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THESIS

Presented by

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To obtain the grade of
DOCTOR

Speciality: **Biochemistry, Cellular and Molecular Biology**

Specialité: **Biochimie, Biologie Cellulaire et Moléculaire**

Natural Molecules Impair the Survival of Triple Negative Breast Cancer Cells: a Focus on Berberine and Artemisinin

Defended the 26th of September 2020 at 10h00 am before the review board:

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Dedication:

It is with a great honour and deep affection that I dedicate this work as a testimony to the dearest people in the world, my parents, may Allah protect and bless them. This work is partly the result of their support; I am sincerely grateful to them. Nothing would have been possible without them, so, this success is a bit mine, but above all very much theirs. Their pride in me today is my best reward. No thanks would be enough. Also, I want to dedicate this work to my lovely brother El Mahdi and my sweet sister Chaïmaâ for their unconditional love and friendship.

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General Introduction:

Breast, a life donor and a life destroyer; this is how women used to see their breasts through history: a breastfeeding and a breast cancer organ at the same time.

Breast cancer is as old as history; many women as Atossa the queen of Persia (475 BC) or Theodora the empress of Byzantium (548 AD) or Anne of Austria the queen of France (1666 AD) have all suffered and hopelessly died from breast cancer [1].Thus, establishing a causal connection between breast cancer and death was relatively simple since practitioners of that time did not understand or find out the remedy to this illness.

In fact, it was announced in an old Egyptian Papyrus (1587-1328 BC) that the disease occurring on the breast is an "evil" without a cure. Afterwards, in ancient Greece, Hippocrates (460-377 BC) proclaimed that cancer is a result of the excess of black bile in the body (based on his four humours theory) and that it is associated with the menstruation arrest [2]. However, medical thinking about the origin of breast cancer has changed in the following centuries with many outstanding physicians and philosophers such as Galen, Al-Razi and Avicenna [3].

As they were investigating the origin of breast cancer, practitioners were also digging far and wide to discover a remedy for this illness. Several treatment approaches were adopted such as surgery, religion and natural medicine. Surgical operations were practiced to eradicate the tumour, as stated in the very first description of breast surgery by Archigenes of Apamea (1^{st}-2^{nd} century) [4]. However, no surgery should have been practiced unless the cancer could be excised completely, as believed by Al-Razi (864-925 AD) [5][6].

Besides surgery, physicians and alchemists spent their lives in discovering, mixing and creating natural remedies to treat breast cancer, as recommended in an ancient Egyptian Papyrus (1587-1328 BC), that using the combination of calamine with the cow brain and wasp thorns may heal breast diseases [2]. Thereafter, new natural remedies and drugs development pursued as the notion of cancer was getting clearer among the scientists.

Nowadays, technology revolution has increased knowledge about breast cancer through diagnosis and treatment advances. Despite the actual breast cancer awareness, world statistics recorded more than 2 million (11.6%) of people have suffered from breast cancer in 2018 in the world, including Morocco with 39.9% new cases which is greater than the world's incidence [7]! In fact, the World Health Organisation reported that breast cancer is increasing

particularly in the developing countries where the majority of cases are diagnosed in late stage [8].

The financial costs are shadowing these numbers as they are high for the patient and for the society. In the United States, direct medical costs for breast cancer in 2009 ranged from 20 000 to 100 000 $US depending on the disease stages [9]. Unfortunately, in Morocco little data are available on cancer economic costs, however “Le Registre de Casablanca” has reported that breast cancer direct medical costs in Morocco were estimated in 2010 by 86.4 million $US [10]. Breast cancer not only costs money but it also costs lives as well, where more than 600 000 people have died from breast cancer in 2018 in the world [7]. Whereas one of the major financial costs of breast cancer is the treatment, several cancer institutions, hospitals and laboratories are mobilised to lead various pre-clinical and clinical researches to improve breast cancer treatment to be efficient and accessible.

Treatment development should take into consideration the heterogeneity of breast cancer, as this disease represents several heterogeneous subtypes distinguished by their proper histological and molecular properties. These differences interfere in the treatment decision; while some breast cancer subtypes respond well to hormone or chemo therapies, other subtypes do not. Triple Negative Breast Cancer (TNBC) represents approximately 10 to 20 % of all breast cancer subtypes and it is an aggressive breast cancer subtype with no targeted therapy due to the absence of estrogen and progesterone hormone receptors with the lack of human epidermal growth factor receptor-2/Neu over-expression. Since targeted therapy is not yet available for TNBC patients, cytotoxic chemotherapy remains the only available way for their treatment.

Unfortunately, chemotherapy carries very serious adverse effects along and after the treatment; such as the high costs of the drugs (one shot of chemotherapy costs approximately 6000 $US), the high incidence of systemic toxicity as it does not only kill cancer cells but also causes normal growing cells to die, leading to severe side effects (hair loss, bone pain, nausea…) and finally the development of resistances in tumour cells (as manifested by the recurrence and metastasis phenomenon). Therefore, finding a potent treatment for TNBC is urgently required to improve the healing and life quality of the patients.

The aim of the study:

Responding to this urgent call of TNBC drugs development, we conducted rigorous investigations in cellular and molecular levels on the effects of four pure natural molecules: carvacrol, thymoquinone, berberine and artemisinin, on a large panel of non-TNBC and TNBC cell lines to add more insights into TNBC treatment problems.

Our study design allied on:

1- Screening the effect of each natural molecule on the viability of a large panel of non-TNBC and TNBC cell lines to further select the most cytotoxic molecules and the most sensitive cell lines;
2- Investigating the mechanisms of their cytotoxicity in both cellular and molecular levels:
    - The cell cycle and its related proteins expression;
    - Apoptosis and its related proteins expression.
3- Studying the interaction of the natural molecules with conventional chemotherapy molecules on the viability of TNBC cells lines;
4- Exploring the effect of the natural molecules on the viability of normal human breast cells.

Chapter I

Presentation of Breast Cancer and Triple Negative Breast Cancer Subtype

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I.1. Incidence and mortality of breast cancer in the world and in Morocco

To understand the behaviour of diseases in groups of people, we need to use statistics that would help us in figuring out the patterns of the disease and finding ways to control it.

As we first look in the official website of the World Health Organization, we learn that breast cancer is the top cancer in women both in the developed and the developing world (Figure 1).

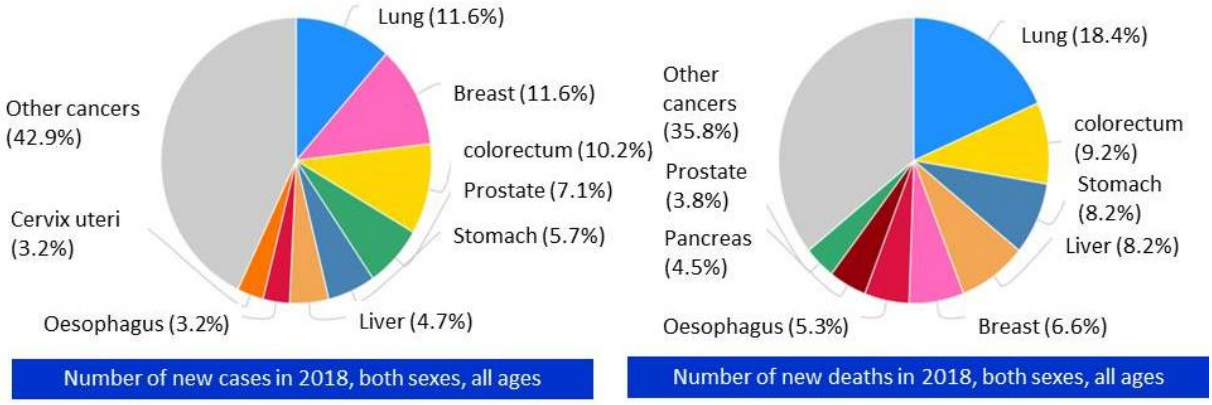

Figure 1: Incidence (number of new cases) and mortality (number of deaths) from cancer in both sexes and all ages in the world, 2018 [7]

According to Globocan 2018 report (Figure 1), we can notice that breast and lung cancers represent the highest rate of the new cases ever diagnosed in 2018 comparing to other types of cancer for both sexes. In the case of mortality, the highest rate of deaths ever registered in 2018 goes to lung cancer with 18.4%. Furthermore, breast cancer caused 6.6% of deaths in the world for both sexes in 2018.

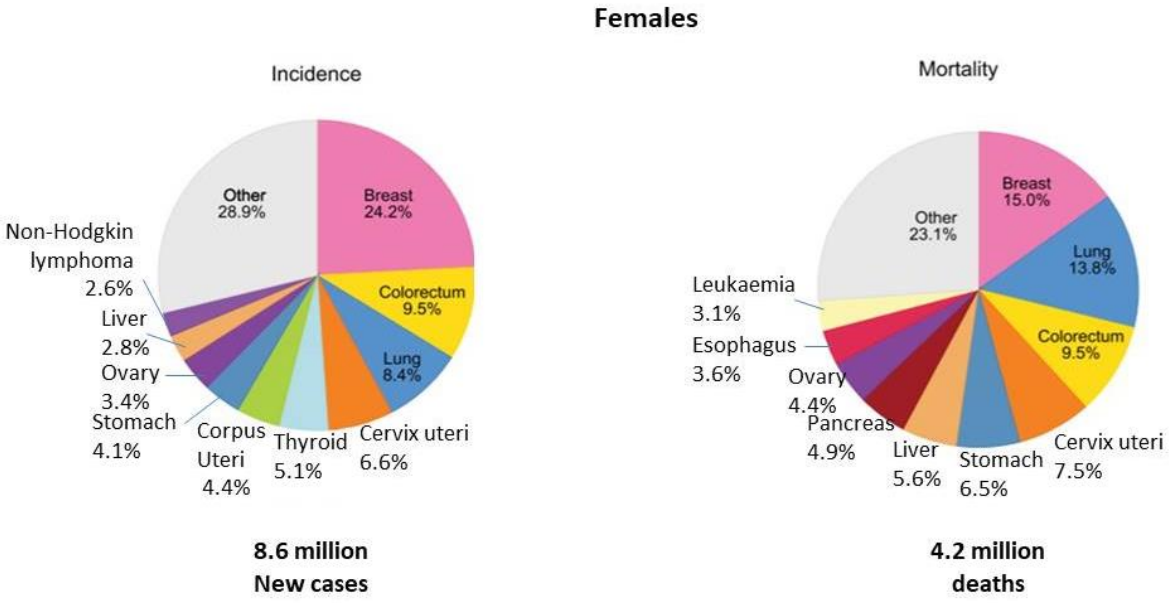

Figure 2: Incidence and mortality from breast cancer among females in the world [11]

Among females, breast cancer is the most commonly diagnosed cancer and the leading cause of death, with 24.2% and 15.0%, as incidence and mortality rates, respectively, in the world in 2018 (Figure 2).

Furthermore, if we look closely to each country in the world (Figure 3 and 4), we can see that among 185 countries, breast cancer incidence in women is the highest in 154 countries, including Morocco with 36.9% of incidence, which is greater than that of breast cancer in the world, and it is the leading cause of death in 103 countries in the world, as well as Morocco with 10.7% of mortality registered in 2018.

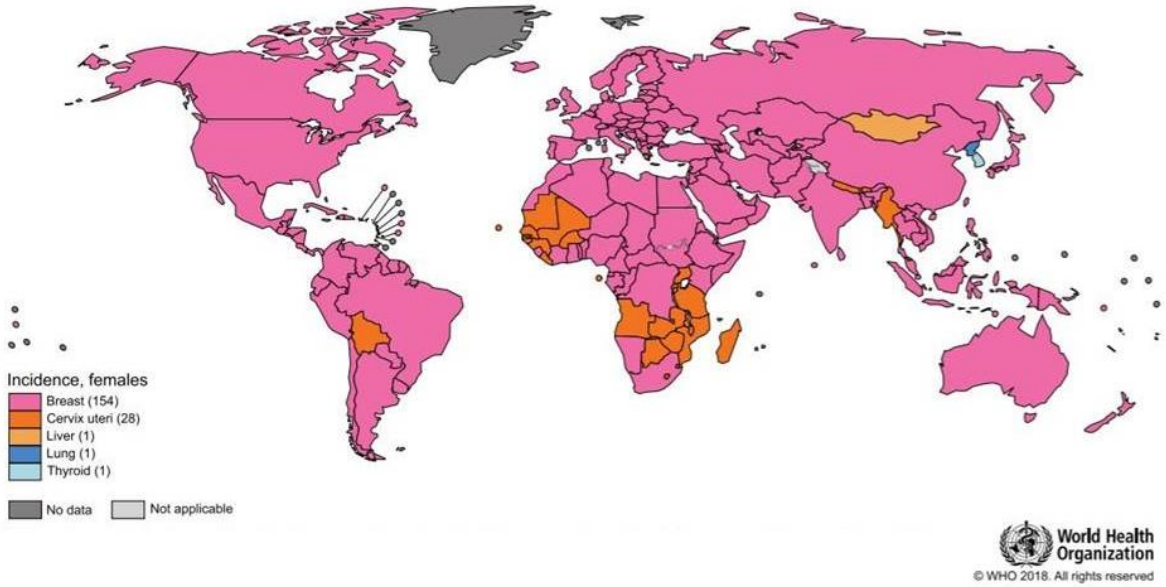

Figure 3: World map representing breast cancer incidence in each country, among females in 2018 [11]

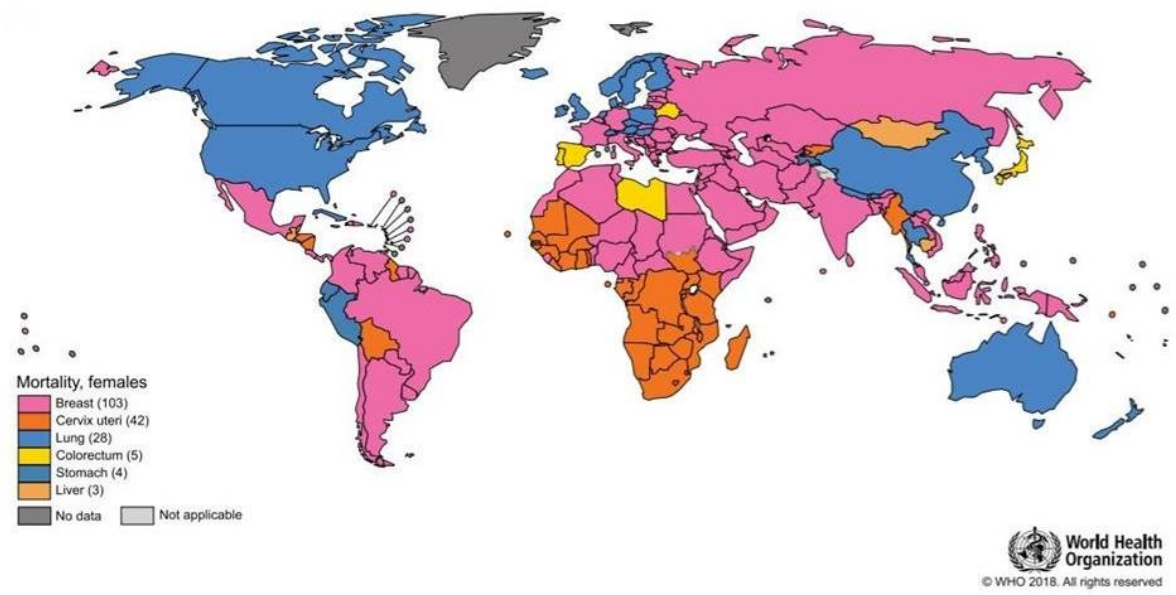

Figure 4: World map representing mortality from breast cancer in each country, among females in 2018 [11]

All these terrifying breast cancer statistics and numbers carry a huge economic burden to the world. The financial expenses of cancer are high for both the patient and the society. In fact, the World Health Organisation has stated that "breast cancer is the top cancer in women worldwide and is increasing particularly in developing countries where the majority of cases are diagnosed in late stage"[8]. Therefore, incidence and mortality from breast cancer solely depend on several parameters including population's lifestyle, genetics, country's economics, treatment and screening programs availability, etc.

Thus, paying more attention to breast cancer education, social, psychological and financial support, as well as screening campaigns is highly required as a strategy for breast cancer management. Additionally, managing breast cancer necessitates a better understanding of the disease as its diversified heterogeneity.

I.2. Breast cancer heterogeneity:

Breast cancer is a heterogeneous disease that can be classified using **histological** and **molecular** features. Most breast cancers are carcinomas, which represent the tumours that start in the epithelial cells. When carcinomas form in the breast, they are usually a more specific type called adenocarcinoma, which start in the cells' ducts (the milk ducts): ductal carcinoma, or the lobules (milk-producing glands): lobular carcinoma.

I.2.1. Histological subtypes of breast cancer:

The type of breast cancer can also refer to whether the cancer has spread (invasive) or not (in Situ):

- **In Situ:** the main type of in situ breast cancer is ductal carcinoma in situ (DCIS) which can be a precursor (or not) to invasive cancer [12].
- **Invasive:** The abnormal cells that were originated from the ducts or the lobules spread and grow into the surroundings tissues. The most common types are invasive ductal carcinoma (IDC) (80%), invasive lobular carcinoma (ILC) (10%), and other less common types of invasive cancer that each represents fewer than 5% of all invasive breast cancers [13].

I.2.2. Molecular subtypes of breast cancer:

Depending on the expression of distinct biological markers, we distinguish four major molecular breast cancer subtypes.
The main biological markers that are currently evaluated by standard clinical practices are: Estrogen Receptors (ER), Progesterone Receptors (PR) and human epidermal growth factor receptor 2 (HER2).

- **Luminal A (ER+/PR+/HER2-):** Because they test positively for both hormone receptors, they are usually responsive to hormonal therapy. Therefore, this subtype is associated with the most favourable prognosis, and it represents 73% of all breast cancer subtypes [12].
- **Luminal B (ER+/PR+/HER2+):** In addition to being positive for both hormones receptors and HER2, recently, this subtype has been defined by being highly positive for the protein Ki67 (a cellular marker for proliferation). This subtype is associated with poorer outcomes and it represents 11% of all breast cancer subtypes [12].
- **HER2 positive (ER-/PR-/HER2+):** Because they test negatively for both hormone receptors, they are usually unresponsive to hormonal therapy. However, this subtype is rich in HER2 which makes it responsive to HER2 targeted therapy and improve the outcomes of the patients. This subtype represents 4% of all breast cancer subtypes [12].
- **Basal-like/Triple Negative (ER-/PR-/HER2-):** Basal-like and Triple Negative breast cancers are both related with high metastasis and poor prognosis. It was reported that 71% of TNBC were found to be basal-like while 77% of basal-like cancers were triple negative

[14]. The differences between Triple Negative and Basal-like cancers are dependent on morphology, immunohisto-chemistry and transcriptional profiles [15]. TNBC subtype represents 10-20% of all breast cancer subtypes. Despite recent advances in diagnosis and treatment, patients with TNBC have been shown to have the highest rate of recurrence within the first 5 years after diagnosis compared with hormone receptor-positive (HR+) and HER2+ breast cancer [16]. This subtype tends to be more common in premenopausal African-American women [17].

I.3. TNBC heterogeneity: Molecular classification

Treatment of patients with TNBC has been challenging due to the heterogeneity of the disease and the lack of its molecular targets. Molecular classification of TNBC provides a better understanding of the tumour mechanisms and a theoritical basis to find any potential targeted therapies. In year 2011, Lehmann et al., [18], using the gene cluster sequence method, established the 6 following molecular subtypes of TNBC: Basal-like 1 (BL1), Basal-like 2 (BL2), Mesenchymal (M), Mesenchymal stem-like (MSL), Immunomodulatory (IM) and Luminal Androgen Receptor (LAR) (Table 1).

Table 1: Each TNBC subtype is represented with their specific characterization according to Lehmann et al using the gene cluster sequence method.

TNBC subtype	Characterization
Basal-like 1	Cell cycle and DNA damage response pathways and Ki67 over expression.
Basal-like 2	Growth factors signaling pathways (EGF, NGF, MET, Wnt/β-catenin and IGF 1R).
Mesenchymal	Cell motility components, extracellular receptor interaction and cell differenciation pathways.
Mesenchymal stem-like	Cell motility components, cell differenciation and growth pathways.
Immunomodulatory	Immune cell signaling factors, cytokine signaling, antigen processing and presentation, and signal transduction pathways.

Luminal Androgen Receptor	Exhibition of Androgen Receptors, Estrogen Receptors ER (but ERα is negative), prolactin and ErbB4 signaling.

In year 2019, Jézéquel et al [19] reported another subtyping results for TNBC using same TNBC gene expression profiling method. They classified TNBC in 3 clusters:

Cluster 1, as the molecular apocrine which is caracterised by Androgen Receptors expression and PI3K mutations.

Cluster 2, Basal-like with low immune response and high M2-like macrophages.

Cluster 3, Basal-like with high immune response and low M2-like macrophages.

These classification methods provide a theoritical basis for exploring effective targeted therapies for TNBC treatment.

Chapter II

Treatment modalities of Triple Negative Breast Cancer subtype

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The choice of breast cancer treatment is based on tumour size, nodal status, histologic grade, and the presence of ER, PR and HER2 expression to predict the response to either hormone therapy and/or trastuzumab (for HER2) [20]. Now, because TNBC lacks of ER, PR and HER2, the treatment used for other breast cancer types is not effective in this case. Generally, and depending on its tumor size and metastasis status, TNBC is treated with surgery (mastectomy or lympectomy), radiotherapy, and chemotherapy. However, TNBC remains more aggressive than other breast cancer subtypes with a higher three year recurrence rate and five-year mortality rate after the treatment. Therefore, in order to improve the prognosis and survival of patients with TNBC, some new treatments have been developed, such as targeted therapy and immunotherapy.

II.1. Current treatment strategies

II.1.1. Surgery:

TNBC involves surgical excision of breast tumour mass by mastectomy or breast-conserving surgery by lympectomy, followed by adjuvant radiotherapy. However, the choice of the operative therapy impacts the outcome of patients with TNBC; where in a study, it was found that the 5-year Overall Survival was better for breast-conserving surgery than for the mastectomy group (89 versus 69%, respectively) [21]. On the other hand, another study found that, after breast-conserving treatment with radiation, women with TNBC had a higher rate of local failure compared with women without TNBC, although the difference is still small [22].

II.1.2. Radiotherapy and chemotherapy:

Radiotherapy implies using high-energy rays to kill cancer cells and inhibit their growth. It was reported that radiotherapy is an essential component of breast-conserving treatment as showed in a previous study; TNBC patients who have received radiotherapy after breast-conserving treatment had 3-year locoregional recurrence free survival of 79.6% compared to 57.9% for those who did not receive adjuvant radiotherapy [23].

In addition to radiotherapy, many studies have shown that chemotherapy was beneficial for TNBC treatment; the response rate of TNBC patients to neoadjuvant chemotherapy was

higher than that of non-TNBC patients [24]. Anthracycline (doxorubicin, epirubicin, etc) and taxane (paclitaxel, docetaxel, etc) –based regimens represent the standard treatment strategies for breast cancer patients and have been shown to be highly active also in TNBC subtype. Furthermore, as TNBC is strongly associated with the Breast Cancer-1 (BRCA-1) gene mutations (cells with BRCA-1 mutations are deficient in DNA repair mechanisms), it makes it sensitive to platinum agents (cisplatin, carboplatin, etc) that bind to DNA and cause DNA double-strand breaks; then, TNBC cells lacking DNA repair mechanisms have no choice than undergoing apoptosis [25].

II.1.3. Targeted therapy and immunotherapy:

There is no damage to the healthy cells by using targeted therapy comparing with that of chemotherapy. Based on the type of TNBC and the components of their main pathways, some inhibitors have been developed and used to treat TNBC, for example:

- **PARP inhibitors**: in cancer cells when BRCA1/2 is mutant, cancer cells rely on Poly (ADP-ribose) polymerase (PARP) to repair their DNA damages. Briefly, PARP detects the single strand break in the DNA, it binds to it, and with the help of NAD+ (Nicotinamide adenine dinucleotide) it begins the synthesis of a polymeric adenosine diphosphate ribose chain, which acts as a signal for other DNA repairing enzymes. PARP inhibitors (e.g, olaparib) are used to work competitively with NAD+ to bind into PARP molecules and consequently inhibit DNA repair [26]. Furthermore, adding a DNA damaging agent (cisplatin) to PARP inhibitors therapy has given promising results for TNBC treatment [27]. In January 2018, the US Food and Drug Administration (FDA) approved Olaparib for the treatment of BRCA1/2 mutant metastatic breast cancers [28].
- **VEGF inhibitors**: Generally, Vascular Endothelial Growth Factor (VEGF) inhibitors block the growth of tumour neovascularisation; the anti-VEGF monoclonal antibody, bevacizumab, has shown some promising results in patients with metastatic breast cancer when added to paclitaxel, a first line chemotherapy drug [29]. However, bevacizumab in breast cancer and TNBC has not been approved by Food and Drug Administration agency, because of the lack of evidence regarding its efficacy and its increased toxicity [30].
- **EGFR inhibitors:** A large proportion of TNBC tumours overexpress the Epithelial Growth Factor Receptor (EGFR) and it is a negative prognostic factor in TNBC [31]. Interestingly, it was reported that the monoclonal antibody cetuximab, an EGFR inhibitor,

has shown some interesting activity in TNBC patients when combined with carboplatin [32].

- **Src tyrosine kinase inhibitors:** Src tyrosine kinase (Rous Sarcoma Virus) is overexpressed in TNBC and has been associated with metastatic disease progression [33]. A previous study has shown that dasatinib, a Src tyrosine kinase inhibitor, inhibited the growth of TNBC and was synergistic when combined to cisplatin [34]. Lehmann et al, have demonstrated that Mesenchymal and Mesenchymal Stem-Like TNBC subtypes were sensitive to dasatinib [18].
- **PI3K/mTOR inhibitors:** Mammalian target of rapamycin (mTOR) is an effector of the phosphatidylinositol 3-kinase (PI3K) signalling pathway, which is a key regulator pathway for promoting the survival and proliferation of tumour cells in many cancer types. Aberrations in the PI3K/protein kinase B/mTOR pathway are common in TNBC; 72% of tumours carried phosphorylated mTOR from 177 patients with TNBC [35]. A previous study has shown that a patient-derived xenograft TNBC model when treated with the mTOR inhibitor exhibited 77 to 99% tumour-growth inhibition [36]. Apitolisib is a PI3K inhibitor that also targets mTOR. In a cell derived TNBC model (MDA-MB-231), mice treated with apitolisib achieved tumour-growth inhibition of over 50% [37]. Several studies are now supporting the entery of PI3K/Akt/mTOR inhibitors to early-phase clinical trials [38].
- **Androgen receptors antagonists:** In the Luminal Androgen Receptor (LAR) TNBC subtype androgen hormone binds to androgen receptors (AR) which induces a translocation of the AR to the nucleus and activates genes transcription. Androgen receptors antagonists such as enzalutamide and bicalutamide competitively target AR to consequently inhibit tumour growth.
- **Immunotherapy:** PD-L1 or Programmed Cell Death Ligand 1 is an immune checkpoint protein that can bind to receptor PD-1 which is located on T cells and then inhibits the activity of the T cell effectors in tissues, to finally tumour cells can escape the T cell immune response [27]. PD-L1 in tumour cells or tumours microenvironment has been positively associated with TNBC [39]. The key tools for cancer immunotherapy are the immune checkpoint inhibitors or antagonists such as immune checkpoint antagonists of the PD-1/PD-L1 (atezolizumab). On March 2019, the FDA approved the immunotherapy drug atezolizumab used in combination with the chemotherapy drug nab-paclitaxel in metastatic TNBC [40].

II.2. Current treatment limitations

II.2.1. Surgery adverse effects

- Imaging: The need to develop more efficient location techniques to allow preoperative localization of non-palpable breast lesions [41].

- Post-surgery quality of life: 19-30% of patients negatively rated their breast cosmesis, arm swelling/pain, limitation of movement, loss of feeling in fingers and breast sensitivity and tenderness, in 5 and 10 years after breast conserving surgery and radiation [42].
- Psychology: A recent study found that women with pronounced breast asymmetry were much more likely to have negative psychological outcomes [43].

II.2.2. Chemotherapy and radiotherapy adverse effects

Systemic toxicity

A recent study found that the most common side effects mentioned by both patients and professionals were skin toxicity, fatigue and breast deformity [44], in addition to others:
- Central and peripheral neurotoxicity: caused by chemotherapy drugs such as cisplatin, paclitaxel, vincristine, gemcitabine… [45].
- Cardiovascular toxicity: as the main mechanisms of action of anthracyclines, especially doxorubicin, are DNA intercalation and free radical formation, the death of myocyte is occurred and leads to cardiac toxicity [46].
- Hematopoietic toxicity: most of chemotherapeutic agents cause a decrease in the bone marrow which leads to a dose limiting complications or an early cessation of chemotherapy. These effects are especially seen with docetaxel, paclitaxel, carboplatin, cisplatin and doxorubicin [45].
- Gastro-intestinal toxicity: nausea, vomiting and diarrhea are frequent adverse of treatments with chemotherapy such as cisplatin, carboplatin and doxorubicin. Pancreatitis was seen with L-asparaginase therapy, and liver injury was seen in the case of cisplatin, gemcitabine and paclitaxel therapies [45].
- Nephrotoxicity: cisplatin is a well-known chemotherapeutic nephrotoxin [45].

Chemo-resistance

Despite the anticancer effects of chemotherapy, cancer cells tend to escape apoptosis and maintain their viability through cellular senescence and cytoprotective autophagy [47]. Chemo-resistance accounts for 90% of drug failures in metastatic cancers [48].
Understanding the mechanisms of chemo-resistance in TNBC has a potential to develop more effective drugs by not only targeting TNBC cancer cells but also targeting cellular chemo-resistance pathways.

II.2.3. Targeted therapy and immunotherapy adverse effects

PARP inhibitors: the most common adverse events of PARP inhibitors are haematological (anaemia), gastrointestinal (nausea), kidney insufficiency (increase in creatinine concentrations) and fatigue [49][50].

VEGF inhibitors: bevacizumab has been shown to be involved in neurotoxicity, cardiovascular and pulmonary complications [45].

EGFR inhibitors: one of the most occurred adverse event in patients treated with cetuximab or panitumumab is hypomagnesemia which can change the duration of the treatment [51]. In a previous study, ocular toxicities were reported to be adverse effects of cetuximab and erlotinib [52].

Src tyrosine kinase inhibitors: dasatinib was reported to be toxic on cardiovascular system, lungs and bone marrow [45].

PI3K/mTOR inhibitors: PI3K/mTOR inhibitors may influence the development of oedema in patients with metastatic breast cancer [53]. Previous findings reported that gastrointestinal complications, thrombocytopenia, diarrhea, nausea, and asthenia are considered as dose-limiting effects of PI3K/mTOR inhibitors treatment [54].

Androgen receptors antagonists: it has been reported in a study that the most common adverse events related to Androgen receptors antagonists treatment were tremor (42%), nausea (42%), vomiting (37%), fatigue (37%), anaemia, delirium, mental status change, and confusional state in breast cancer patients [55].

Immunotherapy: according to preliminary results of a phase 1 trial presented at the American Association for Cancer Research'2015 Annual Meeting in Philadelphia, PA, USA (April 18–22), MPDL3280A is a monoclonal antibody against programmed death ligand-1 (PD-L1) that seems to be safe and tolerated in women with metastatic TNBC [56]. Furthermore, it was reported in a recent study that atezolizumab was an effective and well-tolerated treatment option for metastatic TNBC [57]. However a later study reported that the immune checkpoint targeting agents, PD-L1 inhibitors, are frequently associated with

cutaneous side effects [58]. Additionally, another recent study reported that problems in cutaneous, gastrointestinal, and pulmonary systems are common serious adverse events in the treatment with PD-L1 inhibitors [59].

To conclude, there are still big challenges for the treatment of TNBC, such as the lack of effective targets and the side effects of chemotherapy. However, it is mandatory to take into consideration the diversity of these adverse effects in making clinical decisions when treating patients. Therefore, further trials are warranted for optimal use of such agents in the clinical practice.

II.3. Natural products-based drugs development

II-3.1. Natural anticancer agents in TNBC

Man has always been leaning to nature for his well-being as he has discovered very early the therapeutic properties of certain natural products to overcome his suffering and improve his health. Today, in order to develop more effective drugs with low to no undesirable side effects, pharmaceutical industries and researchers are looking everywhere to improve current treatments or discovering new ones.

Natural product-derived drugs constitute a vast majority (more than 60%) of the chemotherapeutic agents currently in use for all types of cancers such as taxol, vinblastine, doxorubicin and many more [60][61]. For example: vinblastine and vincristine are vinca alkaloids isolated from the pink periwinkle plant *Catharantus rosea* and obtained by simple structural modifications [62]. Another example of the current chemotherapeutic agent, paclitaxel, that was first isolated from the bark of the Western yew tree *Taxus brevifolia* and *Taxus baccata* [63]. These drugs are widely marketed and were successfully used as effective anticancer drugs.

Recently, several reviews have analysed and summarised the anticancer effect of many important natural compounds with different mechanisms on TNBC. Here we are citing some of the most recent preclinical findings (from 2015 to 2018). A.K. Pandurangan and M.R. Mustafa (2018) [64] have reviewed some natural agents tested against TNBC with their proper mechanistic actions. Briefly:

- Caffeic acid (3,4-dihydroxycinnamic acid) phenethyl ester (CAPE) is a natural phenol compound and an active component of propolis from honeybee hives cells which has been found to be cytotoxic to MDA-MB-231 cells.

- Glycyrrhizin is a saponin-like compound that provides the main sweet flavour for *Glycyrrhiza glabra* (licorice) in combination with etoposide inhibits the topoisomerase 2α and induces apoptosis in MDA-MB-231 cells.

- Ampelopsin E an oligostilbenoid constituent found in *Dryobalanops beccarii.* It induces apoptosis and G2/M phase cell cycle arrest in MDA-MB-231 cells.

- Maximiscin is a secondary metabolite from the fungal isolate *Tolypocladiu*. It induces G1 phase cell cycle arrest in MDA-MB-468 cells.

- Coralyne is a heterocyclic analogue of protoberberine alkaloid. Coralyne in combination with paclitaxel exhibits synergistic effect on inhibition of proliferation, migration and induction of apoptosis in MDA-MB-231 cells.

- Vernodalin was isolated from the plant *Centratherum anthelminticum*. It regulates MDA-MB-231 cell apoptosis through activating the expression of Fork-head box (FOX) transcription factors, Bim, $p27^{Cip1}$, $p21^{Waf1/cip1}$, cyclin D1 and cyclin E in MDA-MB-231 cells.

- Norstictic acid metabolite is common in several lichen species of the genera *Usnea, Ramalina* and *Parmelia.* It suppressed the proliferation, migration and invasion with c-Met inhibition in MDA-MB-231 cells.

Furthermore, Elizabeth Varghese et al, (2018) [65] reviewed some natural molecules, found in the common kitchen vegetables and spices, with their targeted pathways in TNBC, both *in vitro* and *in vivo*. Briefly:

- Luteolin is a flavonoid found in some green leaves of vegetables and it is targeting β-catenin, VEGFR2 and PI3K/Akt pathway in MDA-MB-231 and BT-549.

- Chalcones are flavonoids found in some vegetables and are targeting Wnt/β-catenin and VEGF/VEGFR2 in MDA-MB-231, BT-549 and MDA-MB-468.

- Piperine is an alkaloid extracted from pepper and is targeting survivin, p65 and cell cycle components in both MDA-MB-231 and MDA-MB-468.

- Quercetin and Rutin are flavonoids found in apples and onions and are targeting PI3K/ Mitogen-Activated Protein Kinase (MAPK) and Wnt/β-catenin in both MDA-MB-231and MDA-MB-468.

- Fisetin is a flavonoid extracted from some green vegetables and is targeting PI3K/Akt/mTOR in MDA-MB-231 and MDA-MB-468.

- Curcumin is a phytopoly-phenol extracted from turmeric and is targeting VEGFR2/3, EGFR, Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB) in MDA-MB-231 and MDA-MB-468.

In conclusion, based on all these findings, natural compounds may be promising new tools to develop more effective TNBC drugs by identifying their action pathway and targeted proteins. As well as, these observations may help to a better use of these natural compounds in combination therapies to improve efficacy of the treatment with low to no side effects.

II.3.2. Carvacrol, thymoquinone, berberine and artemisinin: as new hopes for breast cancer and TNBC subtype treatment

II.3.2.a. Carvacrol: anticancer activity and toxicity

Carvacrol, $C_{10}H_{14}O$, is a monoterpenic phenol and a major component of the essential oils of many aromatic plants such as *Origanum dictammus, Origanum vulgare, Origanum majorana, Thymbra capitata, Satureja hortensis, Thymus vulgaris, Thymus zygis, Thymus serpyllum*, and *Satureja montana* [66]. Carvacrol is used as a safe additive for food flavouring and preservation, as it has been approved by the Food and Drug Administration [67]. Considerable researches have been led to establish the biological actions of carvacrol for its potential use in clinical applications. Carvacrol has been shown to have several interesting biological activities such as antibacterial, antifungal, acaricidal, antioxidant, antiplatelet, anti-inflammatory, antinociceptive, antidepressant/anxiolytic, organ protection and regeneration, metabolic enzyme modulations, spasmolytic and vasorelaxant [68][69][70].

Anticancer activity

In the last few years, researchers have focused on investigating the anticancer effects of carvacrol. Numerous studies have reported its anticancer effects accounting for human hepatocellular carcinoma cell line HepG-2 [71], breast cancer [72] gastric carcinoma cells, A549 non-small-cell lung cancer cells and human colon cancer cells by inducing strong

cytotoxic, genotoxic, and proapoptotic activities against cancer cells, in a dose-dependent manner [69].

Toxicity

Besides the important anticancer effects of carvacrol, this molecule may have a potential toxic effect on genotoxicity, corrosivity and lethality.

Genotoxicity:

It was reported that mutagenicity and genotoxicity of carvacrol is produced through DNA damage induction in a human colon carcinoma cell line [73], with a low genotoxic potential in the mouse lymphoma [74].

Corrosivity and lethality:

Corrosivity and lethality studies of carvacrol on animals and humans subjects are summarised in the next table (Table 2) as detailed in Andersen et al., review [75].

Table 2: Corrosivity and lethality studies of carvacrol on animals and human subjects.

Subjects	Measured parameter	Administration method	Dose of carvacrol	Observations
Rats	- Median Lethal Dose	- Oral gavage	- 810 mg/kg	- Death
	- Skin corrosivity	- Cutaneous	- (No data)	- Non-corrosive
Mice	Median Lethal Dose	- Intravenous - Intraperitoneal - Subcutaneous	- 80 mg/kg - 73.3 mg/kg - 680 mg/kg	Death
Rabbits	- Median Lethal Dose	- Cutaneous	- 2700 mg/kg	- Death
	- Irritancy	- Cutaneous	- 5000 mg/kg	- Redness and oedema
Dogs	Median Lethal Dose	Intravenous	310 mg/kg	Death
Guinea pigs	General adverse effects	Subcutaneous	1 %	No adverse effects
Humans	General adverse effects	Patch test	- 4 % in petrolatum - 8 % in petrolatum	No adverse effects

Interestingly, carvacrol showed general safety towards human subjects' health besides its important anticancer activities against several types of cancer. However, the studies about its

effect on TNBC subtype are still needed to understand its potential usefulness in TNBC therapy development.

II.3.2.b. Thymoquinone: anticancer activity and toxicity

Thymoquinone, $C_{10}H_{12}O_2$, is one of the volatile bioactive constituents of *Nigella sativa* seed oil also known as black seed. Various studies have reported its antioxidant, analgesic, anti-inflammatory, anti-asthmatic, antipyretic, antimicrobial, antihypertensive, chemo-protective potentials as well as its anticancer activities [76][77].

Anticancer activity

Several studies have revealed the therapeutic effect of thymoquinone in different types of cancers such as bone, cervical, liver, colon, lung, brain, skin and breast cancers through various cellular mechanisms such as cell proliferation inhibition, apoptosis induction, interruption of cell cycle progression, production of reactive oxygen species (ROS), activating caspases pathway, downregulating Wnt/β-catenin, PI3K/Akt and Signal Transducer and Activator of Transcription 3 (STAT3) pathways [76].

Toxicity

Thymoquinone showed no observable cytotoxicity against fibroblast-like synoviocytes in the concentration ranging between 0 to 10 μM, and it was tolerated at a dose of 75 to 2600 mg/day in rats [78].

II.3.2.c. Berberine: anticancer activity and toxicity

Berberine, $C_{20}H_{18}NO_4^+$, is a natural isoquinoline alkaloid compound isolated from the stems and roots of several plants such as *Berberis vulgaris*, *Berberis aristata* and *Coptidis rhizome*. It exerts several pharmacological activities such as antiplatelet effect, antibacterial, anti-inflammatory, immunomodulatory, anti-oxidative, neuroprotective, hypolipidemic, anti-diabetic and anticancer activities [79].

Anticancer activity

Besides to our previous study where we reported that berberine killed MCF-7 breast cancer cells through apoptosis induction (see Annex 1) [80], several preclinical studies have also reported the anticancer effect of berberine where it exhibited its inhibitory effects on a variety of tumours such as hepatoma, leukemia, lung, colon, ovarian, cervical and breast cancer cells through apoptosis induction and cell cycle arrest, inhibition of migration and

invasion, reduction of the expression of VEGF mRNA and inhibition of angiogenesis [81][82][80][83].

Toxicity

Berberine has generally showed different level of toxicity and safety in animals [84] and humans [85] (Table 3 and 4).

Laboratory animals

Table 3: Toxicological effects of berberine on laboratory animals

Subjects	Measured parameter	Administration method	Dose of berberine	Observations
Mice	Median Lethal Dose	- Oral	- 329 mg/kg	- Death
		- Intraperitoneal	- 23 mg/kg	- Death
Mice	Immunocytotoxicity	Intraperitoneal	10 mg/kg	Negative changes in cellular and humoral immune functions
Cats	General adverse effects	Oral	- 100 mg/kg	- Vomiting (in 6-8h)
			- 100 mg/kg	- Death (in 8-10h)
Rats	Liver morphology	Intraperitoneal	50, 100, 150 mg/kg	Liver tissue damages

Human subjects:

Table 4: Toxicological effects of berberine on human subjects

Subjects	Measured parameter	Administration method	Dose of berberine	Observations
Diabetic patients	General adverse effects	Oral	500 mg TD/ 13 weeks	34.5% of patients showed flatulence, diarrhea and constipation
Diabetic patients	General adverse effects	Oral	1 g BD/ 8 weeks	Safe

TD: Three times a day; **BD:** Two times a day.

Furthermore, some clinical trials reported that berberine decreased the incidence and severity of radiation-induced lung injury and acute intestinal symptoms in cancer patients with radiotherapy [86][87].

II.3.2.d. Artemisinin: anticancer activity and toxicity

Discovered in 1972 by Professor Youyou Tu, and her team, one of the 2015 Nobel Prize winners in Medicine, artemisinin, an ancient Chinese herbal therapy extracted from *Artemisia annua* L. (sweet wormwood) has been recognized as a treatment for malaria fevers [88]. In addition to its antimalarial effect, artemisinin, $C_{15}H_{22}O_5$, the sesquiterpene lactone exerted several biological activities such as antiviral effect, antibacterial, antihelminthic, antiprotozoa, antifungal, anti-inflammatory and anticancer activities [89].

Anticancer activity

Artemisinin and its derivatives (dihydroartemisinin, artesunate, artemether, arteether and artemisone) have been recently found to have potent activity against many types of cancer including lung cancer, gastric cancer, brain glioma, colon cancer, leukemia, ovarian cancer, prostate carcinoma and breast cancer [90]. Artemisinin and its derivatives can exert their anticancer effect through arresting cell growth, inducing apoptosis, inhibiting metastasis and causing oxidative DNA damage and double strand breaks. Previous studies have reported that artemisinin-induced cytotoxicity of cancer cells was due to intracellular production of Reactive Oxygen Species (ROS) and this is linked to an endoperoxide bridge in artemisinin molecule which reacts with the presence of intracellular ferrous iron in different cancer types [89][90][91].

Toxicity

Laboratory animals:

There are few available data describing the safety of artemisinins. A clinical toxicity evaluating the activity of artemisinin in dogs has reported that the most frequent adverse event was anorexia but only in a minority of the subjects, concluding that oral artemisinin is generally well tolerated in dogs [92].
Previous literatures showed that artemisinin derivatives, arteether and artesunate, might have adverse effects related to the neurotoxicity and auditory functions in rats [90][93]. Fortunately it was safe on the auditory system of human malaria patients [94].

Human subjects:

More than 2 million malaria patients have been treated with artemisinin or its derivatives with no major or serious adverse events being reported [95].

Furthermore, there was no evidence of clinical brain stem pathology of artemisinin and its derivatives treatments in treated people, the only observed adverse events were low frequencies of abdominal pain, and diarrhea [93].

Chapter III

Material & Methods

III.1. Drugs and cell lines

Berberine chloride hydrate ≥90% (PubChem CID: 2353), artemisinin 98% (PubChem CID: 68827), thymoquinone 98% (PubChem CID: 10281) and carvacrol 98% (PubChem CID: 10364) were all purchased from Sigma Aldrich (USA). Cell culture reagents were all purchased from Life Technologies (USA). All breast cancer cell lines: TNBC (BT-20, HCC70, HCC38, HCC1937, HCC1143, BT-549, MDA-MB-468 and MDA-MB-231), non-TNBC (MCF-7/ER-PR+HER2+ and SKBR3/ER-PR-HER2+) and the immortalized breast epithelial MCF-10A cell lines were purchased from ATCC (USA).

III.2. Cell culture

Cell lines were authenticated in 2018 and they do not exceed 15 passages in cell culture or they were stored in liquid nitrogen for future use.

BT-20 and MCF-7 cell lines were cultured in MEM (Eagle) medium with 10% fetal bovine serum (FBS), 1% glutamax, 1% penicillin/ streptomycin, 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino-acids and 1 mM sodium pyruvate.

HCC70, HCC38, HCC1937, HCC1143 and BT-549 cell lines were cultured in RPMI 1640 medium with 10% FBS, 1% glutamax, 1% penicillin/ streptomycin, 1.5 g/L sodium bicarbonate, 10 mM Hepes and 1 mM sodium pyruvate.

MDA-MB-468 cells were cultured in RPMI 1640 medium with 10% FBS, 1% glutamax and 1% penicillin/ streptomycin.

MDA-MB-231 cells were cultured in DMEM/F12 with 10% FBS and 5% Penicillin/ streptomycin.

SKBR3 cells were cultured in McCoy's 5A medium with 10% FBS, 1% glutamax and 1% penicillin/ streptomycin

MCF10A cells were cultured in DMEM/F12 with 5% horse serum, 1% penicillin/ streptomycin, 20ng/mL EGF, 100ng/mL cholera toxin, 0.01mg/mL insulin and 500ng/mL hydrocortisone.

All the cells were maintained in a humidified 5% CO2 incubator at 37°C.

III.3 Assessment of cell viability using MTT assay

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) (Sigma-Aldrich), a colorimetric assay capable of detecting viable cells by the reduction of the yellow tetrazolium salt to purple formazan. In order to ensure their adhesion and their exponential growth on the day of the treatment, the TNBC and non-TNBC cells were seeded in 96 well plates at the proper density 2 days (48 h) before the treatment (Table 5). In the day of the treatment, the cells were treated with one of the drugs (berberine, artemisinin, thymoquione and carvacrol) already dilluted in Dimethylsulfoxyde (DMSO) (Sigma-Aldrich). After the treatment, the cells were incubated for 4 times their doubling time, as described in [96] (Table 5). DMSO (1%), non-treated cells and Doxorubicin were used as negative and positive controls, respectively. At the end of the incubation period, the cells were treated with MTT (5mg/ml in Phosphate Buffered Saline (PBS)) for 4h at 37°C. 10% Sodium Dodecyl Sulfate (SDS) in 10 mM hydrochlorid acid (HCL) was added to dissolve the formazan, and then the cells were incubated overnight at 37°C. The production of formazan which is directly proportional to the viable cell number was read as absorbance values at 540nm using Infinite 200 (Tecan) with Magellan data analysis software. Experiments were done in triplicate and in three independent assays. Cell viability percentage curves and IC_{50} were calculated using Microsoft Excel 2010. IC_{50} was calculated using the line equation between two "x" and their corresponding "y" values around the 50% of growth inhibition.

Table 5: Seeded cell concentrations per well used in cell viability assay and their doubling time

Cell lines	Cell concentration ($\times 10^3$ cell/mL) 150µL/well in 96 wells plate	Cells doubling time (hour)
HCC1937	10	45
HCC70	20	50
MDA-MB-468	10	45
HCC38	20	60
BT-20	10	50
HCC1143	10	60
BT-549	5	30
MCF-7	10	45
SKBR3	10	60

MDA-MB-231	5	25

III.4 Two-dimensional clonogenic assay

Two-dimensional (2D) clonogenic (or colony formation) assay is an *in vitro* cell survival test based on the ability of a single cell to grow into a colony by undergoing multiple divisions [97]. We choosed to work on the most sensitive cell lines to each treatment, which were: MDA-MB-468, HCC38 and HCC70. These cells were treated with all the molecules (Berberine, artemisinin, thymoquinone and carvacrol) at the doses of 0.5 and 5 µM, these doses are around their IC_{50}. As long as berberine was the most cytotoxic molecule, we enrolled another 2D clonogenic test on the most sensitive cell lines to berberine's treatment BT-20, MDA-MB-468 and HCC70 with berberine at the doses of 0.2, 0.5 and 1 µM. Viable cells were seeded to 6 wells plates (Ø 35mm) at different concentrations depending on the cell line (Table 6), and allowed to attach to the well bottom overnight. Cells were treated then with different doses of the drugs and incubated the time to allow colonies to form in the negative control DMSO (0.005%) (Table 6). The obtained colonies were washed with PBS, fixed and stained with 0.05% of Coomassie® Brilliant Blue R-250 (ICN Biomedicals) solubilized in 50% of methanol, 10% Acetic acid and 40% of Milli-Q water. For each cell line we counted the colonies in each well and compared them to DMSO control treated cells using ImageJ software. All the experiments were carried out in triplicate and in three independent assays.

Table 6: Seeded cell number per well used in 2D clonogenic assay and their colony formation time

Cell lines	The number of cells/ well	Colony formation time (day)
MDA-MB-468	2000	10
HCC70	5000	15
HCC38	7000	15
BT-20	7000	15

III.5 Cell cycle analysis by flow cytometry

Cells were seeded to 6 wells plates (Ø 35 mm) at different concentrations depending on the cell line; MDA-MB-468 and BT-20 as 2×10^5 cells/well, HCC70 and HCC38 as 3×10^5 cells/well. The cells were then allowed to attach to the well bottom overnight.

MDA-MB-468, HCC70 and BT-20 were treated with berberine at 0.2, 0.5 and 1 µM and then incubated for 72 and 120 h.

MDA-MB-468 and HCC38 were treated with artemisinin at 0.5 and 5 µM, and then incubated for 48 h.

DMSO (0.005%) was used as a negative control. All the cells were incubated in 37°C with 5% CO_2.

After the incubation time, both attached and detached cells were harvested, washed three times and centrifuged at 1500 rpm (for 5 min) with PBS (1X). The cells were then centrifuged and fixed with cold ethanol (70%) and incubated for 30 minutes in RT. Next, the cells were washed twice with PBS (1X). After removing ethanol, cells were centrifuged and the pellet was resuspended in PBS containing 10µg/mL of propodium iodide (invitrogen P3566) and 100µg/mL of RNAase (invitrogen 12091-021). The pellet was then incubated for 15 min at RT in the dark. Finally, the cell cycle analysis was carried out using LSRII flow cytometer (Becton Dickinson) with DIVA™ software to determine cellular DNA content. The cell population percentage of each phase was analyzed using FlowJo and ModFit LT software package.

III.6 Apoptosis analyses

Cells were seeded to 6 wells plates (Ø 35 mm) at 2×10^5 cells/well for MDA-MB-468 and BT-20 cells, and at 3×10^5 cells/well for HCC70 cells, and allowed to attach to the well bottom overnight. Cells were treated with berberine at the doses of 0.2, 0.5 and 1µM, and incubated for 120 and 144h in 37°C with 5% CO_2. DMSO (0.005%) was used as a negative control. After incubation, both attached and detached cells were harvested and washed twice by PBS (1X). Next, the cells were then stained with 1% Annexin-V-FITC diluted in 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic (HEPES) buffer for 15min in the dark. Cells were then washed and stained with 1% propodium iodide diluted in HEPES buffer at room temperature (RT) in the dark. The quantification of viable, apoptotic and necrotic cells populations was achieved through LSRII flow cytometer (Becton Dickinson) using DIVA™ and FlowJo softwares.

III.7 Protein analyses by western blotting

1×10^6 of MDA-MB-468 and BT-20 cells, and 1.5×10^6 of HCC70 cells were seeded in (Ø 90mm) Petri dishes and allowed to attach overnight, then treated with berberine at 0.5 and 1 µM, and then incubated for 120 and 144h in 37°C with 5% CO_2. DMSO (0.005%) was used as a negative control. For whole cell protein lysates, the samples were prepared according to the established protocol [96]. The lysates were collected using Laemmli 1X buffer. Proteins dosage was done by BCA Protein assay kit reducing Agent Compatible (Perbio, ref 23250). Proteins concentrations were read as absorbance values at 562 nm using Infinite 200 (Tecan, Lyon, France) with Magellan data analysis software.

Equivalent amounts of protein lysates (20 µg) were separated by electrophoresis in acrylamide gel (Bio-Rad TGX 4-15%) starting with 15 mA then increasing to 25 mA, then blotted onto nitrocellulose membrane (Bio-Rad, Marnes la Coquette, France). The membranes were then saturated with 5% BSA+TBS/0.1% Tween for 1 h at RT.

The blotted membranes were incubated with different primary antibodies for 2 h at RT, and then washed four times with TBS/0.1% Tween 5 minutes each, at RT. The membranes were then followed by incubations with secondary antibodies labeled with horseradish peroxidase (Jackson Immuno Research Laboratories, Interchim, Clichy, France) for 1 h at RT. The proteins were visualized using ECL kit (Amersham Pharmacia Biotech, Orsay, France). The quantification was performed using a LAS-3000 Luminescent Image analyser and Multi Gauge software (Fuji, FSVT, Courbevoie, France).

Actin was detected for normalisation between samples using anti-beta-actin primary antibody at the dilution of 1:2000 (Sigma- Aldrich). MGMT, PCNA, cleaved-PARP (cl-PARP), cleaved-caspase-7 (cl-caspase-7) and cleaved-caspase-8 (cl-caspase-8) antibodies were used at 1:1000 dilution. Cyclin D1 antibody was used at 1:10000 dilution. Histone H2AX and phosphorylated-H2AX (P-H2AX) antibodies were diluted at 1:2000. Cyclin B1 antibody was used at 1:1000 dilution. All the antibodies were purchased from Cell Signaling Technology, Ozyme, Saint Quentin en Yveline, France.

III.8 Drug combination assay

MTT assay was used to measure cell viability of artemisinin/cisplatin and berberine/cisplatin combinations in the same conditions as described above in the cell viability assay.

MDA-MB-468 cells at 1×10^4 cells/mL and HCC38 at 2×10^4 cells/mL were seeded and grown in 96 wells plates, and treated with increasing concentrations of artemisinin from 0 to 10 µM,

in combination with increasing concentrations of the conventional chemothetapy drug cisplatin from 0 to 0.5 µM for MDA-MB-468 and from 0 to 5 µM for HCC38 cells.

MDA-MB-468 and BT-20 cells at 1×10^4 cells/mL, HCC70 at 2×10^4 cells/mL were seeded and grown in 96 wells plates, and treated with increasing concentrations of berberine from 0 to 2 µM for MDA-MB-468 and from 0 to 1 µM for BT-20 and HCC70 cells, in combination with increasing concentrations of cisplatin from 0 to 0.5 µM for MDA-MB-468 and from 0 to 5 µM for BT-20 and HCC70.

The choice of the drugs concentrations ranges was according to their IC_{50} towards the cell lines; IC_{50} of berberine and artemisinin are found in the cells viability assays as shown in this present paper, and cisplatin IC_{50} were found by the Breast Cancer Biology Group team of Institut Curie laboratories (Paris, France).

After 4 doubling-time incubation period for each cell line (table 5), MTT was added and incubated in 37°C for 4 h. Afterwards, 10% SDS in 10 mM hydrochlorid acid was added to dissolve the formazan and incubated overnight at 37°C. Absorbance values were read at 540nm using a plate reader (Infinite 200, Tecan, Lyon, France) with Magellan data analysis software. Experiments were independently done three times in triplicate. DMSO (1%) was used as a negative control. The fraction affected from viability percentages was calculated using an Excel template and the results were analyzed with the web version of Chalice Analyzer (Horizon Discovery) to calculate Loewe model Excess scores [98].

III.9 Three-dimensional cell culture

Three-dimensional (3D) cell culture was conducted with matrigel (BD Biosciences) as described in [99]. Briefly, in 96 well plates, 5200 cell/well of malignant MDA-MB-468 cells and 6500 cell/well of normal Human breast epithelial cells MCF-10A were seeded between two matrigel coats with cell culture medium, for 7 and 10 days, respectively, at 37°C with 5% CO_2. The medium was changed and replaced with a new one every 2/3 days.

Cells were then treated with berberine at various concentrations (0.5 and 5 µM). DMSO (0.025%), non-treated cells and BI2536 drug [100] were used as negative and positive controls, respectively. Cell viability was determined 3 days after the treatment using the WST1 Cell Proliferation Assay Kit (Millipore) which is based on the enzymatic cleavage of the tetrazolium salt WST1 to formazan by cellular mitochondrial dehydrogenases present in viable cells. The absorbance was measured using a microplate reader (Infinite 200, Tecan)

with Magellan data analysis software at a wavelenght of 450nm. Experiments were carried out in triplicate and in three independent assays.

III.10 Statistical analysis

Data are represented as the mean ± standard deviation (SD) of three independent experiments. The statistical significance of the obtained results was evaluated by using a one-way analysis of variance ANOVA at $P<0.05$ (as *), $P<0.01$ (as **) and $P<0.001$ (as ***), using STATA 14.2 software.

Chapter IV

Results & Discussion

IV.1. Screening of berberine, artemisinin, thymoquinone and carvacrol growth inhibition effects of several TNBC cell lines:

In this paper, we first studied the antiproliferative effects of carvacrol, thymoquinone, berberine and artemisinin on eight TNBC (BT-20, HCC70, HCC38, HCC1937, HCC1143, BT-549, MDA-MB-231 and MDA-MB-468) and two non-TNBC (MCF-7/ER-PR+HER2+ and SKBR3/ER-PR-HER2+) cell lines.

Berberine inhibited the cell growth of TNBC and non-TNBC cell lines in a dose dependent manner, with IC_{50} values ranging from 0.21 µM to 26.56 µM (Table 7). According to the IC_{50} values and the curves shape of the cell lines, we noticed that the cells have different responses towards the treatment depending on the doses of berberine, while HCC70 (IC_{50}= 0.21 µM), BT-20 (IC_{50}= 0.21 µM) and MDA-MB-468 cells (IC_{50}= 0.66 µM) were found to be the most sensitive ones to berberine treatment, MDA-MB-231 was the most resistant one (IC_{50}= 26.56 µM) among all the treated cell lines (Figure 5).

Artemisinin inhibited cell growth depending on the dose and also on the cell line type with IC_{50} values ranging from 1.25 µM to 54.59 µM (Table 8). Cells have different responses towards the treatment depending on the doses of artemisinin, while HCC38 (IC_{50}= 1.25 µM), and MDA-MB-468 (IC_{50}= 2.23 µM) were the most sensitive ones, HCC70 was the most resistant one with IC_{50}= 99.60 µM among all the treated cell lines (Figure 6).

Thymoquinone differently inhibited the cell growth of non-TNBC and TNBC cells in a dose dependent manner, with IC_{50} ranging from 0.76 µM to 72.45 µM (Table 9). MDA-MB-468 (IC_{50}= 0.76 µM) and SKBR3 (IC_{50}= 8.30 µM) were the most sensitive cell lines to thymoquinone treatment. However, HCC70 was the most resistant one among the treated cell lines with IC_{50}= 72.45 µM (Figure 7).

Surprisingly, carvacrol did not inhibit the cell growth of all treated cells even at its highest dose (100 µM) except for MDA-MB-468 with IC_{50}= 5.15 µM. (Table 10) and (Figure 8).

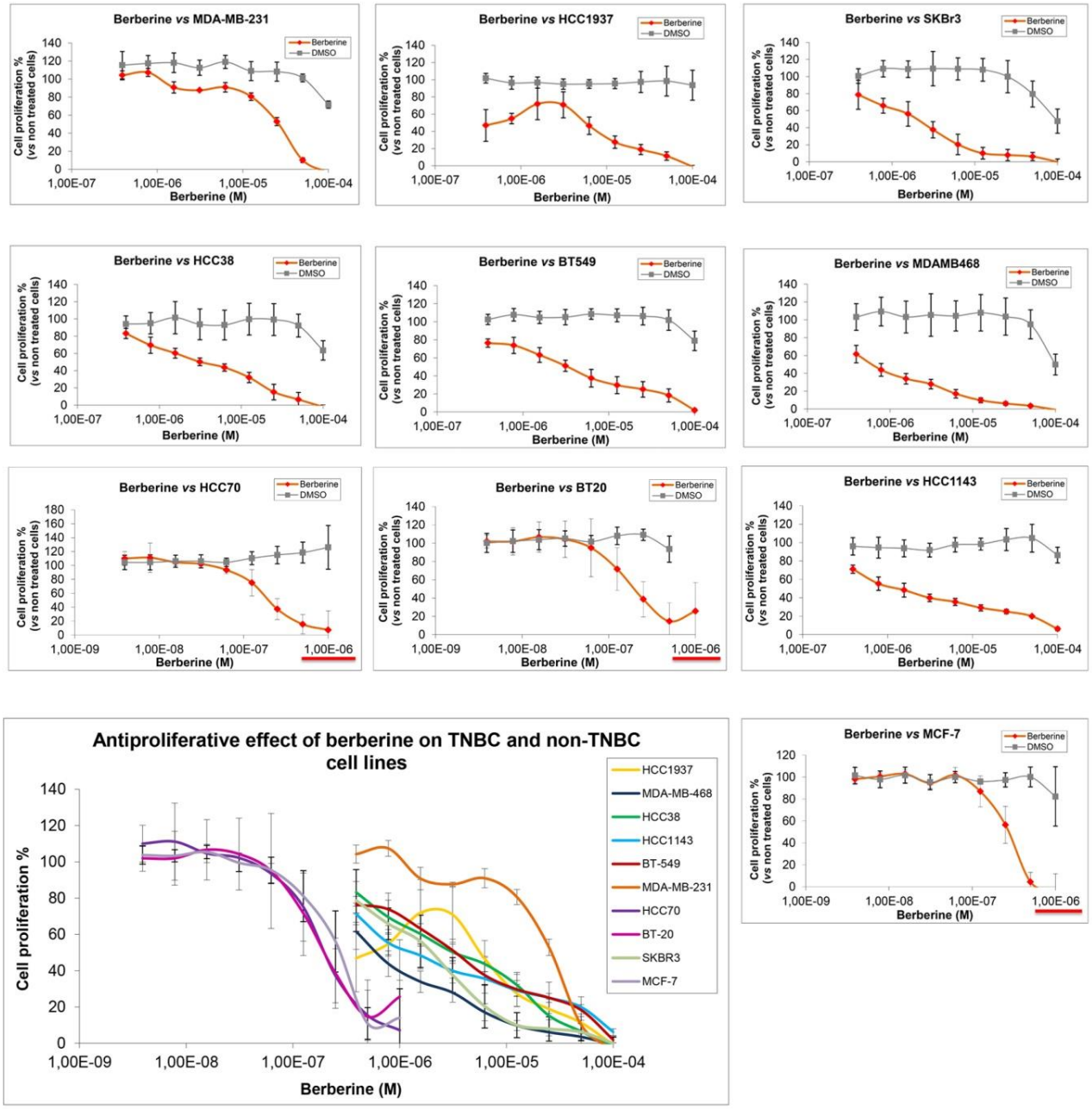


Figure 5: Effect of berberine's treatment on TNBC and non-TNBC cell lines proliferation. The cell lines were seeded and treated with a wide range of berberine's doses. Cell viability was measured by MTT assay and represented as cell proliferation percentage ± Standard Deviation (SD) of three independent experiments carried out in triplicate

Table 7: IC_{50} (µM) values of berberine on TNBC and non-TNBC cell lines ±Standard Deviation.

Cell line	Berberine IC_{50} (µM)
HCC70	0.21 ± 0.06
BT-20	0.21 ± 0.10
MCF-7	0.28 ± 0.08
MDA-MB-468	0.66 ± 0.25
HCC1143	1.56 ± 0.74

SKBR3	2.03 ± 0.94
BT549	3.66 ± 1.29
HCC38	3.78 ± 1.05
HCC1937	5.48 ± 2.09
MDA-MB-231	26.56 ± 2.37

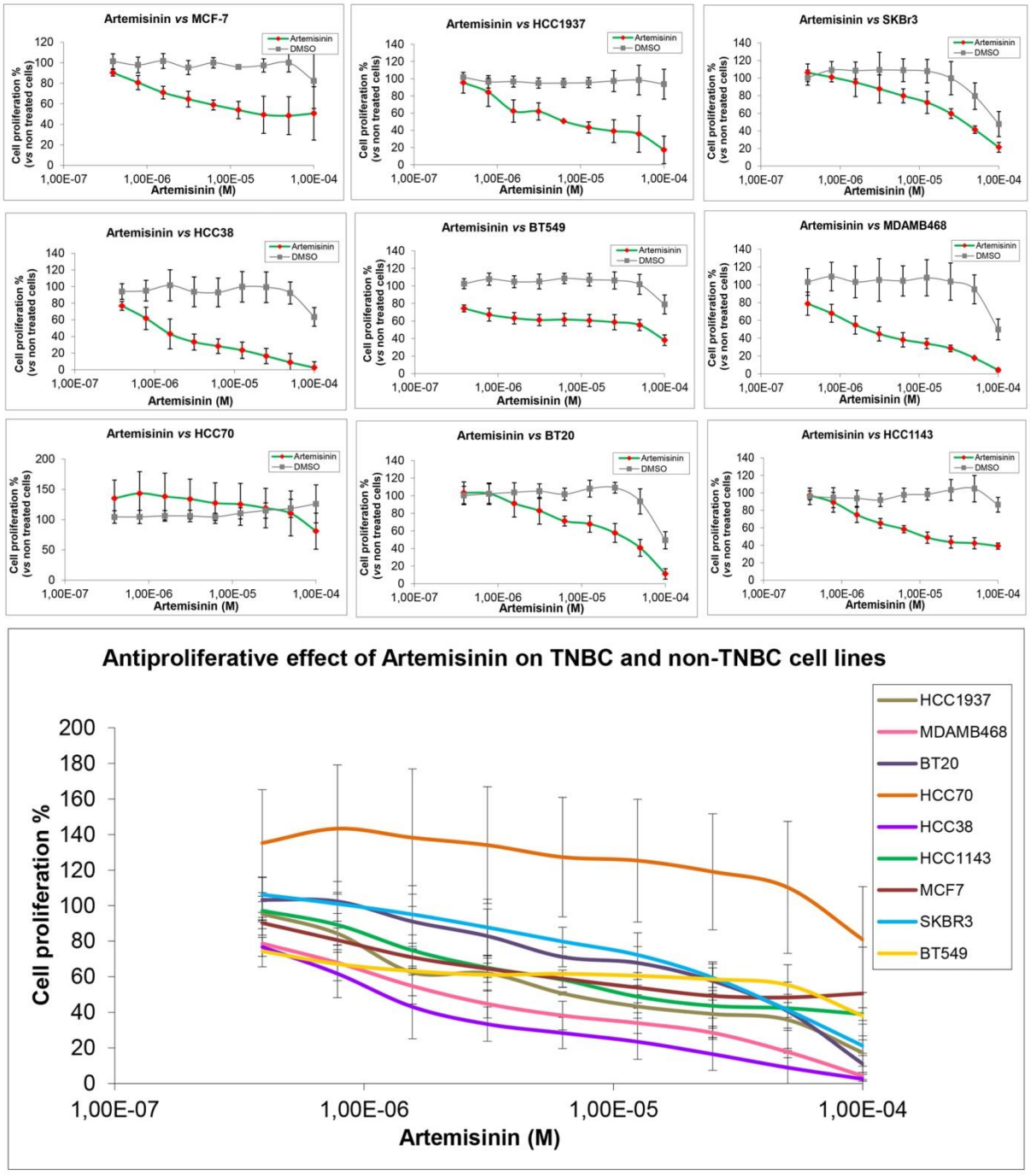


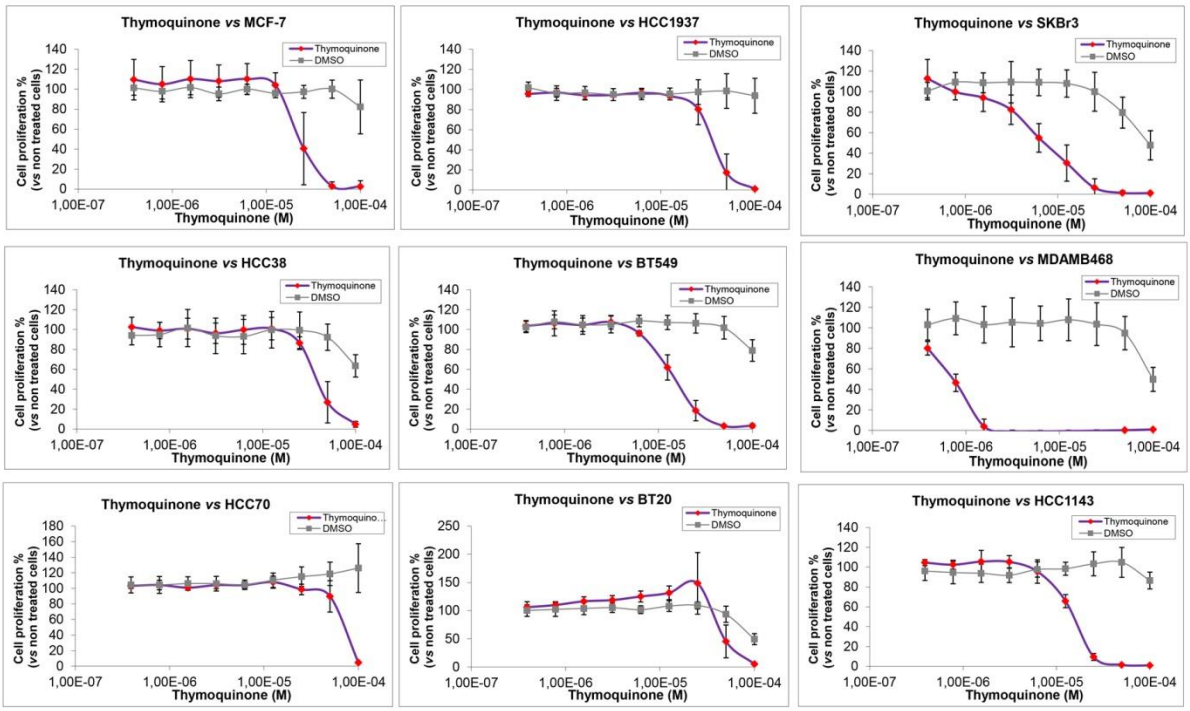
Figure 6: Effect of artemisinin's treatment on TNBC and non-TNBC cell lines proliferation. The cell lines were seeded and treated with a wide range of artemisinin's doses. Cell viability was measured by MTT assay and represented as cell proliferation percentage ± Standard Deviation (SD) of three independent experiments carried out in triplicate.

Table 8: IC_{50} (µM) values of artemisinin on TNBC and non-TNBC cell lines ±Standard Deviation.

Cell line	Artemisinin IC_{50} (µM)
HCC38	1.25 ± 1.31
MDA-MB-468	2.23 ± 3.33
HCC1937	8.01 ± 2.98
HCC1143	15.28 ± 6.14
MCF-7	28.24 ± 24.14
BT-20	35.87 ± 13.25
SKBR3	37.76 ± 6.65
BT-549	54.59 ± 30.75
HCC70	99.60

Table 9: IC_{50} (µM) values of thymoquinone on TNBC and non-TNBC cell lines ±Standard Deviation.

Cell line	Thymoquinone IC_{50} (µM)
MDA-MB-468	0.76 ± 0.11
SKBR3	8.30 ± 3.51
BT-549	15.97 ± 3.32
HCC1143	15.91 ± 0.93
MCF-7	28.52 ± 6.40
HCC1937	37.34 ± 6.62
HCC38	42.37 ± 9.02
BT-20	52.63 ± 13.03
HCC70	72.45 ± 6.17



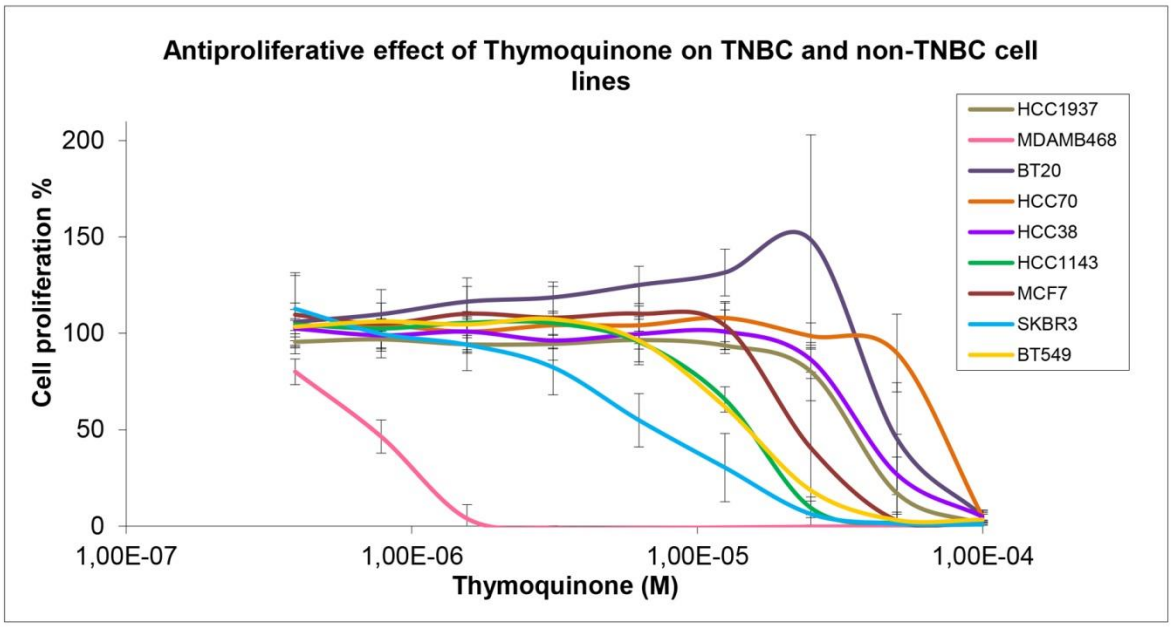


Figure 7: Effect of thymoquinone's treatment on TNBC and non-TNBC cell lines proliferation. The cell lines were seeded and treated with a wide range of thymoquinone's doses. Cell viability was measured by MTT assay and represented as cell proliferation percentage ± Standard Deviation (SD) of three independent experiments carried out in triplicate.

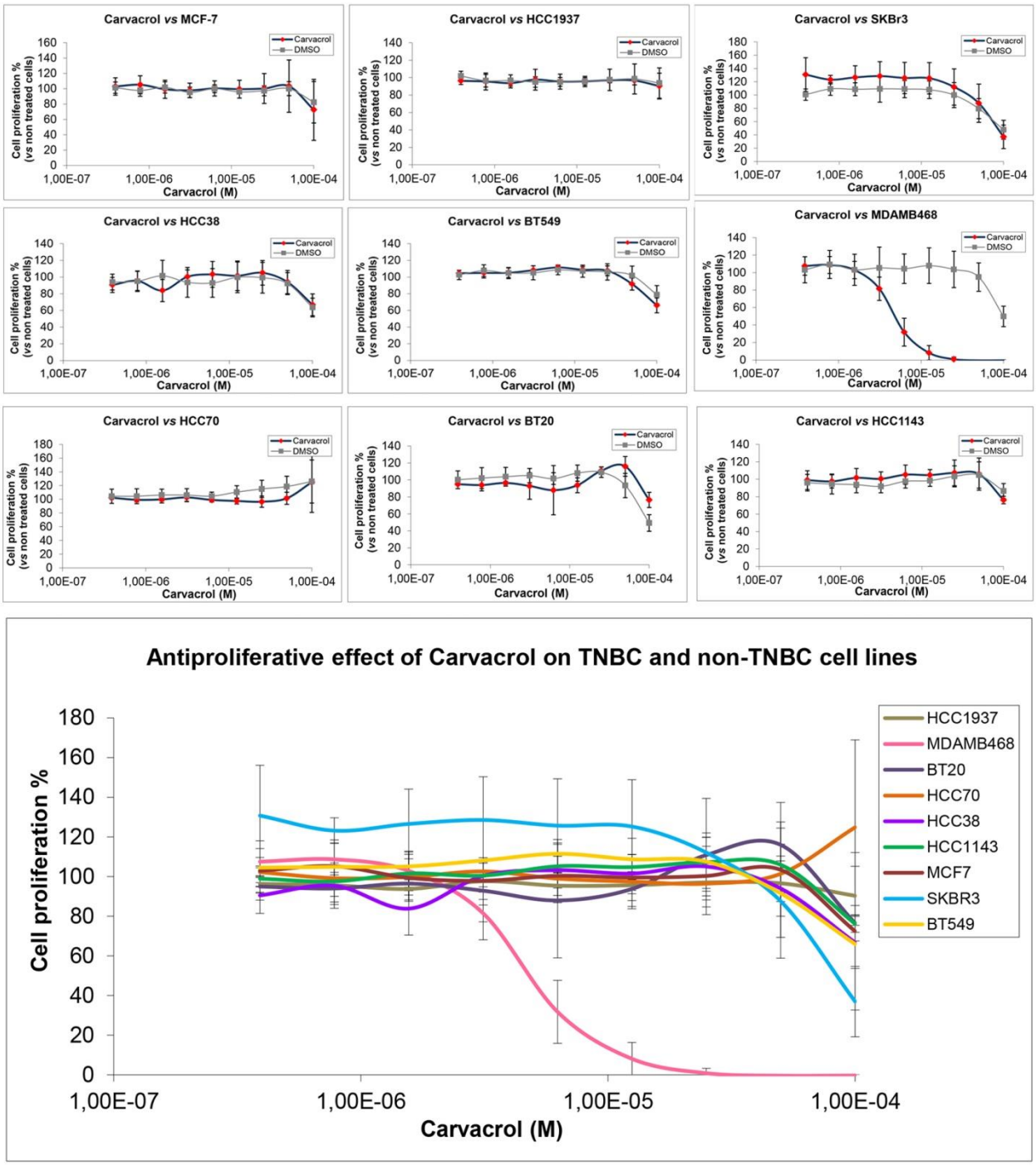


Figure 8: Effect of carvacrol's treatment on TNBC and non-TNBC cell lines proliferation. The cell lines were seeded and treated with a wide range of carvacrol's doses. Cell viability was measured by MTT assay and represented as cell proliferation percentage ± Standard Deviation (SD) of three independent experiments carried out in triplicate

Table 10: IC_{50} (µM) values of carvacrol on TNBC and non-TNBC cell lines ±Standard Deviation.

Cell line	Carvacrol IC_{50} (µM)
MDA-MB-468	5.15 ± 0.69
SKBR3	–

BT-549	**—**
HCC1143	**—**
MCF-7	**—**
HCC1937	**—**
HCC38	**—**
BT-20	**—**
HCC70	**—**

—: No cell growth inhibition was observed

To summarize, berberine and artemisinin were the most effective molecules against TNBC and non-TNBC cell lines in comparison to the other tested drugs, with carvacrol being the least effective one. We also noticed that MDA-MB-468 was the most sensitive cell line towards all the tested natural molecules. Further, to establish the inhibitory role of berberine, artemisinin, thymoquinone and carvacrol on transforming properties of TNBC cells, we performed the 2D-clonogenic assay as described in "Material and Methods" section.

The choice of TNBC cell lines used on the 2D clonogenic assay was based on the sensitivity of a cell line towards the drug, according on the cell viability measurement test results; MDA-MB-468 cell line was sensitive to all tested drugs, HCC70 cells were sensitive to berberine and HCC38 cells were sensitive to artemisinnin treatment.

As shown in figure 9, berberine significantly (p<0.001) inhibited the capacity of MDA-MB-468, HCC38 and HCC70 cells to form colonies on the bottom of the wells at 0.5 µM and 5 µM. Furthermore, since 5 µM is largely superior to the IC_{50} of the treated cells (tables 7), we decreased the doses of berberine to 0.2, 0.5 and 1 µM and added BT-20 to the group of tested cells, as BT-20 is one of the most sensitive cell lines to berberine treatment.

Figure 10 showed that berberine significantly (p<0.001) inhibited colony formation in MDA-MB-468 at all tested concentrations. However, we noticed that BT-20 and HCC70 responded differently to berberine treatment; when berberine at 0.2 µM significantly (p<0.01) inhibited colony formation of BT-20 cells, it exhibited no significant effect at the doses of 0.5 and 1 µM, even though the IC_{50} of berberine on BT-20 is largely under these tested concentrations (tables 7). Moreover, berberine showed a significant (p<0.001) effect on the inhibition of colony formation of HCC70 at 0.2 and 1 µM, but surprisingly, it significantly (p<0.01) induced colony formation at the dose of 0.5 µM.

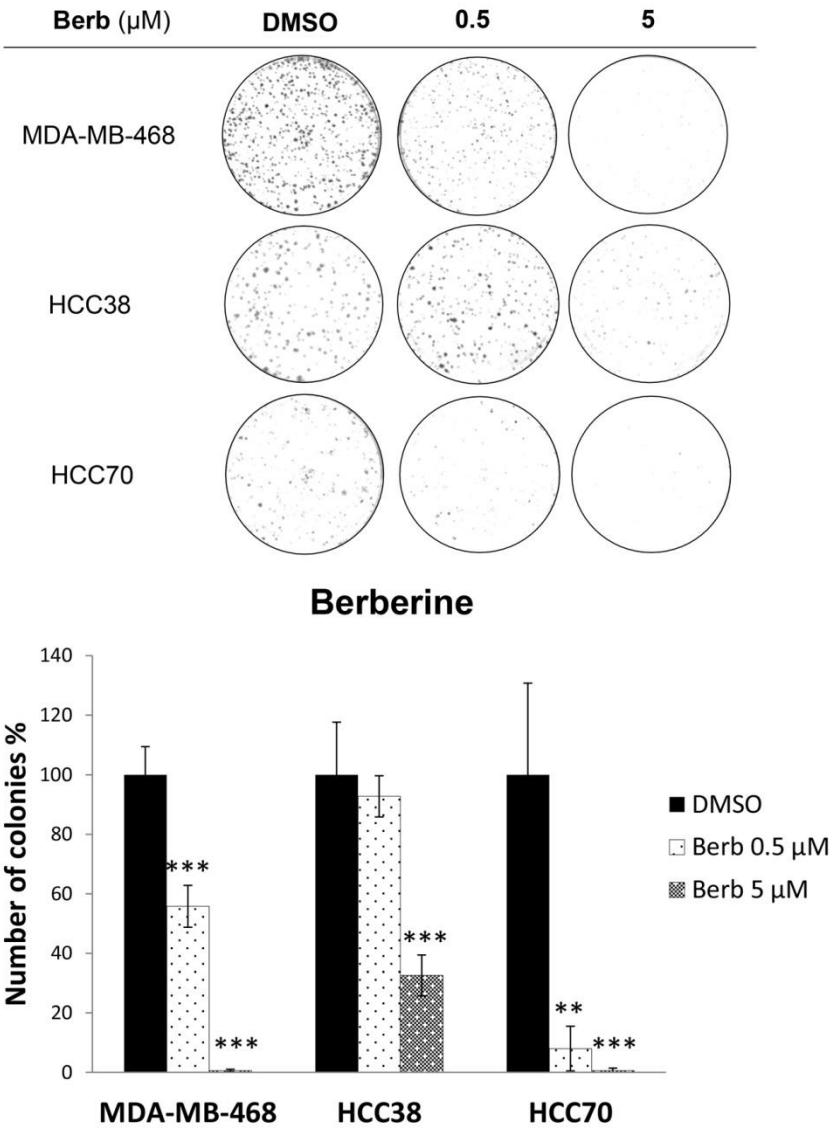


Figure 9: Berberine's inhibition of TNBC cell lines colony formation. Pictures of wells containing colonies of berberine-treated TNBC cell lines and the presentation of the number of colonies % vs control (DMSO) of each treated cell line. Berberine's doses: 0.5 and 5 μM. TNBC cell lines: MDA-MB-468, HCC38 and HCC70. ±Standard Deviation of three independent experiments done in triplicate, p <0.05 (as *), p <0.01 (as **) and p <0.001 (as ***) compared to DMSO. (Berb: Berberine).

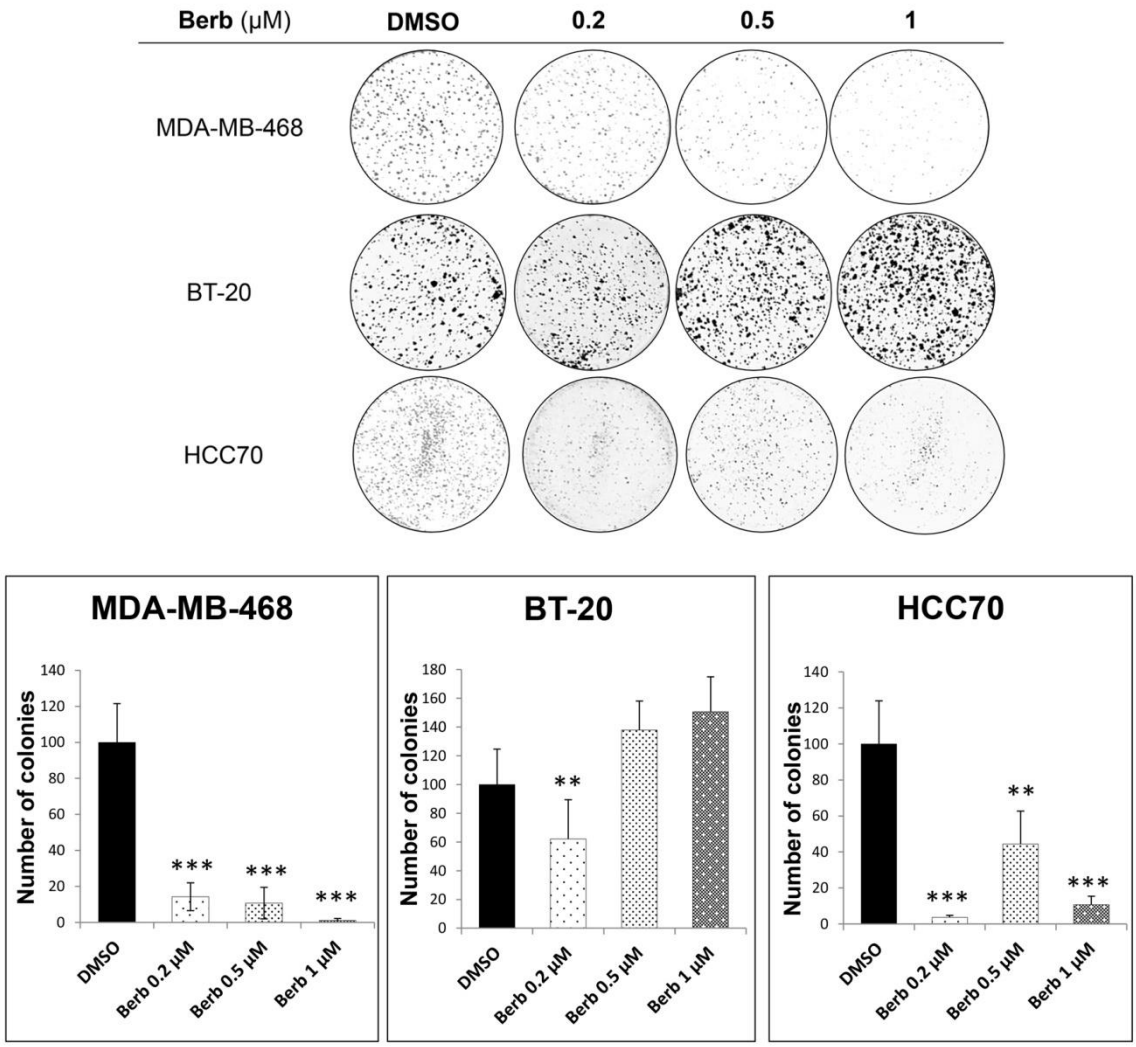


Figure 10: Berberine's inhibition of TNBC cell lines colony formation. Pictures of wells containing colonies of berberine-treated TNBC cell lines and the presentation of the number of colonies % vs control (DMSO) of each treated cell line. Berberine's doses: 0.2, 0.5 and 1 µM. TNBC cell lines: MDA-MB-468, BT-20 and HCC70. ±Standard Deviation of three independent experiments done in triplicate, p <0.05 (as *), p <0.01 (as **) and p <0.001 (as ***) compared to DMSO. (Berb: Berberine).

When cells were treated with artemisinin, the results in figure 11 did not show any inhibition of colony formation in HCC70 as expected; since HCC70 cells were the most resistant cells to artemisinin treatment as shown in the viability test results (Figure 6 and Table 8). However, Arte treatment significantly (p<0.001) inhibited the colony formation capacity of MDA-MB-468 and HCC38 at 5 µM (Figure 11).

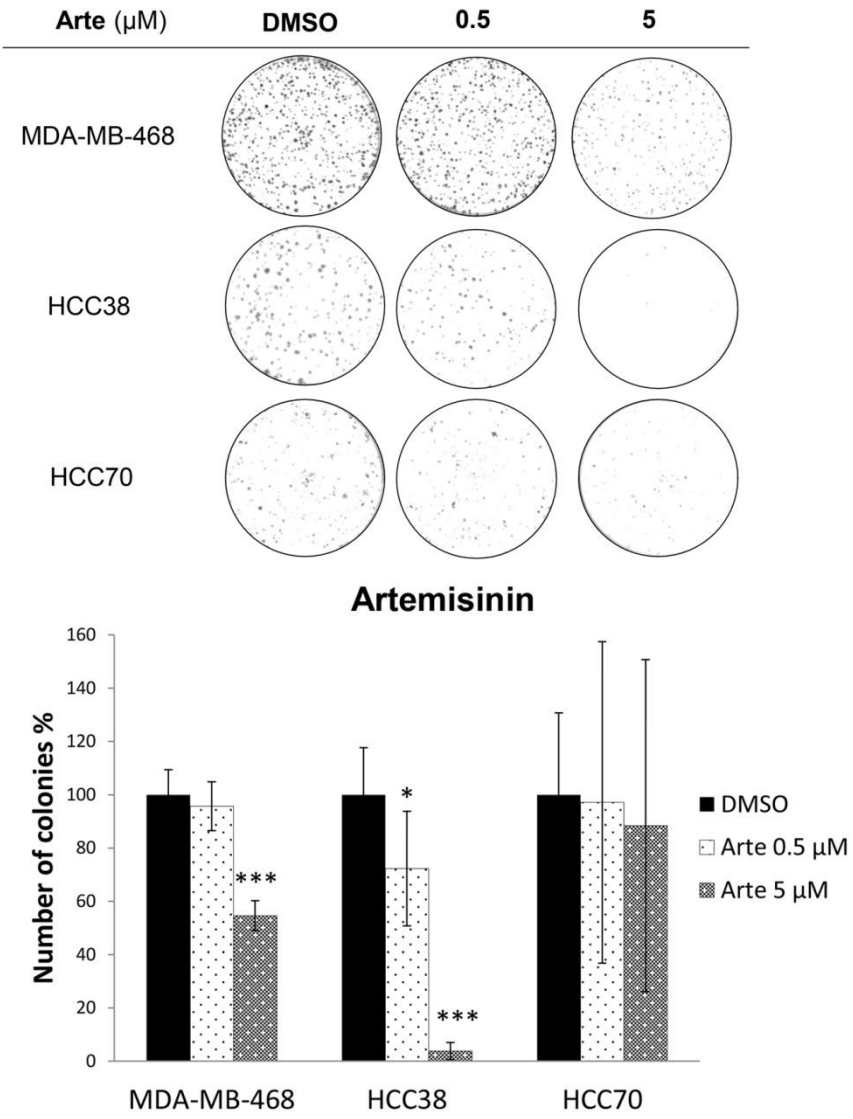


Figure 11: Artemisinin's inhibition of TNBC cell lines colony formation. Pictures of wells containing colonies of artemisinin-treated TNBC cell lines and the presentation of the number of colonies % vs control (DMSO) of each treated cell line. Artemisinin's doses: 0.5 and 5 µM. TNBC cell lines: MDA-MB-468, HCC38 and HCC70. ±Standard Deviation of three independent experiments done in triplicate, p <0.05 (as *), p <0.01 (as **) and p <0.001 (as ***) compared to DMSO. (Arte: Artemisinin).

Figure 12 shows no significant inhibitory effect on colony formation of the HCC38 and HCC70 thymoquinone-treated cell lines, neither at 0.5 or 5 µM. However, it showed a significant (p<0.001) inhibitory effect on MDA-MB-468, the most sensitive cell line towards thymoquinone (figure 7), at 5 µM (figure 12).

On the other hand, carvacrol again did not exhibit any significant effect on HCC38 nor on HCC70 cell lines as expected (Figure 12). Surprisingly, although carvacrol inhibited MDA-MB-468 cells proliferation with IC_{50}= 5.15 µM, it did not show any effect on their colony formation capacity, even at 5 µM (figure 12).

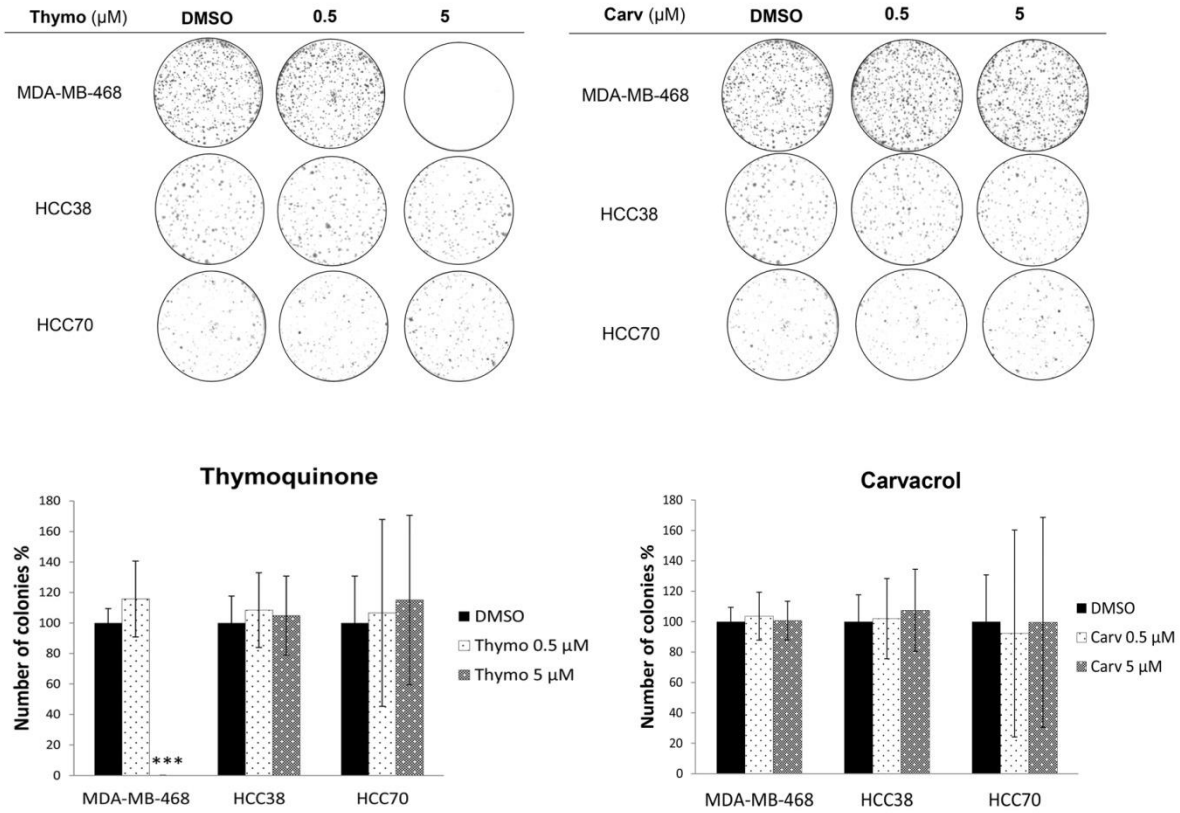


Figure 12: Thymoquione and carvacrol inhibition of TNBC cell lines colony formation. Left: Pictures of wells containing colonies of thymoquione-treated TNBC cell lines and the presentation of the number of colonies % vs control (DMSO) of each treated cell line. Right: Pictures of wells containing colonies of carvacrol-treated TNBC cell lines and the presentation of the number of colonies % vs control (DMSO) of each treated cell line. Thymoquinone and carvacrol doses: 0.5 and 5 µM. TNBC cell lines: MDA-MB-468, HCC38 and HCC70. ±Standard Deviation of three independent experiments done in triplicate, p <0.05 (as *), p <0.01 (as **) and p <0.001 (as ***) compared to DMSO. (Thymo: thymoquinone. Carv: carvacrol).

Conclusion:

Cell growth inhibition and 2D colony formation assays helped us in screening TNBC cell lines and the drugs (berberine, artemisinin, thymoquinone and carvacrol) to finally select berberine and artemisinin as the most effective drugs against the tested TNBC cell lines; since berberine showed significant cell growth and colony formation inhibition on its sensitive cell lines (MDA-MB-468, BT-20 and HCC70) and same for artemisinin which significantly showed same effects on its sensitive cell lines (MDA-MB-468 and HCC38) as well.

Based on these results, we continued our study to investigate the mechanisms of berberine and artemisinin on TNBC cells in both cellular and molecular levels. The results are presented as two original articles:

IV.2: Article 1: Berberine impairs the survival of Triple Negative Breast Cancer cells: cellular and molecular analyses (published)

IV.3: Article 2: Artemisinin impairs the survival of triple negative breast cancer cells alone and in combination with cisplatin (submitted)

IV.2: Article 1

Berberine impairs the survival of Triple Negative Breast Cancer cells: cellular and molecular analyses

molecules

MDPI

Article

Berberine Impairs the Survival of Triple Negative Breast Cancer Cells: Cellular and Molecular Analyses

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Abstract: Triple negative breast cancer (TNBC) is an aggressive breast cancer subtype. Non-available targeted therapy for TNBC represents its biggest treatment challenge. Thus, finding new promising effective drugs is urgently needed. In the present study, we investigated how berberine, a natural isoquinoline, impairs the survival of TNBC cells in both cellular and molecular levels. Our experimental model was based on the use of eight TNBC cell lines: MDA-MB-468, MDA-MB-231, HCC70, HCC38, HCC1937, HCC1143, BT-20, and BT-549. Berberine was cytotoxic against all treated TNBC cell lines. The most sensitive cell lines were HCC70 (IC_{50} = 0.19 μM), BT-20 (IC_{50} = 0.23 μM) and MDA-MB-468 (IC_{50} = 0.48 μM). Using flow cytometry techniques, berberine, at 0.5 and 1 μM for 120 and 144 h, not only induced cell cycle arrest, at G1 and/or G2/M phases, but it also triggered significant apoptosis. At the molecular level, these results are consistent with the expression of their related proteins using Western blot assays. Interestingly, while berberine was cytotoxic against TNBC cells, it had no effect on the viability of normal human breast cells MCF10A cultured in a 3D matrigel model. These results suggest that berberine may be a good potential candidate for TNBC drug development.

Keywords: apoptosis; berberine; cell cycle; cytotoxicity; triple negative breast cancer

1. Introduction

Breast cancer continues to be the most prevalent cancer in the vast majority of countries globally. According to Globocan 2018, new breast cancer cases are estimated by 11.6% worldwide in 2018, and approximately 12% of these cases are triple negative breast cancer (TNBC)[1,2], which is an aggressive breast cancer subtype with no targeted therapy due to the absence of estrogen and progesterone hormone receptors with the lack of human epidermal growth factor receptor-2/Neu over-expression [2]. Since targeted therapy is not yet available for TNBC patients, cytotoxic chemotherapy is the only available way for their treatment. Unfortunately, chemotherapy does not only kill cancer cells but also causes

normal growing cells to die, leading to severe side effects [3]. Thus, new treatments are urgently required to improve the survival rate and patients' life quality.

Berberine (PubChem CID: 2353) [4], is a natural isoquinoline alkaloid compound isolated from the stems and roots of several plants such as *Berberis vulgaris*, *Berberis aristata*, *Berberis asiatica*, *Coptidis rhizome, Coptidis chinensis, Coptidis japonica, Mahonia aquifolium,* and *Mahonia beale* [5,6]. It exerts several pharmacological activities such as antiplatelet, antibacterial, anti-inflammatory, immunomodulatory, anti-oxidative, neuroprotective, anti-diabetic, and hypolipidemic [6,7].

Several preclinical studies have reported the anticancer effect of berberine where it exhibited its inhibitory effects on a variety of tumours such as hepatoma, leukemia, breast, lung, colon, ovarian and cervical cancer cells through apoptosis induction and cell cycle arrest, inhibition of migration and invasion, reduction of the expression of VEGF mRNA and inhibition of angiogenesis [8].

Here, we aimed to explore the mechanisms of berberine's effect on the behavior of several TNBC cell lines, such as proliferation, colony formation, cell cycle progression, DNA damage, and apoptosis in both cellular and molecular levels. Furthermore, and as long as the main problem of chemotherapy regimen is systemic toxicity, we investigated the effect of berberine on the viability of normal human breast epithelial cells.

2. Results

2.1. Berberine Inhibits Proliferation of Triple Negative Breast Cancer (TNBC) Cells

Screening berberine's anti-proliferative activity on 8 different TNBC cell lines, through MTT assay, showed that berberine inhibited their growth in a dose dependent manner, with IC_{50} values ranging from 0.19 μM to 16.7 μM (Figure 1 and Table 1). According to IC_{50} values and the curve shapes of the treated cell lines, we noticed that the cells have different responses towards the treatment depending on the doses of berberine, when HCC70 (IC_{50} = 0.19 μM), BT-20 (IC_{50} = 0.23 μM) and MDA-MB-468 cells (IC_{50} = 0.48 μM) were found to be the most sensitive ones to berberine treatment and inversely, MDA-MB-231 was the most resistant one (IC_{50} = 16.7 μM) among all the treated cell lines (Figure 1 and Table 1).

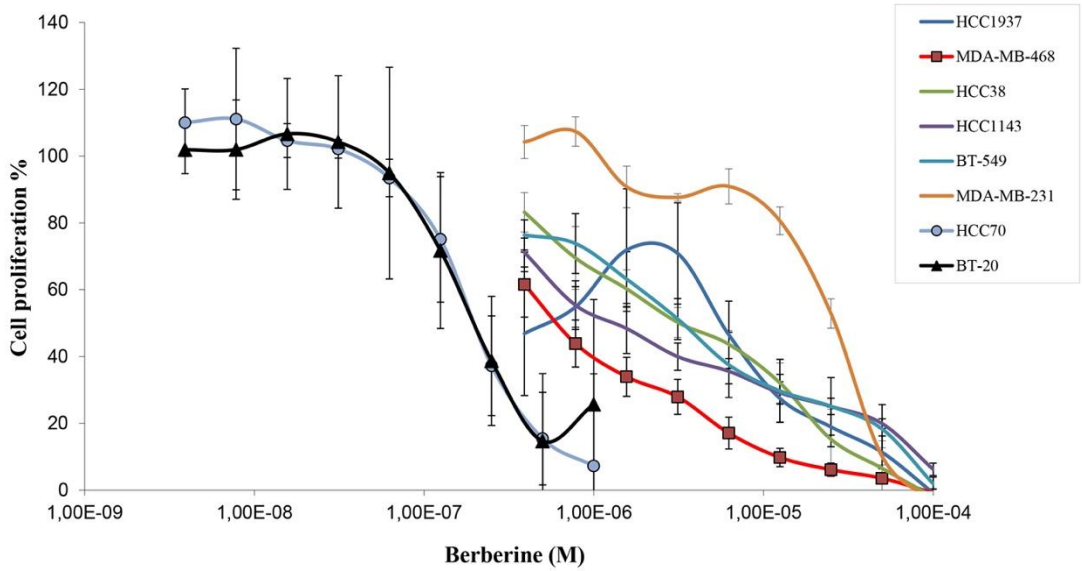


Figure 1. Effect of berberine's treatment on triple negative breast cancer (TNBC) cell proliferation. TNBC cell lines were seeded and treated with berberine. The cell viability was measured by MTT assay. ± Standard Deviation of three independent experiments carried out in triplicate.

Table 1. IC_{50} (μM) values of berberine on TNBC cell lines ± standard deviation.

Cell Line	Berberine IC_{50} (μM)
HCC70	0.19 ± 0.06
BT-20	0.23 ± 0.10
MDA-MB-468	0.48 ± 0.25
HCC1143	1.67 ± 0.74
HCC38	3.24 ± 1.05
BT-549	3.44 ± 1.29
HCC1937	2.30 ± 2.09
MDA-MB-231	16.7 ± 2.37

Further to establish the inhibitory role of berberine on transforming properties of cancer cells, we performed the 2D-clonogenic assay. When compared to DMSO treated cells, results showed that berberine significantly reduced colony formation of MDA-MB-468 ($p < 0.001$), BT-20 ($p < 0.01$) and HCC70 ($p < 0.001$) at 0.2 μM, indicating a potent cell growth inhibition (Figure 2).

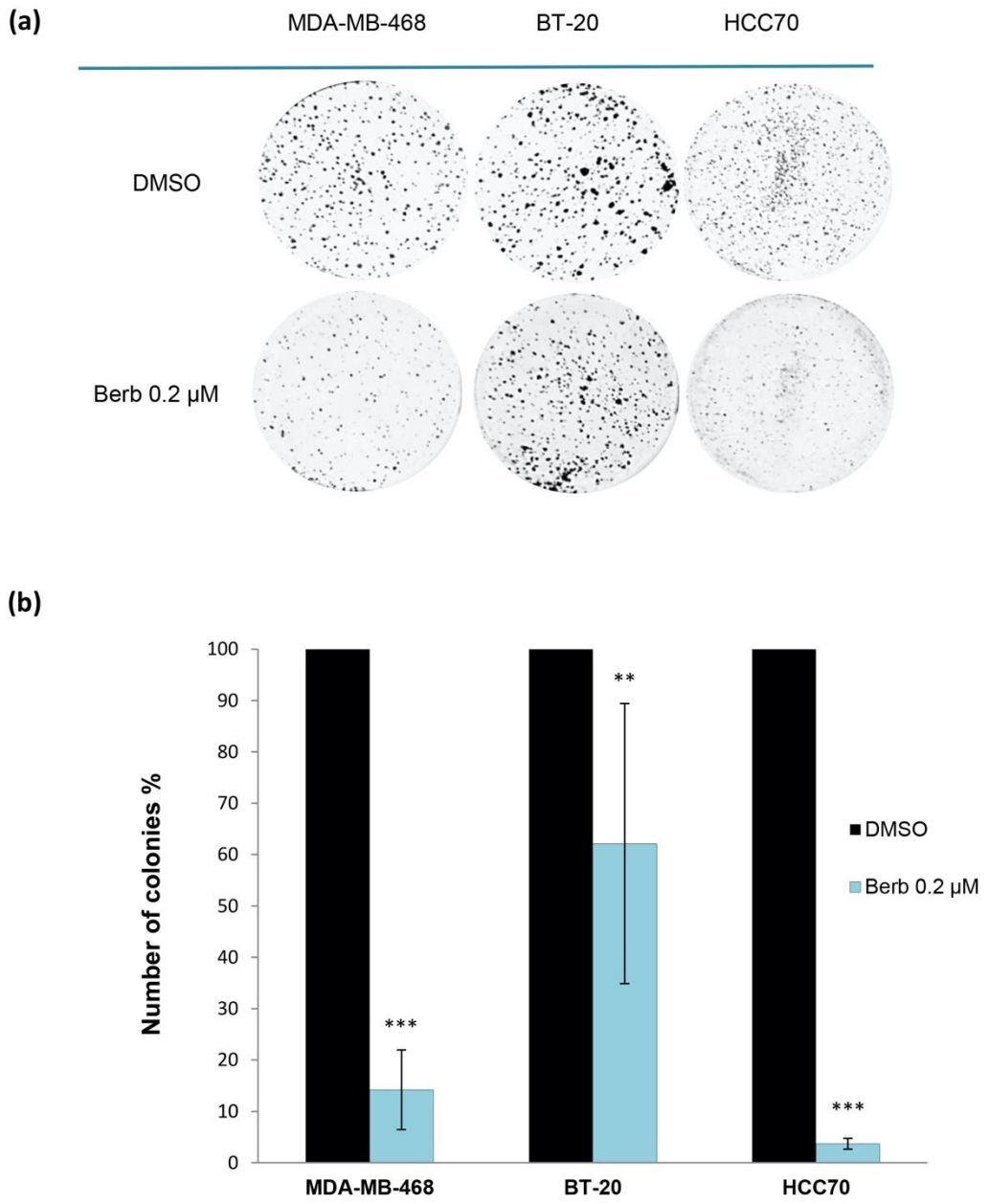
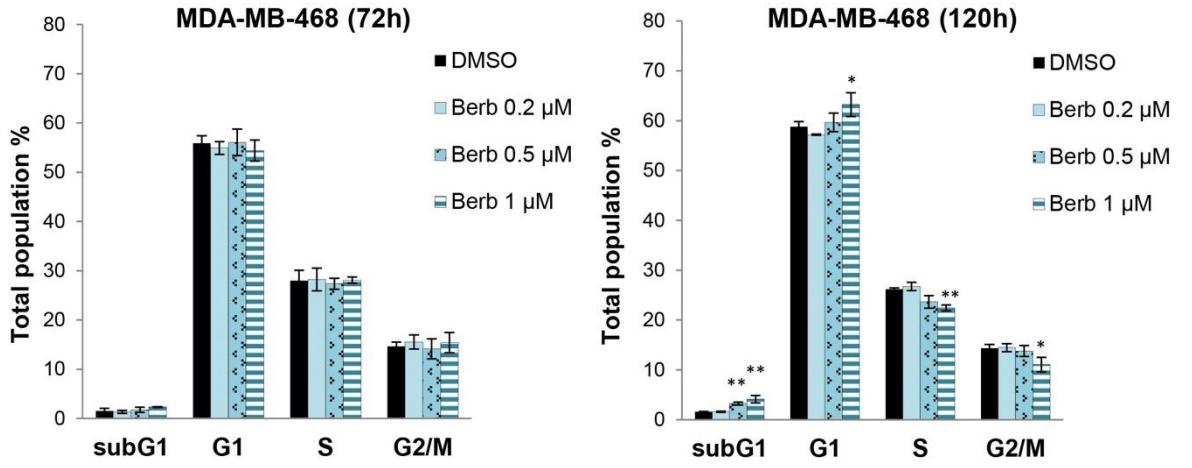


Figure 2. Berberine's inhibition of TNBC cell lines colony formation. (**a**) Pictures of wells containing colonies of berberine-treated TNBC cell lines. (**b**) Number of colonies % vs control (DMSO) of each treated cell line. TNBC cell lines: MDA-MB-468, BT-20 and HCC70. Berb—Berberine. ±Standard Deviation of three independent experiments done in triplicate, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to DMSO.

2.2. Berberine Differentially Affects TNBC Cell Cycle Progression

Since berberine inhibited cell proliferation, we further studied the role of this molecule on cell cycle progression in MDA-MB-468, HCC70 and BT-20 cells by flow cytometry. Results showed that cells had different responses towards berberine's treatment depending on the cell line type (Figure 3). Berberine had no significant effect on MDA-MB-468 cell cycle at 72 h of the treatment. However, it significantly ($p < 0.05$) induced G1 phase arrest in MDA-MB-468 cells at 1 µM and at 120 h, in comparison to DMSO treated control cells (Figure 3a). The figure shows a significant increase in the percentage of cells in G1 phase ($p < 0.05$) with a concomitant significant decrease in the percentage of cells in S ($p < 0.01$) and G2/M ($p < 0.05$)

phases, suggesting the role of berberine in inhibiting the entry into S phase of MDA-MB-468 cells (Figure 3a). This effect correlated with the changes in the amount of cell cycle proteins cyclin D1 and PCNA, at 144 h and not at 120 h (Figure 4a). Western blotting analysis showed a significant decrease in the amount of cyclin D1 ($p < 0.05$) and PCNA ($p < 0.001$) proteins after 144 h in MDA-MB-468 treated cells (Figure 4a).



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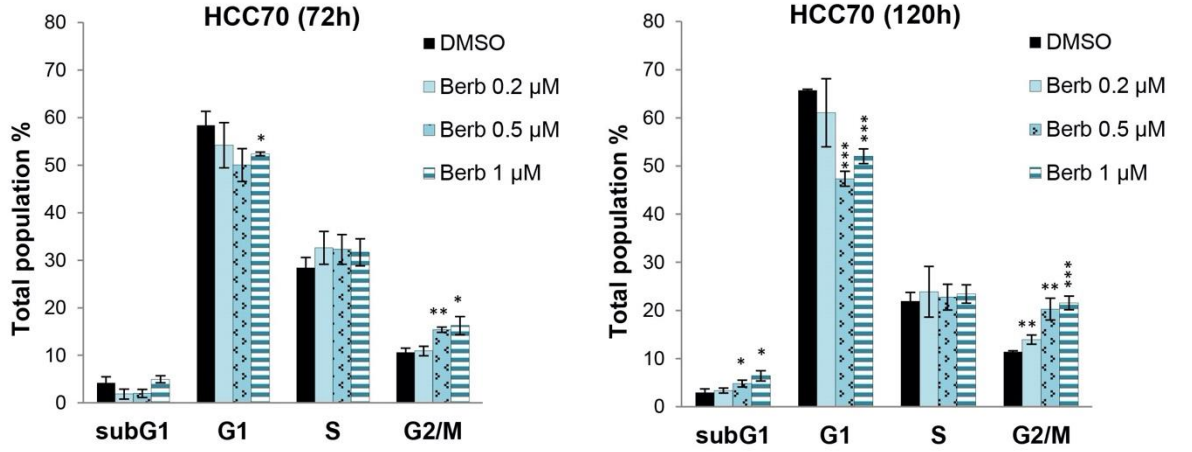

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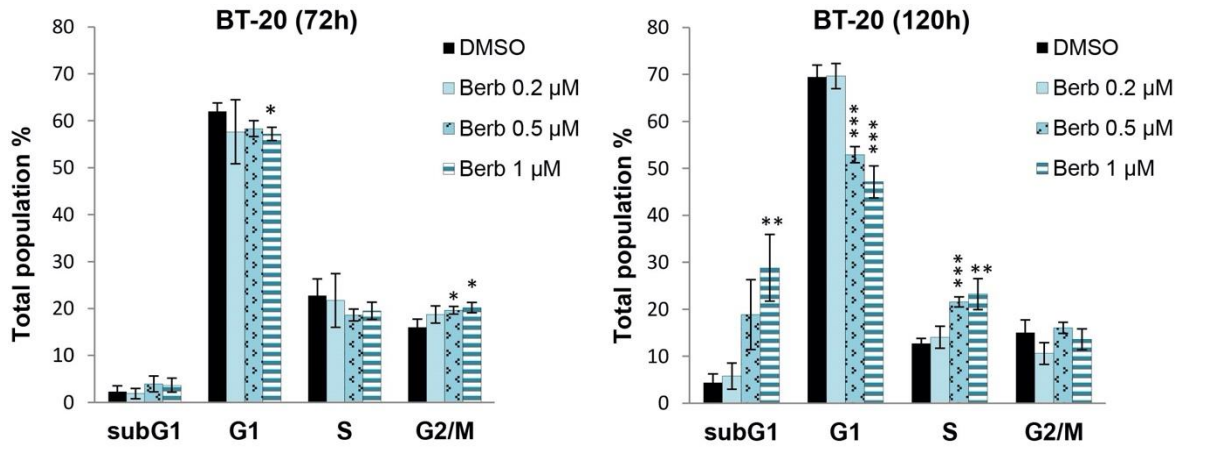
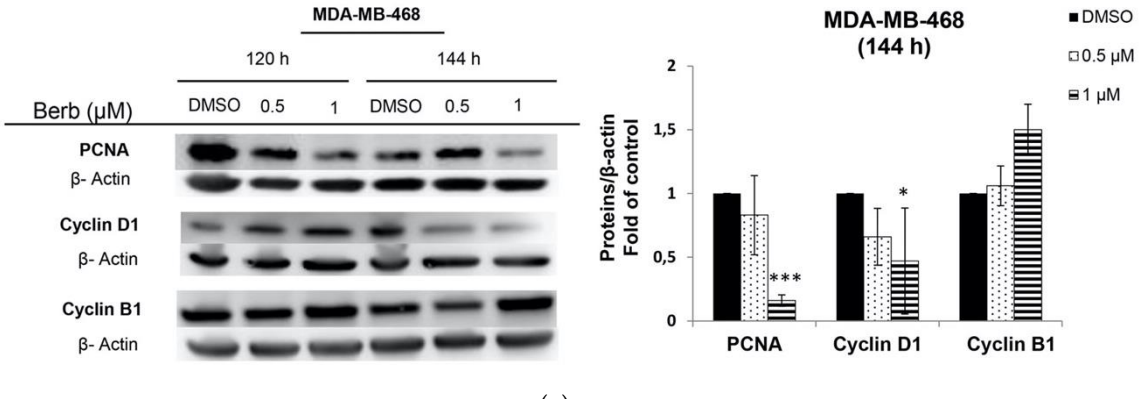


(**c**)

Figure 3. Berberine's effects on TNBC cell cycle progression depending on its doses (μM) 0.2, 0.5 and 1, and time for 72 h and 120 h. Total population % of cell cycle phases (G1, S, G2/M and SubG1) ± SD of three independent experiments, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to DMSO. (**a**) MDA-MB-468, (**b**) HCC70 and (**c**) BT-20. Berb—Berberine.



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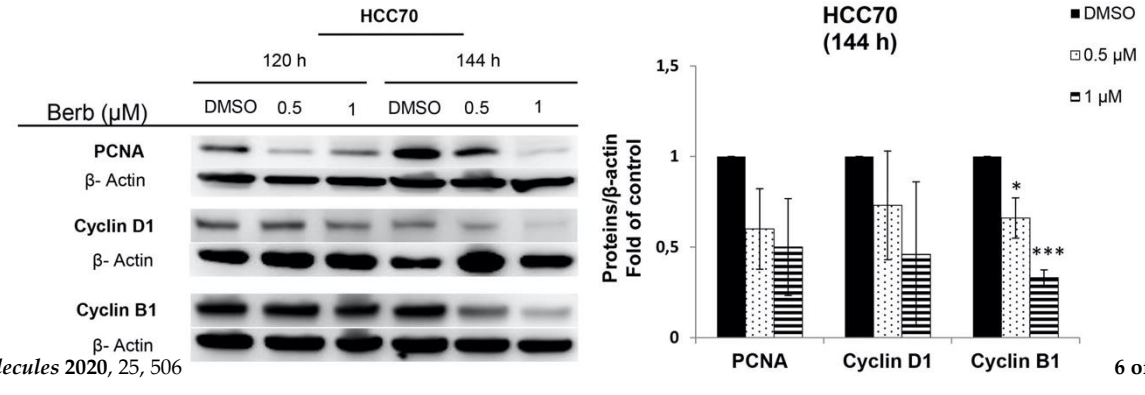

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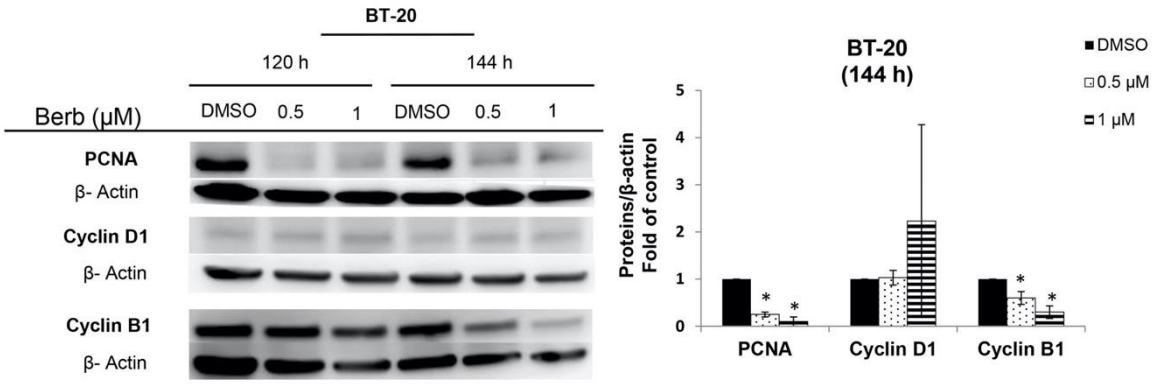


(**c**)

Figure 4. Representative Western blot results, of the expression of cell cycle related proteins PCNA, Cyclin D1 and Cyclin B1 of (**a**) MDA-MB-468, (**b**) HCC70 and (**c**) BT-20 treated with berberine at 0.5 and 1 μM for 120 h and 144 h. Results of three independent experiments ± SD, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to DMSO, Berb—Berberine.

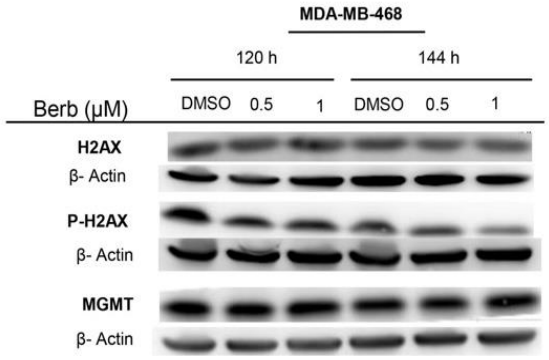
However, in the case of HCC70 and BT-20 cells and at 120 h of the treatment, berberine at 0.5 and 1 μM significantly ($p < 0.001$) decreased their G1 phases with a simultaneous significant ($p < 0.001$) increase in G2/M phase of HCC70 cell line and a significant ($p < 0.001$) increase in S phase in BT-20 cells, compared to DMSO treated cells, as it is shown in Figure 3b,c. These results correlated with the Western blot results at 144 h, whereby cyclin B1 was

significantly down-regulated in both cells, BT-20 ($p < 0.05$), and HCC70 ($p < 0.001$), with a significant ($p < 0.05$) decrease in PCNA expression in only BT-20 cell line, in comparison to DMSO treated cells (Figure 4b,c).

On the other hand, after 120 h of the treatment, we noticed a significant manifestation of the cell death shown by the SubG1 phase increase in all the three tested cell lines MDA-MB-468 ($p < 0.01$) , HCC70 ($p < 0.05$) and BT-20 ($p < 0.01$) in comparison to DMSO treated control cells (Figure 3).

2.3. Berberine Induces DNA Damage in TNBC Cell Lines

Compared to DMSO treated cells, Western blot results showed that BT-20-berberine treated cells exhibited a significant ($p < 0.001$) increase in the phosphorylation of H2AX, both at 120 h and 144 h of the treatment, same with HCC70 cells that showed a significant ($p < 0.05$) dose dependent increase of H2AX phosphorylation at 120 h of the treatment (Figure 5b,c). These results indicate that those cells may be experiencing genotoxic stress under berberine treatment. On the other hand, berberine had no significant effect on H2AX phosphorylation and the expression of MGMT protein in MDA-MB-468 cells (Figure 5a).



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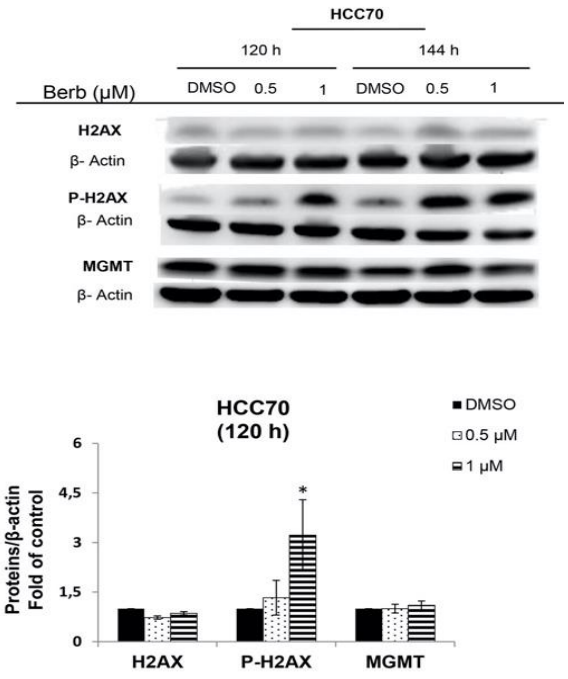

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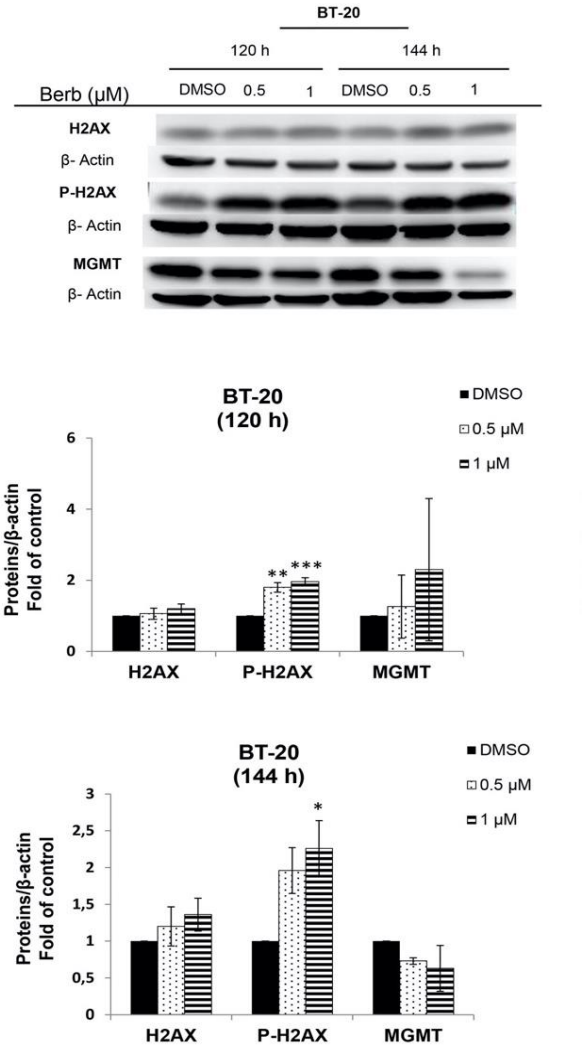


(**c**)

Figure 5. The effects of berberine treatment on the phosphorylation of H2AX and the expression of MGMT proteins of TNBC cell lines (**a**) MDA-MB-468, (**b**) HCC70 and (**c**) BT-20 at 120 h and 144 h. Representative Western blot results of three independent experiments. Berberine doses: 0.5 and 1 μM. ± SD, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to DMSO. Berb—Berberine.

2.4. Berberine Induced Apoptosis on TNBC Cell Lines in a Dose and Time Dependent Manner

Cell cycle results already showed that SubG1 phases of the three tested cell lines (MDA-MB-468, BT-20 and HCC70) significantly increased after 120 h of the treatment with berberine, as compared to DMSO treated control cells (Figure 3). To confirm that berberine-induced loss of the proliferation capacity of TNBC cells was associated with the induction of apoptosis, MDA-MB-468, BT-20, and HCC70 were treated with berberine at 0.2, 0.5, and 1 μM and incubated for 120 h and 144 h. Afterwards, apoptotic cells were counted using Annexin-V and PI staining by flow cytometry (Figure 6, Figure 7). Revelation of the apoptotic marker's expression: cl-PARP, cl-caspase 8, and cl-caspase 7 by Western blot are shown in Figures 7 and 8.

Berberine did not show a significant apoptosis neither in MDA-MB-468 nor in HCC70, but only in BT-20 treated cells at 120 h of the treatment (data not shown). However, Annexin-V staining results indicated that, at 144 h, berberine triggered apoptosis in all TNBC tested cells differently depending on the dose and the cell line (Figures 6 and 7). MDA-MB-468 ($p <$

0.05), BT-20 (p < 0.001) and HCC70 (p <0 .05) cells exhibited a significant increase in the apoptotic populations compared to DMSO control cells (Figure 7). These results correlated with the findings of Western blot on the cleavage of PARP, caspase-7 and caspase-8 depending on the dose, time and the cell line (Figure 8). We noticed that berberine significantly induced the cleavage of PARP (p < 0.05) at 120 h of the treatment and the cleavage of caspase-8 proteins (p < 0.05) lately after 144 h of berberine's treatment in MDA-MB-468 cells (Figure 8a). BT-20 cells exhibited a significant cleavage of caspase-7 (p < 0.05) and caspase-8 (p < 0.01) earlier at 120 h of the treatment (Figure 8b). However, in HCC70 cells, we only noticed a significant (p <0.05) increase in the cleavage of PARP protein upon 144 h of berberine's treatment (Figure 8c). The cleavage of PARP, caspase-7 and caspase-8 indicates the occurrence of apoptosis [9].

These findings demonstrate that berberine engender apoptotic cell death in TNBC cell lines through the cleavage of PARP, caspase-7, and caspase-8 proteins, all depending on the time, dose, and the cell line.

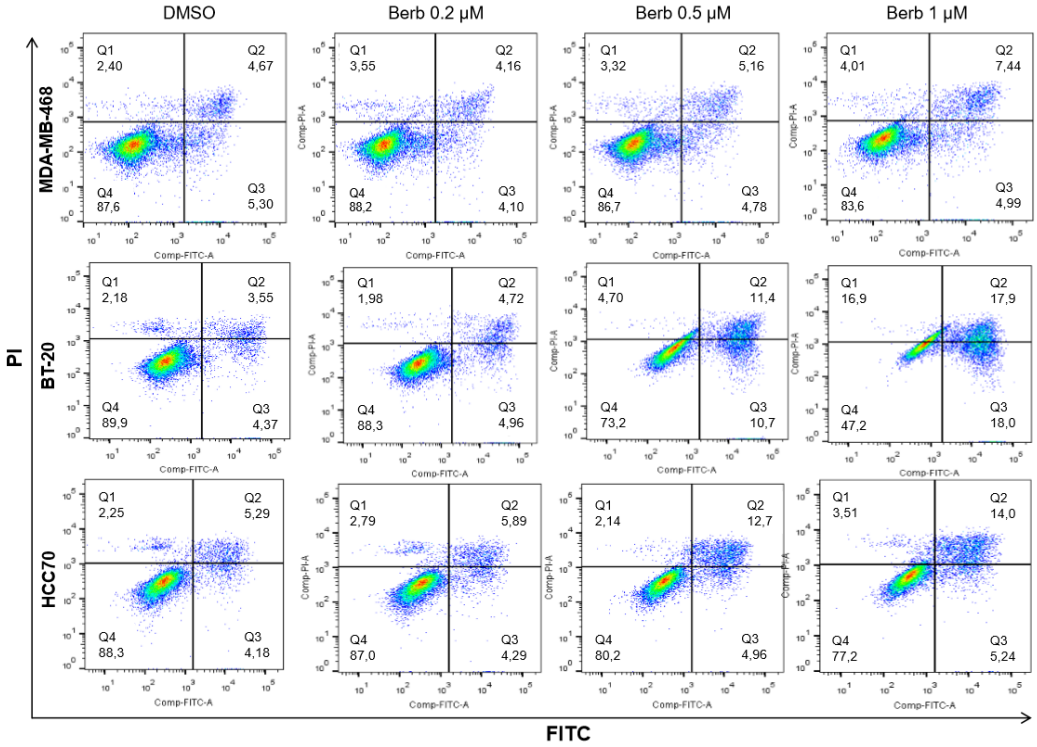
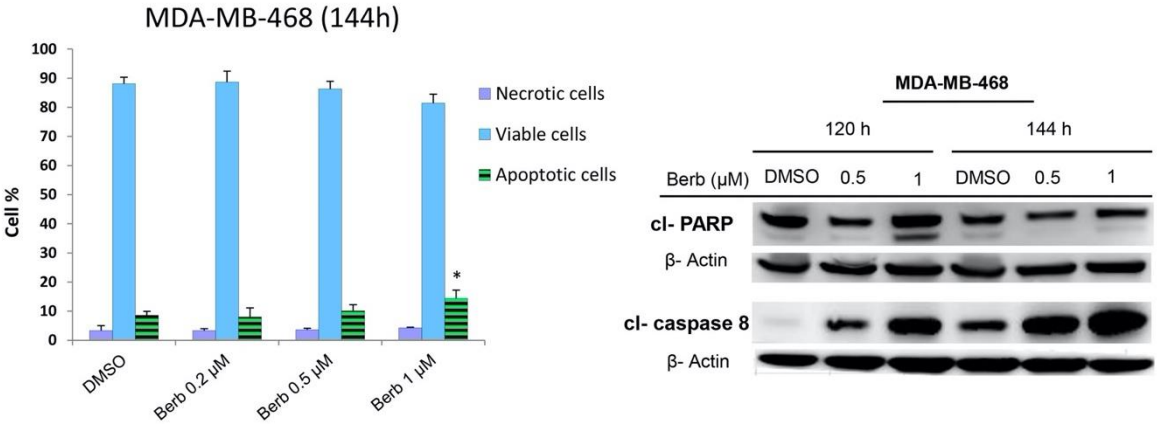


Figure 6. Annexin-V FITC and PI staining on TNBC cell lines: MDA-MB-468, BT-20 and HCC70 following the treatment with berberine (µM) 0.2, 0.5 and 1, at 144h. Q1: PI+/FITC-; Q2: PI+/FITC+; Q3: PI-/FITC+;Q4: PI-/FITC-. Berb—Berberine.



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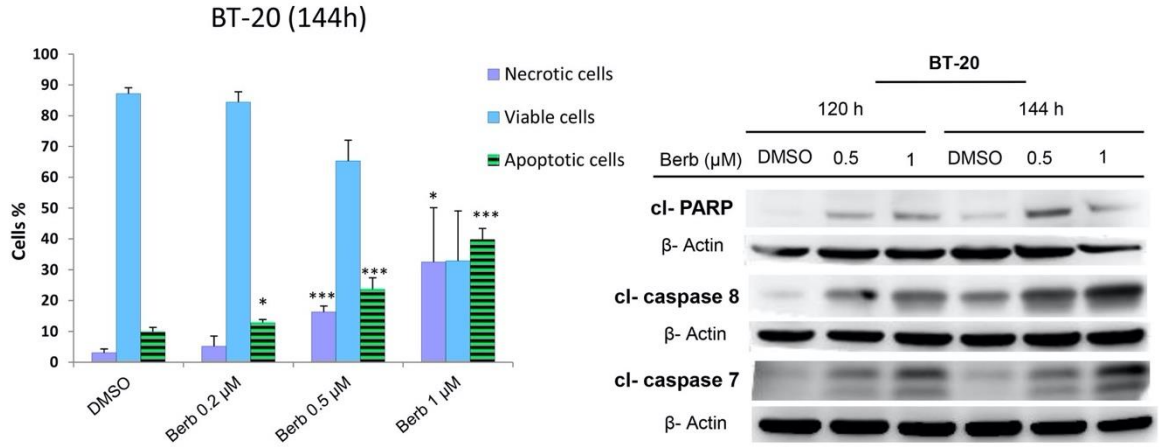
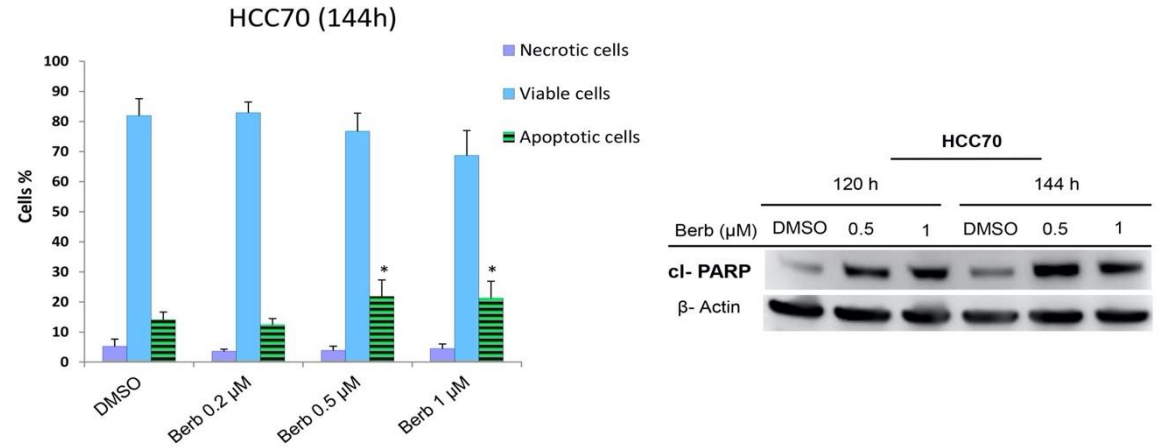


(**b**)



(**c**)

Figure 7. Berberine induced apoptosis in a dose and time dependent manner. Annexin-V and PI staining after 144 h of the treatment with berberine at 0.2, 0.5 and 1 μM. Western blot results of three independent experiments showing apoptosis related proteins expression upon berberine's treatment at 120 h and 144 h. (**a**) MDA-MB-468, (**b**) BT-20, and (**c**) HCC70. ±SD of three independent experiments, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to DMSO. Berb—Berberine.

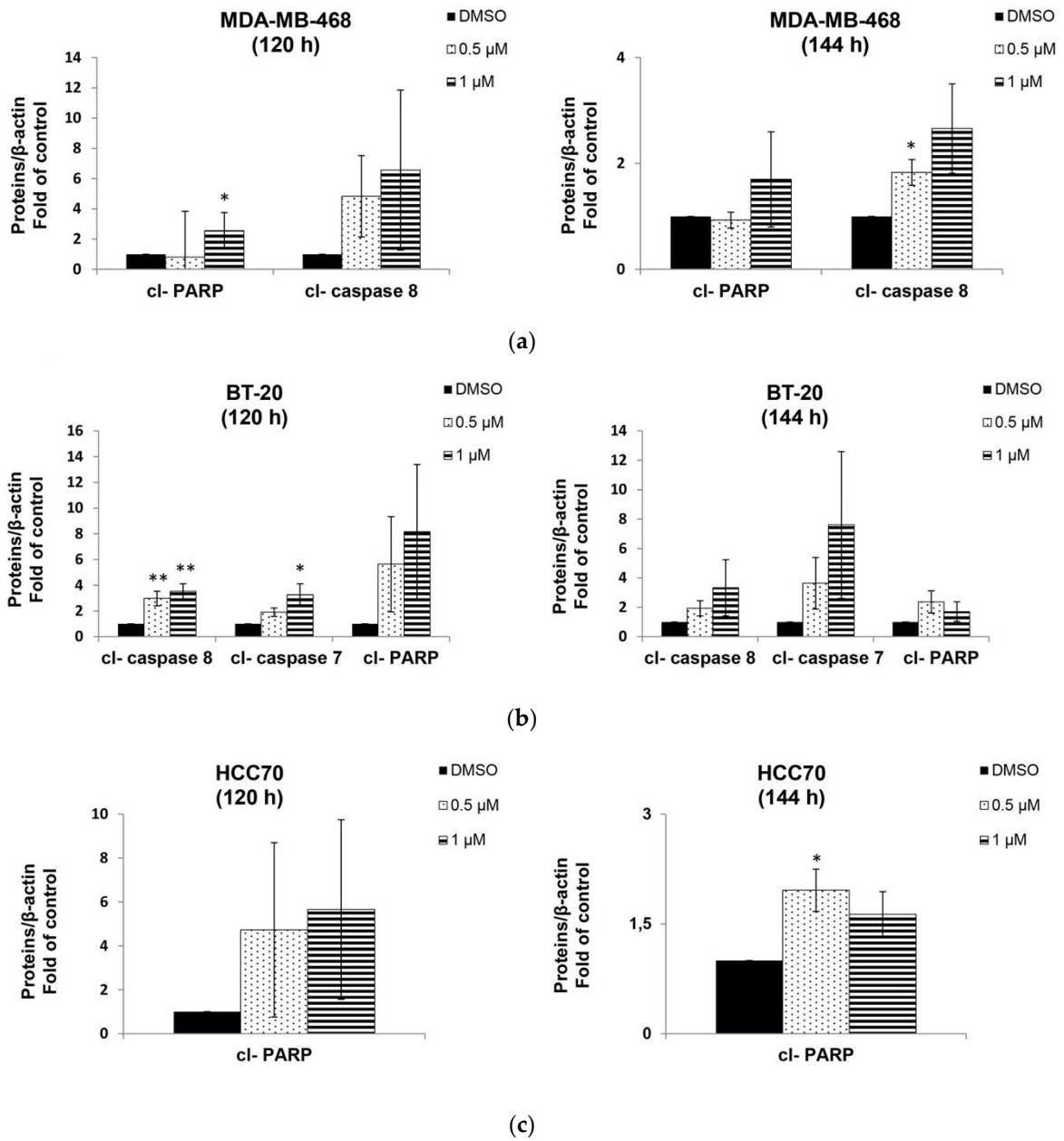


Figure 8. Fold of control of the western blot apoptosis related proteins expression upon berberine's treatment at 120 h and 144 h: cl-PARP, cl-caspase 7 and cl-caspase 8. (**a**) MDA-MB-468, (**b**) BT-20 and (**c**) HCC70. ±SD of three independent experiments, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to DMSO. Berb—Berberine.

2.5. Berberine Does not Affect the Viability of "Normal" Human Breast MCF-10A Cells

Berberine was tested on MDA-MB-468 cell line grown in a more physiological context, in matrigel 3D culture [10]. Under these conditions, non-transformed mammary epithelial cell lines, such as MCF-10A, recapitulate epithelial morphogenesis by forming acinar structures within 10 days of culture, and then stop to grow, whereas cancer cells, such as MDA-MB-468, exhibit disorganized structures and continue to proliferate [11]. Berberine at 5 μM when added to these structures once formed, was significantly ($p < 0.05$) cytotoxic on malignant MDA-MB-468 cells while it had no significant effect on normal Human MCF-10A cells at the same dose of berberine (5 μM) (Figure 9). On the other hand, we didn't notice any effect on MDA-MB-468 cells when treated by 0.5 μM of berberine, and this may be related to the fact

that berberine does not penetrate well into matrigel and that the concentration of the inhibitor reaching the cells is lower.

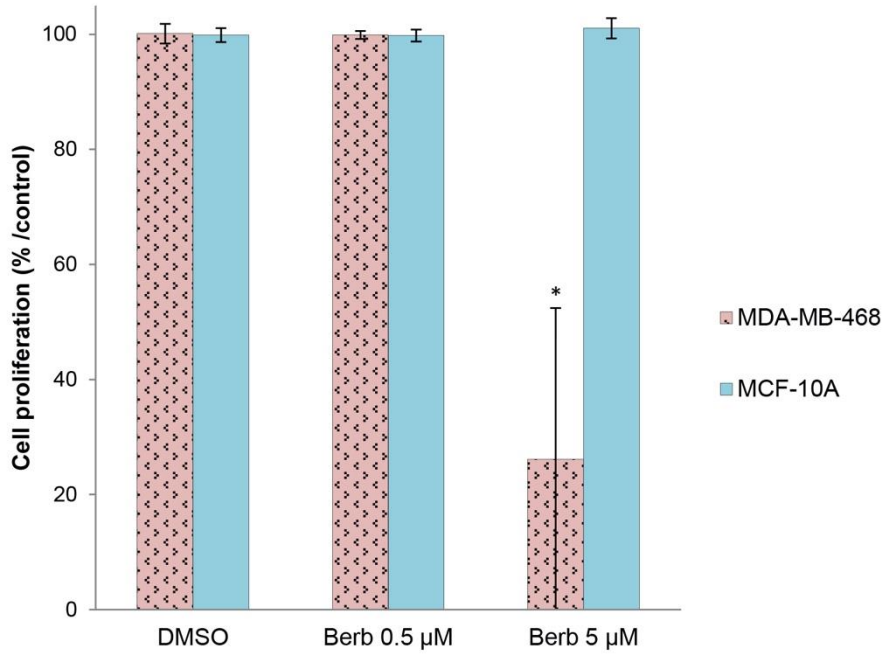


Figure 9. The effect of berberine on the viability of MDA-MB-468 TNBC malignant cells and MCF-10A normal Human epithelial breast cells cultured in matrigel 3D culture. Berberine's doses: 0.5 and 5 µM. ±SD of three independent experiments, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to DMSO. Berb—Berberine.

3. Discussion

A total of 10%–20% of newly diagnosed breast cancers are TNBC [12]. Despite recent advances in diagnosis and treatment, patients with TNBC have been shown to have the highest rate of recurrence within the first five years after diagnosis compared with hormone receptor-positive (HR+) and HER-2/neu receptor-positive (HER2+) breast cancer patients [12]. Thus, underlying molecular mechanisms and new therapeutic strategies are urgently required.

To maintain tissue homeostasis, cell proliferation and death must be regulated. Many studies suggest that the regulation of the process of the cell cycle progression and programmed cell death may be achieved by controlling a set of factors such as cell cycle and apoptosis proteins [13].

Deregulated cell proliferation and inhibition of apoptosis are the main causes of all tumour development, they present two obvious targets for therapeutic intervention: (i) Interfering with the process of DNA synthesis and cell division. (ii) Targeting the suppressors of apoptosis in tumor cells [14]. In this paper, berberine showed an interesting inhibitory effect of the proliferation of several TNBC cell lines (BT-20, HCC70, MDA-MB-468, MDA-MB-231, HCC1143, BT-549, HCC38 and HCC1937) with an IC_{50} ranging from 0.19 µM to 16.7 µM; with BT-20, HCC70 and MDA-MB-468 being the most sensitive ones and BT-549 and MDA-MB-231 being the most resistant ones to berberine treatment. In fact, recent studies have found that berberine inhibits TNBC cell growth and metastasis [15–20].

We continued our study on the three most sensitive cell lines (MDA-MB-468, HCC70 and BT-20) to first explore berberine's antiproliferative effect on their cell cycle progression. Interestingly, we found that berberine blocked cell proliferation in G2/M phases in both MDA-MB-468 and HCC70 cells, and blocked cell proliferation in S phase in BT-20. Interestingly, berberine decreased the expression of PCNA in MDA-MB-468 and BT-20, a proliferating cell nuclear antigen, the concentration of which changes during the cell cycle through its involvement in DNA replication and repair [21].

Mitotic events regulation is linked to the control of the activity of cyclin B1 protein to make cells enter mitosis, skip mitosis or arrest at G2 phase [22]. In our results, we found that cyclin B1 protein was also expressed at lower levels after berberine treatment in BT-20 and HCC70 causing G2/M phase arrest in HCC70. Previous findings report that berberine inhibited the expression of cyclin B1 to induce G2/M phase arrest in numerous cancer types [23,24].

Remarkably, berberine-treated (1 μM) MDA-MB-468 cells showed a significant increase in G1 phase population with a simultaneous significant decrease in S and G2/M phases at 120 h of the treatment. In all known cell cycle proteins, cyclin D1 was the most critical checkpoint protein in regulating G1 to S phase [13]. In previous reports, the effects of berberine were shown to be largely attributed to cell cycle arrest at the G1 phase through the down-regulation of Cyclin D1 [25,26]. Our results are well correlated with these findings when berberine reduced PCNA and cyclin D1 protein expression in MDA-MB-468 to block their proliferation progress in G1 cell cycle phase. On the other hand, using MDA-MB468, Lin, Y.S. et al [20] demonstrated that berberine treatment at 6 and 12 μM for 48h blocked the cell cycle in G0/G1 with a simultaneous decrease in cyclin D1 expression. However, these results are not in agreement with ours, since the treatment of these cells with berberine for 72 h did not induce any significant cell cycle arrest but it did at 120 h (Figure 3), suggesting that the effect of berberine on cell cycle is dose and time dependent.

Targeting cell cycle progression as a therapeutic intervention has a limited efficacy as it could be only cytostatic [14]. Fortunately, berberine treatment not only caused cell cycle arrest on all treated TNBC cell lines but also triggered apoptosis. Apoptosis includes two major signaling pathways: the extrinsic death receptor pathway and the intrinsic mitochondrial one [27]. To induce apoptosis, several biochemical events occur including the cleavage of caspases as indicators of the intrinsic apoptotic pathway besides of the cleavage of PARP protein. PARP cleavage has been considered as a hallmark of apoptosis caused by DNA strand breaks [9]. In a previous study, it has been reported that no apoptosis was observed after treatment with berberine for 48 hours neither against MDA-MB-468 at 12 μM nor toward MDA-MB-231 at 25 μM [20]. However, in our study, no significant apoptosis induction was observed at 72 h and 120 h. Although, when we increased the incubation time to 144 h, we found that berberine significantly induced apoptosis in all studied cells, e.g., MDA-MB-468 at 1 μM, HCC70 at 0.5 and 1 μM, BT-20 at 0.2, 0.5 and 1 μM. At the molecular level, cleaved PARP, cleaved caspase-8 and cleaved caspase-7 proteins expression started earlier at 120h to induce apoptosis in TNBC cells.

Therefore, berberine is also known for its ability to engender apoptosis in several cancer types including breast cancer cells by triggering DNA damage [28]. Moreover, a previous study reported that berberine induces DNA strand breaks in MDA-MB-231 and BT-549 TNBC cell lines at 49.08 μM and 44.58 μM, respectively, in 48h upon the treatment [16]. Some studies suggest that berberine may directly bind to DNA by intercalation [28]. Based on these findings, we led our research to see whether the resulted apoptosis is caused by DNA

damage in TNBC cells, by quantifying the amount of P-H2AX expression. H2AX, a variant form of histone H2A, becomes phosphorylated in response to DNA double-strand breaks [29]. Expectedly, we observed a remarkable accumulation of DNA double-strand breaks in berberine-treated cells, as determined by measuring the phosphorylation of H2AX in both HCC70 and BT-20 cells. Inversely, we didn't notice any effect on the phosphorylation of H2AX in MDA-MB-468 when treated with berberine, suggesting either that berberine induces DNA double-strand breaks depending on the cell line, or either we need to explore the DNA damage in MDA-MB-468 by other techniques in coming perspectives.

Cancer therapy involving cytotoxic drugs kills cells that have a high level of proliferation and regeneration. While this type of therapy targets tumor cells, it affects rapidly proliferating non tumor cells such as skin and hair, accounting for the high level of toxicity associated with such treatments [13].

In our study, we showed that berberine treatment is significantly tolerated by normal human breast epithelial cells MCF-10A when cultured in a 3D matrigel model in comparison to MDA-MB-468 cells cultured in the same conditions. These results are related to some previous studies where it was reported that berberine exhibited no significant effect on the breast normal epithelial cell growth [30], nor in normal human peripheral blood mononuclear cells (PBMC) [31]. Actually, this is important when the systemic toxicity of chemotherapy drugs treatments is the major conventional therapy inconvenient.

The effectiveness of berberine in impairing the growth of TNBC cells without affecting the growth of normal human breast epithelial cells indicates that it may serve as a potential natural compound for TNBC drugs development.

4. Materials and Methods

4.1. Reagents

Cell culture reagents were all purchased from Life Technologies (Camarillo, CA, USA). All human TNBC and the immortalized breast epithelial MCF10A cell lines were purchased from ATCC (Manassas, VA, USA). Cell lines were authenticated in 2018 and they do not exceed 15 passages in cell culture. Berberine chloride hydrate ≥90% was purchased from Sigma Aldrich (Saint-Louis, MO, USA).

4.2. Cell Culture

The TNBC cell line (BT-20) was cultured in MEM Eagle from Sigma Aldrich (Saint-Louis, MO, USA) medium with 10% fetal bovine serum (FBS), 1% glutamax, 1% penicillin/ streptomycin, 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino-acids, and 1 mM sodium pyruvate.

HCC70, HCC38, HCC1937, HCC1143 and BT-549 cell lines were cultured in RPMI 1640 medium from Fisher Scientific (Waltham, MA, USA) with 10% FBS, 1% glutamax, 1% penicillin/ streptomycin, 1.5 g/L sodium bicarbonate, 10 mM Hepes, and 1 mM sodium pyruvate.

MDA-MB-468 cells were cultured in RPMI 1640 medium with 10% FBS, 1% glutamax, and 1% penicillin/streptomycin.

MDA-MB-231 cells were cultured in DMEM/F12 with 10% FBS and 5% Penicillin/streptomycin.

MCF10A cells were cultured in DMEM/F12 with 5% horse serum, 1% penicillin/streptomycin, 20 ng/mL EGF, 100 ng/mL cholera toxin, 0.01 mg/mL insulin, and 500 ng/mL hydrocortisone.

All the cells were maintained in a humidified 5% CO_2 incubator at 37 °C.

4.3. Assessment of Cell Viability Using MTT Assay

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) from Sigma-Aldrich (Saint-Louis, MO, USA), a colorimetric assay capable of detecting viable cells by the reduction of the yellow tetrazolium salt to purple formazan. In order to ensure their adhesion and their exponential growth on the day of the treatment, the cells were seeded in 96-well plates at the proper density 2 days (48 h) before the treatment (Table 1). In the day of the treatment, the cells were treated with berberine already dilluted in Dimethylsulfoxyde (DMSO) (Sigma-Aldrich). After the day of the treatment, the cells were incubated for 4 times their doubling time, as described in [32] (Table 2). DMSO (1%), non-treated cells and Doxorubicin were used as negative and positive controls, respectively. At the end of the incubation period, the cells were treated with MTT (5 mg/mL in Phosphate Buffered Saline (PBS)) for 4 h at 37 °C. Subsequently, 10% Sodium Dodecyl Sulfate (SDS) in 10 mM hydrochlorid acid was added to dissolve the formazan, and then the cells were incubated overnight at 37 °C. The production of formazan which is directly proportional to the viable cell number was read as absorbance values at 540 nm using Infinite 200 (Tecan, Lyon, France) with Magellan data analysis software. Experiments were done in triplicate. Cell viability percentage curves and IC_{50} were calculated using Microsoft Excel 2010 from Microsoft Corporation, Microsoft Casablanca (Casablanca, Morocco). The curves were fitted as logarithmic tendency curves then IC_{50} were calculated using their log equations.

Table 2. Seeded cell concentrations per well used in cell viability assay.

Cell Lines	Cell concentration × 10^3 (cell/mL) *	Cells Doubling Time (h)
HCC1937	10	45
HCC70	20	50
MDA-MB-468	10	45
HCC38	20	60
BT-20	10	50
HCC1143	10	60
BT-549	5	30
MDA-MB-231	5	25

* 150µL/Well in 96 Wells Plate.

4.4. Two-Dimensional Clonogenic Assay

Two-dimensional (2D) clonogenic (or colony formation) assay is an *in vitro* cell survival test based on the ability of a single cell to grow into a colony by undergoing multiple divisions [33].Viable cells were seeded to 6 wells plates (Ø35mm) at 2000 cells/well of MDA-MB-468, at 5000 cells/well of HCC70 and 7000 cells/well of BT-20, and allowed to attach to the well bottom overnight. Cells were treated then with 0.2 µM of berberine, and incubated for 10 days for MDA-MB-468 and for 15 days for both HCC70 and BT-20, time to allow colonies to form in the negative control DMSO (0.005%). The obtained colonies were washed with PBS (1X), fixed and stained with 0.05% of Coomassie® Brilliant Blue R-250 from MPBiomedicals (Aurora, Ohio, USA) solubilized in 50% of methanol, 10% Acetic acid and 40% of Milli-Q water. The colonies number of each line was then compared to DMSO control treated cells using ImageJ software. Experiments were carried out in triplicate and for at least three times.

4.5. Cell Cycle Analysis by Flow Cytometry

Cells were seeded to 6 wells plates (Ø35 mm) at 2 × 10^5 cells/well for the MDA-MB-468 and BT-20 cells, and at 3 × 10^5 cells/well for HCC70 cells, and allowed to attach to the well bottom overnight.

Cells were treated with berberine at the doses of 0.2, 0.5, and 1µM, and incubated for 72 and 120 h in 37 °C with 5% CO_2. DMSO (0.005%) was used as control. After respective incubation times, both attached and detached cells were harvested, washed three times and centrifuged at 1500 rpm (for 5 min) with PBS (1X). The cells were then fixed with 70% of cold ethanol and incubated for 30 min in RT. Next, the cells were washed twice with PBS (1X). After removing ethanol, the pellet was resuspended in PBS containing 10µg/mL of propodium iodide (invitrogen P3566) and 100 µg/mL of RNAase (invitrogen 12091-021). The pellet was then incubated for 15 min at RT. Finally, the cell cycle analysis was carried out with LSRII flow cytometer (Becton Dickinson) using DIVA™ software to determine cellular DNA content. The cell population percentage of each phase was analyzed using FlowJo and ModFit LT software package.

4.6. Apoptosis Annexin-V staining

Cells were seeded to 6 wells plates (Ø35 mm) at 2 × 10^5 cells/well for the MDA-MB-468 and BT-20 cells, and at 3 × 10^5 cells/well for HCC70 cells, and allowed to attach to the well bottom overnight. Cells were treated with berberine at the doses of 0.2, 0.5 and 1μM, and incubated for 120 h and 144 h in 37 °C with 5% CO_2. DMSO (0.005%) was used as control. After incubation, both attached and detached cells were harvested and washed twice by PBS (1X). Next, the cells were then stained with 1% Annexin-V-FITC diluted in 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic (HEPES) buffer for 15 min at RT in the dark. Cells were then washed and stained with 1% propodium iodide diluted in HEPES buffer. The analysis and populations quantification were achieved through LSRII flow cytometer (Becton Dickinson) using DIVA™ and FlowJo softwares.

4.7. Molecular Analysis by Western Blotting and Antibodies

Briefly, 1 × 10^6 of MDA-MB-468 and BT-20 cells, and 1.5 × 10^6 of HCC70 cells were seeded in (Ø90mm) Petri dishes and allowed to attach overnight, then treated with berberine at 0.5 and 1 μM, and then incubated for 120 and 144 h in 37 °C with 5% CO_2. DMSO (0.005%) was used as control. For whole cell protein lysates, the samples were prepared according to the established protocol [32]. Briefly, the lysates were collected using the Laemmli 1X buffer. Proteins dosage was done by BCA Protein assay kit reducing Agent Compatible (Perbio, ref 23250). Proteins concentrations were read as absorbance values at 562 nm using Infinite 200 (Tecan, Lyon, France) with Magellan data analysis software. Equivalent amounts of protein lysates (20 μg), were separated by electrophoresis in acrylamide gel (Bio-Rad TGX 4%–15%) starting with 15 mA then increasing to 25 mA, then blotted onto nitrocellulose membrane (Bio-Rad, Marnes la Coquette, France). The membranes were then saturated with 5% BSA+TBS/0.1% Tween for 1 h at RT.

The blotted membranes were incubated with different primary antibodies for 2 h at RT, and then washed four times with TBS/0.1% Tween 5 min each, at RT. The membranes were then followed by incubations with secondary antibodies labeled with horseradish peroxidase (Jackson Immuno Research Laboratories, Interchim, Clichy, France) for 1 h at RT.

The proteins were visualized using ECL kit (Amersham Pharmacia Biotech, Orsay, France). The quantification was performed using a LAS-3000 Luminescent Image analyser and Multi Gauge software (Fuji, FSVT, Courbevoie, France). Actin was detected for normalisation between samples using anti-beta-actin primary antibody at the dilution of 1:2000 (Sigma-Aldrich, Saint Quentin Fallavier, France). MGMT, PCNA, cleaved-PARP (cl-PARP), cleaved-caspase-7 (cl-caspase-7) and cleaved-caspase-8 (cl-caspase-8) antibodies were used at 1:1000 dilutions. Cyclin D1 antibody was used at 1:10000 dilution. Histone H2AX and phosphorylated-H2AX (P-H2AX) antibodies were diluted at 1:2000. Cyclin B1 antibody was used at 1:1000 dilution. All the antibodies were purchased from Cell Signaling Technology, Ozyme, Saint Quentin en Yveline, France.

4.8. Three-Dimensional Cell Culture

Three-dimensional (3D) cell culture was conducted with matrigel fromBD Biosciences (Le Pont de Claix, France) as described in [10]. Briefly, in 96 well plates, 5200 cell/well of malignant MDA-MB-468 cells and 6500 cell/well of normal Human breast epithelial cells MCF-10A were seeded between two matrigel coats and cell culture medium, for 7 and 10 days, respectively, at 37 °C with 5% CO_2. The medium was changed and replaced with a new one every 2 or 3 days.

Cells were then treated with berberine at various concentrations (0.5 and 5 μM). DMSO (0.025%), non-treated cells and BI2536 drug [34] were used as negative and positive controls, respectively. Cell viability was determined 3 days after the treatment using the WST1 Cell Proliferation Assay Kit (Millipore) which is based on the enzymatic cleavage of the tetrazolium salt WST1 to formazan by cellular mitochondrial dehydrogenases present in viable cells. The absorbance was measured using a microplate reader (Infinite 200, Tecan, Lyon, France) with Magellan data analysis software at a wavelenght of 450 nm. Experiments were carried out in triplicate.

4.9. Statistical Analysis

Data are represented as the mean ± standard deviation (±SD) of three independent experiments. The statistical significance of the obtained results was evaluated by using a one-way analysis of variance ANOVA at * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, using STATA 14.2 software (Lakeway Drive, College Station, Tx, USA).

5. Conclusion

To summarize, our study showed that berberine inhibited TNBC cell growth by inducing cell cycle arrest and apoptosis. The expression pattern of the protein markers involved in the regulation of cell cycle progression, DNA damage, and apoptosis was correlated to the inhibition of cellular proliferation, suggesting that berberine may be a good candidate in one of the challenges of the global health care system in making cancer treatment more personalized and effective with reduced to no side effects.

Authors Contributions: Conceptualization, L.E, V.M, T.D and A.Z; Methodology, L.E, V.M, T.D and A.Z ; Validation, L.E, V.M, T.D and A.Z ; Formal Analysis, T.D and A.Z ; Investigation, L.E and V.M ; Resources, T.D ; Writing—Original Draft Preparation, L.E ; Writing—Review & Editing, L.E, V.M, T.D and A.Z; Supervision, V.M, T.D and A.Z ; Project Administration, T.D and A.Z ; Funding Acquisition, T.D.

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References

1. Torre, L.; Rebecca Siegel, A.J. *Global Cancer Facts & Figures*, 3rd ed.; American Cancer Society: Atlanta, GA, USA, 2015; pp. 1–64
2. Boyle, P. Triple-negative breast cancer: Epidemiological considerations and recommendations. *Ann. Oncol.* **2012**, *23*, 8–13.
3. Jia, L.Y.; Shanmugam, M.K.; Sethi, G.; Bishayee, A. Potential role of targeted therapies in the treatment of triple-negative breast cancer. *Anticancer Drugs* **2016**, *27*, 147–155.
4. Mangal, M.; Sagar, P.; Singh, H.; Raghava, G.P.S.; Agarwal, S.M. NPACT: Naturally occurring plant-based anti-cancer compound-activity-target database. *Nucleic Acids Res.* **2013**, *41*, 1124–1129.
5. Wang, K.; Feng, X.; Chai, L.; Cao, S.; Qiu, F. The metabolism of berberine and its contribution to the pharmacological effects. *Drug Metab. Rev.* **2017**, *49*, 139–157.

6. Imanshahidi, M. Pharmacological and therapeutic effects of Berberis vulgaris and its active constituent, berberine. *Phytother. Res.* **2008**, *22*, 999–1012.
7. Kumar, A.; Ekavali, K.C.; Mukherjee, M.; Pottabathini, R.; Dhull, D.K. Current knowledge and pharmacological profile of berberine: An update. *Eur. J. Pharmacol.* **2015**, *761*, 288–297.
8. Liu, D.; Meng, X.; Wu, D.; Qiu, Z.; Luo, H. A natural isoquinoline alkaloid with antitumor activity: Studies of the biological activities of berberine. *Front. Pharmacol.* **2019**, *10*, 9.
9. Boulares, A.H.; Yakovlev, A.G.; Ivanova, V.; Stoica, B.A.; Wang, G.; Iyer, S.; Smulson, M. Role of Poly(ADP-ribose) Polymerase (PARP) Cleavage in Apoptosis Caspase 3-resistant PARP mutant Increases Rates of Apoptosis in Transfected Cells. *J. Biol. Chem.* **1999**, *274*, 22932–22940.
10. Lee, G.Y.; Kenny, P.A.; Lee, E.H.; Bissell, M.J. Three-dimensional culture models of normal and malignant breast epithelial cells. *Nat. Methods* **2010**, *4*, 359–365.
11. Kenny, P.A.; Lee, G.Y.; Myers, C.A.; Neve, R.M.; Semeiks, J.R.; Spellman, P.T.; Lorenz, K.; Lee, E.H.; Barcellos-Hoff, M.H.; Petersen, O.W.; et al. The morphologies of breast cancer cell lines in three-dimensional assays correlate with their profiles of gene expression. *Mol. Oncol.* **2007**, *1*, 84–96.
12. Reddy, S.M.; Barcenas, C.H.; Sinha, A.K.; Hsu, L.; Moulder, S.L.; Tripathy, D.; Hortobagyi, G.N.; Valero, V. Long-term survival outcomes of triple-receptor negative breast cancer survivors who are disease free at 5 years and relationship with low hormone receptor positivity. *Br. J. Cancer* **2018**, *118*, 17–23.
13. Feitelson, M.A.; Arzumanyan, A.; Kulathinal, R.J.; Blain, S.W.; Holcombe, R.F.; Mahajna, J.; Marino, M.; Martinez-Chantar, M.L.; Nawroth, R.; Sanchez-Garcia, I.; et al. Sustained proliferation in cancer: Mechanisms and novel therapeutic targets. *Semin. Cancer Biol.* **2015**, *35*, S25–S54.
14. Evan, G.I.; Vousden, K.H. Proliferation, cell cycle and apoptosis in cancer. *Nature* **2001**, *411*, 342–348.
15. Kim, J.; Lee, K.-M.; Ko, E.; Han, W.; Lee, J.; Shin, I.; Bae, J.Y.; Kim, S.; Noh, D.Y. Berberine Inhibits Growth of the Breast Cancer Cell Lines MCF-7 and MDA-MB-231. *Planta Med.* **2008**, *74*, 39–42.
16. Zhao, Y.; Jing, Z.; Lv, J.; Zhang, Z.; Lin, J.; Cao, X.; Zhao, Z.; Liu, P.; Mao, W. Berberine activates caspase-9/cytochrome c -mediated apoptosis to suppress triple-negative breast cancer cells in vitro and in vivo. *Biomed. Pharmacother.* **2017**, *95*, 18–24.
17. Kim, S.; Lee, J.; You, D.; Jeong, Y.; Jeon, M.; Yu, J.; Kim, S.W.; Nam, S.J.; Lee, J.E. Berberine Suppresses Cell Motility Through Downregulation of TGF-β1 in Triple Negative Breast Cancer Cells. *Cell Physiol. Biochem.* **2018**, *45*, 795–807.
18. Kim, S.; You, D.; Jeong, Y.; Yu, J.; Kim, S.W.; Nam, S.J.; Lee, J.E. Berberine down-regulates IL-8 expression through inhibition of the EGFR/MEK/ERK pathway in triple-negative breast cancer cells. *Phytomedicine* **2018**, *50*, 43–49.
19. Yao, M.; Fan, X.; Yuan, B.; Takagi, N.; Liu, S.; Han, X.; Ren, J.; Liu, J. Berberine inhibits NLRP3 Inflammasome pathway in human triple-negative breast cancer MDA-MB-231 cell. *BMC Complement. Altern. Med.* **2019**, *19*, 216.
20. Lin, Y.S.; Chiu, Y.C.; Tsai, Y.H.; Tsai, Y.F.; Wang, J.Y.; Tseng, L.M.; Chiu, J.H. Different mechanisms involved in the berberine-induced antiproliferation effects in triple-negative breast cancer cell lines. *J. Cell Biochem.* **2019**, *120*, 13531–13544.

21. Kurki, P.; Vanderlaan, M.; Dolbeare, F.; Gray, J.; Tan, E.M. Expression of proliferating cell nuclear antigen (PCNA)/cyclin during the cell cycle. *Exp. Cell Res.* **1986**, *166*, 209–219.
22. Nakayama, Y. Role of Cyclin B1 levels in DNA damage and DNA damage-induced senescence. *Int. Rev. Cell. Mol. Biol.* **2013**, *305*, 303–337.
23. Zhao, W.; Liu, H.; Wang, J.; Wang, M.; Shao, R. Cyclizing-berberine A35 induces G2/M arrest and apoptosis by activating YAP phosphorylation (Ser127). *J. Exp. Clin. Cancer Res.* **2018**, *37*, 98.
24. Eo, S.H.; Kim, J.H.; Kim, S.J. Induction of G2 /M Arrest by Berberine via Activation of PI3K/Akt and p38 in Human Chondrosarcoma Cell Line. *Oncol. Res.* **2015**, *22*, 147–157.
25. Luo, Y.; Hao, Y.; Shi, T.; Deng, W.; Li, N. Berberine inhibits cyclin D1 expression via suppressed binding of AP-1 transcription factors to CCND1 AP-1 motif. *Acta Pharmacol. Sin.* **2008**, *29*, 628–633.
26. Chai, Y.S.; Hu, J.; Lei, F.; Wang, Y.G.; Yuan, Z.Y.; Lu, X.; Wang, X.P.; Du, F.; Zhang, D.; Xing, D.M.; et al. Effect of berberine on cell cycle arrest and cell survival during cerebral ischemia and reperfusion and correlations with p53/cyclin D1 and PI3K/Akt. *Eur. J. Pharmacol.* **2013**, *708*, 44–55.
27. Matthews, G.M.; Newbold, A.; Johnstone, R.W. Intrinsic and Extrinsic Apoptotic Pathway Signaling as Determinants of Histone Deacetylase Inhibitor Antitumor Activity. *Adv. Cancer. Res.* **2012**, *116*, 165–197.
28. Letašiová, S.; Jantová, S.; Miko, M.; Ovádeková, R.; Horváthová, M. Effect of berberine on proliferation, biosynthesis of macromolecules, cell cycle and induction of intercalation with DNA, dsDNA damage and apoptosis in Ehrlich ascites carcinoma cells. *J. Pharm. Pharmacol.* **2006**, *58*, 263–270.
29. Rogakou, E.P.; Boon, C.; Redon, C.; Bonner, W.M. Megabase chromatin domains involved in DNA double-strand breaks in vivo. *J. Cell. Biol.* **1999**, *146*, 905–916.
30. Patil, J.B.; Kim, J.; Jayaprakasha, G.K. Berberine induces apoptosis in breast cancer cells (MCF-7) through mitochondrial-dependent pathway. *Eur. J. Pharmacol.* **2010**, *645*, 70–78.
31. El Khalki, L.; Tilaoui, M.; Jaafari, A.; Ait Mouse, H.; Zyad, A. Studies on the Dual Cytotoxicity and Antioxidant Properties of *Berberis vulgaris* Extracts and Its Main Constituent Berberine. *Adv. Pharmacol. Sci.* **2018**, *2018*, 1–11.
32. Marty, B.; Maire, V.; Gravier, E.; Rigaill, G.; Vincent-Salomon, A.; Kappler, M.; Lebigot, I.; Djelti, F.; Tourdès, A.; Gestraud, P.; et al. Frequent PTEN genomic alterations and activated phosphatidylinositol 3-kinase pathway in basal-like breast cancer cells. *Breast Cancer Res.* **2008**, *10*, R101.
33. Franken, N.A.P.; Rodermond, H.M.; Stap, J.; Haveman, J.; van Bree, C. Clonogenic assay of cells in vitro. *Nat. Protoc.* **2006**, *1*, 2315–2319.
34. Maire, V.; Némati, F.; Richardson, M.; Vincent-Salomon, A.; Tesson, B.; Rigaill, G.; Gravier, E.; Marty-Prouvost, B.; De Koning, L.; Lang, G.; et al. Polo-like kinase 1: A potential therapeutic option in combination with conventional chemotherapy for the management of patients with triple-negative breast cancer. *Cancer Res.* **2013**, *73*, 813–823.

Sample Availability: Samples of berberine compound are available from the authors.

IV.3: Article 2:

Artemisinin Impairs the Survival of Triple Negative Breast Cancer Cells Alone and In Combination With Cisplatin (submitted)

Article

Artemisinin impairs the survival of triple negative breast cancer cells alone and in combination with cisplatin

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Abstract: Artemisinin is a sesquiterpene lactone compound extracted from Chinese medicinal plants such as *Artemisia annua L.* Artemisinin displays a number of beneficial roles in the treatment of malaria and various types of cancers. Cisplatin is an effective chemotherapeutic agent in triple negative breast cancer (TNBC) treatment; however, systemic toxicity occurring in TNBC patients with cisplatin-based therapy remains the major obstacle to a successful treatment. Thus, the aim of the present study was to first investigate the differential antiproliferative effect of artemisinin towards seven TNBC cell lines: HCC38, MDA-MB-468, HCC1937, HCC1143, BT-20, BT-549 and HCC70, using MTT assay. Then, after selecting the most sensitive cell lines we examined the effect of artemisinin on their cell colony formation, cell cycle progression and its synergism with cisplatin using Loewe Excess model. Interestingly, artemisinin inhibited TNBC cell growth with HCC38 (IC50=1.25 µM) and MDA-MB-468 (IC50= 2.23 µM) being the most sensitive ones. Moreover, artemisinin induced a significant cell cycle arrest in S phase of HCC38. Interestingly, the combination of artemisinin with cisplatin treatment resulted in synergy in treated HCC38 and MDA-MB-468 cells. To the best of our knowledge, these are the first results of artemisinin showing its antiproliferative effect on TNBC cells through cell cycle arrest and synergy when combined to the platinum drug, cisplatin; suggesting that artemisinin may be a good candidate for TNBC drug development.

Keywords: Artemisinin, cisplatin, synergy, triple negative breast cancer

1. Introduction

The battle against breast cancer disease is a worldwide challenge; it costs lives, money and time. According to Globocan 2018, new breast cancer cases are estimated by 11.6% worldwide in 2018 and approximately 12% of these cases are Triple Negative Breast Cancer (TNBC) (Torre et al., 2015)(Boyle, 2012). Based on immunohistochemistry, TNBC is characterized by the absence of expression of estrogen and progesterone hormone receptors

and the lack of epidermal growth factor receptor 2 / Neu overexpression (HER2) and this is responsible for its poor prognosis, lack of targeted therapies and clinical management problems (Papa et al., 2014).

Because patients with TNBC are not likely to benefit from anti-hormones or anti-HER2 therapy, first-line treatment usually consists of conventional cytotoxic chemotherapy using a large number of agents, including platinum-based regimens such as cisplatin.

Cisplatin remains an effective treatment in many forms of cancer therapy including TNBC (Zhang et al., 2015)(Silver et al., 2010). Cisplatin mediates its antitumour effects by inducing DNA damage and apoptosis (Radin et al., 2016)(Al-Bahlani et al., 2018); however, it is a dose-limiting treatment because of its serious adverse side effects such as its systemic toxicity which accounts for central and peripheral neurotoxicity, hematopoietic, gastro-intestinal and nephrotoxicity (Livshits, Rao, & Smith, 2014) (Reilly et al., 2015). Strategies designed to increase the effectiveness of cisplatin treatment in TNBC are still under investigation.

Artemisinin (PubChem CID: 68827) is an ancient Chinese herbal therapy for malaria fevers (Tu, 2016)(Guo, 2016), which has been recently found to have potent antitumor activity (Zhang et al., 2018)(Zyad et al., 2017). Artemisinin and its derivatives can exert their anticancer effect through arresting cell growth, inducing apoptosis and inhibiting metastasis in different types of cancer (Konstat-Korzenny et al., 2018). However, anticancer effect of artemisinin treatment on breast cancer has been only reported in some preclinical studies; in which it was reported that artemisinin and its derivative artemisone arrested MCF-7 cell cycle by reducing the levels of expression of cyclin D1, CDK4 and pRb (Gravett et al., 2011).

Since artemisinin and its derivatives cause oxidative DNA damage and double strand breaks (Berdelle et al., 2011)(Aquino et al., 2013), their combination with DNA-damaging drugs may result in enhanced anticancer effects. Indeed, artemisinin and its derivatives have shown additive to synergistic effects when combined to synthetic chemotherapy drugs such as anthracyclines, anti-metabolites, taxanes and platinum-based drugs in other cancer types (Bhaw-Luximon et al., 2017)(Efferth, 2017). In this paper, and for the first time in literature, we studied the effect of artemisinin on the viability and cell cycle progression of TNBC cell lines, as well as its effect when combined to the platinum-based drug, cisplatin.

2. Materials and Methods

2.1.Reagents

Cell culture reagents were all purchased from Life Technologies (USA). All breast cancer cell lines: TNBC (BT-20, HCC70, HCC38, HCC1937, HCC1143, BT-549 and MDA-MB-468) and non-TNBC (MCF-7/ER-PR+HER2+ and SKBR3/ER-PR-HER2+) were purchased from ATCC (USA). Artemisinin 98% was purchased from Sigma Aldrich (USA).

2.2.Cell culture

Cell lines were authenticated and they do not exceed their 15^{th} passage in cell culture.

BT-20 and MCF-7 cells were cultured in MEM (Eagle) medium with 10% fetal bovine serum (FBS), 1% glutamax, 1% penicillin/ streptomycin, 1.5g/L sodium bicarbonate, 0.1mM non-essential amino-acids and 1mM sodium pyruvate. HCC70, HCC38, HCC1937, HCC1143 and BT-549 cells were cultured in RPMI-1640 medium with 10% FBS, 1% glutamax, 1% penicillin/ streptomycin, 1.5g/L sodium bicarbonate, 10mM Hepes and 1mM sodium pyruvate. SKBR3 cells were cultured in McCoy's 5A medium with 10% FBS, 1% glutamax and 1% penicillin/ streptomycin. MDA-MB-468 was cultured in RPMI-1640 medium with 10% FBS, 1% glutamax and 1% penicillin/ streptomycin. All the cells were maintained in a humidified 5% CO_2 incubator at 37°C.

2.3.Assessment of cell viability using MTT assay

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) (Sigma-Aldrich), a colorimetric assay capable of detecting viable cells by the reduction of the yellow tetrazolium salt to purple formazan. In order to ensure their adhesion and their exponential growth on the day of the treatment, the cells (HCC38, MDA-MB-468, HCC1937, HCC1143, MCF-7, BT-20, SKBR3, BT-549 and HCC70) were seeded in 96 well plates at the proper density two days (48 h) before the treatment (Table 1). Then, they were treated with artemisinin previously dissolved in DMSO (Sigma-Aldrich). The cell lines were incubated for 4 doubling time, as described (Marty et al., 2008). DMSO (1%), non-treated cells and Doxorubicin were used as negative and positive controls, respectively. At the end of the incubation period, the cells were treated with MTT (5mg/ml in PBS) for 4 h at 37°C. 10% SDS in 10 mM hydrochlorid acid was added to dissolve the formazan and incubated overnight at 37°C. The production of formazan which is directly proportional to the viable cell number was read as absorbance values at 540nm using a plate reader (Infinite 200, Tecan, Lyon, France) with Magellan data analysis software. Experiments were independently performed at least three times in duplicate.

Table 1: Seeded cell concentrations per well used in cell viability assay

Cell lines	**Cell number ($\times 10^3$ cell/mL) 150µL/well in 96 wells plate**	**doubling time (hour)**
HCC1937	10	45
HCC70	20	50
MDA-MB-468	10	45
HCC38	20	60
BT-20	10	50
HCC1143	10	60

BT-549	5	30
MCF-7	10	45
SKBR3	10	60

2.4.Two-dimensional clonogenic assay

Two-dimensional (2D) clonogenic (or colony formation) assay is an *in vitro* cell survival test based on the ability of a single cell to grow into a colony by undergoing multiple divisions (Franken, Rodermond, Stap, Haveman, & van Bree, 2006). Experiments were performed with MDA-MB-468 and HCC38 cells because they were the most sensitive cell lines towards artemisinin treatment, as found in the cell growth inhibition assay results. HCC70 cells were also chosen as the most resistant cells to artemisinin. All cells were treated with 0.5 and 5 µM artemisinin, doses around their IC_{50}. Different number of viable cells were seeded onto 6 well plates (Ø 35mm) depending on the cell line (Table 2), and allowed to attach to the well bottom overnight. Cells were then treated with different doses of the drugs and incubated the time to allow colonies to form in the negative control condition (0.005% DMSO) (Table 2). The obtained colonies were washed with PBS, fixed and stained with 0.05% of Coomassie® Brilliant Blue R-250 (ICN Biomedicals) solubilized in 50% of methanol, 10% Acetic acid and 40% of Milli-Q water. For each cell line, we counted the colonies in each well and compared them to DMSO-treated cells using ImageJ software. All the experiments were carried out in triplicate and in three independent assays.

Table 2: Seeded cell number per well used in 2D clonogenic assay and their colony formation time

Cell lines	The number of cells/ well	Colony formation time (day)
MDA-MB-468	2000	10
HCC38	7000	15
HCC70	7000	15

2.5.Cell cycle analysis by flow cytometry

MDA-MB-468 (2×10^5 cells/well) and HCC38 (3×10^5 cells/well) cells were seeded onto 6 well plates (Ø 35 mm). The cells were then allowed to attach to the well bottom overnight. Cells were treated with 0.5 or 5 µM artemisinin or with DMSO (0.005%) as a negative control. Cells were incubated for 48 h at 37°C with 5% CO_2. Both attached and detached cells were then harvested, washed three times and centrifuged at 1500 rpm (for 5 min) with PBS (1X). The cells were then centrifuged and fixed with cold ethanol (70%) and incubated for 30 minutes at RT. Next, the cells were washed twice with PBS (1X). After removing ethanol, cells were centrifuged and the pellet was resuspended in PBS containing 10µg/mL of

propodium iodide (invitrogen P3566) and 100µg/mL of RNAase (invitrogen 12091-021). The pellet was then incubated for 15 min at RT in the dark. Finally, the cell cycle analysis was carried out using LSRII flow cytometer (Becton Dickinson) with DIVA™ software to determine cellular DNA content. The cell population percentage of each phase was analyzed using FlowJo and ModFit LT software package.

2.6.Synergy calculations

MTT assay was used to measure cell viability of artemisinin/cisplatin combination in the same conditions as described above in the assessment of cell viability of artemisinin alone. MDA-MB-468 and HCC38 cells were grown in 96 wells plates at a density of 10×10^3 and 20×10^3 cells/mL, respectively, and treated with increasing concentrations of artemisinin in combination with increasing concentrations of cisplatin. After 4 doubling-time incubation, MTT was added and incubated in 37°C for 4 h. SDS (10% in 10 mM hydrochlorid acid) was then added to dissolve the formazan and incubated overnight at 37°C. Absorbance values were read at 540nm using a plate reader (Infinite 200, Tecan, Lyon, France) with Magellan data analysis software. Experiments were independently done three times in triplicate. DMSO (1%) was used as a negative control. The fraction affected from viability percentages was calculated using an Excel template and the results were analyzed with the web version of Chalice Analyzer (Horizon Discovery) to calculate Loewe model Excess scores (LOEWE, 1953).

2.7.**Statistical analysis**

Data are represented as the mean ± standard deviation (SD) of three independent experiments done in triplicate. The statistical significance of the obtained results was evaluated by using a one-way analysis of variance ANOVA, the difference is considered significant when $p < 0.05$ (as *), $p < 0.01$ (as **) and $p < 0.001$ (as ***), using STATA 14.2 software.

3. Results & Discussion

3.1.Artemisinin differentially inhibits the proliferation of TNBC cell lines

Previous studies have investigated the effect of the anti-malarial drug artemisinin and its derivatives (dihydroartemisinin, artesunate, artemether, arteether and artemisone) on several types of cancer such as leukemia, lung cancer, liver cancer, brain glioma, colorectal and gastric cancer (Zyad et al., 2017)(Zhang et al., 2018). Anticancer effect of artemisinin treatment on breast cancer has been reported by numerous preclinical studies; however its effect on TNBC has not been yet reported in the literature. We only can retrieve studies about artemisinin derivatives such as a new artemisinin dimer piperazine derivative (ADPs) which reduced the wild type epidermal growth factor receptor (EGFR or HER1) in the MDA-MB-231 TNBC cell line (Y. J. Zhang et al., 2013). In addition, Artesunate, another semi-synthetic derivative of artemisinin which causes cell cycle arrest and apoptosis in MDA-MB-468 cells and in the HER2-enriched SKBR3 breast cancer cell line (Greenshields, Fernando, & Hoskin, 2019). Here, we report for the first time the effects of artemisinin on TNBC cell lines and its combination with cisplatin.

We analyzed the antiproliferative effect of artemisinin on seven TNBC (BT-20, HCC70, HCC38, HCC1937, HCC1143, BT-549 and MDA-MB-468) and two non-TNBC (MCF-7/ER-PR+HER2- and SKBR3/ER-PR-HER2+) cell lines. The results showed that artemisinin differentially inhibited cell growth depending on its dose and also on the cell line type with IC_{50} values ranging from 1.2 µM to 54.6 µM (Table 3). HCC70 cells were not sensitive to artemisinin (Table 3). There was heterogeneity in the sensitivity towards artemisinin among the panel of TNBC and non-TNBC cell lines: HCC38 (IC_{50}= 1.2 µM) and MDA-MB-468 (IC_{50}= 2.2 µM) cells were the most sensitive, HCC70 cells were the most resistant (Figure 1). These results are confirmed by the 2D clonogenic assay, where 5 µM artemisinin significantly (p<0.001) reduced the colony formation capacity of MDA-MB-468 and HCC38 cells (Figure 2). In contrast, the treatment of HCC70 cells with 5 µM artemisinin gave highly variable results with no significant effect on colony formation (Figure 2).

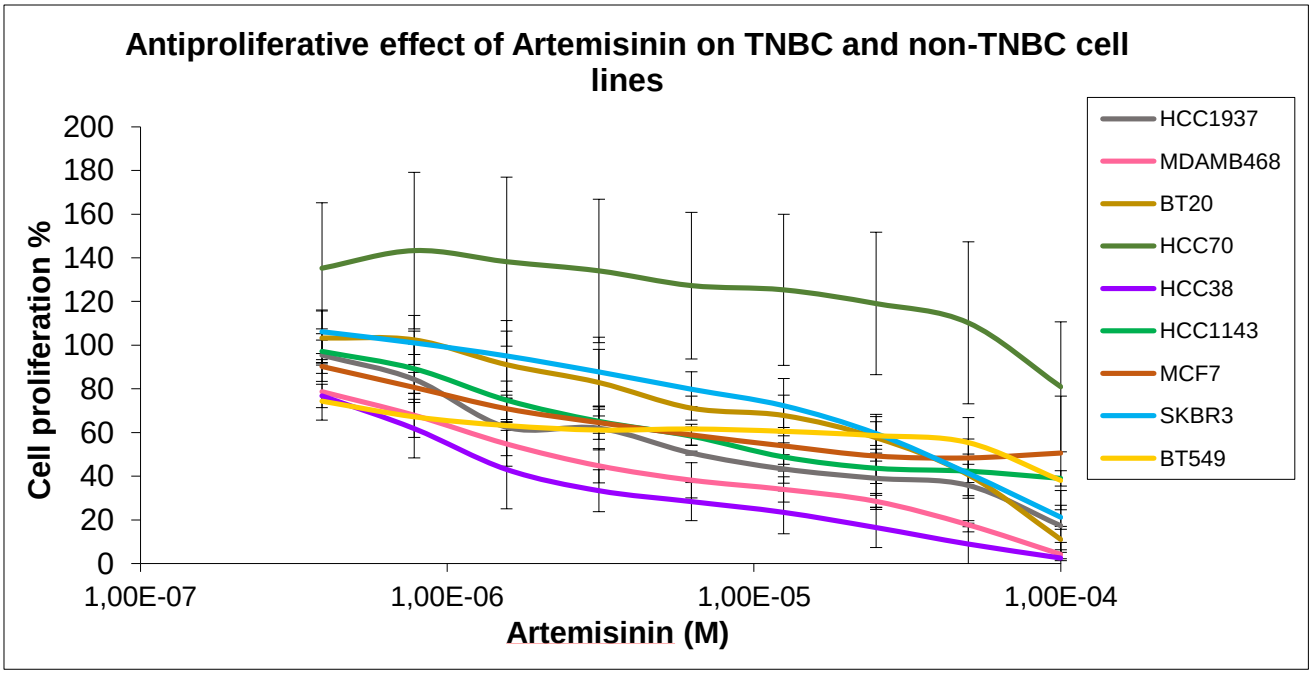

Figure 1: Effect of artemisinin on the proliferation of TNBC and non-TNBC breast cancer cell lines. Cells were seeded and treated with various concentrations of artemisinin. Cell viability was measured by MTT assay and represented as cell proliferation percentage ± Standard Deviation (SD) from three independent experiments carried out in triplicate.

Table 3: IC_{50} (µM) values of artemisinin on TNBC and non-TNBC cell lines

Cell line	Artemisinin IC_{50} (µM)
HCC38	1.2 ± 1.3
MDA-MB-468	2.2 ± 3.3
HCC1937	8.0 ± 3
HCC1143	15.3 ± 6.1
MCF-7	28.2 ± 24.1
BT-20	35.8 ± 13.2
SKBR3	37.8 ± 6.6

BT-549	54.6 ± 30.7
HCC70	>100

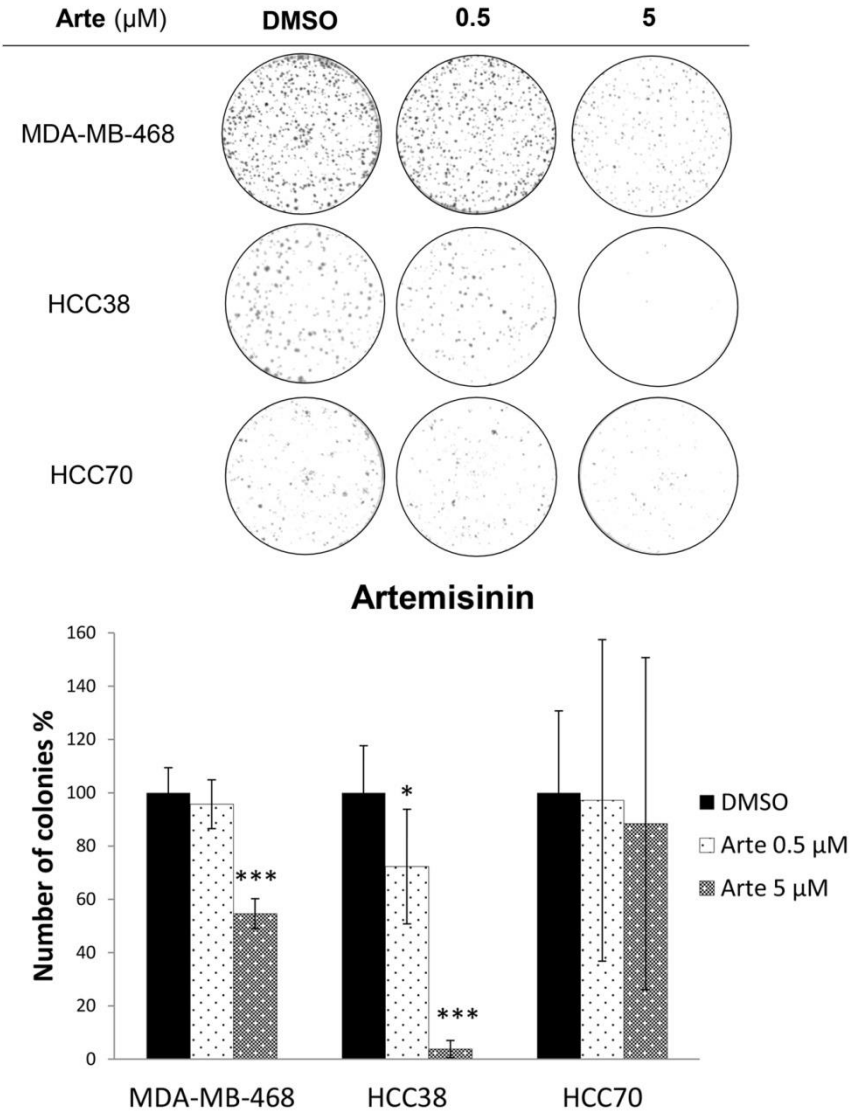

Figure 2: Effect of Artemisinin on the ability of cells to form colonies. Images of wells of DMSO- or artemisinin (0.5, 5 µM)-treated TNBC cells (top). Graph presenting the percentage of colonies compared to the DMSO-treated cells for each cell line (bottom panel). Arte: Artemisinin. Percentage±Standard Deviation of three independent experiments done in triplicate, p <0.05 (as *), p <0.01 (as **) and p <0.001 (as ***) compared to DMSO-treated cells.

3.2.Artemisinin differentially affects TNBC cell cycle progression

Since artemisinin inhibited cell proliferation of MDA-MB-468 and HCC38 cells, we further examined by flow cytometry whether this molecule affected cell cycle progression. We observed that artemisinin affected the cell cycle in a cell type dependent manner (Figure 3). Indeed, the cell cycle of MDA-MB-468 cells was not affected 48 h after treatment with artemisinin (Figure 3-a). In contrast, 5 µM artemisinin for 48 h significantly (p <0.05) induced an S phase arrest in HCC38 cells, in comparison to DMSO-treated cells (Figure 3-b).

This result suggests that artemisinin impairs the entry into the G2/M phase of HCC38 cells (Figure 3-b).

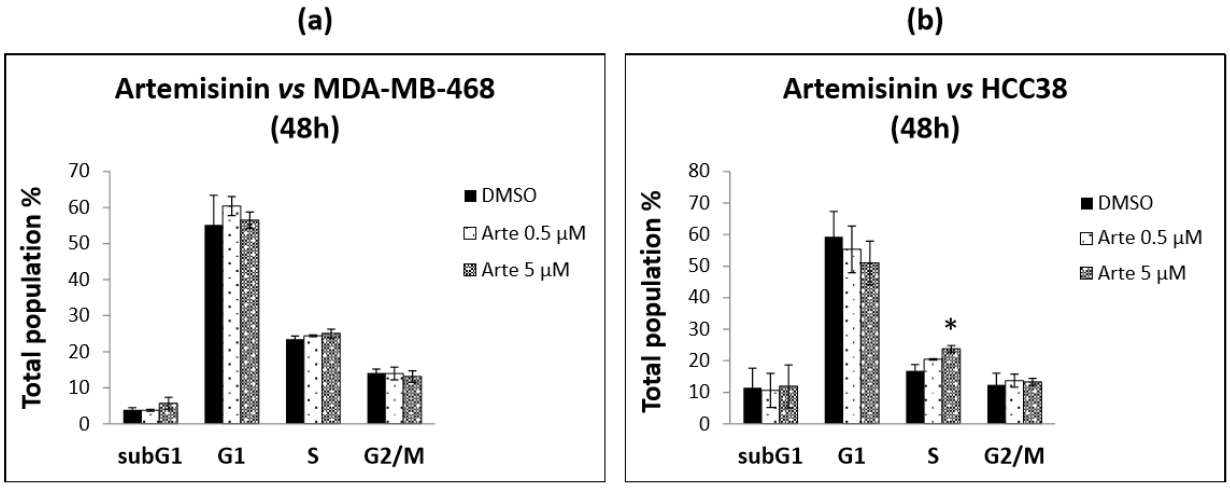

Figure 3: Effects of artemisinin on cell cycle progression of TNBC cells. MDA-MB-468 **(a)** and HCC38 **(b)** cells were treated with DMSO or artemisinin (0.5, 5 µM) for 48 h, and then analyzed by FACS. Total population (%) in the different phases of the cell cycle (G1, S, G2/M, SubG1) are indicated ±SD from three independent experiments, p <0.05 (as *), p <0.01 (as **) and p <0.001 (as ***) compared to DMSO. (Arte: Artemisinin).

3.3.Artemisinin synergizes with cisplatin to inhibit TNBC cell viability

Therapeutic drugs combination is proven to be an effective strategy for TNBC treatment (Chalakur-Ramireddy et al., 2018)(Lee et al., 2018). Drug combination increases the efficacy of the treatment while reducing systemic toxicity and drug resistance problems. Cisplatin, a platinum drug, was reported to be an effective treatment against TNBC but with serious side effects and drug resistance problems (Zhang et al., 2015)(Silver et al., 2010)(Reilly et al., 2015). In our study, we have evaluated the potential of the artemisinin + cisplatin combination on TNBC cell proliferation. These experiments were assessed on the HCC38 and MDA-MB-468 artemisinin-sensitive cells. The cytotoxic effect of cisplatin on our TNBC cells was previously determined in our laboratory, so we had to exploit these results in the purpose of this combination test. To find any synergistic activity between artemisinin and cisplatin, we treated HCC38 and MDA-MB-468 cells in a two-dimensional 6×6 dose matrix assay using Loewe additivity model (Greco et al., 1995)(Vinet et al., 2019). The results are represented as a heatmap of Loewe excess scores for both drugs in the treated cells. Fraction affected percentage was calculated from the viability and used to generate the scores using Chalice Analyzer (http://chalice.horizondiscovery.com/analyzer-server/cwr/analyze.jsp). As shown in figure 4, artemisinin when combined to cisplatin, possessed significant synergistic activity in both cell lines, in the ranges of concentrations of artemisinin from 2.5µM to 10µM with cisplatin at 2.5µM, in HCC38 (P<0.001) (Figure 4-a-b), and when artemisinin ranges from 1.2µM to 10µM with cisplatin at 0.12µM, in MDA-MB-468 (P<0.01) (figure 4-c-d). Our results are in agreement with previous studies of combining therapy of artemisinin derivatives with cisplatin on lung and ovarian cancer (Zhang et al., 2013)(Feng et al., 2014)(Wang et al., 2015).

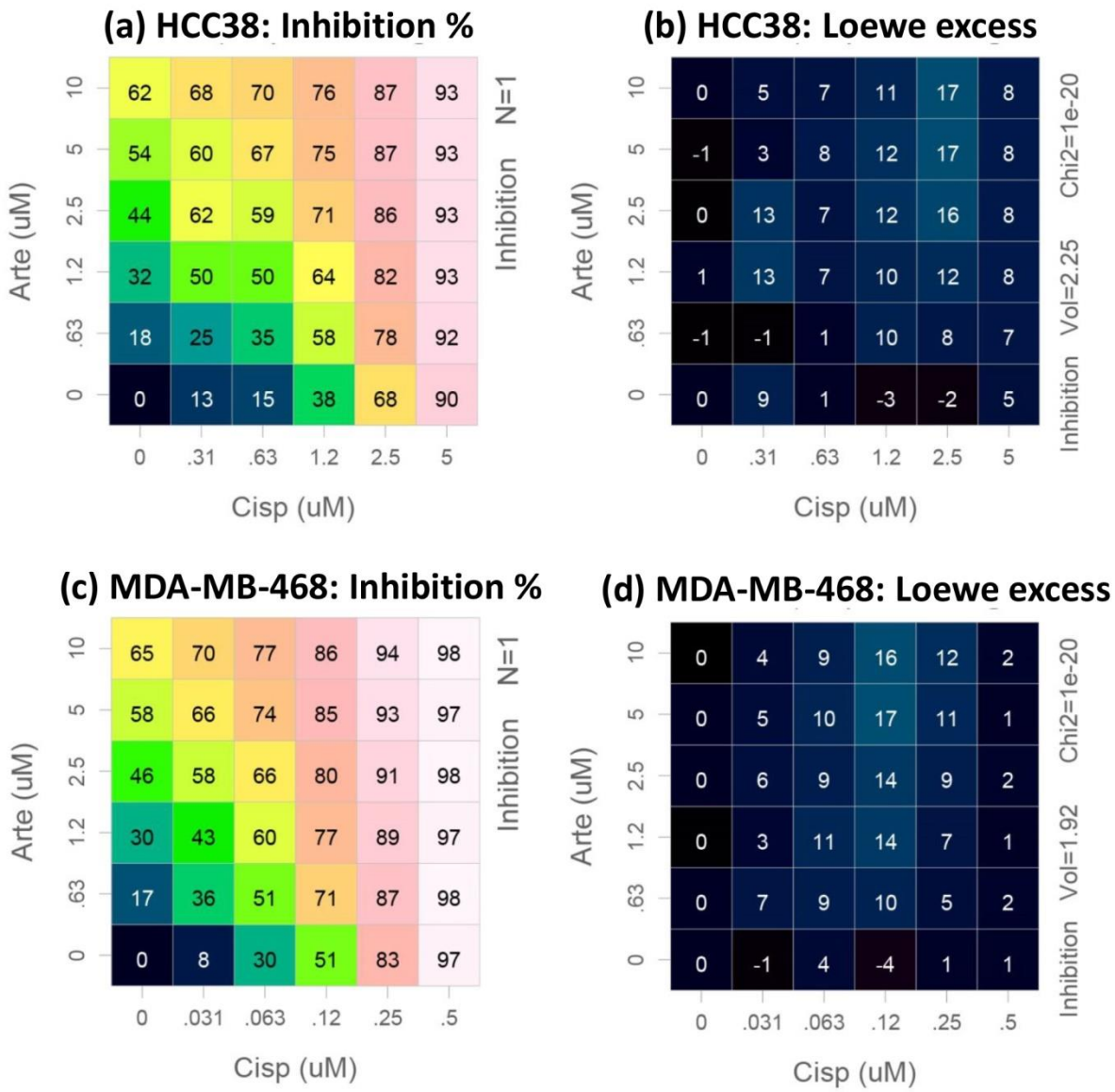

Figure 4: Synergistic interaction between artemisinin and cisplatin. **(a,c)** Cell viability inhibition (% compared to DMSO-treated cells) is indicated for each combination in HCC38 and MDA-MB-468 cell lines. **(b,d)** Chalice Analyzer (http://chalice.horizondiscovery.com/analyzer-server/cwr/analyze.jsp) was used to calculate the Loewe excess. Arte: artemisinin, Cisp: cisplatin.

Conclusions

The anti-proliferative effect of artemisinin derivatives on breast cancer including TNBC was reported to be due to cell cycle arrest and apoptosis (Zhang et al., 2018)(Greenshields et al., 2019). Previous studies have reported that artemisinin-induced cytotoxicity of other cancer cells was due to intracellular Reactive Oxygen Species (ROS) accumulation (Y. Zhang et al., 2018)(Greenshields et al., 2019). It has been reported that the generation of ROS in artemisinin-treated cells is linked to the presence of intracellular ferrous iron in different cancer types (Konstat-Korzenny et al., 2018)(Zhang et al., 2018).

As cancer cells divide rapidly, they typically have high rate of iron uptake through the transferrin receptors (Chen et al., 2019). Transferrin receptors are involved in the transport of

metals into cells such as iron and platinum (Mujika et al., 2011). Hence, the high uptake of iron and the platinum drug cisplatin in the presence of artemisinin may synergistically induce cytotoxicity and generate intracellular ROS. Furthermore, artemisinin in combination with cisplatin induced the generation of ROS treatment in gastric cells (Wu, 2016) and ovarian cancer cells (Wang et al., 2015). This suggests that the synergy effect of artemisinin and cisplatin, in our study, may be due to intracellular ROS generation.

To summarize, our study showed that artemisinin differentially inhibited TNBC cell growth in a dose and cell dependent manner, as well as it induced cell cycle arrest in HCC38 cells. Artemisinin and cisplatin combination synergistically inhibited TNBC cell growth. These findings suggest that artemisinin may be a good candidate in TNBC drug development, used alone or in combination with the conventional anti-cancer drug, cisplatin.

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Conflicts of Interest: The authors of this article declare that they have no conflict of interest.

References

Abdelmajid Zyad, Mounir Tilaoui, Abdeslam Jaafari , Moulay Ali Oukerrou, Hassan Ait Mouse. (2017). More insights into the pharmacological effects of artemisinin. *Phytotherapy Research*. 1–14. https://doi.org/10.1002/ptr.5958

Al-Jaaidi, S. A.-B. and S. (2018). Triple-Negative Breast Cancer, Cisplatin and Calpain-1. *IntechOpen, Chapter 8*. https://doi.org/http://dx.doi.org/10.5772/57353

Aquino, I., Tsuboy, M. S. F., Marcarini, J. C., Mantovani, M. S., Perazzo, F. F., & Maistro, E. L. (2013). Genotoxic evaluation of the antimalarial drugs artemisinin and artesunate in human HepG2 cells and effects on CASP3 and SOD1 gene expressions. *Genetics and Molecular Research*. *12*(3), 2517–2527. https://doi.org/10.4238/2013.July.24.6

Berdelle, N., Nikolova, T., Quiros, S., Efferth, T., & Kaina, B. (2011). Artesunate induces oxidative DNA damage, sustained DNA double-strand breaks, and the ATM/ATR damage response in cancer cells. *Molecular Cancer Therapeutics*. *10*(12), 2224–2233. https://doi.org/10.1158/1535-7163.MCT-11-0534

Bhaw-Luximon, A., & Jhurry, D. (2017, March 1). Artemisinin and its derivatives in cancer therapy: Status of progress, mechanism of action, and future perspectives. *Cancer Chemotherapy and Pharmacology*. https://doi.org/10.1007/s00280-017-3251-7

Boyle, P. (2012). Triple-negative breast cancer: Epidemiological considerations and recommendations. *Annals of Oncology*. *23*(SUPPL. 6), 8–13. https://doi.org/10.1093/annonc/mds187

Chalakur-Ramireddy, N. K. R., & Pakala, S. B. (2018). Combined drug therapeutic strategies for the effective treatment of Triple Negative Breast Cancer. *Bioscience Reports*. *38*, 20171357. https://doi.org/10.1042/BSR20171357

Chen, Y., Fan, Z., Yang, Y., & Gu, C. (2019). Iron metabolism and its contribution to cancer (Review). *International Journal of Oncology*. https://doi.org/10.3892/ijo.2019.4720

Efferth, T. (2017). Cancer combination therapies with artemisinin-type drugs. *Biochemical*

Pharmacology. https://doi.org/10.1016/j.bcp.2017.03.019

Feng, X., Li, L., Jiang, H., Jiang, K., Jin, Y., & Zheng, J. (2014). Dihydroartemisinin potentiates the anticancer effect of cisplatin via mTOR inhibition in cisplatin-resistant ovarian cancer cells: involvement of apoptosis and autophagy. *Biochemical and Biophysical Research Communications*. *444*(3), 376–381. https://doi.org/10.1016/j.bbrc.2014.01.053

Franken, N. a P., Rodermond, H. M., Stap, J., Haveman, J., & van Bree, C. (2006). Clonogenic assay of cells in vitro. *Nature Protocols*. *1*(5), 2315–2319. https://doi.org/10.1038/nprot.2006.339

Gravett, A. M., Liu, W. M., Krishna, S., Chan, W. C., Haynes, R. K., Wilson, N. L., & Dalgleish, A. G. (2011). In vitro study of the anti-cancer effects of artemisone alone or in combination with other chemotherapeutic agents. *Cancer Chemotherapy and Pharmacology*. *67*(3), 569–577. https://doi.org/10.1007/s00280-010-1355-4

Greco, W. R., Bravo, G., & Parsons, J. C. (1995). The search for synergy: a critical review from a response surface perspective. *Pharmacological Reviews*, *47*(2), 331–385. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7568331

Greenshields, A. L., Fernando, W., & Hoskin, D. W. (2019). The anti-malarial drug artesunate causes cell cycle arrest and apoptosis of triple-negative MDA-MB-468 and HER2-enriched SK-BR-3 breast cancer cells. *Experimental and Molecular Pathology*. *107*, 10–22. https://doi.org/10.1016/j.yexmp.2019.01.006

Guo, Z. (2016). Artemisinin anti-malarial drugs in China. *Acta Pharmaceutica Sinica B*. *6*(2), 115–124. https://doi.org/10.1016/j.apsb.2016.01.008

Konstat-Korzenny, E. I., Alberto Ascencio-Aragón, J., Niezen-Lugo, S., & Vázquez-López, R. (2018). Artemisinin and Its Synthetic Derivatives as a Possible Therapy for Cancer. *Medical Sciences*. *6*(1), 19. https://doi.org/10.3390/medsci6010019

Lee, A., & Djamgoz, M. B. A. (2018). Triple negative breast cancer: Emerging therapeutic modalities and novel combination therapies. *Cancer Treatment Reviews*. https://doi.org/10.1016/j.ctrv.2017.11.003

Livshits, Z., Rao, R. B., & Smith, S. W. (2014). An approach to chemotherapy-associated toxicity. *Emergency Medicine Clinics of North America*. https://doi.org/10.1016/j.emc.2013.09.002

Loewe, S. (1953). The problem of synergism and antagonism of combined drugs. *Arzneimittel-Forschung*. *3*(6), 285–290. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/13081480

Marty, B., Maire, V., Gravier, E., Rigaill, G., Vincent-Salomon, A., Kappler, M., … Dubois, T. (2008). Frequent PTEN genomic alterations and activated phosphatidylinositol 3-kinase pathway in basal-like breast cancer cells. *Breast Cancer Research*. *10*(6), R101. https://doi.org/10.1186/bcr2204

Mujika, J. I., Lopez, X., Rezabal, E., Castillo, R., Marti, S., Moliner, V., & Ugalde, J. M. (2011). A QM/MM study of the complexes formed by aluminum and iron with serum transferrin at neutral and acidic pH. *Journal of Inorganic Biochemistry*. *105*(11), 1446–1456. https://doi.org/10.1016/j.jinorgbio.2011.07.019

Papa, A., Caruso, D., Tomao, S., Rossi, L., Zaccarelli, E., & Tomao, F. (2014). Triple-

negative breast cancer: investigating potential molecular therapeutic target. *Expert Opinion on Therapeutic Targets*. *19*(1), 55–75. https://doi.org/10.1517/14728222.2014.970176

Radin, D., Lippa, A., Patel, P., & Leonardi, D. (2016). Lifeguard inhibition of Fas-mediated apoptosis: A possible mechanism for explaining the cisplatin resistance of triple-negative breast cancer cells. *Biomedicine and Pharmacotherapy*. *77*, 161–166. https://doi.org/10.1016/j.biopha.2015.12.022

Reilly, E. A. O., Gubbins, L., Sharma, S., Tully, R., Ho, M., Guang, Z., … Mccann, A. (2015). The fate of chemoresistance in triple negative breast cancer (TNBC). *BBA Clinical*. *3*, 257–275. https://doi.org/10.1016/j.bbacli.2015.03.003

Silver, D. P., Richardson, A. L., Eklund, A. C., Wang, Z. C., Szallasi, Z., Li, Q., … Garber, J. E. (2010). Efficacy of Neoadjuvant Cisplatin in Triple-Negative Breast Cancer. *J Clin Oncol*. *28*, 1145–1153. https://doi.org/10.1200/JCO.2009.22.4725

Torre, L., & Rebecca Siegel, A. J. (2015). Global Cancer Facts & Figures 3rd Edition. *American Cancer Society*. (800), 1–64. https://doi.org/10.1002/ijc.27711

Tu, Y. (2016). Artemisinin-A Gift from Traditional Chinese Medicine to the World (Nobel Lecture). *Angewandte Chemie International Edition*. *55*(35), 10210–10226. https://doi.org/10.1002/anie.201601967

Vinet, M., Suresh, S., Maire, V., Monchecourt, C., Némati, F., Lesage, L., … Dubois, T. (2019). Protein arginine methyltransferase 5: A novel therapeutic target for triple-negative breast cancers. *Cancer Medicine*. *8*(5), 2414–2428. https://doi.org/10.1002/cam4.2114

Wang, B., Hou, D., Liu, Q., Wu, T., Guo, H., Zhang, X., … Shao, C. (2015). Artesunate sensitizes ovarian cancer cells to cisplatin by downregulating RAD51. *Cancer Biology and Therapy*. 16(10), 1548–1556. https://doi.org/10.1080/15384047.2015.1071738

Wu, X. (2016). Effect of artemisinin combined with cisplatin intervention on epithelial-mesenchymal transition, angiogenesis and ATP generation in MGC-803 gastric cancer cell lines. *Journal of Hainan Medical University.* 22(18), 1-5.

Zhang, J.-L., Wang, Z., Hu, W., Chen, S.-S., Lou, X.-E., & Zhou, H.-J. (2013). DHA regulates angiogenesis and improves the efficiency of CDDP for the treatment of lung carcinoma. *Microvascular Research*. *87*, 14–24. https://doi.org/10.1016/j.mvr.2013.02.006

Zhang, J., Wang, Z., Hu, X., Wang, B., Wang, L., Yang, W., … Shao, Z. (2015). Cisplatin and gemcitabine as the first line therapy in metastatic triple negative breast cancer. *UICC International Journal of Cancer IJC*. *136*, 204–211. https://doi.org/10.1002/ijc.28966

Zhang, Y. J., Gallis, B., Taya, M., Wang, S., Ho, R. J. Y., & Sasaki, T. (2013). pH-responsive artemisinin derivatives and lipid nanoparticle formulations inhibit growth of breast cancer cells in vitro and induce down-regulation of HER family members. *PloS One*. *8*(3), e59086. https://doi.org/10.1371/journal.pone.0059086

Zhang, Y., Xu, G., Zhang, S., Wang, D., Saravana Prabha, P., & Zuo, Z. (2018). Antitumor Research on Artemisinin and Its Bioactive Derivatives. *Natural Products and Bioprospecting*. *8*(4), 303–319. https://doi.org/10.1007/s13659-018-0162-1

General Discussion & Perspectives

General Discussion & Conclusion

Looking for an effective treatment as a research strategy to cure cancer disease has shown tremendous results over history of the mankind, when man has been always relying on nature goods to find his relief.

Indeed, throughout our evolution, the importance of natural products has been enormous for our health; several successful cancer drugs are originated from nature products, such as taxol, vinblastine, doxorubicine and many more. All this witnessed success is thanks to modern analytical and structural chemistry that has opened a new generation to study natural products by providing new tools to purify different compounds and to identify their structures, which gave researchers more insights into their mechanisms of action.

Despite all the considerable researchers' efforts around the world to find an efficient cure for Triple Negative Breast Cancer (TNBC), this disease is still being a grim reaper to many vulnerable people. Furthermore, currently available anticancer drugs still need to be developed for a high toxicity against cancer and a greater safety towards patients.

Previous studies have shown the anticancer activities of berberine, carvacrol, artemisinin and thymoquinone, and demonstrated their general safety towards people. In our study, we investigated the effects of these four natural molecules on TNBC cell lines.

Berberine and artemisinin inhibited TNBC cell growth in a dose and cell dependent manners. In a molecular level, berberine induced cell cycle arrest and apoptosis in TNBC cells through changing the expression of their related proteins such as cyclin D1, cyclin B, cl-caspase-7, cl-caspase-8, and cl-PARP. Interestingly, berberine was not toxic against normal human cells, which is interesting as long as systemic toxicity is one of the serious problems of chemotherapy.

Artemisinin, on the other hand, significantly induced cell cycle arrest in TNBC HCC38 cells. Furthermore, it significantly synergized with cisplatin to inhibit the proliferation of TNBC cells. However, when cisplatin was combined to berberine, it resulted in a significant antagonism when treated to TNBC cells; MDA-MB-468, HCC70 and BT-20 (see Annex 2).

These results suggest that berberine and artemisinin may be good candidates in one of the challenges of the global health care system in making cancer treatment more personalized and effective with reduced to no side effects.

Studying on a large panel of TNBC cell lines gives a general idea of the behaviour of the cells when treated with berberine, artemisinin, carvacrol or thymoquinone. We found that the tested cell lines have all exhibited a significant cytotoxicity towards the treatments, but with different degrees of sensitivity. Hypothetically, the tested drugs may have different targeted pathways depending on the cell line type.

On the other hand, berberine and artemisinin showed an interesting cytotoxic effect against TNBC cell lines, unlike thymoquinone and carvacrol that only showed weak to no significant antiproliferative effect in our experimental model. These results may be related to their chemical structures of the drugs, which can be modified to enhance their cytotoxic mechanisms.

Relying research strategy only on finding effective treatments is not largely enough, as breast cancer disease presents a high rate of metastasis and recurrence. Consequently, next to this research approach, early diagnosis is a good solution that can help many women survive this disease in its early stages. Thus, paying more attention to breast cancer education, social, psychological and financial support, as well as screening campaigns is highly required as a strategy for breast cancer management.

Perspectives

Our results suggest that berberine and artemisinin may be good candidates for TNBC drugs development, which open new research approaches as future perspectives.

First, it would be interesting to confirm these *in vitro* results of berberine and artemisinin by an *in vivo* study using an animal model with a TNBC tumour, such as the Patient-Derived Xenograft (PDX) model which represents a direct transfer of human tumours into an immunodefficient mouse and maintained by multiple passaging from a mouse to another. Additionally, using a balb/c mouse model with a murine breast cancer cell line would be another method to continue the *in vivo* studies.

Secondly, studying the toxic effects of berberine and artemisinin on the laboratory animals are also scheduled for the *in vivo* studies.

Finally, focusing research on identifying their targeted pathways (such as EGFR, VEGF, mTOR…) on several TNBC models would extend the efficacy of berberine and artemisinin treatment.

References

[1] J.S. Olsen, Bathsheba's breast. Women, Cancer and History., 2002.

[2] M. Yalom, A History Of The Breast, Galaade éd, 1997.

[3] S.A. Emami, A. Sahebkar, N. Tayarani-Najaran, Z. Tayarani-Najaran, Cancer and its treatment in main ancient books of islamic Iranian traditional medicine (7th to 14th century AD), Iran. Red Crescent Med. J. 14 (2012) 747–57. https://doi.org/10.5812/ircmj.4954.

[4] N. Papavramidou, T. Papavramidis, T. Demetriou, Ancient greek and greco-Roman methods in modern surgical treatment of cancer, Ann. Surg. Oncol. 17 (2010) 665–667. https://doi.org/10.1245/s10434-009-0886-6.

[5] M. Karamanou, G. Tsoucalas, K. Laios, G. Androutsos, History of Oncology - Rhazes' (864-925) views on cancer and the introduction of chemotherapy, Jbuon. 24 (2019) 868–871. https://www.jbuon.com/archive/24-2-868.pdf.

[6] Al-Razi, Al-Hawi fi al-Tibb, (2000).

[7] WHO- International Agency for Research on Cancer (IARC), Globocan 2018-All cancers fact sheet, 2012. 876 (2012) 4. http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx.

[8] WHO | World Health Organization, (n.d.). https://www.who.int/ (accessed December 21, 2019).

[9] J.D. Campbell, S.D. Ramsey, The costs of treating breast cancer in the US: A synthesis of published evidence, Pharmacoeconomics. 27 (2009) 199–209. https://doi.org/10.2165/00019053-200927030-00003.

[10] A. Diarra, Etude économique des cancers au maroc : Estimation à partir des référentiels Internationaux – Centre Hospitalier Universitaire Hassan II, 2010. http://www.chu-fes.ma/etude-economique-des-cancers-au-maroc-estimation-a-partir-des-referentiels-internationaux/ (accessed January 4, 2020).

[11] F. Bray, J. Ferlay, I. Soerjomataram, R.L. Siegel, L.A. Torre, A. Jemal, Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries, CA. Cancer J. Clin. 68 (2018) 394–424. https://doi.org/10.3322/caac.21492.

[12] American Cancer Society, Breast Cancer Facts & Figures 2019-2020, Am. Cancer Soc. (2019) 1–44.

[13] Cancer.org, Invasive Breast Cancer (IDC/ILC) | Types of Invasive Breast Carcinoma, (n.d.). https://www.cancer.org/cancer/breast-cancer/understanding-a-breast-cancer-diagnosis/types-of-breast-cancer/invasive-breast-cancer.html (accessed November 27, 2019).

[14] F. Bertucci, P. Finetti, N. Cervera, B. Esterni, F. Hermitte, P. Viens, D. Birnbaum, How basal are triple-negative breast cancers?, Int. J. Cancer. 123 (2008) 236–240. https://doi.org/10.1002/ijc.23518.

[15] P. Gazinska, A. Grigoriadis, J.P. Brown, R.R. Millis, A. Mera, C.E. Gillett, L.H. Holmberg, A.N. Tutt, S.E. Pinder, Comparison of basal-like triple-negative breast

cancer defined by morphology, immunohistochemistry and transcriptional profiles, Mod. Pathol. 26 (2013) 955–966. https://doi.org/10.1038/modpathol.2012.244.

[16] S.M. Reddy, C.H. Barcenas, A.K. Sinha, L. Hsu, S.L. Moulder, D. Tripathy, G.N. Hortobagyi, V. Valero, Long-term survival outcomes of triple-receptor negative breast cancer survivors who are disease free at 5 years and relationship with low hormone receptor positivity., Br. J. Cancer. 118 (2018) 17–23. https://doi.org/10.1038/bjc.2017.379.

[17] L.A. Carey, C.M. Perou, C.A. Livasy, L.G. Dressler, D. Cowan, K. Conway, G. Karaca, M.A. Troester, K.T. Chiu, S. Edmiston, S.L. Deming, J. Geradts, M.C.U. Cheang, T.O. Nielsen, P.G. Moorman, H.S. Earp, R.C. Millikan, Race, breast cancer subtypes, and survival in the Carolina Breast Cancer Study, J. Am. Med. Assoc. 295 (2006) 2492–2502. https://doi.org/10.1001/jama.295.21.2492.

[18] B.D. Lehmann, J.A. Bauer, X. Chen, M.E. Sanders, A.B. Chakravarthy, Y. Shyr, J.A. Pietenpol, Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies, J. Clin. Invest. 121 (2011) 2750–2767. https://doi.org/10.1172/JCI45014.

[19] P. Jézéquel, O. Kerdraon, H. Hondermarck, C. Guérin-Charbonnel, H. Lasla, W. Gouraud, J.-L. Canon, A. Gombos, F. Dalenc, S. Delaloge, J. Lemonnier, D. Loussouarn, V. Verrièle, M. Campone, Identification of three subtypes of triple-negative breast cancer with potential therapeutic implications, Breast Cancer Res. 21 (2019) 65. https://doi.org/10.1186/s13058-019-1148-6.

[20] A. Society, O.F. Clinical, 2016 ASCO Educational Book, ASCO Educ. B. 34 (2016) 1–931.

[21] C.C. Parker, F. Ampil, G. Burton, B.D.L. Li, Q.D. Chu, Is breast conservation therapy a viable option for patients with triple-receptor negative breast cancer?, Surgery. 148 (2010) 386–91. https://doi.org/10.1016/j.surg.2010.05.018.

[22] L.J. Solin, W.T. Hwang, N. Vapiwala, Outcome after breast conservation treatment with radiation for women with triple-negative early-stage invasive breast carcinoma, Clin. Breast Cancer. 9 (2009) 96–100. https://doi.org/10.3816/CBC.2009.n.018.

[23] A.E. Dragun, J. Pan, S.N. Rai, B. Kruse, D. Jain, Locoregional recurrence in patients with triple-negative breast cancer: Preliminary results of a single institution study, Am. J. Clin. Oncol. Cancer Clin. Trials. 34 (2011) 231–237. https://doi.org/10.1097/COC.0b013e3181dea993.

[24] H.A. Wahba, H.A. El-Hadaad, Current approaches in treatment of triple-negative breast cancer, Cancer Biol. Med. 12 (2015) 106–116. https://doi.org/10.7497/j.issn.2095-3941.2015.0030.

[25] P. Kumar, R. Aggarwal, An overview of triple-negative breast cancer, Arch. Gynecol. Obstet. 293 (2016) 247–269. https://doi.org/10.1007/s00404-015-3859-y.

[26] D. Slade, PARP and PARG inhibitors in cancer treatment, Genes Dev. 34 (2020) 360–394. https://doi.org/10.1101/gad.334516.119.

[27] Y. Chang-qing, L. Jie, Z. Shi-qi, Z. Kun, G. Zi-qian, X. Ran, Recent treatment progress of triple negative breast cancer, Prog. Biophys. Mol. Biol. (2019). https://doi.org/10.1016/j.pbiomolbio.2019.11.007.

[28] K.E. McCann, S.A. Hurvitz, Advances in the use of PARP inhibitor therapy for breast cancer, Drugs Context. 7 (2018) 212540. https://doi.org/10.7573/dic.212540.

[29] K. Miller, M. Wang, J. Gralow, M. Dickler, M. Cobleigh, E.A. Perez, T. Shenkier, D. Cella, N.E. Davidson, Paclitaxel plus Bevacizumab versus Paclitaxel Alone for Metastatic Breast Cancer, N. Engl. J. Med. 357 (2007) 2666–2676. https://doi.org/10.1056/NEJMoa072113.

[30] R.B. D'Agostino, Changing end points in breast-cancer drug approval - The avastin story, N. Engl. J. Med. 365 (2011). https://doi.org/10.1056/nejmp1106984.

[31] M.C.U. Cheang, D. Voduc, C. Bajdik, S. Leung, S. McKinney, S.K. Chia, C.M. Perou, T.O. Nielsen, Basal-like breast cancer defined by five biomarkers has superior prognostic value than triple-negative phenotype, Clin. Cancer Res. 14 (2008) 1368–1376. https://doi.org/10.1158/1078-0432.CCR-07-1658.

[32] L.A. Carey, H.S. Rugo, P.K. Marcom, E.L. Mayer, F.J. Esteva, C.X. Ma, M.C. Liu, A.M. Storniolo, M.F. Rimawi, A. Forero-Torres, A.C. Wolff, T.J. Hobday, A. Ivanova, W.-K. Chiu, M. Ferraro, E. Burrows, P.S. Bernard, K.A. Hoadley, C.M. Perou, E.P. Winer, TBCRC 001: randomized phase II study of cetuximab in combination with carboplatin in stage IV triple-negative breast cancer., J. Clin. Oncol. 30 (2012) 2615–23. https://doi.org/10.1200/JCO.2010.34.5579.

[33] R.S. Finn, J. Dering, C. Ginther, C.A. Wilson, P. Glaspy, N. Tchekmedyian, D.J. Slamon, Dasatinib, an orally active small molecule inhibitor of both the src and abl kinases, selectively inhibits growth of basal-type/"triple-negative" breast cancer cell lines growing in vitro., Breast Cancer Res. Treat. 105 (2007) 319–26. https://doi.org/10.1007/s10549-006-9463-x.

[34] E.M.H. Kim, K. Mueller, E. Gartner, J. Boerner, Dasatinib is synergistic with cetuximab and cisplatin in triple-negative breast cancer cells, J. Surg. Res. 185 (2013) 231–239. https://doi.org/10.1016/j.jss.2013.06.041.

[35] S.H. Ueng, S.C. Chen, Y.S. Chang, S. Hsueh, Y.C. Lin, H.P. Chien, Y.F. Lo, S.C. Shen, C. Hsueh, Phosphorylated mTOR expression correlates with poor outcome in early-stage triple negative breast carcinomas, Int. J. Clin. Exp. Pathol. 5 (2012) 806–813.

[36] H. Zhang, A.L. Cohen, S. Krishnakumar, I.L. Wapnir, S. Veeriah, G. Deng, M.A. Coram, C.M. Piskun, T.A. Longacre, M. Herrler, D.O. Frimannsson, M.L. Telli, F.M. Dirbas, A. Matin, S.H. Dairkee, B. Larijani, G. V Glinsky, A.H. Bild, S.S. Jeffrey, Patient-derived xenografts of triple-negative breast cancer reproduce molecular features of patient tumors and respond to mTOR inhibition, Breast Cancer Res. 16 (2014) R36. https://doi.org/10.1186/bcr3640.

[37] J.J. Wallin, K.A. Edgar, J. Guan, M. Berry, W.W. Prior, L. Lee, J.D. Lesnick, C. Lewis, J. Nonomiya, J. Pang, L. Salphati, A.G. Olivero, D.P. Sutherlin, C. O'Brien, J.M. Spoerke, S. Patel, L. Lensun, R. Kassees, L. Ross, M.R. Lackner, D. Sampath, M. Belvin, L.S. Friedman, GDC-0980 is a novel class I PI3K/mTOR kinase inhibitor with robust activity in cancer models driven by the PI3K pathway, Mol. Cancer Ther. 10 (2011) 2426–2436. https://doi.org/10.1158/1535-7163.MCT-11-0446.

[38] R.L.B. Costa, H.S. Han, W.J. Gradishar, Targeting the PI3K/AKT/mTOR pathway in triple-negative breast cancer: a review, Breast Cancer Res. Treat. 169 (2018) 397–406. https://doi.org/10.1007/s10549-018-4697-y.

[39] D.M. Pardoll, The blockade of immune checkpoints in cancer immunotherapy, Nat. Rev. Cancer. 12 (2012) 252–264. https://doi.org/10.1038/nrc3239.

[40] FDA Approves Atezolizumab for Triple-Negative Breast Cancer - National Cancer Institute, (n.d.). https://www.cancer.gov/news-events/cancer-currents-blog/2019/atezolizumab-triple-negative-breast-cancer-fda-approval (accessed May 30, 2020).

[41] D.O. Jeffries, L.A. Dossett, J.M. Jorns, Localization for breast surgery the next generation, in: Arch. Pathol. Lab. Med., College of American Pathologists, 2017: pp. 1324–1329. https://doi.org/10.5858/arpa.2017-0214-RA.

[42] E. Hau, L. Browne, A. Capp, G.P. Delaney, C. Fox, J.H. Kearsley, E. Millar, E.H. Nasser, G. Papadatos, P.H. Graham, The impact of breast cosmetic and functional outcomes on quality of life: Long-term results from the St. George and Wollongong randomized breast boost trial, Breast Cancer Res. Treat. 139 (2013) 115–123. https://doi.org/10.1007/s10549-013-2508-z.

[43] J.F. Waljee, E.S. Hu, P.A. Ubel, D.M. Smith, L.A. Newman, A.K. Alderman, Effect of esthetic outcome after breast-conserving surgery on psychosocial functioning and quality of life., J. Clin. Oncol. 26 (2008) 3331–7. https://doi.org/10.1200/JCO.2007.13.1375.

[44] D.B. Raphael, J.A. ter Stege, N.S. Russell, L.J. Boersma, T. van der Weijden, What do patients and health care professionals view as important attributes in radiotherapy decisions? Input for a breast cancer patient decision aid, The Breast. 49 (2020) 149–156. https://doi.org/10.1016/j.breast.2019.11.005.

[45] Z. Livshits, R.B. Rao, S.W. Smith, An approach to chemotherapy-associated toxicity, Emerg. Med. Clin. North Am. 32 (2014) 167–203. https://doi.org/10.1016/j.emc.2013.09.002.

[46] L.A. Smith, V.R. Cornelius, C.J. Plummer, G. Levitt, M. Verrill, P. Canney, A. Jones, Cardiotoxicity of anthracycline agents for the treatment of cancer: Systematic review and meta-analysis of randomised controlled trials, BMC Cancer. 10 (2010) 337. https://doi.org/10.1186/1471-2407-10-337.

[47] E.A.O. Reilly, L. Gubbins, S. Sharma, R. Tully, M. Ho, Z. Guang, K. Weiner-gorzel, J. Mccaffrey, M. Harrison, F. Furlong, M. Kell, A. Mccann, The fate of chemoresistance in triple negative breast cancer (TNBC), BBACLI. 3 (2015) 257–275. https://doi.org/10.1016/j.bbacli.2015.03.003.

[48] L.N. Abdullah, E.K.-H. Chow, Mechanisms of chemoresistance in cancer stem cells, Clin. Transl. Med. 2 (2013) 3. https://doi.org/10.1186/2001-1326-2-3.

[49] V.C. Ruiz-Schutz, L.M. Gomes, R.C. Mariano, D.V.P. de Almeida, J.M. Pimenta, G.Z. Dal Molin, F.R. Kater, R. Yamamura, N.F. Correa Neto, F.C. Maluf, F.A. Schutz, Risk of fatigue and anemia in patients with advanced cancer treated with olaparib: A meta-analysis of randomized controlled trials, Crit. Rev. Oncol. Hematol. 141 (2019) 163–173. https://doi.org/10.1016/j.critrevonc.2019.06.012.

[50] C.J. LaFargue, G.Z. Dal Molin, A.K. Sood, R.L. Coleman, Exploring and comparing adverse events between PARP inhibitors, Lancet Oncol. 20 (2019) e15–e28. https://doi.org/10.1016/S1470-2045(18)30786-1.

[51] L. Cosmai, M. Gallieni, C. Porta, Renal toxicity of anticancer agents targeting HER2

and EGFR, J. Nephrol. 28 (2015) 647–657. https://doi.org/10.1007/s40620-015-0226-9.

[52] D.S. Borkar, M.E. Lacouture, S. Basti, Spectrum of ocular toxicities from epidermal growth factor receptor inhibitors and their intermediate-term follow-up: A five-year review, Support. Care Cancer. 21 (2013) 1167–1174. https://doi.org/10.1007/s00520-012-1645-y.

[53] K.M. Daniell, A. Bardia, F. Sun, S.A. Roberts, C.L. Brunelle, T.C. Gillespie, H.E. Sayegh, G.E. Naoum, D. Juric, S.J. Isakoff, D.M. Fitzgerald, A.G. Taghian, Incidence of peripheral edema in patients receiving PI3K/mTOR/CDK4/6 inhibitors for metastatic breast cancer, Breast Cancer Res. Treat. 175 (2019) 649–658. https://doi.org/10.1007/s10549-019-05206-y.

[54] L. Antonuzzo, M. Del Re, V. Barucca, F. Spada, G. Meoni, G. Restante, R. Danesi, F. Di Costanzo, N. Fazio, Critical focus on mechanisms of resistance and toxicity of m-TOR inhibitors in pancreatic neuroendocrine tumors, Cancer Treat. Rev. 57 (2017) 28–35. https://doi.org/10.1016/j.ctrv.2017.05.001.

[55] A. Bardia, A. Gucalp, N. DaCosta, N. Gabrail, M. Danso, H. Ali, K.L. Blackwell, L.A. Carey, J.R. Eisner, E.S. Baskin-Bey, T.A. Traina, Phase 1 study of seviteronel, a selective CYP17 lyase and androgen receptor inhibitor, in women with estrogen receptor-positive or triple-negative breast cancer, Breast Cancer Res. Treat. 171 (2018) 111–120. https://doi.org/10.1007/s10549-018-4813-z.

[56] J. Gibson, Anti-PD-L1 for metastatic triple-negative breast cancer, Lancet. Oncol. 16 (2015) e264. https://doi.org/10.1016/S1470-2045(15)70208-1.

[57] A.S. Heimes, M. Schmidt, Atezolizumab for the treatment of triple-negative breast cancer, Expert Opin. Investig. Drugs. 28 (2019) 1–5. https://doi.org/10.1080/13543784.2019.1552255.

[58] L.K. Collins, M.S. Chapman, J.B. Carter, F.H. Samie, Cutaneous adverse effects of the immune checkpoint inhibitors, Curr. Probl. Cancer. 41 (2017) 125–128. https://doi.org/10.1016/j.currproblcancer.2016.12.001.

[59] J. Yang, X. He, Q. Lv, J. Jing, H. Shi, Management of Adverse Events in Cancer Patients Treated With PD-1/PD-L1 Blockade: Focus on Asian Populations, Front. Pharmacol. 10 (2019). https://doi.org/10.3389/fphar.2019.00726.

[60] D.J. Newman, G.M. Cragg, K.M. Snader, Natural Products as Sources of New Drugs over the Period 1981−2002, J. Nat. Prod. 66 (2003) 1022–1037. https://doi.org/10.1021/np030096l.

[61] M. Gordaliza, Natural products as leads to anticancer drugs, Clin. Transl. Oncol. 9 (2007) 767–776. https://doi.org/10.1007/s12094-007-0138-9.

[62] T. Efferth, P.C.H. Li, V.S.B. Konkimalla, B. Kaina, From traditional Chinese medicine to rational cancer therapy., Trends Mol. Med. 13 (2007) 353–61. https://doi.org/10.1016/j.molmed.2007.07.001.

[63] D.G.I. Kingston, The shape of things to come: Structural and synthetic studies of taxol and related compounds, Phytochemistry. 68 (2007) 1844–1854. https://doi.org/10.1016/j.phytochem.2006.11.009.

[64] A.K. Pandurangan, M.R. Mustafa, Therapeutic strategies of natural agents 14 on triple-

negative breast cancer, in: Anticancer Plants Nat. Prod. Biotechnol. Implements, Springer Singapore, 2018: pp. 321–341. https://doi.org/10.1007/978-981-10-8064-7_14.

[65] E. Varghese, S.M. Samuel, M. Abotaleb, S. Cheema, R. Mamtani, D. Büsselberg, The "Yin and Yang" of natural compounds in anticancer therapy of triple-negative breast cancers, Cancers (Basel). 10 (2018). https://doi.org/10.3390/cancers10100346.

[66] G. Kiskó, S. Roller, Carvacrol and p-cymene inactivate Escherichia coli O157:H7 in apple juice, BMC Microbiol. 5 (2005). https://doi.org/10.1186/1471-2180-5-36.

[67] P. Georgiana, S. Muste, C. Mureșan, E. Mureșan, R. Vlaic, S.C.H.I. Ș, A. Sturza, Flavour Compounds of Lavandula Angustifolia L. To Use in Food Preservation, Hop Med. Plants. 25 (2017) 38–46.

[68] Z.E. Suntres, J. Coccimiglio, M. Alipour, The Bioactivity and Toxicological Actions of Carvacrol, Crit. Rev. Food Sci. Nutr. 55 (2015) 304–318. https://doi.org/10.1080/10408398.2011.653458.

[69] M. Sharifi-Rad, E.M. Varoni, M. Iriti, M. Martorell, W.N. Setzer, M. del Mar Contreras, B. Salehi, A. Soltani-Nejad, S. Rajabi, M. Tajbakhsh, J. Sharifi-Rad, Carvacrol and human health: A comprehensive review, Phyther. Res. 32 (2018) 1675–1687. https://doi.org/10.1002/ptr.6103.

[70] E.R. Silva, F.O. de Carvalho, L.G.B. Teixeira, N.G.L. Santos, F.A. Felipe, H.S.R. Santana, S. Shanmugam, L.J. Quintans Júnior, A.A. de Souza Araújo, P.S. Nunes, Pharmacological Effects of Carvacrol in In vitro Studies: A Review, Curr. Pharm. Des. 24 (2018) 3454–3465. https://doi.org/10.2174/1381612824666181003123400.

[71] Q.H. Yin, F.X. Yan, X.Y. Zu, Y.H. Wu, X.P. Wu, M.C. Liao, S.W. Deng, L.L. Yin, Y.Z. Zhuang, Anti-proliferative and pro-apoptotic effect of carvacrol on human hepatocellular carcinoma cell line HepG-2, Cytotechnology. 64 (2012) 43–51. https://doi.org/10.1007/s10616-011-9389-y.

[72] K.M. Arunasree, Anti-proliferative effects of carvacrol on a human metastatic breast cancer cell line, MDA-MB 231, Phytomedicine. 17 (2010) 581–588. https://doi.org/10.1016/j.phymed.2009.12.008.

[73] M. LLana-Ruiz-Cabello, S. Maisanaba, M. Puerto, A.I. Prieto, S. Pichardo, Á. Jos, A.M. Cameán, Evaluation of the mutagenicity and genotoxic potential of carvacrol and thymol using the Ames Salmonella test and alkaline, Endo III- and FPG-modified comet assays with the human cell line Caco-2, Food Chem. Toxicol. 72 (2014) 122–128. https://doi.org/10.1016/j.fct.2014.07.013.

[74] S. Maisanaba, A.I. Prieto, M. Puerto, D. Gutiérrez-Praena, E. Demir, R. Marcos, A.M. Cameán, In vitro genotoxicity testing of carvacrol and thymol using the micronucleus and mouse lymphoma assays, Mutat. Res. - Genet. Toxicol. Environ. Mutagen. 784–785 (2015) 37–44. https://doi.org/10.1016/j.mrgentox.2015.05.005.

[75] Andersen, Final Report on the Safety Assessment of Sodium p -Chloro- m -Cresol, p -Chloro- m -Cresol, Chlorothymol, Mixed Cresols, m -Cresol, o -Cresol, p -Cresol, Isopropyl Cresols, Thymol, o -Cymen-5-ol, and Carvacrol1, Int. J. Toxicol. 25 (2006) 29–127. https://doi.org/10.1080/10915810600716653.

[76] M. Imran, A. Rauf, I.A. Khan, M. Shahbaz, T.B. Qaisrani, S. Fatmawati, T. Abu-Izneid, A. Imran, K.U. Rahman, T.A. Gondal, Thymoquinone: A novel strategy to

combat cancer: A review, Biomed. Pharmacother. 106 (2018) 390–402. https://doi.org/10.1016/j.biopha.2018.06.159.

[77] A. El-Far, Thymoquinone Anticancer Discovery: Possible Mechanisms, Curr. Drug Discov. Technol. 12 (2015) 80–89. https://doi.org/10.2174/1570163812666150716111821.

[78] M.A. Barkat, Harshita, J. Ahmad, M.A. Khan, S. Beg, F.J. Ahmad, Insights into the Targeting Potential of Thymoquinone for Therapeutic Intervention Against Triple-negative Breast Cancer, Curr. Drug Targets. 19 (2018). https://doi.org/10.2174/1389450118666170612095959.

[79] M. Imanshahidi, Pharmacological and therapeutic effects of Berberis vulgaris and its active constituent, berberine., Phyther. Res. 22 (2008) 999–1012. https://doi.org/10.1002/ptr.2399.

[80] L. El Khalki, M. Tilaoui, A. Jaafari, H. Ait Mouse, A. Zyad, Studies on the Dual Cytotoxicity and Antioxidant Properties of Berberis vulgaris Extracts and Its Main Constituent Berberine, Adv. Pharmacol. Sci. 2018 (2018). https://doi.org/10.1155/2018/3018498.

[81] D. Liu, X. Meng, D. Wu, Z. Qiu, H. Luo, A natural isoquinoline alkaloid with antitumor activity: Studies of the biological activities of berberine, Front. Pharmacol. 10 (2019). https://doi.org/10.3389/fphar.2019.00009.

[82] J.B. Patil, J. Kim, G.K. Jayaprakasha, Berberine induces apoptosis in breast cancer cells (MCF-7) through mitochondrial-dependent pathway, Eur. J. Pharmacol. 645 (2010) 70–78. https://doi.org/10.1016/j.ejphar.2010.07.037.

[83] J. Kim, K.-M. Lee, E. Ko, W. Han, J. Lee, I. Shin, J.-Y. Bae, S. Kim, D.-Y. Noh, Berberine Inhibits Growth of the Breast Cancer Cell Lines MCF-7 and MDA-MB-231, Planta Med. 74 (2008) 39–42. https://doi.org/10.1055/s-2007-993779.

[84] N. Singh, B. Sharma, Toxicological Effects of Berberine and Sanguinarine, Front. Mol. Biosci. 5 (2018). https://doi.org/10.3389/fmolb.2018.00021.

[85] M. Imenshahidi, H. Hosseinzadeh, Berberine and barberry (Berberis vulgaris): A clinical review, Phyther. Res. 33 (2019) 504–523. https://doi.org/10.1002/ptr.6252.

[86] G.H. Li, D.L. Wang, Y. De Hu, P. Pu, D.Z. Li, W.D. Wang, B. Zhu, P. Hao, J. Wang, X.Q. Xu, J.Q. Wan, Y.B. Zhou, Z.T. Chen, Berberine inhibits acute radiation intestinal syndrome in human with abdomen radiotherapy, Med. Oncol. 27 (2010) 919–925. https://doi.org/10.1007/s12032-009-9307-8.

[87] Y. Liu, H. Yu, C. Zhang, Y. Cheng, L. Hu, X. Meng, Y. Zhao, Protective effects of berberine on radiation-induced lung injury via intercellular adhesion molecular-1 and transforming growth factor-beta-1 in patients with lung cancer, Eur. J. Cancer. 44 (2008) 2425–2432. https://doi.org/10.1016/j.ejca.2008.07.040.

[88] Y. Tu, Artemisinin-A Gift from Traditional Chinese Medicine to the World (Nobel Lecture), Angew. Chemie Int. Ed. 55 (2016) 10210–10226. https://doi.org/10.1002/anie.201601967.

[89] Abdelmajid Zyad, Mounir Tilaoui, Abdeslam Jaafari , Moulay Ali Oukerrou, Hassan Ait Mouse, More insights into the pharmacological effects of artemisinin, Phyther. Res. (2017) 1–14. https://doi.org/10.1002/ptr.5958

[90] Y. Zhang, G. Xu, S. Zhang, D. Wang, P. Saravana Prabha, Z. Zuo, Antitumor Research on Artemisinin and Its Bioactive Derivatives, Nat. Products Bioprospect. 8 (2018) 303–319. https://doi.org/10.1007/s13659-018-0162-1.

[91] A.M. Gravett, W.M. Liu, S. Krishna, W.C. Chan, R.K. Haynes, N.L. Wilson, A.G. Dalgleish, In vitro study of the anti-cancer effects of artemisone alone or in combination with other chemotherapeutic agents, Cancer Chemother. Pharmacol. 67 (2011) 569–577. https://doi.org/10.1007/s00280-010-1355-4.

[92] K. Hosoya, C.G. Couto, C.A. London, W.C. Kisseberth, M.A. Phelps, J.T. Dalton, Comparison of high-dose intermittent and low-dose continuous oral artemisinin in dogs with naturally occurring tumors, J. Am. Anim. Hosp. Assoc. 50 (2014) 390–395. https://doi.org/10.5326/JAAHA-MS-6145.

[93] T. Gordi, E.I. Lepist, Artemisinin derivatives: toxic for laboratory animals, safe for humans?, Toxicol. Lett. 147 (2004) 99–107. https://doi.org/10.1016/j.toxlet.2003.12.009.

[94] M. Van Vugt, B.J. Angus, R.N. Price, C. Mann, J.A. Simpson, C. Poletto, S.E. Htoo, S. Looareesuwan, N.J. White, F. Nosten, A case-control auditory evaluation of patients treated with artemisinin derivatives for multidrug-resistant Plasmodium falciparum malaria, Am. J. Trop. Med. Hyg. 62 (2000) 65–69. https://doi.org/10.4269/ajtmh.2000.62.65.

[95] S.R. Meshnick, T.E. Taylor, S. Kamchonwongpaisan, Artemisinin and the antimalarial endoperoxides: From herbal remedy to targeted chemotherapy, Microbiol. Rev. 60 (1996) 301–315.

[96] B. Marty, V. Maire, E. Gravier, G. Rigaill, A. Vincent-Salomon, M. Kappler, I. Lebigot, F. Djelti, A. Tourdès, P. Gestraud, P. Hupé, E. Barillot, F. Cruzalegui, G.C. Tucker, M.-H. Stern, J.-P. Thiery, J.A. Hickman, T. Dubois, Frequent PTEN genomic alterations and activated phosphatidylinositol 3-kinase pathway in basal-like breast cancer cells, Breast Cancer Res. 10 (2008) R101. https://doi.org/10.1186/bcr2204.

[97] N. a P. Franken, H.M. Rodermond, J. Stap, J. Haveman, C. van Bree, Clonogenic assay of cells in vitro., Nat. Protoc. 1 (2006) 2315–9. https://doi.org/10.1038/nprot.2006.339.

[98] S. LOEWE, The problem of synergism and antagonism of combined drugs., Arzneimittelforschung. 3 (1953) 285–90. http://www.ncbi.nlm.nih.gov/pubmed/13081480 (accessed November 5, 2018).

[99] G.Y. Lee, P. a Kenny, E.H. Lee, M.J. Bissell, Three-dimensional culture models of normal and malignant breast epithelial cells, Nat. Methods. 4 (2010) 359–365. https://doi.org/10.1038/nmeth1015.Three-dimensional.

[100] V. Maire, F. N?mati, M. Richardson, A. Vincent-Salomon, B. Tesson, G. Rigaill, E. Gravier, B. Marty-Prouvost, L. De Koning, G. Lang, D. Gentien, A. Dumont, E. Barillot, E. Marangoni, D. Decaudin, S. Roman-Roman, A. Pierr?, F. Cruzalegui, S. Depil, G.C. Tucker, T. Dubois, Polo-like kinase 1: A potential therapeutic option in combination with conventional chemotherapy for the management of patients with triple-negative breast cancer, Cancer Res. 73 (2013) 813–823. https://doi.org/10.1158/0008-5472.CAN-12-2633.

ANNEXES

ANNEX 1

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Research Article

Studies on the Dual Cytotoxicity and Antioxidant Properties of *Berberis vulgaris* Extracts and Its Main Constituent Berberine

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The present study attempts to investigate the cytotoxic activity of ethanol and ethyl acetate extracts of the Moroccan *Berberis vulgaris* and its major component berberine, together with exploring their antioxidant properties. It also consists of studying the combination effect of berberine and S-nitroso-N-acetylpenicillamine (SNAP), a nitric oxide (NO) donor, against the human breast adenocarcinoma cell line (MCF-7). Using the MTT assay, we report a differential cytotoxic effect of ethanol and ethyl acetate extracts since the ethanol extract is more cytotoxic than the ethyl acetate one, with $IC_{50} = 3.54\,\mu g/mL$ and $596.71\,\mu g/mL$, respectively. Interestingly, no cytotoxic effect was observed against normal cells. Furthermore, these extracts showed a remarkable antioxidant activity as measured by the DPPH free radicals scavenging assay. In fact, the IC_{50} values are $69.65\,\mu g/mL$ and $77.75\,\mu g/mL$ for the ethanol and ethyl acetate extracts, respectively. In addition, several concentrations of berberine, when combined with the NO donor used at IC_{30}, induced a synergistic cytotoxic activity at concentrations ranging from $8.40\,\mu M$ to $33.60\,\mu M$, as revealed by the combination index values, using the Chou–Talalay method. However, at the other concentrations tested, an antagonistic effect was observed. The observed cytotoxicity was related to apoptosis induction as demonstrated by the annexin-V-streptavidin FITC-staining analysis.

1. Introduction

Our knowledge of plants and their benefits is as old as mankind. Man discovered very early the therapeutic properties of certain plants to overcome his suffering and improve his health. Thus, we chose to work on *Berberis vulgaris* from Oujda, east of Morocco, a plant of the Berberidaceae family locally named "Aghriss," "Izergui," and "Bou-Semmane" and used in traditional medicine for its antipyretic, hepatoprotective, and anti-inflammatory properties [1]. Several studies have been conducted on its biological activities and more specifically on berberine, an isoquinoline alkaloid, considered as an active molecule with many properties such as hypoglycemic, antibacterial, antifungal, anti HCV, and anticancer activities [2–8]. Indeed, despite the significant advances of modern medicine, we always note a slight failure of conventional drug treatments in the case of high incidence of side effects and development of resistance. Therefore, the first part of this work consists of comparing the cytotoxic activity of *Berberis vulgaris* extracts against breast cancer cells and normal human cells, as the nonselectivity of chemotherapy treatment is what causes the systemic toxicity. We also investigated the molecular mechanisms of the cytotoxicity, as recent knowledge on molecular carcinogenesis has provided the potential for therapeutic intervention in cancer by specifically targeting and sensitising cancer cells to apoptosis [9].

In the second part, this work aims to explore the combination effect of berberine and S-nitroso-N-acetylpenicillamine (SNAP), a nitric oxide (NO) donor, against breast cancer cells. Knowing that NO is a free radical synthesised from L-arginine by NO synthase (NOS), three isoforms of NOS (neuronal (nNOS), endothelial NOS (eNOS), and inducible

NOS (iNOS)) are expressed in various tissues and cells. NO plays an important role in different biological responses, including the regulation of vascular tone, neurotransmission, antiviral defence, and immune responses [10]. Recent studies have also demonstrated that NO is an interesting regulator of cell death [11]. This part of the study was designed to explore the effect of NO on the viability of breast cancer cells, alone and in combination with berberine.

2. Materials and Methods

2.1. Berberis vulgaris and Its Extracts. The plant used in this study was collected at the end of autumn to early winter (December 2014), in the region of Oujda, east of Morocco. The part used in this study is the root barks of the plant. The root barks were isolated and dried in the shade at room temperature and then crushed and ground. Then, the extraction was performed with a Soxhlet extractor using two types of solvents of different polarities: ethyl acetate and ethanol. The extracts obtained were concentrated using a rotary evaporator until the total evaporation of the solvent.

2.2. Chemicals. Dulbecco's modified Eagle's medium (DMEM), dimethyl sulfoxide (DMSO), ethylenediaminetetraacetic acid (EDTA), phosphate-buffered saline (PBS), methyl tetrazolium (MTT), crystal violet, ethyl acetate, ethanol, methanol, trifluoroacetic acid and acetonitrile (HPLC–MS grade), Ficoll, isopropanol, hydrochloric acid (HCl), sodium dodecyl sulfate (SDS), annexin-V-FITC, trypan blue, S-nitroso-N-acetylpenicillamine (SNAP), berberine, and cisplatine were purchased from Sigma-Aldrich (Saint Quentin, France).

2.3. Cell Line and Culture. The tumour cell line used in this study was the human breast adenocarcinoma (MCF-7) generously provided to our laboratory by the Gustave Roussy Institute (Villejuif, France). These cells are cultured at 37°C in a humidified atmosphere with 5% CO_2 in a culture medium (DMEM) supplemented with 5% of fetal bovine serum, 100 UI/mL of penicillin, 100 μg/mL streptomycin, and 0.2% sodium bicarbonate.

2.4. Cytotoxicity Measurement. The cytotoxic activity was studied against the MCF-7 tumour cell line using the colourimetric methyl tetrazolium (MTT) test as described by Mosmann [12] and modified in our laboratory [13]. The target cells were washed twice and placed on 96-well microtiter plates (Bioster, Italy) at a density of 3×10^4 cells/mL in 100 μl/well of the completed culture medium. Then, 100 μl of the culture medium, containing decreasing doses of the ethanol or ethyl acetate extracts already solubilised in the DMSO, was added in each well of the microtiter plates containing cells for a final volume of 200 μl, with 400 μg/mL as the initial concentration, to reach a final concentration of 0.78 μg/mL. The cisplatine was used as a positive control. After exposure of the cells to serial concentrations of tested products for 48 h at 37°C and 5% CO_2, 100 μl of medium was carefully aspirated from each well and replaced by 20 μl of MTT solution (5 mg/mL of PBS). After incubation in the same conditions for 3 h, the plates were treated with 100 μl of isopropanol/HCL (10 mL: 2.5 μl) solution to dissolve the blue intracellular formazan product. 30 min later at 37°C, the solubilised formazan produced by the metabolically active cells was measured by scanning the 96-well plates at dual wavelength of 540–630 nm using a Multiskan apparatus (Labsystems, Helsinki, Finland). Thus, the cytotoxic effect was calculated using the following formula:

$$\%\ \text{Cell viability} = \left(\frac{\text{OD}}{\text{OD}_0}\right) * 100, \quad (1)$$

where OD_0 and OD are the optical density obtained, respectively, for the negative control cells receiving DMSO (0.5%) alone and the ethanol extract- or ethyl acetate extract-treated cells. Three independent sets of experiments performed in duplicate were evaluated.

2.5. Cytotoxic Effect against Human Peripheral Blood Mononuclear Cells (PBMCs). This test was realised in order to evaluate the effect of the tested extracts on normal human cells. To isolate the PBMCs, blood samples (10 mL) in sterile heparinised tubes were collected under medical and ethical committee control from healthy volunteer donors.

Peripheral blood mononuclear cells were isolated using standard Ficoll-hypaque density centrifugation. The interface lymphocytes were harvested and washed twice with sterile phosphate-buffered saline (PBS). The cytotoxic effect was measured by the MTT test in the same conditions and concentrations as detailed above for the tumour cells.

2.6. Chemical Analysis of Berberis vulgaris Extracts. This analysis was conducted by a chromatography coupled to a mass spectrometry (HPLC-MS) of the National Centre for Scientific and Technical Research (CNRST) laboratories (Rabat, Morocco). The HPLC-MS analysis was conducted at 279 nm and 30°C using an RP-C18 column (150×4.6) $\times$ 5 μm, with a Thermo Fisher apparatus equipped with a surveyor pump coupled to the PDA detector (diode array detector: 200–600 nm) and a mass spectrometry-ion trap LCQ advantage (ESI) (Thermo Finnigan, San Jose, CA, USA). A constant flow rate of 0.5 mL/min of a polar mobile phase (Solution 1: water/0.05% trifluoroacetic acid; Solution 2: acetonitrile/0.05% trifluoroacetic acid) polarity changed during 76 min of the analysis, pushing 20 μl of the extract to be analyzed in an apolar stationary phase column. The sample passed directly to a mass spectrometer where its principle resided in the separation in the gas phase-charged molecules (ions) based on their mass/charge ratio m/z. The full scan mass data m/z were obtained in both positive and negative modes.

2.7. Analysis of Apoptosis Induction. The apoptosis analysis was performed using the annexin V-streptavidin FITC test. Briefly, MCF-7 cells (4×10^4 cells) were treated in the 24-well plates with the IC_{30} of ethanol extract (0.2 μg/mL) or the IC_{30} of berberine (0.67 μg/mL) or grown under serum starvation conditions (used as a positive control). After 6 h, 12 h, and

24 h incubations in the same culture conditions as the above, the cells were washed with PBS, stained with annexin V-fluorescein isothiocyanate (FITC), and treated with streptavidin conjugated to FITC. The fluorescence was visualised using an Olympus BX51 microscope equipped with fluorescence filter and CytoVision software in order to detect apoptosis induction. The assay is based on the ability of annexin V (green fluorescence) to bind to phosphatidylserine exposed on the surface of cells undergoing apoptosis [14].

2.8. Antioxidant Activity Measurement. The studied extracts and berberine were diluted in methanol, and 150 μl of 0.004% DPPH was added in each well containing the serial concentrations of extracts, berberine, and the positive control (vitamin C). After incubation in darkness at room temperature for 30 min, the absorbance was measured at 515 nm [15]. The presence of DPPH radicals resulted in a dark purple colour of the solution. Reducing DPPH radicals by an antioxidant results in the discolouration of the solution. The percentage of inhibition is calculated using the following equation:

$$\%\ \text{Inhibition} = 100 * \left(\frac{(A_0 - A_s)}{A_0} \right), \tag{2}$$

where at 515 nm wavelength A_0 is positive control absorbance and A_S is extracts and berberine absorbance.

2.9. Nitric Oxide Effect on the MCF-7 Tumour Cell Line Using the Crystal Violet Test. We used S-nitroso-N-acetylpenicillamine (SNAP) as a nitric oxide (NO) donor since it releases NO under physiological conditions, making it a useful tool for studying the pharmacological and physiological actions of NO [10]. The cytotoxic activity was studied against the MCF-7 tumour cell line and was measured using the crystal violet staining test as described by Zyad et al. [16]. The target cells were washed twice and placed on 96-well microtiter plates (Bioster, Italy) at a density of 6×10^3 cells/mL in 100 μl/well of the completed culture medium. Then, 100 μl of the culture medium containing decreasing concentrations of SNAP, already solubilised in the DMSO and PBS, was added in each well of microtiter plates containing cells for a final volume of 200 μl. After 48 h incubation in a humidified atmosphere at 37°C and 5% CO_2, the medium was removed and replaced with 100 μl of 0.5% crystal violet solution. After 10 min incubation at room temperature, the plates were washed carefully and viable crystal violet stained cells were lysed with 1% SDS solution. Absorbance at 540 nm was then measured in each well using a Multiskan apparatus (Labsystems, Helsinki, Finland). Thus, the cytotoxic effect was measured using the following formula:

$$\%\ \text{Cell viability} = \left(\frac{\text{OD}}{\text{OD}_0} \right) * 100, \tag{3}$$

where OD_0 and OD are the optical density obtained, respectively, for untreated cells and SNAP-treated cells. Three independent sets of experiments performed in duplicate were evaluated.

2.10. Combination Effect of Nitric Oxide and Berberine against the MCF-7 Cell Line. After measurement of the cytotoxic effect of berberine against the MCF-7 cell line using the crystal violet staining assay, the degree of synergism between berberine and SNAP was determined by using the combination index (CI) analysis at a nonconstant ratio; that is, drug combinations were made by varying the concentrations of one drug (berberine) while keeping the second drug (SNAP) concentration fixed at IC_{30}.

The combination effect is measured as described by Chou and Talalay [17] using the following formula:

$$\text{CI} = \frac{D1}{(Dx)1} + \frac{D2}{(Dx)2}, \tag{4}$$

where $D1$ is dose of drug 1 to produce x% cell death in combination with drug 2, $(Dx)1$ is dose of drug 1 to produce x% cell death alone, $D2$ is dose of drug 2 to produce x% cell death in combination with drug 1, and $(Dx)2$ is dose of drug 2 to produce x% cell death alone. The CI was calculated using Microsoft Excel 2010.

An average $CI < 1$ indicates synergism, $CI > 1$ indicates antagonism, and an average $CI = 1$ indicates additivity effect.

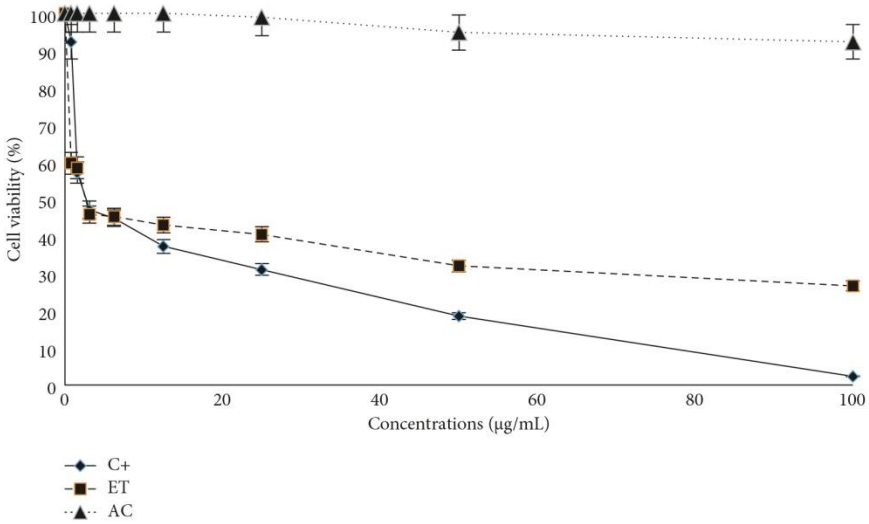
2.11. Statistical Analysis. The results are presented in the form of averages ± standard deviation for assays in triplicate. The comparison of the averages is made by Microsoft Office Excel software. The differences are considered significant at $p < 0.05$.

3. Results

3.1. In Vitro Cytotoxic Effect of Berberis vulgaris Extracts. The in vitro cytotoxic activity of *Berberis vulgaris* extracts was measured by the MTT assay against the MCF-7 tumour cell line at various concentrations. This activity was evaluated for the ethyl acetate and ethanol extracts of root barks. The results are summarised in Figure 1.

The cells were incubated for 48 h with increasing concentrations of extracts of *Berberis vulgaris*, and the cytotoxic activity was measured by the MTT test as described in the Materials and Methods section. It is shown in this figure that the cytotoxic activity of the various extracts was dose dependent. In fact, as long as the concentration increased, the percentage of cell viability decreased. However, this effect was different from one extract to another; the percentage of viable MCF-7 cells treated by ethanol extract has decreased quickly to only 50% at a concentration of 3.54 μg/mL. However, the ethyl acetate extract was less cytotoxic, with the concentration leading to only 50% of viable population equal to 596.7 μg/mL. It is noteworthy that the cytotoxic effect of the ethanol extract was comparable to that of the positive control (Figure 1).

3.2. Evaluation of Berberis vulgaris Extract Cytotoxicity against Normal Human Peripheral Blood Mononuclear Cells (PBMCs). In order to investigate the effect of the ethanol and ethyl acetate extracts and berberine molecule against normal human cells, normal human PBMCs were incubated



Berberis vulgaris extracts	IC_{50} µg/mL
ET	3.54
AC	596.71
C+	3.87

FIGURE 1: Cytotoxic effect and IC_{50} values of different concentrations of *Berberis vulgaris* extracts against the MCF-7 tumour cell line. ET: ethanol extract, AC: ethyl acetate extract, C+: cis-platin.

with increasing concentrations of these extracts in the same conditions as those used against the MCF-7 tumour cells. The obtained results are shown in Figure 2.

The cells were incubated for 48 h with increasing concentrations of extracts of *Berberis vulgaris* and berberine molecule, and then the cytotoxic activity was measured by the MTT test as described in the Materials and Methods section.

This figure shows that despite the increasing concentrations of the stimuli, the viability percentage of PBMCs is substantially constant (almost 100%). The results indicate that these products are tolerated by normal human cells and that the cytotoxicity of studied products is specific for tumour cells. These results are interesting since nontargeting of the tumour cells by actual anticancer products is the origin of their systemic toxicity.

3.3. Chemical Composition Analysis of the Ethanol Extract. As long as we found that the ethanol extract of *Berberis vulgaris* had the most cytotoxic effect against the MCF-7 cell line, unlike the ethyl acetate one, we decided to explore the chemical composition of this extract using the high performance liquid chromatography coupled to mass spectrometry (HPLC/MS) method. The obtained results are shown in Figure 3. Given the retention time and the mass/charge (m/z) ratio of the various peaks in the chromatogram, we detected five main components: jatrorrhizine, palmatine, columbamine, berberine, and epiberberine, as shown in Table 1.

3.4. Cytotoxic Effect of Berberine against the MCF-7 Cell Line. Given that berberine is the most representative molecule in the ethanol extract of *Berberis vulgaris*, we queried whether the cytotoxic activity of this extract was due

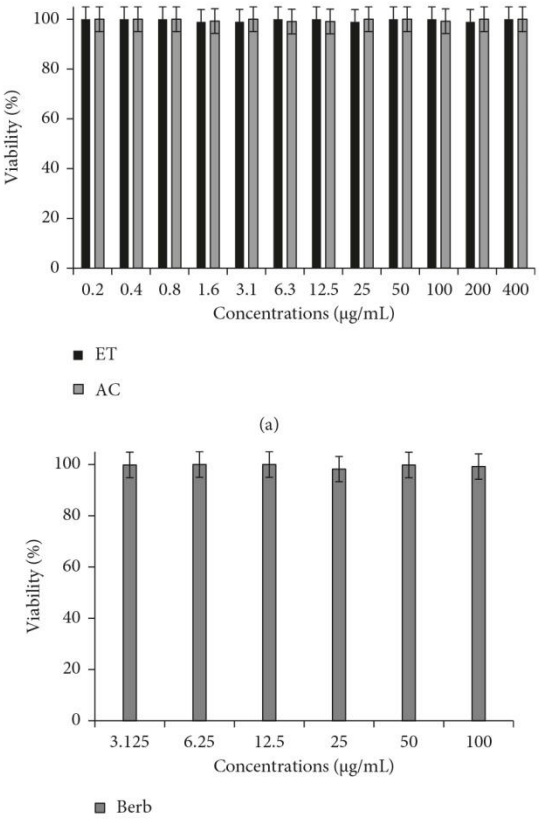


FIGURE 2: Cytotoxic effect of *Berberis vulgaris* extracts (a) and berberine (b) against normal human cells PBMCs. ET: ethanol extract, AC: ethyl acetate extract, Berb: berberine.

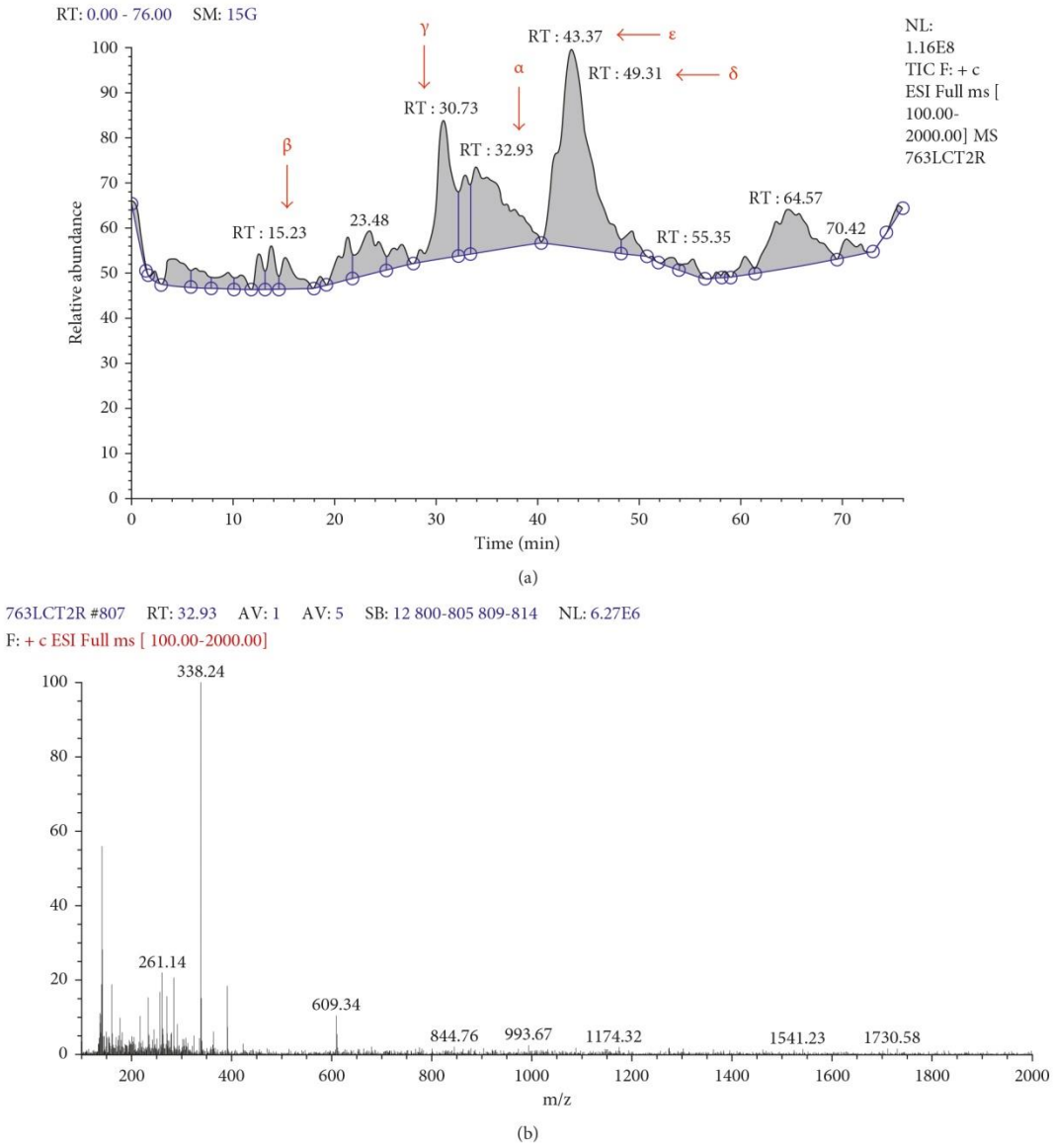


Figure 3: Chemical composition of the ethanol extract of *Berberis vulgaris*. (a) Ethanol extract's chromatogram. (b) Example of the mass spectra of molecule *α* of *Berberis vulgaris* ethanol extract. The molecules are presented as peaks characterised by mass/charge (*m/z*) ratio and retention time.

to this molecule. Then, we conducted a comparison test of the cytotoxic effect of berberine and ethanol extract. The test was carried out using the MTT assay under the same conditions as the previous cytotoxicity tests described above. The results are shown in Figure 4. As shown in this figure, berberine has a similar effect against MCF-7 tumour cells as the ethanol extract with nonsignificant differences, suggesting that the cytotoxic effect of the ethanol extract might be mediated mainly by berberine.

The cells were incubated for 48 h with increasing concentrations of berberine, cisplatin, or ethanol extract of *Berberis vulgaris*, and the cytotoxic activity was measured by the MTT test as described in the Materials and Methods section.

3.5. Evaluation of Berberine and Nitric Oxide (NO) Combination Effect against Tumour Cells. Nitric oxide (NO) is a potent antitumour product [23–25]. In order to determine the effect of berberine and NO combination against the MCF-7 tumour cell line (synergy, additivity, or antagonism), we used the combination index (CI) analysis by the Chou–Talalay

Table 1: Summary of the major components of *Berberis vulgaris* ethanol extract.

Molecule number	RT (min)	Molecules	$[M+H]^+$ (m/z)	References
α	32.93	Jatrorrhizine	338.24	[18, 19]
β	15.23	Palmatine	342.23	[20, 21]
γ	30.73	Columbamine	338.24	[18, 19]
δ	49.31	Berberine	336.22	[18, 19, 22]
ε	43.37	Epiberberine	336.26	[19]

method. Next, we evaluated the cytotoxic effect of S-nitroso-N-acetylpenicillamine (SNAP) as a NO donor alone and that of berberine alone against the MCF-7 cell line under the same conditions (Table 2). Then, we evaluated the effect of their combination when the SNAP concentration was constant at the IC_{30} values and the berberine concentration varied from 0.525 μM to 1075 μM (Table 3).

It is shown in Table 2 that the cytotoxic activities of NO and berberine alone increased in a dose-dependent manner. In fact, as long as the concentration increased, the cytotoxicity also increased showing a lysis percentage that increased quickly and reached 50% at a concentration of 92.4 μM and 60 μM for SNAP and berberine, respectively.

It is noteworthy that when combined, berberine and SNAP induced a strong cytotoxic activity as it is shown in Table 3. The synergistic activity was observed at concentrations of berberine from 8.4 μM to 33.6 μM combined with the IC30 of SNAP (15.84 μM). However, at high or very low concentrations (<8.4 μM) of berberine, an antagonistic effect was observed (Table 3).

3.6. Antioxidant Activity of Berberis vulgaris Extracts and Berberine. To investigate whether berberine and *Berberis vulgaris* extracts show antioxidant activity, we analyzed the percentage of free radical scavenging under the effect of these products using the DPPH (1.1-diphenyl-2-picrylhydrazyl) technique [15]. The results are expressed as a percentage of inhibition of the free radical DPPH (Figure 5).

This figure showed the scavenging percentage of the free radical (DPPH) with increasing concentrations of the studied products (ethanol extract, ethyl acetate extract, and berberine). It is indicated in this figure that as the concentration of these products increased, the trapping percentage of the DPPH also increased. Interestingly, when the ethanol and ethyl acetate extracts showed a comparative antioxidant activity with an $IC_{50} = 69.65\,\mu$g/mL and 77.75 μg/mL, respectively, with no significant differences, berberine showed only a low antioxidant activity.

3.7. Apoptotic Cell Death Induction by Ethanol Extract of Berberis vulgaris and Berberine against Tumour Cells. In order to contribute to the understanding of molecular mechanisms involved in the observed cytotoxic activity of *Berberis vulgaris* ethanol extract and its major component, a kinetic study of berberine-induced apoptosis assay was performed using the annexin V-FITC test for 6 h, 12 h, and 24 h. Indeed, utilisation of fluorescein isothiocyanate (FITC)-conjugated annexin V is a standard procedure for monitoring the progression of apoptosis. In fact, early apoptotic cells are annexin V-positive with an intact membrane permeability, whereas late (end-stage) apoptotic cells are annexin V-positive and lose their membrane permeability. Viable cells remained unstained (annexin V-FITC-negative) [14] (Figure 6).

The results showed that berberine and ethanol extract induced MCF-7 cells' killing via apoptosis mechanism. Interestingly, cells treated with berberine are undergoing an early apoptosis unlike the cells treated with ethanol extract that show a late (end-stage) apoptosis at 6 h of stimulation since they are both annexin V-positive with the differences in membrane alteration. Furthermore, over time, berberine and ethanol extract increased the cell membrane alteration as shown at 12 h and 24 h of stimulation (Figure 6).

4. Discussion

This study aims to evaluate the antitumour and antioxidant properties of Moroccan *Berberis vulgaris*. The barberry plant was collected in Oujda, east of Morocco, at the end of autumn to early winter (December 2014). We performed the extraction from its root barks using two solvents with different polarities: ethyl acetate and ethanol.

In this work, and for the first time, we evaluated the effect of these two extracts on the human breast adenocarcinoma cell line (MCF-7). Although they exhibited prominent cytotoxic activity against these tumour cells, the ethanol extract was more cytotoxic than the ethyl acetate one with $IC_{50} = 3.54\,\mu$g/mL and 596.71 μg/mL, respectively. This dissimilarity in their effects is probably due to the different polarities of the two solvents. Furthermore, as the ethanol solvent is more polar than the ethyl acetate one, the phytochemical composition of the ethanol extract has particular molecules that gave it its cytotoxic power against the MCF-7 target cells.

In addition, the phytochemical examination by HPLC/MS performed on the ethanol extract, because of its large cytotoxic activity against the MCF-7 cell line unlike the ethyl acetate extract, revealed the presence of several principal molecules including jatrorrhizine, palmatine, columbamine, berberine, and epiberberine. According to our ethanol extract chromatogram analysis and the results of Ghareeb et al. [3] who found that 1 mg of the ethanol extract of the root barks of *Berberis vulgaris* contains 0.62 mg of berberine, our ethanol extract may be composed mainly of the berberine molecule. These results are in agreement with other findings reporting the presence of these molecules, amongst others depending on the extraction solvent type and the considered part of the plant [26, 27]. Several factors may be responsible for the variation in chemical composition and thus the biological activities of extracts of *Berberis vulgaris* such as climate, soil, cultivation period, and the preservation and extraction methods. Nevertheless, the involvement of minor products in these activities is not to be neglected.

Our results also demonstrated that when comparing the cytotoxic effects of the ethanol extract and berberine, we

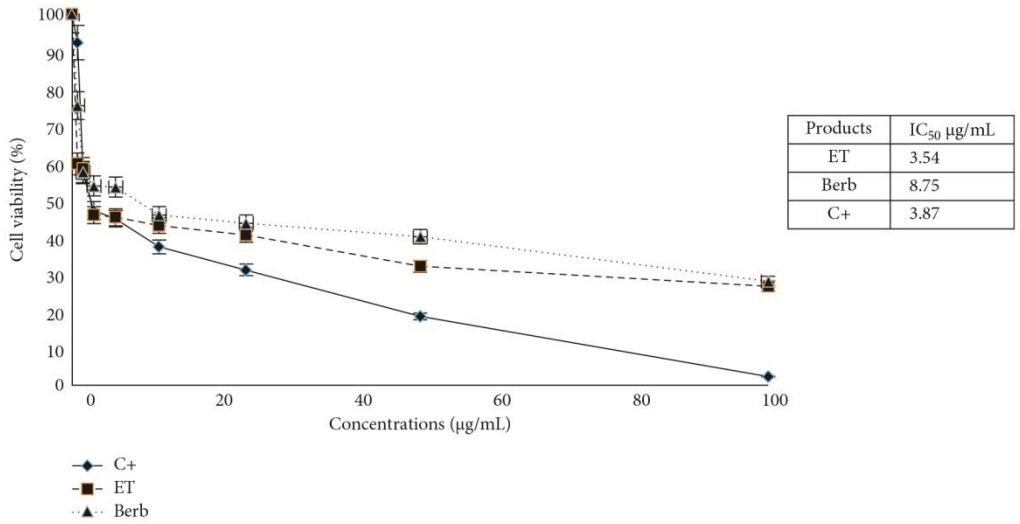


Figure 4: Cytotoxic effect of berberine against the MCF-7 cell line. ET: ethanol extract, Berb: berberine, C+: positive control (cisplatin).

Table 2: Cytotoxic activity of berberine and SNAP alone against the MCF-7 tumour cell line.

	Berberine Concentrations (μM)					SNAP Concentrations (μM)				
	0	100	200	400	600	0	100	200	400	600
Lysis (%)	0	43.2 (±0.81)	66.4 (±1.8)	75.81 (±1.3)	81.32 (±0.6)	0	50.9 (±9.8)	58.76 (±10.5)	66.62 (±8.2)	71.22 (±9.4)

noted that their effects are similar with nonsignificant differences. This suggests that the cytotoxic effect of the ethanol extract is mainly mediated by berberine, which has a cytotoxic activity with $IC_{50} = 8.75\,\mu g/mL$ against the MCF-7 cells using the MTT test.

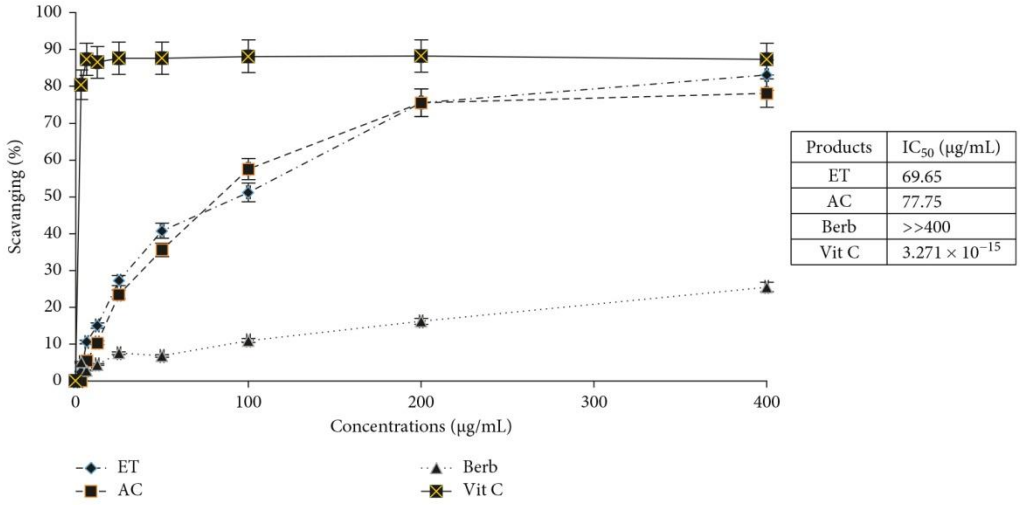
Since one of the major problems in cancer chemotherapy is the systemic toxicity due to damaged normal cells by anticancer drugs, we tested our products on normal human peripheral blood mononuclear cells (PBMCs). The results showed that all of the stimuli studied at the inducing toxicity concentrations on the tumour cells have no cytotoxic effect on normal cells. These results correlate with those of Ghareeb et al., who found that the ethanol extract and berberine have no antiproliferative effect on normal PBMCs, although they reported that after incubation of the normal cells for 72 h with ethanol extract or berberine, a slight stimulation of the proliferation of PBMC was observed [3]. This result is very interesting since nontargeting of the tumour cells by anticancer products is the greatest problem causing systemic toxicity. These results corroborate the findings that ethanol extract has an antiviral (especially against hepatitis C) and antifungal effect (against *Aspergillus flavus*), probably due to its immunostimulatory capacity and increasing the activity of phagocytic cells [3] also due to its antioxidant effect for the Malaysian barberry [28].

The tissues and cells exposed to oxidative stress show an alteration of their membrane, DNA damage and reduced repair capacity of the DNA that may contribute to the development of cancer [29]. Thus, the research of powerful antioxidants contributes to the subject of prevention against cancer. In fact, the infusion of *Berberis vulgaris* collected in Peru possesses an interesting antioxidant activity [30]. Similarly, berberine extracted from another barberry plant collected in India has an interesting antioxidant property that

Table 3: Combination index determination for the combined effect of berberine and NO donor (SNAP).

Berberine (μM)	SNAP ($IC_{30}\,\mu$M)	Fa (B + S)	CI	Description[a]
0.525	15.84	0	14.7	Antagonism
1.050	15.84	0.755	13.8	Antagonism
2.10	15.84	6.410	8.8	Antagonism
4.20	15.84	22.381	2.6	Antagonism
8.40	15.84	39.555	0.7	Synergy
16.80	15.84	49.361	0.5	Synergy
33.60	15.84	52.010	0.6	Synergy
67.20	15.84	52.285	1.2	Antagonism
134.40	15.84	53.103	2.1	Antagonism
268.80	15.84	73.204	1.4	Antagonism
537.60	15.84	80.507	1.1	Antagonism
1075.20	15.84	81.749	1.9	Antagonism

Fa: affected fraction; B: berberine; S: SNAP; B + S: berberine + SNAP; CI: combination index. [a]CI = 1.00: additive effect; CI < 1.00: synergistic effect; CI > 1.00: antagonistic effect.



Products	IC_{50} (μg/mL)
ET	69.65
AC	77.75
Berb	>>400
Vit C	3.271×10^{-15}

FIGURE 5: In vitro antioxidant effect of berberine and *Berberis vulgaris* extracts. ET: ethanol extract, AC: ethyl acetate extract, Berb: berberine, Vit C: vitamin C.

gives it its antitumour activity [29]. On the other hand, other investigations found that berberine has an oxidizing activity (production of reactive oxygen-derived ROS) against tumour cells [31–33]. These different results led us to evaluate the antioxidant potential of our extracts and berberine in vitro. Our results showed that ethanol and ethyl acetate extracts have a very large antioxidant potential, and these results confirm the studies of Hanachi and Golkho in reporting the potent antioxidant activity of *Berberis vulgaris* ethanol extract [28].

However, at the same concentrations as the tested extracts, berberine has only 25% inhibition of free radical scavenging compared to both extracts, as measured by the DPPH test, demonstrating that berberine is not very antioxidant. This is in agreement with several reports such as the work of Keawpradub et al. reporting that berberine has a low antioxidant activity ($EC_{50} > 100\,\mu g/mL$) [34] and the work on the CaSki human cervical cancer line, where Lin et al. found that berberine has an oxidizing activity by increasing the levels of ROS (reactive oxygen derivatives) that destabilizes the potential mitochondrial membrane, thereby dropping cytochrome C in the cytosol that will go into the activation of caspase-3, eventually causing the phenomenon of apoptosis [35]. This is also in agreement with the results of Ho et al., using the human tongue cancer SCC-4 line [36]. In this context, our work suggests that the antioxidant effect of the ethanol and ethyl acetate extracts was due to molecules other than berberine. Furthermore, the results of Hanachi and Golkho showed that the antioxidant effect of the ethanol extract of barberry is probably due to its richness in phenolic compounds [28].

On the other hand, we examined the molecular mechanisms of the cytotoxicity effect of ethanol extract and berberine using annexin-V binding assay at 6 h, 12 h, and 24 h. Phosphatidylserine externalisation was assessed by observing with fluorescence microscopy the extent of streptavidin-fluorescein isothiocyanate (FITC) annexin-V binding. We found that the apoptosis phenomenon starts earlier, at 6 h of stimulation by IC_{30} of both berberine and ethanol extract, noting that the cells treated by ethanol extract showed a damaged membrane unlike the ones treated by berberine at that time. The difference in the membrane shape between the cells treated with ethanol extract and berberine is probably due to other molecules contained in the ethanol extract which have damaged the cell membrane (late apoptosis). Indeed, it has been recently reported that palmatine hydrochloride, one of the ethanol extract compounds, significantly increases the early and late apoptotic rates of the MCF-7 cells [37].

Furthermore, several findings have reported berberine-induced apoptosis in different cell lines, for example, non-small cell human lung cancer, human epidermoid carcinoma cells, A431, U937, B16, HL-60, and MCF-7 cells [38–42].

Berberine treatment could, in a dose-dependent and time-dependent manner, increase Fas protein expression and induce FasL expression in tumour cell lines [43, 44]. It also increases Bax gene protein expression in cancer cells [33, 39, 45–47]. The alteration of berberine on proapoptotic and antiapoptotic gene expressions might be partly mediated by the generation of reactive oxygen species (ROS) [43]. In this work, berberine exhibited apoptosis-promoting and antiproliferative effects in the breast cancer MCF-7 cell line. This finding is in agreement with that of Lin et al. and Patil et al. [42, 48], who reported that the induction of apoptosis by berberine is through cell cycle disruption and DNA fragmentation in a mitochondria-dependent pathway by increasing levels of cytoplasmic cytochrome C, caspase-9 activity, and cleavage of PARP, while decreasing levels of Bcl-2 in MCF-7 cells. It has also been reported that berberine inhibits COX-2 transcriptional activity in a dose-dependent and time-dependent manner in MCF-7 cells [42]. It is possible that berberine may serve as a potential naturally occurring compound for breast cancer therapy.

On the other hand, the evaluation of synergy or antagonism of agents used in combination is an integral part

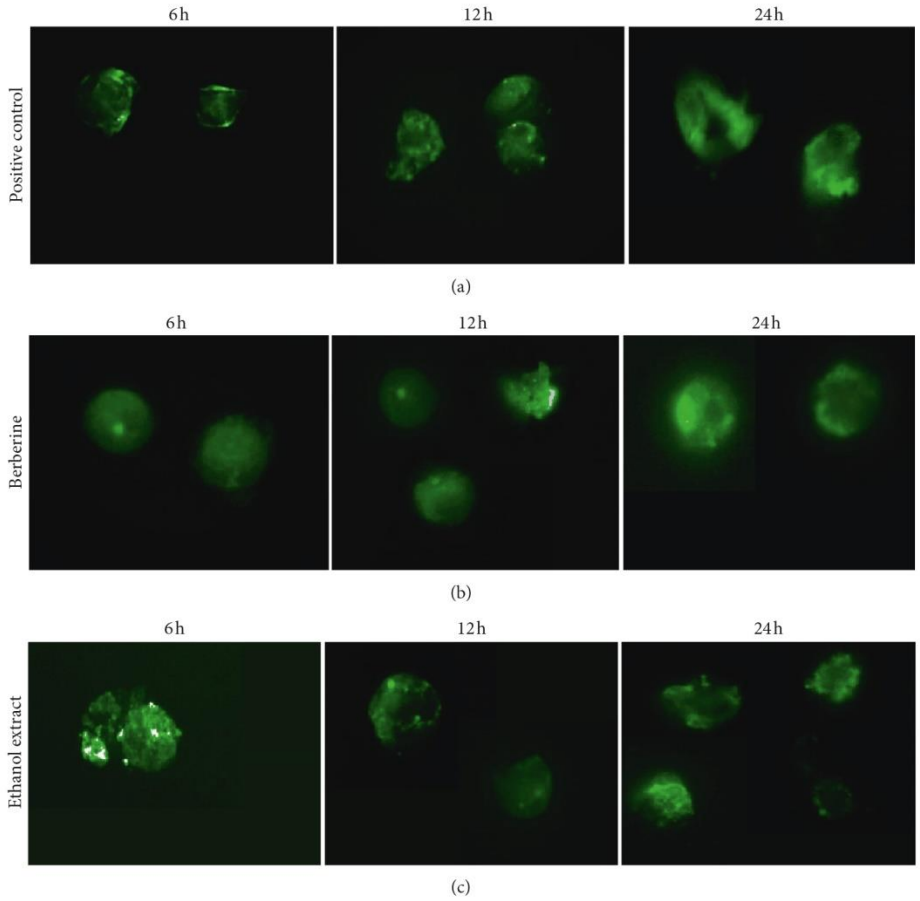


FIGURE 6: Photographs of annexin V-FITC-stained MCF-7 cells treated with the IC_{30} of berberine and ethanol extract at 6 h, 12 h, and 24 h. (a) Positive control; (b) Berberine; (c) Ethanol extract.

of cancer chemotherapy development. In this paper, we examined the combination effect of berberine combined with S-nitroso-N-acetylpenicillamine (SNAP), a nitric oxide (NO) donor, under physiological conditions. This assay was conducted using the Chou–Talalay method [17]. The choice of NO was based on the fact that this molecule has been described as a potent physiological antitumour agent [23–25]. In addition, NO has been shown to have both protective and deleterious functions [49, 50]. We investigated the effect of NO on the breast cancer cells and found that it exhibited a prominent in vitro cytotoxicity against the MCF-7 cell line in a dose-dependent manner.

To our knowledge, the interaction between berberine and a NO donor has not been previously described in the literature. However, NO can damage cells in many ways involving oxidative stress, DNA damage, protein modification, disruption of energy metabolism, interference with calcium homeostasis, and mitochondrial dysfunction. Depending on the context and severity of the damage, such disturbances may result in cell death either by necrosis or apoptosis, or in a successful repair and cell survival [49]. In our study, berberine, when combined with the NO donor (SNAP used at IC_{30}), induced a synergistic cytotoxic activity at concentrations of berberine ranging from 8.40 μM to 33.60 μM. However, at the other tested concentrations, an antagonistic effect was observed. These results may suggest the existence of differential mechanisms of berberine and SNAP interactions in MCF-7 cells in a dose-dependent manner. In fact, preventing or inducing apoptosis by NO may be modulated by dose-dependent interactions; at low concentrations, NO protects cells by inhibiting TNFα-induced apoptosis, but at high concentrations, NO induces apoptosis in endothelial cells [10, 50]. This double-edged effect of NO may have significant implications for the interaction with berberine, resulting in the different combination effects (antagonism and synergy) in the breast MCF-7 cancer cells. To our knowledge, this is also the first time where the interaction of berberine and SNAP has been reported.

5. Conclusion

In summary, our study demonstrates for the first time the selective cytotoxic effect of the ethanol extract of Moroccan

Berberis vulgaris against the MCF-7 tumour cell line depending on the dose of exposure without affecting the normal cells and that this cytotoxic effect may be due to its main compound berberine. We also reported that the ethanol extract and berberine display cell lysis by the apoptosis pathway and highlighted their antioxidant actions. On the other hand, this is the first report on the in vitro interaction between berberine and a NO donor.

Our study provides a basis for future clinical studies of berberine in patients with cancer, used alone or in combination with NO-inducer drugs. An adjuvant mechanism-based therapy with berberine compound may significantly improve clinical efficacy. This research, together with the previously reported findings in literature, will help improve our understanding about the molecular mechanisms of berberine as an anticancer agent.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

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References

[1] J. Belakhdar, *La Pharmacopée Marocaine Traditionnelle*, Ibis Press, MA, USA, 1998.

[2] G. Cui, X. Qin, Y. Zhang, Z. Gong, B. Ge, and Y. Q. Zang, "Berberine differentially modulates the activities of ERK, p38 MAPK, and JNK to suppress Th17 and Th1 T cell differentiation in type 1 diabetic mice," *Journal of Biological Chemistry*, vol. 284, no. 41, pp. 28420–28429, 2009.

[3] D. A. Ghareeb, A. E. A. El-Wahab, E. E. M. Sarhan, M. M. Abu Serie, and M. A. El Demellawy, "Biological assessment of BV and its active constituent, berberine: antibacterial, antifungal and antihepatitis C virus (HCV) effect," *Journal of Medicinal Plants Research*, vol. 7, pp. 1529–1536, 2013.

[4] S. Letasiova, S. Jantova, L. Cipak, and M. Muckova, "Berberine—antiproliferative activity in vitro and induction of apoptosis/necrosis of the U937 and B16 cells," *Cancer Letters*, vol. 239, no. 2, pp. 254–262, 2006.

[5] K. C. Karnam, M. Ellutla, L. N. Bodduluru et al., "Preventive effect of berberine against DMBA-induced breast cancer in female Sprague Dawley rats," *Biomedicine and Pharmacotherapy*, vol. 92, pp. 207–214, 2017.

[6] T. Singh, M. Vaid, N. Katiyar, S. Sharma, and S. K. Katiyar, "Berberine, an isoquinoline alkaloid, inhibits melanoma cancer cell migration by reducing the expressions of cyclooxygenase 2, prostaglandin E2 and prostaglandin E2 receptors," *Carcinogenesis*, vol. 32, no. 1, pp. 86–92, 2011.

[7] N. Wang, H.-Y. Tan, L. Li, M.-F. Yuen, and Y. Feng, "Berberine and *Coptidis rhizoma* as potential anticancer agents: recent updates and future perspectives," *Journal of Ethnopharmacology*, vol. 176, pp. 35–48, 2015.

[8] F. Tang, D. Wang, C. Duan et al., "Berberine inhibits metastasis of nasopharyngeal carcinoma 5-8F cells by targeting rho kinase-mediated Ezrin phosphorylation at threonine 567," *Journal of Biological Chemistry*, vol. 284, no. 40, pp. 27456–27466, 2009.

[9] E. Bremer, G. van Dam, B. J. Kroesen, L. de Leij, and W. Helfrich, "Targeted induction of apoptosis for cancer therapy: current progress and prospects," *Trends in Molecular Medicine*, vol. 12, no. 8, pp. 382–393, 2006.

[10] Y. H. Shen, X. L. Wang, and D. E. L. Wilcken, "Nitric oxide induces and inhibits apoptosis through different pathways," *FEBS Letters*, vol. 433, no. 1-2, pp. 125–131, 1998.

[11] M. P. Murphy, "Nitric oxide and cell death," *Biochimica et Biophysica Acta*, vol. 1411, no. 2-3, pp. 401–414, 1999.

[12] T. Mosmann, "Rapid colourimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays," *Journal of Immunological Methods*, vol. 65, no. 1-2, pp. 55–63, 1983.

[13] M. Tilaoui, H. Ait Mouse, A. Jaafari, and A. Zyad, "Comparative phytochemical analysis of essential oils from different biological parts of *Artemisia herba alba* and their cytotoxic effect on cancer cells," *PLoS ONE*, vol. 10, p. e0131799, 2015.

[14] I. Vermes, C. Haanen, H. Steffens-Nakken, and C. Reutelingsperger, "A novel assay for apoptosis flow cytometric detection of phosphatidylserine early apoptotic cells using fluorescein labeled expression on Annexin V," *Journal of Immunological Methods*, vol. 184, no. 1, pp. 39–51, 1995.

[15] K. Soon Kim, S. Lee, Y. Lee et al., "Anti-oxidant activities of the extracts from the herbs of *Artemisia apiacea*," *Journal of Ethnopharmacology*, vol. 85, no. 1, pp. 69–72, 2003.

[16] A. Zyad, D. Branellec, Y. Mahé, T. Tursz, and S. Chouaib, "The development of human tumour-cell resistance to TNF-alpha does not confer resistance to cytokine-induced cellular cytotoxic mechanisms," *International Journal of Cancer*, vol. 52, no. 6, pp. 953–958, 1992.

[17] T.-C. Chou and P. Talalay, "Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors," *Advances in Enzyme Regulation*, vol. 22, pp. 27–55, 1984.

[18] L. Grycova, J. Dostal, and R. Marek, "Quaternary protoberberine alkaloids," *Phytochemistry*, vol. 68, no. 2, pp. 150–175, 2007.

[19] X. Luo, B. Chen, and S. Yao, "Simultaneous analysis of protoberberine, indolequinoline and quinolone alkaloids in coptis–evodia herb couple and the Chinese herbal preparations by high-performance liquid chromatography–electrospray mass spectrometry," *Talanta*, vol. 66, no. 1, pp. 103–110, 2005.

[20] J.-M. Huang, G.-Q. Wang, Y.-E. Jin, T. Shen, and W. Weng, "Determination of palmatine in canine plasma by liquid chromatography-tandem mass spectrometry with solid-phase extraction," *Journal of Chromatography B*, vol. 854, no. 1-2, pp. 279–285, 2007.

[21] Q.-F. Liu, X.-J. Shi, Z.-D. Li, M.-K. Zhong, Z. Jiao, and B. Wang, "Pharmacokinetic comparisons of berberine and palmatine in normal and metabolic syndrome rats," *Journal of Ethnopharmachology*, vol. 151, no. 1, pp. 287–291, 2014.

[22] D. Patil, M. Gautam, S. Mishra et al., "Quantitative determination of protoberberine alkaloids in tinosporacordifolia

by RP-LC-DAD," *Chromatographia*, vol. 71, no. 3-4, pp. 341–345, 2009.

[23] A. Orucevic and P. K. Lala, "Effects of N(g)-methyl-L-arginine, an inhibitor of nitric oxide synthesis, on interleukin-2-induced capillary leakage and antitumour responses in healthy and tumour-bearing mice," *Cancer Immunology and Immunotherapy*, vol. 42, no. 1, pp. 38–46, 1996.

[24] D. C. Jenkins, I. G. Charles, L. L. Thomsen et al., "Roles of nitric oxide in tumour growth," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 92, no. 10, pp. 4392–4396, 1995.

[25] K. Kashfi, Y. Ryan, L. L. Qiao et al., "Nitric oxide-donating nonsteroidal anti-inflammatory drugs inhibit the growth of various cultured human cancer cells: evidence of a tissue type-independent effect," *Journal of Pharmacology and Experimental Therapeutics*, vol. 303, no. 3, pp. 1273–1282, 2002.

[26] N. Mokhber-Dezfuli, S. Saeidnia, A. Reza Gohari, and M. Kurepaz-Mahmoodabadi, "Phytochemistry and Pharmacology of Berberis Species," *Pharmacognosy Reviews*, vol. 8, no. 15, p. 8, 2014.

[27] M. ImanShahidi and H. HosseinZadeh, "Pharmacological and therapeutic effects of *Berberis vulgaris* and its active constituent, berberine," *Phytotherapy Research*, vol. 22, no. 8, pp. 999–1012, 2008.

[28] P. Hanachi and S. H. Golkho, "Using HPLC to determination the composition and antioxidant activity of *Berberis vulgaris*," *European Journal of Scientific Research*, vol. 29, pp. 47–54, 2009.

[29] C. J. Thirupurasundari, R. Padmini, S. Chithambaram, and N. Devaraj, "Effect of berberine on the antioxidant status, ultrastructural modifications and protein bound carbohydrates in azoxymethane-induced colon cancer in rats," *Chemico-Biological Interactions*, vol. 177, no. 3, pp. 190–195, 2009.

[30] A. Berłowski, K. Zawada, I. Wawer, and K. Paradowska, "Antioxidant properties of medicinal plants from Peru," *Food and Nutrition Sciences*, vol. 4, no. 8, pp. 71–77, 2013.

[31] M. S. Choi, D. Y. Yuk, and J. H. Oh, "Berberine inhibits human neuroblastoma cell growth through induction of p53-dependent apoptosis," *Anticancer Research*, vol. 28, no. 6A, pp. 3777–3748, 2008.

[32] S. Jantova, L. Cipak, and S. Letasiova, "Berberine induces apoptosis through a mitochondrial/caspase pathway in human promonocytic U937 cells," *Toxicology in Vitro*, vol. 21, no. 1, pp. 25–31, 2007.

[33] J. P. Lin, J. S. Yang, J. H. Lee, W. T. Hsieh, and J. G. Chung, "Berberine induces cell cycle arrest and apoptosis in human gastric carcinoma SNU-5 cell line," *World Journal of Gastroenterology*, vol. 12, no. 1, pp. 21–28, 2006.

[34] N. Keawpradub, S. Dej-adisai, and S. Yuenyongsawad, "Antioxidant and cytotoxic activities of Thai medicinal plants named Khaminkhruea and Fibraureatinctoria," *Songklanakarin Journal of Science and Technology*, vol. 27, no. 2, pp. 455–467, 2005.

[35] J.-P. Lin, J. S. Yang, N. W. Chang et al., "GADD153 mediates berberine-induced apoptosis in human cervical cancer Ca ski cells," *Anticancer Research*, vol. 27, no. 5A, pp. 3379–3386, 2007.

[36] Y.-T. Ho, C. C. Lu, J. S. Yang et al., "Berberine induced apoptosis via promoting the expression of Caspase-8, -9 and -3, apoptosis-inducing factor and endonuclease G in SCC-4 human tongue squamous carcinoma cancer cells," *Anticancer Research*, vol. 29, no. 10, pp. 4063–4070, 2009.

[37] J. Wu, Q. Xiao, N. Zhang et al., "Palmatine hydrochloride mediated photodynamic inactivation of breast cancer MCF-7 cells: effectiveness and mechanisms of action," *Photodiagnosis and Photodynamic Therapy*, vol. 15, pp. 133–138, 2016.

[38] S. K. Katiyar, S. M. Meeran, N. Katiyar, and S. Akhtar, "p53 cooperates berberine-induced growth inhibition and apoptosis of non-small cell human lung cancer cells in vitro and tumour xenograft growth in vivo," *Molecular Carcinogenesis*, vol. 48, no. 1, pp. 24–37, 2009.

[39] S. K. Mantena, S. D. Sharma, and S. K. Katiyar, "Berberine inhibits growth, induces G1 arrest and apoptosis in human epidermoid carcinoma A431 cells by regulating Cdki-Cdk-cyclin cascade, disruption of mitochondrial membrane potential and cleavage of caspase 3 and PARP," *Carcinogenesis*, vol. 27, no. 10, pp. 2018–2027, 2006.

[40] H. L. Wu, C. Y. Hsu, W. H. Lin, and B. Y. Yung, "Berberine-induced apoptosis of human leukemia HL-60 cells is associated with down-regulation of nucleophosmin/B23 and telomerase activity," *International Journal of Cancer*, vol. 81, no. 6, pp. 923–929, 1999.

[41] K. Fukuda, Y. Hibiya, M. Mutoh, M. Koshiji, S. Akao, and H. Fujiwara, "Inhibition by berberine of cyclooxygenase-2 transcriptional activity in human colon cancer cells," *Journal of Ethnopharmacology*, vol. 66, no. 2, pp. 227–233, 1999.

[42] J. Lin, C. He, K. Zhou, J. Wang, and J. X. Kang, "Coptis extracts enhance the anticancer effect of estrogen receptor antagonists on human breast cancer cells," *Biochemical and Biophysical Research Communications*, vol. 378, no. 2, pp. 174–178, 2009.

[43] W. H. Hsu, Y. S. Hsieh, H. C. Kuo et al., "Berberine induces apoptosis in SW620 human colonic carcinoma cells through generation of reactive oxygen species and activation of JNK/p38 MAPK and FasL," *Archives of Toxicology*, vol. 81, no. 10, pp. 719–728, 2007.

[44] J. M. Hwang, H. C. Kuo, T. H. Tseng, J. Y. Lin, and C. Y. Chu, "Berberine induces apoptosis through a mitochondria/caspases pathway in human hepatoma cells," *Archives of Toxicology*, vol. 80, no. 2, pp. 62–73, 2006.

[45] S. K. Mantena, S. D. Sharma, and S. K. Katiyar, "Berberine, a natural product, induces G1-phase cell cycle arrest and caspase-3-dependent apoptosis in human prostate carcinoma cells," *Molecular Cancer Therapy*, vol. 5, no. 2, pp. 296–308, 2006.

[46] K. S. Eom, J. M. Hong, M. J. Youn et al., "Berberine induces G1 arrest and apoptosis in human glioblastoma T98G cells through mitochondrial/caspases pathway," *Biological and Pharmaceutical Bulletin*, vol. 31, no. 4, pp. 558–562, 2008.

[47] C. C. Lin, S. T. Kao, G. W. Chen, H. C. Ho, and J. C. Chung, "Apoptosis of human leukemia HL-60 cells and murine leukemia WEHI-3 cells induced by berberine through the activation of caspase-3," *Anticancer Research*, vol. 26, no. 1A, pp. 227–242, 2006.

[48] J. B. Patil, J. Kim, and G. K. Jayaprakasha, "Berberine induces apoptosis in breast cancer cells (MCF-7) through mitochondrial-dependent pathway," *European Journal of Pharmacology*, vol. 645, pp. 70–78, 2010.

[49] C.-Q. Li and G. N. Wogan, "Nitric oxide as a modulator of apoptosis," *Cancer Letters*, vol. 226, no. 1, pp. 1–15, 2004.

[50] J. Li, T. R. Billiar, R. V. Talanian, and Y. M. Kim, "Nitric oxide reversibly inhibits seven members of the caspase family via S-nitrosylation," *Biochemical and Biophysical Research Communications*, vol. 240, no. 2, pp. 419–424, 1997.

ANNEX 2

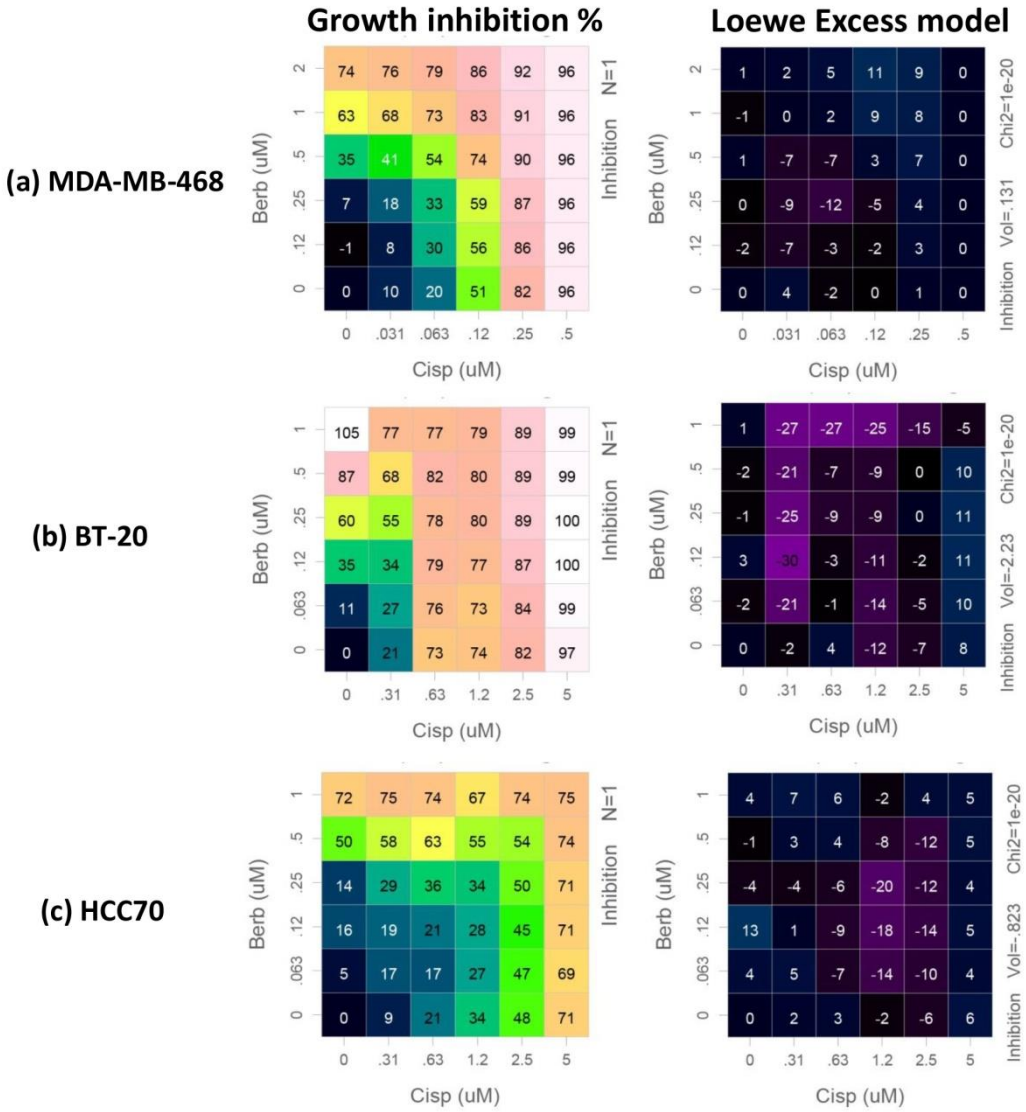

Figure Annex 2: Berberine and cisplatin combination resulted in antagonism when treated to TNBC cells. Loewe models of the combination effect of berberine and cisplatin. Respresentative growth inhibition percentage and Loewe model matrix representing synergy scores through the intensity of the colours. **(a)** MDA-MB-468, **(b)** BT-20 and **(c)** HCC70. (Berb: berberine, Cisp: cisplatin).

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Résumé:

Le cancer du sein triple négatif (TNBC) est un sous-type de cancer du sein agressif sans traitement ciblé en raison de l'absence d'expression des récepteurs de l'œstrogène et de la progestérone (ER^- et PR^-) et le manque de surexpression du récepteur-2 / Neu du facteur de croissance épidermique humain. ($HER2^-$). La non disponibilité de la thérapie ciblée pour TNBC représente son plus grand défi de traitement. Il est donc urgent de trouver de nouveaux médicaments efficaces et prometteurs. Dans la présente étude, nous avons étudié sur les cellules TNBC les effets potentiels de quatre médicaments naturels purs, la berbérine, l'artémisinine, le carvacrol et la thymoquinone, au niveau cellulaire et moléculaire.

Notre modèle expérimental était basé sur l'utilisation de sept lignées cellulaires TNBC: MDA-MB-468, HCC70, HCC38, HCC1937, HCC1143, BT-20 et BT-549, et deux lignées cellulaires non TNBC: MCF-7 ($ER^- PR^+ HER2^+$) et SKBR3 ($ER^- PR^- HER^+$).

Nous avons d'abord commencé par cribler l'effet antiprolifératif de la berbérine, de l'artémisinine, du carvacrol et de la thymoquinone sur le grand panel de lignées cellulaires TNBC et non TNBC, en utilisant des tests clonogéniques 2D et le test MTT. Ensuite, l'arrêt du cycle cellulaire et l'apoptose avec l'expression de leurs protéines ont été analysés en utilisant la cytométrie en flux et les tests de Western blot. L'effet de la berbérine sur l'analyse de viabilité des cellules humaines normales MCF-10A a été analysé en utilisant une culture en matrigel 3D. La synergie de l'artémisinine avec le cisplatine a été mesurée en utilisant le modèle Loewe Excess.

Le dépistage du test d'inhibition de la croissance a montré que la berbérine et l'artémisinine étaient les médicaments les plus efficaces, avec le carvacrol et la thymoquione étant les moins efficaces. Toutes les lignées cellulaires TNBC traitées n'ont pas survécu au traitement par la berbérine. Cependant, les lignées cellulaires les plus sensibles à la berbérine étaient HCC70 (IC_{50} = 0,21 µM), BT-20 (IC_{50} = 0,21 µM) et MDA-MB-468 (IC_{50} = 0,66 µM). La berbérine a ciblé de manière significative les marqueurs du cycle cellulaire PCNA, cycline D1 et cycline B1 pour provoquer un arrêt du cycle cellulaire en phase G1 dans les MDA-MB-468 et en phase G2 / M dans les HCC70 et BT-20. De plus, la berbérine a ciblé de manière significative l'expression de marqueurs d'apoptose: la caspase-8 clivée, la caspase-7 clivée et le PARP clivé pour induire une apoptose significative dans toutes les lignées cellulaires traitées. Alors que la berbérine était cytotoxique contre les cellules TNBC, elle n'a eu aucun effet significatif sur la viabilité des cellules humaines normales MCF-10A.
D'autre part, l'artémisinine a inhibé la croissance cellulaire des HCC38 (IC_{50} = 1,25 µM) et MDA-MB-468 (IC_{50} = 2,23 µM), ces lignées cellulaires étaient les plus sensibles parmi toutes celles traitées par l'artémisinine. De plus, l'artémisinine a induit un arrêt significatif du cycle cellulaire en phase S des HCC38. Enfin, la combinaison de l'artémisinine avec le traitement au cisplatine a entraîné une synergie significative lorsqu'elle a été administrée aux cellules HCC38 et MDA-MB-468.

Ces résultats suggèrent que la berbérine et l'artémisinine peuvent être de bons candidats pour le développement des médicaments pour le TNBC. En plus, cette étude représente une étape parmi d'autres dans les études précliniques visant à améliorer les stratégies de traitement du TNBC dans le monde.

Mots clés: Cancer du Sein Triple Négatif, berberine, artemisinin, thymoquinone, carvacrol, cycle cellulaire, apoptose, expression des protéines, synergie.

ملخص

سرطان الثدي ثلاثي السلبية (TNBC) هو نوع فرعي من سرطان الثدي دون علاج مستهدف بسبب نقص مستقبلات هرمون الاستروجين والبروجستيرون (-ER) و (-PR) مع عدم وجود إفراط في إفراز مستقبلات -2 / نيو من عامل نمو البشرة البشرية (HER2) .يمثل عدم توفر العلاج المستهدف لـ TNBC التحدي الأكبر في العلاج. لذلك من المستعجل إيجاد أدوية جديدة فعالة واعدة. في هذه الدراسة ، درسنا الآثار المحتملة لأربعة عقاقير طبيعية ، بربارين ، أرتيميسينين ، كارفاكرول و ثيموكينون ، على خلايا TNBC ، على المستوى الخلوي والجزيئي.

استند نموذجنا التجريبي إلى استخدام سبعة سلالات خلوية TNBC: MDA-MB-468 و HCC70 و HCC38 و HCC1937و HCC1143 و BT-20 وBT-549 ، وسلالتين من الخلايا (+HER2 + PR- ER-) MCF-7و SKBR3 (ER- PR- HER).

لقد بدأنا أولاً بفحص التأثير المضاد للتكاثر لبربارين والأرتيميسينين والكرافاكول والثيموكينون على مستوى كبير لخلايا TNBC وغير TNBC ، باستخدام اختبارات مستعمرة ثنائي الأبعاد واختبار MTT بعد ذلك ، تمت دراسة توقف الدورة الخلوية وموت الخلايا المبرمج مع تعبير البروتينات باستخدام قياس التدفق الخلوي واختبارات ويسترن بلوت. تمت دراسة تأثير البربرين على خلايا MCF-10A البشرية الطبيعية باستخدام الزراعة على matrigel ثلاثية الأبعاد. تم قياس تآزر الأرتيميسينين مع سيسبلاتين باستخدام نموذج لوي اكسيس.

أظهر فحص اختبار تثبيط النمو أن البربرين والأرتيمسينين كانا من أكثر الأدوية فاعلية ، وكانت كارفاكيرول وتيموكويون الأقل فعالية. ليس كل الخلايا TNBC المعالجة نجا من العلاج بالبربارين. ومع ذلك ، كانت الخلايا الأكثر حساسية للبربارين (IC50 = 0.21µM) ، HCC70 (IC50 = 0.21µM) ، BT-20 و MDA-MB-468 (IC50=0.66µM). استهدف بربرين بشكل ملحوظ بروتينات PCNA و cyclin D1 و cyclin B1 لتسبب توقيف دورة الخلية عند المرحلة G1 في MDA-MB-468 وعند المرحلة G2 / M في HCC70 و BT-20. بالإضافة إلى ذلك ، استهدف البربرين بشكل ملحوظ التعبير عن بروتينات موت الخلايا المبرمج: cl-caspase-8 ,cl-caspase-7 و cl-PARPللحث على موت الخلايا المبرمج الكبير في جميع الخلايا المعالجة. بينما كان البربرين سامًا ضد خلايا TNBC ، لم يكن له تأثير كبير على صلاحية خلايا MCF-10A البشرية الطبيعية.

من ناحية أخرى ، عرقل الأرتيميسينين نمو خلايا HCC38 (IC50= 1.25µM) و MDA-MB-468 (IC50= 2.23µM)، وكانت خطوط الخلايا هذه هي الأكثر حساسية بين جميع الخلايا المعالجة بواسطة الأرتيميسينين. بالإضافة إلى ذلك ، تسبب الأرتيميسينين في توقيف كبير لدورة الخلية في المرحلة S في HCC38. أخيرًا ، أدى مزيج الأرتيميسينين مع علاج سيسبلاتين إلى تآزر كبير عند علاج خلايا HCC38 و MDA-MB-468.

تشير هذه النتائج إلى أن البربرين والأرتيميسينين قد يكونا مرشحين جيدين لتطوير عقاقير TNBC. بالإضافة إلى ذلك ، تمثل هذه الدراسة واحدة من عدة خطوات في الأبحاث قبل السريرية التي تهدف إلى تحسين استراتيجيات العلاج ل TNBC في جميع أنحاء العالم.

الكلمات الدالة : سرطان الثدي ثلاثي السلبية، بربارين، أرتيميسينين، كارفاكرول، ثيموكينون، الدورة الخلوية، موت الخلايا المبرمج، تعبير البروتينات، تآزر كبير عند العلاج.

Abstract:

Triple negative breast cancer (TNBC) is an aggressive breast cancer subtype with no targeted therapy due to the absence of expression of estrogen and progesterone hormone receptors (ER- and PR-) and the lack of human epidermal growth factor receptor-2/Neu over-expression (HER2-). Non-available targeted therapy for TNBC (except for PDL1 and PARP inhibitors, recently approved by FDA) represents its biggest treatment challenge. Thus, finding new promising effective drugs is urgently needed. In the present study we investigated on TNBC cells the potential effects of four pure natural drugs berberine, artemisinin, carvacrol and thymoquinone, in both cellular and molecular level.
Our experimental model was based on the use of seven TNBC cell lines: MDA-MB-468, HCC70, HCC38, HCC1937, HCC1143, BT-20 and BT-549, and two non-TNBC cell lines: MCF-7 ($ER^-PR^+HER2^+$) and SKBR3 ($ER^-PR^-HER2^+$).

We first started by screening the antiproliferative effect of berberine, artemisinin, carvacrol and thymoquinone on the large panel of TNBC and non-TNBC cell lines, using MTT and 2D clonogenic assays. Afterwards, cell cycle arrest and apoptosis with their related proteins were analyzed using flow cytometry and Western blot assays. The effect of berberine on normal human cells MCF-10A viability analysis was analyzed using Matrigel 3D culture. Artemisinin synergism with cisplatin was measured using Loewe Excess model.

The screening of growth inhibition assay showed that berberine and artemisinin were the most effective drugs, with carvacrol and thymoquione being the least effective ones. All treated TNBC cell lines failed to survive berberine's treatment. However, the most sensitive cell lines to berberine were HCC70 (IC_{50}=0.21 µM), BT-20 (IC_{50}=0.21 µM) and MDA-MB-468 (IC_{50}=0.66 µM). Berberine significantly targeted cell cycle markers PCNA, cyclin D1 and cyclin B1 proteins to cause cell cycle arrest at G1 phase in MDA-MB-468 and at G2/M phase in HCC70 and BT-20. Furthermore, berberine significantly targeted the expression of apoptosis markers: cleaved-caspases-8, cleaved-caspases-7 and cleaved-PARP to induce significant apoptosis in all treated cell lines. Interestingly, while berberine was cytotoxic against TNBC cells, it had no significant effect on the viability of normal human cells MCF-10A.

In the other hand, Artemisinin inhibited the cell growth of HCC38 (IC50=1.25 µM) and MDA-MB-468 (IC50= 2.23 µM), these cell lines were the most sensitive ones among all artemisinin-treated cells. Additionally, artemisinin induced a significant cell cycle arrest in S phase of HCC38. Finally, the combination of artemisinin with cisplatin treatment resulted in a significant synergy when treated to both HCC38 and MDA-MB-468 cells.

These results suggest that berberine and artemisinin may be good candidates for TNBC drug development, and this present study is one of multiple steps of preclinical investigations towards improving TNBC treatment strategies in the world.

Key words: Triple Negative Breast Cancer, berberine, artemisinin, thymoquinone, carvacrol, cell cycle, apoptosis, protein expression, synergy.