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## RESEARCH ARTICLE

# THE ANTIOXIDANT, ANTIBACTERIAL, ANTIHEMOLYTIC AND EPITHELIAL OVARIAN CANCER ANTIPROLIFERATIVE ACTIVITIES OF THE LEBANESE PLANT *SALVIA LIBANOTICA*

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## ABSTRACT

**Background:** Salvia libanotica is an endemic plant species in the Mediterranean region. It is widely used in Lebanon to treat abdominal pain and colds. Objective: This study aimed to evaluate the global nutritional value of this plant. **Methods:** The total phenol content and the antioxidant activity were determined using the Folin-Ciocalteau and DPPH radicals assay, respectively. The antiproliferative (determined by Neutral red and MTT assay) effect of S. libanotica was tested on the epithelial ovarian cancer (SKOV-3) cell line. Microtiter broth dilution method was employed to assess the antibacterial activity. The antihemolytic activity of S. libanotica was assessed using H2O2 hemolysis test. **Results:** Our results showed that S. libanotica extracts contained high amount of polyphenols. The plant showed also an antioxidant activity, it has inhibited the viability of SKOV-3 cell line in a dose-dependent manner. The extracts were potent against hemolysis of the erythrocytes and showed high bacteriostatic action toward different bacterial species. Finally, S. libanotica showed that itleads to apoptotic death in SKOV-3 by the upregulation of the pro-apoptotic proteins p53 and Bax. Conclusion: The results suggested that S. libanotica could be used in treating for several diseases naturally with the least side effects on human health.

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## INTRODUCTION

Epithelial ovarian cancer (EOC) is the sixth most common type of cancer in the world and the second most common gynecological malignancy(Permuth-Wey and Sellers, 2009).EOC is the fifth leading cause of death among women, largely due to the difficulty to detect early stage disease(Siegel *et al.,* 2012). As EOC has an early dissemination to the peritoneal cavity, intraperitoneal (IP) chemotherapyis needed to reduce the chance of recurrence.But many patients showed the development of a resistance to thechemotherapeuticdrugs associated with toxic side effects. For this reason, it is important to identify certain new agents that have better anticancer effect without toxic effects in *healthy cells.* Long ago, plants were used for treatment of certain disorders (Gupta and Sharma, 2006).

Even today, the *use* of this *natural* resource continues through the non-industrialized societies, and is often less expensive than modern pharmaceuticals. In the Asian and African countries, as much as 80% of the population uses herbal medicine for some part of primary health care(Shinwari and Qaiser, 2011).The use of herbal medicinal products and supplements has increased vastly over the past three decades in the United States and Europe because of the effectiveness of herbal products in some diseases.The biological activities of any medicinal plant are due to the presence of secondary metabolites such as phenolic compounds, alkaloids, tannins, saponins, flavonoids and steroids (Kurmukov, 2012).Herbal medicines are commonly used in the Middle East to treat various diseases such as infertility, depression, epilepsy and abdominal pain(Alzweiri *et al.,* 2011; Baydoun *et al.,* 2015; Dirani *et al.,* 2014; Farhan*et al.,* 2013;Rammal *et al.,* 2013). In the region, mainly in Jordan and Syria, over 100 nativespecies are used in traditionalmedicine (Alzweiri *et al.,* 2011; Abu-Irmaileh and Afifi, 2003). Lebanon contains over 2,607

species of flowering plants, which includes over78 endemic species and more than 138 species of medicinal plants (Nehme, 1978; Post, 1932; Abu Chaar, 2004; Deeb *et al.,* 2013; Abu-Darwish and Efferth, 2018). As traditional medicine becomes more and more important to treat different diseases, our studyis focused on the endemic medicinal plants in Lebanon and more especially on the plants that have not been investigated before. In this study, we are interested on *S.libanotica* species. *S.libanotica*, an endemic plantofthe Mediterranean region, is commonly used inthe Middle East to treat colds and abdominal pain(Gali-Muhtasib*et al.,* 2000)To date, there have not been enough studies onthe biological effects of this Lebanese plant. For this reason, thisstudy aimed to determine the total phenol contents of *S. libanotica* and to evaluate itsantibacterial, antioxidant, anti-hemolytic and antiproliferative effect on the human epithelial ovarian cancer cell line (SKOV-3). Therefore, the anticancer mechanism of the water extract of *S. libanotica* via apoptosis induction was investigated.

## MATERIALS AND METHODS

**Plant collection and extraction:** The fresh plant was gathered from Mount Lebanon- Bchamoun, then, cleaned and dried in the shade at room temperature. After drying, the plants have been grinded and transformed into small pieces for further extraction. The grinded plantwas preserved in clean plastic containers, kept away from light, heat and moisture until extraction. Solvents used for extraction were distilled water and ethanol. For the water extract,a total of 15 g of the plant dry weight of *S.libanotica* was dissolved in 250 ml distilled water and agitated for 3 hours at room temperature, and inthe last 10 minutes the temperature was increasedto 60°C. For the ethanol extract, 25 g of the plant dry weight was dissolved in 300 ml ethanol and agitated overnight at room temperature.
Both, ethanol and water extracts, were concentrated using rotary evaporator and lyophilisator, respectively.

**Estimation of total phenolic content (TPC):** The method of *Folin-Ciocalteau* reagent has been used to estimate the TPC (Farhan, Malli*et al.,* 2012).100µl (1 mg/ml) of both, water and ethanol extracts, were prepared and mixed with 0.5 ml of *Folin–Ciocalteau* reagent (1/10 dilution) and 1.5 ml of a 2% $Na_2CO_3$ (Sodium Bicarbonate). The absorbance of theblue-colored liquidextract was measured at 760 nm using a Gene Quant 1300 UV-Vis spectrophotometers. The results were expressed in mg of Gallic acid equivalent (GAE) per g of dry weight of plant powders. The TPC in extracts was determined as mg of gallic acid equivalents (GAE) per g of extract (mg of GAE/g of extract) using the equation obtained from the standard gallic acid calibration curve. The blank was preparedby adding 0.5 mL water-MeOHto1.5 mL of $Na_2CO_3$ (2%).

**Evaluation of the antioxidant activity by DPPH assay:** Antioxidant activity was determined by DPPH radical scavenging activity according to the method of Rammal et al. (Farhan, Rammal *et al.,* 2012).Samples with increased concentrations (10, 50, 50, 80 and 100µg/ml) were prepared. 1 ml of each prepared dilution of water and ethanol extracts of *S.libanotica*was added to 1ml of DPPH (0.15 mM/L in ethanol). Control was prepared by mixing 1 ml DPPH with 1 ml of selected solvent. The solutions were incubated in the darknessand at room temperature for 30 minutes. Then the absorbance was measured at 517 nm by a Gene Quant 1300 UV-Vis spectrophotometer. The ascorbic acidwas used as a positive control. Ethanol and distilled water were used as blanks for ethanol and water extracts, respectively. The DPPH scavenging ability of the samples was calculated according to the following equation:% Radical scavenging activity = [(Abs control − Abs sample)]/ (Abs control)] ×100.

**Cells, Cell Cultureand Bacterial Culture :** Human epithelial ovarian carcinoma cell line SKOV-3 was purchased from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in RPMI-1640 medium (Sigma Chemical Company) supplemented with 0.1mg/ml streptomycin, 100 U/ml penicillin and 10% fetal bovine serum (FBS). SKOV-3 was maintained at 37°C, under an atmosphere containing 5% $CO_2$. Four referenced bacterial specieswere used in this study. One Gram-positive species, *Staphylococcus aureus*; three Gram-negative species, *Escherichia coli, E. coli* multidrug resistance and *Pseudomonas aeruginosa.* All bacterial specieswere stored at -80°C in glycerol stocks and used as required. Bacterial cultures used in this research were: Brain heart infusion broth/agar (BHI/BHA), tryptone soya broth (TSB) and Mueller-Hinton broth (MHB).

**Cells culture to cytotoxic assays:** The cytotoxicity test was performed onSKOV-3 cell line. SKOV-3 was cultured in 75 $cm^2$ sterile flasks. When the cellswereapproximately 75% confluent, the cell monolayer was washed with a culture medium, trypsinized, distributed in a flat-bottomed 96- well plate ($8 \times 10^3$ cells/well), and incubated for 24hrsat 37°C for cell adherence. After 24 hrs, the cells were treated with five different concentrations of water extract of *S.libanotica*: 50, 100, 150, 200 and 250 µg/ml each being tested in three replicates. The plates were incubated for 24, 48 and 72hrs at 37°C in a 5% $CO_2$ incubator. The untreated cells were usedas a negative control, while the cells incubatedonly with distilled water were used as a vehicle control. No effect due to the distilledwater was observed. The results were used to construct a graph of the cell viability inhibition percentage against extract concentrations.

**Cytotoxic activity:** A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/ml; 20 µl/well) was added to evaluate mitochondrial viability (Varache-Lembège *et al.,* 2008); after 4 hrsincubation, the supernatants were removed, 100 µl of isopropanol was added to each well, and the reactions were mixed to solubilize the formazan crystals. The optical density was determined at 595 nm to measure the signal and the background, respectively. The experiment was repeated three times independently, each time in triplicates. Cell viability was also performed using Neutral Red assay based on the protocol described earlier (Borenfreund and Puerner, 1985; Malek *et al.,* 2011; Nasreddine *et al.,* 2015). Neutral Red, a chromogenic dye, is an indicator of lysosomal activity. Lived cells demonstrated a chromogenic change with Neutral Red that is detected spectrophotometrically. After24, 48 and 72hrs, the old medium was replaced by100 □l of fresh medium containing 40 □g/ml neutral red and incubated for 3 hrs. This final step was done to allow the uptake of the vital dye into the lysosomes of viable and undamaged cells. Then, the media was discarded and cells were washed twice with 100 □l of 1X PBS. The intracellular accumulation of neutral red dye was extracted using200 µl of 50% ethanol-1% acetic acid lysing solution. The optical

density (OD) of the eluted neutral red dye was red at 490 nm using a microplate reader. The experiment was repeated three times independently, each time in triplicates. The percentage of inhibition of each of the test samples was calculated according to the following formula using the OD values obtained: Percentage of inhibition (%) = (OD control − OD sample)/OD control × 100. The cytotoxicity of *S. libanotica*is expressed as $IC_{50}$ value. The $IC_{50}$ value isthe concentration of test agents that cause 50% inhibition or cell death, averaged from at least three separate experiments, and was obtained by plotting the percentage inhibition versus concentration of water extract.

**Hemolytic activity**

Thehemolytic activity was determined according to the method of D. Malagoli(Malagoli, 2007). Fresh human blood sample was centrifuged at 2500 rpm for 10 min and erythrocytes were separated from the plasma and were washed three timeswith 1X-Phosphate Buffered Saline (PBS Buffer) atpH7.4. After each washing, cells were pelleted by centrifugation at 2500 rpm for 12 min at 4°C and the supernatant was discarded. The obtained pellet was diluted with PBS to give a suspension of 5%. To 1 ml of the erythrocyte suspension, 100 µl of ethanol and water extracts were added at differentconcentrations (10, 50 and 100 µg/ml diluted in PBS-1X). The mixture was incubated at 37°C for 1 hr and a half, with shaking every 30 minutes, then the tubes were centrifuged at 2500 rpm for 10 min at 4°C and the color density of the supernatant was measured spectrophotometrically at 540nm. The absorbance value of the erythrocytes maintained exclusively in PBS has been utilized to set the 0 value before reading the samples that contained the extract. 1% SDS was used as a positive control.For each concentration and for the control, the experiments were set in triplicates. A total of three independent experiments have been performed. Hemolyticlevels were expressed as apercentage of hemolysis according to the following equation:% Hemolysis = Absorbance of control – absorbance of extract / Absorbance of control X 100.

**Anti-hemolytic activity**

The anti-hemolytic activity was determined according to the method of Omale James (James, 2014). Fresh human blood sample was centrifuged at2500 rpm for 10min and erythrocytes were separated from the plasma and were washed three timeswith 1X-Phosphate Buffered Saline (PBS Buffer) at pH7.4. After each washing, cells were pelleted by centrifugation at 2500 rpm for 12 min at 4°C and the supernatant was discarded. The obtained pellet was diluted with PBS to give a suspension of 5%. To 1 ml of the erythrocytes suspension, 100 µl of ethanol and water extracts were added at differentconcentrations (10, 50 and 100 µg/ml diluted in PBS-1X). The mixture was incubated for 5 min at room temperature and then 100 µl of $H_2O_2$(75%)(Hydrogen peroxide diluted in PBS) was added to induce oxidative degradation of the lipids membrane (hemolysis). The tubes were incubated at 37°C for 1 hr and a half, with shaking every 30 minutes, then the tubes were centrifuged at 2500 rpm for 10 min at 4°C and the color density of the supernatant was measured spectrophotometrically at 540nm. To obtain 100% hemolysis (control), 100 µlof distilled water (for water samples) or 100 µlof ethanol (for ethanol samples) wereadded to 1 ml of the RBCs suspension. The relative hemolysis in the control was taken as 100%.For each concentration and for the control, the experiments were set in triplicates. A total of three independent experiments have been performed. Inhibitory activity of the extracts on hemolysis was calculated and expressed as percent hemolysis.

% Hemolysis = Absorbance of control – absorbance of extract / Absorbance of control X 100.

**Minimal inhibitory concentration (MIC):** MICs were determined using the microtiter broth dilution method as recommended by the Clinical and Laboratory Standards Institute. The different bacterial specieswere grown in BHI overnight and then bacterial suspensions of each species were prepared using MHB at $10^4$ CFU/ml to be used for inoculation. 100 µl (of 48 mg/ml) of each extract (water or ethanol)were used to perform serial two-fold dilutions onMHB using a 96-well flat-bottom polystyrene tissue culture-treated microtiter plate then 100 µl of the previously prepared suspensions were inoculated into each well. Positive growth control well was treated by antibiotic Triaxone and negative growth control lacking a bacterial inoculum were taken into account. The plates were then incubated at 37°C for 24 h, the MIC of each extract was the lowest concentration with no visible growth in its corresponding well.

**Western blot analysis:** SKOV-3 cells were seeded ata density of0.7 x $10^6$cellsin a25 $cm^2$cell culture flask. After an overnight incubation, cellsweretreatedwith50, 150 and 250µg/mlof *S.libanotica*for 72 h. Total cell lysate was prepared using the lysing buffer RIPA (150 mMNaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40, 50 mMTris, pH 8, 20 mM, NaF, 2 mM EGTA, 0.5% levamisole, 1 mM $NaVO_4$) (Roche). The proteinconcentration was determined using Bradford method (Bio-Rad). Protein complexes were separated on a gradient of 10% of SDS-PAGE gel. The membrane was incubated with *Bax* (abcam) and *p*53 (abcam) primary antibodies. Proteins were detected using ECL detection kit (Bio- Rad) according to the manufacturer's protocol.The immune complexes were visualized with the use of the ECL Plus kit (Amersham) according to the manufacturer's protocol. The autoradiographs obtained were scanned and the bands intensity was quantified using ImageJ software 1.48 (National Institutes of Health, Bethesda, MD, USA). All bands were normalized with respect to GAPDH.

**Statistical analysis:** All results were presented as a mean ± standard error of the mean (SEM). Statistical analysis wasperformed using GraphPad Prism 7 (GraphPad Software Inc., CA, USA). Two-way ANOVA was used to calculate  values (p). Groups that are significantly different from control are indicated in the figures as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $P < 0.0001$.

## RESULTS

**Total phenolic content (TPC):** Many studies have demonstrated that phytophenols have antioxidant, antiproliferative and antimicrobial activities (Parry *et al.,* 2006; Zhang*etal.,* 2008; Yang*etal.,* 2009). Natural phytophenolsare considered good antioxidant compounds having the ability to damage free radicals in the organisms and this is related to their bioactivity to inhibit lipoxygenase, to

scavenge free radicals and to chelate metals (Chung*et al.,* 1998).The bioactive components of *S.libanotica* are not well known. For this reason, a decision was taken to estimate the amount of phenol in thisplant. Estimation of total phenol in the extracts was determined using FolinCiocalteu method and the standard compound (Gallic acid). The results obtained showed that *S. libanotica* extracts contain large amount of TPCwith116± 2.6mg/g (in water extract) and 99± 5.6 mg/g (in ethanol extract).

**Evaluation of the antioxidant activity by DPPH assay:** Free radicals and oxidants can be harmful or helpful to the body, but an excess of these radicals generates an oxidative stress, which is harmful to the human. This process plays a major role in the development of several diseases as cardiovascular, neurodegenerative, autoimmune diseases, rheumatoid arthritis and cancer (Rahman *et al.,* 2012). Oxidative stress is inhibited by antioxidants, which are molecules produced naturally in situ, or provided externally by foods and/or supplements. Hence, there is a need to identify plants with potent antioxidant capacity. An easy, rapid and sensitive method to measure antioxidant capacity of plant extract involves the use of the free radical, 2, 2-Diphenyl-1- picrylhydrazyl (DPPH) (Brand-Williams *et al.,* 1995).The antioxidant activity of the *S.libanotica* (water and ethanol extracts)increased in a dose-dependent manner from >15% at 10 µg/ml to >90% at 100µg/ml (Figure 1). In order to determine the concentration required to inhibit the *50%* of DPPH radicals by both water and ethanolic extracts, the dose response curve was plotted. The results of the cytotoxic assay were mentioned as $IC_{50}$(µg/ml). The results obtained showed that water and ethanol extracts of *S.libanotica* have high and very similar antioxidant activity with an $IC_{50}$ of 29 ± 1.67 to 31.2 ± 1.29 µg/ml, respectively. This strong antioxidant activity of *S. libanotica* could be attributed to the presence of phenolic compounds and can have future therapeutic implications by protecting cells against oxidative stress.

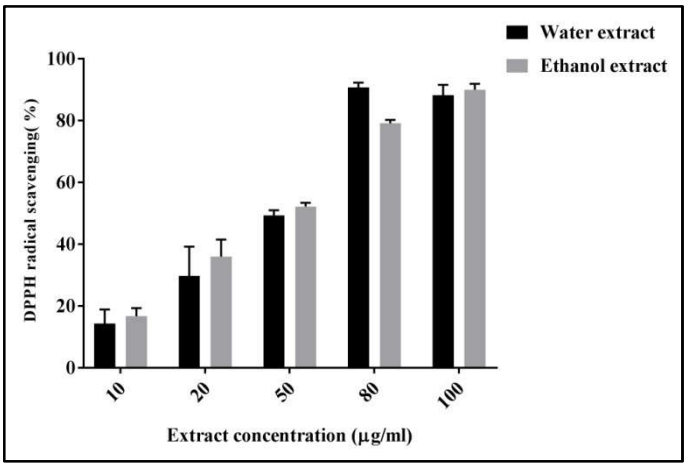


**Figure 1. Antioxidant activities at different concentrations of water and ethanol extracts of*S.libanotica*.The samples were prepared in triplicates. Each value represents the mean ± SEM of triplicate**

**Hemolytic activity:** As the effectiveness of the drugs is better by infusion, it is important to monitor if *S.libanotica* has any effect on RBCs lysis. For this aim, a simple hemolytic assay was done. The results obtained showed that the different concentrations of water and ethanol extracts of *S.libanotica* did not have any hemolytic effect (Data not presented). The applied protocol confirms that *S.libanotica*is able tobe taken by an infusion.

**Anti-hemolytic effect**

*S. libanotica* didn't show a high hemolytic effect suggesting testing for an anti-hemolytic effect. For this purpose, hydrogen peroxide ($H_2O_2$) has been used. Results obtained showed a marked reduction in hemolysis of the RBCs treated with $H_2O_2$ and with *S.libanotica*extracts. Hemolysis of the RBCs decreased with increasing the extract concentrations (Figure 2). The % of hemolysis has been decreased only by 26.61% at 100 µg/ml for the ethanol extract and by 33.87% for the water extract at the same concentration.

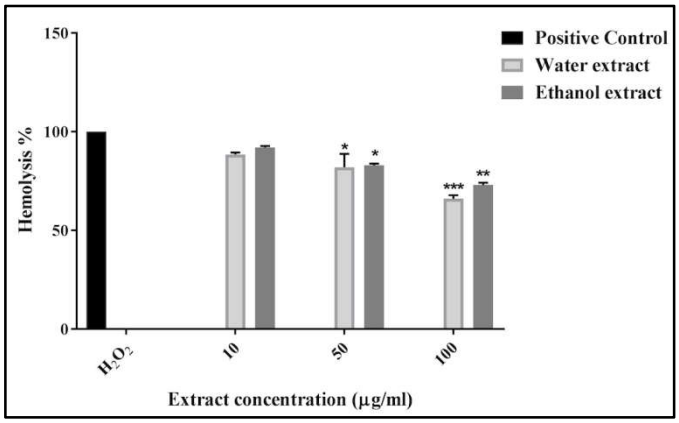


**Figure 2. Antihemolytic activity at different concentrations of water and ethanol extracts of*S.libanotica*.The samples were prepared in triplicates. Each value represents the mean ± SEM of triplicate.* P <0.05, ** P <0.01,*** P <0.001**

**Antibacterial Activity**

The antibacterial activity was determined according to the method described above. Results obtained showed that the two extracts (ethanol and water) of *S.libanotica* have the same antibacterial activity on the four bacterial species (Table 1). The MICs was about1.5 mg/ml for water and ethanol extractson *E. coli, P. aeruginosa* and *S. aureus* and 3 mg/ml for water and ethanol extracts on *E.coli multi-resistance*. These results agreed with thoseobtainedbyIbrahim et al.who hastested the effect of ethanol extracts of *S.libanotica*on *methicillin-resistant S.aureus* (MRSA) andin which *S.libanotica*showed its antibacterial effect against MRSA with MIC= 4 mg/ml (Abdallah Ibrahim *et al.,* 2013).

**Table 1. Minimal Inhibitory concentration (MIC) for water and ethanol extracts of *S.libanotica*on the four bacterial species**

| Bacterial species | MIC (mg/ml) for water extract | MIC (mg/ml) for ethanol extract |
|---|---|---|
| *Escherichia coli* | 1.5 | 1.5 |
| *Pseudomonas aeruginosa* | 1.5 | 1.5 |
| *Staphylococcus aureus* | 1.5 | 1.5 |
| *E. coli multi-resistance* | 3 | 3 |

**Cell viability and cytotoxicity effect for water extract of *S.libanotica*on SKOV-3 cancer cells:** The results obtained on *S.libanotica*showed that this plant contains a large amount of phenols. These compounds are known for their antiproliferative effects (Teleszko and Wojdyło, 2015; Šavikin *et al.,* 2014).

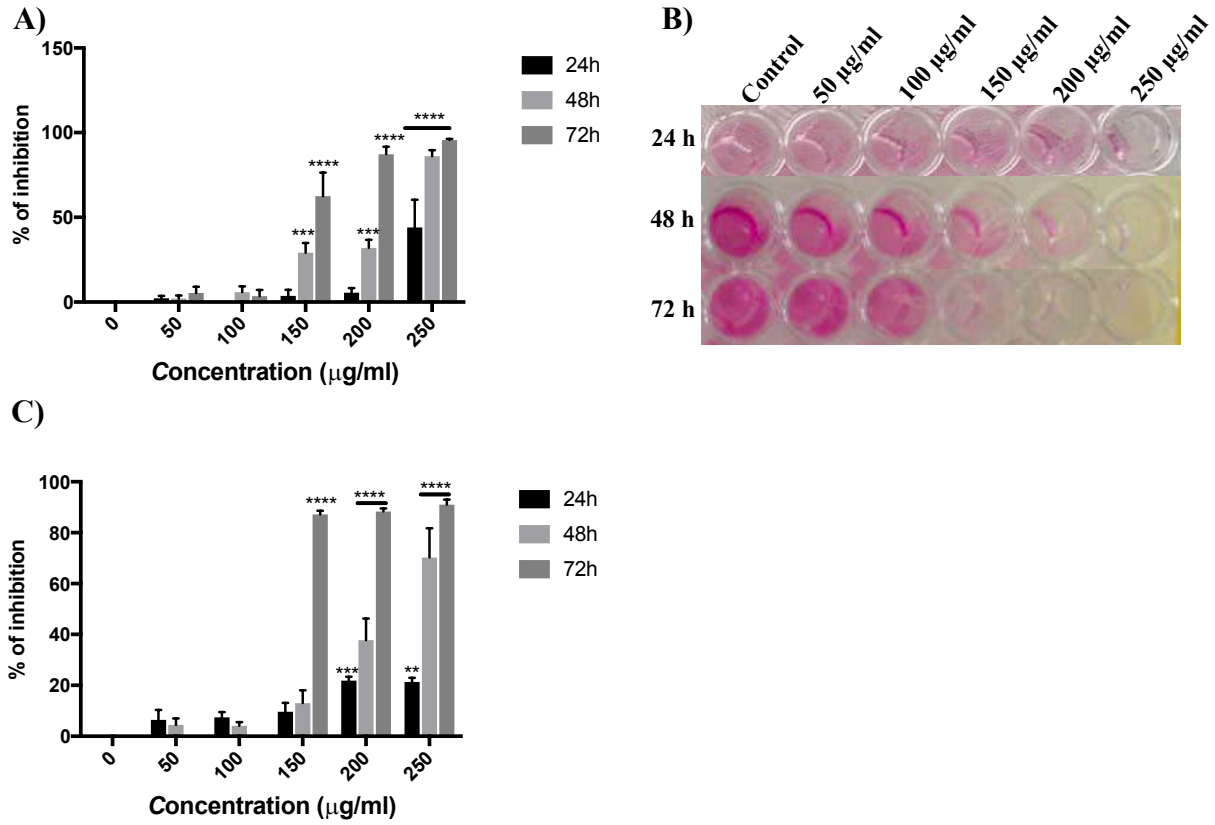


**Figure 3. Effect of water extract of *S. libanotica* plant on the viability of SKOV-3 cancer cells. A, C. SKOV-3 cells were incubated at different concentrations of water extract of *S.libanotica* plant (0-250 µg/ml) for 24, 48 and 72 hours. Cell viabilitywasestimated by theneutralred (NR) test (A) or by MTT test (C)**

**Table 2. Concentration of water extract of *S. libanotica* which induced 50% decrease in SKOV-3 cancer cell survival and determined by Neutral red and MTT cytotoxicity assay.**

| | | 24 hours | 48 hours | 72 hours |
|---|---|---|---|---|
| Neutral Red test | IC50 [µg/ml] ±SEM | - | 193.3 ± 7.06 | 143.3 ± 4.25 |
| MTT test | IC50 [µg/ml] ±SEM | - | 216 ± 7.03 | 132.8± 8.27 |

In addition, the previous results obtained showed that the amount of phenol in water and in ethanol extracts of *S.libanotica* was very similar. For this reason, a decision was taken to determine the antiproliferative effects of one of these two extractions. Because the antitumor effect of *S.libanotica*on the ovarian cancer isnot known, examination of its antiproliferative effects on the epithelial ovarian cancer cells (SKOV-3) will be done. Evaluation of the antiproliferative and cytotoxic activity of water extract of *S.libanotica* was performed by measuring the viability of human epithelial ovarian cancer cells (SKOV-3) using the neutral red (NR) and the MTT Cytotoxicity/ Viability assay. SKOV-3 was treated for 24, 48 and 72 hours with increasing concentrations (50, 100, 150, 200, and 250 µg/ml) of *S. libanotica*. The effect of inhibition by *S.libanotica*was dose and time-dependent (Figures 3 A, B and C and figure 4). The percentage of inhibition was calculated according to the formula mentioned above. The neutral red and MTT assays showed that the water extract of *S.libanotica*has no significant antiproliferative effect after 24, 48 and 72hrs of treatment at a concentration of50 and 100 µg/ml(Figure 3 A and C). In addition, the proliferation of SKOV-3 was not affected by the addition of 150 µg/ml of *S.libanotica*for 24 hrs (Figure 3).The percentage of survival inhibition was significantly increased after 24hrsof treatment at a concentration of 200 µg/ml (Figures 3 C).

In addition, the percentage of survival inhibition was highly significant after 48 and 72 hrs of treatment at a concentration of 200 and 250 µg/ml. The highest antiproliferative activity ($p<0.0001$) on the SKOV-3 cell line was approximately 95-fold increase at 250 µg/ml of *S.libanotica* after 72 hrs (Figure 3 A and C). The percentage of survival inhibition was compared to untreated cells. In order to determine the concentration required to achieve a 50% inhibition of cells induced by *S.libanotica*, the dose response curve was plotted. The results of the cytotoxic assay were mentioned as $IC_{50}$(µg/ml) in Table 2. From the results obtained, it was clear that, at 72 hrs, *S.libanotica*has the highest antiproliferative activity on the SKOV-3 cell line with an $IC_{50}$ of 143.1± 4.25 (NR assay) and 132.8 ± 8.27(MTT assay). Percentinhibition of viablecells is calculated using the formula: % Inhibition = OD (opticaldensity) of non-treatedcells - OD oftreatedcells / OD of non-treatedcells × 100. Resultsrepresent % inhibtionof cell survivalof SKOV-3 cellscompared to control. B).The NR assay plate of stained cells. Each column represents different concentrations of water extract of *S.libanotica* plant and each line represents different incubation time. Experiments were done in triplicates and results represent the mean ± SEM of 3 independent experiments. *** $P < 0.001$ was considered to be statistically highly significant and **** $P < 0.0001$ extremely significant (Two-way ANOVA).

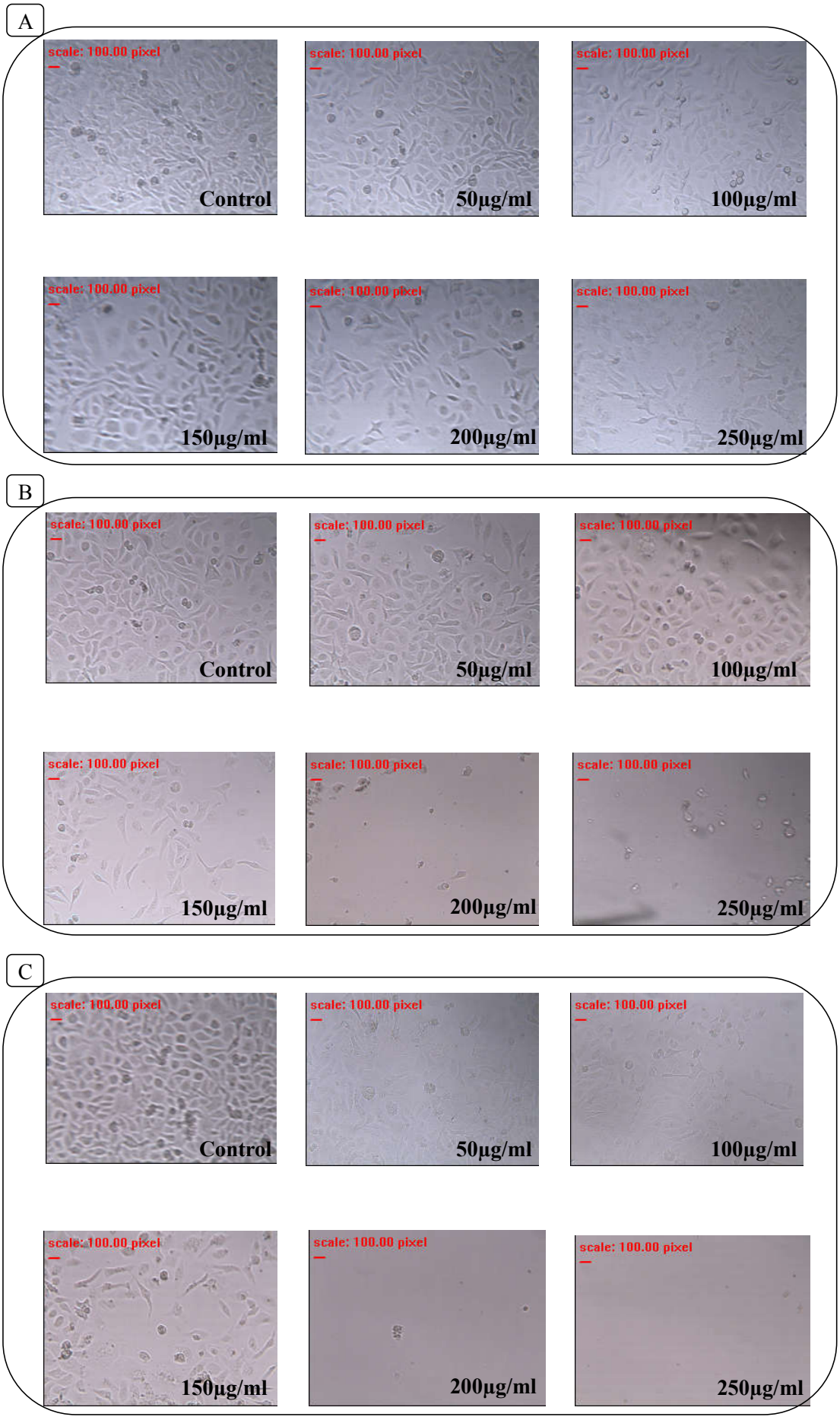


**Figure 4. Microscopicviewof SKOV-3 cells after 24 (A), 48 (B) and 72 hours (C) incubationwithincreasingconcentrations (50, 100, 150, 200 and 250 µg/ml) ofwater extract of *S.libanotica*.Theresultspresented are fromone experiment outoffourthatwerecarriedout and photographedmicroscopically (× 40)**

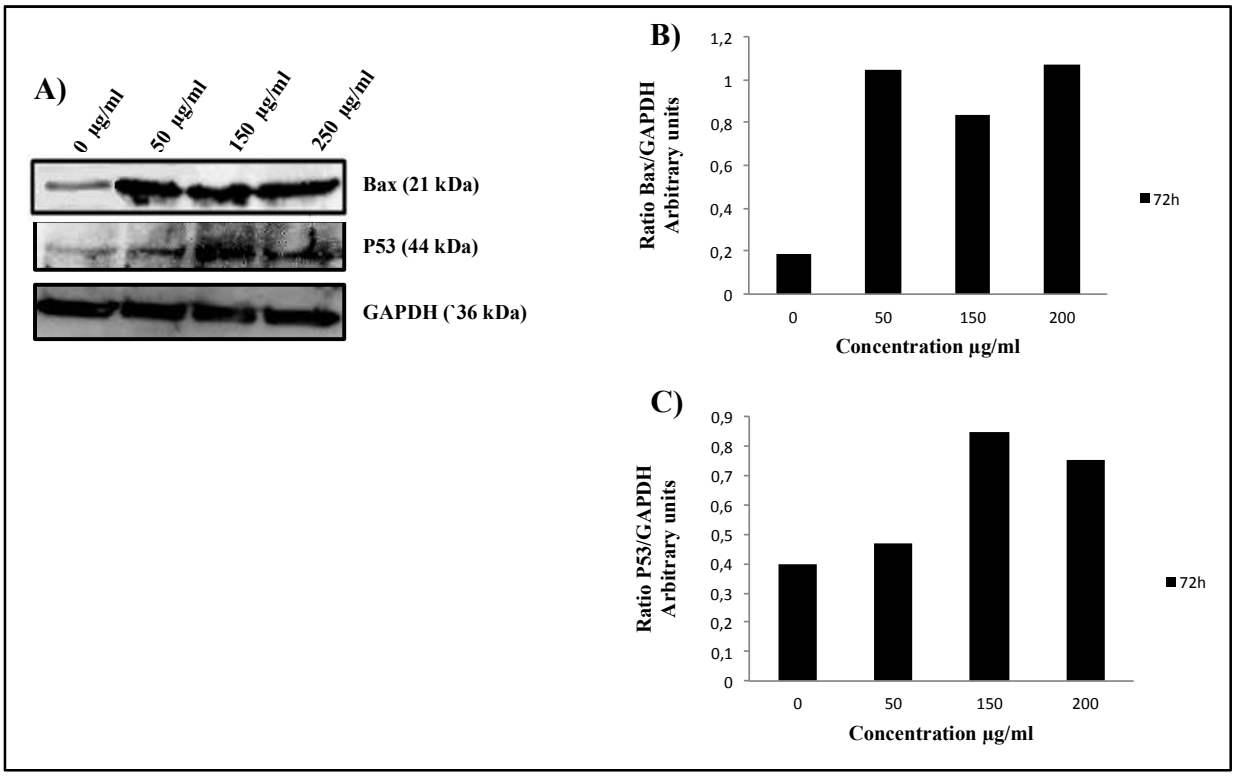


**Figure 5. Expression of apoptosis indicators is altered in SKOV-3 cells.SKOV-3 cells were incubated with water extract of *S.libanotica* (50, 150 and 250 µg/ml) for 72 hrs. (A) Representative western blots showing the expression of *p53* and *Bax*, in SKOV-3. (B, C). The expression of target proteins was normalized to that of GAPDH, and the ratios were presented in arbitrary units. Band intensities of *p53* and *Bax* were measured by densitometry using Image J**

### *Salvia libanotica* induces apoptosis in SKOV-3 cells

To evaluate the mode of cell death induced by *S.libanotica* in SKOV-3cells, western blot experiment was carriedout tomeasurepro-apoptotic proteins.The tumor suppressor gene, *p53*, is known to be responsible for the inhibition of cell growth and/or the involvement to apoptosis. *p53* regulates apoptosis via upregulation of the expression of *Bax*and blocks the function of *Bcl-2*. The level of the expression of *p53* and *Bax* have been examined and the results obtained showed that both *p53* and *Bax* proteins were increased in a dose-dependent manner following treatment with the water extract of *S.libanotica* (50, 150 and 250 µg/ml) for 72 hrs(Figure 5), suggesting that the apoptosis of SKOV-3 cells by*S.libanotica*is mediated by *p53* and *Bax* activation.

## DISCUSSION

Ovarian cancer is the fifth most deadly type of cancersaffecting women according to the American Cancer Society(Siegel*et al.,* 2012). Advanced ovarian cancer can be treated by chemotherapy, but this treatment must be adjusted because of its severe side effects. Therefore, identifying new andmore effective treatments than the chemotherapy and without toxic or other side effects are highly needed. Current medicine aims to treat several diseases through traditional medicine with plants (Schmidt*et al.,* 2008). As medicinal plants are commonly used in the Middle East to treat various human diseases (Alzweiri *et al.,* 2011; Baydoun *et al.,* 2015; Dirani *et al.,* 2014; Farhan *et al.,* 2013; Rammal *et al.,* 2013; Farhan *et al.,* 2013; Abu-Irmaileh and Afifi, 2003), the presented study was focused on the use of an endemic plant from Lebanon and more especially on *S. libanotica* whose biological effects are not well studied. It is usually believed that secondary metabolites present in different plants are responsible for their therapeutic effects. Phenolic compounds (polyphenols) constitute a group of secondary metabolites, which have important functions for the well being of the human. Phenolic compounds are present in most medicinal plants. Several data showed that phenols have protecting effects againstcardiovascular, neurodegenerative diseases, diabetes, osteoporosisand against the development of cancers due to their potent antioxidant properties (Graf *et al.,* 2005; Arts and Hollman; 2005). For this reason, was decided to determine the total phenol content of *S.libanotica* and to evaluate its effects in different diseases. Theresults obtained showed that *S.libanotica* extracts (water and ethanol) contain high amount of polyphenols. These results led us to focus on the idea of studying the antibacterial, antioxidant, anti-hemolytic and antiproliferative effects of *S.libanotica*. Using the DPPH method, the resultsobtained showed a high antioxidant activity for*S. libanotica*. Recent studies have shown the *antibacterial activities* of *various* species of *medicinal plants against* many infectious bacteria (gram-positive and gram-negative bacteria) (Singh *et al.,* 2005; Al-Hebshi *et al.,* 2006; Moon *et al.,* 2006; Salazar*et al.,* 2006). However, the antibacterial activity of *S. libanotica has* never been investigated. The presented results showed that *S.libanotica* extracts (water and ethanol) have antibacterial activities on four bacterial species; *E. coli, P. aeruginosa*, *S. aureus*and and *E.coli multi-resistance.* Theseresults can make this plant a good alternative for many traditional antibiotics. The anticancerous effect and antitumor mechanisms of *S. libanotica* are not well known, its *anti-tumor* effect in ovarian cancer had not yet been studied. In *vivo*, the oil extract of *S.*

*libanotica* had potent suppressive activities against chemically induced mouse skin tumorigenesis (Gali-Muhtasib and Affara, 2000). In vitro, the bioactive compounds of *S. libanotica* had shown to inhibit growth of two isogenic human colon cancer cell lines HCT-116 (p53+/+ and p53-/-). In p53+/+, apoptosis was associated with increased Bax/Bcl-2 ratio and pp53/p53 ratio, cytochrome c release, cleavage of poly(ADP-ribose)-polymerase (PARP), cleavage and activation of caspase-3. In contrast, the apoptosis in p53-/- cells is caspase-independent, mediated by cytochrome c release and PARP cleavage (Itani *et al.,* 2008). This study showed that the water extract of *S. libanotica* has an antiproliferative effect inepithelial ovarian cancer (SKOV-3) cell line. The apoptosis of SKOV-3 cells is associated with an increase in expression of both genes, p53 and Bax. In addition, the study showedthat water and ethanol extracts of *S.libanotica* have protective effects against erythrocytes hemolysis. Taken together, the presented datashowed that *S.libanotica*can be used in the treatment of human EOC. In the future, studies will be conductedto gaugethe effects of the combination of *S. libanotica* extracts with standard chemotherapy drugson killing ovarian cancer cells. It will also be necessary to conduct in *vivo* studies to further identify the antioxidant and antiproliferative effects of this plant.

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**Conflict of Interest**

The authors declare that they have no conflicts of interest.

**Abbreviations**

Epithelial ovarian cancer (EOC), Estimation of total phenolic content (TPC), *Salvia libanotic*a (*S. libanotica*),fetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Neutral Red (NR),2, 2-Diphenyl-1- picrylhydrazyl (DPPH), Gallic acid equivalent (GAE), Brain heart infusion broth (BHI), Mueller-Hinton broth (MHB),absorbance (Abs), Phosphate Buffered Saline (PBS), Minimal inhibitory concentration (MIC),the half maximal inhibitory concentration ($IC_{50}$), optical density (OD), standard error of the mean (SEM).

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