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Assessment of the Potential Anti-tumour Effect of Salvia triloba and the Combination of Salvia triloba and Paclitaxel on Ovarian Cancer: SKOV3 Cell Line

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THE AMERICAN UNIVERSITY IN CAIRO
الجامعة الأمريكية بالقاهرة

School of Sciences and Engineering

Graduate Program

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A Thesis Submitted to Biotechnology Master's Program

In partial fulfilment of the requirements for the Master of Sciences in Biotechnology

By:

Naela Adel Mohammed Saleh

Under the supervision of:

Dr. Asma Amleh

Dec 2020

The American University in Cairo (AUC)

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Has been approved by

Thesis Committee Supervisor/Chair_____

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Affiliation _____

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Dept. Chair/Director Date Dean Date

Dedication

To the children of Yemen, who are dying from hunger, and have not seen schools for years.

To the people of Yemen, who are facing the world's worst humanitarian crisis.

To those people in Yemen, who are suffering from hunger and malnutrition.

To the millions of Yemenis who do not know when they will eat next.

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Abstract

Cancer remains one of the most lethal diseases in the world, and ovarian cancer is the primary cause of mortality among gynecologic cancer cases. Most existing chemotherapies, when applied individually and separately, are both toxic and non-effective. Herbal plants have been studied for decades to be employed in anti-cancer medication due to their efficacy. *Salvia triloba*, generally known as Greek Sage, has been traditionally used in the Middle East for medical treatment. There have been recent studies associated with the anti-cancer ability of *Salvia triloba* against both breast cancer and skin cancer. However, no evidence has been found for a study that associates *Salvia Triloba* with anti-cancer ability for ovarian cancer.

In this study, we investigated the anticancer effects of *Salvia triloba* using SKOV3 ovarian cancer cell line. Cell viability and cell migration were both studied through applying *Salvia triloba* acetone crude extract as a single treatment and, as well, in combination with Paclitaxel, using MTT, Trypan Blue Exclusion test of cell viability, and Trans-well assays. Gene expression was also studied using quantitive PCR. The CompuSyn software was used to obtain data regarding the type of combination effect.

The results obtained proposed that *Salvia triloba* in combination with Paclitaxel could potentially reduce both cell viability and migration on SKOV3 cell line through controlling the expression of key genes in signaling pathways, which play a critical role in ovarian cancer progression.

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List of Abbreviations

ANOVA	Analysis of Variance
APC	adenomatous polyposis coli
AR	Androgen receptor
β-catenin	Beta Catenin
BAX	BCL2 Associated X
BRM	Biological response modifiers
PTEN	Phosphatase and tensin homolog
CI	Combination Index
COX-2	Cyclooxygenase-2
DMEM:	Dulbecco's Modified Eagle's medium
DMSO	Dimethyl Sulfoxide
DNA:	Deoxyribonucleic acid
DRI	Dose Reduction Index
E2	Estradiol (E2)
ER	Estrogen Receptor
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
FA	Fraction Affected
FBS	Fetal bovine serum
GSK3β	Glycogen Synthase Kinase 3 beta
IC50	Half-maximal inhibitory concentration
JNKs	c-Jun N-terminal kinases
LEF	lymphoid enhancement factor
MAPKs	Mitogen-activated protein kinases
MDM2	the murine double minute 2
MDR	Multi-drug resistant
MEP	Median-effect principle
mRNA:	Messenger ribonucleic acid
MTT:	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
N-cadherin:	Neural cadherin
OC	Ovarian cancer
qPCR	Quantitative polymerase chain reaction (qPCR)
p53	Tumor protein p53

Paclitaxel	PTX
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
PEG-2	Polyethylene glycol -2
PI3KR1	Phosphatidylinositol 3-kinase regulatory subunit alpha
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PR	Progesterone receptor
PTEN	Phosphatase and Tensin Homolog
RPMI	Roswell Park Memorial Institute Medium
RNA	Ribonucleic acid
RUNX2	Runt-related transcription factor 2
SET7	SET Domain-Containing Protein 7
S. triloba	*Salvia triloba*
TCF	T-cell factor
TLC	Thin Layer Chromatography
TNF	Tumor necrosis factor
UV	Ultraviolet

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1. Introduction

1.1 Cancer

Cancer is one of the most lethal diseases in the globe. The figures show that more than 16% of the deaths in the world are due to cancer, and most of the deaths take place in poor and middle-income countries (WHO, 2018). It is estimated that there were a total number of 18.1 million new cancer cases, with 9.6 million cancer deaths in 2018 (WHO, 2018). Number of cancer cases increases each year. Heart related diseases are the leading cause of deaths now. However, it is expected that cancer would become the top cause of deaths in 21st century according to experts in the field (HPW, 2018). The risk of incidence of cancer is very high, and it is estimated that 1 in 8 males and 1 in 10 females will develop cancer during their lifetime (HPW, 2018). The three leading types of cancer, in terms of occurrence, are respectively: lung, women breast, and colorectal cancers, and they are among the top five lethal cancers.

Lung cancer is the most fatal type of cancer, and colorectal cancer is the second most lethal one while breast cancer is the fifth (Bray et al., 2018). Cancer is a disease in which cells grow abnormally without control. The cells in the human body are constantly renewed to replace the mutated and damaged cells (Bollinger, 2015). This is generally the normal process inside the human body, but if the mutated cells accumulate without disposal, then they grow and build-up tumor, which could cause cancer. Any human at any age, regardless of location and ethnicity, is susceptible to cancer, but elderly people have generally higher chances of exposure. This is because more mutated cells accumulate with age, and cause cancer. Almost 90% of cancer cases in the UK are for people, who are 50 or more, and this is applicable to nearly all cancer types (CR-UK, 2016).

Many factors are associated with cancer risk, and some of the factors can be controlled while other cannot. The factors that can be controlled mainly relate to the lifestyle, such as: weight and obesity, smoking and consuming alcohol (Bollinger, 2015). The factors, which are beyond a person's control, are: age, sex and family history (Bollinger, 2015). There are also other considerable environmental elements, such as: immunity system, air pollution, exposure to chemicals and radiation. There are more than 200 different kinds of cancer, but there are 5

major cancer groups based on the cell where cancer starts (CRUK, 2014). The five main groups are: carcinomas, lymphomas, leukaemias, brain tumors, and sarcomas (CRUK, 2014).

1.2 Cancer Cells

Normal cells are generally regulated by proper process signals that govern cell growth, division, differentiation, and death. However, cancer cells avoid such regulating signals, as they grow and proliferate with no control. Once this proliferation pursues to grow, extends and spread, then it leads to tumor spread, which is lethal and leads to deaths (Hejmadi, 2010).

Carcinogenesis is a process that takes place before cancer cells appear. Carcinogenesis is a process that has multiple steps through which the architecture of tissues is altered, and preneoplastic nodules are formed (Feitelson et al., 2015). There have been evidences that the human tumorigenesis is a process of multiple steps that are associated with genetic mutations that lead and transform progressively normal cells in humans into malicious ones (Hanahan & Weinberg, 2000). Cancerous cells characteristically proliferate excessively in most cancers (Sever & Brugge, 2015).

1.3 Ovarian Cancer:

OC is the 7th most common cancer in females, and 300,000 females are diagnosed with cancer each year (WOCC, 2018a). OC is the primary cause of mortality among gynecologic cancer cases (Ushijima, 2010). OC is considered the most risky diagnosis among female associated cancers with the highest death rate in the globe (Sak, 2015). Estimated figures show that approximately 180,000 females die each year due to OC (WOCC, 2018a). This is despite the advances made in cancer treatment. It, therefore, has a high mortality rate, and this could be attributed to several reasons. One of the reasons is that it is commonly not diagnosed at an early stage but rather at a later or advanced stage, and this is mainly because OC does not bear symptoms at early stages (Shaw et al., 2004).

Another reason is that repetitive occurrence of OC becomes highly resistant to chemo-therapy (Shaw et al., 2004), bearing in mind that OC has a high reoccurrence rate. Commonly, 70% of the recurrent advanced phase cases deteriorates, and even up to 25% of the cases deteriorates in stage I and stage II of the disease (Ushijima, 2010). OC is a chronic disease

whose cure involves administrating sequentially various and multiple agents (Herzog, 2004). Each of these sequential agent may or may not cause a tumor response (Herzog, 2004).

The figures will get worse by 2040. It is predicted that the number of new cases diagnosed with OC will incline to almost 435,000 cases, which is an increase of 47% (WOCC, 2018a). The number of predicted deaths in 2040 is more than 293,000 females (WOCC, 2018a). The figures in the US show that 2.5% of all malignancies among women is due to OC, but it accounts for 5% of all cancer associated deaths, and of the reasons is its high fatality rate (Torre et al., 2018). It is found that 80% of the cases in the US are diagnosed with the disease at an advanced stage (Torre et al., 2018).

OC generally progresses in older women. It is found in the US that 50% of the women who have OC are older than 62 years (ACS, 2020). Each female has a risk of developing OC during her entire life at about 0.013%, and the chance of losing life due to this cancer is almost 0.01% (ACS, 2020). This is without taking into consideration the low malignant ovarian tumors (ACS, 2020). OC is associated with several main symptoms, such as: pain, bloating, eating complications and urinary signs (WOCC, 2018b). There are also other occasionally correlated symptoms and signs for OC, including: extreme fatigue, abnormally unusual bleeding, changes in bowel movements and weight loss (WOCC, 2018b).

1.4 Treatment of Ovarian Cancer

OC has been evidenced to be a molecular disease, and this is because identically similar alternations in p53 gene have been found in advanced OC stages (Berchuck et al., 1994). A study has revealed that ERβ1 could function as a tumor suppressor on SKOV3 OC cell lines regardless of ERα (Treeck et al., 2007). This was achieved through upregulation of ERβ1, which resulted in solid anti-cancerous effects (Treeck et al., 2007). This was resulted through inhibiting both growth and motility, and inducting of apoptosis, which was accompanied by certain alterations in gene expression (Treeck et al., 2007). Hormone levels in the body in OC, like some other cancer types, contributes a significant role (Meehan & Sadar, 2003).

The cause of and the factors that lead to OC are believed to be amongst the most of non-understood main malignancies in humans (Meehan & Sadar, 2003). Both estrogen receptor

(ER) and progesterone receptor (PR) are identified in less than half of all ovarian tumors. Among all gynecological malignancies, OC remains the most dreaded diagnosis with the highest mortality rate worldwide (Sak, 2015). Ovulation by (or inclusion-cyst formation) could play some role in the pathogenic development of OC, but obviously it is inadequate by itself (Risch, 1998).

Various studies have demonstrated that the use of estradiol after menopause increases the chance of developing OC (Treeck et al., 2007). This has brought more attention to the role of estrogens in OC. It has been noticed that about 10 – 15% of early stage and 40 – 50% of advanced stage of OC cases have alterations in p53 (Berchuck et al., 1994). The research techniques for treatment are, therefore, based on gene transfer via recombinant adenoviruses (Shin et al., 2008). It has been demonstrated that SKp2 can be a newly established target for treatment and cure of OC (Shin et al., 2008).

1.5 SKOV3 Cell Line

SKOV3 (also known as SKOV-3 and SK-OV-3) is a human cell line that was initially isolated and established in 1973 from a 64-year old Caucasian women who had adenocarcinoma of the ovary (MSKCC, 2020). SKOV3 cell line resist tumor necrosis factor (TNF) and also other cytotoxic (antineoplastic) chemotherapy, such as: cisplatin, Doxorubicin (Adriamycin, Rubex) and diphtheria toxin (MSKCC, 2020). SKOV3 cell lines are believed to exhibit minimum levels of ERβ, and at the same time, they exhibit dysfunctional and truncated ERα and, therefore, they do not respond to estrogen (Jones et al., 1994). It has been evidenced that the expression of high levels of ERβ1 indicates a lower proliferation activity of SKOV3 even if E2 does not exist (Treeck et al., 2007).

This agrees with previous studies which indicate that ERβ1 possesses anti-proliferation effects on many types of cancer, including OC as it inhibits tumorigenesis (Lazennec et al., 2001). SKOV3 has a variant that is multi-drug resistant (MDR), which is SKOV3TR (Abouzeid et al., 2014). It is SKOV3-paclitaxel-resistant (TR). The CDX (Cell Line Derived Xenograft) SKOV3 mouse model is generated from the SKOV3 human ovarian cell line (Altogenlabs, n.d.). SKOV3 cell line is hypo-diploid. It is suitably employed as a transfection host for OC studies (Altogenlabs, n.d.). It has been indicated that the SKOV3 line can be employed as a prototype for antiestrogen resistant with functional ER in OC (Hua et al., 1995).

1.6 Herbal Plants and Their Anti-cancer Effects

People have used herbal plants for medical treatments for a long time. Historic records indicate that people used herbal treatments prior to recorded history, as related proofs were found in old civilizations, namely: Egypt, Sumeria (Iraq) and China (Houghton, 2007). All nations and cultures have possibly developed knowledge and methods to utilize local plants and herbs for treatment during their history. The cure of a disease through the use of plant or herbal extracts is called "herbalism", but recently the term "phytotherapy" is more popular and more frequently used (Houghton, 2007). It is worth noticing that the use of herbals and plants in pharmaceuticals has gained more attention, in the last few decades, in the more industrialized nations in the West. The interest was mainly among scientists, researchers and pharmaceutical manufacturers but lately the general public started to pay more attention to herbal plants (Houghton, 2007).

It is worth mentioning that some of the vital drugs used currently in modern treatments contain the "active principles" of traditional treatment styles, such as: codeine and morphine (Houghton, 2007). There has been renewed interests in plants and herbs in the pharmaceutical industry in developed countries, and the interests have been fueled by the need to discover new "biologically active molecules" and to adapt plants crude extracts to be used by the public (H. Gali-Muhtasib, 2006). New development and advances in spectroscopic and chromatographic technology will certainly help in the separation and technical analysis of useful plants biological components, which exist in tiny amounts that could not been identified previously (Balandrin et al., 1985). Cancer is a disease that is caused due to disturbances in the human body, and herbal drugs can play a role in fixing such disruption (Sakarkar & Deshmukh, 2011).

1.7 Mechanism of Anti-cancer Herbal Treatment

Anti-cancer medicines, made from extracts of natural products, have been considered as the most powerfully active drugs considering their anti-cancer ability by altering the function of associated proteins in cancerous cells (H. Gali-Muhtasib, 2006). To cure effectively cancer with minimal side effects, it would be required to target the regulating signaling pathways, which are involved in growth and proliferation of cancer cells (H. Gali-Muhtasib, 2006). It is obvious that chemotherapy has been successful in the types of tumor that are caused by cell types that could be ready to die due to apoptosis (Hickman, 1992). On the other hand, the cell types that are of germ cell or hematopoietic origin will need further studies based on the

apoptosis and its molecular basis, in order to gain more knowledge for fighting chemo-resistant cancer (Hickman, 1992).

Anticancer drugs are majorly more powerful when used against tumor cells in just one specific phase of the cell cycle (H. Gali-Muhtasib & Bakkar, 2002). There, understanding the molecular players of cell cycle process along with regulation, pathways and mechanism is crucial. It is worth noting that clinical studies show that bioactive compounds have proven to possess anticancer abilities that act through a mechanism (Gulfishan et al., 2018). Some types of herb help the body in improving the detoxification process, and therefore, they safeguard the body from cancer (Sakarkar & Deshmukh, 2011). Furthermore, there are some herb types that decreases side effects resulted from radiotherapy and chemotherapy (Sakarkar & Deshmukh, 2011). There are specific biological response modifiers[1] (BRM) extracted from herbs function as growth inhibitors of cancer cells through the modulation and control of certain enzymes and hormones (Sakarkar & Deshmukh, 2011).

Various research and study experiments have revealed that herb-based medicines contribute a role against cancer through the following activities: immunity system improvement, apoptosis induction, multi-drug resistance reversal and angiogenesis inhibition (Romero-Jiménez et al., 2005). Even though herbal medicines have greatly helped patients with advanced cancer, the exact anti-cancer mechanism and pathways associated with herbal drugs are not fully understood, however (Sakarkar & Deshmukh, 2011). Many substances and compounds derived from natural plants and aquatic source have associated cytotoxic abilities with distinguished pathways and mechanisms, including: apoptosis induction, tumor progress inhibition, DNA damage, topoisomerases inhibition (Lichota & Gwozdzinski, 2018).

When compounds extracted from herbs are used together with anti-cancer medicines, they work together to fight cancer without affecting normal cells (Lichota & Gwozdzinski, 2018). The side effects caused by the use of anticancer medicines could be lowered through the use of nanoparticle encapsulations to pass the medicines to the specific target sites (Lichota & Gwozdzinski, 2018). Herbal derivatives have been used for chemo-preventative purposes in different types of cancer, and they have demonstrated their anti-proliferation ability via multiple mechanisms, such as: JNKs and MAPKs activation, oncogene activation, arrest of cell

[1] Also known as immune-modulators or cytokine inhibitors

cycles, PEG-2 suppression, escalation of ROS, decrease of mitochondrial membrane, and inhibition of COX-2 (N. Singh et al., 2018).

1.8 Salvia Triloba (S. Triloba)

The extracts of *S. triloba*, utilized for medical purposes, are water and oil (H. U. Gali-Muhtasib & Affara, 2000). The water extract is produced by boiling the leaves, while oil extract is produced via a basic distillation process (H. U. Gali-Muhtasib & Affara, 2000). Extracted oil from *S. triloba* is rich with contents, such as: ketones, terpenes, alcohol, oxides, and esters (H. U. Gali-Muhtasib & Affara, 2000). It has been evidenced that *S. triloba* has a potentially active suppression ability against tumor progress and development in mouse skin cancer, and it demonstrated anti-proliferation activities (H. U. Gali-Muhtasib & Affara, 2000). This has suggested that the oil extract could potentially be used as a chemo-preventive remedy (H. U. Gali-Muhtasib & Affara, 2000). *S. Triloba* leaves were analyzed in 1949, and the oil yield was measured to be in the range of 21 to 26 ml per 1 Kg of leaves (H. Gali-Muhtasib et al., 2000).

1.9 Drug Combination

It has recently been agreed that drug combination has a better anti-cancer effect than single drugs due to multiple factors (Lu et al., 2017). It should, however, be highlighted that only a small number of anti-cancer mixtures have been scientifically studied in order to understand their anti-cancer mechanisms (Lu et al., 2017). The use of anti-cancer drug combinations has increased in the last few years, because effects of single anti-cancer drug are weak against advanced cancer cases (Lu et al., 2017). This is mainly due to the fact that a single drug cannot work through the different cancer processes (Lu et al., 2017). A combination of anti-cancer drugs is commonly more effective than single drugs, but the cost of drug experimental results have shown that anti-cancer drug combinations are effective in the control of both tumor progression and metastasis development (Lu et al., 2013).

One of the benefits of drug combinations is that they can be used to avoid treatment resistance (Jardim et al., 2020). They also improve tumor response, in general, and they are employed to delay cancer progression as they have the ability to affect multiple cancer development pathways (Jardim et al., 2020). It is worth noting that the synergistic anti-cancer drug mixtures (combinations) have two main advantages, as they have the ability to reduce drug resistance, and at the same time, they are effective and could help avoid toxicity (X. Li et al., 2017). The

problem is that their availability is limited and they are costly (X. Li et al., 2017). Researches and studies on cancer drug combinations and target genes have significantly advances in the last few decades, and this is due to increase and growth of information on drug physicochemical features, pharmacogenomics details, protein pathways, and tumor progress (X. Li et al., 2017).

Anti-cancer drug combinations, in theory, have the ability to be more beneficial and more effective than individual drugs, which are limited in functionality (Celebi et al., 2019). The identification of efficient anti-cancer drug combinations is challenging and complicated and, as a result, the real development of such combinations is much far beyond theoretical assumptions (Celebi et al., 2019).

2. Aim

2.1 Aim and Objectives

The main aim of this study was to examine the effect of *S. triloba* acetone crude extract and the combined treatment of *S. triloba* and Paclitaxel (PTX) on SKOV3 ovarian cancer cell line viability, migration, and gene expression.

To achieve the main aim, four objectives were addressed:

Objective 1: to investigate the cytotoxic effect of *S. triloba* and the combination on SKOV3 cells' viability using the MTT and Trypan Blue assays.

Objective 2: to explore the synergetic effect of the combination (*S. triloba* with PTX)

Objective 3: to explore the cytotoxic effect of *S. triloba* and the combination on SKOV3 cells' migration ability using the Trans-well Migration assay.

Objective 4: to research the effect of *S. triloba* and the combination on the gene expression in SKOV3 cells using quantitative polymerase chain reaction (qPCR) assay.

3. Methodology

3.1 Plant Material:

The plant utilized in the experiment of our study was *S. triloba*. The plant leaves were gathered and collected in spring 2018 from cultivations that are in Hebron, Palestine. The GPS coordinates of the locations are: (31° 32' 4.2216" N, 35° 6' 4.302" E, Lat Long: (31.534506, 35.101195)). The species were identified and confirmed based on the "Flora of Syria, Palestine and Sinai" famous handbook (Post, 1896).

3.2 Crude Extract Preparation (Maceration):

S. triloba was extracted and prepared utilizing a maceration process, whereby the leave samples, which were finely ground and weighted 1.0 gram, were immersed in acetone. The suspension was then incubated for a total duration of 24 hours at room temperature, and the suspension was homogenized through regular blinding. The suspension was then filtered by glass separation funnel with a tight cotton plug to produce the extract. Determination of the extract yield was made through the method of Zhang et al (2007) with some minimum alterations as required. The extracted *S. triloba* crude was first dried by removing the solvent using a rotary evaporator (rotavap) under vacuum, and then lyophilized. The dry crude extracts were weighted, and the formula (K. Zhang, 2014; S. Zhang et al., 2007):
Extraction Yield (%) = (Dry weight of extract ÷ Weight of original plant material) × 100.

The acetone crude extract has been selected as preliminary data in our lab suggested that it exhibited good results. Acetone has many advantages compared to other solvents. It is volatile, it has low toxicity to biocides as it is volatile, it is miscible with water, and it dissolves numerous lipophilic and hydrophilic compounds (Eloff, 1998). Its yield is higher than water (Eloff, 1998). Acetone is lower in polarity than water (Sepahpour et al., 2018). Furthermore, a previous study showed that using acetone solvent to extract turmeric had resulted in a higher phenolic content since they are made of nonpolar covalent bonds that are attached to a phenolic group, which helps them to be easily dissolved in acetone (Sepahpour et al., 2018).

The extract and maceration related process was performed by our collaborator. The extract samples were received in dry-freeze status. A solution of 150 mg/ml concertation was formed through the use of dimethyl sulfoxide (DMSO) to liquefy the lyophilized extracts (Ben-Arye et al., 2016). Several concentrations (150, 75, 37.5, 18.75, and 9.375 µg/ml) were prepared using serial dilutions using RPMI media.

3.3 Cell Culture:

The SKOV3 OC cell line was used in the experiments of the present study. The cell line was kindly provided by Dr. Anwar Abdelnaser, Department of Biology, AUC). Invitrogen media used for cell culture is RPMI (Roswell Park Memorial Institute) from Life Technologies. The RPMI media was supplemented with 10% fetal bovine serum (FBS) (Invitrogen), streptomycin (100 mg/ml) (Invitrogen) and penicillin (100 units/ml) (Invitrogen). The cells were incubated at 37°C and 5% CO2.

3.4 MTT Assay:

MTT assay was employed to evaluate the cytotoxic effect on SKOV3 cell line of *S. triloba* acetone crude extracts. MTT is a colorimetric assay in which the yellow tetrazolium dye, 3-(4, 5 dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide, is reduced into the purple formazan product due to the activity of the oxidoreductase enzyme in the mitochondria of the living organism. The assay basically involves assessing the metabolic activity of cells by quantifying the amount of purple formazan formed after cells reduce the yellow MTT tetrazolium reagent and thus providing a reflection of the amount of present viable cells (Hua Chen et al., 2018). The viability of the cells is measured by using a spectrophotometer. The more colour intensity indicates the more cell viability (Bahuguna et al., 2017). The SKOV3 cell line was incubated for a duration of 48 hours in the 96 well plate with *S. triloba* acetone crude extract. The untreated SKOV3 cells were used as a positive control.

After 48 hours incubation time, the media was discarded, and then 10 µl (5 mg/ml) of MTT was added to each well. After that, all was incubated for 3 hours. Then, 100 µl of DMSO (dimethyl sulfoxide) was added to each well. DEMSO is generally employed as dissolvent to liquefy the formed formazan crystals. SPECTROstar-Nano microplate reader (BMG LABTECH) is the instrument which was used to measure the absorbance readings at 570 nm.

To calculate the cells viability percentage, the absorbance of the treated cells was normalized to the absorbance of the untreated cells multiplied by 100. DMSO was used as a solvent for the acetone crude extract so, DMSO was used as a negative control to assess if it has any cytotoxic effects on the cells.

3.5 IC50:

The values of cells viability percentage, which were obtained from the MTT assay, were used for further analysis. The non-linear regression analysis was used to generate the dose response curve for *S. triloba* acetone crude extract for 48 hours (Figure 1). The obtained IC50 value for *S. triloba* was 75.96 µg/ml. The equation used to generate the IC50 curve is:

log(inhibitor) vs. normalized response -- Variable slope
{Y=100/(1+10^((LogIC50-X)*HillSlope))}
Where: X = log of concentration of treatment and Y = % Viability

Using the logarithmic values in the equation is to display the obtained values of the nonlinear equation on the graph. The cells viability percentage values were analyzed by GraphPad Prism software and normalized. Then, the equation was applied to calculate the IC50 for *S. triloba* and PTX individually.

3.6 Drug Combination Index:

In our study, we calculated the IC50 for both *S. triloba* and PTX by preparing a serial dilution for each using MTT assay. Then, the diluted IC50 for both was combined according to the ratio used in the Chou and Talalay method (Chou, 2006). We used IC50 values according to the ratios stated in (Table 1). The dose combination for both *S. triloba* and PTX was calculated as follows: Combination 1 represents the IC50 multiplied by 4, combination 2 represents the IC50 multiplied by 2, combination 3 represents the IC50 value for each treatment, combination 4 represents the IC50 multiplied by 0.5, and combination 5 represents the IC50 multiplied by 0.25 (Table 2). Therefore, we obtained five different concentrations for the combination doses based on Chou and Talalay approach.

Three experiments were carried out and each had three replicates. SKOV3 cells were seeded in 96 well plates with 5,000 cells per well and cultured for 48 hours. The cells were treated with *S. triloba*, and PTX. The different concentrations were used as recommended by the

ComuSyn software designers (v 1.0 by ComboSyn, Inc) (Table 1). Untreated cells were used as a control. MTT was added to the cells and incubated for three hours. Plates had been shaken for five minutes, then the optical density was measured at 570 nm using a SPECTROstar Nano Microplate Reade (BMG LABTECH).

Drug combination has been used recently in chemotherapy, as its synergetic effect is a potent anticancer treatment, and it helps to overcome the dilemma of chemoresistance. Chou and Talalay (2006) presented an evaluation method that quantifies the efficacy of the combined treatment using the median effect analysis. They developed an analytic software that can mathematically determine the nature of the combination drug effect. The combined drug could have one of three types: a synergistic, additive, or antagonistic effect on cancer cells (Chou, 2006). This mean that the CompuSyn software reports whether the effect type of the different combinations is synergistic, additive, or antagonistic as shown in (Table 2). The software was used in the present study to calculate the combination index (CI), and, as well, the dose reduction index (DRI), according to the median effect principle of Chou and Talalay.

Table 1: Treatment Combination
The obtained IC50 for each treatment was multiplied by: 4, 2, 0.5, and 0.25 to find out the combinations that would be employed in the experiments of the present study.

Drug 1

Drug 2	IC50 X 4	IC50 x 2	IC50	IC50 X 0.5	IC50 X 0.25
IC50 X 4	Combination 1				
IC50 X 2		Combination 2			
IC50			Combination 3		
IC50 X 0.5				Combination 4	
IC50 X 0.25					Combination 5

Based on the CompuSyn software and as illustrated in (Table 2), the combined treatment is supposed to have a very strong synergetic effect if the combination index value is < 1, and it is strong if the value is between 0.1 and 0.3, while it is just a synergetic effect if the value is between 0.3 and 0.7. On the other hand, if the value is between 0.7 and 0.85, then the effect is moderate, while if the value is between 0.85 and 0.90m this indicates slight synergism. The

other CI values indicate that they have either additive or antagonistic effects, and each effect type has various sub-classifications.

To explore the cytotoxic effect of the combined treatment of *S. Triloba* and PTX on SKOV3 cells, in the present study, individual treatment of each and the combined treatment of both were applied on SKOV3 cells using MTT Assay. CompuSyn Software was used to analyze the dose effect for all the treatments both individual and combined. Five combined treatment concentrations were used as recommended by the software designers to find out the optimum drug combination that provides a synergetic effect, which is proposed to improve cancer chemotherapy effectiveness. A significant decrease in cell viability was observed for all concentrations especially in the higher ones. A report was generated by the CompuSyn software with the required information regarding the optimum combined drug dose.

CompuSyn can be used for dose effect analysis for single drugs using the median-effect equation, and for multiple drug combinations, as well, using both the median-effect equation and the combination index equation, as follows:

- **Median-effect equation: $fa/fu = (D/Dm)^m$**

  fa = Function affected by dose, fu= function unaffected,

  Fu = 1 – FA, D = Dose, Dm= Median-effect dose

The median effect equation is a combination of many equations, and it is derived from the mass action low principle. This allows finding out the effect of a single drug. The combination index equation (CIE) is based on the theory of the median effect equation to figure out the effectiveness of individual drugs in a combination (Chou, 2006).

- **Combination index (CI) = $(D)1/(D\chi)1 + (D)2/(D\chi)2$**

  Combination index (CI): A quantitative measure based on the mass-action law of the degree of drug interaction in terms of synergism and antagonism for a given endpoint of the effect measurement.

The durable effects of some combined treatments are, usually, more active than a single therapy. There are three known mechanistic interactions of combinations, which are: additive, antagonistic, and synergetic. In the **Additive effect,** the combined effect is predicted by the mass-action law principle in the absence of synergism or antagonism; in the **Antagonism** effect, it is smaller than the expected additive effect based on the mass-action law; while in the **Synergism** effect, it is greater than the expected additive effect based on the mass-action law.

Drug combination analysis by CompuSyn software (Paramus, NJ) was used to quantitatively measure the dose-effect relationships of each drug alone and in combination, and to determine if the combinations have a synergistic, additive, or antagonistic effect. Automated and computerized values were obtained for the Combination Index (CI) for the combined treatment used.

Table 2: Description of Combination Index Values

Description and symbols of synergism, antagonism or additivity in drug combination studies analysed with the combination index method. Source (Chou, 2006).

Range of Combination Index	Description	Graded Symbols	Graphic Symbols
<0.1	Very strong synergism	+++++	
0.1–0.3	Strong synergism	++++	
0.3–0.7	Synergism	+++	
0.7–0.85	Moderate synergism	++	
0.85–0.90	Slight synergism	+	
0.90–1.10	Nearly additive	±	
1.10–1.20	Slight antagonism	–	
1.20–1.45	Moderate antagonism	– –	
1.45–3.3	Antagonism	– – –	
3.3–10	Strong antagonism	– – – –	
>10	Very strong antagonism	– – – – –	

3.7 Cell Morphology:

Treated cells with *S. triloba* were observed for 48 hours under a normal inverted microscope at 10X and 20X magnification. The cellular morphological changes were assessed and compared with the untreated viable cells. Treated SKOV3 cells morphology was affected, and their epithelial shape was changed into a spherical shape as their cell membrane blabbed, and number of cells was sharply decreased, which indicated growth inhibition. In contrast, the untreated SKOV3 cells remained healthy with an intact structure. Incubation time for cells was 48 hours. (Figure 6A) shows the effect of *S. Triloba* acetone crude extract on SKOV3 cell morphology, while (Figure 6B) shows the effect of the combined *S. triloba* IC50 and PTX IC50 treatment on SKOV3 cells morphology using the different concentrations.

3.8 Trypan Blue Exclusion Assay:

The Trypan Blue Exclusion Assay is used to estimate cell viability and evaluate the cytotoxic effect of a treatment (Altman et al., 1993). Therefore, equal volumes of 0.4% (w/v) trypan blue solution (Serva, Germany) and treated SKOV3 cells with acetone crude extract of *S. triloba* were counted using a dual-chamber haemocytometer and a light microscope. The following equations were used to calculate the number of viable and nonviable cells (Strober, 2015):

Viable cells/mL = (Total number of viable cells/total number of squares counted) x dilution factor x 10,000

Non-viable cells/mL = (Total number of non-viable cells/total number of squares) x dilution factor x 10,000

The mean values of the three experiments were analysed using Prism software. The IC50 values, obtained from the MTT assay for both *S. triloba* and PTX, were used in this assay to assess the effect of *S. triloba* and PTX single treatments on SKOV3 cells. On the other hand, the dose for the combination used in this assay was obtained from CompuSyn.

3.9 RNA Extraction:

Trizol reagent (Invitrogen, USA) was used to extract total RNA from SKOV3 cells according to the manufacturer's instructions. RNA extraction experiment was carried on an RNAase-free environment. Nuclease-free water (Thermo Scientific, USA) was used to dissolve the extracted RNA. The RNA concentration and purity were measured at both 260 and 280 nm using a

SPECTROstar Nano Absorbance Plate Reader (BMG Labtech). RNA concentration was automatically calculated by the devise using the following formula:

RNA concentration (µg/mL) = OD at A260 X Dilution Factor X 40

3.10 Trans-well Migration Assay:

The trans-well assay was utilized to examine the effect of *S. triloba* acetone crude extract on the migration and invasion ability of SKOV3 cells. Typsinized SKOV3 cells with seeding density of 200,000 cells per insert were seeded in the top chamber of 8 µm diameter pore sized. The cells were seeded in the hydrated inserts (GBO) along with IC50 acetone crude extract. RPMI medium (Roswell Park Memorial Institute) supplemented with 1% FBS was added to the treated cells in total volume of 300 ul per insert. The to-be-seeded cells and media suspension in the inserts were finally placed in the 24-well plate. RPMI media (400 ul) supplemented with 10% FBS were added to 24-well plate below each insert, and cells were incubated for 24 hours at 37C and 5% CO2. Migrated cells fixation was performed by immersing the bottom chamber of each insert in 400 ul of 4% formaldehyde mixed with PBS and 1% triton X-100. Then, migrated cells were stained with fluorescent DAPI stain (KPL, 71-03-01) (1:1000 dilution of DAPI 10 mg/mL stock in PBS for a final concentration of 10 ug/mL). Visualization of the migrated cells was achieved by using fluorescent microscope at 100X magnification. Images from different fields were taken by the Olympus IX70 inverted microscope. The images of the treated cells and the control (untreated cells) were analysed by ImageJ software to calculate the average number of migrated cells per field.

3.11 qPCR:

The quantitative polymerase chain reaction (qPCR) was used to assess the gene expression in the cell samples treated with the *S. triloba* IC50. The RNA amplification was done by using real time PCR, and specific primers for each gene were employed for the purpose of gene amplification. The fluorescence quantity of the amplification in each cycle within the amplification process was identified using Cyber green dye. For the quantification of genes expression, the data obtained was analysed by $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001).

The investigated gene in the sample is normalized to the indigenous gene. The fold change in the expression of the amplified gene was compared to the untreated cells control. The melt

curve is used in the analysing to verify that the amplification product is specific for the gene of interest (Zihlif et al., 2013). (Table 3) shows the list of investigated genes along with primers sequences used.

Table 3: qPCR Primer Sequences

Gene		Primer Sequence
β-catenin	F:	5'-GAGGAGCAGCTTCAGTCCCC-3'
	R:	5'-GCCATTGTCCACGCTGGATT-3'
RUNX2	F:	5'-GATGGGACTGTGGTTACTGTCA-3'
	R:	5'-CTCAGATCGTTGAACCTTGC-3'
MDM2	F:	5'-CCCAAGACAAAGAAGAGAGTGTGG-3'
	R:	5'-CTGGGCAGGGCTTATTCCTTTTCT-3'
N-cadherin	F:	5'-GCGTCTGTAGAGGCTTCTGGT-3'
	R:	5'-TCTGCAGGCTCACTGCTCTC-3'
SETD7	F:	5'-F-CGAATTACACACCAAGAGGTT-3'
	R:	5'-TAGGCAACGGTGAGCTCTTC-3'
p53	F:	5'-F-TCAACAAGATGTTTTGCCAACTG-3'
	R:	5'-ATGTGCTGTGACTGCTTGTAGATG-3'
BAX	F:	5'-F-GATGGACGGGTCCGGGGAG-3'
	R:	5'-GATGCGCTTGAGACACTCGC-3'
PTEN	F:	5'-F-TGACCAATGGCTAAGTGAAG-3'
	R:	5'-CGCCTCTGACTGGGAATA-3'
PI3KR1	F:	5'-F-CGCCTCTTCTTATCAAGCTCGTG-3'
	R:	5'-GAAGCTGTCGTAATTCTGCCAGG-3'
GAPDH	F:	5'-AAGGTCATCCCTGAGCTGAAC-3'
	R:	5'-ACGCCTGCTTCACCACCTTCT-3'

3.12 Data Statistical Analysis

All the relevant data analysis, in the present study, was made through GraphPad Prism Software v 7. All the various comparisons used in this study were based on either one-way ANOVA or two-way ANOVA followed by Dunnett's test as implemented by Prism. Multiple T-tests were also performed as required. Furthermore, ImageJ software was used to determine the count of cells. Dose response curves and IC50 values were generated automatically by GraphPad Prism v7 as follows:

(log(inhibitor) vs. normalized response -- Variable slope)

Data were presented as mean ± SD and considered as statistically significant at a P-value <0.05(*), <0.1 (**), and <0.001(***).

4. Results

4.1 MTT Viability and IC50:

The MTT assay was used to determine the cytotoxic effect of *S. triloba* by applying different concentrations on SKOV3 cells. The acetone crude extract was diluted starting from 150 µg/mL to 9.375 µg/mL in a serial manner. Then, the treated cells were incubated for 48 hours. *S. triloba* reduced the cells viability compared to the control (untreated cells). The viability was not of much reduction for the low concentrations, which are: 9.375, 18.75, 37.5 µg/mL. Interestingly, these three low *S. triloba* concentrations had similar effect trends on SKOV3 cells. The significant reduction was observed at the two highest concentrations:150 µg/mL and 75 µg/mL, respectively, as illustrated in (Figure 1A). The highest reduction was observed when 150 µg/mL concentration was used with (*** p-value < 0.001). The use of 75 µg/mL concentration had reduced the cells viability with p value of (*** p-value < 0.001). There was a significant difference between the 75 µg/mL concentration and the 150 µg/mL with (*** p-value < 0.001). DMSO was used as a solvent in which the acetone crude extract was dissolved. It was used to prepare the working solution of the acetone crude extract as well. Therefore, DMSO with 0.2 % concentration was applied on SKOV3 cells to ensure the safety of the compound on cells as shown in (Figure 1A). The cells viability was 18.082% only when using 150 µg/mL of *S. triloba*, while it was 65.640%, when applying the 75 µg/mL of *S. triloba* concentration.

The obtained results from the MTT assay were used to determine the IC50 value for *S. triloba.* The data were submitted to the Graphpad Prism software, and the non-linear regression analysis was used to calculate the IC50 value (Figure 1B). Dose response curves for *S. triloba* was plotted for 48 hours incubation time to generate IC50 values for each treatment. The IC50 value was as follows: 75.96 µg/mL for *S. triloba.* The obtained hillslope coefficient for *S. triloba* was > 1 (6.678) (Table 4), which indicates a positive cooperativity and interaction between *S. triloba* and the cell receptors.

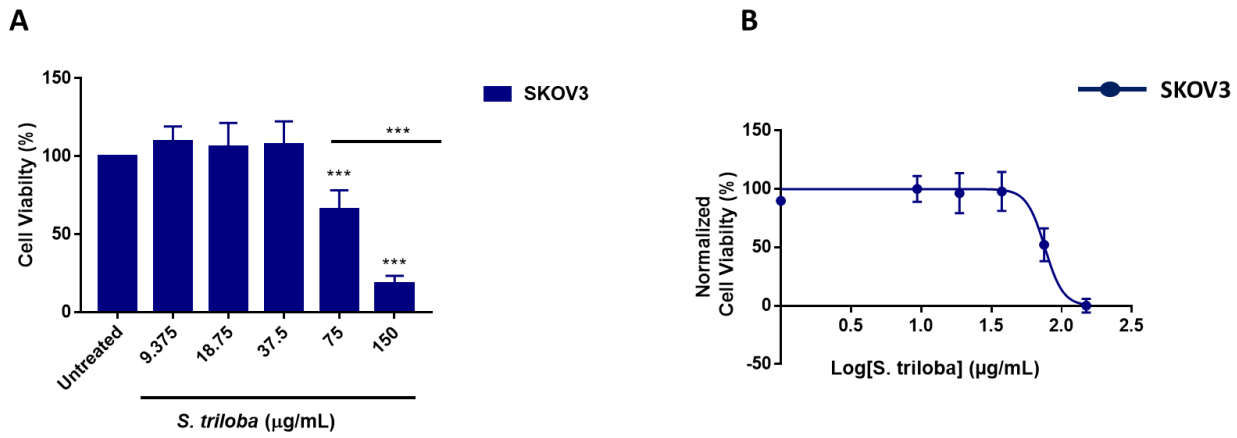

Figure 1: Effect of *S. triloba* acetone extract on SKOV3 cells viability.
***P ≤ 0.001, **P ≤ 0.01, *P ≤ 0.05. The error bars represent the standard deviation of the data from the mean. The results were compared to the untreated cells. A) The 9.375, 18.75, and 37.5 µg/mL concentrations had similar effect on SKOV3 cells as they were almost like the untreated cells. The 76 µg/mL concentration had a significant reduction with P value of ***P ≤ 0.001. The highest concentration 150 µg/mL had a greater reduction with P value of ***P ≤ 0.001. There is a significant difference between the 75 µg/mL concentration and the 150 µg/mL. B) Dose response curve for the effect of *S. triloba* on SKOV3 cells, the viability percentage was normalized to a scale from 0 to 150. The *S. triloba* IC50 value was 76 µg/mL.

The MTT Assay was, also, used to determine the cytotoxic effect of PTX on SKOV3, with different concentrations. PTX was diluted in a serial manner starting from 36 µg/mL to 1.5 µg/mL. Treated cells were incubated for 48 hours. PTX reduced the cells viability compared to the untreated cells. All used concentrations, significantly, reduced cells viability with (***P ≤ 0.001) (Figure 2A). The most significant reduction was observed at 36 µg/mL and 24 µg/mL, about 80 and 70%, respectively. When applying 12 µg/mL concentration, the reduction was almost 40%. When applying the concentrations 1.5, 3, and 6 µg/mL, the reduction in cells viability was about 50%. It is worth mentioning that there was a significant difference between concentrations 12 and 36 µg/mL with (**P ≤ 0.01).

The obtained results from the MTT assay were used to determine the IC50 value for PTX, as well. The data were submitted to the Graphpad Prism software, and the non-linear regression analysis was used to calculate the IC50 value (Figure 2B). Dose response curves for PTX was plotted for 48 hours incubation time to generate the IC50 value. The IC50 value was 3.148 µg/mL. The hillslope coefficient was about one (1.043) (Table 4). PTX dose response curve had values ranging from 36 µg/mL to 1.5 µg/mL. PTX doses 1.5, 3, and 6 µg/mL effects were similar as they reduced about 40% of SKOV3 viability. The highest concentration (36 µg/mL)

decreased cells viability by about 80%, the 24 µg/mL reduced it by 70%, and the 12 µg/mL concentration reduced viable cells by about 60% (Figure 2B).

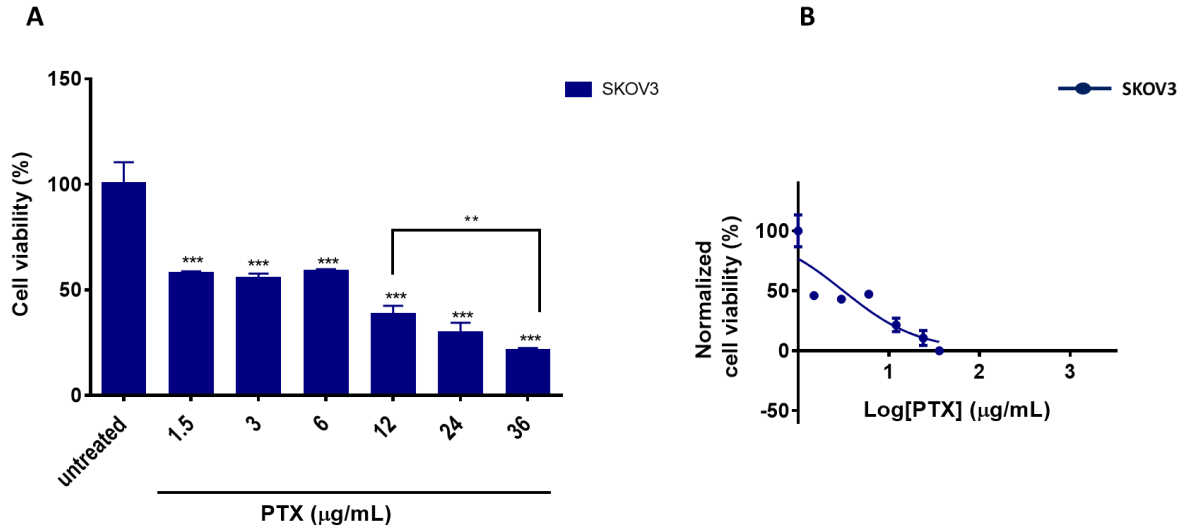

Figure 2: Effect of PTX on SKOV3 cell viability.
***P ≤ 0.001, **P ≤ 0.01, *P ≤ 0.05 A) Treating SKOV3 cells with PTX reduced thier viability. The 1.5, 3, and 6 µg/mL concentrations reduced cells viability by about 50%, while the 12 µg/mL concentration reduced cells viability by about 60%. The 24 µg/mL reduced viability by about 70%, and the 36 µg/mL made the highest reduction, about 80%. The error bars represent the standard deviation of the data from the mean. The results were compared to the untreated cells. There is a significant difference between the 12 and 36 µg/mL. B) Figures shows the dose response curve for the effect of PTX on SKOV3 cells, and the cell viability percentage was normalized to a scale starting from 0 to 100. PTX C50 value was (3.148 µg/mL)

Furthermore, the MTT assay was used to determine the cytotoxic effect of the combined treatment of *S. triloba* and PTX by applying the concentrations, based on the recommended process, on SKOV3 cells. The calculated concentrations for the *S. triloba* and PTX combined treatment were obtained, as shown in (Table 6). The acetone crude extract was diluted starting from 304 µg/mL to 19 µg/mL and PTX was diluted from 12.592 µg/mL to 0.787 µg/mL. Then, both treatments were combined and applied on the cells. Treated cells were incubated for 48 hours. The combination reduced cells viability, and the reduction intensified while applying the highest concentration compared to the untreated cells.

The greatest reductions were observed at the (304 µg/mL *S. triloba* + 12.592 µg/mL PTX) concentrations. The percentage of viable cells was 13.38% only. However, the effect of the other used concentrations was like each other, in general. The other concentrations used were: (152 µg/mL *S. triloba* + 6.296 µg/mL PTX), (76 µg/mL *S. triloba* + 3.148 µg/mL PTX), (38 µg/mL *S. triloba* + 1.574 µg/mL PTX), and (19 µg/mL *S. triloba* + 0.787 µg/mL PTX). The

viable cells percentage was as follows: 29.8%, 27.7%, 25.7%, and 23.4%, respectively. All reductions by the different concentrations were significant with (*** p-value < 0.001) as shown in (Figure 3). Moreover, there was a significant difference between C4 and C5 with (*P ≤ 0.05).

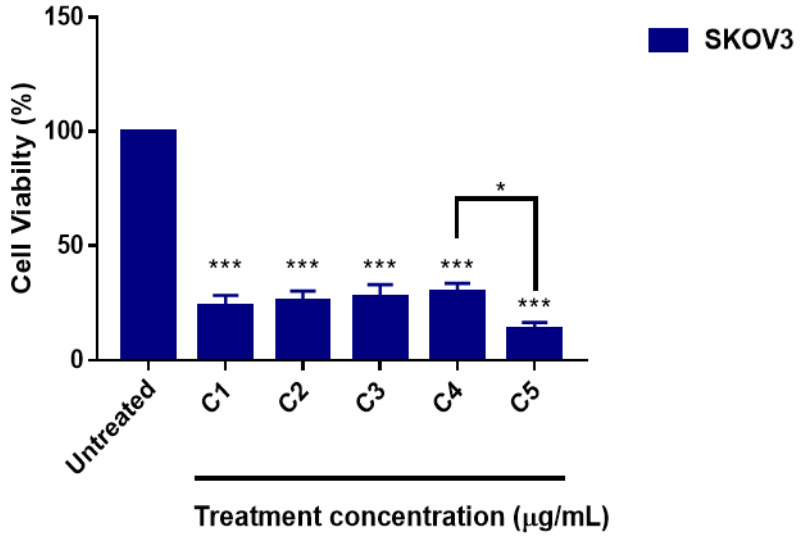

Figure 3: Effect of the combined treatment of *S. triloba* and PTX on SKOV3 cells viability.
P ≤ 0.001, **P ≤ 0.01, *P ≤ 0.05. The error bars represent the standard deviation of the data from the mean. The obtained results were compared to the untreated cells. The combined dose dramatically decreased the number of viable cells with P value of (P ≤ 0.001). The highest decrease in number of cells was when using 304 µg/mL + 12.592 µg/mL concentration (C1). The effect of the remaining concentrations C2: 152 µg/mL + 6.296 µg/mL, C3: 76 µg/mL + 3.148 µg/mL, C4: 38 µg/mL + 1.574 µg/mL, and C5: 19 µg/mL + 0.787 µg/mL was similar to each other with a reduction of more than 50%. There is a significant difference between C4 and C5.

Table 4: Summary of IC50, Hill coefficient and R2
Summary of IC50, Hill coefficient and R2 values obtained for acetone crude extract of *S. triloba* and PTX. R2 is a standard measure that statistically indicates the goodness and accuracy of the obtained plotted data for linear regression models.

Treatment	Time (hours)	IC50± std error (µg/mL)	HillSlope± std error	Sy.x (%)	R Square
S. triloba	48	75.96 ± 1.0237	6.678 ± 3.928	12.86	0.8902
PTX	48	3.148± 1.197	1.043± 0.202	15.53	0.771

4.2 Data Obtained from CompuSyn Software:

To determine the synergetic effect for the drug combination, it is required to get the dose effect curves for each drug, separately. The median effect dose values (Dm) for each drug dose is an

important parameter for calculating the CI values. m-value is the shape parameter for the dose-effect curve. The m value is the slope of the median-effect plot. Value m = 1 indicates a hyperbolic curve, m > 1 signifies a sigmoidal curve, while m < 1 indicates a flat sigmoidal dose effect. The slope means the change in response per unit dose. The sigmoidal curve indicates that the effect increases with the dose unit. This indicates that the curve agrees with the mass-action low. The flat curve indicates that the effect is not changing with dose increases, which indicates that the curve is not in line with the mass-action low. The mass-action low states that the rate of the reaction is directly proportional with the concentration of the products.

The r-value indicates the conformity parameter for goodness of fit to the median-effect principle (MEP) of the mass-action law. It is the linear correlation coefficient of the plot, where r = 1 indicates a perfect conformity. The Median-effect dose (Dm) indicates the dose that produces 50% effect, such as: IC50, ED50, or LD50. It is a potency parameter, and it is obtained from the antilog of the x-intercept of the median-effect plot. The Dose Reduction Index (DRI) is a measure of how many folds the dose of each drug in a synergistic combination may be reduced at a given effect level compared with the doses of each single drug.

4.2.1 Dose Effect Response Curves

The dose effect of *S. triloba*, PTX, and the combined treatments were plotted into a dose effect curve that represents the relationship between the drug dose for each treatment and cells lethality. The resulted curves indicate the shape and the potency of the treatment used. The dose-effect curve for each drug is determined and then the combined effect of the two drugs is determined, consequently. The combined effect for both drugs is the combination index values that are obtained by the CompuSyn software. Finding the combined effect is the first step to determine the additive, synergistic, or antagonistic effect of the combination on cells. Furthermore, it is important to determine the potency and the shape of dose-effect curve for each drug through identifying drug parameters, including m, Dm, and r (Table 5). The degree of sigmoidicity for each curve is signified by the curve slope (m value) where m value means the change in response per unit dose as explained previously. The graph, generated by the CompuSyn software, represents the dose-effect curve for the treatments and the combination (Figure 4A).

The dose effect curve of S. triloba, PTX, and the combined treatment was transformed into a linear form where the values were transformed and plotted into straight lines (Figure 4B). The five data point values that are represented in the dose-effect curves for the single treatments and the combined treatment were linearized and plotted in a graph. The straight lines are obtained by transforming the median effect equation into a logarithmic form. Then the software uses the parameters that are obtained from the median effect plot to calculate the CI and DRI values by using the CI equation.

Therefore, the five data points were plotted into a linear form using the median-effect plot, whereby y = log (f a /f u) vs. x = log (D) as shown in (Figure 4B). The Dm value, which signifies the potency of each drug is obtained from the anti-log of the x-intercepts and the m value. The graph, generated by the CompuSyn software, represents the linearized dose-effect for single and combined treatments. The Dm values obtained, in our study, for the combined treatment (Tables 5 and 8) show that the potency of every treatment in the combination was much greater than the potency of each single treatment. This indicates that adding *S. triloba* to PTX could potentiate it and make it a much more powerful drug.

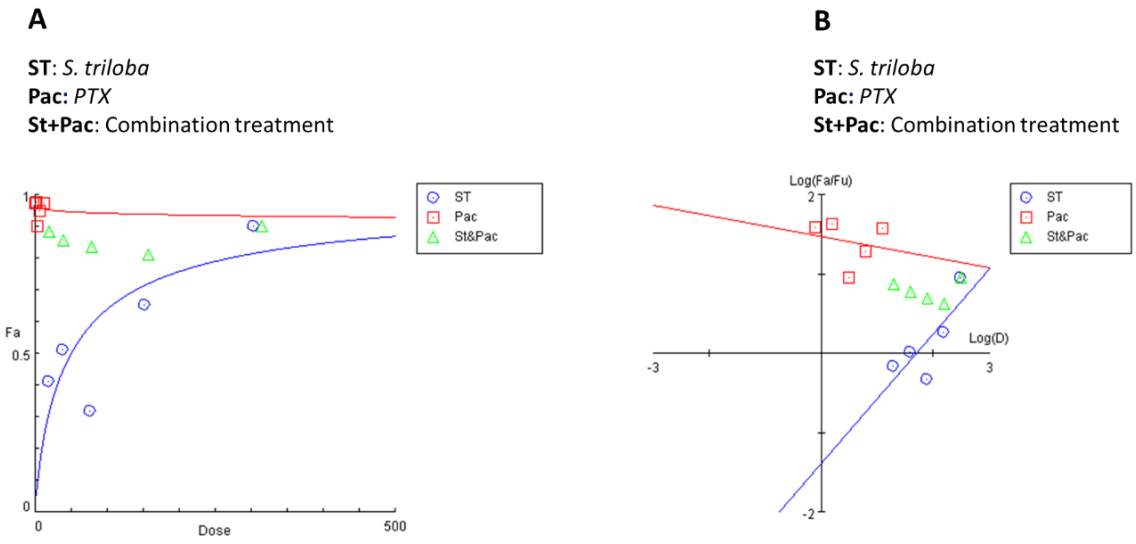

Figure 4: Dose Effect Curve of *S. triloba* Acetone Crude Extract & PTX on SKOV3 Viability
4A) The does effect of *S. triloba*, PTX, and the combined treatment. It indicates the relationship between drug dose and the stress caused on cells. The graph describes both the "potency" and the "shape" of the dose-effect curve for each drug. Dm indicates the potency of the treatment, while m indicates the shape of the dose curve. (4B) The plot shows the dose effect of *S. triloba*, PTX, and the combined treatment values that are transformed and plotted into straight lines. The straight lines are obtained by calculating the logarithmic values of x-intercepts data on the axes for both drugs and the combination. Fa is the fraction affected. Fu is the fraction unaffected. Log D is the log of the dose.

Table 5: A summary for the Dm, m, r values for both treatments

Drug/Combo	Dm	m	r
S. triloba	49.6650	0.82220	0.78140
PTX	1.93E11	-0.1307	-0.2136

Dm = The median effect dose value or the drug dose required for 50% inhibition
m = The shape parameter for the dose-effect curve
r = indicates conformity

4.2.2 Combination Index (CI)

The potency (Dm) and the shape of the dose response curve were both taken into consideration in calculating the combination index which is a quantitative representation of the pharmacological inter-activity between the two drugs. In the present study, five different values were obtained from CompuSyn using the recommended process. As a result, five Combination Index (CI) values were generated by the software. The CI value indicates the type of the combination effect. (Table 8) shows the synergetic effect for the five concentrations used in the combined treatments. The CI column in (Table 8) illustrates that there is an obvious synergetic effect in the five different concentrations used for both treatments, because each value is < 1. The "effect" column shows that the cell death is very high with a minimum of 81% (Table 8). As shown in the table, there is neither antagonistic nor additive effect for the different combinations used.

As indicated, CompuSyn software was used, in the present study, to obtain the Combination Index (CI) values and the effect of the combinations on SKOV3 cell line. After entering the data, the effect and CI values were obtained (Table 8). The effect, which means the percentage of cell death due to the combined treatments, illustrated that the highest concentration, which is (304.0 µg/mL *S. triloba* + 12.592 µg/mL PTX), has the highest cell death effect being 0.9, which is 90%. All the other combinations of *S. triloba* and PTX have an effect between 0.81 and 0.88, which means between 81 and 88%. All the CI values of the different combinations obtained are < 1, which indicated synergetic effect for all the five combinations used (Figure 5A). Synergism induces apoptosis more than cell growth inhibition. These results might indicate that the combination of *S. triloba* and PTX is potentially a promising anti-cancer medicinal therapy.

Table 6: The Recommended Treatment Combinational Design Used in the Study

S. triloba

Paclitaxel	304 µg/ml	152 µg/ml	76 µg/ml	38 µg/ml	19 µg/ml
12.592 µg/ml	Combination 1				
6.296 µg/ml		Combination 2			
3.148 µg/ml			Combination 3		
1.574 µg/ml				Combination 4	
0.787 µg/ml					Combination 5

Table 7: Concentrations (combinations) used:

List of the he obtained concentrations after IC50 multiplications by the recommended values

Concentration of combination 1 (C1)	304 µg/ml + 12.592 µg/ml
Concentration of combination 2 (C2)	152 µg/ml + 6.296 µg/ml
Concentration of combination 3 (C3)	76 µg/mL + 3.148 µg/mL
Concentration of combination 4 (C4)	38 µg/mL + 1.574 µg/mL
Concentration of combination 5 (C5)	19 µg/mL + 0.787 µg/mL

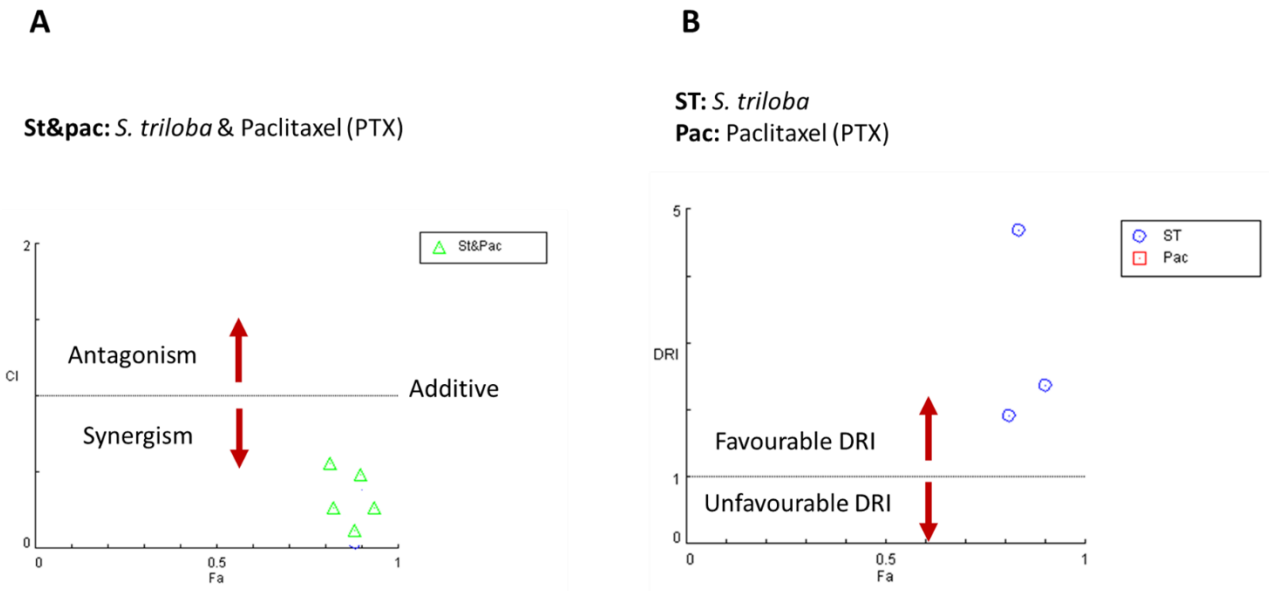

Figure 5: Combination Index (CI) Plot and Dose Reduction Index (DRI) Plot *S. triloba* and PTX
(5A) The synergetic effect for the five combined concentrations used. The horizontal line indicates the additive effect of the combination with value = 1. Combination index of value that is > 1 indicates antagonistic effect, while combination index value < 1 indicates a synergetic effect of the drug combination. All combined treatments used in the study have a synergetic effect. The values for PTX are not shown in the graph because they are out of scale as the numbers range from 769.36 to 46,459.6 folds. (5B) The plot shows that the dose reduction index values (DRI) being > 1, and it shows that all used combinations are favourable for dose reduction. All The obtained DRI values for both *S. triloba* and PTX are favourable for dose reduction as they are all > 1. DRI values, which are < 1, are not favourable for dose reduction. Fa is the fraction affected.

Table 8: CI Data for The Combined treatment (*S. triloba* and PTX):
The effect (cell death%) of the combined treatment indicates the highest concentration (304.0 µg/mL *S. triloba* and 12.592 µg/mL PTX) has the highest cell death effect which is 90%. All the other combinations have an effect between 81 and 88%. All the CI values obtained are less than 1, which indicated synergetic effect for all the five combinations used.

Dose St (µg/mL)	Dose PTX (µg/mL)	Effect	CI	CI Index
19.0	0.787	0.88325	0.03267	Very strong synergism
38.0	1.574	0.85706	0.08664	Very strong synergism
76.0	3.148	0.83451	0.21388	Strong synergism
152.0	6.296	0.81048	0.52268	Synergism
304.0	12.592	0.9	0.42419	Synergism

St= *S. triloba*
CI= combination Index

4.2.3 Dose Reduction Index (DRI)

"The DRI is a measure of how many -fold the dose of each drug in a synergistic combination may be reduced at a given effect level compared with the doses of each drug alone." (Chou, 2006). This means that DRI > 1 indicates the dose for a given drug combination can be reduced. The aim of synergistic drug combinations is to reduce the dose of the used drugs. Reducing the doses, while maintaining efficacy, is very important to reduce drugs toxicity especially in anti-cancer treatments. (Table 9) shows the dose reduction index (DRI), in which the dose could be reduced by the specified fold for both *S. triloba* and PTX in the combination. The DRI fold number for *S. triloba* is between 2.36 and 30.63 folds, while the PTX DRI is much greater with a value between 769.36 and 46,459.6 folds. The combination therapy is highly effective as the lowest dose inhibited cells viability by 88%, and the highest dose had an inhibition percentage of 90% (Table 9).

(Table 9) shows that all the doses were reduced because all the obtained DRI values are > 1, which indicates that the doses could be lowered while maintaining the same drug efficacy. (Figure 5B) also show that all the obtained results have DRI > 1, which indicates they are favorable for dose reduction. It is worth mentioning that the major objective of having synergistic drug combination is to reduce the dose of the drug used; thereby, reducing the toxicity while sustaining efficacy. Our findings exhibited that doses of *S. triloba* and PTX could be significantly reduced, as illustrated by the DRI calculations.

Table 9: DRI Data Obtained from CompuSyn for both *S. triloba* and PTX
The Fa values range from 0.81 to 0.9 (81% to 90%) indicating high cell deaths percentage. The DRI (Dose Reduction Index) are between 2.36 and 30.63 folds for *S. triloba*, and between 769.36 and 46,459 folds for PTX.

Fa	Dose St	Dose PTX	DRI St	DRI PTX
0.88325	581.995	36,563.7	30.6313	46,459.6
0.85706	438.648	216,434.	11.5434	137,506.
0.83451	355.350	813,738.	4.67566	25,8494.
0.81048	290.809	2,870,223	1.91321	455,880.
0.9	718.858	9,687.78	2.36466	769.360

Fa: the fraction of cells affected
DRI: the dose reduction index
St: *S. triloba*

4.3 Cell Morphology:

Morphological alternations associated with apoptosis were observed on SKOV3 cells upon applying *S. triloba* treatment. Moreover, the changes in cells morphology were noticed to be greater in the high concentrations of the treatment, while in the low concentrations, the significant morphological changes were not observed. Using higher concentrations (75 and 150 µg/mL), cells were noticed to be less in their density compared to the untreated cells. Moreover, the cells lost their epithelial structure and turned to be contracted and rounded in their shape as shown in (Figure 6).

The effect of the various combined concentrations of *S. triloba* and PTX on SKOV3 cell morphology is illustrated in (Figure 6B). The combined concentrations are based on the recommended process (Table 1). The concentrations used are shown in the images as follows: C5 (Image f): 304 µg/mL *S. triloba* + 12.592 µg/mL PTX, C4 (Image e): 152 µg/mL *S. triloba* + 6.296 µg/mL PTX, C3 (Image d): 76 µg/mL *S. triloba* + 3.148 µg/mL PTX, C2 (Image c): 38 µg/mL *S. triloba* + 1.574 µg/mL PTX, and C1 (Image b): 19 µg/mL *S. triloba* + 0.787 µg/mL PTX. Image (a) is for the untreated cells. Images (e & f) shows that there were morphological changes when the highest concentration (304 µg/mL *S. triloba* + 12.592 µg/mL PTX) and (152 µg/mL *S. triloba* + 6.296 µg/mL PTX) were applied, and the cells blebbed and became spherical. Images (b – d) show that the alterations occurred to the cells were less severe as their epithelial shape changed, and the cells shrunk when less concentrated combinations were used. In addition, number of treated SKOV3 cells was smaller when compared to the untreated cells.

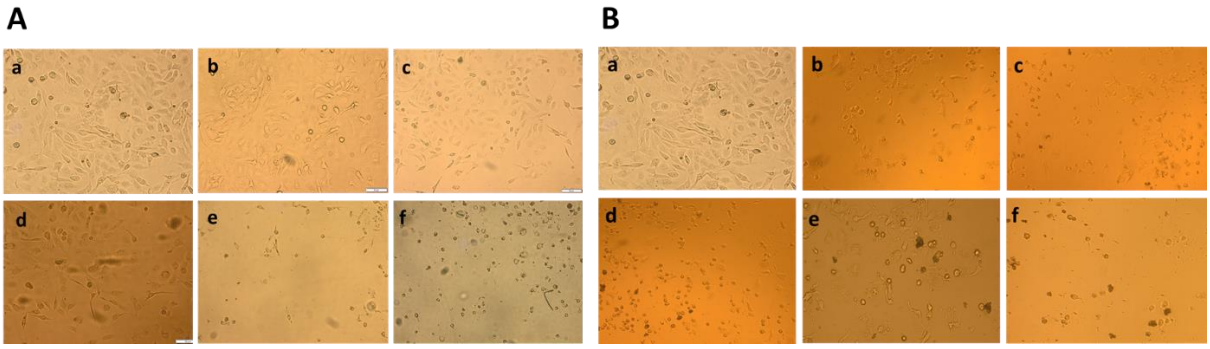

Figure 6: Effect of *S. triloba* acetone extract and the combined treatment on SKOV3 cells morphology
Images were taken with 10X magnification. (A) Different concentrations were used in the MTT assay to investigate the effect of *S. triloba* on SKOV3 cells. Images (a - f) show the effect of different concentrations of *S. triloba*. a is untreated cells, b: concentration of 9.375 µg/mL, c: 18.75 µg/mL, d: 37.5 µg/mL, e: 76 µg/mL, and f: 150 µg/mL. The highest concentration was 150 µg/mL, while the lowest concentration was 9.375 µg/mL. The 75 µg/mL concentration affected the cells morphology and turned their shape to be smaller and shrunk, while the higher concentration 150 µg/mL turned the epithelial shape of the cells to be rounded and spherical. (B) The images show the effect of the different combined concentrations of *S. triloba* IC50 and PTX IC50 on cells morphology. They illustrate the effects of the different concentrations of the combined treatments on SKOV3 cells. Images (a – f) show the different concentrations of the combined treatment. Image (a) for untreated cells. Images (e and f) show that morphological changes occurred when applying the highest concentration (152 µg/mL + 6.296 µg/ml) and (304 + 12.592 µg/mL), respectively, and the cells became spherical and blebbed. Images (b, c, and d) show that less severe alterations occurred to the cells as their epithelial shape changed, and the cells shrunk when less concentrated combinations were applied. Furthermore, number of cells was smaller compared to untreated cells. C5 (Image f): 304 + 12.592 µg/mL, C4 (Image e): 152 µg/mL + 6.296 µg/mL, C3 (Image d): 76 µg/mL + 3.148 µg/mL, C2 (Image c): 38 µg/mL + 1.574 µg/mL, and C1 (Image b): 19 µg/mL + 0.787 µg/mL.

4.4 Trypan Blue Exclusion Assay:

Three treatments were used in the Trypan Blue assay experiment: the CompuSyn best obtained Combination Index (CI) value for the combined treatment (19 µg/mL *S. triloba* + 0.787 µg/mL PTX), the acetone crude extract with IC50 value concentration (76 µg/mL), and the PTX IC50 (3.148 µg/mL). All the three treatments were applied on SKOV3 cells and incubated for 48 hours. After incubation time, the cells were harvested and counted using Trypan Blue dye. The percentage of live and dead cells was calculated. The percentage of dead cells due to the treatment was compared to the percentage of untreated cells. In the present study, a decrease in the number of viable cells was observed. Furthermore, the decrease was statistically significant with p value > 0.001 for all the treatments used.

The percentage of viable cells that were treated with *S. triloba* IC50 was 55.6%, while for the PTX IC50 treatment, it was 27.6%. The highest effect on cell viability was caused by the combined treatment, with a cell viability of 8% only (Figure 7). This means that the combined treatment caused the highest effect, followed by PTX IC50, and then *S. triloba* IC50 being the lowest effect on SKOV3 cells viability. In summary, the combined treatment had stronger

cytotoxic effect than *S. triloba* and PTX single treatments, as it terminated most of the cells (Figure 7). In addition, there was a significant difference between the effect of *S. triloba* Acetone crude extract IC50 (76 µg/mL) and the combination with (***P ≤ 0.001).

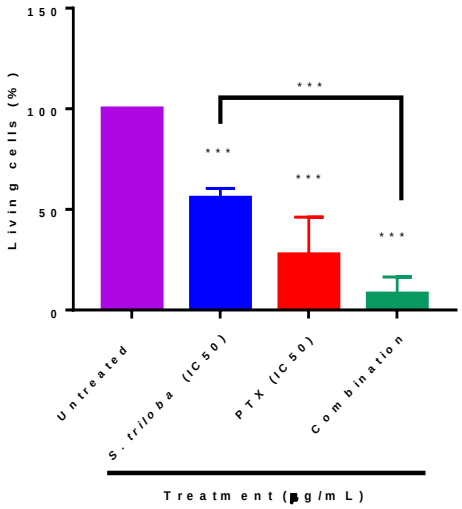

Figure 7: Effect of the three treatments on SKOV3 cell viability ability
***P ≤ 0.001, **P ≤ 0.01, *P ≤ 0.05. Effect of *S. triloba* Acetone crude Extract IC50 (76 µg/mL), PTX IC50 (3.148 µg/mL), and the CompuSyn best obtained Combination Index (CI) value for the combined treatment (19 µg/mL *S. triloba* + 0.787 µg/mL PTX) on SKOV3 cells using trypan blue assay. *P<0.05, **P<0.01, ***P<0.001). the results were compared to the untreated cells .The cell viability percentage resulted from *S. triloba* IC50 treatment was the highest (55.6%), followed by PTX (27.5%), while the combined treatment had the lowest viability percentage (8%). There is a significant diference between the effect of *S. triloba* Acetone crude Extract IC50 and the combination. The error bars represent the standard deviation of the data from the mean.

4.5 Trans-well Migration Assay:

The Trans-well migration assay was used to assess the effect of *S. triloba* acetone crude extract, PTX, and the combined treatment on the migration ability of SKOV3 cells. In the assay, *S. triloba* IC50, PTX IC50, and the combined treatment CI value were applied. The CompuSyn best obtained Combination Index (CI) value for the combined treatment (19 µg/mL *S. triloba* + 0.787 µg/mL PTX) was used. The migrated cells from the upper chamber to the lower chamber were stained with DAPI stain to be observed when using the fluorescent microscope (Figure 8B). After analyzing the obtained data, we found that the migration percentage of the treated cells by *S. triloba* was the highest (87%), followed by the treated cells with PTX (41%), whereas the lowest migration percentage was for the cells that were treated with the combined treatment (0%) as illustrated in (Figure 8A). The combined treatment eliminated the migration ability. The effects of the combined and PTX treatments were significant compared to the untreated cells. The P value was < 0.001. In contrary, the effect of *S. triloba* acetone crude

extract was not statistically significant. Nevertheless, there were significant differences between the used treatments concentrations.

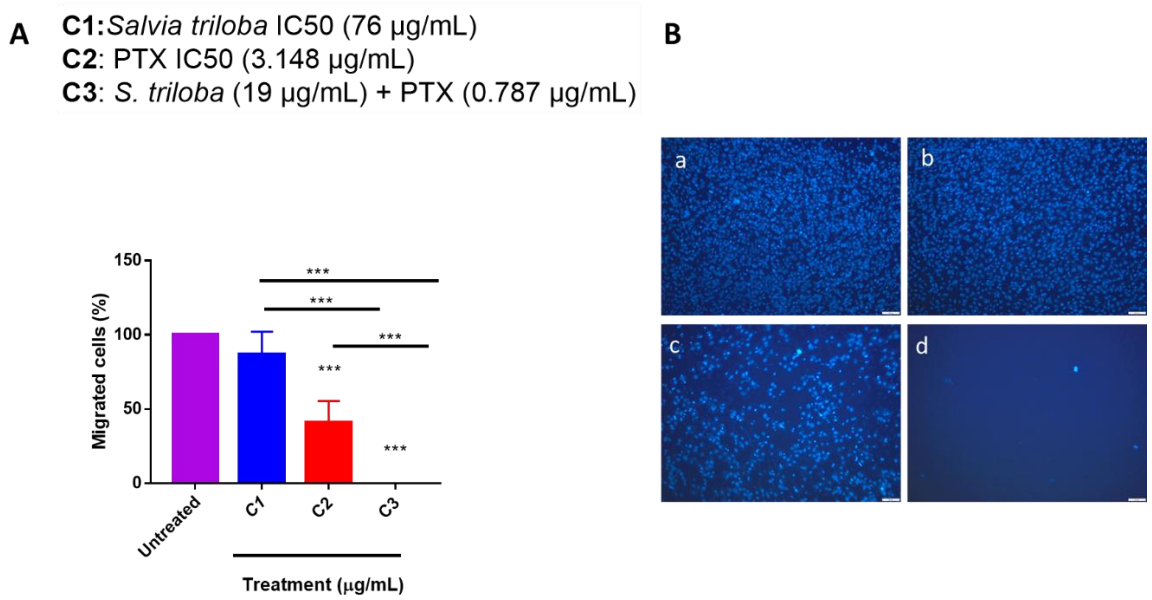

Figure 8: Effect of *S. triloba* acetone crude extract, PTX, and the combined treatment on SKOV3 cell migration.
***P ≤ 0.001, **P ≤ 0.01, *P ≤ 0.05. The error bars represent the standard deviation of the data from the mean. A) The graph represents the effect of [C1] *S. triloba* acetone crude extract IC50 (76 µg/mL), [C2] PTX IC50 (3.148µg/mL), and [C3] the combined treatment (19 µg/mL *S. triloba*+ 0.787 µg/mL PTX) on SKOV3 cell migration as a percentage compared to untreated cells. The graph was generated by GraphPad Prism software. Both PTX and the combined treatment had significant effects on SKOV3 cell migration with p value of ***P<0.001. The combined treatment had the highest effect on SKOV3 cells migration ability. There are significant differences between the used concentrations for the three treatments. B) Representative images illustrating DAPI-stained nuclei of SKOV3 cells that migrated to the lower chamber of the trans-well insert. Image (a) is for untreated cells, image (b) is for *S. triloba* IC50 (76 µg/mL), image (c) is PTX IC50 (3.148 µg/mL), and image (d) is for the combined treatment. Results are a representation of three independent experiments.

4.6 qPCR:

Several genes that are engaged in cancer proliferation, apoptosis, and migration were examined in our study to assess the effect of *S. triloba* extract and the combined treatment on gene expression on SKOV3 cells in both treated and untreated cells. Quantitative PCR (qPCR) was employed in our study for this purpose. It was used for the following genes: RUNX2, PI3KR1, β-catenin, PTEN, SETD7, MDM2 for cell proliferation; p53, and BAX for cell apoptosis; and N-cadherin for cell migration. The genes studied are listed in (Table 3).

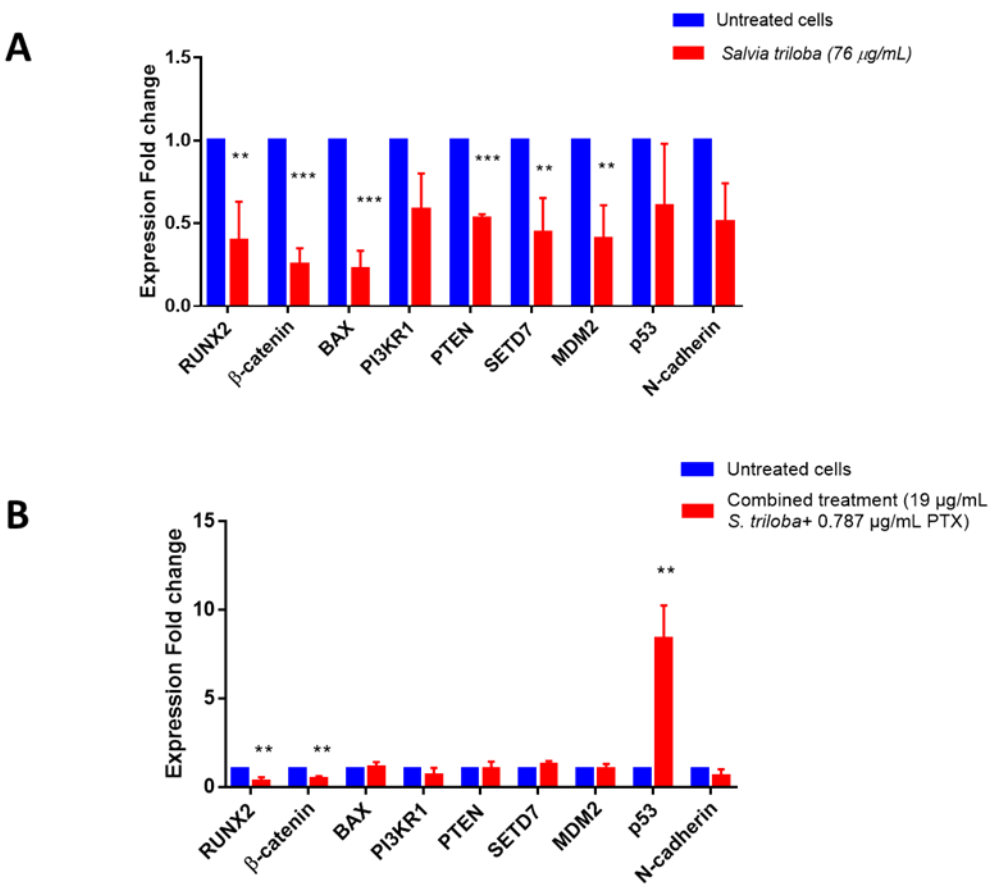

Figure 9: Effect of *S. triloba* IC50 and the combined treatment on gene expression in SKOV3 Cells. ***P ≤ 0.001, **P ≤ 0.01, *P ≤ 0.05. A) *S. triloba* acetone extract IC50 (76 µg/mL) affected gene expression in SKOV3 cells, and several genes were deregulated. The most affected genes were β-catenin, BAX, and PTEN with P value of ***P<0.001, and they were downregulated significantly. Other genes were affected less significantly, as well. RUNX2, SETD7, and MDM2 were downregulated with p value of **P<0.01. B) The combined treatment effect on SKOV3 cells shows that two genes were downregulated significantly, which are RUNX2 and β-catenin, with P value of **P<0.01, whereas p53 was significantly upregulated with **P<0.01. Other investigated genes were downregulated insignificantly by the combined treatment such as PI3KR1 and N-cadherin. Furthermore, other genes were not affected when applying the combined treatment, such as MDM2, PTEN, and BAX genes. On the other hand, SETD7 gene was slightly upregulated. The error bars represent the standard deviation of the from the mean.

All the investigated genes, in the treated cells with the *S. triloba* IC50 *(*76 µg/mL), were downregulated compared to the untreated cells. Expression of all genes was reduced by varying folds (Figure 9A). Using the acetone crude extract, the genes: β-catenin, BAX, and PTEN were the most downregulated ones with ***P value < 0.001, while RUNX2, SETD7, and MDM2 were downregulated with less P value of **P<0.01. On the other hand, p53, N-cadherin, and PI3KR1 were downregulated, but the reduction was not significant as shown in (Figure 9A). On the other hand, the effect of the combined treatment on SKOV3 cells caused RUNX2 and β-catenin genes to significantly downregulate with **P value < 0.01 (Figure 9B). Furthermore, p53 was upregulated significantly with **P value < 0.01. PI3KR1 and N-cadherin genes were

downregulated, but not significantly. Moreover, the following genes were unaffected by the combined treatment: BAX, PTEN, and MDM2. However, there was a slight increase in SETD7 expression as shown in (Figure 9B).

5. Discussion:

5.1 MTT Assay

In the present study, SKOV3 cells viability was affected by *S. triloba*, PTX and the combination treatments, as indicated in the result section. The reduction in cells viability was significant for *S. triloba* as a single treatment when higher concentrations were used. This result is in an agreement with previous studies that demonstrated *S. triloba* acetone crude extract as a treatment known for its cytotoxic effects on some types of cancer cells. For instance, *S. triloba* was reported to be cytotoxic for prostate cancer cells, but not toxic to normal cells, as it reduced cancer cells viability (Atmaca & Bozkurt, 2016). Furthermore, it was reported that it supressed cancer cell proliferation and had a modulating effect on the apoptotic signalling pathways and their downstream targets (Abu-Dahab et al., 2014; Rupasinghe et al., 2016) In a review paper (Rupasinghe et al., 2016), Salvia species including *S. triloba* found to inhibit PI3K/AKT survival pathway, and this may eventually lead to cancer cells angiogenesis inhibition and apoptosis. Since *S. triloba* acetone crude extract encompasses a variety of compounds, including β-caryophyllene, 1,8- cineole, β -pinene and camphor, it could be possible that it affects tumour cell proliferation and survival pathways (Atmaca & Bozkurt, 2016)

Furthermore, PTX is known for its anti-cell division effect. The combined treatment of *S. triloba* and PTX inhibited cancer cell growth and cancer progression, and it promoted cell death. The reduction of cell viability for combined treatment was observed to be much higher compared to monotreatment. The combined treatment is believed to be more effective on cancer cells viability and core molecular pathways than the monotreatment because advanced cancer diseases involve multiple pathogenesis and various pathways, and this is complicated for single treatments to inhibit (Lu et al., 2017). Furthermore, in our study, the used combination had synergetic effect which means that it was more powerful than the single treatment. Inhibition of cancer cell proliferation due to the effect of the combined treatment has been reported to inhibit critical pathways that are responsible for cell viability and proliferation such as Wnt/β-catenin and PI3K/AKT pathways (Islam & Aboussekhra, 2019; Sferrazza et al., 2020). Therefore, the reduction in cell viability, in the present study, might be due to the effect of the used combination on the signalling pathways.

The CompuSyn results, in the present study, exhibited that the combined treatment of *S. triloba* and PTX could be a promising anticancer therapy, as synergism was the type of the interaction between those two treatments. Both treatments were integrated, synergistically, to inhibit cell proliferation with all doses and concentrations used in this study. Moreover, the CI showed a very strong synergism using minimum doses with a very high efficacy which was 88% lethal effect on SKOV3 OC cells viability. The DRI values for *S. triloba* as obtained by CmopuSyn indicated that the effective concentration could be reduced by many folds, while for PTX it was reduced by thousands of folds. This means that the combination therapy is highly effective with minimum dose. Maintaining treatment efficacy while reducing the drug doses is the core purpose of using combined treatment, as this minimizes treatment side-effects and cytotoxicity but, at the same time, maintaining high efficacy.

DMSO is the solvent that *S. triloba* acetone crude extract was dissolved in. In the present study, the used DMSO concentration was 0.2%. DMSO showed no cytotoxic effect on SKOV3 cells viability (Figure 1). This indicated that the DMSO concentration used, in our study, did not affect SKOV3 growth. In contrast, the number of viable cells increased compared to the untreated cells, and this could be explained as DMSO at low concentrations (less than 0.5%) increases cell proliferation (Song et al., 2012). This could explain why it enhanced cell proliferation and cells viability.

5.2 IC50:

IC50 is defined as the extract concentration of treatment that is required to inhibit 50% of the cell population. Dose response curves were generated based on the results that were obtained from the MTT assay experiment for *S. triloba*. The results were determined after incubation for 48 hours, which is the incubation period in our lab for SKOV3 cell line, which has been determined based on literature. The IC50 value obtained is: 76 µg/mL. The R^2 for the analyzed data equals 0.8902 which indicates the goodness of the obtained MTT data, because its value is larger than 0.5. MTT data which is > 0.5 has a good fitness for the non-linear regression analyzing model used for IC50 determination in our study. The effect of the treatment is almost equal at the low concentrations 37.5µg/mL, 18.75µg/mL, and 9.375 µg/mL, but dose response started to be more effective at the two highest concentrations: 150 µg/mL to 75µg/mL, with the concentration 150 µg/mL showing the most cytotoxic effect on cell viability.

S. triloba extract was reported to be one of the most anticancer cytotoxic medicinal plants among several selected plants tested using MTT for human breast cancer cell lines (Atmaca & Bozkurt, 2016). *S. triloba* IC50 values are reported to vary depending on various factors, such as: concentrations used, cell line, and cancer type. For instance, previous studies reported that in the MCF-7 breast cancer cells, IC50 was found to be 250 µg/mL, and in MDA-MB-231, IC50 was 290 µg/mL, and all was for 24 hours (Atmaca & Bozkurt, 2016). Furthermore, different IC50 values were, as well, reported. For instance, it was found to be 287 µg/mL in the PC-3 prostate cancer cell line, and it was 287 µg/mL in the DU-145 prostate cancer cell line, and all was 72 hours incubation (Atmaca & Bozkurt, 2016). In another study IC50 for *S. triloba* was reported to be 25.55 µg/mL for MCF-7 cell line for 72 hours (Abu-Dahab et al., 2014). The *S. triloba* IC50 in our study was 75.96 ± 1.0237 µg/mL with incubation time of 48 hours, which is within the range of values stated in those studies. *S. triloba* in higher concentrations could induce comparable antitumor activity with much less cytotoxic effects on normal cells compared to Toxol (brand name for PTX) treatment (Zare Shahneh et al., 2013).

IC50 for PTX depends on cell line and incubation time. In the present study, PTX IC50 was found to be 3.148 µg/mL. In the literature, PTX IC50 was found to be 1.2 µg/mL for SKOV3 cells for 24 hours incubation time (Smith et al., 2005). It is reported that PTX suppresses 90% of cell growth on some cell lines, such as: Human Umbilical Vein Endothelial (HUVEC), human acute myelocytic leukemia (KG-1A), and human leukemic monocyte lymphoma (U937) (Zare Shahneh et al., 2013). Therefore, applying PTX on SKOV3 cells might have induced more cell deaths. In addition, applying the synergetic combined treatment on SKOV3 cells, considering the dual synergetic action of *S. triloba* and PTX on multiple signaling pathways, such as: the p53 and PI3K/AKT pathways, might have led to the higher cytotoxic effect observed in this study.

The results showed that the combination synergistically had a potent capability against cancer. This agrees with what the literature proposes. Many previous experiments advised that various plant derivatives have potential treatment effects against multiple diseases and disorders, including cancer, and they could be used as single treatments or in combinations with the regular cancer medications (Pezzani et al., 2019). For instance, a previous study exhibited that Zoledronic acid was used in combination with plumbagin, which is derived from a plant used in Chines traditional medicine. The combination was used in vitro on the MDA-MB-231SArfp

human breast cancer cell line (Qiao et al., 2015). In conclusion, the combination potency and the *S. triloba* accompanying toxicity might lead to a greater efficacy on SKOV3 cells viability.

5.3 Selection of the Combined Dose

The results of the dose reduction value for using combined treatment, in the present study, agree with previous studies. For instance, a previous study demonstrated that a combination treatment of dasatinib chemotherapy with the herbal plant, curcumin, was used for colon cancer, and it was found that the dasatinib concentration could be reduced by 13 – 25 folds since the DRI value was between 13 and 25 (Nautiyal et al., 2011). It is worth mentioning that the combination of the mentioned study was synergistic, as well. The obtained values of the reduction fold for PTX, in the present study, is much greater than the values mentioned in the previous dasatinib-curcumin study. The range of fold reduction for treatment doses is extremely important for cancer therapy, as it reduces the drugs cytotoxicity, which is clinically critical for patient's safety and wellbeing. It is a great beneficial strategy to combine dietary compounds, such as *S. triloba* with conventional chemotherapy, such as PTX, to treat cancer using dramatically reduced doses, to obtain a better therapeutic effect.

Since it is critical in cancer treatment to have high effects with the lowest possible doses in order to minimize health concerns, we selected in our study the lowest concentration (19.0 µg/mL *S. triloba* + 0.787 µg/mL PTX). Among the five combined concentrations, the lowest concentration demonstrated a high effect on cell viability with the minimum dose. Its effect is 88%, which is almost the same as the highest concentration (304.0 µg/mL *S. triloba* + 12.592 µg/mL PTX), which has a 90% effect on SKOV3 cells viability. Therefore, the lowest concentration (19.0 µg/mL *S. triloba* + 0.787 µg/mL PTX), obtained from CompuSyn, was used to investigate the effects of the combined treatment on SKOV3 cell viability using Trypan Blue exclusion test of cell viability and to investigate the effect of the combined treatment on SKOV3 cells migration.

On the other hand, in the present study, the obtained median dose value for PTX was very high (1.93E11) compared to *S. triloba* crude extract median dose which was 49.6650 (Table 5). This might be attributed to high PTX concentrations. This might be the reason that caused it to be not in line with the median effect equation. The used PTX concentrations could be reduced in further experiments.

5.4 Cell Morphology:

Using *S. triloba,* whether as a single treatment or combined with PTX was associated with cell morphology changes. In a previous study, *Angelica shikokiana* (Japanese medicinal herb) bioactive compounds, triterpenes and flavonoids, inhibited tubulin polymerization (Mira & Shimizu, 2015). Tubulin is associated with cytoskeleton structure and shape, and as a result, impacting tubulin stabilization might affect cell morphology. The acetone crude extract of *S. triloba* contains bioactive compounds including triterpenes and flavonoids compounds (Lopresti, 2017). The *S. triloba* bioactive compounds might have interacted with cytoskeleton components, such as tubulin. As a result, this could have affected SKOV3 cells cytoskeleton and morphology.

The changes in the SKOV3 cells shape, in our study, due to *S. triloba* acetone crude extract and the combined treatments suggested an effect on cell membrane morphology. Cell membrane morphological changes, as a result, could have been responsible for possible modifications that might have led to the induction of related pathways that resulted in the high rate of cell cytotoxicity. It has been reported in the literature that herbs could cause cell morphological changes. For instance, a previous study stated that plant-bioactive compounds targeted the cell cytoskeleton, and affected plasma membrane and cell signals (Hermenean & Ardelean, 2017). In our study, the effect of the combined treatment was stronger on SKOV3 cell line than the effect of *S. triloba* acetone crude extract as a single treatment. This could be attributed to the dual effect of the combined treatment on the cell cytoskeleton and, as well, on the critical signaling pathways.

5.5 Trypan Blue Exclusion

Trypan Blue assay was used in our study to determine the percentage of both living and dead cells. When we added the *S. triloba* acetone crude extract, PTX, and the combined treatments to the cells, they affected cell viability significantly. The treated cells start to lose the cell membrane which protects the intracellular components from being lost. Upon staining the cells with trypan blue dye, the dead cells are stained in blue as they lack their membrane (Strober, 2015). When applying the combined treatment of *S. triloba* and PTX were applied to SKOV3 cells in the present study, the number of dead cells was significantly higher than those of the living cells. This indicated that the combined treatment diminished the number of living cells dramatically, as it may affect the associated intracellular cancer pathways that are responsible

for cell growth and proliferation. This might be due to the action of bioactive/phytochemical components present in both *S. triloba* and PTX.

The combination affected cell viability, led to cancer cell death, and dramatically reduced the number of living cells. It is reported that some plants bio-active compounds bind to β-tubulin near the lumen of microtubule, which is a different site that taxol (PTX) binds to, and causes microtubules formation, which leads to cell cycle arrest (Hermenean & Ardelean, 2017). The combination might have a robust effect on SKOV3 cells viability much more than the single treatment as the bioactive molecules synergistically led to cancer cell death. This suggested that the combined treatment acted synergistically to hinder cancer development.

5.6 Trans-well Migration Assay

The synergetic effect of the *S. triloba* acetone crude extract and PTX was investigated on the migration ability of SKOV3 cells using the Trans-well migration assay. Our study demonstrated that the combined treatments abolished the migration capability of SKOV3 cells. Our results are more significant than a previous study that used a combined treatment of PTX and another plant. The previous study showed a significant reduction in the cancer cells migration upon adding the combined treatment compared to individual treatments (Xiaomeng et al., 2020). However, the effect of *S. triloba* on its own on SKOV3 cells migration was not significant, in our study. This was not the expected scenario as it is reported that it blocks the migration ability in several cancer cell lines (Atmaca & Bozkurt, 2016).

Moreover, Sugiol which is a diterpene that is isolated from *S. triloba* induced cell apoptosis and inhibited RAF/MEK/ERK signaling pathway, and inhibited SKOV3 cells migration (Y. Wang et al., 2017). Our result contradicted with the reported migration inhibitory effect of *S. triloba.* The reason behind this result could be that the SKOV3 cells proliferated during the incubation time (24 hours), as it was long enough for the cells to proliferate. In the literature, the doubling time for SKOV3 cells is 24 hours (Paramee et al., 2018). This could have led to the insignificant reduction in SKOV3 cell migration using *S. triloba* as a single treatment. The cells might have proliferated during the incubation time, which could have affected the number of migrated cells. Therefore, an optimization and a shorter incubation period might change the result.

5.7 Gene Expression

The literature states the health benefits of bioactive compounds of plant extracts, such as phenols, as they have anti-inflammatory, antioxidant, and anti-carcinogenic effects due to their various therapeutic uses, as they are able to: inhibit oncogenic pathways that control cell apoptosis, proliferation and angiogenesis; promote tumor suppressor proteins induction; enhance cell cycle arrest; and modulate ROS levels (Anantharaju et al., 2016). In the present study, the molecular basis by which the expression of several markers involved in cell proliferation, apoptosis, and cell migration was studied. Most of the investigated genes in this study were downregulated by the *S. triloba* acetone crude extract. PIK3R1, RUNX2, p53, BAX, β-catenin, MDM2, PTEN, SETD7, and N-cadherin were the genes of interest. They can be categorized into three groups based on their molecular pathways, which are affected by *S. triloba*, namely: PI3KR1/AKT, Wnt/β-catenin and RUNX2. β-catenin is the key player in the Wnt/β-catenin pathway, PIK3R1is the main factor in the PI3KR1/AKT pathway, and RUNX2 is a crucial player in RUNX2 pathway.

5.7.1 PI3K/AKT Pathway

The PI3KR1/AKT is a conserved signalling pathway which is activated via a process that constitutes multiple steps (Hemmings & Restuccia, 2012). The PI3K/AKT signalling pathway is mutated or over-activated in various types of cancer, and this pathway regulates a wide array of cell associated processes, such as: proliferation, growth, angiogenesis, survival, metastasis and metabolism (Ersahin et al., 2015). Two important proteins are involved, namely: phosphatidylinositol 3-kinase (PI3KR1) and Akt (Protein Kinase B). PI3KR1 has the ability to phosphorylate the 3 location hydroxyl group of the phosphatidylinositol's inositol ring (CD, 2020). The other protein AKT, which is a serine/threonine-specific protein kinase, plays an important role in many cell processes (CD, 2020). AKT, upon activation, controls functions either activating phosphorylation or inhibiting a group of proteins that are associated with cell activities, such as: cell proliferation, adhesion, neovascularization, motility, and cell death (CD, 2020).

The PI3K/AKT pathway has numerous downstream impacts. This pathway can be negatively regulated through inactivation of AKT protein, and targeting PIP3 level (Nagata et al., 2004). One of the major downregulation proteins is PTEN (Phosphatase and tensin homolog), which has the ability to convert PIP3 into PIP2, and this causes inhibition of the PI3K/AKT pathway,

which is an anticancer activity (Nagata et al., 2004). PI3K/ AKT/mTOR pathway is a central regulator of OC in which AKT phosphorylates the BAD gene in OC cells (Aziz et al., 2018). PI3KR1 is known to be upregulated in OC, and it contains a missense mutation on chromosome 3 in SKOV3 cells (Ghoneum & Said, 2019). However, it has been a challenge to target this pathway in OC as several trials were not clinically successful (Aziz et al., 2018).

Tectorigenin, which can be isolated from some flower types, decreases the PI3K/AKT pathway in human OC by down-regulating AKT (Yang et al., 2012). In addition, it has been reported, in many studies, that the bioactive components from Salvia plants modulate the cell signaling pathways including PI3K/AKT, which indicates its anticancer effect (Rupasinghe et al., 2016). In our study, the PI3KR1 mRNA expression was downregulated upon adding the acetone crude extract suggesting that the *S. triloba* acetone crude extract affected the PI3KR1 gene and the PI3K/AKT pathway, consequently. The findings of our study showed a down-regulation in PIK3R1 expression. This is similar and goes in accordance with previous studies in which PI3K was downregulated when a plant treatment was added. When the ethanolic extract of the neem leaf was added to the PC-3 and LNCaP prostate cancer cell lines, it affected the PI3K/Akt pathway (Gunadharini et al., 2011). The inhibition of AKT activity stimulates apoptosis in a wide range of mammalian cells (Gunadharini et al., 2011). This might be explained by the interconnection between signaling pathways.

PTEN is a wild type in SKOV3 cells (Phadngam et al., 2016). The mRNA level of PTEN was significantly downregulated with P value > 0.001 when the acetone crude extract was added to SKOV3 cells. The acetone crude extract might be targeting the transcriptional activity of combination of different molecules. As a consequence, it impaired the PI3K/AKT pathway by downregulating PTEN gene at the same time (Silva et al., 2008). It is reported that PI3K/AKT pathway is a central regulator of OC.

Hyperactivation of PI3K/AKT pathway due to mutations lead to ovarian tumorigenesis; and therefore, it is considered a promising target. In the present study, it seemed that the PI3K/AKT pathway was targeted by *S. triloba* when applied as a single treatment, and by the combined treatment, as well. This could be observed from the downregulation of mRNA expression of the PI3KR1 gene in this study. The downregulation was not significant in both cases, individual *S. triloba* and combined treatments. This could be made more significant if the acetone crude extract was fractionated.

The combined treatment has a great therapeutic value as it targets signalling pathways that are essential for cancer progression (Islam & Aboussekhra, 2019). The present study showed that using the combined treatment affected the critical signalling pathways in OC, such as PI3K pathway. Most importantly, both drugs in the combination integrated to cause a greater effect on SKOV3 cells. Our result agrees with another study (Davies, 2013) which states that the synergy effects of the combined treatment can be achieved by multi-target mechanism. Therefore, other signalling cascades might be affected by applying the combination as the cross talk between signalling pathways is reported in the literature (S. K. Singh et al., 2019).

Targeting PI3K/AKT pathway affects other cascade pathways because PI3K/AKT pathway has a crosstalk with multiple pathways that are involved in OC. It is worth mentioning that in OC cells, PI3K/AKT/mTOR network inhibits the P21 and P27 expressions. This suggested that the crosstalk of pathway PI3K/AKT signalling has a regulatory role in OC (Aziz et al., 2018). There is another PI3K/AKT crosstalk with p53 pathway as AKT participates in the degradation of p53 via MDM2 phosphorylation (CD, 2020). Furthermore, Wnt/β-catenin Pathway is strongly interconnected with PI3K/AKT pathway; therefore, the latter deregulation affects cellular β-catenin signalling.

5.7.2 Wnt/β-catenin Pathway

Wnt/β-Catenin is a critical pathway in cell process, such as the regulation of crucial phases in the cell, including cell fate, cell polarity, cell migration, neural development, and organogenesis throughout embryonic progress. β-catenin is a protein that has multiple functions and plays a major role in physiological normal homeostasis in the cell, and it is an adaptor protein for intracellular adhesion (van Ooyen & Nusse, 1984). Wnt is the main regulator of β-catenin. Wnt is a protein family of 19 glycoproteins that has a core role in regulating canonical Wnt (the β-catenin-dependent) and non-canonical Wnt (β-catenin independent) pathways (van Ooyen & Nusse, 1984). In canonical Wnt pathway, Glycogen Synthase Kinase 3 beta (GSK3β), AXIN, Dsh, β-catenin, T-cell factor (TCF)/lymphoid enhancement factor (LEF) and adenomatous polyposis coli (APC) have been recognized as signal transducers (Guo & Wang, 2009).

β-catenin overexpression has been linked to many diseases including cancer (van Ooyen & Nusse, 1984). When β-catenin is combined with T-cell factor (TCF), the complex activates β-catenin target gene expression, and this would help in supporting the progression of

carcinogenic cells (Hlubek et al., 2007). It has been demonstrated that the β-catenin apparent availability and distribution contribute significantly to both behavior and phenotype of tumor cells (Brabletz et al., 2001). It has been evidenced that β-catenin is linked to cell proliferation and growth forms in differentiated colorectal adenocarcinomas in invasive fronts, primary tumors, and lymph node metastases (Brabletz et al., 2001).

It has been observed that there is deregulation in the Wnt/β-catenin signaling pathway in the majority of colorectal carcinomas cases (Hlubek et al., 2007). It has been reported that the Wnt/β-catenin signaling pathway has been correlated to OC tumorigenesis and metastasis, and this is associated with its crosstalk with other cancer signaling pathways, such as PI3K/AKT pathway (Gatcliffe et al., 2008) (Niu et al., 2012). β-catenin deregulation is reported in SKOV3 cells, and mechanisms for deregulating β-catenin include mutations in the CTNNB1 gene that encodes β-catenin, or mutational inactivation of APC, AXIN1, and AXIN2 (Wu et al., 2001).

In our study, the mRNA expression of β-catenin was significantly downregulated by *S.* triloba with P value > 0.001 which is consistent with other studies. One of these studies describes the downregulation mechanism in OC upon adding *Salvia miltiorrhizae* as a treatments as it reverses the decreased ratio of p-glycogen synthase kinase 3β (GSK3β) and increased ratio of p-β-catenin to β-catenin in ovariectomized rats (Liu et al., 2018). Targeting this pathway leads to decreasing its downstream genes, which can reduce the OC invasiveness and progression (Shanmugam et al., 2016). In another study, curcumin diminished the osteosarcoma cells ability to migrate and proliferate by suppressing the Wnt/β-catenin pathway (Shanmugam et al., 2016). PI3K/AKT pathway downregulation might affect β-catenin mRNA expression level and cause its downregulation consequently.

In our study, the combined treatment of *S. triloba* and PTX significantly downregulated the β-catenin expression levels. This is in accordance with other studies. (Sferrazza et al., 2020) demonstrated that the combined treatment of curcumin and irinotecan inhibited β-catenin, GSK3β, and N-cadherin expression through the prevention of β-catenin/TCF DNA binding and transactivation. This downregulation might be explained by the activation of the tyrosine kinase receptor and the activation of GSK3β and degradation of β-catenin (Shang et al., 2017).

In comparison between the results of the *S. triloba* alone, on one hand, and the combination treatment, on the other hand, the present study showed that the acetone crude extract

downregulated β-catenin expression more significantly than the combined treatment. This might be due to an increase in Wnt signaling because of PTX treatment as it activates the Wnt signaling pathway (X. Wang et al., 2019). As a result, this could have led to the less significance decreasing of β-catenin compared to *S. triloba* alone.

5.7.3 RUNX2 Pathway

The RUNX2 protein is a transcription factor. It binds to specific regions of DNA and helps control the activity of a group of genes. RUNX2 mediates cellular proliferation and cell cycle control. RUNX2 is a major factor in osteoblast division, and found to control cell division, and proliferation during formation of endochondral bone (Haiyan Chen et al., 2014). It has been demonstrated that RUNX2 is a major player in cancer progression and invasion. Some studies have shown that RUNX2 is a player in some cancer types, such as: acute myeloid leukemia, and multiple myeloma (Hay et al., 2019; Kuo et al., 2009).

RUNX2 is upregulated in tissues of epithelial OC (EOC) when compared to normal ones, and this upregulation is largely associated with poor EOC patients' diagnosis (W. Li et al., 2012). RUNX2 was associated with a variety of luteal function traits of the rat ovaries by regulating the transcription of multiple luteal genes in luteinizing granulosa cells (Park et al., 2010). Our study showed a significant decrease in the expression of RUNX2 gene with $P < 0.05$. These data are compatible with other studies. For instance, Chloranthus multistachys-ISTA (istanbulin A) inhibited RUNX2 activation and inhibited its transcriptional activity as well (Fu et al., 2015).

RUNX2 is reported as a novel transcriptional regulator of tumor-induced EMT (Fu et al., 2015). RUNX2 is a substrate for PI3K/AKT and Wnt/β-catenin signal pathways as they decrease its expression and its target genes expression upon adding a plant treatment, which is Ephedra Foeminea, on human bone osteosarcoma U2OS cell line (Mpingirika et al., 2020). This might be the reason behind RUNX2 down-regulation due to using *S. triloba* treatment in our study. In the literature, it is well documented that AKT activates the RUNX2 mRNA expression and its downstream genes (Cohen-Solal et al., 2015). Interestingly, these two pathways are evidenced to be activated by RUNX2 (Cohen-Solal et al., 2015). There is a feedback loop between these pathways, as they are overlapped and strongly linked inside the cell. Targeting

RUNX2 and its crosstalk partners would be a beneficial therapeutic way to treat OC, as it targets the genes that are involved in OC angiogenesis, chemo-resistance, and metastasis.

N-cadherin is involved in the Wnt/β-catenin and other critical pathways in OC. This gene actively promotes cancer metastasis. It is reported that N-cadherin positively and actively regulates cell migration, cell invasion, EMT and stemness properties of prostate cancer cells (M. Wang et al., 2016). It was downregulated by *S. triloba* in our study, and this could possibly be due to the downregulation of β-catenin and PI3K/AKT pathways. The PI3K/AKT pathway can overexpress the mRNA level of N-cadherin via its transcriptional regulation of the two genes Twist and Snail (B. Li et al., 2017). Therefore, the downregulation of PI3KR1, in our study, could have caused the downregulation of N-cadherin. Moreover, the downregulation of β-catenin could have caused the downregulation of RUNX2 and other transcriptional factors, such as Twist, and this might have led to the downregulation of N-cadherin.

SETD7/9 oncogene, which is important in the development of OC, activates RUNX2. SETD7 (SET Domain-Containing Protein 7) is an enzyme that transfers a methyl group to a lysine residue of various substrates. Its function is the acetylation or deacetylation of proteins. Therefore, it plays a major role in the epigenetic gene expression modulation, which is a hallmark of tumorigenesis.

In the present study, SETD7/9 was significantly downregulated by *S. triloba.* As a result, this might have affected SKOV3 cells proliferation, migration, and invasion. This might have led to the downregulation of RUNX2 and β-catenin in the transcription level. A previous study indicated that Berberine acts as a putative epigenetic modulator by affecting the SETD7 positively as a strategy treating multiple myeloma via epigenetic modifications (Z. Wang et al., 2016). When the combination treatment of *S. triloba* and PTX were used in the present study, the effect on the mRNA level on RUNX2 and N-cadherin was similar. However, there was a minor increase in SETD7 expression. The genes downregulation might be because of the effect of *S. triloba* and not due to PTX. Moreover, the slight increase in SETD7 upregulation might be because of PTX. It is worth mentioning that, SETD7 methylates and stabilizes p53 and inhibits its nuclear export, which is crucial in cancer treatment (Campaner et al., 2011).

5.7.4 p53, MDM2 and BAX genes:

The p53 is a critical tumor suppressor. It regulates various biological functions, such as: DNA repair and apoptosis and, generally, it ensures genome stability. It is described as the "guardian of the genome", and its protein could be induced to respond to many types of stress signals (Zhao et al., 2014). It has been found to be the most frequently down regulated gene in human malignancies that have been studied so far (Zhao et al., 2014). BAX (BCL2 Associated X) is a protein coding gene, and it encodes a protein that belongs to the BCL2 protein family and it is regulated by p53 gene. p53 status in SKOV3 cells is null[2] (i.e. functionless); therefore, its regulation of BAX gene is lost, which leads to cancer invasion and chemoresistance due to an inefficient mechanism for controlling BAX (Ahn et al., 2017). This might explain the downregulation of BAX gene in SKOV3 cells, as its regulator gene is lost. Therefore, the expression of BAX will be diminished as p53 is lost in SKOV3 cells.

MDM2 (the murine double minute 2) is an oncogene, and it has shown malignant activities in various tumors. The oncogenic structural features are attributed to the fact that it has multiple conserved functional domains (Zhao et al., 2014). There is a feedback loop relationship between MDM2 and P35. MDM2 negatively regulates p53, which reduces apoptosis, while p53 decreases MDM2 expression (Cohen-Solal et al., 2015). In this study MDM2 expression was down regulated upon addition of the acetone crude extract to the cells, which indicates the ability of the extract to reduce its levels and, therefore, decrease cancer progression (X. Zhang et al., 2010). MDM2 downregulation is most likely due to the targeting of β-catenin and PI3KR1/AKT pathways by the extract.

There was no effect of the combined treatment in this study on mRNA level of MDM2 gene, as its level was the same as the untreated cells (control cells). This might be because the acetone crude extract downregulated MDM2, while PTX upregulated it. This could be attributed to the interruption of the MDM2-p53 feedback loop, as p53 expression was elevated but MDM2 expression was not affected, while it was supposed to be downregulated (Shen et al., 2012). Furthermore, modulating MDM2 ubiquitination activity by *S. triloba* and PTX might have led to decrease its ability to downregulate itself and p53, as well, as they might have altered it

[2] Null here means functionless. This means it exists but with no function due to inhibition.

conformation, modification, and interaction with P53 (Nag et al., 2014). It is worth mentioning that phosphorylated p53 could escape the MDM2 binding and act as a transcription factor.

The same applies to the two genes: PTEN and BAX. *S. triloba* might have antagonized the PTX effect upon using the combined treatment. The increase of the expression of the apoptotic molecule BAX, induces the occurrence of cell apoptosis (Hao Chen et al., 2016); this was explained as the treatments promote the apoptosis of tumor cells mainly by inhibiting NFκB and the activity of transcription factor AP1 lead to the opening of the mitochondrial inner membrane, which led to the cytochrome C release of proapoptotic proteins into the cytoplasm. and the starting of the apoptosis (Hao Chen et al., 2016).

According to a previous study, *S. triloba* induced apoptosis in breast cancer cell lines, but it did not affect p53 expression levels (Abu-Dahab et al., 2014). In the present study, p53 gene was downregulated, but the downregulation was not significant, when *S. triloba* was applied on SKOV3 cells, but the combined treatment upregulated p53 significantly. It has been demonstrated before that the combination of Resveratrol with etoposide in Colon Rectal Cancer cell lines has a synergistic effect on cell growth inhibition via downregulation of mitogen-activated protein kinase signaling pathways and an increase in apoptosis via activation of p53 (Ahmed et al., 2019). It is known that chemoresistance is linked to p53 mutations, and this led to escape cell death, as PI3K/Akt signaling pathway is the major mechanism that growth factors promote cell survival and blocking caspase 9 activity (Takeuchi et al., 2006). Thus, using the combined treatment of *S. triloba* and PTX can potentially overcome chemotherapy resistance which is responsible for the high mortality incidence among OC patients.

There is an ongoing argument over the effectiveness of plants treatments as whole extracts or as isolated components. Some studies have proposed that the isolated components of a plant are more effective than the entire extract of the plan, while other studies have suggested that the reverse by indicating that the whole extract is more effective than the individual components because the extract enhances the metabolism absorption of the various bioactive elements (Atmaca & Bozkurt, 2016). It is believed that the various bioactive components of the extract could provide more synergistic effects as they could impact multiple molecules and different pathways (Atmaca & Bozkurt, 2016). It is a matter of fact that *S. triloba* extract constitutes various phytochemical components, including: a1,8- cineole, β-caryophyllene, β -pinene and camphor. This makes it possible that it might act on different cancer signaling

pathways, and it could possibly induce cancer apoptosis and inhibition of angiogenesis (Atmaca & Bozkurt, 2016). It has been discovered that *S. triloba* extract functions as tumor specific, as it was cytotoxic on human PC (prostate cancer), but it did not affect the non-transformed breast epithelial cells (Atmaca & Bozkurt, 2016).

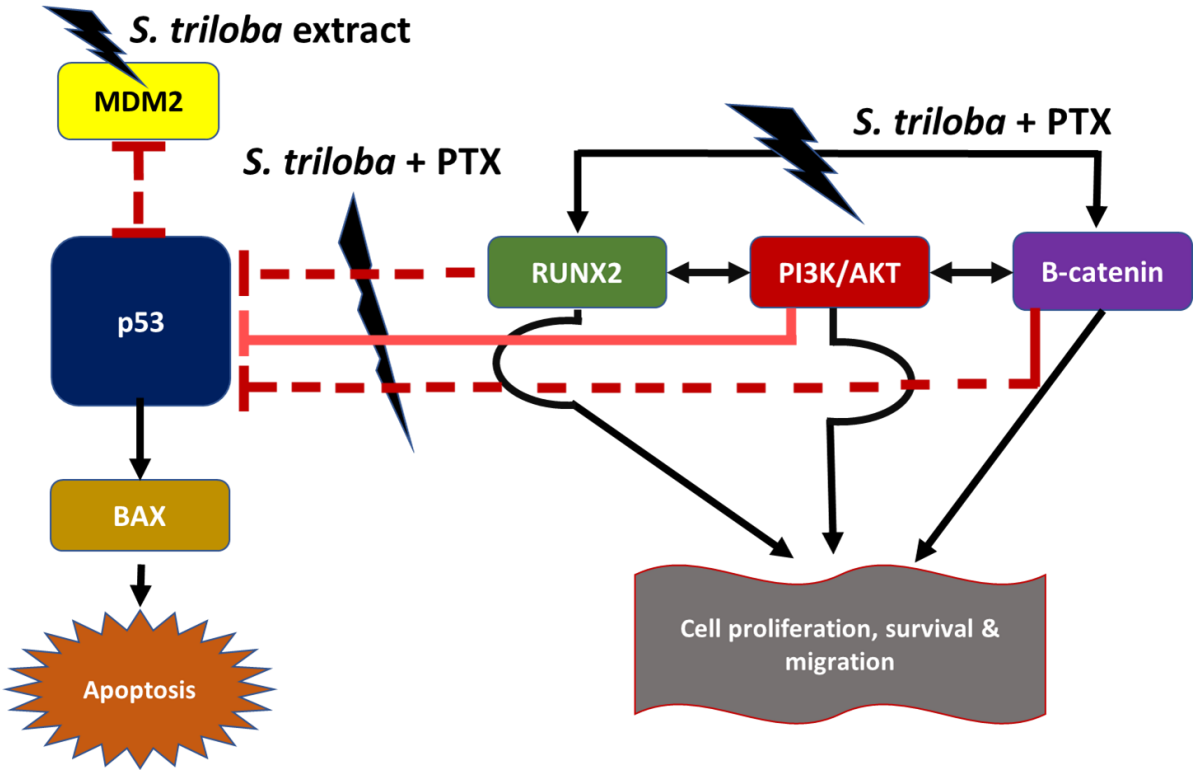

Figure 10: Proposed model of the effect of *S. triloba* extract and the combination (*S. triloba* and PTX) on the steady-state mRNA expression of key markers involved in OC.
The figure shows a proposed model by which *S. triloba* extract and the combination might have affected the steady-state mRNA expression of key markers involved in OC cell proliferation, survival, and apoptosis. The figure shows the crosstalk between the pathways involved. The deregulation in the three pathways PI3K/AKT, RUNX2, and β-catenin in cancer can cause mutations in several gens such as p53 (the guardian of the genome). Inhibition of p53 by the three pathways can lead to p53 dependent apoptosis inhibition which promotes cancer progression. The combined treatments downregulated β-catenin and RUNX2 significantly, while PI3KR1 was downregulated. Furthermore, the combined treatment upregulated P53 significantly by about 8 folds. On the other hand, *S. triloba* extract downregulated MDM2 significantly and upregulated both RUNX2 and β-catenin considerably.

5.8 Conclusion

To conclude, this study showed that *S. triloba* acetone crude extract combined with PTX significantly reduced SKOV3 cell viability and migration ability. Furthermore, the combined treatment decreased the steady-state mRNA expression of key markers involved in promoting both proliferation and migration in SKOV3 cells and activated apoptosis pathway. The use of *S. triloba* in the combination treatment might help in amplifying the apoptotic signals in SKOV3 cells. It is likely that *S. triloba* contains bioactive compounds that have anti-OC

properties, as they targeted critical pathways that have central role in OC proliferation and migration.

Moreover, *S. triloba* in the combinations appeared to potentiate PTX efficacy against OC. This suggests that *S. triloba* combined with PTX might have strong therapeutic potentiality via targeting crucial pathways involved in OC.

The bioactive compounds in the *S. triloba* acetone crude extract have pleiotropic effects; therefore, they might exert synergistic chemo preventive actions.

5.9 Future Recommendations

Based on the results of the present study, the following recommendations are proposed:

- Explore the apoptosis signaling induction via the apoptosis assays and the levels of phosphorylated p53 in treated SKOV3 cells using Western Blot assay.
- It would be required to identify key modulators of the main pathways that inhibit apoptosis in OC.
- Further studies are required for the identification of anti-OC bioactive compounds of the *S. triloba* extract.
- *S. triloba* alone or in combination with other treatments may be a potential therapeutic agent for other types of cancer.
- Finding out the effect of *S. triloba* on other critical pathways in cancer would be useful.
- In this study, it has been demonstrated that *S. triloba* acetone crude extract possesses anti OC therapeutic properties in vitro. It is recommended that in vivo experiments (using mouse models) be conducted to assess the effects better and validate the results.

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