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In vitro Evaluation of Cancer Chemopreventive Activity of *Seriphidium herba-alba* Extracts

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Abstract: ***Seriphidium herba-alba*** family *Asteraceae* has been used in the folk medicine by many cultures for treatment of various ailments since ancient times, in the current research we were aiming to evaluate the cancer chemopreventive activity of two crude extracts of *S.herba-alba* ([A] CH_2Cl_2−MeOH (1:1) and [B] MeOH−H_2O (7:3)) on two cell lines MCF-7 and Hep-G2. Assessment of cytotoxicity (MTT assay) indicated that both extracts were safe (IC_{50} >20µg/ml. GSTs activity was enhanced by treatment with three different doses of extracts. Analysis of cell mode of death revealed that apoptosis/necrosis ratio was >1 in MCF-7 and <1 in Hep-G2. Assessment of the anti-angiogenic effect of extracts showed that VEGF and PDGF levels were decreased, especially VEGF levels in Hep-G2, were greatly decreased. **In Conclusion**: both extracts may be cancer chemopreventive agents since they had anti- initiating activity via their enhancing of carcinogen detoxification, and anti-promoting activity via their anti-angiogenic effect and encouraging apoptosis**.**

Key words: Apoptosis, chemoprevention, GSTs, necrosis, PDGF, VEGF Seriphidium herba-alba

1. Introduction

In Egypt, incidence rates of cancer per 100,000 people/year were 166.6 populations(age-standardized rate) from both sexes in 2013, and by 2050, a 3-fold increase in incident cancer relative to 2013 was estimated ***(Ibrahim et al., 2014)***. Cancer Chemoprevention is defined as the use of natural, biological or synthetic agents to prevent, reverse, suppress or halt either the initial phases of carcinogenesis or the progression of premalignant cells to invasive disease ***(Sporn et al., 1976)***. Carcinogenesis is a multistage process includes: initiation, promotion, and progression processes. Understanding cellular and molecular basis of the carcinogenesis provides a targeted approach for cancer chemoprevention ***(Gamal-Eldeen et al., 2006)***.

Seriphidium herba-alba (previously named Artemisia herba-alba) is a medicinal, aromatic, greenish-silver perennial herb grows 20-40 cm in height and grows wildly in arid areas of the Mediterranean basin. This plant is abundant in Saint Catharine, Sinai. Egypt. And used widely in folk medicine for treatment of diarrhea and abdominal cramps and in the healing of external wounds. The vapors generated by heating the leaves and flowers in hot water relieve symptoms of colds and coughs. It's also used for the treatment of diabetes mellitus,

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neurological disorders as epilepsy, Alzheimer's disease, depression, and jaundice. It also exhibited pronounced anti-spasmodic and mild antibiotic activities ***(Mohamed et al., 2010, Boutkhil et al., 2011)***.

This prompted us to investigate the cancer chemopreventive activities of Seriphidium herba-alba extracts using in vitro bioassays in human cell lines, Breast adenocarcinoma cells (MCF-7), and Hepatocellular carcinoma cells (Hep-G2).For initiation stage, the effect of the extract products on carcinogen detoxification was assessed through investigating their effect on the induction of carcinogen conjugation with the detoxification enzymes; a reaction catalyzed by glutathione S-transferases (GSTs).For the promotion and progression stages, the cytotoxic effect of the extract products was tested on both cell lines, the cell death type (apoptosis and/or necrosis) was examined, and the anti-angiogenic effect of the extract products was tested through assessment of both vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF).

Figure (1): Seriphidium herba-alba -family: Asteraceae (Flickr - Photo Sharing).

2. Materials and Methods

I. Plant extracts:

Two batches of the aerial parts (100 g) of *Seriphidium herba-alba* were powdered and extracted at room temperature with CH_2Cl_2−MeOH (1:1) to produce extract **A** and MeOH−H_2O (7:3) to produce extract **B**. The filtrate solvent extract were concentrated in vacuo using a rotary evaporator to obtain a crude extract***(Hamed et al., 2016)***, Plant extracts were prepared as stock solutions in dimethyl sulfoxide (DMSO), and then freshly diluted in the cell culture medium.

II. Cell lines:

Breast adenocarcinoma cells (MCF-7) and Hepatocellular carcinoma cells (Hep G2) were used through this work and were purchased from American Type Culture Collection (ATCC, NY, USA).

MCF-7 cells were routinely cultured in tissue culture flasks and maintained in RPMI 1640 media supplemented with 10% fetal bovine serum, 2 μmol/ml L-glutamine, 250 ng/ml fungizone, 100 units/ml penicillin, and streptomycin 100 μg/ml. Cells were maintained in a humidified air containing 5% CO2 at 37°C. The cultures were passaged every four days by trypsinization using 1.5 ml trypsin/EDTA solution for 5 min at 37°C

Hep G2cells were routinely cultured in tissue culture flasks and maintained in low glucose DMEM media supplemented with 10% fetal bovine serum, 2 μmol/ml L-glutamine, 250 ng/ml fungizone, 100 units/ml penicillin,

and streptomycin 100 µg/ml. Cells were maintained in a humidified air containing 5% CO2 at 37°C. The cultures were passaged every four days by trypsinization using 1.5 ml trypsin/EDTA solution for 5 min at 37°C.

All cell culture materials were obtained from Cambrex, BioScience (Copenhagen, Denmark), while all other chemicals were from Sigma–Aldrich, NY, USA and Antibodies for ELISA were obtained from abcam – Cambridge – UK.

The whole research and experiments were carried out in the Cancer Biology Lab., Center of scientific excellence, National Research Center, Cairo, Egypt.

III. Assessment of Cytotoxicity [MTT assay]:

MTT assay is based on the cleavage of the tetrazolium salt by mitochondrial dehydrogenases in viable cells. Cells (5 ×104 cells/well) of both cell lines were incubated with various extracts concentrations at 37◦C in a serum-free medium, before being submitted to MTT assay according to ***(McHale and McHale, 1988)***. The relative cell viability was expressed as the mean percentage of viable cells compared with DMSO-treated cells. The half maximal inhibitory concentration of the cell growth (IC_{50}) was calculated from the curve equation of the cell growth and in the later assays we used three consecutive doses from each extract as following:

Extract A	Extract B
A_{25} = ¼ IC_{50}	B_{25}= ¼ IC_{50}
A_{50} = ½ IC_{50}	B_{50}= ½ IC_{50}
A_{75} = ¾ IC_{50}	B_{75}= ¾ IC_{50}

IV. Assessment of Tumor anti-initiation activities:

Glutathione-S-transferases (GSTs):

GSTs activity was measured according to the method described by ***(Habig et al., 1974)***. Assay kit was supplied from Biodiagnostic co. Egypt; Cells (5×10^4 cells / well) were plated at in 96 well-microplates. Wells were treated with doses A_{25}, A_{50}, A_{75}, B_{25}, B_{50} and $B_{75}$of *Seriphidium herba-alba* extracts, specifying a well for eachconcentration. Triplicate wells were prepared for each concentration. The plates were incubated for 24 h in a humidified 5% CO_2 atmosphere at 37ºC without changing the media. After incubation, cells were washed 3 times with PBS and lysed in 125 µl PBS by 3 repetitive freezing-thawing cycles (thawing at 37ºC for 2 min and freezing at -80ºC for 15 min) and cell lysate was used in assay according to kit instructions.

V. Assessment of Tumor anti-promotion and anti-progression activities:

a- Analysis of cell mode of death (Necrosis /Apoptosis ratio):

To screen the effect of the extracts on the mode of cell death, apoptosis and necrosis ratios were investigated using double staining with acridine orange / ethidium bromide ***(Ribble et al., 2005)***. Cells were cultured in 8 Well Cell Culture Chamber Slide at a density of (5×10^5 cells / well) and treated with doses A_{25}, A_{50}, A_{75}, B_{25}, B_{50} and $B_{75}$of *Seriphidium herba-alba* extracts, specifying a well for each concentration. After incubation for 48 h, Cells were stained using the nucleic acid-binding dye mixture of 100 µg/ml Acridine orange and 100 µg/ml Ethidium bromide in PBS, And were examined by fluorescence microscope (Carl Zeiss , Axiostar plus. HBO 50/ AC).

b- Effect on Angiogenesis: Assessment of Vascular endothelial growth factor [VEGF] and Platelet derived growth factor [$PDGF_{BB}$]:

The Extracellular assay is based on the principle of sandwich enzyme-linked immunosorbent assay ELISA ***(Goldman et al., 1993)***. Cells were cultured in 6 well-microplates at density of (5×10^5 cells / well) and Wells were treated with doses A_{25}, A_{50}, A_{75}, B_{25}, B_{50} and $B_{75}$of *Seriphidium herba-alba* extracts, specifying a well for each concentration. After incubation for 48 h The medium and monolayer cells were harvested and the medium from each well was clarified by centrifugation (2000 rpm, 10 min, 4°C) in 1.5 ml polypropylene microcentrifuge tubes, Individual aliquots (700 µl) were stored frozen (-20°C) in separate microcentrifuge tubes until analyzed.

96 well-maxisorp microplate was coated with relevant monoclonal antibody, diluted in PBS to the working concentration required for each procedure as mentioned above, using 50µL per well, and the plate was incubated overnight at 4ºC. After incubation, the plate was washed once using 200 µL washing buffer per well. Then, blocking buffer was added (200 µL per well) and incubated for 1.5 h at 37º C followed by one time washing (200 µL washing buffer per well). Each standard was freshly prepared, each of relevant standard and sample were dispensed onto coated wells (50 µL/well), serial dilutions of standard were performed in six repeated wells and the plate was sealed and incubated at 37ºC for 1.5 h with gentle agitation. After incubation, the plate was washed three times using 200 µL washing buffer per well each time. The relevant secondary biotinylated antibody was added (50 µL per well) and the plate was incubated for 1.5 h at 37ºC followed by three times washing. Conjugated Streptavidin horseradish Peroxidase was added (50 µL per well) then the plate was incubated for 1 h at 37 ºC followed by four times washing. Substrate of TMB/ H_2O_2 equal volumes was added (50 µL per well) and the plate was protected from light and incubated for 20 minutes at room temperature. The reaction was stopped by 1N HCL (50 µL per well) and absorbance at 450 nm was determined.

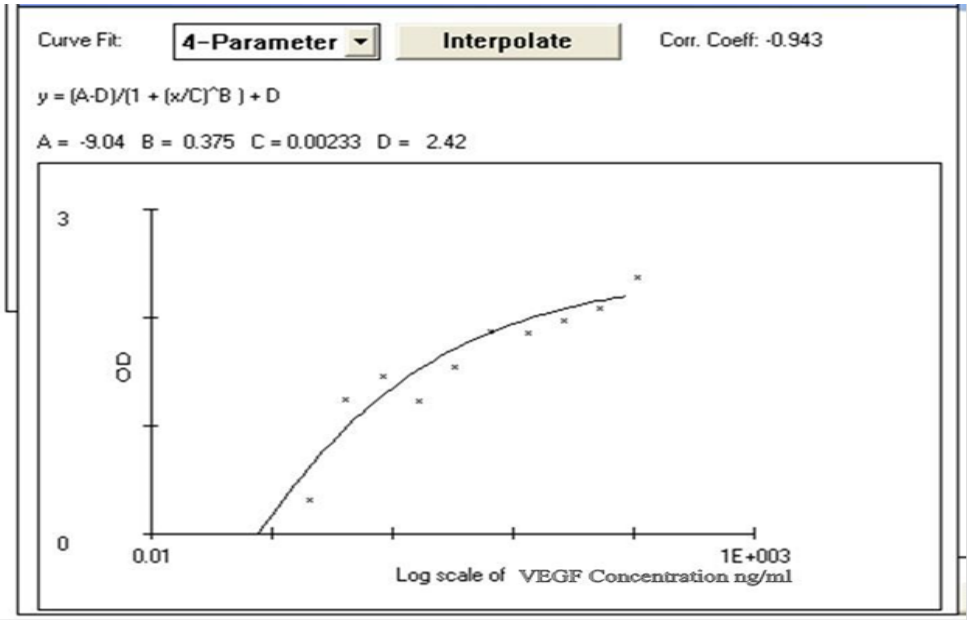

Figure (3): VEGF standard curve - ELISA

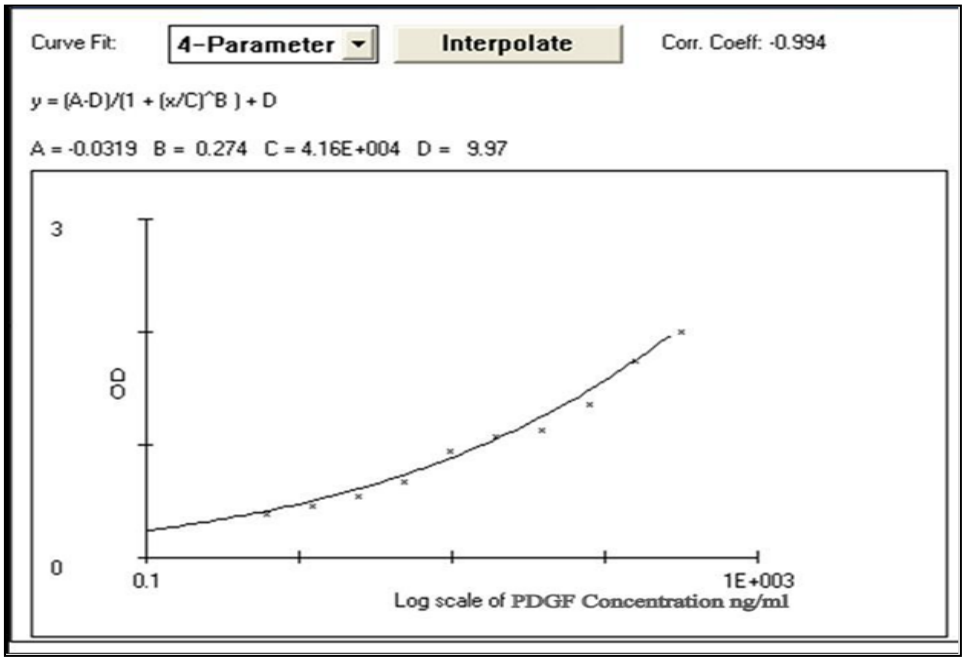

Figure (4): $PDGF_{BB}$ standard curve - ELISA

VI. Statistical analysis

The data obtained from *in vitro* studies were expressed as Mean $\pm$ SD. Differences between control cells and treated cells were analyzed using an unpaired student t test.

The result were considered to be significant when *p<0.05, **p<0.01, ***p<0.001.

Descriptive statistics were performed using Microsoft Excel 2010. All analysis and graphics were performed using **Graphpad prism (windows version 6; Graphpad software 2012)** and **SOFTMAX® PRO SOFTWARE Version 5.**

3. Results and Discussion

A- Cytotoxicity assessment [MTT assay]:

The treatment of MCF-7 and Hep-G2 cells with the two extracts showed decrease of cell viability in a concentration-dependent manner and the calculated IC50 values were (81.45, 116.67 µg/ml) and (112, 178.94 µg/ml) respectively.

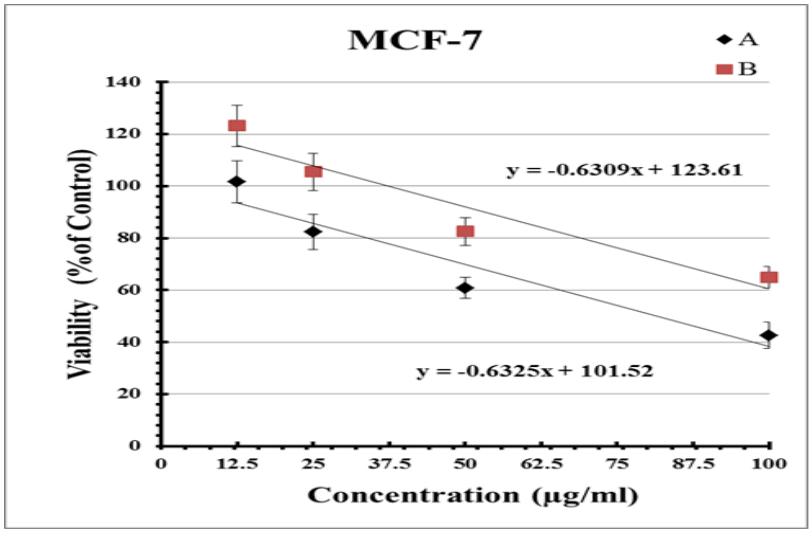

Figure (5): Relationship between different doses of S. herba-alba extracts A and B (0-100 µg/ml) and the growth of MCF-7 cells (% of control) using MTT assay.

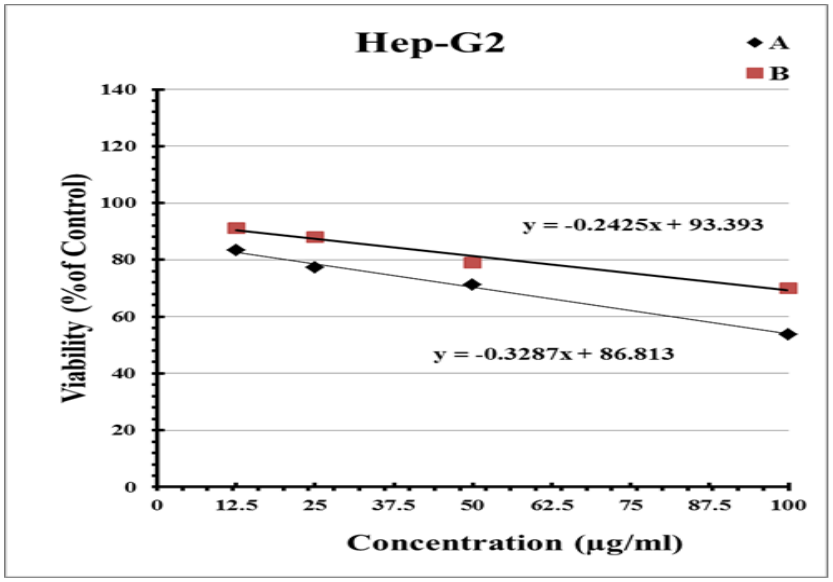

Figure (5): Relationship between different doses of S. herba-alba extracts A and B (0-100 µg/ml) and the growth of Hep-G2 cells (% of control) using MTT assay.

Unlike chemotherapy, chemopreventive agents are targeted at healthy asymptomatic persons who are at high risk for cancer. So, it is essential that agents should be non-toxic and administered according to a route and pattern which is compatible with lifestyle.

From our results, it is obvious that both Seriphidium herba-alba extracts that were used in this research were safe and possessed no cytotoxicity in MTT assay, According to the U.S. NCI plant screening program (crude extract is considered to have in vitro cytotoxicity with an IC50 value ≤ 20 µg/mL) ***(Graidist et al., 2015)*** and these

results were in agreement with***(Lupidi et al., 2011)*** who reported MTT assay for Seriphidium herba-alba aqueous and ethanolic extracts and both IC50 were > 20 µg/mL.

Table (1): Doses of S. herba-alba extracts A and B which were used in the later assays on both cell lines MCF-7 and Hep-G2.

MCF-7	Hep-G2
A25 = ¼ IC50 = 61.09 µg/ml	A25 = ¼ IC50 = 84 µg/ml
A50 = ½ IC50 = 40.37 µg/ml	A50 = ½ IC50 = 56 µg/ml
A75 = ¾ IC50 = 20.36 µg/ml	A75 = ¾ IC50 = 28 µg/ml
B25= ¼ IC50 = 87.5 µg/ml	B25= ¼ IC50 = 133.8 µg/ml
B50= ½ IC50 = 58.34 µg/ml	B50= ½ IC50 = 89.47 µg/ml
B75= ¾ IC50 = 29.17 µg/ml	B75= ¾ IC50 = 44.74 µg/ml

B- Assessment of Tumor anti-initiation activities:

Effect on GSTs:

In MCF-7 cell line, The phase 2 enzyme GSTs were significantly enhanced by A25,A50 ($P<0.05$) and by A75 and B75 treatment to 1.16-fold ($P < 0.001$) and 1.56-fold ($P < 0.001$) of the control cells respectively, while the doses B25 and B50 showed non-significant enhancement on the activity of GSTs.

In Hep-G2 cell line, The phase 2 enzyme GSTs were significantly enhanced by A25,B25 treatment ($P<0.05$) and by A50,A75 and B75 treatment to 1.215-fold ($P<0.01$),1.256-fold ($P<0.01$) and 1.251-fold ($P<0.01$) of the control cells respectively, while the B50 dose showed non-significant enhancement on the activity of GSTs.

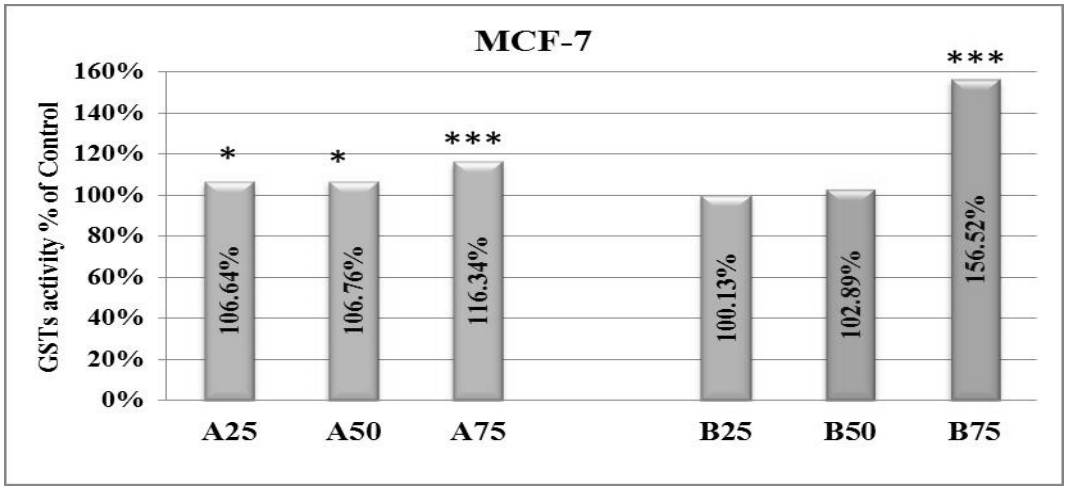

Figure (7): Histogram representing GSTS activity(% of control) in MCF-7 after treatment with 3 different doses of both S. herba-alba extracts A and B.

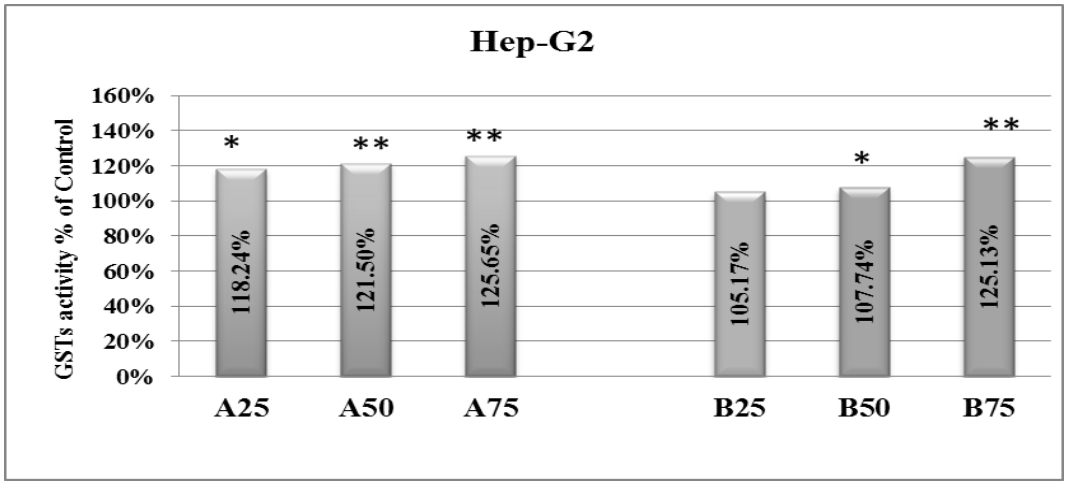

Figure (8): Histogram representing GSTS activity(% of control) in Hep-G2 after treatment with 3 different doses of both S. herba-alba extracts A and B.

One of the most ubiquitous detoxification enzyme classes; the glutathione S-transferases (GSTs), these enzymes have a pivotal role in inhibiting cellular damage produced by diverse carcinogens, Induction of these protective enzymes represents a promising chemopreventive strategy ***(Clapper and Szarka, 1998)***

The principal function of (GSTs) is to catalyze the conjugation of GSH to reactive electrophiles as part of the cellular adaptive response to chemical toxins and oxidative stress ***(Henderson et al., 2014)***.

From our results, the phase 2 enzyme GSTs were significantly enhanced by treatment of both cell lines (MCF-7 and HepG2) by different doses of both Seriphidium herba-alba extracts, and these results indicate that both extracts have anti-tumor initiating activity.

C- Assessment of Tumor anti-promotion and anti-progression activities:

a- Analysis of cell mode of death (Necrosis /Apoptosis ratio):

For both extracts A&B the mode of cell death in MCF-7 was apoptosis at low doses and by increasing the dose the rate of necrosis was increased against apoptosis in comparison with control untreated cells. The rate of necrosis reaches the highest percentage at A75 and B75 doses with a priority of apoptosis

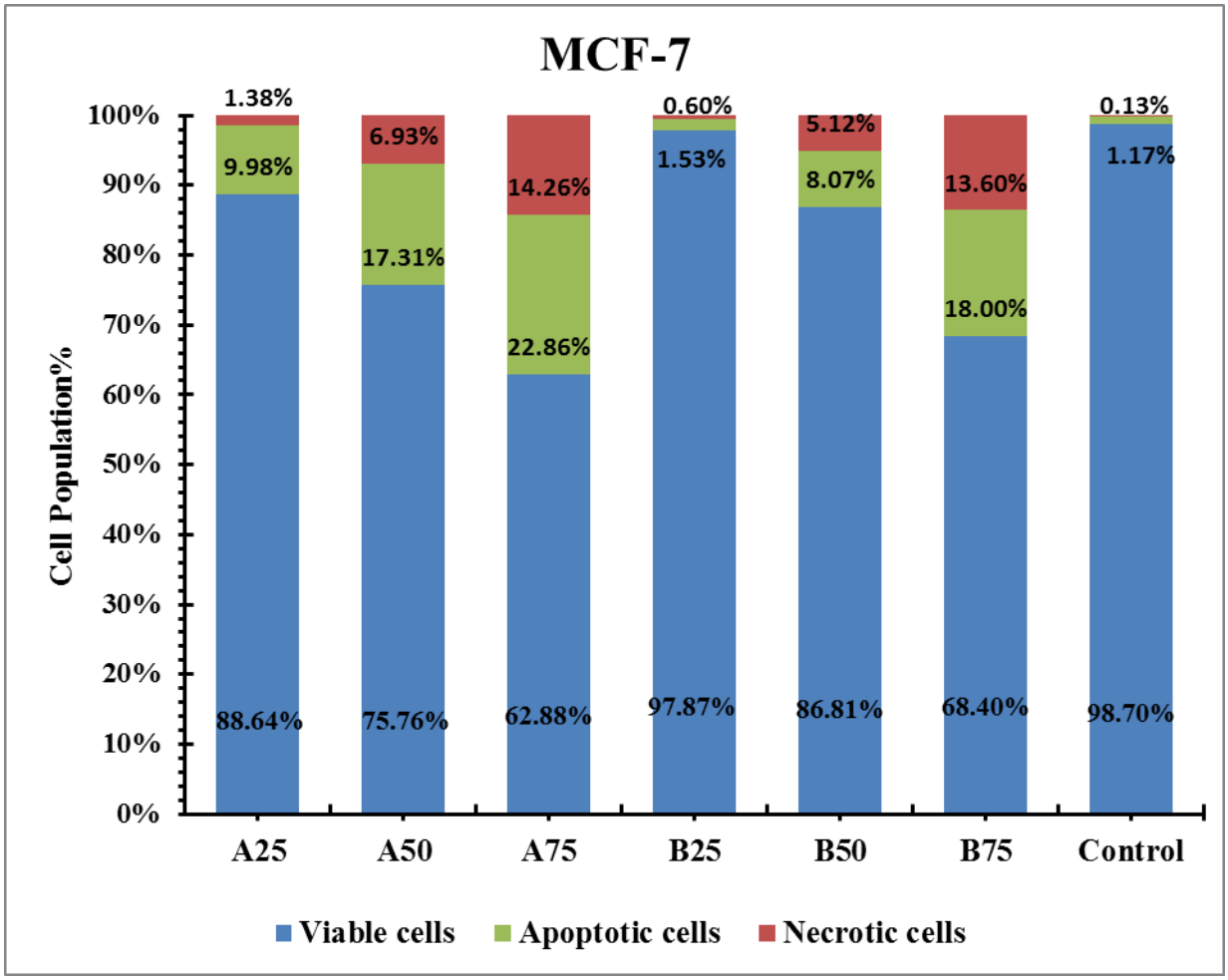

Figure (9): Histogram represened the percentage of viable,apoptotic and necrotic cells from the whole cell population in MCF-7 cells treated with different doses of extracts A and B

For both extracts A&B the mode of cell death in Hep-G2 was allocated between apoptosis and necrosis at low doses and by increasing the dose the rate of necrosis was increased over apoptosis in comparison to the control untreated cells. The rate of necrosis reaches the highest percentage at A75 and B75 doses exceeding the apoptosis percentage.

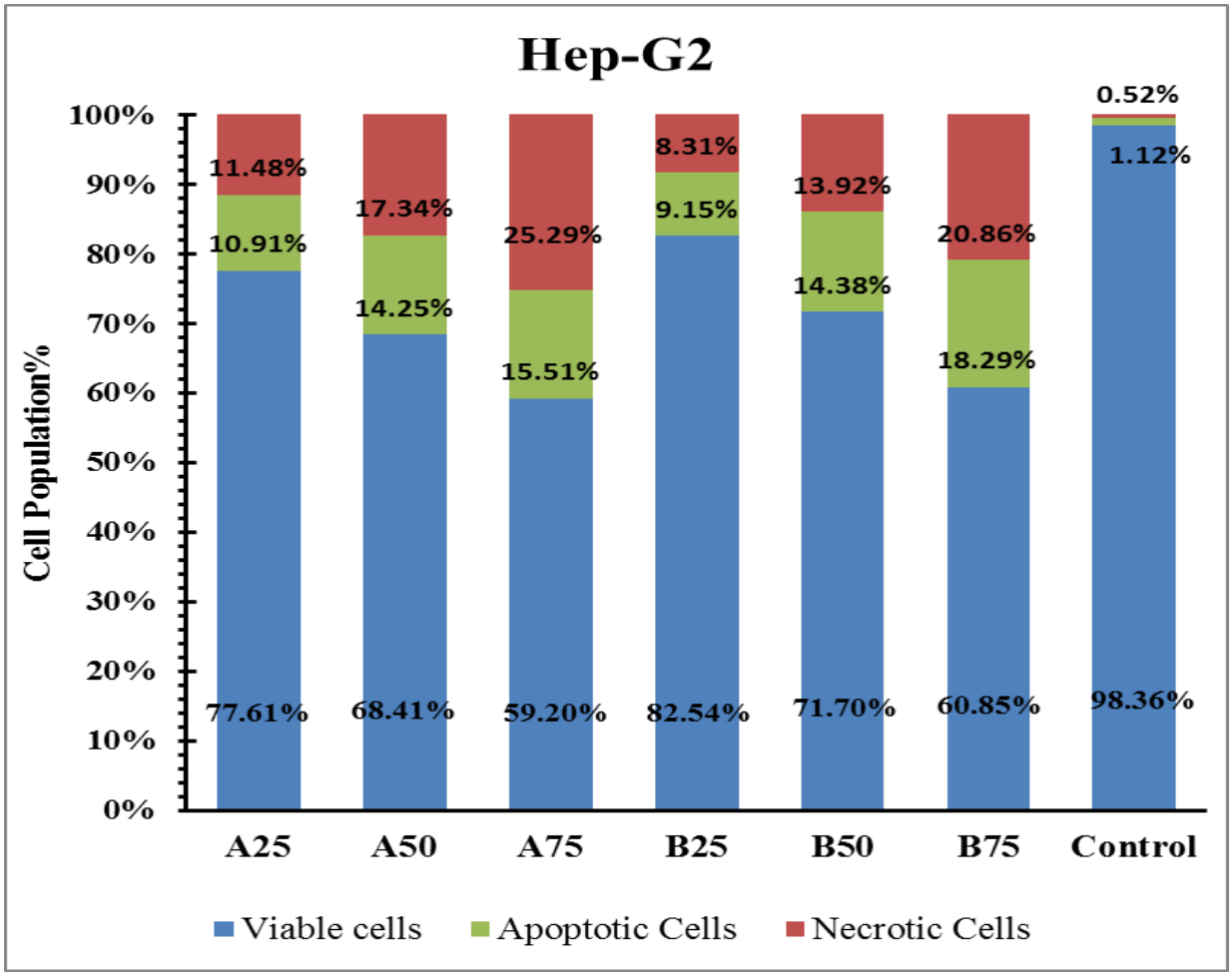

Figure (10): Histogram represened the percentage of viable,apoptotic and necrotic cells from the whole cell population in Hep-G2 cells treated with different doses of extracts A and B

Although the final results of apoptosis and necrosis are the same (death of the affected cells), they differ significantly. In necrosis, cell death occurs because of adverse effects or changes in the cell's environment. So, necrosis can be viewed as the consequence of a "biological accident" which leads to death of an "innocent victim" In apoptosis, cells actively participate via activating a cascade of biochemical reactions which result in cell death. Accordingly, apoptosis has been called "cell suicide"***(Nanji and Hiller-Sturmhofel, 1997)***.

Necrosis and Apoptosis are two different modes of cell death, which are distinguished by well defined biochemical and morphological features. In apoptosis, the cells undergo nuclear and cytoplasmic shrinkage, then the chromatin is condensed and fragmented, and finally the cells are broken into multiple membranes surrounded bodies (apoptotic bodies). In contrast, during Necrosis cell swells, disruption and disintegration of the cell membrane rapidly occur ***(Gamal-Eldeen et al., 2009)***.

The induction of apoptosis is a novel target for cancer chemoprevention as anti-promotion anti-progression strategy.

In the present study, the mode of cell death in MCF-7 cells was apoptosis at low doses and upon increasing the dose the rate of necrosis was increased against apoptosis in comparison with control untreated cells. The rate of

necrosis reaches the highest percentage at A75 and B75 doses with apriority of apoptosis. And the mode of cell death in Hep-G2 cells was allocated between necrosis and apoptosis at low doses and by increasing the dose the rate of necrosis was increased over apoptosis in comparison to the control untreated cells. The rate of necrosis reaches the highest percentage at A75 and B75 doses exceeding the apoptosis percentage. the activation of several kinases by caspase cleavage leads to the membrane remodeling and active blebbing observed in apoptotic cells which may be one of the explanation for the induced apoptosis ***(Herr and Debatin, 2001)***, And this was in agreement with ***(Lupidi et al., 2011)*** who reported that aqueous and ethanolic extracts of S. herba-alba induce apoptosis via the activation of caspase-3.

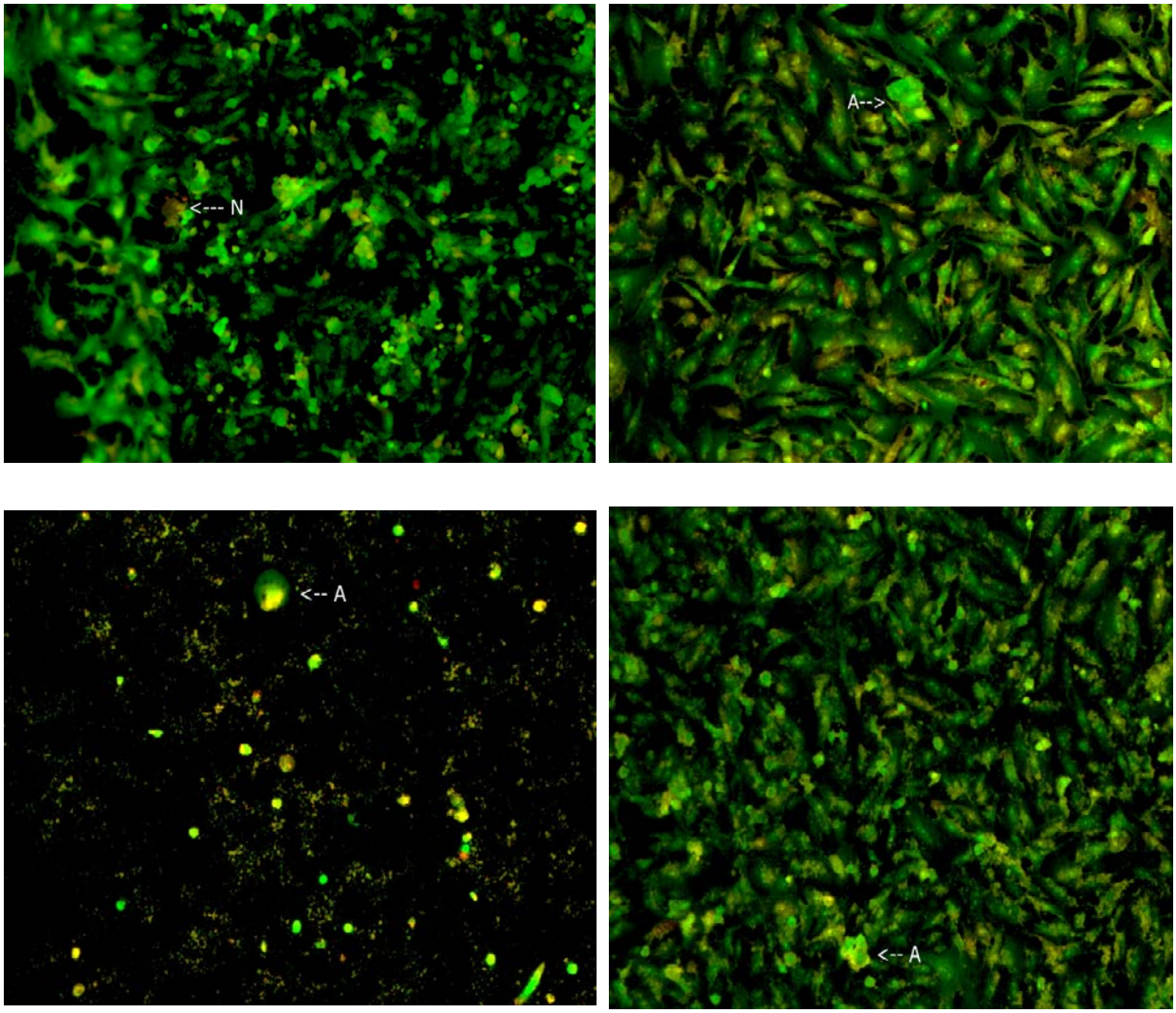

Figure (11): Representative photos for AO/EB stained and treated MCF-7 cells under fluorescence microscope (x40) (A= apoptotic cell, N= necrotic cell).

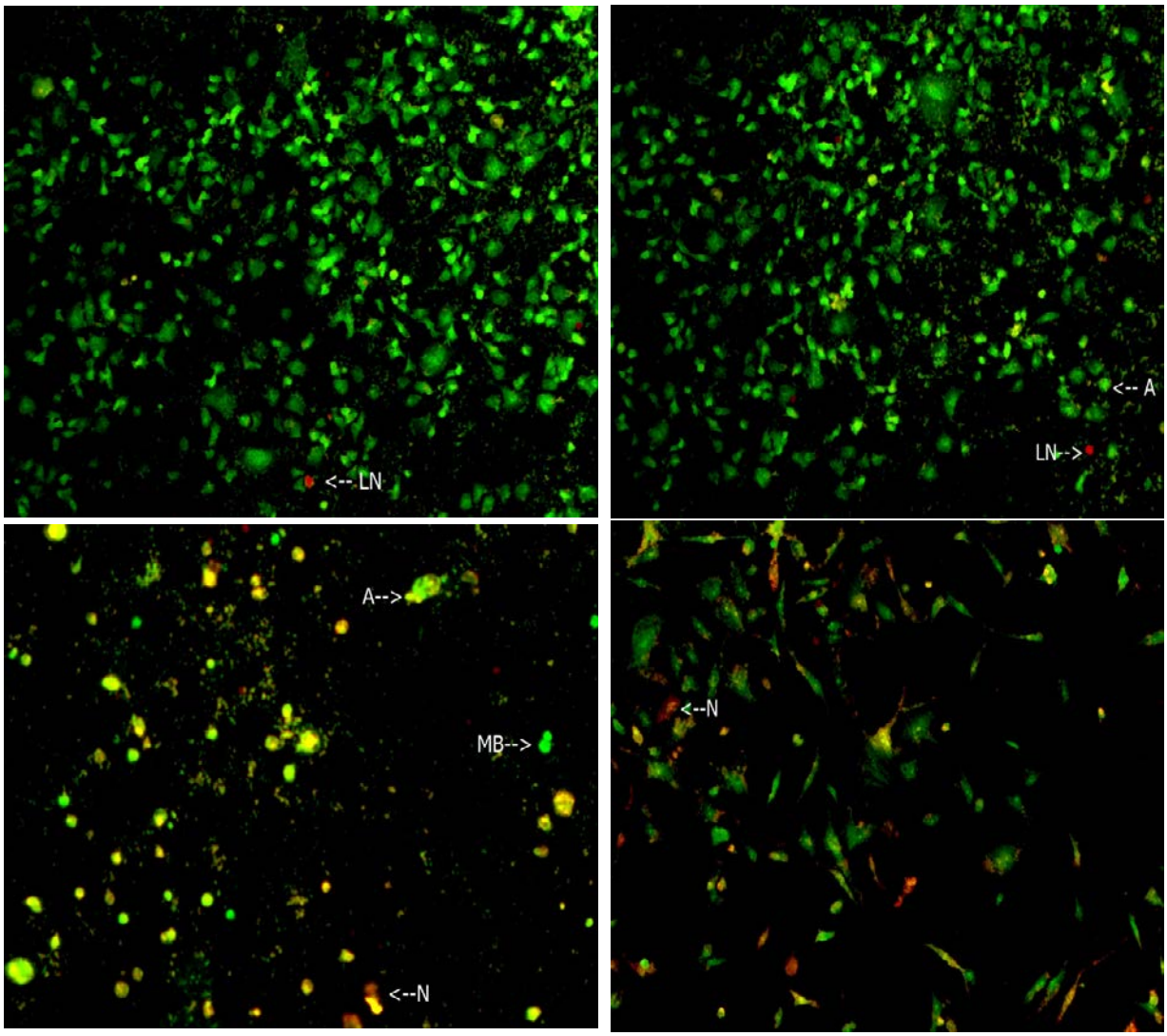

Figure (12): Representative photos for AO/EB stained and treated Hep-G2 cells under fluorescence microscope (x40) (A= apoptotic cell, N= necrotic cell, LN= late necrotic cell, MB=membrane blebbing).

b- Effect on Angiogenesis: Assessment of Vascular endothelial growth factor [VEGF] and Platelet derived growth factor [PDGF]:

1- VEGF:

Results revealed that in MCF-7 cell lines, concentrations of VEGF were significantly ($p<0.05$) decreased with doses of A75, B25, B50 and B75, but with doses A25 and A50, the decrease was non-significant.

In Hep-G2 cell lines, all doses showed significant great decrease ($p<0.05$)

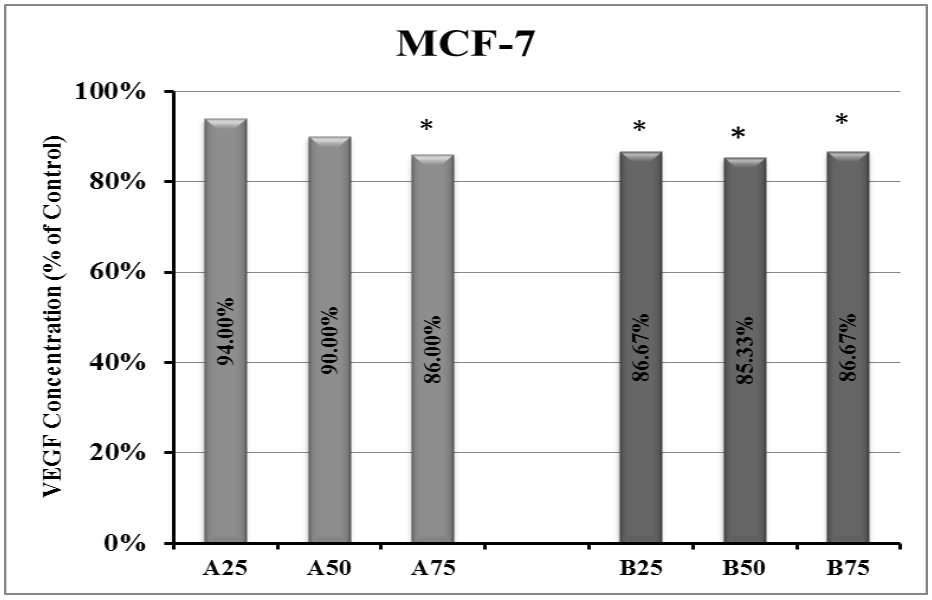

Figure (13): Histogram representing VEGF concentration in MCF-7 cell lines as percent of control after treatment with 3 different doses of S. herba-alba extracts A and B.

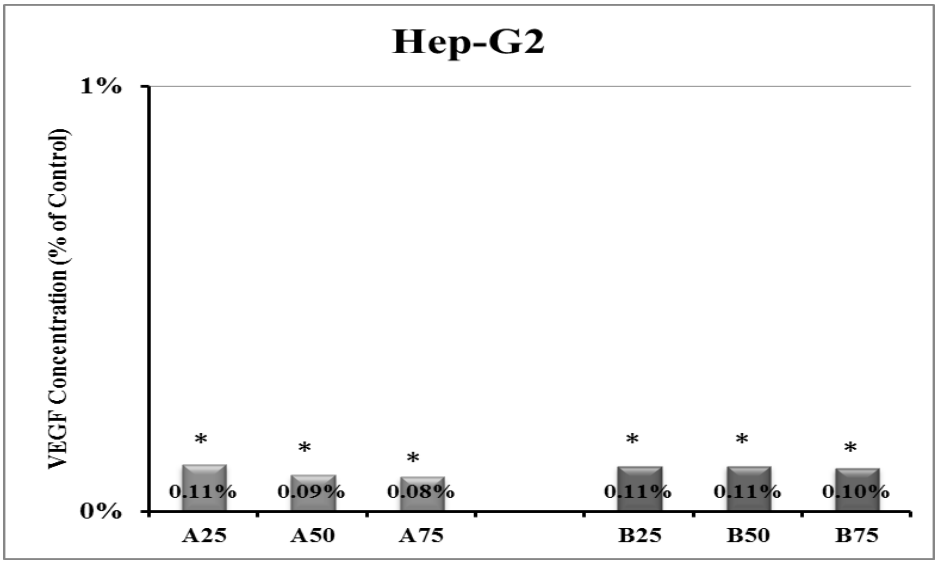

Figure (14): Histogram representing VEGF concentration in Hep-G2 cell lines as percent of control after treatment with 3 different doses of S. herba-alba extracts A and B.

2-PDGFBB:

In MCF-7 cell line, concentrations of PDGFBB were significantly decreased ($p<0.01$) after treatment with 3 different doses of S. herba-alba extract B, while S. herba-alba extract A showed significant decrease ($p<0.05$) for dosed A75 and A50 and dose A25 showed non-significant decrease.

In Hep-G2 cell line, concentrations of PDGFBB were significantly decreased ($p<0.05$) with doses of S. herba-alba extract A and extract B except B75, which showed non-significant decrease.

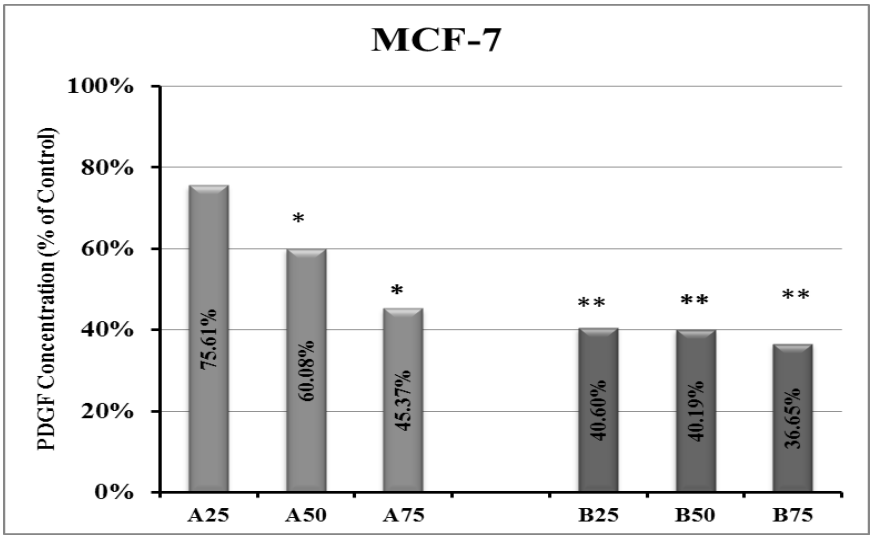

Figure (15): Histogram representing PDGFBB concentration in MCF-7 cell lines as percent of control after treatment with 3 different doses of S. herba-alba extracts A and B.

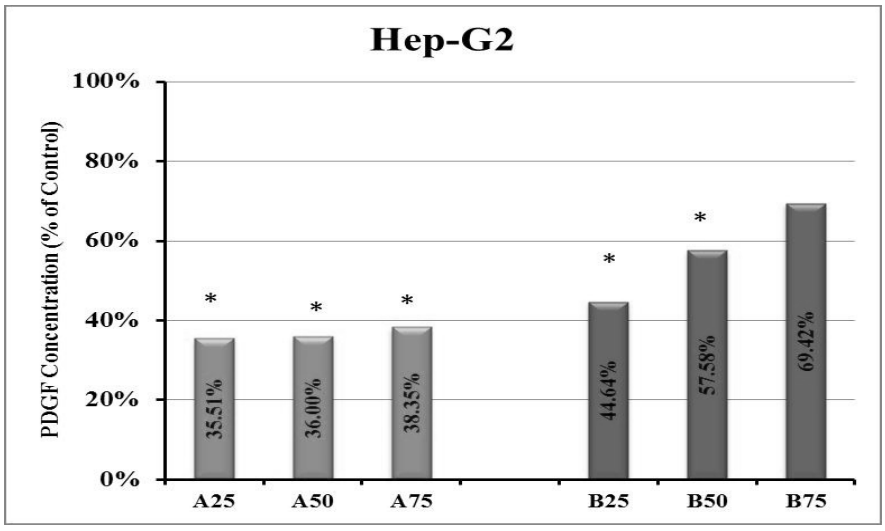

Figure (16): Histogram representing PDGFBB concentration in Hep-G2 cell lines as percent of control after treatment with 3 different doses of S. herba-alba extracts A and B.

Tumor growth is dependent on angiogenesis which is the growth of new blood vessels, anti-angiogenesis is a strategy for preventing benign microtumors from progressing to invasive cancer ***(Li et al., 2012)***.

In the current study, two of the major angiogenic factors (VEGF and PDGF) were assessed after treatment with S.herba-alba extracts, and it has been shown that treatment with different doses of extracts cause significant inhibition of both angiogenic factors especially VEGF in Hep-G2 cells which was greatly inhibited.

4. Conclusion

In this work we evaluate the cancer chemopreventive activity of two Seriphidium herba-alba extracts; Methylene chloride and Methanolic extract, both were safe, enhanced the GSTs activity, and decreased GSH levels, induce apoptosis moreover necrosis and possessed anti-angiogenic activity. S. herba-alba is widely used in folk medicine and the extracts used in this research may be potential cancer chemopreventive agents but after further investigations and need to be proved in in-vivo models.

Acknowledgments

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