AMERICAN UNIVERSITY OF BEIRUT

POTENTIAL OF ANTI-NEOPLASTIC THERAPEUTICS IN TARGETING HUMAN OVARIAN CANCER CELLS

by
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ABSTRACT
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Title: Potential of Anti-neoplastic Therapeutics in Targeting Human Ovarian Cancer Cells.

Background: The ovary is characterized by three different cell types which are epithelial, germ, and stromal cells. Depending on the cell origin of the tumor, several types of ovarian cancers emerge. The most common type is the epithelial ovarian cancer which arises from the epithelium and comprises 95% of all ovarian cancer types. The germ-cell tumors and stromal tumors account for 2-3% and 1.2% respectively. The epithelial ovarian cancer is divided in multiple subtypes and the most common is the high grade serous ovarian cancer. Although the pathogenesis of ovarian cancer at the molecular level is still not well exhibited, recent researches and studies link certain pathways and alterations directly to ovarian cancer.

Aim: This proposal aims to study the effect of Thymoquinone (TQ), the main component of black seed on blocking the epithelial ovarian cancer cells' proliferation and migration, and targeting the ovarian cancer stem cells.

Methods: Two human ovarian cancer cell lines OVCAR-420 and SKOV-3 were used to assess the effect of Thymoquinone on cell viability and proliferation using MTT and Trypan Blue assays. Moreover, we used the wound healing assay to study the effect of TQ on the migration of the cells. Finally, the effect of the drugs on ovarian cancer stem cells, progenitor cells and stem cell characteristics and properties was evaluated using the 3D spheres formation assay.

Results: Our results showed a decrease in cell viability and proliferation in both cell lines after treatment with TQ using the Trypan Blue and MTT assays respectively. Moreover, TQ was also successful in reducing the migratory potential of both cell lines. Furthermore, the sphere formation assay showed a significant effect of the treatment on the count, shape and size of the spheres for both OVCAR-420 and SKOV-3.

Conclusion: This study demonstrates TQ as a potential treatment against ovarian cancer.

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ABBREVIATIONS

2D: Two-dimension

3D: Three-dimension

Bcl-2: B-cell lymphoma 2

CCC: Clear cell carcinoma

CSC: Cancer Stem Cell

DNA: Deoxyribonucleic Acid

FBS: Fetal bovine serum

HGSOC: High grade serous ovarian cancer

LGSOC: Low grade serous ovarian cancer

MTT: 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide

NF-kB: Nuclear factor kappa

PBS: Phosphate buffered saline

SEM: Standard error of the mean

SFU: Sphere formation unit

TQ: Thymoquinone

VEGF: Vascular Endothelial Cell Growth Factor

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CHAPTER I

INTRODUCTION

A. Ovarian Cancer

1. Epidemiology

Gynecological tumors now pose a major threat to women worldwide, mostly because of lack of knowledge and because of environmental deterioration. Ovarian cancer is the most lethal gynecological disease with over 22000 diagnosed cases and a mortality rate of around 14 000 women per year making it the 7th most common type of cancer and 8th most common type of cancer causing female death [1]. Therefore, ovarian and gynecological tumors constitute a major threat to women health worldwide. Statistically, most Ovarian Cancer diagnoses occur in women above 30 years and mostly at an age between 50 and 70 years [2]. In addition to the difference in occurrence of ovarian cancer among different ages, several studies have also shown region-related differences. Figure 1 shows that the incidence of ovarian cancer is highest among northern and eastern Europe, and lowest among Asians and Africans [3]. Interestingly, latest studies also show differences in occurrence within the same country such as the difference in the risk between white women and African-American women with a higher risk among white women [4].

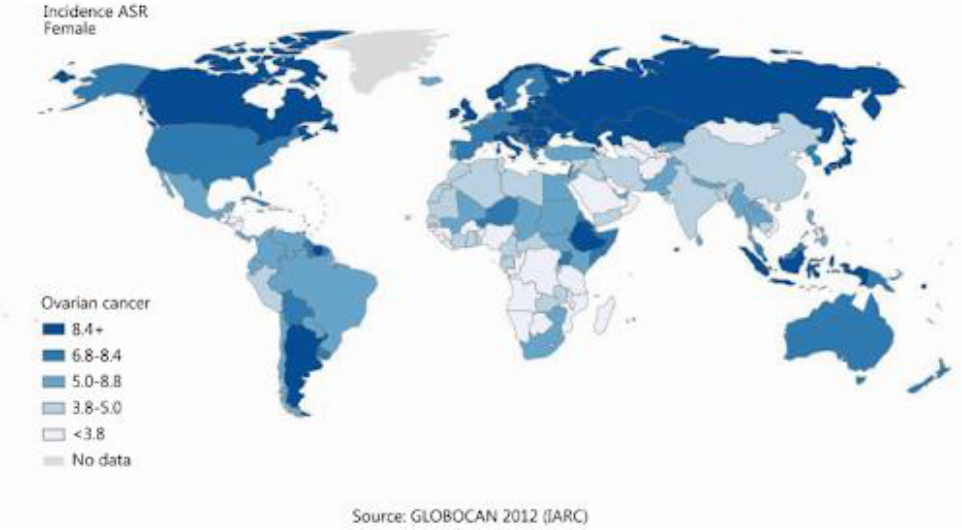


Figure 1. Rate of ovarian cancer occurrence across the globe.

Clinically, ovarian cancer can be classified either as acute or subacute. Acute cases are usually presented with venous thromboembolism while subacute cases are presented with symptoms such as abdominal floating, pain, and other gastrointestinal symptoms [5].

Despite many medical advances in therapeutics and radiation, ovarian cancer remains a difficult type of cancer with a five-year survival rate that is barely experienced by 30% of the patients, mainly due to the delay in diagnosis, this is mostly because of the lack of obvious symptoms throughout the early stages of ovarian cancer where roughly 20% of the patients are diagnosed during an early stage while the rest are diagnosed during later advanced stages [6]. Therefore, detecting early-stage ovarian cancer symptoms has become an important topic in the ovarian cancer research field and continuous efforts are being put into explaining the mechanisms and factors affecting early ovarian cancer progression. Although the pathogenesis of Ovarian cancer at the molecular level is still not well

exhibited, recent researches and studies link certain pathways and alterations directly to this type of tumor [5]. Also, there are many emerging treatment methods that are being developed and are under experimentation. Usually, treatment of ovarian cancer involves a surgical procedure in order to remove any visible tumors, followed by chemotherapy to destruct any residual tumor cells that have spread in the body. Today, intensive research is being made in order to establish the perfect drug combination that would allow to target the most probable cause of ovarian cancer tumors [7].

2. Diagnosis

When symptoms are present, diagnosis of ovarian cancer involves imaging tests such as ultrasonography, CT scans, and X-ray scans. Laparoscopies and blood tests are also used for diagnosis [8].

Ovarian cancer is categorized into four main stages depending on where and how far the tumor spread in the body. At stage I, the tumor is still confined in the ovaries and fallopian tubes. This stage is divided into stage IA where one of the ovaries or fallopian tubes are affected with cancer and stage IB where both of the ovaries or both fallopian tubes are affected with the cancer, while stage IC is where one of the ovaries or fallopian tubes are affected along with ruptured ovarian capsule. For this stage, surgery alone is enough to treat the tumor [9]. Stage II is when the tumor has spread from one or both ovaries or fallopian tubes into the pelvis and is further divided into stage IIA where the cancer has reached the uterus and stage IIB where the cancer has spread to other organs of the pelvis such as the bladder or rectum. Stage II requires surgery as well as up to 6 cycles of chemotherapy [10].

When the cancer has spread out of the pelvis into lymph nodes or omentum that would be stages IIIA and IIIB of ovarian cancer, respectively [11]. Stage IV is when cancer has spread outside of the pelvis and abdomen into distant organs. It is subdivided into stage IVA where the tumor has spread into the extracellular fluid surrounding the lungs, and into IVB where the tumor has reached organs such as the lung, spleen, and liver [12]. For stages III and IV, removal of the ovarian and fallopian tubes as well as the uterus and the omentum is required followed by chemotherapy [13]. In some cases, 2-3 cycles of chemotherapy could also be performed before surgery in order to reduce the size of the tumor and make it easier to remove [4]. Several risk factors are currently being associated with epithelial ovarian cancer, which explains the variety of targets when it comes to treatment and therapy, including genetic factors, age, postmenopausal hormonal therapy use, and infertility [14].

Stage	Description of Tumor Stage
IA	Tumor is confined to one ovary or one fallopian tube.
IB	Tumor is present in both ovarian or both fallopian tubes.
IC	Tumor is present in either one or both ovaries or fallopian tubes, with the presence of a rupture in the ovarian capsule
IIA	The tumor extends into the uterus.
IIB	Tumor extends to other pelvic intraperitoneal tissues.
IIIA	Tumor metastasis in lymph nodes.
IIIB	Tumor metastasis into omentum.
IIIC	>2 cm tumor metastasis into omentum.
IV	Tumor metastasis into distinct organs such as spleen, liver, and extraabdominal organs.

Table 1. Description of the different stages of ovarian cancer.

3. Risk Factors

A wide variety of risk factors have been associated with ovarian cancer and proved to have correlations with the probability of its occurrence, the most significant of those risk factors would be genetics. Women with a family history of ovarian cancer are at a higher risk of developing the disease than women with no family history [15]. Mainly, hereditary ovarian cancer is associated with mutations in BRCA1 and BRCA2 genes, specifically those occurring somatically with women possessing BRCA1 mutations exhibiting a more significant increase in the risk of ovarian cancer than those with BRCA2 mutations [16].

Also, studies show that mutations of certain oncogenes play a role in epithelial ovarian cancer[17].

Age is also a potential ovarian cancer risk factor. Multiple studies were performed in order to study the link between age and ovarian cancer, however the results were opposing, where some statistics showed a direct correlation between age and the risk of ovarian cancer [18], while others did not show any significant correlation [19]. Moreover, researchers hypothesize that with an early monarch age and a late menopause age, the risk for ovarian cancer increases. This is mainly related to the increase in number of ovulatory cycles. However, more research is required in order to clarify this issue [20].

Limited data also shows that the higher the weight and BMI the higher the risk for ovarian cancer. This stems from the fact that fat cells are associated with the conversion of androstenedione to oestrone and elevated levels of oestradiol because of the relative decrease in sex-hormone-binding globulin levels [21]. This is consistent with several studies that show a high BMI directly linked to a 5-29% higher ovarian cancer risk per $5kg/m^2$[22].

An important ovarian cancer risk factor is hormonal therapy. It is currently a common medication used to treat different hormonal imbalances and related conditions. Estrogen hormone injections are mainly used among menopausal women experiencing vaginal dryness or hot flushes, or even among young women suffering from shortage of estrogen [23]. Researchers claim that the hormonal therapy and estrogen injections are associated with a higher risk of ovarian cancer. This effect is most significant when the estrogen therapy has been used for over 10 years [24, 25], and it has been shown that an over 40% increase in ovarian cancer risk is observed even after the therapy is stopped. Lacy Jr, et al

and Riman et al, showed that the risk increases by 20% every five years and continues to increase as the duration of use of hormonal therapy increases [3, 26]. This would explain how statistical literature shows that the use of contraceptives, which block and reduce ovulation, is inversely related to the risk of ovarian cancer. Interestingly, oral contraception is estimated to cause the prevention of 300,000 cases every year by reducing the risk of developing ovarian cancer by 40% [27].

A number of studies have also been done in order to find out how pregnancy and breastfeeding could affect ovarian cancer risk and the results remain mostly inconsistent where although certain studies show that giving birth is associated with a reduced risk of ovarian cancer, there are many studies that suggest that it has no effect on ovarian cancer risk [28]. Also, there are several analyses that claim a positive relation between breastfeeding and ovarian cancer risk showing that women who breastfed had a 20-25% decrease in ovarian cancer risk [29]. In contrast, a recent statistical study showed no significant decrease in the risk as opposed to breastfeeding duration [30].

As for the relation between diet and ovarian cancer risk, it remains unclear and limited. However, a humble association can be drawn between the intake of nutrients and ovarian cancer risk. Some studies show that vegetable intake strongly affects ovarian cancer risk, showing that ovarian cancer risk decreases with higher vegetable intake while the risk is elevated when accompanied by lower vegetable intake [31]. Moreover, lactose is hydrolyzed into glucose and galactose which is toxic to oocytes. Several studies reported a correlation between fat intake and ovarian cancer risk. They showed that fat intake is correlated with a higher ovarian cancer risk, and interestingly, this idea was further

supported by a study showing a reduced risk of ovarian cancer among lactose-intolerant women [32, 33].

4. Types of Ovarian Cancer

The ovary is characterized by three different cell types which are epithelial cells, germ cells, and stromal cells. Depending on the origin of the tumor from any of these cell types, several types of ovarian cancers emerge, the most common being the epithelial ovarian cancer which arises from the epithelium and comprises 95% of all ovarian cancer types, while the other two types, account for 2-3% and 1.2% of ovarian cancer cases, respectively [34].

Germ-cell tumors arise from the reproductive cells of the ovaries, and even though it mostly affects young women and premenarchal girls, it could also occur in postmenopausal women, and this type of tumor mainly arises in the ovaries but can also arise externally throughout the migratory pathway of the eggs towards the fallopian tube [35]. Like all types of ovarian tumors, symptoms are not present throughout very early stages, but the tumor could be discovered during regular gynecological examinations [36].

Stromal tumors arise from the granulosa and sertoli-leydig cells and are distinguished from the other two types of ovarian tumors by being characterized with signs of excessive hormonal production such as early puberty and changes in menstruation [37]. Sertoli-leydig tumors usually appear in the second and third decades of a woman's life and can also uncommonly produce testosterone which results in virilization and hirsutism [38]. The granulosa stromal tumors mostly occur in middle-aged and old women but can also affect premenarchal girls to a much lesser extent, and because of this variety in the age of

diagnosis, this type of cancer is classified into adult granulosa cell tumors and juvenile granulosa cell tumors [39]. Both stromal and germ-cell ovarian tumors are considered low malignant tumors, are often diagnosed at an early stage and are most often successfully removed by surgery [40].

While epithelial cancer is the most common and widespread type of ovarian cancer, making 90% of total ovarian cancer cases, its molecular transformation and progression remains a mysterious topic till today, with the molecular events and pathways leading to this type of cancer being unclear and not understood [40]. Epithelial ovarian cancer is derived from the surface epithelium of the ovary, peritoneum, or fallopian tube. Most studies and researches to date have shown that hormones, glands and other factors that play a role in ovulation and reproduction play a role in this transformation [41].

Epithelial ovarian cancer is divided into two types: type I and type II. Type I tumors are often linked to natural risk factors such as ovulation, inflammation, and endometriosis. Type II ovarian cancer is often lethal and is presented at even later stages than type I ovarian malignancies. This type is associated with genetic mutations of BRCA genes and mutations affecting p53 activation [15]. Within these two types of epithelial ovarian cancer, there are several subtypes that arise from different origins and differ morphologically and molecularly, but despite the differences in morphology and prognosis, those subtypes of EOC are considered and dealt with as one type of cancer and a single entity. The different classifications are named serous, mucinous, endometrioid, clear cell, and brenner tumors (Figure. 3) [42].

Serous ovarian cancer originates in the fallopian tube and is further divided into high grade serous ovarian cancer (HGSOC) and low grade serous ovarian cancer (LGSOC) [33].

High grade serous ovarian cancer is considered the most common of the subtypes and makes up 75% of total epithelial ovarian cancer cases. From a histological perspective, high grade serous ovarian carcinoma is characterized by large nuclei that are irregular in shape and ciliated columnar cells that form papillae along with the presence of mitotic figures as a result of its high mitotic index [43]. The LGSOC cancer is often identified for younger women and is very rare making less than 5% of total ovarian cancer cases, however, it is known for being highly chemoresistant [44]. Unlike, HGSOC, LGSOC arises from a preexisting lesions such as serous cystadenomas and is considered an invasive tumor, and is known for its small and uniform nuclei and a micro-papillary growth pattern [45].

Mucinous ovarian tumors usually originate from the endocervical epithelium and make up a very small percentage of 3% of the epithelial ovarian cancer types [46]. It is usually the most frequent subtype occurring in women below 40 years of age where most of the cases are diagnosed at an early stage with an excellent survival rate. Mucinous ovarian carcinoma range from benign to malignant, and from invasive to noninvasive, and are histologically characterized by atypia, stratification, and papillary growth [47].

Endometrioid tumors, which account for 10% of total ovarian cancer tumors, are sometimes associated with endometriosis and are most frequently seen in patients with Lynch Syndrome [48]. This subtype of epithelial ovarian cancer possesses squamous differentiation with some cystic and solid architecture [49], is associated with a relatively good prognosis, and patients usually present a slightly higher chance of survival than patients of other types [50].

Clear cell carcinoma (CCC) which also arises from endometriosis is a rare yet aggressive type of cancer making up 10% of ovarian tumors most often presented at a

young age. This subtype characterized by poor prognosis and is associated with thromboembolic complications and hypercalcemia [51]. Histologically, CCC is characterized by a clear cytoplasm and glycogen-containing cells and represent a cystic, papillary, solid, and mixture patterns [52].

Brenner tumors are also quite rare and make up 2 % of ovarian tumors, and are associated with the presence of transitional epithelial cells in fibrous stroma making it possess a histology similar to that of bladder transitional epithelium [53].

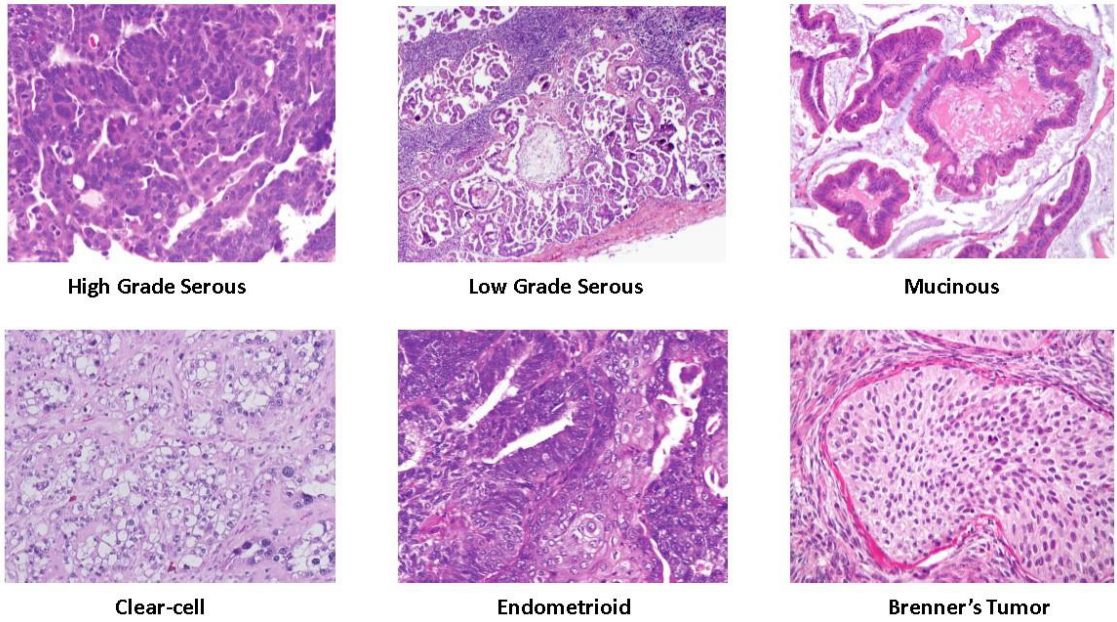


Figure 2. Illustration of the different types of epithelial ovarian cancer.

5. Novel Therapeutics

Ovarian cancer chemotherapy is currently based on the platinum derivatives cisplatin and most recently carboplatin which is an analogue of cisplatin. Those derivatives are used

in combination with the taxane derivatives paclitaxel and docetaxel [54]. This combination of drugs was shown to effectively eliminate the ovarian cancer cells through inducing DNA damage. However, cancer recurrence is very common among ovarian cancer patients and different side effects and complications are obtained [55]. Therefore, along with the increasing need to overcome the limitations of currently used ovarian cancer chemotherapy, and along with the rise in understanding of ovarian cancer progression and mechanisms of action, an increase in the development of new therapeutics is taking place. Those novel drugs and targeted therapies are under intensive evaluation and many of them display promising results in inhibiting cancer progression and reducing chemoresistance when used alone or in combination [56].

Tyrosine kinase inhibitors

Some of those chemotherapeutics belong to the family of tyrosine kinase inhibitors. Tyrosine kinase is often being targeted for ovarian cancer treatment by oral tyrosine kinase inhibitors which are considered multi-target inhibitors given the variety of downstream effects caused by tyrosine kinases [57]. However, for some types of tyrosine kinase inhibitors, clinical trials do not show significant results unlike the *in vitro* trials. That is why, tyrosine kinase inhibitors are being tested in combination with other therapeutic drugs in order to overcome this case and revitalize the tyrosine kinase inhibitors through the synergistic effect of another therapeutic drug [58]. For example, tyrosine kinase inhibitor Nintedanib, showed significant results in phase II and III clinical trials when administered in combination with standard first-line treatment of carboplatin and paclitaxel, with a 18-28% response rate, and it even maintained a tolerable level of toxicity [59]. Cabozatinib,

another tyrosine kinase inhibitor, showed similar results in phase II trials when it comes to efficiency and toxicity levels [60]. Other tyrosine kinase inhibitors such as lapatinib, geifitinib, and erlotinib, are all under clinical trials and display a significant increase in response rate when used as part of first-line therapy, however considerable side effects such as rashes, diarrhea, and other toxicities are also obtained. Overall, more research must be conducted in order to determine the extent of efficiency resulting from the use of different tyrosine kinase inhibitors [58].

Aromatase inhibitors

Aromatase inhibitors, which target aromatase- synthase enzyme involved in estrogen production, are a novel approach nowadays [61]. Anastrazole, an aromatase inhibitor, was shown to be a highly efficient therapeutic oral drug. A phase II clinical trial performed on patients with persistent ovarian cancer, displayed that the majority showed upon its usage a tolerant condition of the disease, with a prolonged survival [62].

VEGF Inhibitors

VEGF is a major component of angiogenesis that allows tissue vascularization and angiogenesis and is known to play a major role in ovarian cancer progression and tumor development and is often being targeted in cancer research [63].

Bevacizumab, a promising monoclonal antibody targeting VEGF, lead to a prolonged survival rate for advanced epithelial ovarian cancer patients by 4 months [64]. This drug inhibits the binding of VEGF to its receptor inhibiting the activation of any downstream effectors leading to vascularization and further cell proliferation and metastasis and does

not show any associated cytotoxic effects as has been concluded in phase III clinical trials. Bevacizumab is currently being studied as a drug used in combination with other cancer therapeutics such as gemcitabine and carboplatin as a first-line treatment [65].

Other advanced therapeutic drugs would include verticillin A, which was recently shown to act as a cytotoxic agent against HGSOC cells. Salvi et al, showed that it acts by causing DNA damage to the tumor cells' genome. They also showed that when introducing the verticillin A to the cells, its encapsulation would lead to an enhanced efficiency and an uplift in its cytotoxic advantages [64], and this makes vertcillin A a considerable approach to ovarian cancer treatment [66]. However, targeting the stem cell population of the ovarian tumors should also be considered when assessing the potency of a possible anticancer agent.

B. Stem Cells

Understanding stem cell biology and its contribution to tumorigenesis is considered one of the most important fields in order to properly target and treat tumors. Cancer stem cells (CSCs) are a small population of cells residing within tumors with the potential of self-renewal, differentiation, and unlimited proliferation. Stem cells possess anti-apoptotic proteins, express DNA repair mechanisms and have several features that allow them to be highly resistant to chemotherapeutic agents [67].

Several studies report that an important feature of ovarian tumorigenesis and progression would be the potential of CSCs present among epithelial tissue cells to acquire mesenchymal characteristics through Epithelial Mesenchymal Transitions (EMTs). This

transition contributes to the invasion and metastasis of the tumor [24]. Unlike normal cancer cells which are influenced and destructed by chemotherapy, cancer stem cells remain unaffected, and this is attributed to their quiescent state and inactivity knowing that chemotherapeutics target active cells present in the S phase of the cell cycle, while cells in the quiescent state are typically present in the G0 phase. Tumor recurrence occurs when the previously dormant and inactive cancer cells wake up even more resistant to therapy than the primary ovarian tumor cells. All of those features play a role in the tumorigenesis, invasion, and chemoresistance of CSCs [68].

Several surface markers characterize ovarian cancer stem cells. CD133 is considered the most common CSC surface biomarker and is associated with tumorigenesis, chemoresistance, and tumor recurrence [69]. Other surface markers include CD44, CD24, EpCAM and ROR1 [70].

C. Natural Products

Phytochemicals are mainly primary and secondary metabolites produced by plants and serve as defensive agents against different pathogens. Those plant extracts are currently a major interest in the field of cancer research, mainly because they are considered accessible and uncostly and are of low toxicity to human beings. Several phytochemicals and natural extracts are currently being investigated for the purpose of cancer treatment and chemoprevention [71], those natural agents include resveratrol, a compound found in grapes which acts as an anti-skin cancer agent [72]. Another phytochemical would be curcumin, also known as diferuloylmethane, which is a phytochemical that has shown impressive results in targeting cell growth and proliferation, and cell survival through

multiple pathways [73]. Flavonoids are also important and ubiquitous phytochemicals, they are considered a structurally related family of metabolites found in fruits and vegetables and have also shown efficiency in treating and preventing different types of cancer [74]. Another natural plant component, TQ, also happens to be considered a promising anticancer agent.

D. Thymoquinone

1. Overview

Nigella Sativa, is an herbal plant originated in West Asia and is generally known as the black cumin seed. This plant has been in the medicinal field for a very long time and has a unique history in traditional and folk medicine mainly in Egypt, Greece, Turkey, Asia, Africa, and the Mediterranean region, and both the seeds and oils from Nigella Sativa are being used for healing and medicinal purposes. TQ, a Nigella Sativa oil component, is the most prominent constituent present in the Nigella Sativa, making up around 50% of its components [75].

TQ holds $C_{10}H_{12}O_2$ as a chemical formula, and its structure consists of a benzene ring bound to a methyl group at side 2 and an isopropyl group at side 5, and a double oxygen bond at positions 1 and 4 of the benzene ring [76]. It is currently being studied widely and shows a broad spectrum of pharmacological properties, and shows impressive effects as an antioxidant, anticancer, antibacterial, hypoglycemic, hepatoprotective, and renal protective agent. Those properties mainly arise from its phytochemical nature, since phytochemicals are known for their ability to interfere with signaling pathways and protect vital cellular

targets from the destructive actions of reactive oxygen species which are characteristics of an anticancer agent [77].

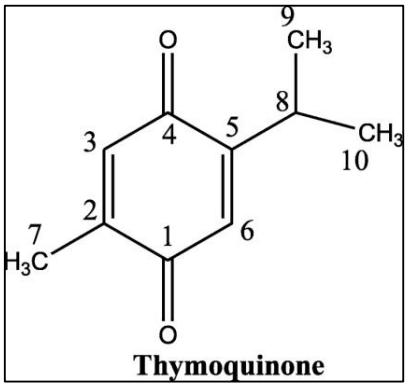


Figure 3. Chemical structure of TQ [Adopted from 14].

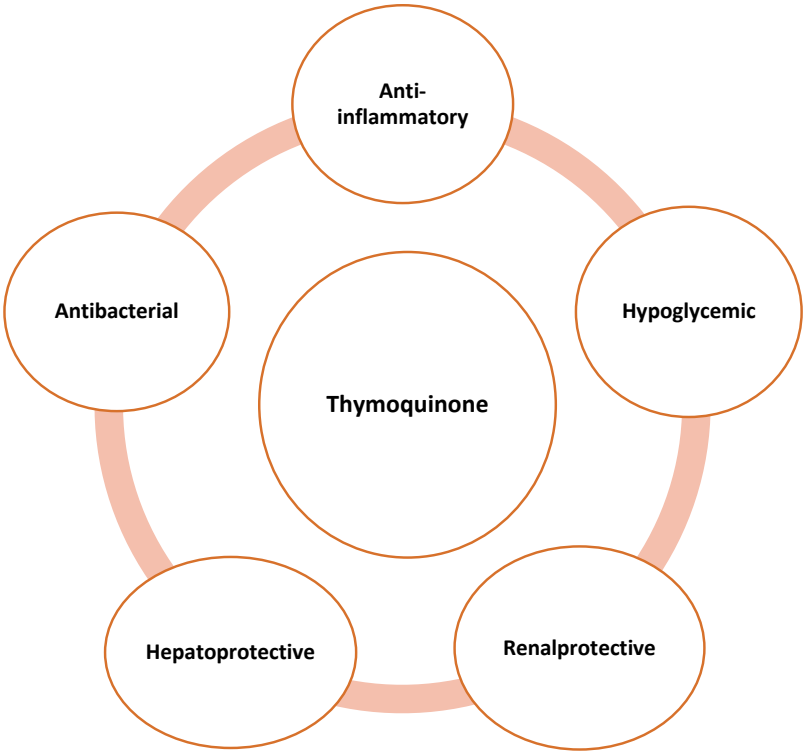


Figure 4. The different medicinal actions performed by TQ.

2. TQ and Cancer

TQ has been under the spotlight as a potential anticancer agent for quite some time, mainly because of its wide variety of anticancer activities, and because of its phytochemical nature and its influence at very small concentrations, mainly around <10 mg/kg, which favors it as a drug of low toxicity and safer to use than other drugs which perform at high concentrations [78].

There are many ways in which TQ has shown anticancer properties, and that is by inducing apoptosis, inhibiting cell proliferation and protein synthesis, arresting cell cycle and modulating different molecular targets [79].

In a recent studies performed on colorectal cancer cells, TQ was shown to induce apoptosis through acting on p-53 [80], and to cause oxidative DNA damage as it has shown on

5-Fluoroacil-resistant colorectal cancer and stem cells [81].

Similar results were also performed by TQ on lung cancer cells. The studies reported by Keat Ng et al, showed a significant elevation in p53 expression and downregulation of Bcl-2 expression as well [82]. In addition to this mode of action, TQ also promoted apoptosis on lung cancer cell lines in another study through activating the p-53 pathway and activating caspases- 3 and -9 [83].

For glioblastoma cells, TQ treatment showed a significant decrease in focal adhesion kinase (FAK) expression, leading to a reduction of extracellular signal-related kinase ERK phosphorylation and consequently a decrease in the secretion of metalloproteinases (MMP-2 and MMP-9) [84]. It also resulted in DNA damage by inhibiting telomerase enzyme activity and therefore inhibiting the growth of glioblastoma cancer cells [85].

TQ was also impressively efficient on renal cancer cells, where it was able to decrease mTOR signaling and activity, therefore, inhibiting migration, invasion, and EMT of the cancer cells [86]. TQ also contributed to the attenuation of the metastasis of renal cancer cells through the Wnt/Beta-Catenin pathway as well by downregulating the expression of mesenchymal markers while upregulating epithelial markers [87].

As for breast cancer cell lines, TQ has been evident to inhibit the expression of the transcription factor TWIST1 by inhibiting its promoter leading to an attenuation of EMT and inhibition of cancer growth and metastasis [88].

Despite the advancements and growing interest in TQ, its contribution to ovarian cancer cells remains. TQ has been shown to induce apoptosis in ovarian cancer cell lines by regulating Bcl-2 and Bax genes [89]. In another study, TQ was reported to exhibit its anti-cancer effects on ovarian cancer cell lines through inducing oxidative stress along with apoptosis [83]. Moreover, an enhanced cytotoxicity, and a more elevated chemotherapeutic action was exhibited when TQ was combined with cisplatin both in vitro and in vivo [90]. This synergism was also obtained when combing TQ, cisplatin, oxilaplatin and the phytochemical quercetin, where the use of TQ and quercetin was shown to sensitize the cells, including cisplatin-resistant cells, to the platinum drug combination [91]. However, the data regarding TQ's effect on ovarian cancer progression and prognosis remains very limited till today.

E. Aim of the Study

In our study, we aimed to assess the effect of TQ on OVCAR-420 and SKOV-3 cell lines. Both cell lines are human epithelial ovarian carcinoma cells, frequently used in

ovarian cancer research. OVCAR-420 originates from serous ovarian carcinoma while SKOV-3 originates from the ascites of a clear cell ovarian tumor, and is resistant to tumor necrosis factor and cisplatin. Therefore, we aimed to measure and analyze the ability of TQ to suppress the viability, proliferation, and migration of the OVCAR-420 and SKOV-3 ovarian cancer cell lines. Moreover, we want to evaluate the potency of TQ in targeting the CSC population for these two ovarian cancer cell lines by assessing the sphere-forming capacity and volume of the formed spheres using our previously established 3D sphere formation assay model [92].

CHAPTER II

MATERIALS AND METHODS

A. Cell Culture

Two ovarian cancer cell lines OVCAR-420 and SKOV-3 , were maintained and cultured in McCoy media (Sigma-Aldrich) supplemented with 10% of heat inactivated fetal bovine serum (FBS) (Sigma-Aldrich), 1% Penicillin/Streptomycin (Sigma-Aldrich) and Plasmocin™ prophylactic (Invivogen). Cell lines were checked using the ICLAC Database of Cross-contaminated or Misidentified Cell Lines confirming they are not misidentified or contaminated. Cells were incubated at 37°C in a humidified incubator containing 5% CO_2.

B. Drug Preparation and Treatment

TQ (Sigma-Aldrich: CAS: 490-91-5; 99.5% purity) was reconstituted in methanol prepared as per manufacturer's instructions into aliquots of concentration 10mM stored at 4 °C.

C. MTT Assay \Cell Proliferation

The anti-proliferative effects of TQ on OVCAR-420 and SKOV-3 were measured *in vitro* using MTT ([3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide]) assay according to the manufacturer's instructions (Sigma-Aldrich). In summary, cells (2×10^3 cells in 100µL full media, per well) were seeded in triplicates into 96-well culture plates (one for every time point: 24h, 48h and 72h) per each condition and incubated overnight at

37°C and 5% CO_2. Wells were randomly distributed across different treatment conditions – 3 wells/condition (Control: media only, Vehicle: Media + 0.1% Methanol, treatment groups: 5 μM, 25 μM, and 50 μM TQ in full media). Every 24 hours, media was removed and substituted with fresh media. At each time point, 10μL of MTT yellow dye (5 mg/mL in DMSO) was added per well and then the cells were incubated for 4 hours. After that, 100 μL of the solubilizing agent (isopropanol) was added to each well. After an hour, the absorbance intensity of every well was measured by the microplate ELISA reader (Multiscan EX) at 595 nm providing the optical density (OD) ratio of the cells for each condition. The percentage of cell proliferation was demonstrated as ratio of OD of treated wells to untreated ones (control).

D. Trypan Blue Assay /Cell Viability

Trypan Blue assay was used to study the effect of TQ on cell viability *in vitro*. OVCAR-420 and SKOV-3 cells (20×10^3 cells/well in 500 μL full media) were seeded in 3 different wells in 24-well culture plates (one for each time point: 24h, 48h, and 72h). Cells were incubated overnight at 37°C and 5% CO_2. Different conditions were applied on cells in duplicates (Control: media only, Vehicle: Media + 0.1% Methanol, treatment groups: 5 μM, 10 μM, 20 μM, and 50uM TQ in full media). At every time point, cells from each well were washed and treated with Trypsin and then cells were stained with Trypan Blue and counted on a hemocytometer under an inverted light microscope. The percentage of cell viability was determined as a ratio of viable cells counted in treated conditions to the count of viable cells in untreated conditions

E. Wound Healing Assay / Cell Migration

To assess the effect of TQ on the cell migration for both ovarian cancer cell lines, Wound Healing assay was used. OVCAR-420 and SKOV-3 (200×10^3 cells/well) were seeded in 12-well culture plates and incubated at 37℃ and 5% CO_2 until they were 90% confluent. Cells were then treated with 5 mg/mL of Mitomycin C (Sigma) for 1h in order to inhibit cellular proliferation. Two cross scratches were performed on each well using a sterile 200 µL pipet. Then, phosphate buffer saline (PBS) was used to wash the monolayer of cells twice in order to remove any detached cells and debris. The remaining wells were then distributed into duplicates of the following conditions: control containing complete media, vehicle containing 0.1% Methanol, and treated cells (in full media). Photographs of the wounds were taken using inverted light microscopy at the following time points: 6 hrs, 18 hrs, 24 hrs, and 48 hrs. The distance between the scratches was measured using ZEN Microscope Software (Zen 2.3).

F. 3D culture and Sphere-Formation Assay

1000 cells were seeded in a Matrigel™/serum free McCoy solution. 50µL of the mixture was carefully plated around the rim of each well of a pre-heated 24-well plate. The mixture was allowed to solidify at 37°C in a humified incubator for an hour. Wells were distributed into the following conditions each containing 500µL of its corresponding media: control (McCoy containing 3% FBS), vehicle (0.1% Methanol), treated wells (1µM, 2.5µM, and 5µM of TQ in McCoy with 3% FBS). The 500µL of each solution was gently added in the middle of the well and was changed regularly every 2 days. Spheres were counted and pictured after 5 and 10 days for OVCAR-420 and SKOV-3, respectively.

G. Statistical Analyses

Statistical analysis was achieved using GraphPad Prism 7 software. The significance of the data was determined using proper statistical tests, including the student t-test. P-values of $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***) were classified as significant, highly significant and very highly significant, respectively.

CHAPTER III

RESULTS

A. TQ reduces the cell proliferation index of OVCAR-420 and SKOV-3 cell lines *in vitro* in a time- and dose- dependent manner

The effect of TQ on the cellular proliferation of OVCAR-420 and SKOV-3 ovarian cancer cell lines *in vitro* was evaluated using the MTT assay. Our results showed that ovarian cancer cell lines exhibit a time- and dose-dependent reduction in proliferation when treated with TQ. A significant 60% inhibition of proliferation for OVCAR-420 ($p<0.05$) **(Figure 5)** and 50% inhibition for SKOV-3 **(Figure 6)** was achieved at 72hr when cell lines were treated with 50μM of TQ. Lower concentration of TQ (20μM) performed an anti-proliferative effect of 40% for OVCAR-420 and SKOV-3 cells. Based on the results obtained from both Trypan Blue and MTT assays, we decided to carry on the Wound Healing assay based on TQ concentration of 20μM.

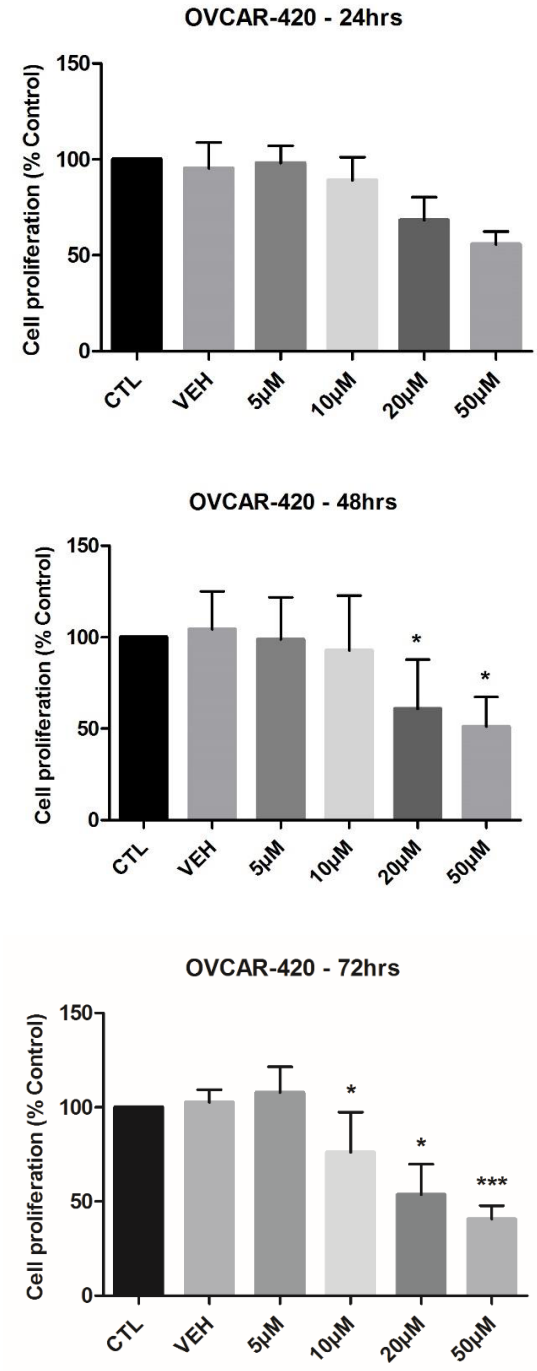


Figure 5. TQ reduces cell proliferation of OVCAR-420 cancer cells in a time- and dose-dependent manner. Cell proliferation was determined using the MTT assay after incubation for 24, 48, and 72 hrs. Results are expressed as percentage of the studied group compared to its control. Data represents an average of 4 independent experiments and are reported as mean

± SEM (SEM being represented by error bars; *P<0.05, **P<0.01, ***P<0.001).

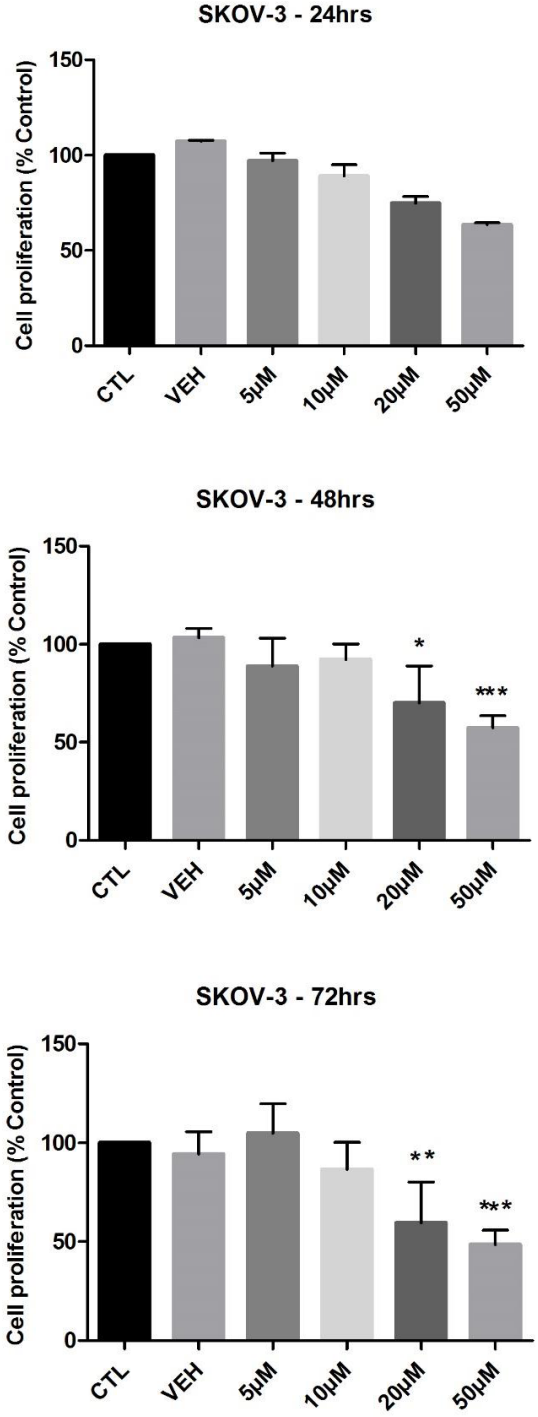


Figure 6. TQ reduces cell proliferation of SKOV-3 cancer cells in a time- and dose-dependent manner. Cell proliferation was determined using the MTT assay after incubation

for 24, 48, and 72hrs. Results are expressed as percentage of the studied group compared to its control. Data represents an average of 4 independent experiments and are reported as mean ± SEM (SEM being represented by error bars*P<0.05, **P<0.01, ***P<0.001).

B. TQ inhibits the cell viability of OVCAR-420 and SKOV-3 cell lines *in vitro* in a dose-dependent manner

Our aim was to investigate the effect of TQ on the cell viability of OVCAR-420 and SKOV-3 ovarian cancer cell lines *in vitro*. To achieve this aim, Trypan Blue exclusion assay was used. First, we monitored the culture conditions for cells to reach 100% confluency after 72hrs in culture as shown in Figures 7A and 7B. OVCAR-420 and SKOV-3 cells were then seeded to achieve 50%, 70%, and 100% confluency after 24, 48, and 72hr in culture. Cells were treated with TQ concentrations of 5, 10, 20, 50μM and counted after 24, 48, and 72hr. Our results showed that the ovarian cancer cell lines exhibit a dose-dependent reduction in viability with a significant reduction of 80% for both cell lines at an early time point of 24hrs when treated with a TQ dosage of 20μM ($p<0.05$). It should be noted that SKOV-3 were more resistant to treatment than OVCAR-420 as shown at 5μM and 10μM. Interestingly, 50μM of TQ achieved almost complete elimination of viable cells for both OVCAR-420 and SKOV-3 starting at 24 hrs ($p<0.05$) **(Figures 8A, 9A)**. Representative phase contrast images of both cell lines treated with 20μM TQ for 24, 48 and 72hrs are presented in **Figures 8B and 9B**.

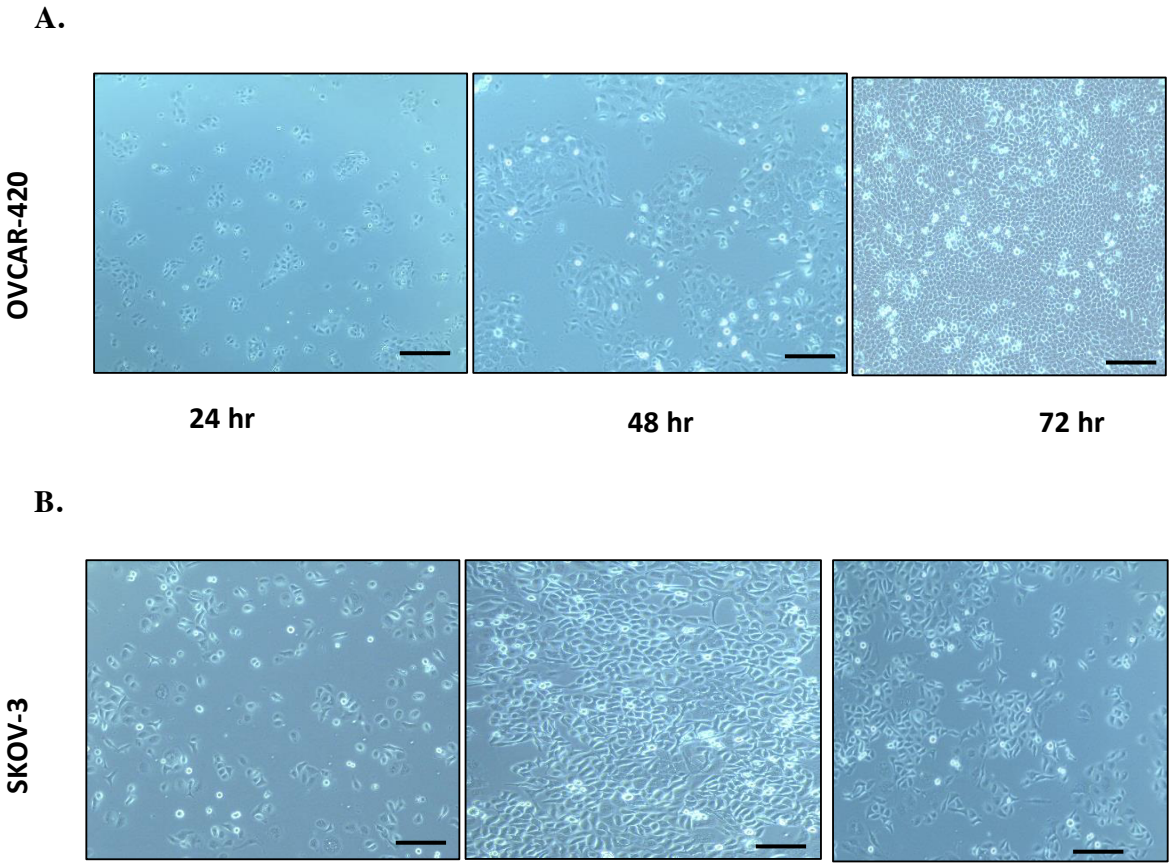
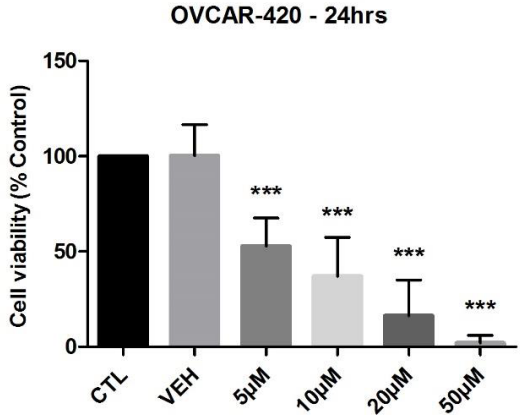


Figure 7. Representative images of OVCAR-420 cell lines (A) and SKOV-3 cell lines (B) at 24, 48, and 72hrs. Images were acquired using ZEN axiovert light microscope. Scale bar = 100µM.

A.


OVCAR-420 - 24hrs
Cell viability (% Control)
150
100
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CTL
VEH
5μM
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20μM
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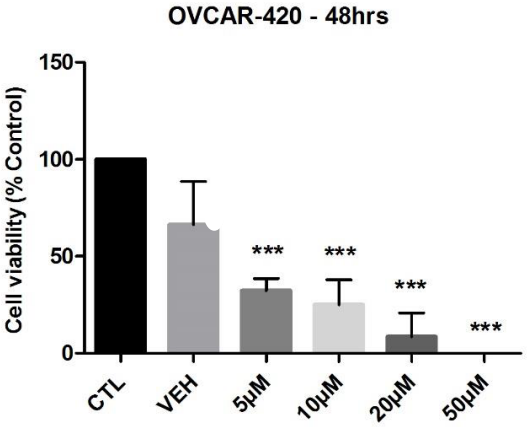
OVCAR-420 - 48hrs
Cell viability (% Control)
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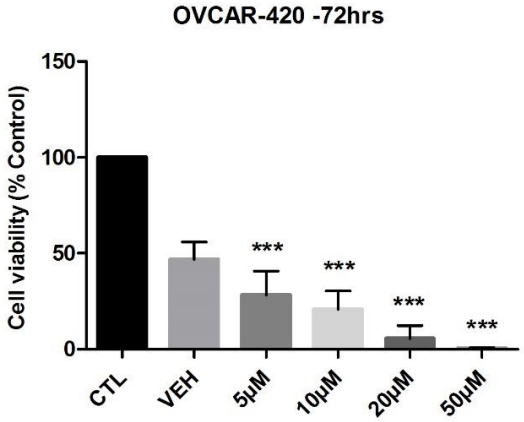
CTL
VEH
5μM
10μM
20μM
50μM


OVCAR-420 -72hrs
Cell viability (% Control)
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B.

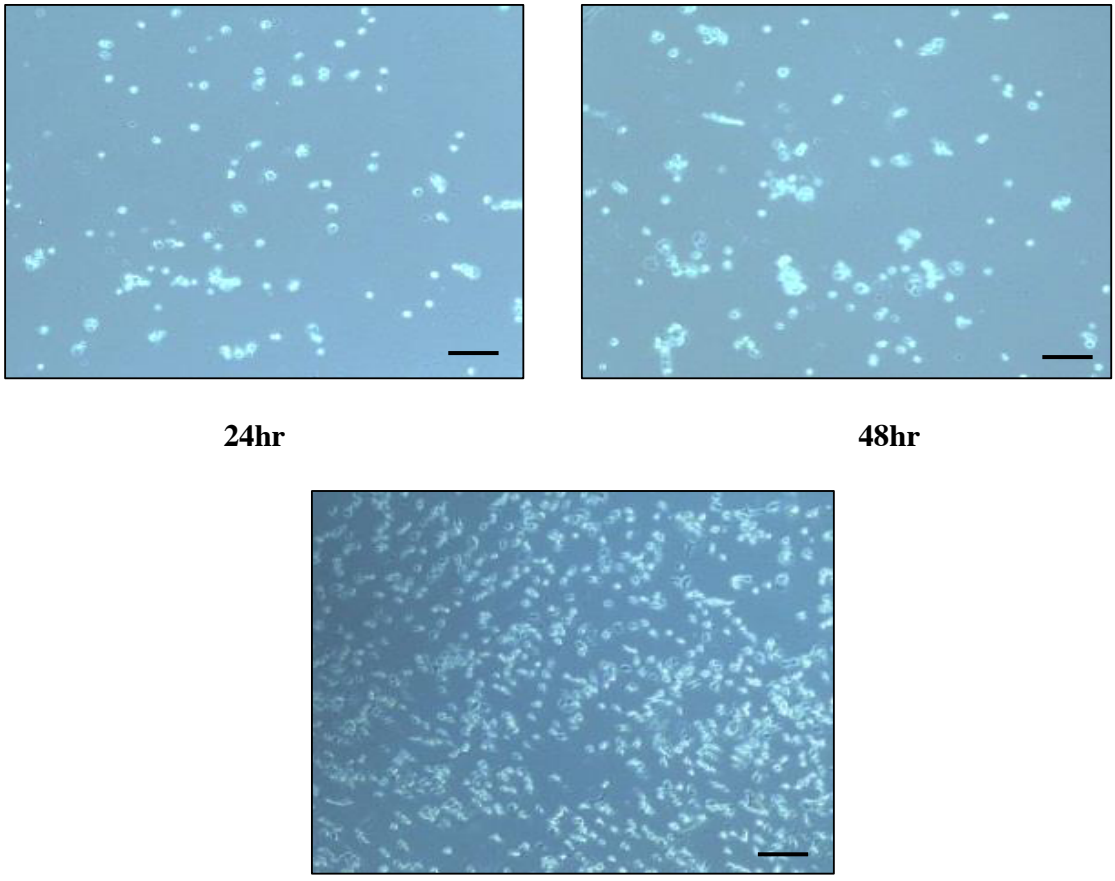
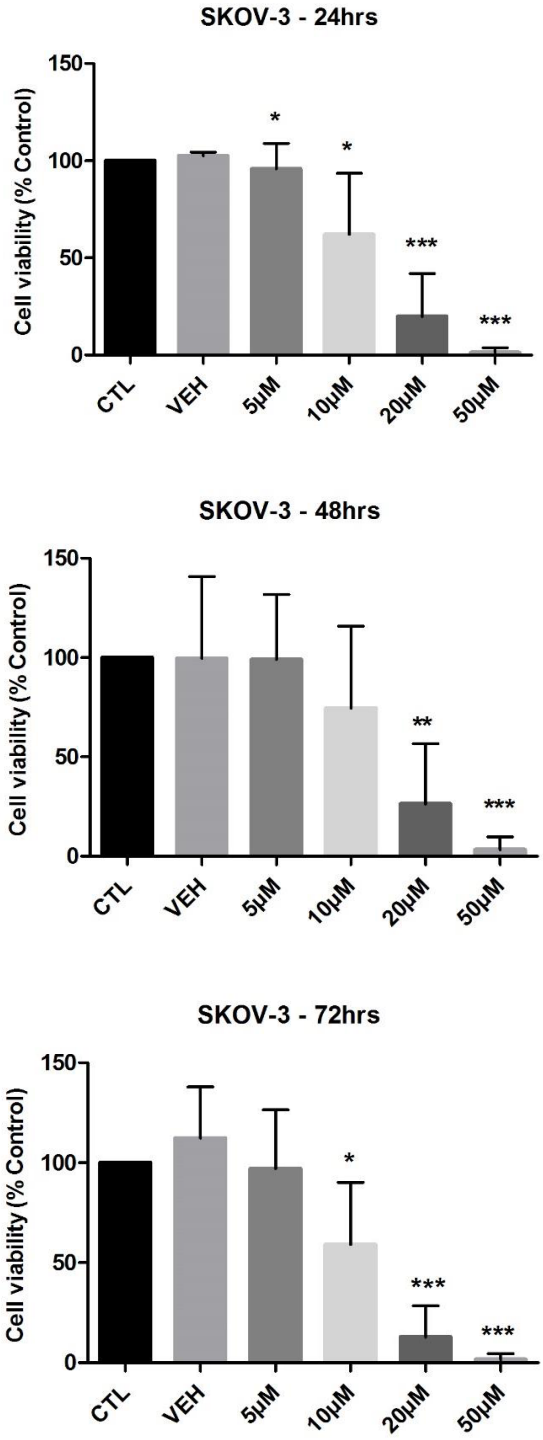


Figure 8. TQ reduces the cell viability of OVCAR-420 cancer cells in a dose-dependent manner. Cell viability was determined using the trypan blue assay for 24, 48, and 72 hrs. TQ decreases the percentage of viable cells in a dose-dependent manner where cells were reduced significantly upon treatment with 20μM of TQ at an early stage of 24hr time point. Results are expressed as percentage of the studied group compared to its control **(A)**. Representative images at each time point were taken for the cells treated with 20μM TQ using ZEN axiovert light microscope. Scale bar = 100μm **(B).** Data represents an average of 4 independent experiments and are reported as mean ± SEM (SEM being represented by error bars; *P<0.05, **P<0.01, ***P<0.001).

A.


SKOV-3 - 24hrs
Cell viability (% Control)
150
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SKOV-3 - 48hrs
Cell viability (% Control)
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SKOV-3 - 72hrs
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B.

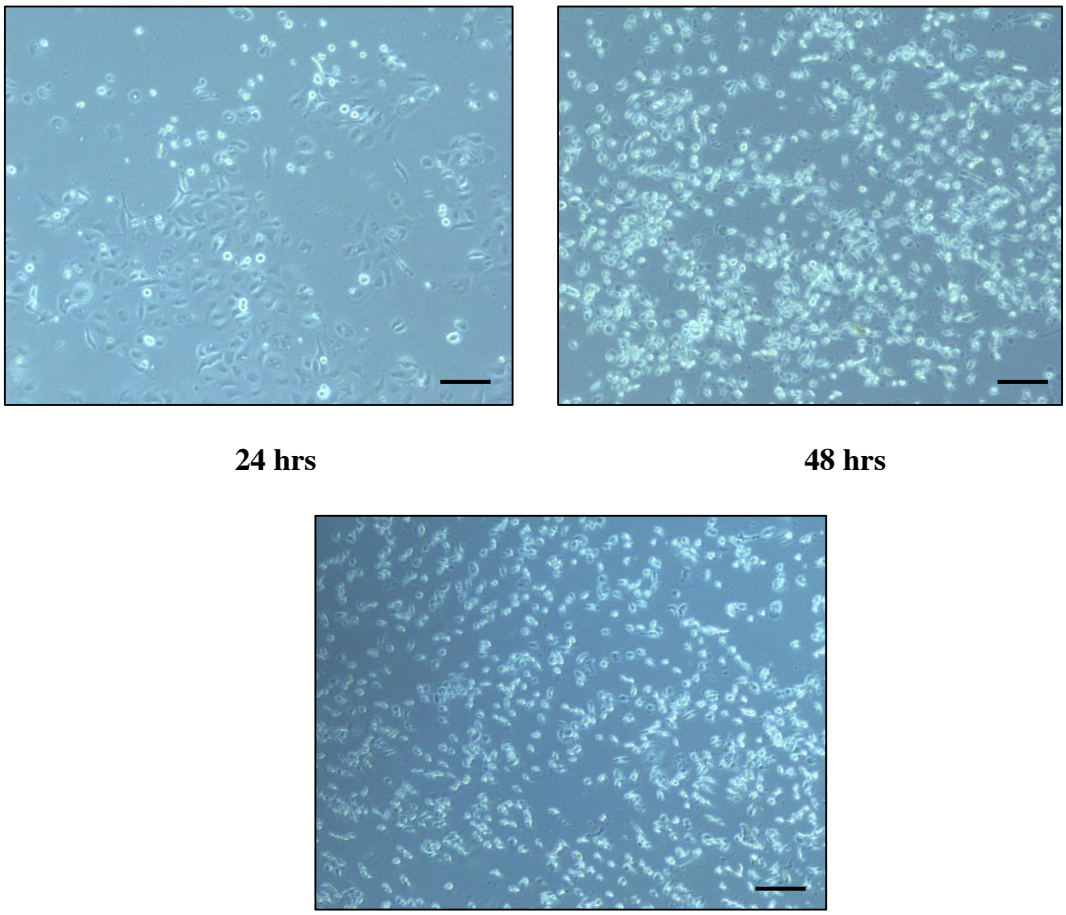


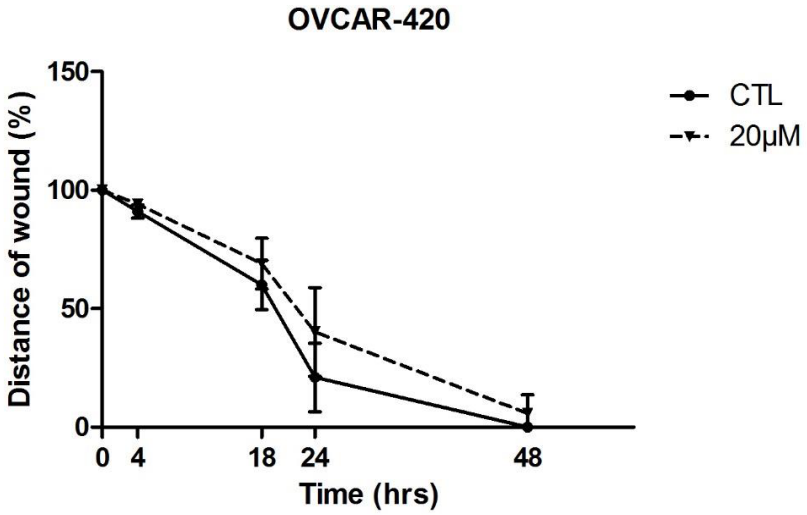
Figure 9. TQ reduces the cell viability of SKOV-3 cancer cell lines in a dose-dependent manner. Cell viability was determined using the trypan blue assay for 24, 48, and 72 hrs. TQ decreases the percentage of viable cells in a dose-dependent manner where cells were reduced significantly upon treatment with 20μM of TQ at an early stage of 24hr time point. Results are expressed as percentage of the studied group compared to its control **(A).** Representative images at each time point were taken for the cells treated with 20μM TQ using ZEN axiovert light microscope. Scale bar = 100μm **(B).** Data represents an average of 4 independent experiments and are reported as mean± SEM (SEM being represented by error bars; *P<0.05, **P<0.01, ***P<0.001).

C. TQ reduces the migration of OVCAR-420 and SKOV-3 cell lines *in vitro*

Ovarian cancer cells most often metastasize into other tissues such as the lymph nodes, spleen and liver. We used wound healing assay, which mimics the migration of cells, to study the effect of TQ on cellular migration of OVCAR-420 and SKOV-3 which is the main reason behind the aggressive invasion of ovarian cancer. As mentioned earlier, we treated the wounded cells with 20μM TQ as opposed to a control group.

Our results showed that the wound was almost completely closed for both cell lines within 48 hours for the control group **(Figure 10, 11, and 12)**. However, 20μM of TQ suppressed cell migration and the closure of the wound starting at 4 hours with a 20% suppression observed at 24hrs for both cell lines as opposed to the control group. Interestingly, even after 48hrs, wound closure remained suppressed specifically for the SKOV-3 cell lines despite complete wound closure for untreated cells **(Figure 10).** This implies that TQ has the potential to inhibit the migration of ovarian cancer cells and therefore influence the invasion of ovarian cancer.

A.



B.

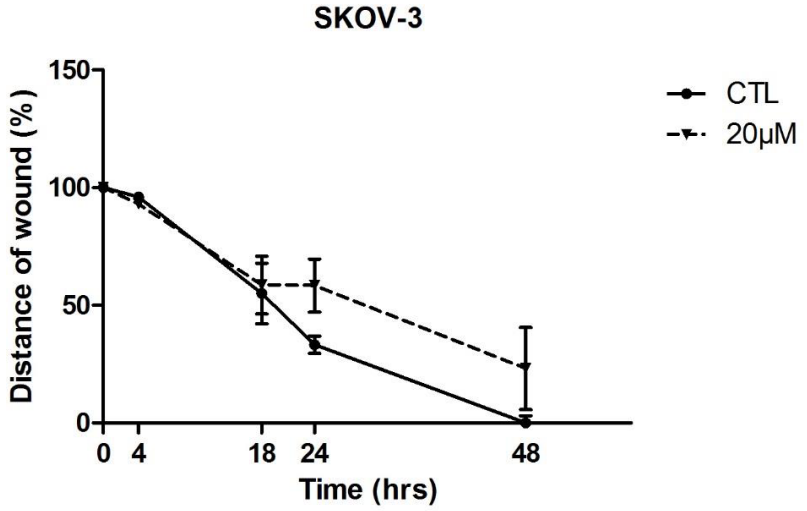


Figure 10. TQ reduces the migratory potential of OVCAR-420 and SKOV-3 cell lines. Results indicate the ability of TQ to inhibit the migratory potential of OVCAR-420 and SKOV-3 cell lines at different time points (4, 18, 24, 48hrs). At 24hrs post-treatment, TQ inhibited cell migration of OVCAR-420 and SKOV-3 cell lines by 20% with respect to the control and the inhibition remained even upon 48hrs post-treatment for the SKOV-3 cell lines. Data are reported as mean ±SEM.

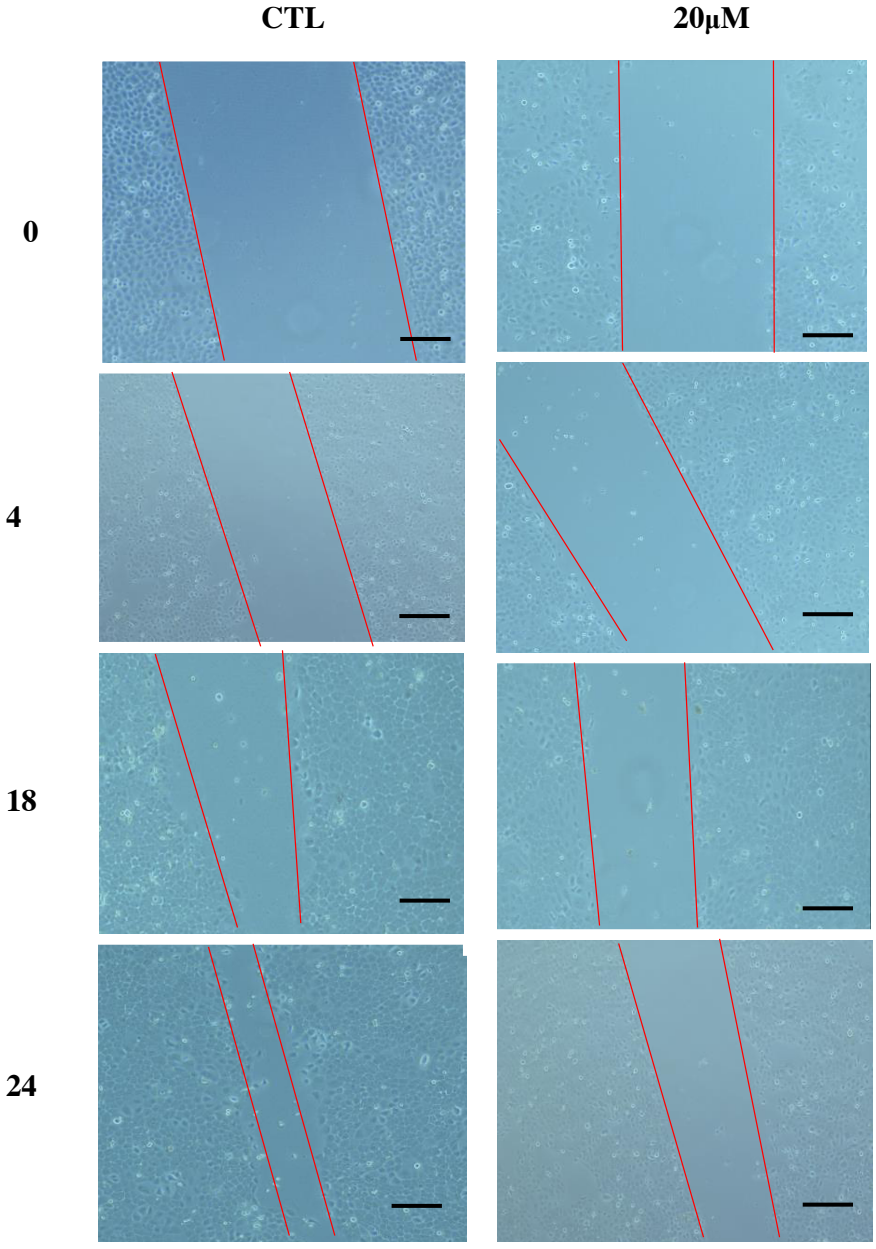


Figure 11. Representative bright field images of OVCAR-420 cells showing effect of 20µM TQ on cell migration. A scratch was applied to the monolayer of confluent cells seeded in 12-well plates with Mitomycin using a 20µL pipette tip. The distance between the cells with or without treatment was assessed at 4 time points: 4, 18, 24, and 48hrs. Scale bar= 100µm.

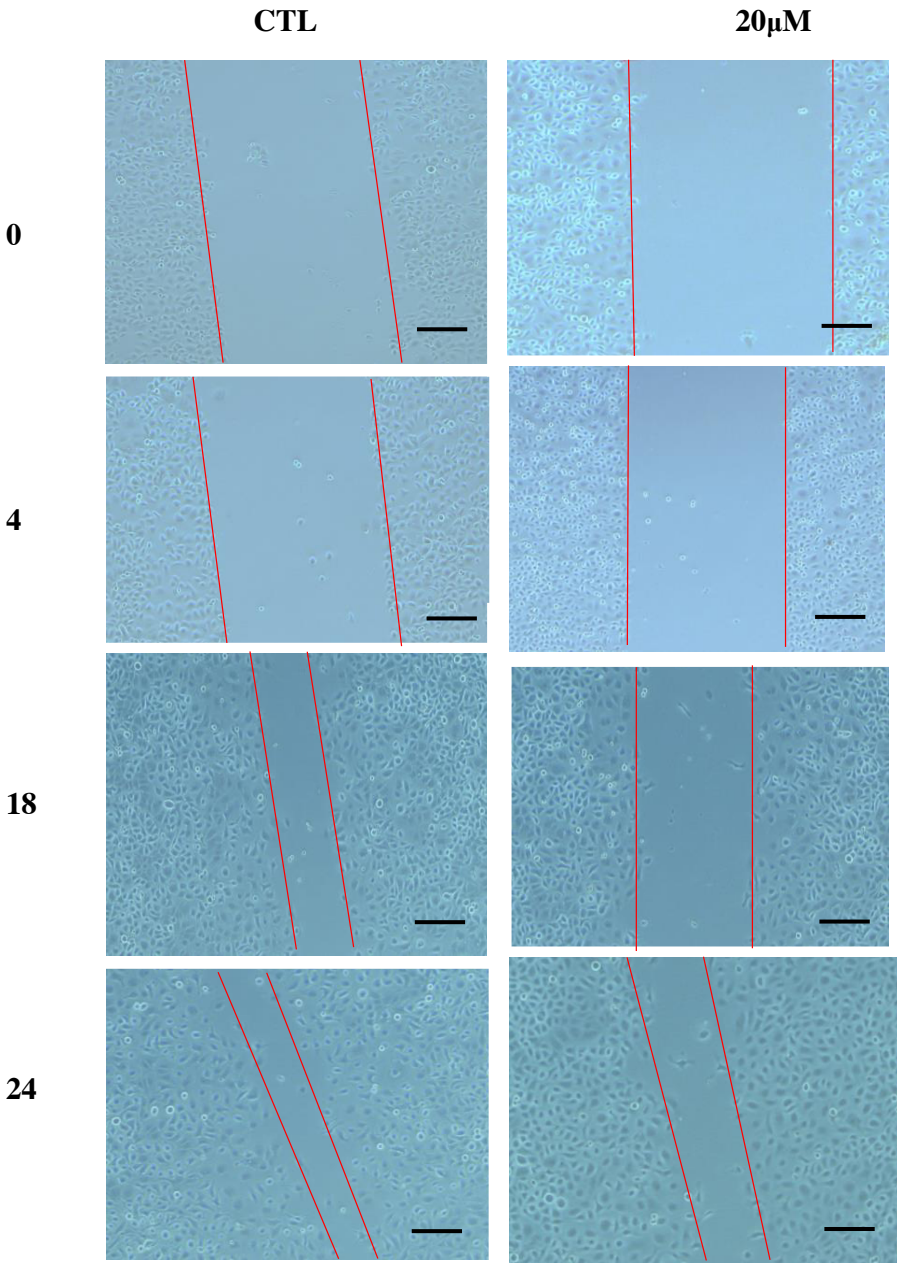
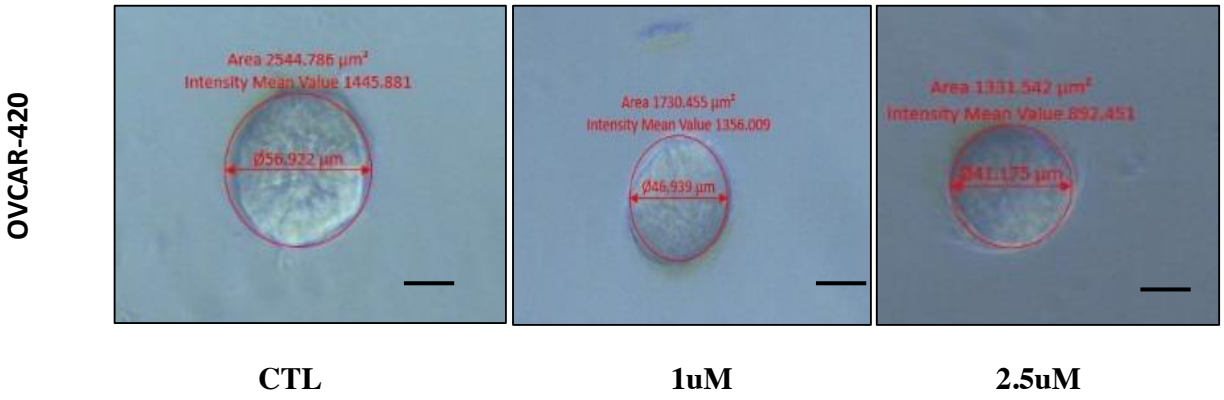


Figure 12. Representative bright field images of SKOV-3 cells showing effect of 20µM TQ on cell migration. A scratch was applied to the monolayer of confluent cells seeded in 12-well plates with Mitomycin using a 20µL pipette tip. The distance between the cells with or without treatment was assessed at 4 time points: 4, 18, 24, and 48hrs. Scale bar = 100µm.

D. TQ inhibits the sphere- forming ability of OVCAR-420 and SKOV-3 cell lines in 3D

Furthermore, we aimed to study the effect of TQ on cancer stem cells using the Spheres Formation assay. Single cell suspensions of OVCAR-420 and SKOV-3 cell lines were seeded in Matrigel™ and cultured. Then, the formed spheres were counted and images were taken at day 5 for OVCAR-420 and day 10 for SKOV-3 using ZEN software (**Figure 13**). This was followed by analyzing the count and size of spheres and calculating the SFU for each cell line using the formula (number of spheres counted \ Number of input cells) × 100. Treatment with TQ resulted in a decrease in the count and size of the obtained spheres in a dose dependent manner compared to the control. While using 1μM of TQ did not influence the sphere forming ability of the cells, a 2.5μM dosage exhibited a dramatic effect on the SFU and the size of the spheres. It should be noted that the inhibition of spheres formation and growth was achieved at a significantly low TQ concentration of 2.5μM compared to 2D culture assays such as in the MTT, Trypan Blue, and Wound Healing assays, which required a much higher concentration (20μM) to achieve significant differences.
Our results showed that 2.5μM of TQ resulted in a significant 70% and 90% decrease in the SFU of OVCAR-420 and SKOV-3, respectively (**Figure 14**). Similarly, a significant decrease in the diameter of the formed spheres was obtained upon treatment with 2.5μM of TQ. The formed spheres belonging to the OVCAR-420 showed a reduction in diameter by 50% while those belonging to SKOV-3 showed a 40% reduction (**Figure 15**). Interestingly, a higher concentration of 5μM resulted in complete absence of spheres.

A.



B.

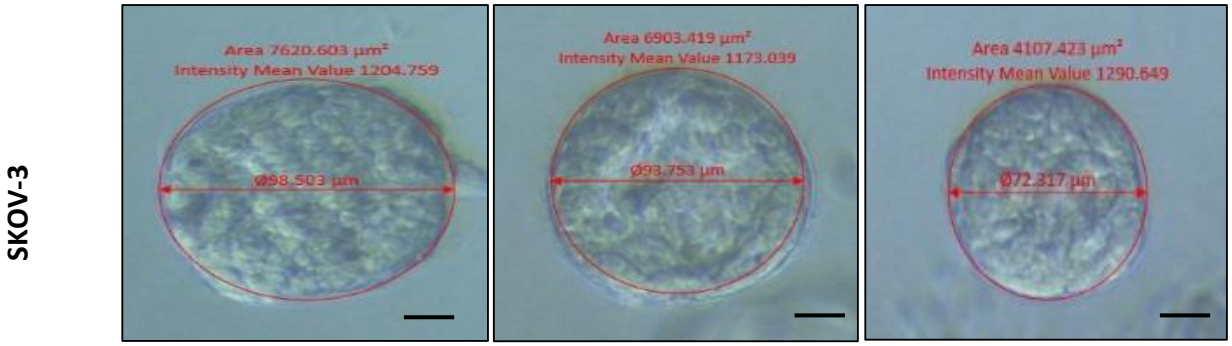


Figure 13. Effect of TQ on the sphere-forming ability of OVCAR-420 (A) and SKOV-3 (B).

Representative images were taken for the G1 spheres of both cell lines with and without treatment with TQ. Images were visualized by Axiovert inverted light microscope and analyzed by ZEN image software. Scale bar = 100µm.

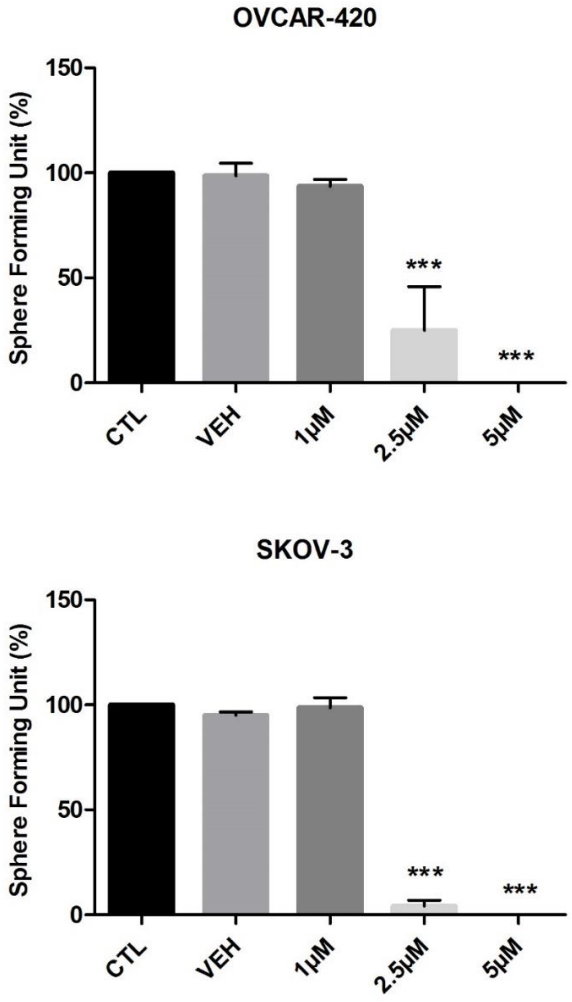


Figure 14. TQ reduces the sphere-forming units of the OVCAR-420 and SKOV-3 cultured spheres in a dose-dependent manner. Cells were seeded in Matrigel™ and G1 (generation 1) spheres were formed and counted for the OVCAR-420 and SKOV-3 cell lines at day 5 and day 10 respectively. Results are expressed as SFU which is calculated according to the formula: number of spheres counted \ Number of input cells) × 100. 2.5μM was able to achieve 70% and 90% reduction of SFU for the OVCAR-420 and SKOV-3 cell lines respectively. A higher dose of 5μM resulted in complete elimination of the SFU of both cell lines. Data is reported as mean ± SEM (SEM represented by error bars; *P<0.05, **P<0.01, ***P<0.001).

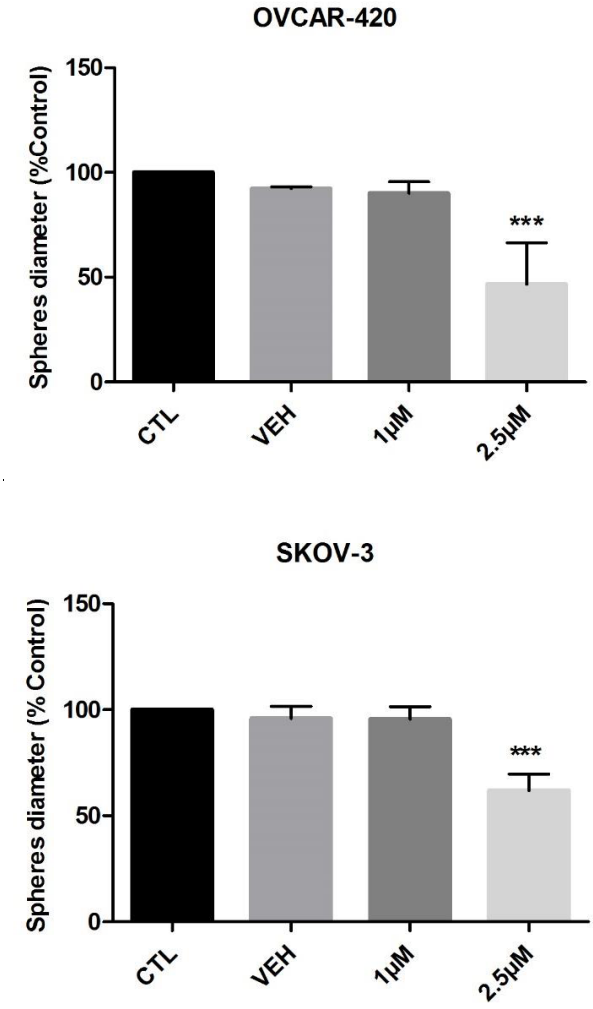


Figure 15. TQ diminishes the size of OVCAR-420 and SKOV-3 cultured spheres in a dose-dependent manner. Using an inverted light microscope, images of the formed OVCAR-420 and SKOV-3 spheres were taken, and the images were analyzed using ZEN software. 2.5μM was able to achieve 50% and 40% reduction of in the size of spheres for the OVCAR-420 and SKOV-3 cell lines, respectively. Results represent the average diameter of 30 fully formed spheres. Values are presented as percentages with respect to the size of spheres for the control. Data is reported as mean ± SEM (SEM represented by error bars; *P<0.05, **P<0.01, ***P<0.001).

CHAPTER IV

DISCUSSION

Ovarian cancer is a lethal malignancy mostly arising from the surface epithelium of the ovaries and fallopian tubes [93]. The standard treatment involves both surgery and the use of chemotherapeutic drugs in order to eradicate all cancer cells. However, recurrence most often takes place despite the combination of treatments [94]. Therefore, a novel therapeutic inhibiting the growth and proliferation, preventing migration and metastasis, and targeting the stem cell population is much needed for effective treatment of ovarian cancer. Other important features that are also widely considered for an effective therapeutic agent are its cost, availability, and biosafety which are all characteristics of natural agents, mainly plant extracts such as TQ. TQ has shown promising action against different types of cancers. However, the effect of TQ on ovarian cancer cells remains vague and more research is required in order to comprehend it [95]. Our study aimed to investigate the effect of TQ on the proliferation, migration, and stemness of OVCAR-420 and SKOV-3 ovarian cancer cells. Our results showed positive outcomes in several different assays that represent different cellular mechanisms and pathways, and we displayed an inhibition in viability and proliferation of the ovarian cancer cell lines upon treatment with TQ, and an inhibition in cellular migration and in the sphere-forming ability of the cells. So, it's considerable that TQ can act on different pathways and affect a variety of cellular proteins corresponding to ovarian cancer cell lines or that it acts on a meeting point between those pathways to generate such effects.

Apoptosis, also known as programmed cell death, is a gene-regulated phenomenon associated with multiple proteins and transcription factors. In our study, TQ showed significant inhibition of the survival and proliferation of both OVCAR-420 and SKOV-3 cell lines. This indicates that TQ may act through inducing apoptosis or DNA damage to the cancer cells. This hypothesis is consistent with several studies performed on different types of cancer that have proven TQ to be an effective apoptotic agent [96]. Results of the study done by Liu et al., on SKOV-3 cell lines showed that TQ effectively activates apoptosis by regulating Bcl-2 and Bax genes [90]. Similarly, downregulation of Bcl-2 gene was also obtained for renal carcinoma Caki cells upon treatment with TQ [97]. In another study done on osteosarcoma cell lines, 25μM of TQ was able to induce both apoptosis and cell-cycle arrest 6 hours after treatment. The concentration used by this study is similar to the one used in our MTT and trypan blue assays which showed a reduction in cell viability and cellular proliferation 24hrs after treatment [98]. Similar results were also obtained by Gali et al, but attributed those results to TQ acting via p53-dependent mechanisms [80]. Our results are also supported by few studies showing how TQ acts through inducing DNA damage. For example, both double-strand breaks and single-strand breaks were observed on glioblastoma cells upon treating those cells with 25μM of TQ which is also similar to the concentration we used to display significant inhibitory results in cell viability and proliferation [99]. What should be noted is that the SKOV-3 cell lines showed more resistance to treatment than the OVCAR-420 cell lines, and this could be ascribed to the fact that the SKOV-3 cell lines belong to advanced stage tumor ascites [100].

Even though there are currently no investigations around the effects of TQ on ovarian cancer cells migration specifically, some studies do display the anti-metgratory role for TQ

on other types of cancer. Potent dose-dependent inhibition of migration and invasion was obtained in a study performed on liver, colon, skin, and breast cancer cells when those tumor cells were treated with TQ. In another in vitro and in vivo study, inhibition of migration and invasion was also obtained on human pancreatic carcinoma cell lines by TQ [101]. Those results are consistent with ours where 20% suppression of migration was achieved at 24hrs by 20uM of TQ for both SKOV-3 and OVCAR-420. Interestingly, in previous studies, the diminished migratory ability was attributed to inhibition of NF-kB activity. NF-kB is a transcription factor that mediates the gene expression of a variety of proteins and is involved in cellular proliferation and survival, so it is safe to hypothesize that TQ can induce multiple inhibitory effects through acting on NF-kB. Inhibition of metastasis was also correlated in several studies with the inhibition of EMT, which is a key process in the invasion of cancer cells [88, 102]. Knowing that EMT and invasion are also associated with angiogenesis, it should be noted that several studies also showed an inhibition of angiogenesis and a suppression of the activity of the angiogenic factor VEGF by TQ. TQ was able to potently reduce the production of VEGF in different types of cancer cells such as human pancreatic carcinoma cells and multiple myeloma cells [103, 104]. Similar results were also obtained *in vivo* when treating rat models bearing colon carcinogenesis [105]. In a study done by Seth et al, 25μM of TQ resulted in downregulation of VEGF in chronic myeloid leukemia cells which may indicate that TQ suppresses migration and invasion by targeting VEGF angiogenic factor through the NF-kB pathway [106].

Knowing that the recurrence of ovarian cancer is mainly attributed to the presence of the slowly dividing dormant CSCs [107], we tested the effect of TQ on the ovarian cancer stemness and showed promising results in inhibiting the SFU of ovarian cancer cells and

diminishing the size of the formed spheres. Interestingly, unlike the results obtained for the 2D assays, TQ showed more efficiency when targeting the SFU of SKOV-3 compared to OVCAR-420 cell line. We also noted that the concentration of TQ required to exert inhibitory effects on the ovarian cancer spheres is distinguishingly lower than that required to exert inhibitory effects on cellular viability, proliferation, and migration. Moreover, despite the many studies showing impressive results for TQ on cellular proliferation, growth, and migration, studies tackling the effect of TQ on cancer stem cells remain limited. Recent experiments performed by our lab displayed the effect of TQ in 3D culture on colorectal cancer cells. Those experiments showed that a small concentration of TQ (3μM) established an inhibitory effect on the sphere-forming ability of colorectal cancer cells. Furthermore, the results also showed that TQ reduced the expression of CD44 which is a multifunctional cell surface molecule and an important stem cell marker. A further investigation done on the formed spheres also showed reduced expression of tumor cell proliferation markers along with a reduced expression of cell adhesion molecules which therefore indicates a reduction in the oncogenic potential of the tumor cells and EMT [81]. This implies that the suppression of the sphere-forming ability of those cells is associated with a reduction in cellular proliferation and stem cell markers. Besides, the results were also associated with an upregulation of cytokeratin epithelial markers, indicating an inhibition in epithelial mesenchymal transitions as well [81].

In summary, our investigation showed that TQ can exhibit anti-proliferative and anti-migratory effects on ovarian cancer cells. We also showed that TQ can induce inhibition of the sphere-forming ability of the cells and suppression in the size of the formed spheres, indicating a promising effect in targeting the stem cell population of ovarian tumors. This

research along with previous experimental data suggests that this natural, non-toxic, and inexpensive plant extract is a promising anti-cancerous agent that should be under more intensive research and investigation in order to further understand its mechanism of action as a potential therapeutic agent to be used as treatment alone or in combination with other therapeutic drugs.

Future perspectives: we will detect the expression of the different proteins that could be potential TQ targets such as Bcl-2, Bax, NF-kB in order to establish the mechanism by which TQ acts on ovarian cancer cells and find out its correlation to the different signaling pathways that are associated with TQ such as the Wnt pathway and Akt\PI3 pathway. To achieve this aim, we will be using PCR, Western Blot, and Immunofluorescence techniques. Finally, in order to validate its prognostic role, TQ must be applied in clinical trials. Moreover, despite the many advantages of TQ, its poor bioavailability remains a limitation that should be kept under consideration. Many approaches to enhance the bioavailability of TQ revolve around the development of nanoparticles which effectively deliver TQ and therefore contribute to an increase in its bioavailability [96] and to further enhance this delivery process, intravenous administration is also encouraged. This carrier system is considered to be efficient and safe, suggesting that TQ with its promising potential and mode of action could be effectively delivered to act accordingly [108].

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