycoprotein (MDR1) is the most widely known ABC-transporter associated with clinical MDR. Carotenoids, especially some of the 40C category to which β-carotene belongs were competitive inhibitors of ABC-transporter. They could synergistically revert the MDR process and enhance the cytotoxicity of chemotherapeutic drugs in human MDR1 expressing cell. Depending on their chemical structures, they amplified drug accumulation in drug-resistant cells, in variable degrees. This study suggested the use of carotenoids in combination with chemotherapeutics in cancer treatment to enhance the efficacy of chemotherapy and reduce the influence of MDR (Figure 9) (Bolhassani et al., 2014).

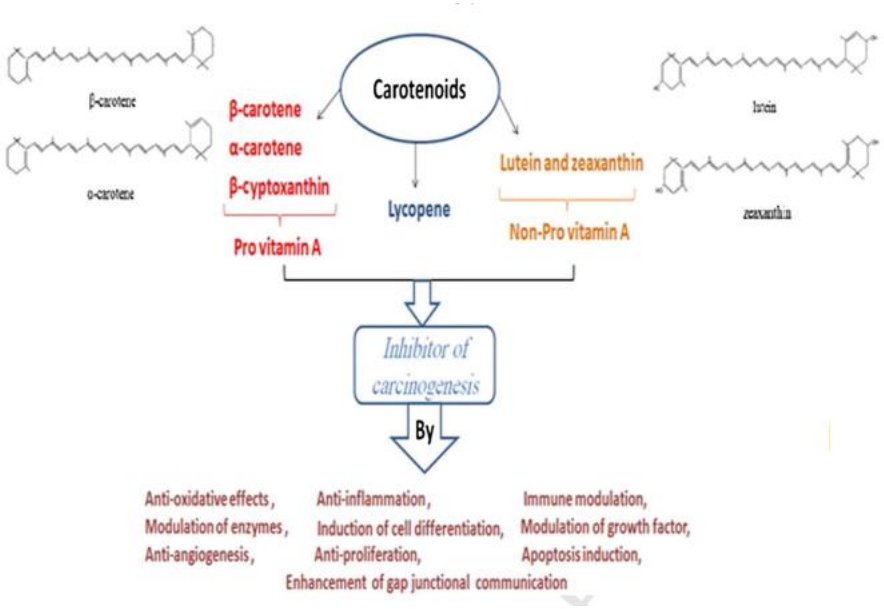


Figure 9: *Inhibitory effects of carotenoids on carcinogenesis. (Bolhassani et al., 2014)*

IV.2.2Anti-inflammatory effect

The radical scavenging property of saffron is associated with its anti-inflammatory activities (Table 6). Studies showed that the anti- inflammatory effects of saffron's constituents are due to its significant inhibitory effects against cycloxoygenase 1 and 2 enzymes and prostaglandin E2 production. Furthermore, attenuating endoplasmic reticulum stress signalling, blocking pro-inflammatory cytokines production such as TNF-α, inhibiting transcription factors like NF-κB which intensifies chronic inflammation, as well as suppressing inflammatory genes expression via raising histone deacetylase activity are the most important causes of saffron's anti-inflammatory property.

Cancer frequently develops in inflamed tissues, suggesting that the inflammatory condition is closely related to carcinogenesis. Experimental data showed that dietary crocin suppresses carcinogenesis in mice by inhibiting inflammation, the mRNA expression of certain pro-inflammatory cytokines and inducible inflammatory enzymes.

Another study (Godugu et al., 2020) stated that Crocin ameliorate pancreatic edema, modulates pancreatic inflammatory cytokines, disturbs nuclear translocation of p65-NF-κB, upregulate Nrf-2 expression and downregulates TNF-α, IL-6, NF-κB and nitrotyrosine expression in mice with acute pancreatitis.

Inflammation also plays an essential role in the atherosclerosis, which is the dominant cause of CVDs, cardiovascular diseases, known as "the world's first killer", especially for middle-aged and elderly subjects. The available data seem to support that the antioxidant, hypolipidemic and hypotensive effects of saffron attenuate atherosclerosis, myocardial injuries and cardiotoxcity. (Ghaffari & Roshanravan, 2019; Xing et al., 2021).

Table 6: *some of the anti-inflammatpry effects of saffron. (Kianmehr & Khazdair, 2020)*

Extract	Effect	Experimental model
Macerated extracts	Inhibited cell viability of lymphocytes and secretion of IFN-γ	Peripheral blood mononuclear cells
Methanol extract	Decreased NO, and iNOS levels, and also prevented cytochrome c releases	Human bronchial epithelial cells
Aqueous extract	Reduced iNOS levels and inflammatory cytokines such as; L-5 and IL-13 levels in the lung tissue. airway hyper-responsiveness and airway cellular infiltration to the lungs	Murine model of asthma
Hydroethanolic extract	Reduced serum levels of endotheline-1 (ET-1) and total protein (TP)	Sensitised guinea pigs
	Reduced total WBC, eosinophil and lymphocyte counts in blood and lung lavage	
	Ameliorated lung pathological indices	
	Decreased tracheal responsiveness to both methacholine and OVA and serum levels of inflammatory mediators	
	Reduced WBC number and decreased the percentage of neutrophils and eosinophils in lung lavage	Sensitised rats
	Reduced WBC, RBC and platelet count, Reduced Eosinophil and neutrophil percentage. Increased Lymphocyte percentage	
Ethanolic extract	Attenuation of pro-inflammatory factors (TNF-α, IL-6 and IL-1β)	Chronic constriction injury in rat
Ethanolic extract	Reduced ear edoema and showed antinociceptive effects	Acetic acid-induced writhing in mice
Aqueous extract of petal	Ameliorated paw edoema	Formalin-induced paw edoema
Ethanolic extract	Delayed disease onset, Elevated antioxidant Capacity and reduced leukocyte infiltration to CNS	Mice model of autoimmune encephalomyelitis
C. sativus capsule	Increased WBC	Schizophrenia patients
Constituent of *C. sativus*	enhanced Amyloid-β 42 (Aβ42) degradation in Alzheimer's Disease monocytes	Human monocytes

IV.2.3Neuro and psychopathology:

The brain is rich in lipids and polyunsaturated fatty acids (PUFA) which renders it sensitive to peroxidation, and so, this central nervous system is susceptible to oxidative stress conditions where metabolic demands of oxygen are high. The excessive production of ROS is responsible for various neurodegenerative diseases. A rise in free radical production in mitochondria causes degeneration of brain tissue leading to aging of brain. Saffron exhibited various neuroprotective and therapeutic effects on the brain (Figure 10). (Nanda & Madan, 2021)

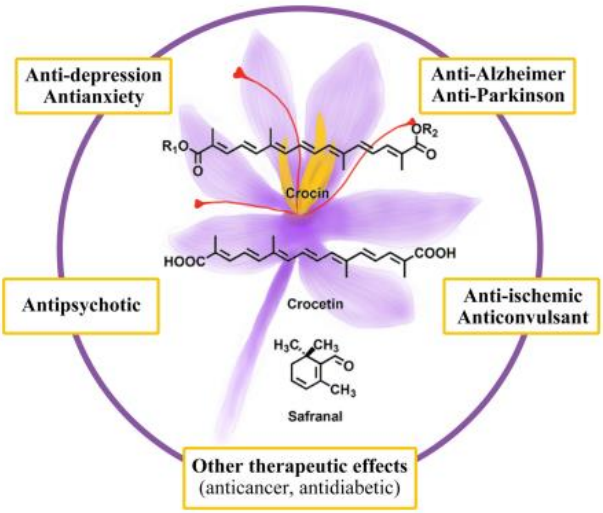


Figure 10: *Examples of the therapeutic effects of saffron extract on the central nervous system. (Bian et al., 2020)*

IV.2.3.1 Depression and anxiety:

The antidepressant effect of saffron stigmas can be mediated via safranal and crocin. Crocin can inhibit the uptake of dopamine and norepinephrine, whereas safranal may inhibit that of serotonin. Studies have shown that saffron may be used to treat mild to moderate depression. In placebo-controlled trials, saffron exhibited a greater therapeutic effect compared with antidepressants. The antidepressant effects of saffron are possibly due to its serotonergic, antioxidant, anti-inflammatory, neuro-endocrine, and neuroprotective. Saffron could also improve depressive symptoms in adults and older people with mild to moderate depression in clinical studies.

A meta-analysis indicated that saffron has a noticeable effect on mild to moderate major depressive disorders of adults is comparable to citalopram and fluoxetine. The anti-depressive mechanisms of saffron may be due to enhanced levels of nerve growth factor inducible (VGF) neuropeptide, cyclic-AMP response element binding protein (CREB), phospho-CREB (p-CREB) and brain-derived neurotrophic factor (BDNF) (Mohtashami et al., 2021)

Overall, saffron has positive effects on the treatment of depression

Anxiety is the most common form of neurosis. The efficiency of saffron in reducing anxiety has been demonstrated in clinical trials, saffron as an add-on therapy to sertraline, might attenuate symptoms of generalized anxiety disorder based on the Hamilton Anxiety Rating Scale total score. At the same time, the side effects were tolerable(Xing et al., 2021)

Poor sleep quality was significantly interrelated with greater symptoms of depression and anxiety,

saffron extract could relieve anxiety and sleep disturbances in patients with mild to moderate depression-anxiety (Mohtashami et al., 2021)

Because of its effective in the context of depression and anxiety, saffron could improving sleep quality. Primary data indicated that a saffron extract could be a natural and safe nutritional strategy to improve sleep duration and quality. However, the mechanisms of saffron extract affect on sleep quality and sleep duration is unclear (Pachikian et al., 2021; Xing et al., 2021).

IV.2.3.2 Neurodegenerative diseases:

Alzheimer's disease (AD) is a central nervous system degenerative disease and the most common type of senile dementia. Many neuropsychiatric signs manifest themselves, such as progressive memory disorders, cognitive dysfunction, personality changes, language barriers, and other symptoms. Saffron inhibits the aggregation and accumulation of amyloid β in the human brain. Randomized double-blind experiments have shown that saffron extracts (extract with 80% ethanol) could reduce the cognitive decline of patients with moderate to severe AD(Xing et al., 2021)

A meta-analysis study have shown that saffron may improve cognition and daily activities of patients with mild cognitive impairment and Alzheimer's disease (Mohtashami et al., 2021)

The neuromodulatory effect of saffron comes essentially from crocetin. It is attributed to its strong antioxidant properties and to the fact that C20-dicarboxylic acid transcrocetin is the only

active metabolite of crocin which could cross the blood-brain barrier (BBB) after saffron administration.(Hashemi & Hosseinzadeh, 2019)

Another angle to understand neurodegenerative diseases was the study of the microglial activation in the brain; it influences heavily the pathological state of the nervous tissue. A recent study showed that both crocin and crocetin were effective in the inhibition of pro-inflammatory mediators including LPS-induced nitric oxide (NO), interleukin-1β (IL-1β), TNF-α, and ROS from microglial cells. These results point out that intake of saffron extract might help restore homeostasis in the brain. (Hashemi & Hosseinzadeh, 2019).

Parkinson's disease PD is a common neurodegenerative disease, characterized by the loss of dopaminergic neurons. The symptoms of PD include tremor, bradykinesia, rigid muscles, impaired balance, and loss of automatic movements. Saffron deserves more interest as a potential therapeutic for PD given its antidepressant and anxiolytic effects since patients suffering from PD show non-motor symptoms such as sleep disorder, depression, and anxiety in the early stages.

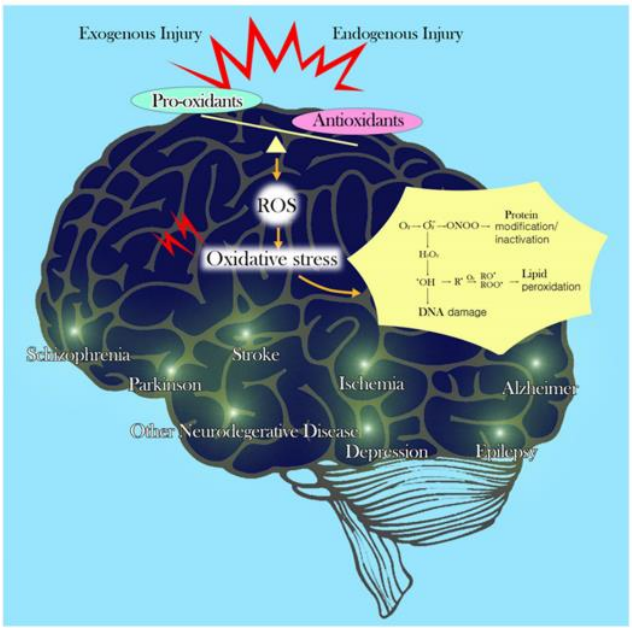


Figure 11: *pathophysiology of oxidative stress in the brain.*

Balance and imbalance between pro-oxidants and antioxidants against ROS species production induce oxidative stress and are consequently involved in neuronal tissue damage resulting in the neurodegenerative diseases. (Lee et al., 2020)

Preclinical studies demonstrated that crocin improved motor deficits and reduced inflammatory cytokines in a rat model. The antioxidative and antiapoptotic effects of safranal were also investigated, safranal protected primary dopaminergic cells against oxidative stress and apoptosis via the Keap1/Nrf2 signaling pathway. (Figure 11)

Saffron and its constituents crocin and crocetin were also shown to exert neuroprotective effects by inhibiting the aggregation and accumulation of α-Synuclein. (Bian et al., 2020)

IV.2.4 Anti-aging effect:

Saffron is rich in carotenoids; zeaxanthin have been considered as a protective agent against aging macular degeneration and senile cataracts. It has been suggested that β-carotene suppresses the increment of hormones related to stress syndrome (Bolhassani et al., 2014).
Safranal was established to be effective in protection of susceptible aged brain from oxidative damage by increasing antioxidant defences(Nanda & Madan, 2021).
Saffron treatment significantly improves memory and learning function by decreasing caspase-3 and lipid peroxidation activity. Hydro-ethanolic extract of saffron (5, 10, and 20mg/kg, for 4 weeks) also ameliorates the oxidative stress and pro-inflammatory indices in aged rat kidney. The findings indicate that saffron ameliorates the aging-related oxidative damage and inflammatory indices, as evident by the decrease in pro-inflammatory gene expression (IL-6, IL-1β, and TNF-α) and increase in some major antioxidant enzymes in the cell (Figure 12).

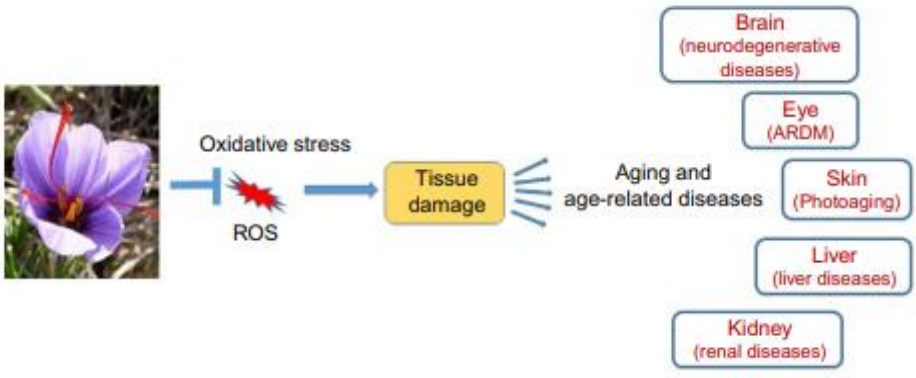


Figure 12: *the protective, anti-aging effect of saffron. (Samarghandian et al., 2020)*

The induction of oxidative stress and the blood-brain barrier (BBB) impairment during cerebral ischemia is deteriorated by aging. Crocin has the ability to ameliorate cerebral ischemia by

middle cerebral artery occlusion (MCAO)-induced damage in aged rats via preserving the BBB integrity.

Crocin, through modulating NF-kB expression and glycosylation-related genes, prevents human dermal fibroblasts (HDF) and human epidermal keratinocytes (NHEK) against UVA-induced oxidative stress and inflammation. The interaction of crocin with osidic receptors of keratinocytes and protein O-glycosylation is responsible for its protective effects against skin photo-aging(Samarghandian et al., 2020)

IV.3 Viral, bacterial and parasitic inhibition:

Saffron also demonstrated antibacterial and anti-parasitic activities; C. sativus has anti-leishmanial activity by induce apoptosis. Crocin, safranal, the most two important bioactive components of saffron, exhibited anti-leishmanial, anti-malarial, anti-Helicobacter pylori, against Candida spp. effects and human carbonic anhydrase inhibition activity. Based on its efficacy in anti-inflammatory, antioxidant, and depression management, saffron could be a potential nutraceutical or drug supplement to alleviate the magnitude of COVID-19 symptoms in patients. (Xing et al., 2021)

V. Materials and methods

V.1 Introduction

The aim of the following study is to obtain an appreciation of the benefits of the Moroccan saffron stigma by studying a few key aspects of its extract: characterize the antibacterial potential of the saffron's methanolic extract against some gram-negative and gram-positive potentially pathogenic bacteria available at the microbiology and molecular biology laboratory at the faculty of sciences of Rabat. Alongside exploring the antibacterial potential, we will quantify the radical scavenging capacity and assess the content in polyphenols, in order to get a round appreciation of the antioxidant potential and therefore, a sense of its impact on the human health, it being part of the culinary culture of many countries around the world and especially the Moroccan one.

V.2 Biological materials

V.2.1 The saffron stigma

The *crocus sativus* stigma was harvested from the Talouine region in autumn 2020.

Figure 13: *picture of crocus sativus flower before stigma harvest, taken by Pr. Sbabou*

The stigma was traditionally harvested and conserved in dark containers after it was dried according to the quality standards. The saffron stigma was conserved hermetically and in darkness until extraction.

Figure 14: *harvest of saffron stigma, picture taken by Pr. Sbabou*

V.2.2 Bacterial samples

The bacterial strains used are from the LMBM collection, isolated from soil:

Gram-negative bacteria	**And Gram-positive bacteria**
Alcaligenes faecalis strain MZ853461	Bacillus sp. strain MT560201
Escherichia coli DH5[alpha] txid668369	Microbacterium resistens strain MZ853466
Enterobacter aerogenes MT560199	

V.3 Extraction of bioactive compounds from saffron stigma

Five grams of stigmas were transferred into a flask containing 100 mL of the methanol (95%). The materials were stirred at room temperature in darkness for 24 h using a magnetic stirrer (Figure 15). The sample was filtered and the filtrate was then left in an oven etuve to desiccate at 37 °C.

Figure 15: *Extraction of secondary metabolites on a magnetic stirrer in dark conditions*

The concentrated dried extract was transferred into a sealed micro tube, weighed and stored at 4 °C for further analysis.

V.4 The bacterial growth inhibition

V.4.1 The Kirby-Bauer Disk Diffusion Test

Disc diffusion method was used to evaluate the antibacterial activity of Saffron extracts on agar culture (Bouillon Medium).

1 Different concentrations of extract (22.5; 11.25; 5.6 and 2.8 mg/mL) was filtered through 0.45 µm sterile syringe filter.

2 Then 30 µL of each dilution was impregnated into sterile, blank discs 6 mm in diameter.

3 Distilled water loaded discs were used as negative controls. All discs were dried before the application on bacterial lawn (100 µL, [0,1]).

4 The positive controls were Piperacillin + Tazobacteam (TZP) and Cefoxitin (FOX) antibiotic discs.

Antibacterial activity was evaluated by measuring the diameter of the inhibition zone (IZ) around the discs.) (Kirby, 2009)

Add schema of the protocol with details on control

V.4.2 Test on Liquid medium

The protocol followed was the Eloff, 1998 method with slight modifications.

The extract was diluted to a 5mg/ml concentration then mixed (v/v) with bacterial suspension of a 0,1 concentration. The mix was then incubated for 24h in darkness and under agitation. The optical density was measured at 620nm; the control consists of the bacterial suspension mixed with dilution medium.

All tests were performed in three replicas. The extract dilution was prepared aseptically and instantly to preserve its bioactive potential.

The Optical Density reads were recorded and transferred into an Excel file. Data was processed using R to get an exact appreciation of any significant variation in the growth rate of for the 5 bacteria confronted to our extract against the control.

All statistical analysis were run using R. version 4.03. Packages: ggplot2; multcompView.

V.5 The total phenolic content

To quantify the amount of polyphenols present in the stigma extract we followed the method by Ainsworth & Gillespie, 2007 (detailed in the Annexe 1):

- Mix 25 µl of sample (10mg/ml) in 95% (vol/vol) methanol.
- Add 200 ml 10% (vol/vol) F–C reagent and vortex thoroughly.
- Add 800 µl 700 mM Na2CO3 into each tube and incubate the assay tubes at room temperature for 2 h.
- Read the absorbance at 765 nm.

Total phenolics calculation

Calculate a standard curve from the blank-corrected Absorbance of the Gallic acid standards.

Calculate total phenolics as Gallic acid equivalents using the regression equation between Gallic acid standards and Absorbance at 765 nm

V.6 Radical scavenging capacity: DPPH assay

To obtain an appreciation of the antioxidant potential of the extract, we used the colorimetric DPPH test (Annexe 2). The protocol is as it follows:

- 0.06 mmol/L solution of DPPH in methanol was prepared and left for 1 h in the dark at 4 °C.

- 0.06 mM methanolic DPPH solution (3.5 mL) was added to methanolic extracts (0.5 mL) and to hydrolysed samples at different concentrations (0.062, 0.125, 0.187, and 0.250 mg/mL).
- solutions were vortexed and left for 30 min in the dark, and the absorbance was measured at 517 nm using a Jasco 7850 UV–Vis spectrophotometer.

The percentage of antioxidant activity (AA%) for each different concentration was calculated using the following equation:

$$AA\% = [(C_{OD} - S_{OD} / C_{OD})] \times 100$$

Where:

- AA% is the antioxidant activity.
- C_{OD} is the absorbance of the control solution (containing only DPPH ·) .
- And S_{OD} is the absorbance of the DPPH · solution containing sample. (Urbani et al., 2016)

V.7 *Crocus sativus* ESTs sequence screening:

V.7.1 Introduction

The goal of this study is to perform a full functional analysis of the available ESTs off the NCBI database. Considering the rarity of molecular data on this species, we will use 3 different annotation programs, in order to retrieve as many predictions and alignments as possible. Based on the results, we will try to harvest sequences unique to *crocus sativus and* use them as a template to design biomarkers that can be used for authentication and traceability.

List of sofwares and programs used for this study:

NCBI database : https://www.ncbi.nlm.nih.gov

Eg assembler : https://www.genome.jp/tools/egassembler/

Augustus : http://bioinf.uni-greifswald.de/augustus/submission.php

Omicsbox's : https://www.biobam.com/

Mercator4 : https://plabipd.de/portal/mercator4

KAAS : https://www.genome.jp/kaas-bin/kaas_main?mode=partial

Primer3 web interface : https://primer3.ut.ee/

V.7.2 Collection, assembly and filtering of ESTs

From the NCBI nucleotide database (https://www.ncbi.nlm.nih.gov/) we collected 6767 nucleotide sequences of *Crocus sativus* ESTs. The collection was carried out on Friday April 10th, 2022, and the collected sequences were put together in a multi-FASTA file.

The assembly was performed through the web server Eg assembler (https://www.genome.jp/tools/egassembler/) which allows the analysis of various types of nucleotide sequences (EST, GSS, cDNA, gDNA) in FASTA format. This program enabled cleaning; by eliminating polyA and polyT tails, low quality and short reads, repeat sequences, microsatellites, and pseudogenic RNAs, as well as vectors, adapters, contaminations, and organelle sequences. The results obtained are assembled sequences "contigs" and unassembled sequences "singletons". The overlapping threshold was set at 80, the other parameters were kept by default.

V.7.3 Annotation and functional analysis of *crocus sativus* ESTs available in NCBI database

V.7.3.1 Structural annotation

Structural annotation was done via Augustus (http://bioinf.uni-greifswald.de/augustus/submission.php). It is a server that allows the prediction of genomic sequences in eukaryotes. This program is based on the Hidden Markov Model (HMM) which describes the characteristics of different regions of the genomic sequence, such as introns, exons, ORFs, promoters, coding and non-coding regions and protein sequences. This server is able to annotate a 3 Mbp sequence and accepts data in FASTA format. Arabidopsis thaliana was chosen as the reference plant and the other parameters were kept as default.

V.7.3.2 Functional annotation: OmicsBox functional analysis module

Omicsbox's (https://www.biobam.com/) Functional Analysis module which is a comprehensive bioinformatics tool for the functional annotation, was used for analysis (Figure 16).

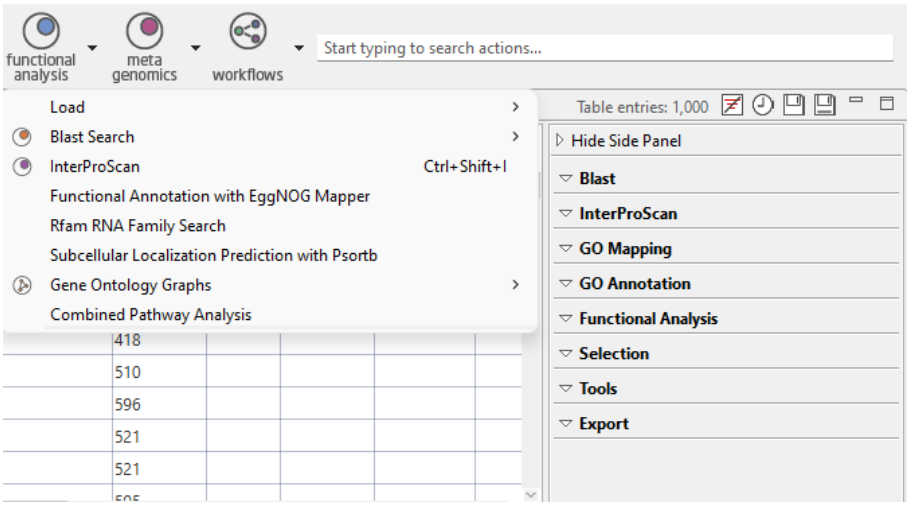


***Figure 16**: The Functional analysis module's menu and side panel from the OmicsBoc software*

The following workflow was followed:

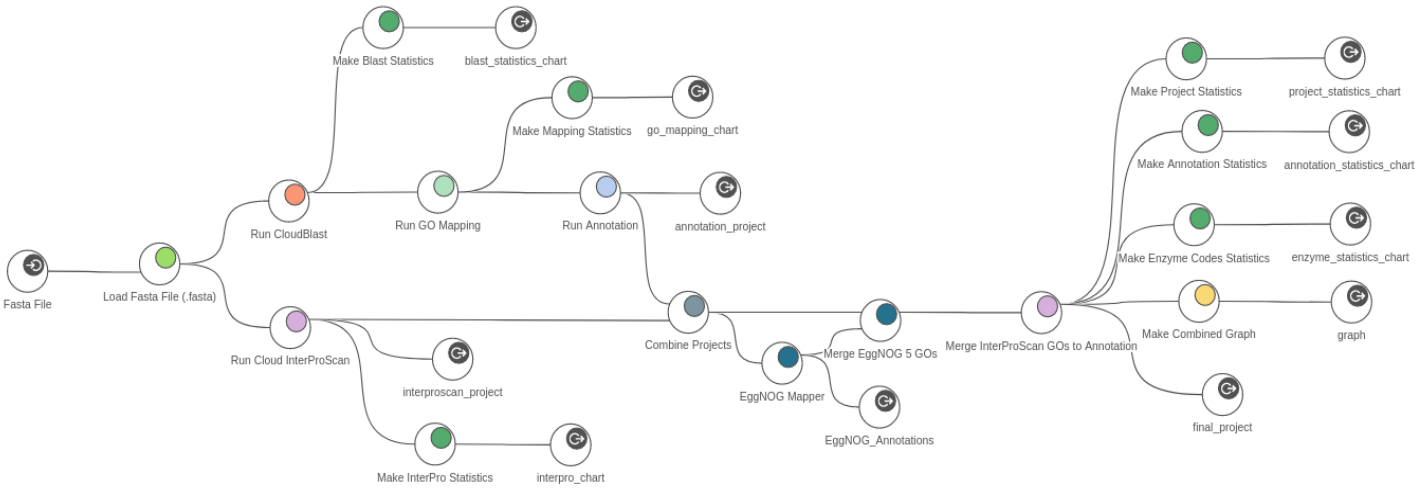


***Figure 17**: The functional analysis workflow followed for this job*

V.7.3.3 Functional Analysis

Functional annotation corresponds to the process of assigning a biological role to each of the genes studiedThe process of assigning a biological function to each of the genes investigated is known as functional annotation. It is based on looking for similarity between the unknown sequence in question and a group of annotated sequences to obtain the most relevant information about the gene's function and predicted outcome.

V.7.3.4 Functional annotation by NCBI Blast+

OmicsBox offers the possibility to run a standard BLAST algorithm against NCBI molecular sequence databases. For our query, the search was laumched against two databases: NR (non-redundant) DB and the SwissProt DB. The BLASTp algorithm was used for the contigs, who were already translated in-silico. The E-value was set to 0.00001. Only the top 20 hits were retrieved for analysis (Figure 18).

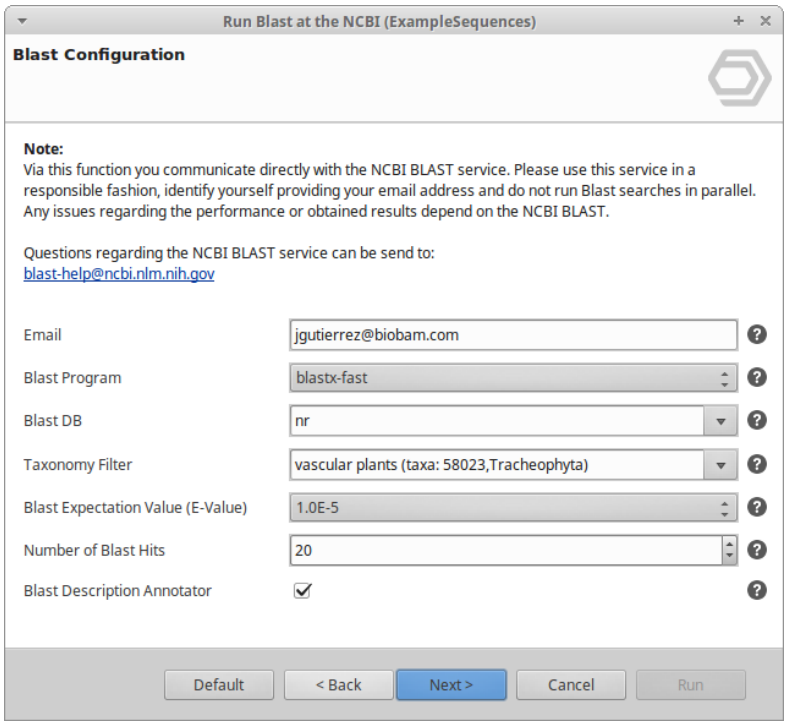


***Figure 18**: The BLAST configuration page*

V.7.3.5 Functional annotation: InterProScan

Proteins are functionally analyzed by categorizing them into families and predicting domains and key locations. InterPro uses predictive models known as signatures offered by multiple separate databases (referred to as member databases) that comprise the InterPro consortium to classify proteins in this manner. CATH-Gene3D , the Conserved Domains Database (CDD) , HAMAP , PANTHER , Pfam , PIRSF , PRINTS , PROSITE Patterns, PROSITE Profiles, SMART, the Structure–Function Linkage Database (SFLD) , SUPERFAMILY and TIGRFAMs are the 13 protein signature databases integrated into InterPro. InterPro combines protein signatures from several member databases into a single searchable resource, leveraging their respective strengths to provide a strong integrated database and diagnostic tool.(Blum et al., 2021)

The output files are in XML format by default (Figure 19).

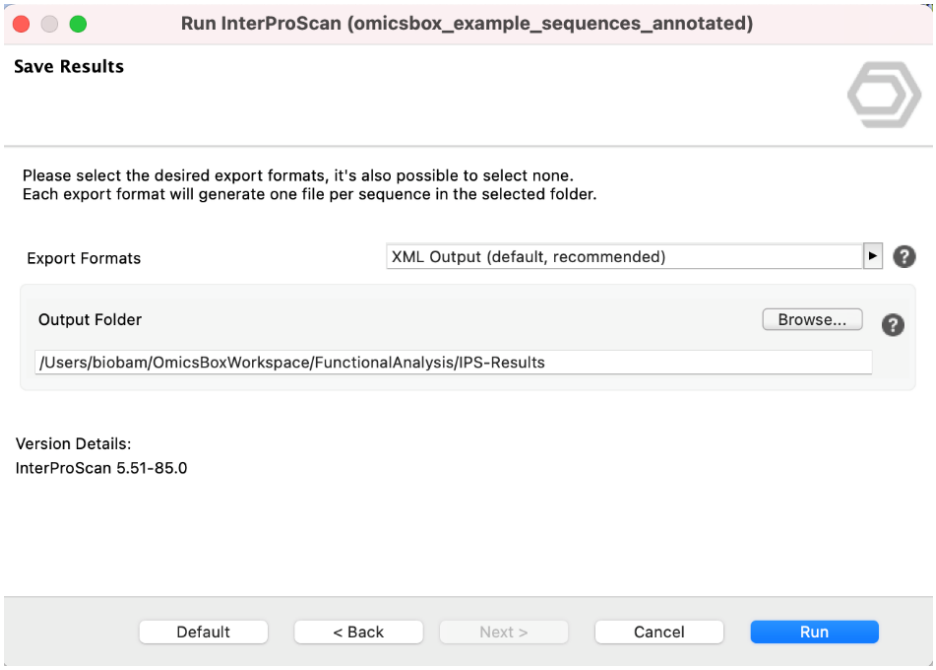


***Figure 19**: InterProScan output results configuration page*

V.7.3.6 Gene Ontology

The GO is a vocabulary of terms that is organized and controlled. Molecular Function (MF), Biological Process (BP), and Cellular Component (CC) are three non-overlapping ontologies that partition the terms. Each ontology defines a specific component of the functionality of a gene or gene product, as well as the relationships between the terms.

Each gene has a collection of terms that explain its function in the most explicit way possible. By definition, if a gene is linked to a term, it is likewise linked to all the parents of that term. (du Plessis et al., 2011)

The GOs are first retrieved during the Mapping step then are subjected to a selection following an annotation rule:

V.7.3.6.1 GO Mapping

Mapping is the process of retrieving GO terms associated with the BLAST search's Hits. OmicsBox goes through four mapping steps:

1. BLAST result accessions are used to retrieve gene names or symbols by utilizing two NCBI mapping files (gene info, gene2accession). The identified gene names are then searched in the GO database's gene-product table for species-specific entries.

2. GeneBank identifiers (gi), the primary blast Hit ids, are used to retrieve UniProt IDs from the PIR (Non-redundant Reference Protein Database), which includes PSD, UniProt, Swiss-Prot, TrEMBL, RefSeq, GenPept, and PDB.

3. Accessions are found directly in the GO database's dbxref table.

4. Accessions from BLAST results are directly searched in the GO database's gene-product table.(Götz et al., 2008)

It is important to note that the mapping requires protein IDs, that is why it is essential to run the BLAST search against protein databases.

V.7.3.6.2 GO Annotation:

An annotation rule is the process of selecting GO terms from the GO pool created by the Mapping step and assigning them to query sequences. This is the primary type of functional annotation in the current OmicsBox version.

An annotation rule (AR) (Figure 19) is applied to the retrieved ontology terms to perform GO annotation. The rule seeks the most specific annotations with a high degree of reliability. The specificity and stringency of this process can be adjusted.

- An annotation score (AS) is computed for each candidate GO. AS is made up of two additive terms.
- The first, direct term (DT), represents this GO's highest hit similarity weighted by an EC-related factor (Enzyme classes).
- The possibility of abstraction is provided by the AS's second term (AT). This is defined as an annotation to a parent node when many child nodes are present in the GO candidate collection. The number of total Gos unified at the node is multiplied by a user-defined GO weight factor, which controls the possibility and strength of abstraction. When the GO weight is set to 0, no abstraction occurs.

Finally, the AR chooses the lowest term per branch that exceeds a user-specified threshold. The terms DT, AT, and AR are defined in the figure 20. (Götz et al., 2008)

$$DT = \max(similarity \times EC_{weight})$$
$$AT = (\#GO - 1) \times GO_{weight}$$
$$AR : lowest.node(AS(DT + AT)) \geq threshold$$

***Figure 20**: The OmicsBox annotation rule*

The annotation configuration for our analysis was set at the default settings (Figure 21). A later filtering process was executed to the output to limit the GOs to the plants GOs, in order to retrieve the relevant annotations.

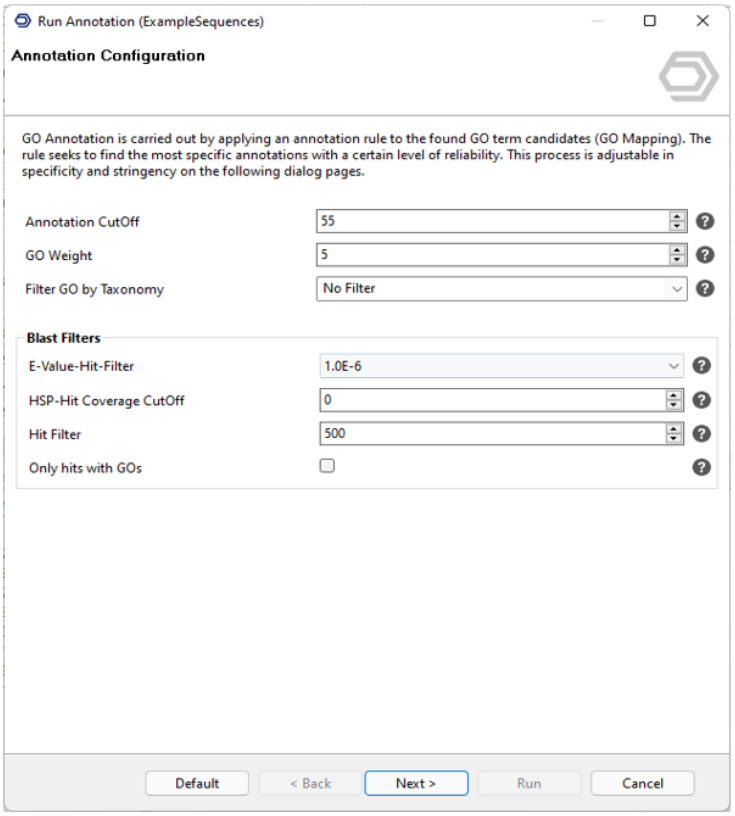


***Figure 21**: The annotation configuration page*

V.7.3.7 EggNOG Mapper

Another functional annotation tool is used for our query to retrieve more functional annotations from a different comprehensive database, using ortholog predictions rather than alignments. Because it avoids transferring annotations from paralogs, the use of orthology predictions for functional annotation is thought to be more precise than traditional homology searches.

Eggnog-mapper is a tool that uses precomputed EggNOG-based orthology assignments to perform fast functional annotation on novel sequences, transcriptomes or metagenomic gene catalogues (genes or proteins).

EggNOG employs two parallel approaches, first summarizing known attributes of group members and then determining which annotations can be robustly propagated to the group as a whole.

- Functional descriptions

Functional descriptions are provided using a heuristic procedure that seeks the most informative description that characterizes the annotated members of the group. This task builds primarily on associated free text descriptions from available public databases, but in the absence of inferences from this source will use GO assignments or construct descriptions based on protein domains characteristic of each group.

- Functional categories

The functional categories introduced in COG, KOG and arCOG are utilized. This is a controlled vocabulary of 20 functional categories to which those databases' orthologous groups are assigned, and non-supervised orthologous groups (NOGs) are similarly assigned to these categories using support vector machine classification with available annotation pathway or module membership, SMART or Pfam domain content, and Gene Ontology annotations as a feature space.(Huerta-Cepas et al., 2019)

For our query, the search was launched with the default settings, since it automatically adjusts to the input sequences (Figure 22). (Huerta-Cepas et al., 2019)

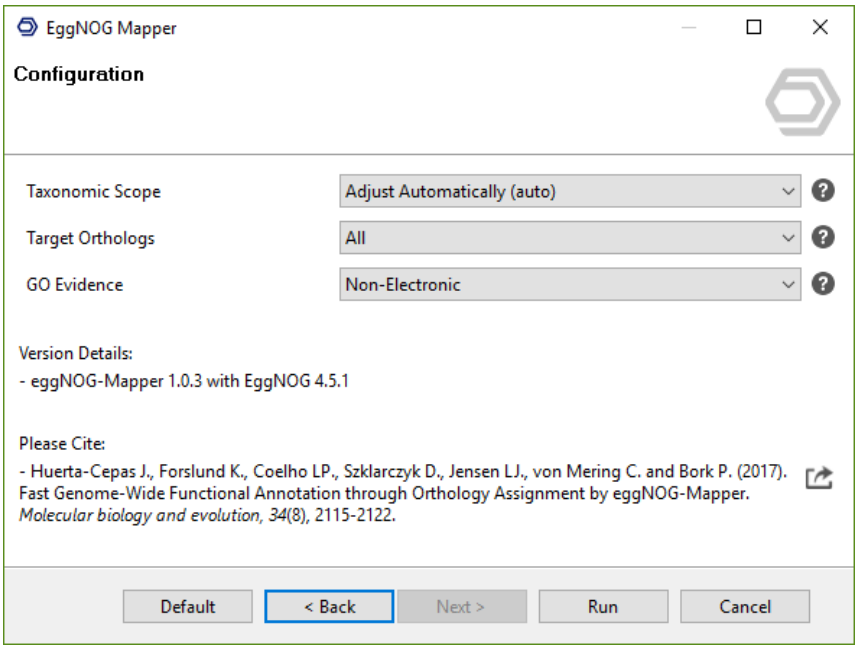


Figure 22: *The EggNOG search configuration page*

V.7.3.8 Mercator

Mercator4 (https://plabipd.de/portal/mercator4) is an online tool for annotating land plant protein sequences with functional annotations. The biological context of a protein is included in

the functional annotation, which concludes with a description of the protein's function. Mapman4 is a framework that organizes all accessible plant protein categories (called BINs) into a hierarchical structure of context descriptions.(Schwacke et al., 2019).

A third functional annotation was performed using the Mercator server (https://www.plabipd.de/portal/web/guest/mercator4) which makes it possible to predict the functions of protein in FASTA format.

V.7.3.9 KEGG pathway analysis

KEGG (Kyoto Encyclopedia of Genes and Genomes) is a database that combines genomic, chemical, and systemic functional data. Gene catalogues in fully sequenced genomes, in particular, are linked to higher-level systemic functions of the cell, organism, and ecosystem .(Kanehisa, 2000)

For genome/metagenome annotation, the KEGG database includes three main components:

- the KEGG GENES database contains annotated gene catalogs for complete genomes (referred to as KEGG organisms) as well as additional protein sequences.
- the KEGG PATHWAY, BRITE, and MODULE databases contain a knowledge base of high-level functions represented as molecular interaction, reaction, and relation networks
- the knowledge base of molecular-level functions associated with ortholog groups in the KO database, where the majority of KO entries are defined as nodes of the KEGG molecular networks in a context-dependent manner. (Kanehisa et al., 2016)

KAAS (KEGG Automatic Annotation Server) (https://www.genome.jp/kaas-bin/kaas_main?mode=partial) compares amino acid sequences against a carefully selected list of ortholog groups in KEGG GENES to provide functional annotations of genes in a genome. The result includes KO (KEGG Orthology) assignments as well as KEGG pathways and functional categories generated automatically.

The KAAS service (https://www.genome.jp/kaas-bin/kaas_main?mode=partial) was used to annotate the *crocus sativus* contigs. The GHOSTZ tool was set as a search program. GHOSTZ which is a homology search tool which can like BLAST, detect remote homologues. It is about 200 times more efficient than BLAST because it uses database subsequence clustering. The representative set is for Eukaryotes and the organisms selected included *Arabidopsis thaliana,* and all the monocots of the gene set, given that they share an ancestral link with *crocus sativus*, since it is itself a monocot (Figure 23).

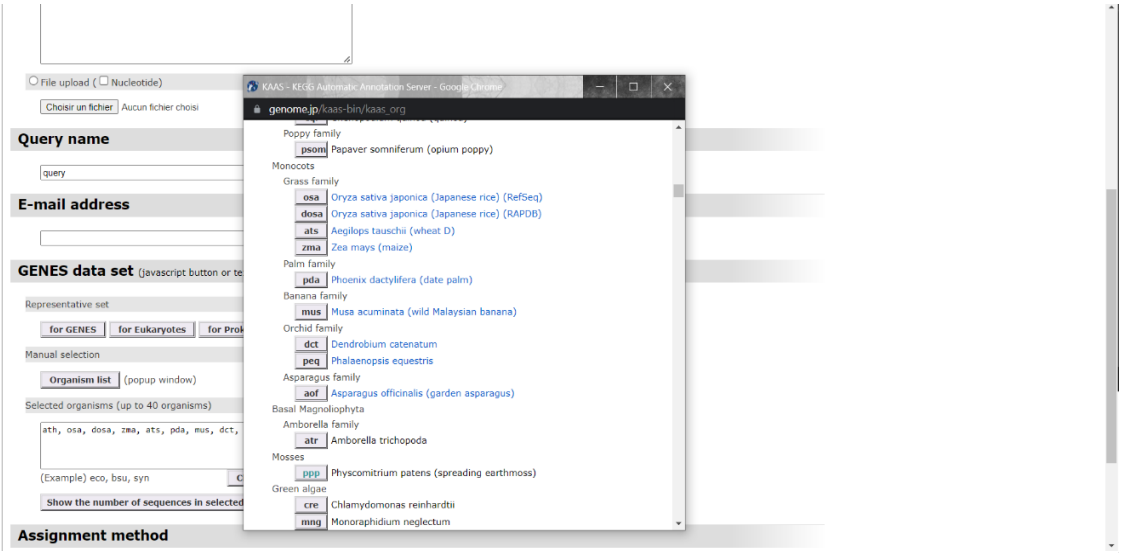


***Figure 23**: Configuration page of the KAAS search*

V.7.3.10 Primers design by Primer3

Among the purposes of our study is to design primers to amplify *crocus sativus* specific genes, to trace authentic saffron from counterfeits.

The design of the primers was conducted using the Primer3 web interface (https://primer3.ut.ee/) . This program picks primers relying on more several C++ libraries, such as libraries that calculate the melting temperatures of oligos and libraries that calculate the propensity of oligos to form hairpins or dimers or to hybridize or prime from unintended sites in the genome.

The design was set with default parameters except for Tm, where we set the conditions to :

- Min Tm: 55 Cº
- Opt. Tm: 60 Cº
- Max Tm: 65 C

VI. Results

VI.1 The saffron extract

The extraction process resulted with a paste-like extract, highly pigmented with a strong fragrance (Figure 24).

The micrp-tube containing the extract was sealed and wrapped in aluminium foil to prevent the bioactive continence from reacting with light, since all the biochemical species present in the stigma are light sensitive and degrade easily from long exposure to air and light.

Figure 24 *The saffron extract*

VI.2 Bacterial inhibition:

Both quantitative and qualitative growth tests were performed on five bacteria isolated from soil, who have recorded pathogenicity for the Human. The stock solution was prepared under aseptic conditions (Figure 25).

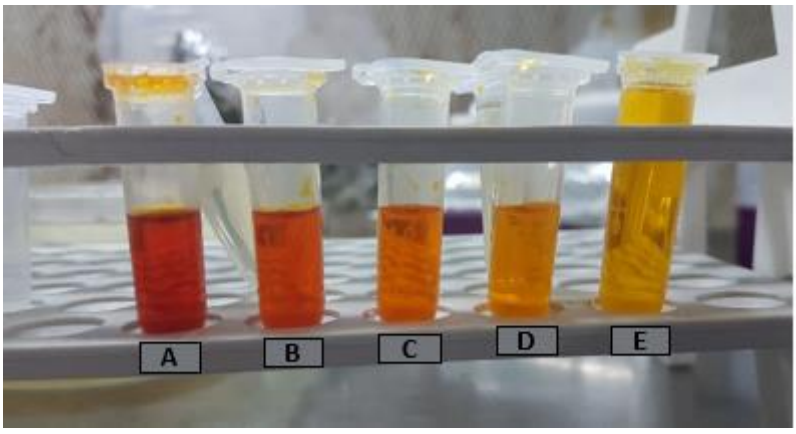


Figure 25: *The different dilutions (D) of the saffron extract*

A: mother solution [C]= 45mg/ml

B: [D1]= 22.5mg/ml *D: [D3]= 5.6mg/ml*

C: [D2]= 12.25mg/ml *E: [D4]= 2.8mg/ml*

The extract was diluted in sterile distilled water; the solution was then filtered (0,45nm filters) in a micro-tube. For the series of dilutions of the extract, the concentrations varied from 22mg/ml to 28mg/ml (Figure 25).

VI.2.1Qualitative test

After a 24h incubation, in 37°C, we compared the results of our tests against the positive and negative controls. The comparison consists of checking the inhibition zones around the disks containing the studied sample against the positive control.

An inhibition zone represents the diffusion of an agent that inhibited the growth of a bacteria (Figure 26).

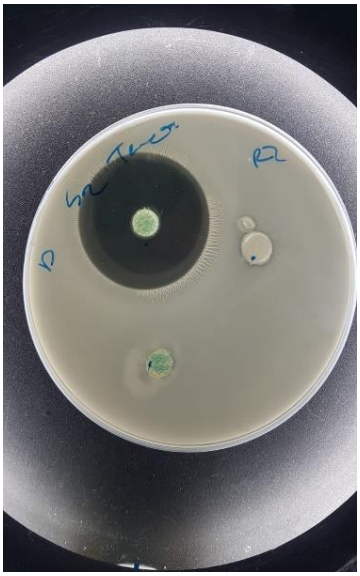


Figure 26*: an example of a clear inhibition zone against FOX antibiotic*

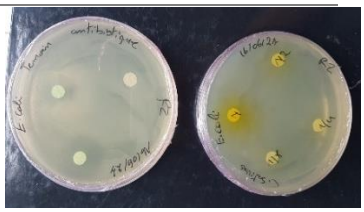
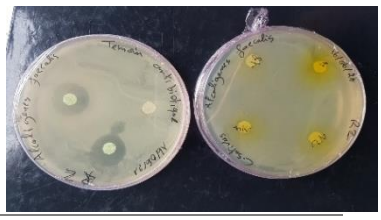
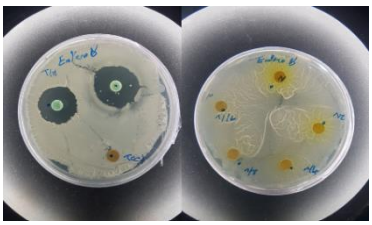
The study of the effect of any substance is coupled with a control, where known antibiotics are used as a reference, to compare the diameter of inhibition zones to, if present. This is what a positive control represents. The absence however of an inhibition zone, means that the bacteria was able to adapt to the added agent and grow.

For this experiment, the tested substances are not directly discharged on the solid medium but are dispensed on sterile disks. The negative control consists of an empty sterile disk that will provide a visual guarantee to the sterility and so, the absence of potential contaminating agents from the used disks. Making sure that only our strain is present in the Petri Dish.

In our experiment, compared to the positive control, there was a total absence of any inhibition, since we did not observe the apparition of an inhibition zone around the extract-soaked disks, implicating that these strains were unaffected by the saffron extract (Table 7).

After a 24h incubation, in 37°C we obtained the following results (Table 7) with saffron extract, all bacteria, compared to control of the tested bacteria exhibited any inhibition.

Table 7 : *The results of the antibacterial test on solid medium*

Bacteria	Test against stigma extract	control			**Pictures**
		TZP (+)	**FOX(+)**	**Disk (-)**	
Escherichia coli	(-)	(+)	(+)	(-)	
Alcaligenes feacalis	(-)	(+)	(+)	(-)	
Enterobacter aerogenes	(-)	(+)	(+)	(-)	
Microbacterim resistens	(-)	(+)	(+)	(-)	
Bacillus subtilis	(-)	(+)	(+)	(-)	

VI.2.2 On liquid medium:

After a 24h incubation under agitation, in darkness, the optical density of all the replicas was measured against control (Figure 27). The same procedure was followed for the positive control (bacteria + extract dilution medium).

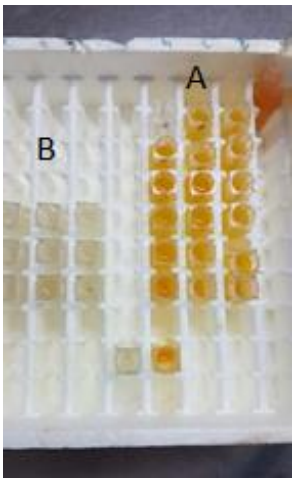


Figure 27 *antibacterial test on liquid medium.*

A: Extract + bacteria; B: control (bacteria + dilution medium)

After gathering all the data in a tabular form, as an Excel file, we ran the results through a statistical analysis using R. As represented in the figure below (Figure 28). The variations in growth of the bacteria+ extract in comparison with the control shows that the *crocus sativus* stigma extract did influence the growth of the different bacteria but not in a statistically significant way (p< 0,5).

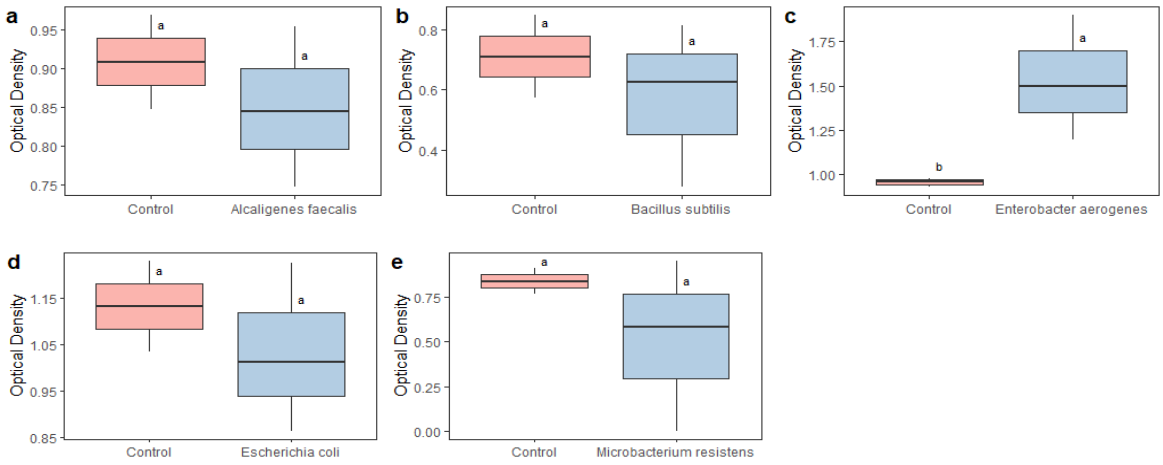


Figure 28 *: Boxplot representing the effect of the saffron extract on the different bacteria against control.*

The totality of the information yielded from the two tests permits us to conclude that the extract had no significant effect on these particular bacterial strains.

VI.3 The biochemical characterization:

VI.3.1The polyphenolic profile

The first step in determining the phenolic content of the extract is to realize a standard curve using Gallic acid, in varied concentrations.

For the standard curve, the Gallic acid is used as a positive control. Different dilutions are necessary to prepare the standard curve. Dilutions of galllic acid

To ensure that the experimental conditions are similar, we prepared the control alongside the test with the extract (Figure 29).

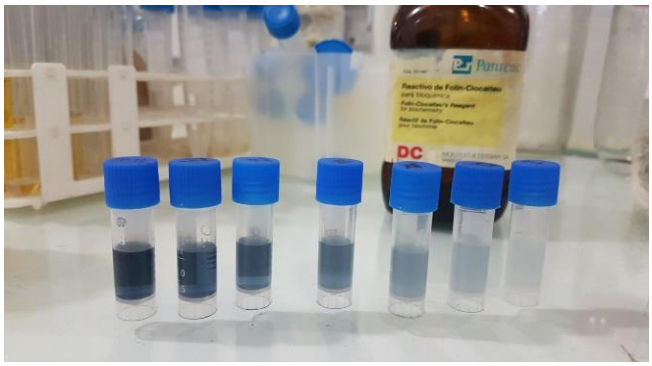


***Figure 29**: The control prepared in different dilutions as indicated by the gradual blue coloration, the colour intensifies, as the concentration gets higher.*

The reaction requires room temperature and obscurity. The incubation time must be respected, and the tubes must remain immobile, since at the end of the reaction a pellet forms. We collected the supernatant to measure the optical density (Figure 30).

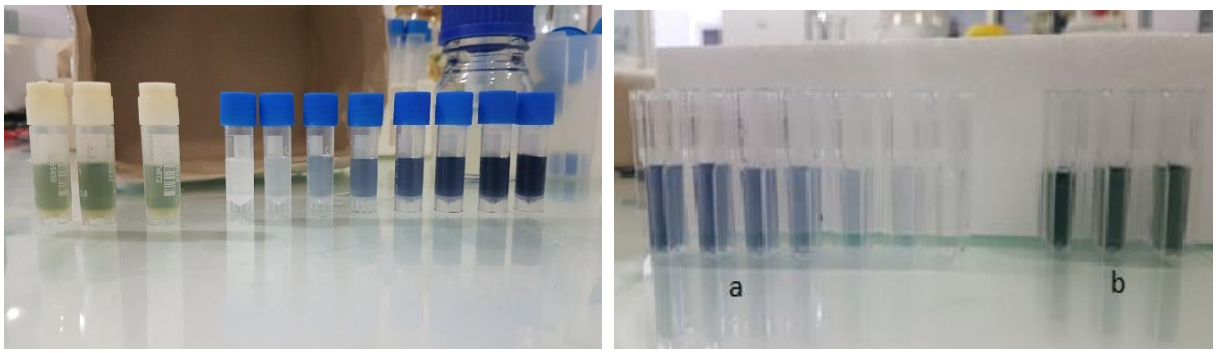


***Figure 30**: The F-C reagent reaction with Gallic acid and saffron Extract after incubation time. The Gallic acid control (a); the extract mixed with the F-C reagent (b).*

After the measurement of the Optical Densities of the different concentrations of the Gallic acid, the recorded OD were transferred on an Excel sheet. The measures were organised in a table then, using the Charts module on Excel, were used to trace a standard linear curve (Figure 31).

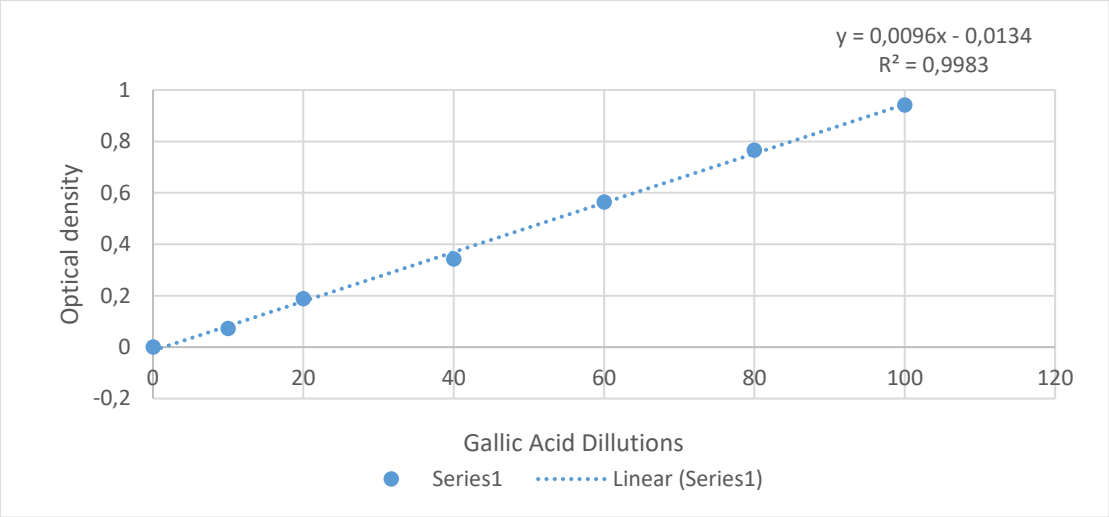


Figure 31 *: Standard Curve*

Total phenolic Content (TPC) results are usually expressed as Gallic acid equivalent, by extracting the Trend Line equation and replacing the 'y' with the OD average (Table 8) of the extract and finding 'x'.

Table 8: *The extract Optical Densities reads*

Optical Density reads of the Extract+ F-C reagent	**OD values**			**Average**
	0.694	0.714	0.833	0.7472

The average OD for saffron extract is: 0,747 . So, x= 79,20.

This means that the polyphenol content of the saffron extract is **31.68 mg GAE/g Extract**

Meaning: TPC = 6.33 mg GAE/g DW

VI.3.2 The radical scavenging activity

Optimizing the reaction in the laboratory setting:

The DPPH scavenging reaction is quite sensitive to its environment. It needs a cool and very dark setting, and a rapidity in mixing the different components, since the DPPH powder is dissolved in Methanol, rendering it easily and rapidly subject to evaporation as soon as it encounters air. Keeping in mind that DPPH is costly; the limited availability leaves no room for error.

The DPPH solution is of a purple colour, indicating a reduced form of the DPPH· molecule. The positive control, where the totality of the solution is oxidized, loses the purple colour and becomes yellow to transparent, signifying that the totality with the DPPH solution has been oxidized. The substance of reference for the positive control is ascorbic acid.

The test was performed in three replicas, with the ascorbic acid as a positive control. After incubation time, the completely discoloured controls indicate that the ascorbic acid was able to completely neutralize the DPPH radicals (Figure 32).

The recorded optical densities were transferred to an Excel sheet where the further calculations and graphs were made

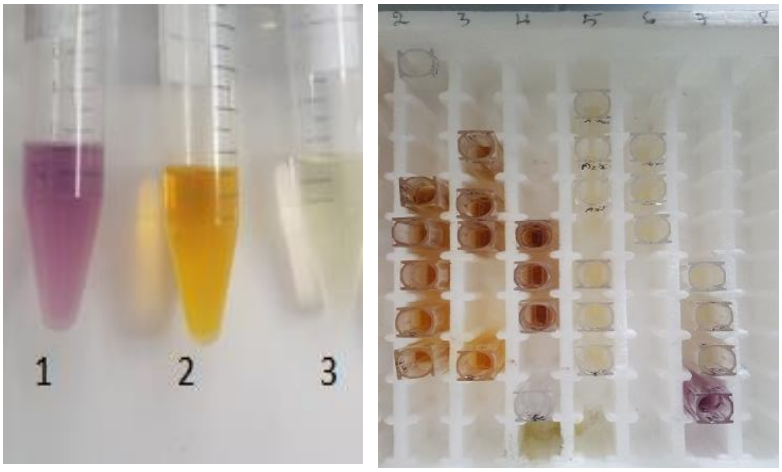


***Figure 32**: The scavenging activity of the saffron extract and ascorbic acid as positive control.*

1: DPPH negative control; 2: DPPH+ Extract; 3: Positive control Asc acid+ DPPH

Antioxidant activity quantification:

After collecting the data, this equation was used to calculate the percentage of antioxidant activity for each concentration:

$$AA\% = [(C_{OD} - S_{OD} / C_{OD})] \times 100$$

Where:

- AA% is the antioxidant activity.
- C_{OD} is the absorbance of the control solution (containing only DPPH ·) .
- And S_{OD} is the absorbance of the DPPH · solution containing sample.

The results are presented below (Table 9):

Table 9: *DPPH scavenging activity of the saffron extract*

Extract concentration mg/ml	**DPPH scavenging capacity of c.sativus(%)**
0,25 mg/ml	29%
0,5 mg/ml	32%
1 mg/ml	36%
2 mg/ml	48%

Antioxidant activity by the DPPH neutralisation method is often reported as IC50, which is defined as the efficient concentration of the antioxidant necessary to reduce the initial DPPH concentration by 50%.(Munteanu & Apetrei, 2021)

For this purpose and using the data above, we traced the following scatter plot diagram:

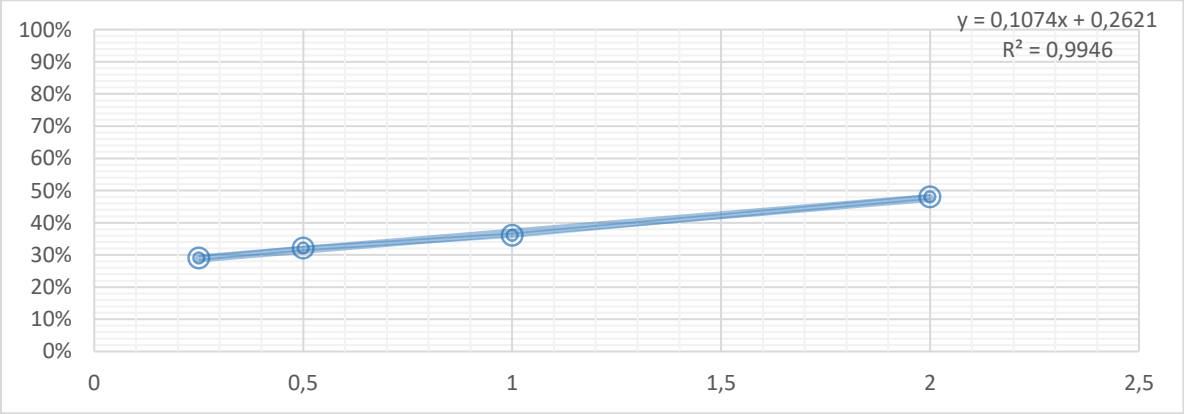


Figure 33: *DPPH scavenging capacity of the methanolic extract of crocus sativus stigma*

Using this diagram, we are able to calculate the IC50, by extracting the Trend Line equation. We obtain:

IC50 = 2.21 mg/ml

This result means that, to be able to reduce half of the free radicals existing in the solution, we will need 2.21 mg of saffron.

VI.4 ESTs screening results

VI.4.1Results of filtering and assembly

A total of 6767 *crocus sativus* ESTs were downloaded in a FASTA file from NCBI database on 11th April 2022.

EG assembler generated 612 contigs and 737 singletons. Statistics on assembled contigs were estimated through the web interface of the Quast program (http://cab.cc.spbu.ru/quast/) (Table 10). The assembled contigs were uploaded in multi FASTA format.

Table: Statistics of contigs assembled by EG assembler estimated by Quast

Table 10: *Results of EG assembler by Quast*

Features	Numerals
EST sequences	6767
Overall assembly size (pb)	330,403
Number of contigs	655
Longest contig size (bp)	1807
Percentage of GC (%)	45.95

VI.4.2Structural annotation results

The Augustus program allowed the prediction of 527 coding sequences which were translated in silico. Protein sequences were downloaded in multi-FASTA format.

VI.4.3 Functional analysis results

The different modules used in the OmicsBox software integrate the individual outputs of each algorithm to the contigs list, where they are manually fused in a single table by uploading the outputs of the queries into the one output file (Figure 34).

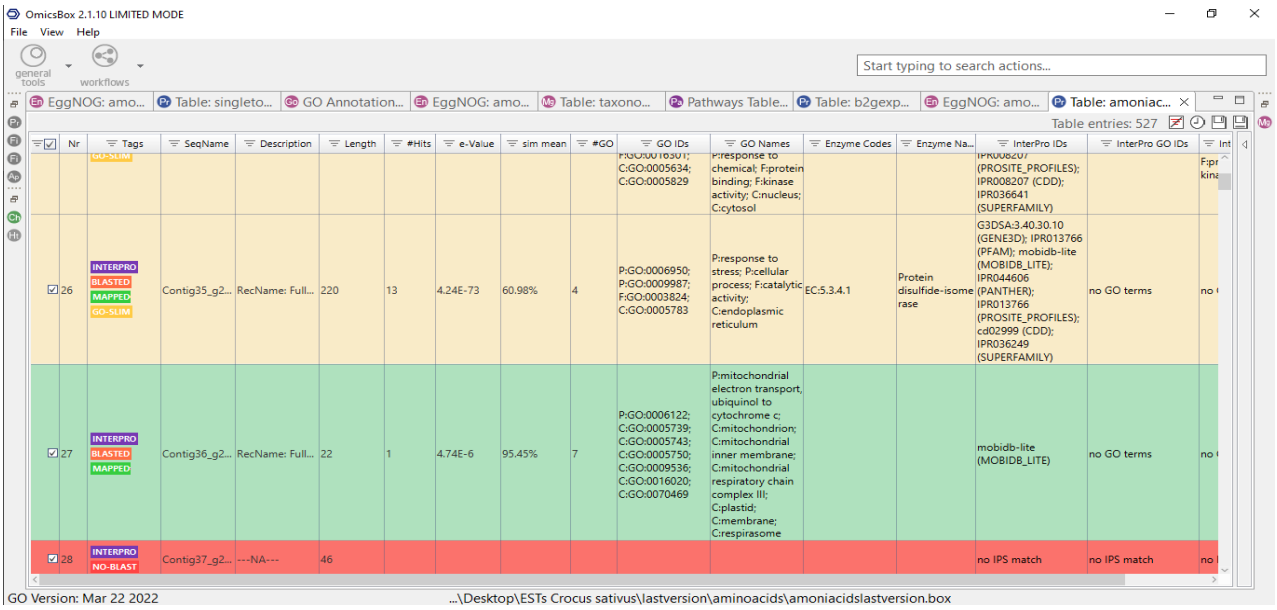


***Figure 34**: The OmicsBox's different outputs merged together*

- Blast annotation results

BLAST+ search resulted in 364 blasted sequences out of the 527 putative protein sequences, which represents 69.07% of the input sequences. For our data set, 42.85% of the top blasted EST-contigs from *crocus sativus* were conserved to Arabidopsis thaliana, followed by Oryza sativa (Figure 35).

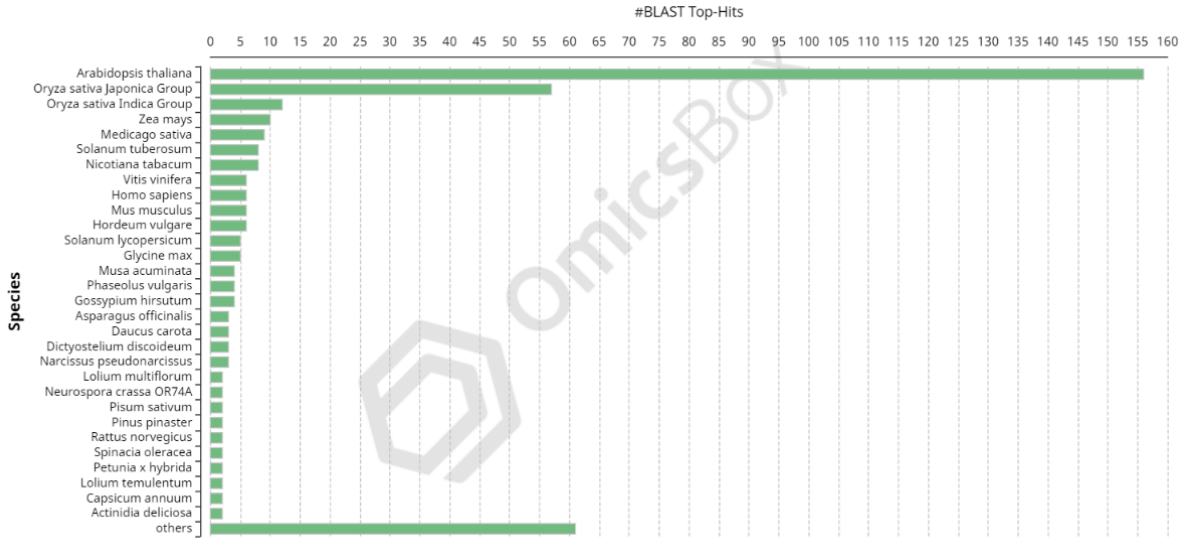


***Figure 35**: Top Hit distribution for our ESTs putative proteins*

- InterProScan Results

For our query sequences, the IPS analysis showed: 70 seq. without IPS, 457 seq. with IPS, and 246 seq. with GOs (Figure 36).

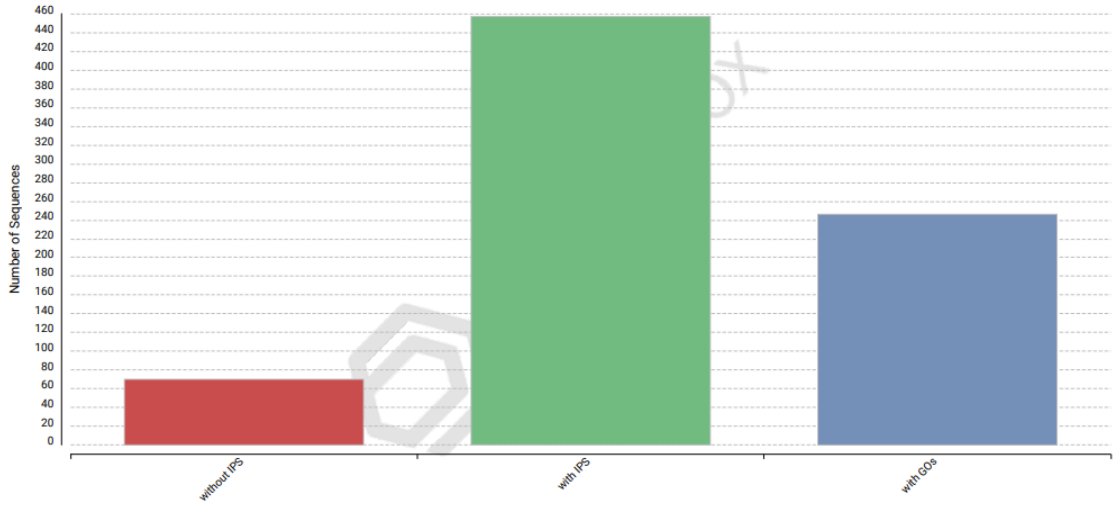


***Figure 36**: InterProScan annotation results*

- Gene Ontology results

The assembled EST-contigs (527) were further use for gene ontology (GO) based functional characterization though omics box. There were 1,890 gene ontology terms recovered for 362 sequences. To predict the exact function of these sequences, contigs are grouped as per the gene ontology terminology though omics box at level 3 scheme. (Figure 37).

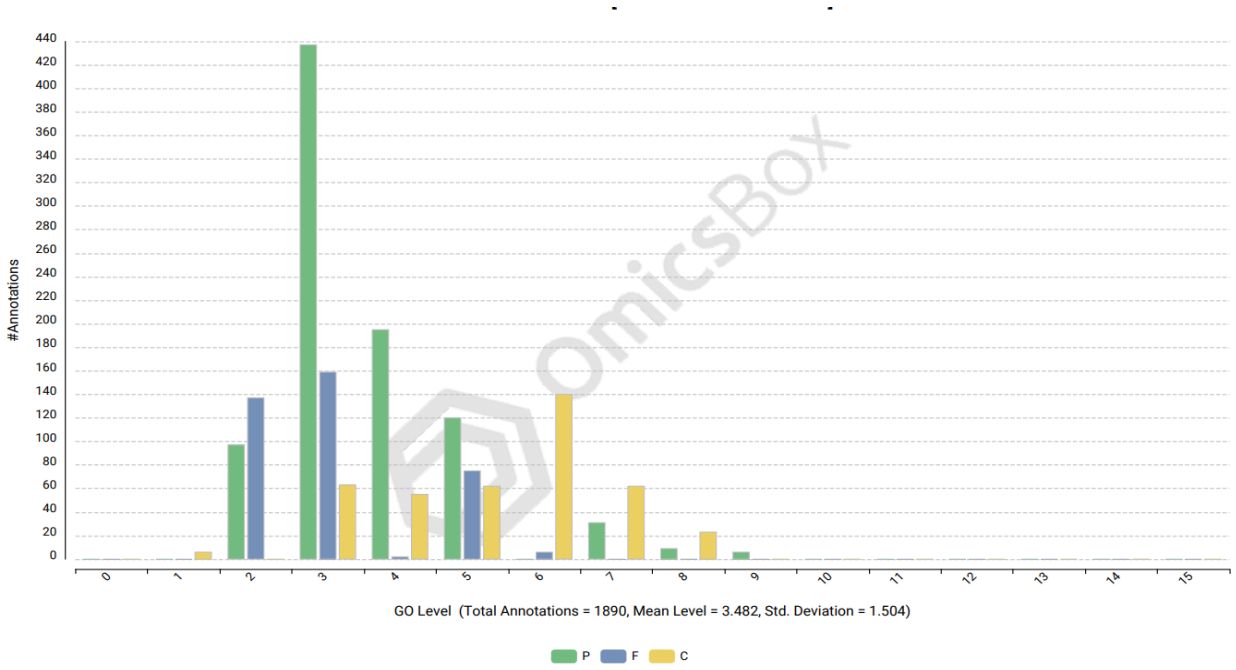


***Figure 37**: GO Mapping results*

P: Biological Process *F: Molecular Function* *C: Celula Component*

The ontology levels being BP (Biological process) MF (Molecular function), and CC (Cellular component) . The top 10 classes from each category are shown in Figure 38. The most abundant molecular function to which our annotated sequences belong is catalytic activity, protein binding, binding and hydrolase activity. Correspondingly, the top biological process to which the highest number of sequences belong is response to stress, followed by response to chemical, cellular process and biosynthetic process. Further classifications of these GOs are listed in Annexe 3.

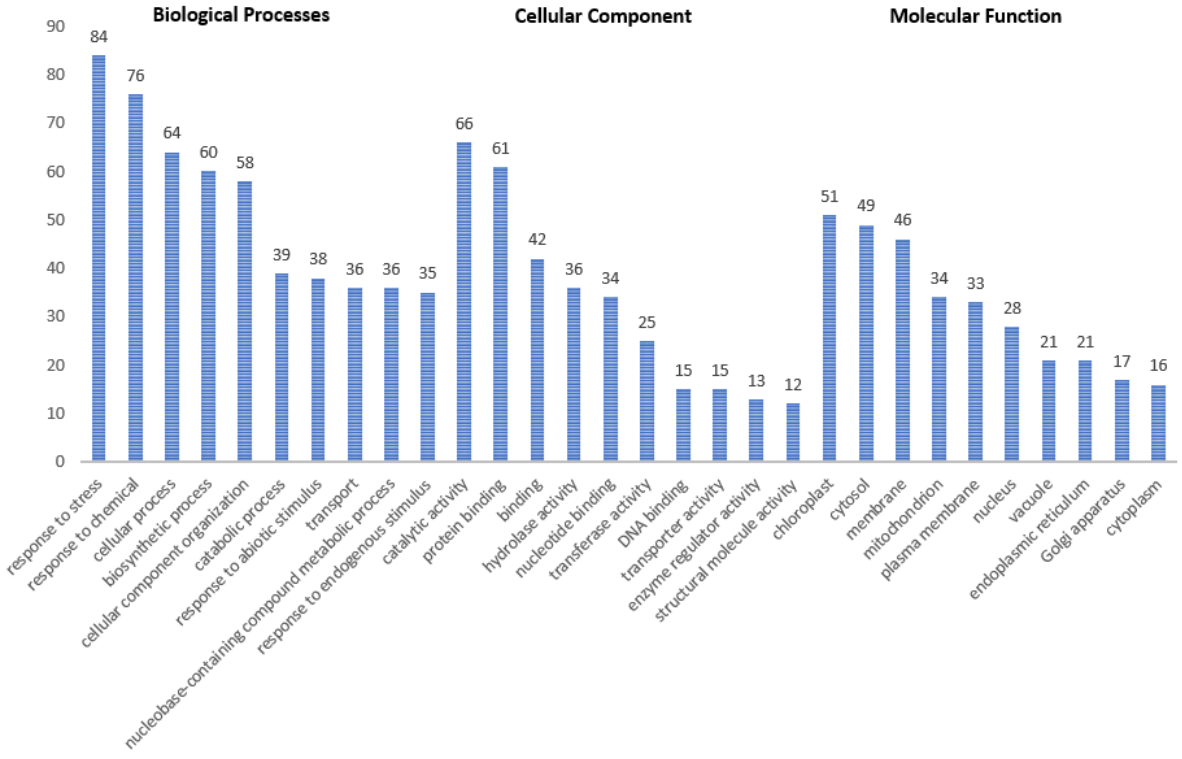


Figure 38: *GO classification of crocus sativus ESTs, Bar chart represents number of sequences belonging to top 10 biological process, cellular component and*

- EggNOG annotation:

The output of the EggNOG analysis can be visualized in a tabular form, as represented in Figure 39:

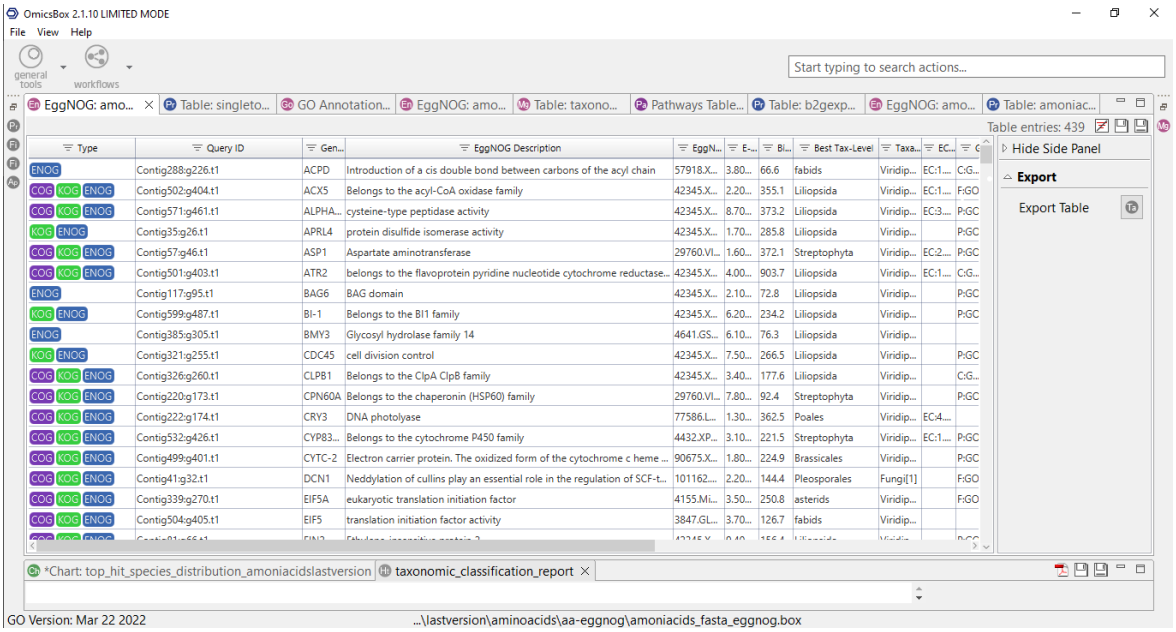


***Figure 39**: EggNOG's output, presented in tabular form in the OmicsBox Metagenomic module*

Total amount of input sequences being 527, the number of GO annotated sequences equalled to 236 / 44.78%.

The number of GO annotations: 1775 with an average GOs per sequence of 7.52. Clusters of Orthologous Groups of proteins (COGs), a phylogenetic classification of the proteins, show that the Metabolism category is the richest in number of input sequences (Figure 40) .

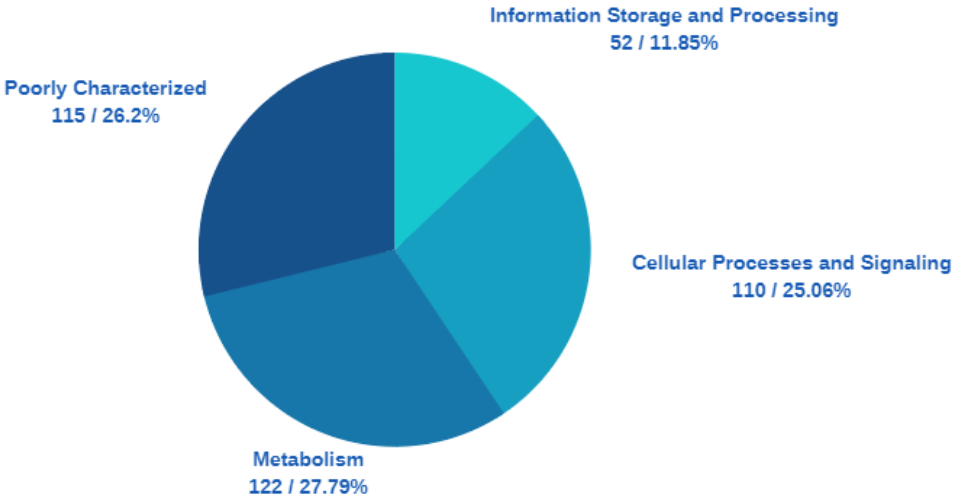


***Figure 40**: COG Categories Distribution chart*

In a close second, Cellular processes and Signalling and finally, Information Storage and Processing. About 26,2 % of the input sequences could not be properly characterized.

Zooming in the Metabolism category, detailed in Figure 41, we find that 17 contigs were predicted to have functions related to secondary metabolism, representing 15.57% of the category. The EggNOG algorithm generated the largest number of predictions related to secondary metabolism, compared to the other functional annotators used in this study.

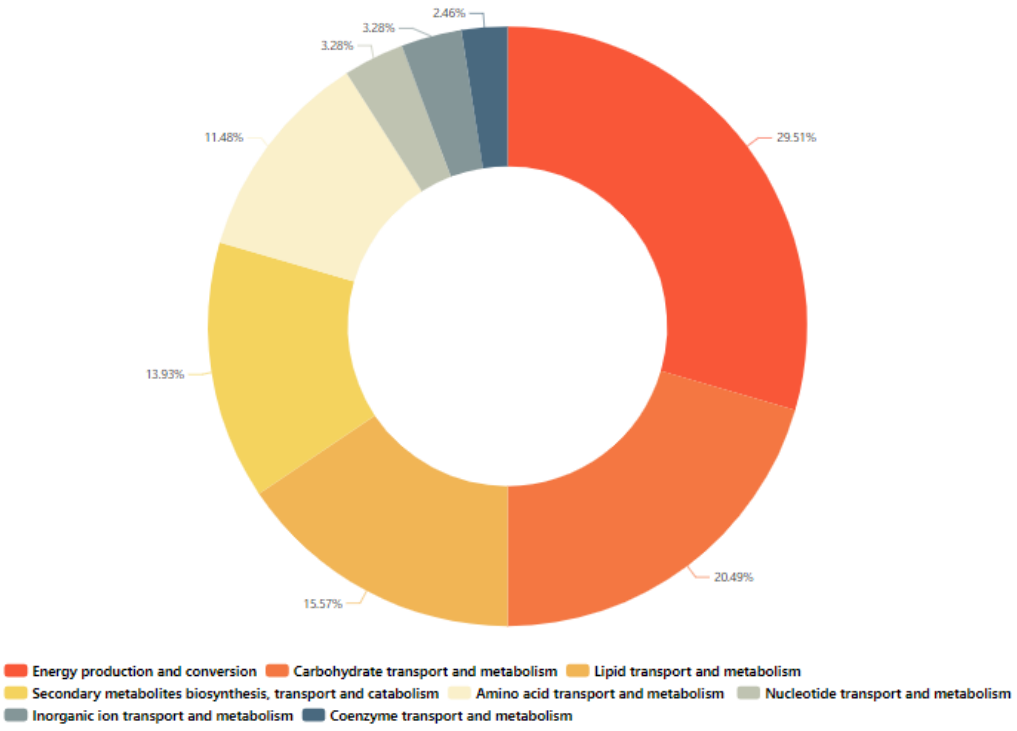


Figure 41: *Metabolism subcategories distribution chart*

VI.4.4 Mercator results

The Mercator analysis annotated 289 of the 527 sequences of the input. The 289 annotated sequences were classified into 186 classes. They are attributed to 152 of the 5251 bins in the Mercator database (Figure 42).

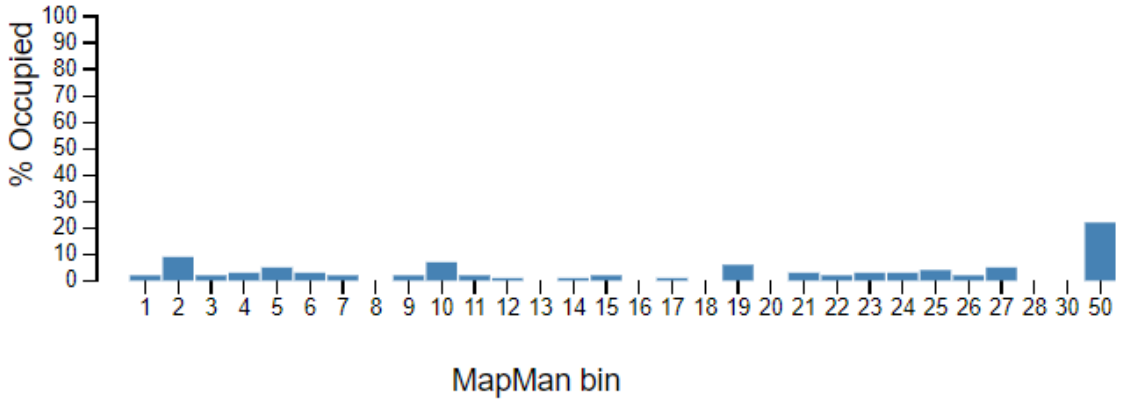


***Figure 42**: Mercator MapMan bin occupancy rates*

The secondary metabolism (Bin 9) was occupied at 2.46% with 2 out 81 Bins occupied:

- 9.1.2: Secondary metabolism.terpenoids.methylerythritol phosphate (MEP) pathway
  - ⇒ 9.1.2.7: Secondary metabolism.terpenoids.methylerythritol phosphate (MEP) pathway.4-hydroxy-3-methylbut-2-enyl diphosphate synthase' ►'contig63:g51.t1'
- 9.1.4: Secondary metabolism.terpenoids.terpene biosynthesis'
  - ⇒ Secondary metabolism.terpenoids.terpene biosynthesis.mono-/sesquiterpene-/diterpene synthase' ► 'contig127:g102.t1'
  - ⇒ Secondary metabolism.terpenoids.terpene biosynthesis.mono-/sesquiterpene-/diterpene synthase' ► 'contig446:g357.t1'

VI.4.5 KEGG pathway analysis

A total of 276 pathways were generated from the search, a screenshot of the online output list is shown in Figure 43. The KEGG pathway analysis showed 74 matches for the ko01100 pathway, the Metabolic pathway. Within that, the biosynthesis of secondary metabolism with 37 hits.

***Figure 43**: KEGG mapped pathways online output*

The KEGG mapper contains in its database manually gathered pathways, exploiting the existing data, to provide a better understanding of the possible roles a protein or an enzyme in the pathway they belong to, as well as how it relates to adjoining pathways (predecessors, products and others).

For our query, the mapped contigs can be seen as the red pathways in on the pathway module (Figure 44). On top of these direct secondary metabolism hits, we can trace back more pathways involved or leading into the secondary metabolism, more results are undergoing analysis for publication.

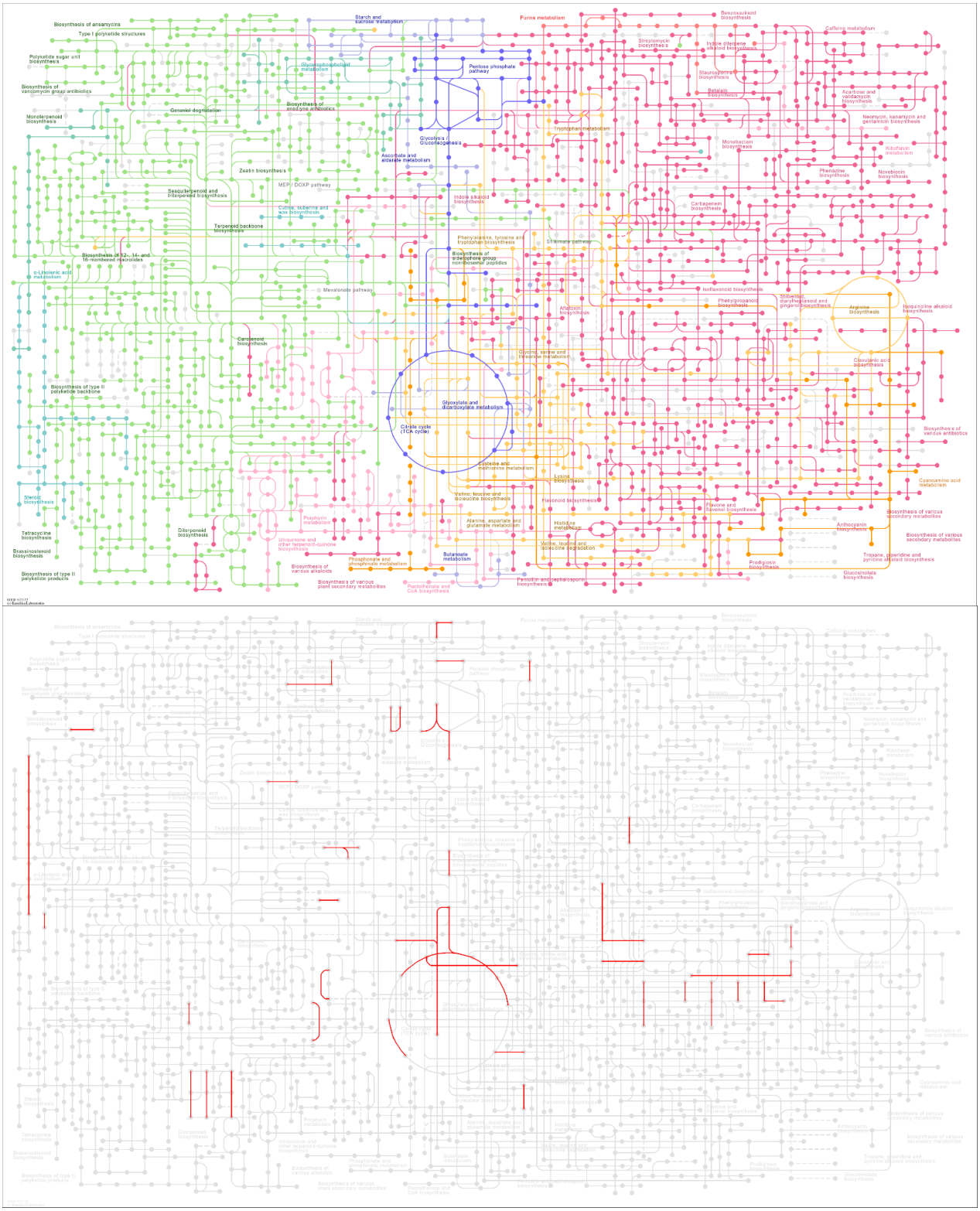


Figure 44: *Biosynthesis of secondary metabolites map (ko01110) and the matched alignments from our query.*

KEGG mapper results, illustrating the secondary metabolism positive matches (red pathways on the second map, rendered colourless for a better visualization) on the general map (upper map), representing the available mapped data on plant secondary metabolism.

Going from the more common phenols and flavonoids biosynthetic pathways to the more special saffron metabolites, the full pathway leading to their biosynthesis is unknown. A lack that is mainly due to the absence of the full genome sequence, as well as a limited cDNA database. Through the KAAS, relying only on positive alignment, with sequences from other species with fully sequenced genome, we were able to trace the crocin, picrocrocin and safranal's parent biosynthetic pathway, the terpenoid backbone biosynthesis, through a distinct enzyme match, present in our query (Figure 45). The product of the pathway, Geranyl diphosphate, is the precursor for crocin biosynthesis, Zeaxanthin.

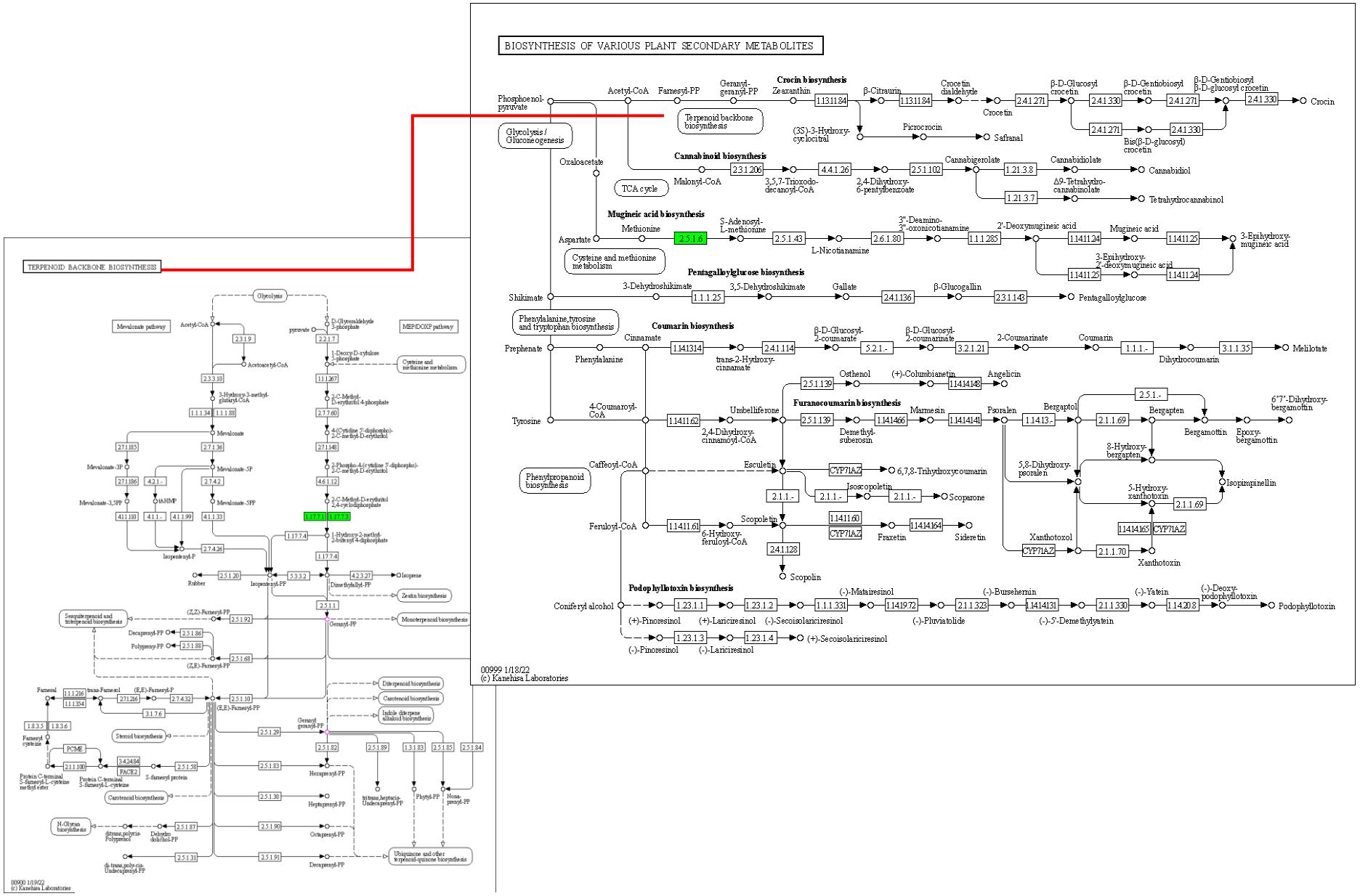


Figure 45: *On the left: Terpenoid Backbone Biosynthesis map, with a positive alignment coloured green, enzyme that catalyses the production of Geranyl-PP, coloured in pink. Linked to the genesis of crocin biosynthesis pathway, in the second map.*

PRIMERS DESIGN

The *crocus sativus* secondary metabolites

To design protocols for identification and authentication of saffron samples, we will extract all the contigs related to secondary metabolism and proceed into filtering them and boil down to the putative proteins identified in the *crocus sativus* species, with best scores of alignment to increase the specificity, and use the said sequences to design primers.

Isolation of target sequences

The goal of our study is to identify, trace, and map the ESTs from NCBI database that relate to secondary metabolism. To do so, we ran different functional annotations, against different functional annotation databases to extract as many positive alignment, and functional prediction as possible.

- From the Gene Ontology groups, we were able to isolate 7 sequences
- From the EggNOG annotation, 17 sequences were retrieved
- From Mercator, 3 sequences were attributed to the secondary metabolism bin.

On top of these automatically generated sequences, we ran a manual search against known proteins identified in the *crocus sativus* species, we selected as reference studies Baba et al., 2015 and Baba & Ashraf, 2016.

Table 11: *List of contigs generated from the different functional annotators as well as the manually selected*

Gene Ontology	Contig501:g403.t1	Contig557:g448.t1
	Contig589:g477.t1	Contig94:g78.t1
	Contig74:g58.t1	Contig532:g426.t1
	Contig587:g475.t1	
EggNOG	Contig12:g9.t1	Contig409:g325.t1
	Contig84:g69.t1	Contig605:g492.t1
	Contig249:g193.t1	Contig642:g512.t1
	Contig257:g199.t1	Contig541:g434.t1
	Contig286:g224.t1	Contig96:g80.t1
	Contig317:g251.t1	Contig29:g22.t1
	Contig332:g264.t1	Contig587:g475.t1
	Contig341:g272.t1	Contig532:g426.t1
Mercator	Contig63:g51.t1	Contig127:g102.t1
	Contig446:g357.t1	
Manually selected	Contig269:g209.t1	Contig7:g6.t1
	Contig439:g350.t1	Contig189:g149.t1
All annotations combined without repetitions = 28 contigs	Contig7:g6.t1	Contig317:g251.t1
	Contig12:g9.t1	Contig332:g264.t1
	Contig29:g22.t1	Contig341:g272.t1
	Contig63:g51.t1	Contig409:g325.t1
	Contig74:g58.t1	Contig439:g350.t1
	Contig84:g69.t1	Contig446:g357.t1
	Contig94:g78.t1	Contig501:g403.t1
	Contig96:g80.t1	Contig532:g426.t1
	Contig127:g102.t1	Contig541:g434.t1
	Contig189:g149.t1	Contig557:g448.t1
	Contig249:g193.t1	Contig587:g475.t1
	Contig257:g199.t1	Contig589:g477.t1
	Contig269:g209.t1	Contig605:g492.t1
	Contig286:g224.t1	Contig642:g512.t1

Filtering direct BLAST hits to *crocus sativus* NCBI sequence

Off these 24 sequences, we successfully found 6 direct BLAST hits to *crocus sativus,* listed in Table 12. These contigs, scoring the highest and with lowest e-value, against identified genes in the saffron flower. The designed primers listed in Table 13

***Table 12**: The list of contig sequences and corresponding best scored hit*

Contig7:g6.t1 ADVPYFQRVAAAHQIHHSEKFEGVPYGLFMGPKELEEIGG LKELEKEVSRRIKAYNNSAEIKT	**>CAC95130.2 beta-carotene hydroxylase [*Crocus sativus*]**
Contig189:g149.t1 HETREEFLICVEEVTEGEKAERIRERSSEMKRAATEAVAEG GPSDKNIQAIVDEIMAIHE	**>CAE48292.1 glucosyltransferase 3 [*Crocus sativus*]**
Contig249:g193.t1 KDYFVDERKKLASTRAMDNAGLKCAIDHILEAEKKGEINE DNVLYIVENINVAAIETTLWSIEWGLAELVNHPDIQQKLRH ELDTILGPGVQVTEPDIQKLPYLQAVVKETLRLRMAIPLLV PHMNLNDAKLGGYDIPAESRILVNAWWLANNPAHWKDPE EFRPERFLEEEAKVEASGNDFRYIPFGVGRRSCPGIILALPIL GITIGRLVQNFELSPPPGQSKIDTSEKGGQF	**>QBF29351.1 cinammate 4-hydroxylase [*Crocus sativus*]**
Contig269:g209.t1 RSRFKTVGSNTLSAIIQNLEHRGRKVSCVIYTFFVSWAADV ARQHAIPSVQYWIQPATVFAIYYHYFHGYESVVAAHSHDPS YPINLPGLPPVQVRDLPSFLTIKPDDPYAVVLSMIRDSFEGL DREGTKTKVLVNTFGQLEADAILAVDKMDIIPVGPILPCKG GVSRGDLLKEDEKGYMEWLDSKPENSVVYVSFGSLAVL	**>ACM66950.1 flavonoid glucosyltransferase [*Crocus sativus*]**
Contig439:g350.t1 MAMNNVRQFLCMVGGDGETSYAKNSRIPEKAIMRTKPIVE EAIKEVYNSLQPKSLVVADLGCSSGPNTFLVISEIVEAIGDH CRKLGHNPPEIQYILNDLHENDFNTLFDYSEKFKEKLKEVE EEVVPYVVGVPGSFYGRLFPQSSVHFIHSSYSLHWLSQGL MNEAKVEDFNLPIYAASMEEVMTIVETIGLFHVEQVEIFET NWDPFDDSSDDDESAFDNFASGKNVVNCSIRAVVEPMFEK YFGEAIMDELFSRYAKNVAKHLLGEKGKHVVFMMALRK	**>CAD70566.1 carboxyl methyltransferase [*Crocus sativus*]**
Contig587:g475.t1 ARVGTGFLTGEKVRSPGEEFDKVFVAICEGKAIDPLLECLK EWNGAPLPIS	**>QBF29348.1 phenylalanine ammonia lyase [*Crocus sativus*]**

Table 13: *List of primers designed using Primer3*

CAC95130.2 **beta-carotene hydroxylase**	OLIGO start tm gc% any_th 3'_th hairpin seq LEFT PRIMER 651 20 55.09 45.00 0.00 0.00 0.00 gatcacgctgtttggtattg RIGHT PRIMER 860 20 55.03 45.00 0.00 0.00 0.00 tccagctcttttaatccacc
CAE48292.1 **glucosyltransferase 3**	OLIGO start len tm gc% any_th 3'_th hairpin seq LEFT PRIMER 304 20 60.03 50.00 10.34 9.47 0.00 atagcaattgacccggccaa RIGHT PRIMER 479 20 59.97 60.00 9.00 9.80 0.00 cagtcatggctagcgggtag
QBF29351.1 **cinammate 4-hydroxylase**	OLIGO start len tm gc% any_th 3'_th hairpin seq LEFT PRIMER 500 20 59.88 55.00 0.00 0.00 0.00 tggaggctgagaagaaggga RIGHT PRIMER 716 20 60.11 60.00 0.00 0.00 0.00 gggagtttctggatgtcggg
ACM66950.1 **flavonoid glucosyltransferase**	OLIGO start len tm gc% any_th 3'_th hairpin seq LEFT PRIMER 1137 20 59.97 55.00 0.00 0.00 0.00 ttatcgcatccttcggtcgg RIGHT PRIMER 1381 20 60.03 60.00 0.00 0.00 0.00 acacctctctccccatctcc
CAD70566.1 **carboxyl methyltransferase**	OLIGO start len tm gc% any_th 3'_th hairpin seq LEFT PRIMER 175 20 60.04 55.00 0.00 0.00 0.00 ggatgctcttcgggtccaaa RIGHT PRIMER 395 20 59.90 60.00 0.00 0.00 0.00 ggcactcctaccacatacgg
QBF29348.1 **phenylalanine ammonia lyase**	OLIGO start len tm gc% any_th 3'_th hairpin seq LEFT PRIMER 718 20 60.03 50.00 0.00 0.00 0.00 aatttccaagggacgccgat RIGHT PRIMER 894 20 59.96 55.00 0.00 0.00 0.00 gcctttgaacccgtagtcca

VII. Discussion:

Antibacterial resistance to saffron extract

Humans have always used medicinal plants as a natural remedy for different bacterial infections and related diseases. The high antibacterial potential of some medicinal plants and plant-based foods is due to the enormous variety of their constituents. However, the potential of plant secondary metabolites, being a rich source for drug leads, is still not fully known due to huge diversity of these metabolites.

While few papers reported a recorded antibacterial activity of the saffron flower generally and the stigma extract specifically, the strains of gram positive and gram-negative bacteria we tested were not inhibited by the saffron extract at the different concentrations, the highest being 22.5 mg/ml.

Parray et al. (2015) reported antibacterial properties for the methanolic extract of the stigma and corms of saffron of Indian origin. The stigma showed effective inhibition of pathogenic bacterial strains *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, and Shigella flexneri*. With a particular effect, at a low concentration (200 µg/ml), against *S. aureus*.

Zara et al. (2021) demonstrated that other parts of the flower also have antibiotic potential such as stamens, the most sensitive strain was S. aureus (MIC, 4.5 mg/mL); extracts of saffron petals also exerted important antimicrobial activity against Staphylococcus aureus(Gandomi Nasrabadi et al., 2012). Similarly, Jafari-Sales & Pashazadeh, (2020) reported antibacterial effects of the methanolic extract of saffron petal on the tested bacteria. The highest effect was observed again with *Staphylococcus aureus.*

Many theories may explain why the tested bacteria resisted to our extract while the saffron stigma reportedly harbours an antibacterial potential. First, it can be due to intrinsic resistance, a trait that is shared universally within a bacterial species, independent of previous antibiotic exposure, and not related to horizontal gene transfer.

Several hypotheses might explain the resistance to the saffron extract: The most common bacterial mechanisms involved in intrinsic resistance are reduced permeability of the outer membrane (most specifically the lipopolysaccharide, LPS, in gram-negative bacteria) and the natural activity of efflux pumps as well as the Multidrug-efflux pumps. Gaining genetic material that confers resistance is also a possibility. The acquisition may be temporary or permanent. The most common

route for acquisition of outside genetic material being the plasmid-mediated transmission of resistance genes.

The antimicrobial resistance mechanisms that might also be responsible for our particular case could be by limitation of the uptake of an inhibiting molecule or its inactivation . There is a natural difference in the ability of bacteria to limit the uptake of antimicrobial agents. The structure and functions of the LPS layer in gram-negative bacteria provides a barrier to certain types of molecules. This gives bacteria innate resistance. As for the molecule inactivation process, there are two main ways: by actual degradation of the molecule, or by transfer of a chemical group to it. Drug inactivation by transfer of a chemical group to the drug most commonly uses transfer of acetyl, phosphoryl, and adenyl groups (Reygaert, 2018).

To conclude with certainty the reasons why these bacteria resisted to the saffron stigma extract, a pathway analysis should be conducted on the interactions between the extract and these bacterial strains to pin out the cellular mechanisms responsible of this phenomenon.

The phenolic content and scavenging capacity of the saffron extract

The amount and type of the active secondary metabolites has an apparent effect on the medical properties of saffron. The concentration and distribution of the secondary metabolites present in the *Crocus sativus* stigma, including phenols, are heavily influenced by two factors: the geographical provenance and the extraction method used. Phenolic compounds are good oxygen radical scavengers, since the electron reduction potential of phenolic radical is lower than that of oxygen radicals and also, phenoxyl radicals are less reactive than oxygen radicals (Bibi Sadeer et al., 2020).

Therefore, there is a positive correlation between the concentration of phenols present in the extract and the radical scavenging capacity. However, the availability of the metabolite's reactive sites is governed by the solvent and method of extraction since it influences the bioavailability of the secondary metabolites.

The nature solvent of influences the detected countenance in phenols as well as the antioxidant capacity of the said extract.

For our assays, the results demonstrated earlier showed that our extract has a high phenolic content. For 1g of dried stigma, it has 6.33 mg of polyphenols, as well as a scavenging capacity, for 50 percent inhibition activity, of 2.21 mg.

Starting with the phenolic content, Lahmass et al. (2018) found for an ethanolic extraction of a Moroccan saffron grown in Oujda, an estimated Total Phenolic Content of 16,63 µg GAE/mg extract, which is lower than the TPC estimated in our extract (29,88 µg GAE/mg extract). This notable difference is due to both the nature of the solvent and the slightly different plant growth conditions. While both the extracts are Moroccan, the microclimate of the two regions (altitude, temperature…) are very different. The Taliouin site, where our saffron is harvested from, is at an altitude of 1630m while the region of Oujda is at a distinctly lower altitude, lower than 730m (*Carte topographique Oujda, altitude, relief*, n.d.) .

Similarly, we find variable phenolic content values for saffron stigma. Karimi et al. (2010) found for an Iranian saffron, with a methanolic extraction a TPC value of 6,5 mgGAE/gDW. Moreover, they further support the solvent effect theory, when they demonstrated that the methanolic extract gave the best TPC values, compared with water and ethanol extractions.

In another Iranian study, Ghanbari et al. (2019) reported TPC values varying from 2.96 to 6.17 mg GAE/ g DW for a methanol/water 80/20 extraction.

For the DPPH scavenging activity, few papers have described the antioxidant activity of the saffron stigma. For the major saffron producing countries, like India, where Parray et al. (2015) stated that Stigma extract exhibited significant scavenging of OH· radicals and overall the scavenging activity of stigma extract (87.0%) at the concentration of 500µg ml−1 for a 0.1 mM DPPH solution. For the same DPPH molarity, Karimi et al. (2010) reported high DPPH· scavenging activity of Iranian saffron stigma (68.2 % for the methanolic extract at a concentration of 300 µg/mL). Likewise, a Greek study by Assimopoulou et al. (2005) established, the IC50 for Greek saffron methanol extract to be 2.5 mg/mL.

Consequently, for our sample, an IC50 of 2,21 mg/ml is a satisfactory amount for a natural product with diverse compounds, both active and inactive. Keeping in mind that due to DPPH unavailability, we chose to work with a smaller molarity in the assay (0,006mM DPPH solution), meaning that we can only get an appreciation of the close variations in the antioxidant potential of the saffron stigma and not really a proper comparison.

Results from a metabolomics study by Gikas et al. (2021) showed that chemical characteristics of saffron were diverse, which mainly arose from the different geoclimatic characteristics inherent to the territory of cultivation. Interestingly, crocus from distant areas e.g., Greece and Morocco, exhibit more pronounced similarities compared to neighbouring regions such as Greece and Italy.

This could be attributed to the pivotal impact of microclimatic conditions rather than considering the wider geographical area of cultivation. The Moroccan climate is typically Mediterranean, resembling the Greek weather, even in the Atlantic coast of the country.

Additionally, changes in the preparation procedures, flower collection, separation/drying and conservation of saffron, can strongly modify the final composition in chemical components present in the stigma (Gikas et al., 2021).

A study by Lage & Cantrell (2009) quantified the main saffron components: crocins, picrocrocin, and safranal. They reported a metabolite screening using HPLC method for the Talouine saffron. It showed that the Talouine saffron was especially rich (% dry weight) in crocins with 36.27%, safranal 0.17 % and picrocrocin 24.52%. These findings further support our results and correlates with the important antioxidant activity as well as the high phenolic content.

Saffron stigma is an interesting source of antioxidants and of generally health promoting, beneficial compounds such as phenols. The information we established from our study suggests that the saffron spice, being a part of the Moroccan cuisine, and an important food additive, affects the human diet positively. More importantly, the saffron especially harvested from Morocco, is very rich in secondary metabolites, a statement supported by our findings, in agreement with previously published findings. For these reasons, it is crucial to set more molecular, metabolomic and transcriptomic studies of the Moroccan saffron, to help put forward its unique value on an international scale.

In-silico study:

The predicted EggNOG sequences

EggNOG annotations, based on predictions rather than alignments, generated a list of predictions, linking a list of 17 contigs to secondary metabolisms. 2 of these predictions are already characterized genes in the EggNOG database (Contig587:g475.t1 analogous to Phenylalanine ammonialyase PAL2 gene and Contig532:g426.t1 to CYP83B1 which Belongs to the cytochrome P450 family) , leaving 15 uncharacterized predictions. These predictions can be then analysed by studying the preserved regions on the sequences and classify them with the purpose of identifying their exact role in the general processes to which belong. Further results and a more detailed analysis are reserved for publication.

Functional analysis of the *crocus sativus* ESTs and primers design:

The compilation of contigs that we generated from the different functional annotators allowed us to trace back, or at least form an idea of the categories of metabolism present in the *crocus sativus* species, as a general screening of the ESTs present in the database. It is important to note that this categorization is built on alignment scores. Traces of molecules involved in these pathways represent molecular evidence of their presence/activity within the *crocus sativus* metabolism. The presence of at least 1 match in a pathway can lead us to putatively indicate the presence of the said pathway. However, the absence of a match in related metabolisms does not mean necessarily absence/inactivity, since the quantity of molecular data isn't enough for us to conclude a full coverage of all transcriptomes , and therefore the inclusion of all pathways. These matches are indicators of the involvement of a specific pathway in the general metabolism of the plant. But then, the substantial number of unique sequences, unmapped and unannotated contigs (31.3% with no Blast, no GOs), allows us to appreciate the distinctiveness of the metabolism. The absence of these sequences, or similar ones, in the 3 main databases used in this study highlights the importance of conducting a full genome sequencing. Keeping in mind the important value of the secondary metabolites of saffron flower, and the different biotechnological applications they can potentially be used for, having such singular sequences, especially involved in the secondary metabolism, can only limit the possibility of making use of such rare beneficial compounds.

One way to exploit the available molecular information is to fight fraudulent saffron. Running PCR quality controls will give a decisive result as to the authenticity of the studied sample. Many flowers that are sold as saffron, such as gardenia flower, or corn stamens , sometimes going as far as mixing these plants with plastic.

The proposed primers are designed using specific sequences coding for molecules of the *crocus sativus* secondary metabolism. These molecules, unique to saffron will amplify during PCR only authentic samples, giving positive results.

VIII. Conclusion and perspectives

The objective of our study was to quantify the radical scavenging capacity of the saffron extract, as well as the phenolic content and its antibacterial potential against specific bacteria. While our extract revealed a relatively high antioxidant capacity as well as a rich phenolic content, complimenting the previously reported data in literature. The concentrations tested for these bacterial strains did not yield any positive results. To further study our extract and fully assess its therapeutic potential, a metabolomics study would help uncover the type and proportion of secondary metabolites existing in the stigma, as well as the other organs of the plant. The extract should also be tested against a larger selection of bacteria, including bacteria pathogenic to humans, modifying the extraction methods and used solvents, to perhaps find an antibacterial activity.

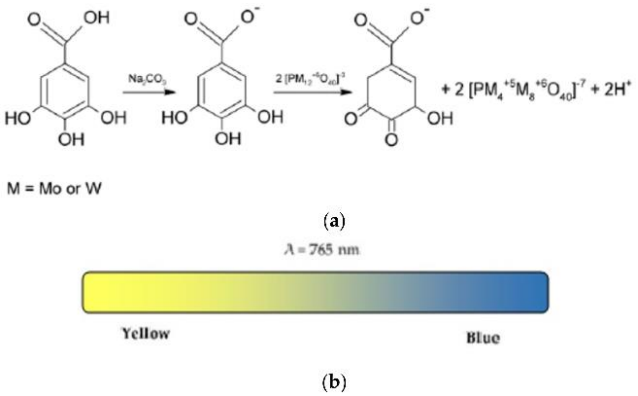
The second objective was to provide a functional analysis and screening of the *crocus sativus* ESTs available in the NCBI database. The analysis allowed us to gather, annotate and assign functions and metabolic pathways to the putative proteins we generated. A supplementary analysis of the unique un-aligned contigs will enrich the current databases. Furthermore, a screening for more putative genes, and allocating them specifically to *crocus sativus* will help offer more biomarkers, perhaps even biomarkers specific to Moroccan saffron, to acclaim the value and quality of the Moroccan red gold.

The biomarkers constructed for this study can be tested in-vivo to further optimize the design parameters for a high-quality PCR output.

Our studies lay the ground to a more in-depth studies of the discussed aspects, and perhaps generate more information to open the accessibility to use this precious spice to biotechnological and pharmacological use.

ANNEXE 1

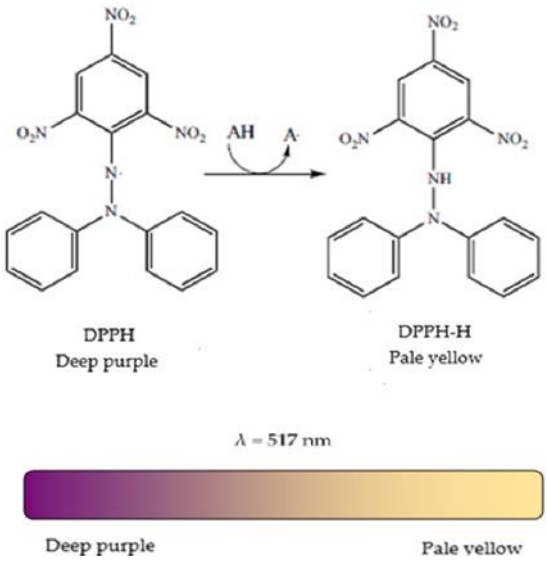
The total phenolic content was used is determined using the Folin-Ciocalteu method. Briefly, this reaction is based on reducing the Folin–Ciocalteu reagent with phenolic compounds in an alkaline state where a complex of the phosphomolybdic/phosphotungstic acid are reduced to obtain a blue chromophore with the maximum absorption at 765 nm. (Munteanu & Apetrei, 2021)



The Reaction between the phenolic compounds and the derivatives of the phosphotungstic and phosphomolybdic acids in the presence of Na2CO3, causing the formation of a blue colour by the Folin–Ciocalteu method (a); Colour variation of the reaction (b). (Munteanu & Apetrei, 2021)

ANNEXE 2

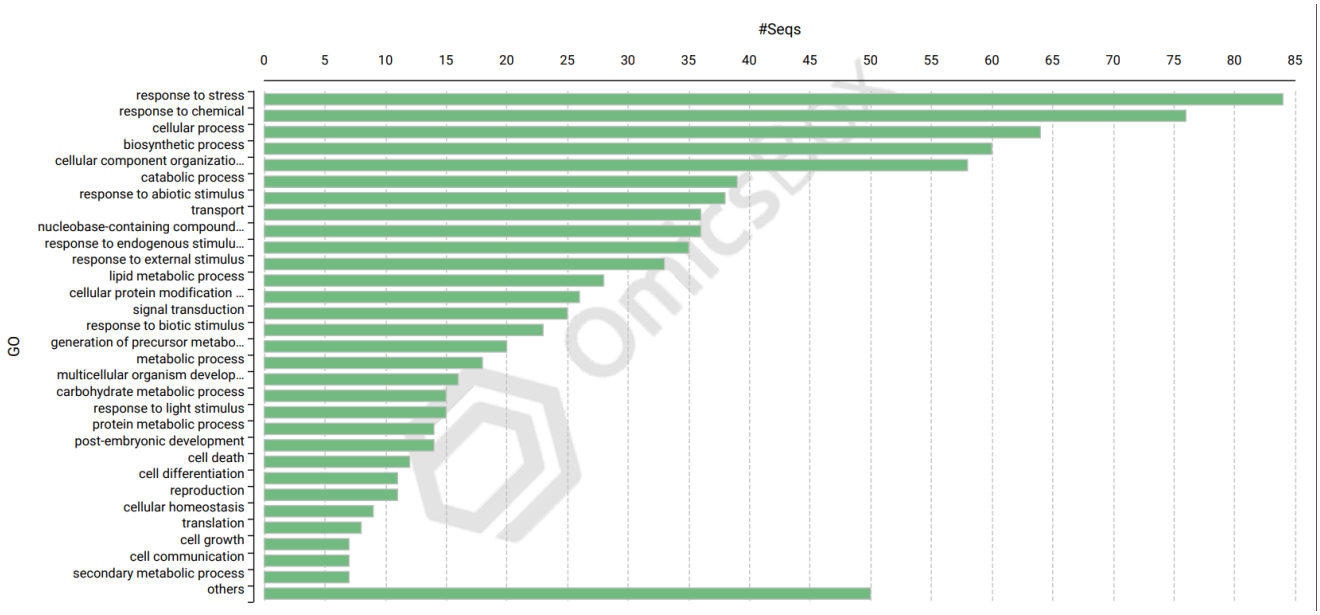
The DPPH reaction is based on donating electrons from the antioxidants in order to neutralise the DPPH radical (2,2-di(4-tert-octylphenyl)-1-picrylhydrazyl). The reaction is accompanied by changing the DPPH colour measured at 517 nm , and discoloration acts as an indicator of antioxidant activity (Munteanu & Apetrei, 2021)



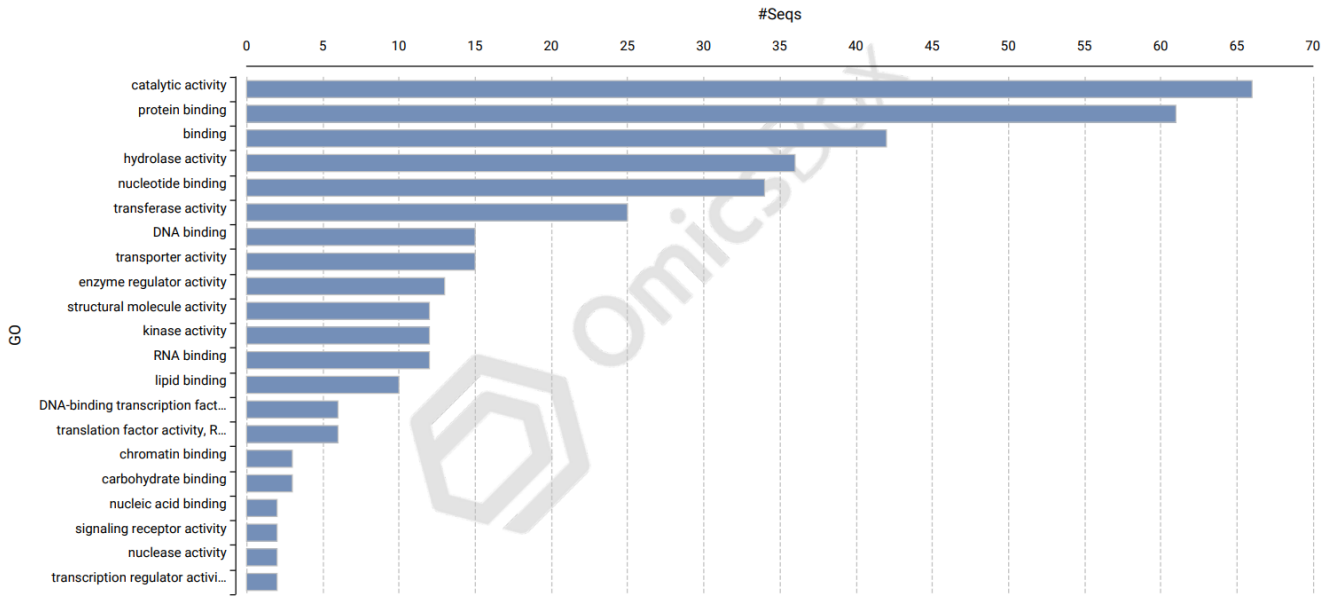
DPPH reaction mechanism by an antioxidant. (Munteanu & Apetrei, 2021)

ANNEXE 3

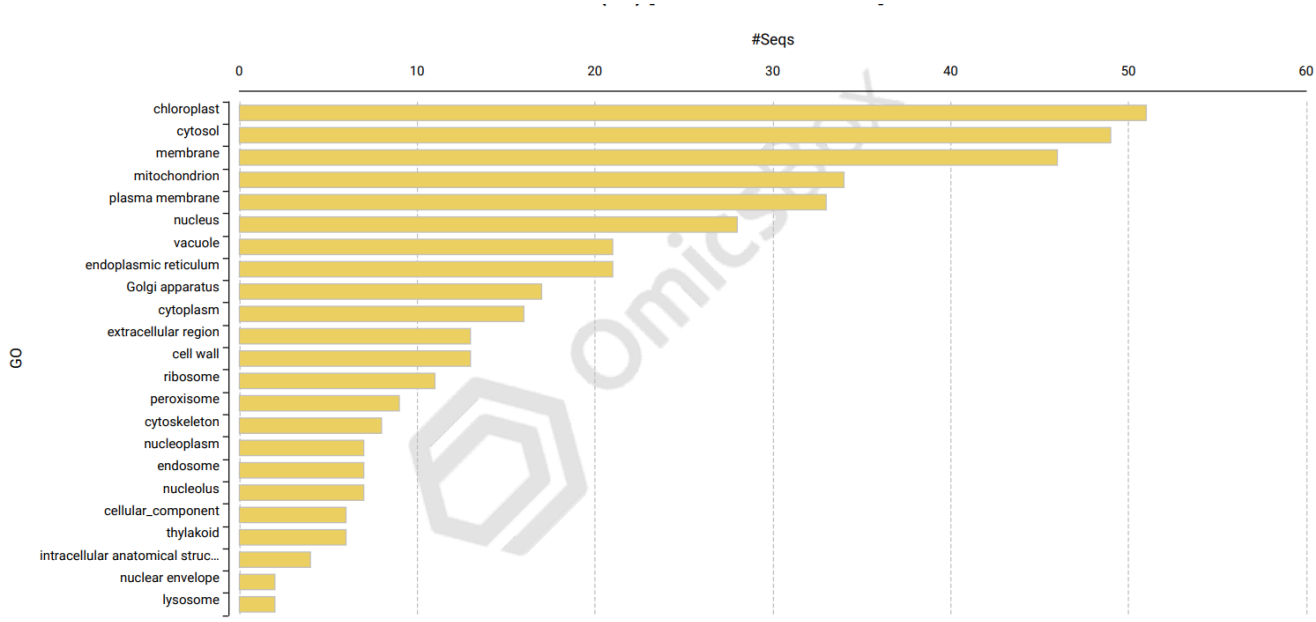
Direct GO Count (Biological Processes):



Direct GO Count (Molecular Function):



Direct GO Count (Cellular Component)



IX. References:

A. Mir Shafat, Javeed I. A. Bhata, Rouf Ahmad Bhata, Bilal A. Beighb, Hafiz ul Islamb, Shakeel Ahmad Dara, Ishrat Bashira, & Gowhar Rashid. (2020). *Chapter 7—Cytogenetic and bioactive attributes of Crocus sativus (Saffron): A tool to unfold its medicinal mystery*. 23. https://doi.org/10.1016/B978-0-12-819590-1.00007-0

AgriMaroc.ma, R. (n.d.). *Maroc: La culture du safran génère plus de 139 millions de dirhams*. Retrieved 14 December 2021, from https://www.agrimaroc.ma/maroc-culture-safran/

Ahmed, N., Anwar, S., Al-Sokari, S. S., Ansari, S. Y., & Wagih, M. E. (2016). Saffron Crocus (Crocus sativus) Oils. In *Essential Oils in Food Preservation, Flavor and Safety* (pp. 705–713). Elsevier. https://doi.org/10.1016/B978-0-12-416641-7.00080-8

Ainsworth, E. A., & Gillespie, K. M. (2007). Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin-Ciocalteu reagent. *Nature Protocols*, *2*(4), 875–877. https://doi.org/10.1038/nprot.2007.102

Akhtar, M. S., & Swamy, M. K. (Eds.). (2019). *Natural Bio-active Compounds: Volume 3: Biotechnology, Bioengineering, and Molecular Approaches*. Springer Singapore. https://doi.org/10.1007/978-981-13-7438-8

Angaji, S. A., Mousavi, S. F., & Babapour, E. (2012). Antioxidants: A few key points. *Annals of Biological Research*, *3*(8), 3968–3977.

Assimopoulou, A. N., Sinakos, Z., & Papageorgiou, V. P. (2005). Radical scavenging activity ofCrocus sativus L. extract and its bioactive constituents. *Phytotherapy Research*, *19*(11), 997–1000. https://doi.org/10.1002/ptr.1749

Baba, S. A., & Ashraf, N. (2016). *Apocarotenoids of Crocus sativus L: From biosynthesis to pharmacology*. Springer Singapore. https://doi.org/10.1007/978-981-10-1899-2

Baba, S. A., Mohiuddin, T., Basu, S., Swarnkar, M. K., Malik, A. H., Wani, Z. A., Abbas, N., Singh, A. K., & Ashraf, N. (2015). Comprehensive transcriptome analysis of Crocus sativus for discovery and expression of genes involved in apocarotenoid biosynthesis. *BMC Genomics*, *16*(1), 698. https://doi.org/10.1186/s12864-015-1894-5

Bastola, K. P., Guragain, Y. N., Bhadriraju, V., & Vadlani, P. V. (2017). Evaluation of Standards and Interfering Compounds in the Determination of Phenolics by Folin-Ciocalteu Assay Method for Effective Bioprocessing of Biomass. *American Journal of Analytical Chemistry*, *08*(06), 416–431. https://doi.org/10.4236/ajac.2017.86032

Bathaie, S. Z., & Mousavi, S. Z. (2010). New Applications and Mechanisms of Action of Saffron and its Important Ingredients. *Critical Reviews in Food Science and Nutrition*, *50*(8), 761–786. https://doi.org/10.1080/10408390902773003

Bellakhdar, J. (1997). *Pharmacopée marocaine traditionnelle*. Ibis press. https://scholar.google.com/scholar_lookup?title=Pharmacope%CC%81e+marocaine+traditionnelle&author=Bellakhdar%2C+Jamal.&publication_year=1997

Bian, Y., Zhao, C., & Lee, S. M.-Y. (2020). Neuroprotective Potency of Saffron Against Neuropsychiatric Diseases, Neurodegenerative Diseases, and Other Brain Disorders: From Bench to Bedside. *Frontiers in Pharmacology*, *11*, 1602.

Bibi Sadeer, N., Montesano, D., Albrizio, S., Zengin, G., & Mahomoodally, M. F. (2020). The Versatility of Antioxidant Assays in Food Science and Safety—Chemistry, Applications, Strengths, and Limitations. *Antioxidants*, *9*(8), 709. https://doi.org/10.3390/antiox9080709

Blum, M., Chang, H.-Y., Chuguransky, S., Grego, T., Kandasaamy, S., Mitchell, A., Nuka, G., Paysan-Lafosse, T., Qureshi, M., Raj, S., Richardson, L., Salazar, G. A., Williams, L., Bork, P., Bridge, A., Gough, J., Haft, D. H., Letunic, I., Marchler-Bauer, A., … Finn, R. D. (2021).

The InterPro protein families and domains database: 20 years on. *Nucleic Acids Research*, *49*(D1), D344–D354. https://doi.org/10.1093/nar/gkaa977

Bolhassani, A., Khavari, A., & Bathaie, S. Z. (2014). Saffron and natural carotenoids: Biochemical activities and anti-tumor effects. *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer*, *1845*(1), 20–30. https://doi.org/10.1016/j.bbcan.2013.11.001

Carte topographique Oujda, altitude, relief. (n.d.). topographic-map.com. Retrieved 20 February 2022, from https://fr-fr.topographic-map.com/maps/jgup/Oujda/

Dhiman, N., & Kharkwal, H. (2020). Biosynthesis and Derivatization of the Major Phytoconstituents of Saffron. In *Saffron* (pp. 83–92). Elsevier. https://doi.org/10.1016/B978-0-12-818462-2.00007-3

du Plessis, L., Škunca, N., & Dessimoz, C. (2011). The what, where, how and why of gene ontology—A primer for bioinformaticians. *Briefings in Bioinformatics*, *12*(6), 723–735. https://doi.org/10.1093/bib/bbr002

El Aymani, I., El Gabardi, S., Artib, M., Chliyeh, M., Selmaoui, K., Ouazzani Touhami, A., Benkirane, R., & Douira, A. (2019). Effect of the Number of Years of Soil Exploitation by Saffron Cultivation in Morocco on the Diversity of Endomycorrhizal Fungi. *Acta Phytopathologica et Entomologica Hungarica*, *54*(1), 9–24. https://doi.org/10.1556/038.54.2019.002

Eloff, J. (1998). A Sensitive and Quick Microplate Method to Determine the Minimal Inhibitory Concentration of Plant Extracts for Bacteria. *Planta Medica*, *64*(08), 711–713. https://doi.org/10.1055/s-2006-957563

Finley, J. W., & Gao, S. (2017). A Perspective on *Crocus sativus* L. (Saffron) Constituent Crocin: A Potent Water-Soluble Antioxidant and Potential Therapy for Alzheimer's Disease.

Journal of Agricultural and Food Chemistry, *65*(5), 1005–1020. https://doi.org/10.1021/acs.jafc.6b04398

Gandomi Nasrabadi, H., Azami Sarokelaei, L., Misaghi, A., Abbaszadeh, S., Shariatifar, N., & Tayyar Hashtjin, N. (2012). Antibacterial effect of aqueous and alcoholic extracts from petal of saffron (Crocus sativus L.) on some foodborne bacterial pathogens. *Journal of Medicinal Plants*, *11*(42), 189–196.

Ghaffari, S., & Roshanravan, N. (2019). Saffron; An updated review on biological properties with special focus on cardiovascular effects. *Biomedicine & Pharmacotherapy*, *109*, 21–27. https://doi.org/10.1016/j.biopha.2018.10.031

Ghanbari, J., Khajoei-Nejad, G., & van Ruth, S. M. (2019). Effect of saffron (Crocus sativus L.) corm provenance on its agro-morphological traits and bioactive compounds. *Scientia Horticulturae*, *256*, 108605. https://doi.org/10.1016/j.scienta.2019.108605

Gikas, E., Koulakiotis, N. S., & Tsarbopoulos, A. (2021). Phytochemical Differentiation of Saffron (Crocus sativus L.) by High Resolution Mass Spectrometry Metabolomic Studies. *Molecules*, *26*(8), 2180. https://doi.org/10.3390/molecules26082180

Godugu, C., Pasari, L. P., Khurana, A., Anchi, P., Saifi, M. A., Bansod, S. P., & Annaldas, S. (2020). Crocin, an active constituent of Crocus sativus ameliorates cerulein induced pancreatic inflammation and oxidative stress. *Phytotherapy Research*, *34*(4), 825–835. https://doi.org/10.1002/ptr.6564

Gómez-Gómez, L., Parra-Vega, V., Rivas-Sendra, A., Seguí-Simarro, J., Molina, R., Pallotti, C., Rubio-Moraga, Á., Diretto, G., Prieto, A., & Ahrazem, O. (2017). Unraveling Massive Crocins Transport and Accumulation through Proteome and Microscopy Tools during the Development of Saffron Stigma. *International Journal of Molecular Sciences*, *18*(1), 76. https://doi.org/10.3390/ijms18010076

Götz, S., García-Gómez, J. M., Terol, J., Williams, T. D., Nagaraj, S. H., Nueda, M. J., Robles, M., Talón, M., Dopazo, J., & Conesa, A. (2008). High-throughput functional annotation and data mining with the Blast2GO suite. *Nucleic Acids Research*, *36*(10), 3420–3435. https://doi.org/10.1093/nar/gkn176

Gulcin, İ. (2020). Antioxidants and antioxidant methods: An updated overview. *Archives of Toxicology*, *94*(3), 651–715. https://doi.org/10.1007/s00204-020-02689-3

Hashemi, M., & Hosseinzadeh, H. (2019). A comprehensive review on biological activities and toxicology of crocetin. *Food and Chemical Toxicology*, *130*, 44–60.

Huerta-Cepas, J., Szklarczyk, D., Heller, D., Hernández-Plaza, A., Forslund, S. K., Cook, H., Mende, D. R., Letunic, I., Rattei, T., Jensen, L. J., von Mering, C., & Bork, P. (2019). eggNOG 5.0: A hierarchical, functionally and phylogenetically annotated orthology resource based on 5090 organisms and 2502 viruses. *Nucleic Acids Research*, *47*(D1), D309–D314. https://doi.org/10.1093/nar/gky1085

Jafari-Sales, A., & Pashazadeh, M. (2020). Antibacterial Effect of Methanolic Extract of Saffron Petal (Crocus sativus L.) on Some Standard Gram Positive and Gram Negative Pathogenic Bacteria In vitro. *Current Perspectives on Medicinal and Aromatic Plants (CUPMAP)*, *3*(1), 1–7. https://doi.org/10.38093/cupmap.692879

José Bagur, M., Alonso Salinas, G., Jiménez-Monreal, A., Chaouqi, S., Llorens, S., Martínez-Tomé, M., & Alonso, G. (2017). Saffron: An Old Medicinal Plant and a Potential Novel Functional Food. *Molecules*, *23*(1), 30. https://doi.org/10.3390/molecules23010030

Kanakis, C. D., Tarantilis, P. A., Pappas, C., Bariyanga, J., Tajmir-Riahi, H. A., & Polissiou, M. G. (2009). An overview of structural features of DNA and RNA complexes with saffron compounds: Models and antioxidant activity. *Journal of Photochemistry and Photobiology B: Biology*, *95*(3), 204–212. https://doi.org/10.1016/j.jphotobiol.2009.03.006

Kanehisa, M. (2000). *Post-genome Informatics*. OUP Oxford.

Kanehisa, M., Sato, Y., Kawashima, M., Furumichi, M., & Tanabe, M. (2016). KEGG as a reference resource for gene and protein annotation. *Nucleic Acids Research*, *44*(D1), D457-462. https://doi.org/10.1093/nar/gkv1070

Karimi, E., Oskoueian, E., Hendra, R., & Jaafar, H. Z. E. (2010). Evaluation of Crocus sativus L. Stigma Phenolic and Flavonoid Compounds and Its Antioxidant Activity. *Molecules*, *15*(9), 6244–6256. https://doi.org/10.3390/molecules15096244

Kirby, B. (2009). Kirby-Bauer Disk Diffusion Susceptibility Test Protocol. *American Society for Microbiology*, *66*, 208.

Lage, M., & Cantrell, C. L. (2009). Quantification of saffron (Crocus sativus L.) metabolites crocins, picrocrocin and safranal for quality determination of the spice grown under different environmental Moroccan conditions. *Scientia Horticulturae*, *121*(3), 366–373. https://doi.org/10.1016/j.scienta.2009.02.017

Lahmass, I., Ouahhoud, S., Elyoubi, M., Benabbas, R., Sabouni, A., Asehraou, A., & Saalaoui, E. (2018). Evaluation of antioxidant activities of saffron stigma and spath as by-product of Crocus sativus L. *MOJ Biol Med*, *3*(4), 29.

Liu, T., Yu, S., Xu, Z., Tan, J., Wang, B., Liu, Y.-G., & Zhu, Q. (2020). Prospects and progress on crocin biosynthetic pathway and metabolic engineering. *Computational and Structural Biotechnology Journal*, *18*, 3278–3286. https://doi.org/10.1016/j.csbj.2020.10.019

Mitra, A. K. (2020). Antioxidants: A Masterpiece of Mother Nature to Prevent Illness. *Journal of Chemical Reviews*, *2*(4), 243–256. https://doi.org/10.22034/jcr.2020.112730

Mohtashami, L., Amiri, M. S., Ramezani, M., Emami, S. A., & Simal-Gandara, J. (2021). The genus Crocus L.: A review of ethnobotanical uses, phytochemistry and pharmacology.

Industrial Crops and Products, *171*, 113923. https://doi.org/10.1016/j.indcrop.2021.113923

Moratalla-López, Bagur, Lorenzo, Salinas, & Alonso. (2019). Bioactivity and Bioavailability of the Major Metabolites of Crocus sativus L. Flower. *Molecules*, *24*(15), 2827. https://doi.org/10.3390/molecules24152827

Munteanu, I. G., & Apetrei, C. (2021). Analytical Methods Used in Determining Antioxidant Activity: A Review. *International Journal of Molecular Sciences*, *22*(7), 3380. https://doi.org/10.3390/ijms22073380

Nanda, S., & Madan, K. (2021). The role of Safranal and saffron stigma extracts in oxidative stress, diseases and photoaging: A systematic review. *Heliyon*, *7*(2), e06117. https://doi.org/10.1016/j.heliyon.2021.e06117

Negbi, M. (1999). *Saffron Crocus sativus L.* Harwood Academic. http://www.crcnetbase.com/isbn/9780203303665

Pachikian, B. D., Copine, S., Suchareau, M., & Deldicque, L. (2021). Effects of Saffron Extract on Sleep Quality: A Randomized Double-Blind Controlled Clinical Trial. *Nutrients*, *13*(5), 1473.

Parray, J. A., Kamili, A. N., Hamid, R., Reshi, Z. A., & Qadri, R. A. (2015). Antibacterial and antioxidant activity of methanol extracts of *Crocus sativus* L. *c.v*. Kashmirianus. *Frontiers in Life Science*, *8*(1), 40–46. https://doi.org/10.1080/21553769.2014.951774

Razak, S. I. A., Anwar Hamzah, M. S., Yee, F. C., Kadir, M. R. A., & Nayan, N. H. M. (2017). A Review on Medicinal Properties of Saffron toward Major Diseases. *Journal of Herbs, Spices & Medicinal Plants*, *23*(2), 98–116. https://doi.org/10.1080/10496475.2016.1272522

Reygaert, W. C. (2018). An overview of the antimicrobial resistance mechanisms of bacteria. *AIMS Microbiology*, *4*(3), 482.

Rodriguez-Ruiz, V., Barzegari, A., Zuluaga, M., Zunooni-Vahed, S., Rahbar-Saadat, Y., Letourneur, D., Gueguen, V., & Pavon-Djavid, G. (2016). Potential of aqueous extract of saffron (Crocus sativus L.) in blocking the oxidative stress by modulation of signal transduction in human vascular endothelial cells. *Journal of Functional Foods*, *26*, 123–134. https://doi.org/10.1016/j.jff.2016.07.003

Samarghandian, S., Farkhondeh, T., & Zeinali, T. (2020). Crocus sativus L. (saffron) extract antioxidant potential and use in aging. In *Aging* (pp. 193–200). Elsevier. https://doi.org/10.1016/B978-0-12-818698-5.00019-5

Saxena, R. B. (2010). Botany, Taxonomy and Cytology of Crocus sativus series. *Ayu*, *31*(3), 374–381. https://doi.org/10.4103/0974-8520.77153

Schwacke, R., Ponce-Soto, G. Y., Krause, K., Bolger, A. M., Arsova, B., Hallab, A., Gruden, K., Stitt, M., Bolger, M. E., & Usadel, B. (2019). MapMan4: A Refined Protein Classification and Annotation Framework Applicable to Multi-Omics Data Analysis. *Molecular Plant*, *12*(6), 879–892. https://doi.org/10.1016/j.molp.2019.01.003

Seifi, A., & Shayesteh, H. (2020). Molecular biology of Crocus sativus. In *Saffron* (pp. 247–258). Elsevier. https://doi.org/10.1016/B978-0-12-818638-1.00015-0

Sung, H., Ferlay, J., Siegel, R. L., Laversanne, M., Soerjomataram, I., Jemal, A., & Bray, F. (2021). Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA: A Cancer Journal for Clinicians*, *71*(3), 209–249. https://doi.org/10.3322/caac.21660

Urbani, E., Blasi, F., Simonetti, M. S., Chiesi, C., & Cossignani, L. (2016). Investigation on secondary metabolite content and antioxidant activity of commercial saffron powder. *European Food Research and Technology*, *242*(6), 987–993.

Vahedi, M., Karimi, R., Panigrahi, J., & Gantait, S. (2019). Salient Biotechnological Interventions in Saffron (Crocus sativus L.): A Major Source of Bio-active Apocarotenoids. In M. S. Akhtar & M. K. Swamy (Eds.), *Natural Bio-active Compounds: Volume 3: Biotechnology, Bioengineering, and Molecular Approaches* (pp. 205–223). Springer. https://doi.org/10.1007/978-981-13-7438-8_8

Xing, B., Li, S., Yang, J., Lin, D., Feng, Y., Lu, J., & Shao, Q. (2021). Phytochemistry, pharmacology, and potential clinical applications of saffron: A review. *Journal of Ethnopharmacology*, *281*, 114555. https://doi.org/10.1016/j.jep.2021.114555

Zaazaa, L., Mohammed, I., & Lage, M. (2017). Contribution to the study of historical introduction about saffron (*Crocus sativus* L.) culture in Morocco. *Acta Horticulturae*, *1184*, 185–188. https://doi.org/10.17660/ActaHortic.2017.1184.27

Zara, S., Petretto, G. L., Mannu, A., Zara, G., Budroni, M., Mannazzu, I., Multineddu, C., Pintore, G., & Fancello, F. (2021). Antimicrobial Activity and Chemical Characterization of a Non-Polar Extract of Saffron Stamens in Food Matrix. *Foods*, *10*(4), 703. https://doi.org/10.3390/foods10040703