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# Immunomodulatory and anticancer activities of herbal drinks consumed in Jordan

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**Abstract**
In this study immunomodulatory and antitumor activity of five herbal drinks consumed in Jordan were evaluated. The antiproliferative activity was determined using MTT assay. The degree of apoptosis induction was detected by TUNEL colorimetric assay. ELISA was used to measure VEGF expression in tumor cells and levels of cytokines secreted by splenocytes. The effect of the extracts on splenocytes proliferation was measured using MTT assay. Macrophage function was evaluated using nitro blue tetrazolium assay. The growth of breast cancer cell lines was inhibited by herbal drinks in dose dependent manner. Ginger and lemon verbena were the most potent, they target cancer cells through the induction of apoptosis and suppression of breast cancer angiogenesis. An increase in Th1 cytokines level and decrease inTh2 cytokine level were evident after lymphocytes stimulation by herbal drinks. The consumption of different herbal drinks provides variable health benefits. Ginger and lemon verbena herbal drinks exhibit anticancer activities. Jordanian zhourat is a potent stimulator of innate and acquired immunity.

**Keywords:** Anticancer, MCF-7 and T47D, immunomodulatory, Jordanian herbal drinks

## 1. Introduction
Within the increasing interest to survive a healthy life, using traditional plants is presented as an alternative medicine and most of the people use these plants for their everyday health care needs, to reduce weight and high cholesterol level, manage diabetes, treat microbial infections, and provide protection against cancer and augmenting the function of the immune system [1]. Natural products are an attractive source of therapies; they are relatively safe, effective, and suitable for large scale production and are less expensive [2]. The use of natural products in treating different ailments is as old as civilization. Avicenna was the first who used the Hindiba (*Cichorium intybus*) for treatment of different diseases including cancer [3]. In Islam, *Nigella sativa* is regarded as one of the greatest forms of healing medicine available. The prophet Mohammad (PBUH) stated, "The black seed can cure all diseases except death" [4]. The most practical and frequently used traditional remedy is preparing an infusion from the valuable parts of plants and herbs such as flowers, leafs and roots or other plant material in hot water, letting them steep for a few minutes The herbal tea is then strained, sweetened if so desired, and served either hot or cold [5]. Many companies produce herbal tea bags for such infusion. In the current study immunomodulatory and antitumor activity of aqueous extracts of herbal drinks that include: ginger (*Zingiber officinale*, Zingiberaceae**)**, hibiscus (*Hibiscus sabdariffa*, Malvaceae), lemon verbena (*Aloysia triphylla*, Verbenaceae), Jordanian zhourat is a combination of sage (*Salvia officinalis*, Lamiaceae), lemon verbena, cardamom (*Elettaria cardamomum*, Zingiberaceae) and cinnamon(*Cinnamomum zeylanicum*, Lauraceae), Syrian zhourat is a combination of chamomile (*Chamomilla recutita*, Compositae**)**, marshmallow (*Althaea officinal*, Malvaceae), green tea *Camellia sinensis*, Theaceae), damask rose (*Rosa damascena*, Rosaceae) and lemon verbena were evaluated.

## 2. Materials and Methods
### 2.1 Reagents
In this study, two different types of tissue culture media were used: Dulbecco's
Modified Eagle Medium (DMEM) and Roswell Park Memorial Institute Medium (RPMI) 1640, L-glutamine, gentamycin and penicillin streptomycin solution were used as supplements to the tissue culture media. Phosphate buffer saline PBS and trypsin-ethylene diamine tetra acetic acid were used to detach adherent cells in the tissue culture flask. Trypan blue 0.4% was

used to assess cell viability and count cells they were purchased from Sigma-Aldrich (USA). Fetal bovine serum was obtained from Sigma-Aldrich (Germany).Ginger herbal tea, lemon verbena, hibiscus, Jordanian zhourat and Syrian zhourat were purchased from Al- Attar for herbal drinks (Jordan). Concanavalin A (Santa Cruz Biotechnology, USA), NH4Cl RBC lysis buffer for human (Bioworld, USA), NBT (nitro tetrazolium blue Chloride, AstaTech, USA). Dimethyl sulphoxide (DMSO) (AZ Chem for chemicals, Canada). Doxorubicin hydrochloride (Ebewe Pharma, Austria). Active dry yeast (Asrico, Jordan) Lipopolysaccharides from *Escherichia coli* (LPS) (Sigma, USA). All other chemicals used in the study were of reagent grade.

**2.2 Commercial kits**

MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide) assay kit (Sigma-Aldrich, USA) was used for antiproliferative assay. Dead End Colorimetric TUNEL System G7362 (Promega, USA) was used to detect apoptosis. Mouse VEGF Elisa kit (R&D Systems, USA) was used to measure VEGF expression in cancer cells, Mouse Th1/Th2 ELISA kit (affymetrix eBioscience, USA) was used to evaluate the immune system response.

**2.3 Sample Preparation**

Several dry herbs (ginger, lemon verbena, hibiscus, Jordanian zhourat, Syrian zhourat) used to prepare herbal drinks were purchased from the local market. All herbs included in the study were provided from the same source (Alattar herbal drinks). Aqueous extracts were prepared by soaking herbal bags (1500 g) of each herb
in hot water for 5 min followed by complete dryness of each extract by using incubator at 40 °C. The dried extracts were weighed and kept at -20 °C.

**2.4 Phytochemical screening**

A qualitative chemical screening for identification of various classes of active chemical constituents such as carbohydrates, tannins, saponins, terpenoids, cardiac glycosides, steroids, flavonoids and alkaloids were performed. Detection of the extracted compounds was done according to standard methods described by [6-8].

**2.5 Biological evaluation**

**2.5.1 Cell lines and cell culturing condition**

Three cell lines were used to investigate the effect of the selected herbal drink aqueous extracts against their survival. The cells were cultured in complete medium and incubated at 37 °C in 5% CO2, 95% humidity incubator. Two human epithelial breast cancer cell lines (MCF-7 and T47D) were provided by the University of Jordan. Both cell lines were cultured in complete RPMI1640 Medium, and incubated at 37 °C in 5% CO2, 95% humidity incubator. Kidney epithelial cells from African green monkey (Vero cell line) were used as a normal control and were also obtained from the University of Jordan. The cells were cultured in complete DMEM Medium and incubated at 37 °C in 5% CO2, 95% humidity incubator.

**2.6 Cytotoxicity assays**

**2.6.1 Trypan blue dye exclusion assay**

Partially confluent cells (80-90%) grown in tissue culture flask were treated with trypsin/EDTA and PBS to detach cells from flask surface. Then the surface of flask was washed with 5 ml of complete tissue culture medium, transferred into sterile centrifuge tube and centrifuged at 1500 RPM and 4 °C for 10 minutes forming a pellet which was later re-suspended in 5 ml of new complete tissue culture medium after decanting the supernatant layer of media. Of the resulting suspension, 100 µl was mixed in vortex mixer with 100 µl of Trypan blue dye and appropriate volume was added to counting chamber (Hemocytometer) in order to be examined under the light microscope where viable cells (clear cells that exclude the blue dye) in the 25 squares were counted.

**2.6.2 (Methyl tetrazolium-MTT assay)**

Cells were dispensed (100 µl/well) into 96-well tissue culture plates (flat bottom) at an optimized concentration of 15000 cells/well in complete tissue culture medium. After 24 h, the media in each well were completely removed and the attached cells were treated in triplicates with decreasing concentrations of aqueous In MTT assay, 200µl were removed from each well containing culture media and followed by washing wells using 100µl of PBS added for each well, then 100µl of culture media was added, then10 µl of thiazolyl blue tetrazolium solution was added to the remaining100 µl and incubated in CO2 incubator for 3 h. The reaction was stopped by adding MTT solubilization solution (100 µl/ well) mixed well and incubated for another one hour. At 550 nm the absorbance was measured by microplate reader. Cell viability (% of survival rate) was calculated compared to untreated cells which represent the negative control (tissue culture media + 0.1% DMSO).

**2.7 Calculation of inhibitory concentration (IC50)**

IC50 represent the concentration at which there is 50% of cell death in comparison to cells of negative control. The process of calculation was conducted by non-linear regression in statistical package for the social sciences (SPSS) version 21(Chicago, illinois).

**2.8 Apoptosis detection in cultured cells**

Degree of apoptosis induced by each treatment was detected using Dead End
Colorimetric TUNEL System. According to kit instructions, cells were fixed by immersing slides in 10% buffered formalin in PBS for 25 minutes. Slides were washed by immersing in fresh PBS for 5 minutes. Cells were then permeabilized by immersing the slides in 0.2% Triton® X-100 solution in PBS for 5 minutes. Slides were rinsed by immersing in fresh PBS for 5 minutes. End-labeling reaction occurred through the even distribution of rTdT reaction mixture on the cells while incubating for 60 min at 37 °C in a humidified chamber, termination of the reaction occurred when slides are immersed in 2X SSC termination solvent. Endogenous peroxidases were blocked by 0.3% hydrogen peroxide. Horseradish Peroxidase-labeled streptavidin was then added to bind to the biotinylated nucleotides for 30 min followed by incubation with DAB components for 10min in the dark. Afterwards slides were mounted with glycerol and observed under the light microscope equipped with computer controlled digital camera. Detailed and step by step procedure was done in accordance to Dead End Colorimetric TUNEL System G7362 (Promega, USA).

**2.9 Determination of VEGF expression in T47D cells**

T47D cells were dispensed into four separated tissue culture flasks at an optimized concentration of 1500000 cells/10ml of complete tissue culture medium. After 24 h, the media in each flask was completely removed and the attached cells were

treated with one of the following treatments: 6.5 mg/ml of aqueous extract of ginger, 5mg/ml of aqueous extract of lemon verbena, 250nM of doxorubicin hydrochloride and blank media as a negative control. Cells were incubated for 48 h, after that the media of each flask were transferred into sterile tubes and the attached cells were harvested by employing trypsinization technique using Trypsin/EDTA and PBS for 2-3 minutes, washed, transferred to the existing sterile tubes and centrifuged at 1500 RPM and 4 °C for 10 min. After decant the supernatant layer, cells were allowed to stand on ice for 30 min with vortex every 10 min. After that the resulting mixture was centrifuged. The supernatant was transferred to a new tube VEGF expression in cancer cells was measured using mouse VEGF enzyme-linked immunosorbent assay (Elisa) kit (catalogue # MMV00; R&D Systems, USA).

**2.10 Preparation of murine splenocytes**

Balb/C mouse was sacrificed and the spleen was removed aseptically. The cells of the spleen were passed through the mesh of a tissue grinder and the suspension was prepared in RPMI-1640. The cell suspension was washed thrice for 10 min using RPMI-1640 (2000 r/min) and then re-suspended in 1 mol/L NH4Cl to eliminate red blood cells (RBC). After 10 min the cells were again centrifuged and resuspended in RPMI-1640 media. Splenocytes then washed, counted and used in other assays.

**2.11 Mitogen proliferation assay**

The mitogen proliferation assay was performed using MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide) assay kit (Sigma-Aldrich, USA) according to the manufacturer's instructions. Briefly, splenocytes suspension were made (2×106 cells/mL) in RPMI-1640 (supplemented with 50 U/mL penicillin, 50 U/mL streptomycin, and 10%FBS) and were seeded into different wells of 96 well culture plates in presence of 5 µg/mL Con A and 4µg/mL LPS. To this, 50 µL of different concentrations (0.625-80mg/mL in RPMI-1640) of aqueous extracts of the ginger, lemon verbena, hibiscus, Jordanian zhourat and Syrian zhourat were added. The plate was incubated for 24 hr under 5% CO2 and humidified atmosphere of 95% air at 37 ◦C temperature. After the incubation 10 µL MTT (5 mg/mL) solution was added to each well. The plate was wrapped with aluminum foil to avoid exposure to light and incubated for 4 h. Then 100 µL solubilizing reagent was added to each well and the absorbance was measured at 550 nm using ELISA microplate absorbance reader.

**2.12 Determination of cytokines (IFN-γ, IL-2, IL-4 and IL-10) levels in activated lymphocytes**

Levels of IFN-γ, IL-2, IL-4 and IL-10 were measured for representative samples of mouse splenocytes, which were cultured with one of the aqueous extract of ginger, lemon verbena, hibiscus, Jordanian zhourat and Syrian zhourat with concentration of 15mg/mL, using affymetrix ELISA mouse IFN-γ, IL-2, IL-4 and IL-10 kit. Briefly, splenocyte suspension was made (2 × 106 cells/mL) in RPMI-1640 (supplemented with 50 U/mL penicillin, 50 U/mL streptomycin, and 10% FBS) and the cells were seeded into different wells of 96 well culture plates. Mouse splenocytes were cultured with concentrations 15mg/mL of ginger, lemon verbena, hibiscus, Jordanian zhourat and Syrian zhourat extract for 48h. After incubation culture supernatants were collected to estimate the concentrations of IL-2, IFN-γ, IL-4 and IL-10. Detailed and step by step procedure was done in accordance to Th1/Th2 ELISA kit (catalogue # 88-7711-44; Affymetrix eBioscience, USA)

**2.13 Macrophage isolation from peritoneal fluid**

Peritoneal macrophages (PEM) were isolated from peritoneal mice cavities using ice-cold sterile phosphate-buffered saline (pH 7.4). Mice were euthanized by cervical dislocation and their abdominal cavities were visualized and 5 ml ice-cold PBS was introduced into the cavity. After gentle massaging the fluid was withdrawn and placed in a centrifuge tube held on ice. The process was repeated five times and fluids were pooled. After centrifugation of the pooled fluid (3000 rpm, 10 min, 4 °C), each cell pellet was suspended in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin (100 U/ml) (all chemical supplies from Sigma, Chennai) and allowed to adhere for 3 hr at 37 °C in 5%CO2 humidified incubator. Thereafter, non-adherent cells were washed away with medium and the adherent cells then collected for use in the various assays outlined below.

**2.14 *In vitro* phagocytic assay (nitro blue tetrazolium (NBT) reduction test)**

NBT reduction assay was carried out according to the method of Rainard [9]. In brief, PEM (5 x 106 cells/well of a 96-well plate) were cultured with different concentrations of aqueous extracts of five herbal plants (2.5-10 mg/ml) for 48 h at 37 °C. Thereafter 20 µl yeast suspension (5 × 107 cells/ml in PBS) and 20 µl nitroblue tetrazolium (1.5 mg/ml) (NBT (Hi-Media, Mumbai) in PBS were added to each well. Wells that received PBS+DMSO (adjusted to 0.1% (v/v)) were used as controls. Cells were then incubated for 60 min at 37 °C; the supernatant was then removed and the adherent macrophages rinsed with RPMI 1640. The cells were air dried before 120 µl of 2M KOH and 140 µl DMSO were added to each well. The absorbance of the turquoise blue solution was measured at 550 nm in the plate reader. The percentage of NBT reduction reflects phagocytic activity.

**2.15 Statistical analysis**

Data are presented using mean ± SEM (Standard Error of Mean). The statistical significance among the groups was determined by using SPSS (Statistical Package for the Social Science, Chicago, Illinois), one-way analysis of variance (ANOVA). A p-value < 0.05 was considered significant.

**3. Results**

**3.1 Percentage yield of Aqueous Extract**

High variation in percentage yield was observed among different extracts. The highest yield was reported for hibiscus aqueous extract with a percentage yield of 24.730% while the lowest yield was observed in ginger extract which produced a yield of 7.156% (Table.1).

**Table 1:** Initial weight and percentage yield of the analyzed plants

| Name of plants | Weight of the plants analyzed | % of dried extracts Yields |
|---|---|---|
| Ginger | 1500 g | 7.156% |
| Hibiscus | 1500 g | 24. 730% |
| Lemon Verbena | 1500 g | 15.498% |
| JordanianZhourat | 1500 g | 7.296% |
| Syrian Zhourat | 1500 g | 8.146% |
| $\% \text{ Yield} = \frac{\text{weight of evaporated aqueous extracts}}{\text{weight of plants analyzed}} * 100\%$ | | |

### 3.2 Phytochemical Screening of Aqueous Extract

The medicinal value of plants lies in some chemical constituents that have a definite physiological action on the human body. These secondary metabolites are generally superior in their biological activities and their strength is dependent on diversity and quantity of such constituents. The phytochemical screening aqueous extracts revealed the presence of some secondary metabolites and the results are shown in (Table.2).

**Table 2**: Phytochemical screening of aqueous extracts of the analyzed plants

| Phytochemical Tests | Ginger | Hibiscus | Lemon Verbena | Jordanian Zhourat | Syrian Zhourat |
|---|---|---|---|---|---|
| Alkaloids | + | + | + | + | + |
| Carbohydrate | + | + | + | + | + |
| Flavonoids | + | + | + | + | + |
| Glycosides | - | + | + | + | + |
| Saponins | + | + | + | + | + |
| Tanins | + | + | + | + | + |
| Terpenoids | + | + | + | + | + |
| Steroids | - | - | + | + | + |
| Present (-) Absent(+) | | | | | |

### 3.3 Antiproliferative assay

A dose dependent response was observed after treatment of breast cancer cell lines with aqueous extracts. Applying of gradual decreasing concentrations of aqueous extracts (0.3125-40 mg/ml) on T47D cell line showed an increase in the average percentage of survival (Fig.1).T47D respond similarly to hibiscus, lemon verbena and Jordanian zhourat with 75% inhibition at concentration 40 mg/ml. While the % of inhibition in ginger and Syrian zhourat was 65% and 60% respectively at the same concentration (Fig.1). On the other hand, a dose-dependent inhibition was observed in MCF-7 cell line treated with increasing concentration of the extracts. The % of inhibition at concentration 40 mg/ml were (48%, 47.95%, 40%, 55%, 44%) in ginger, hibiscus, lemon verbena, Jordanian zhourat, Syrian zhourat respectively (Fig.2). Based on these *in vitro* results, ginger and lemon verbena exhibited the most effective activity against T47D and MCF-7 cell lines with $IC_{50}$ values of 6.5 and 11.5 mg/ml for ginger against T47D and MCF-7, respectively and $IC_{50}$ values of 5 and 14.7 mg/ml for lemon verbena were recorded against T47D and MCF-7, respectively (Table.3). Whereas other extracts were effective only against T47D cell line with $IC_{50}$ values of 4, 6 and 22mg/ml for Jordanian zhourat, hibiscus and Syrian zhourat, respectively. While MCF-7 was the least sensitive to these extracts with $IC_{50}$ values of 37.6, 23 and 108mg/ml for hibiscus, Jordanian zhourat, and Syrian zhourat, respectively (Table.3). Vero cell line showed more resistance to the extracts. At the highest concentration (40 mg/ml) the percentage of survival was 70% (Fig.3). The toxicity of these extracts against Vero cell line was limited with $IC_{50}$ of 226, 230,356, 322,467 mg/ml for ginger, hibiscus, lemon verbena, Jordanian zhourat, Syrian zhourat, respectively (Table. 3).

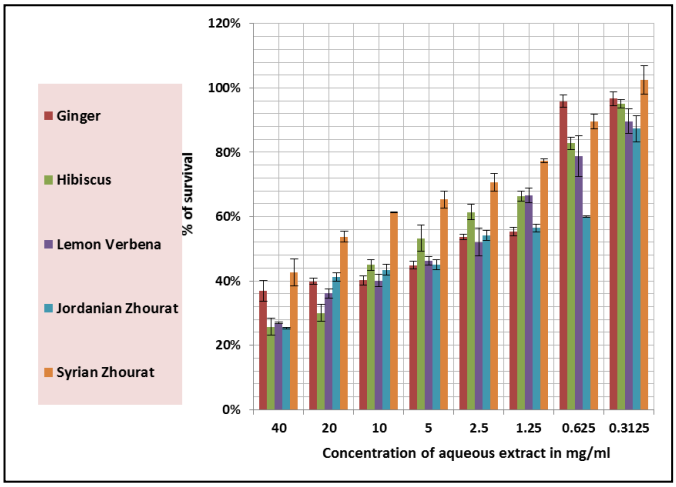


**Fig 1**: Antiproliferative activity of different concentrations of aqueous extracts of the analyzed plants against T47D cell line

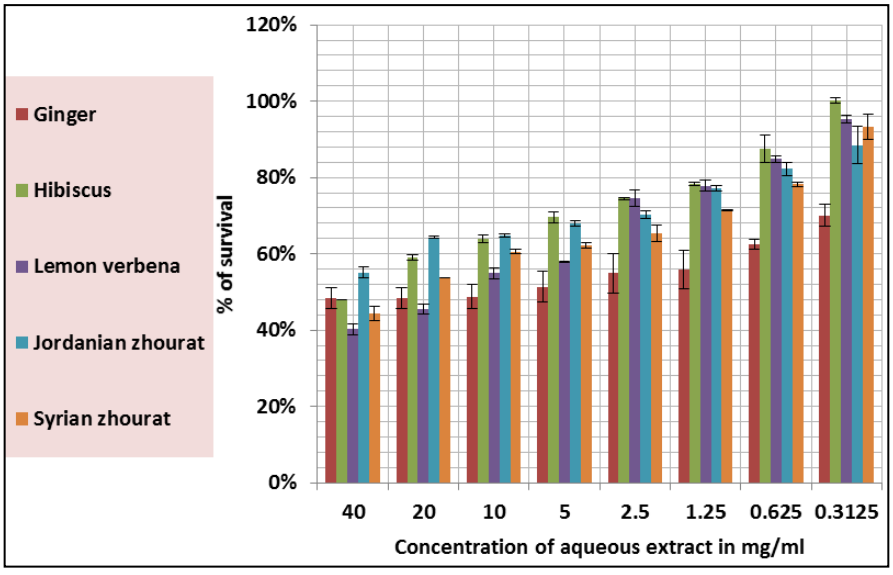


**Fig 2**: Antiproliferative activity of different concentrations of aqueous extract of the analyzed plants against MCF7 cell line

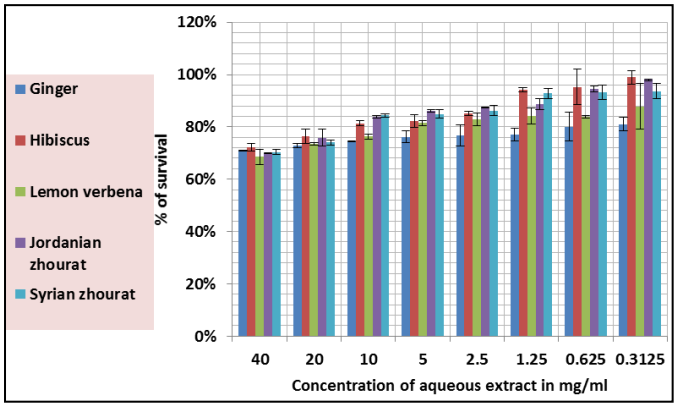


**Fig 3:** Antiproliferative activity of different concentrations of aqueous extract of the analyzed plants against Vero cell line

**Table 3:** The $IC_{50}$ in three cell lines (MCF-7, T47D and Vero) for aqueous extracts of the analyzed plants

| Cell line | Ginger $IC_{50}$ (mg/ml) | Hibiscus $IC_{50}$ (mg/ml) | Lemon verbena $IC_{50}$ (mg/ml) | Jordanian Zhourat $IC_{50}$ (mg/ml) | Syrian Zhourat $IC_{50}$ (mg/ml) |
|---|---|---|---|---|---|
| MCF-7 | 11.5 | 37.6 | 14.7 | 108 | 23 |
| T47D | 6.5 | 6 | 5 | 4 | 22 |
| Vero | 226 | 230 | 356 | 322 | 467 |

### 3.4 Effect on VEGF expression

The expression of VEGF was measured *in vitro* against T47D cell line in order to investigate whether the inhibition of angiogenesis may contribute to the observed antiproliferative effect. In the negative control group that received only tissue culture media, VEGF was highly expressed **(**400 pg/ml, p=0.04) (Fig.4). On the other hand, treating cells with 6.5 (mg/ml) of ginger aqueous extract showed significantly higher reduction in VEGF expression compared with the normal control (100pg/ml, p=0.03). Similar results were obtained when cells were treated with 5(mg/ml) of lemon verbena aqueous extract with VEGF expression level of 140 pg/ml (p= 0.01), while cells treated with doxorubicin showed VEGF expression level of 160 pg/ml (p=0.04) (Fig.4).

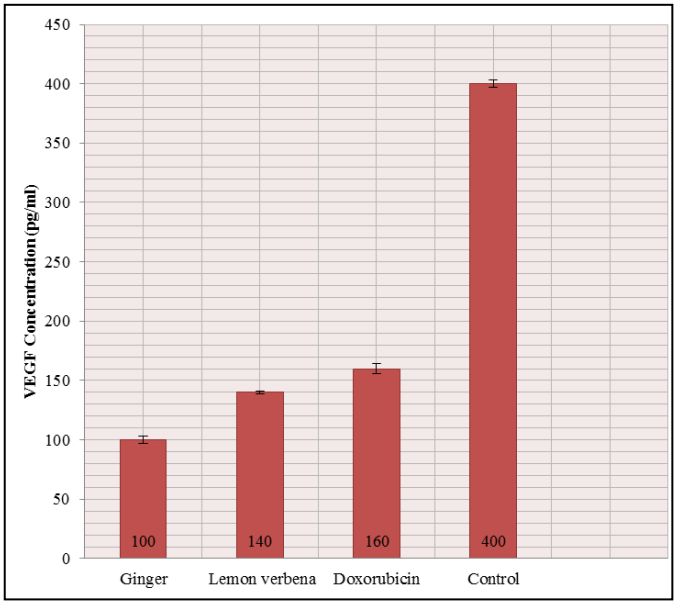


**Fig 4:** The effect of different treatments on the expression of vascular endothelial growth factor (VEGF).Concentration of VEGF (pg/ml) in T47D cells treated with 6.5 mg/ml of Ginger, 5mg/ml of Lemon Verbena and 250 nM of Doxorubicin, as well as in untreated control cells. Where pg/ml= picogram per milliliter Results are expressed as means (bars) ± SEM (lines).

### 3.6 Apoptosis induction ability

In order to gain further details about the mechanism of action of our analyzed plants, TUNEL colorimetric assay was used to detect the DNA fragmentation following treating of T47D with ginger, lemon verbena, doxorubicin and negative control. Apoptosis was detected as dark brown area as shown in (Fig.5). Based on DNA fragmentation, ginger and lemon verbena induced a programmed cells death compared to untreated cells. The highest degree of apoptosis was observed in cells treated with lemon verbena (Fig.5).

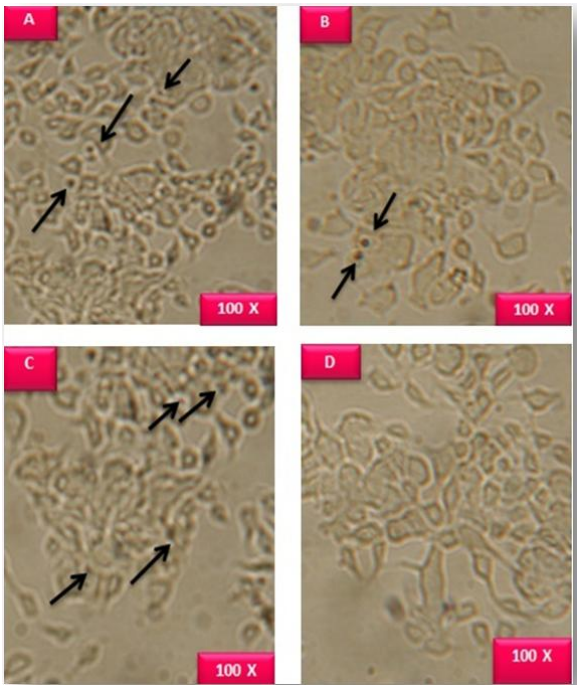


**Fig 5:** Colorimetric TUNEL assay to detect the induction of apoptosis in cultured T47D cells after different treatments. Cancer cells treated with 5mg/ml of Lemon Verbena (A), 6.5 mg/ml of Ginger (B), 250 nM of Doxorubicin (C) and Negative Control (D). Apoptotic nuclei are stained dark brown

### 3.7 The effect on the levels of IFN- γ, IL-4, IL-2 and IL-10

The immune modulation effect of different treatments was evaluated by measuring levels of IFN- γ, IL-4, IL-2 and IL-10 in lymphocytes supernatants stimulated by these extracts using standard ELISA kit. The result showed that there was a significant up-regulation in IFN- γ and IL-2 compared to

control, and significant down-regulation IL-4 and IL-10 in comparison to the control group (Fig.6). All of the extracts have increased IFN- γ concentration compared to control group (Fig.6). At 15mg/mL dose, the IFN- γ concentration was the highest in the supernatants of splenocytes that were cultured with lemon verbena extract of (425.00 ± 2.60) pg/mL, which was significantly higher than control group (280.00±1.6) pg/mL, followed by ginger extract that increased IFN- γ concentration to (400.00±2.60)pg/mL whereas hibiscus, Syrian zhourat and Jordanian zhourat induced similar increasing for IFN- γ concentration and the concentrations were (308.00±4.20)(304.00±1.70) and (300.00±2.50) pg/mL respectively. Accounting for (107.143-151.786) % increase in the IFN- γ secretion.

At dose of 15 mg/mL, the concentration of IL-2 was significantly higher than that of the control (275 ± 2.3) pg/mL, accounting for (104.364 - 109.818) % increase in the IL-2 secretion. Lemon verbena extract, ginger and Syrian zhourat increased IL-2 concentration by the same range as follows (304.00±3.30), (302.00±4.30) and (302.00±4.40) pg/mL, the lowest increase was in the extract of hibiscus which was (287.00±3.30), while Jordanian zhourat extract increased IL-2 concentration to (296.00±2.80). All of the extracts considerably down-regulated IL-4 secretion accounting for (102.740 - 138.249) % decrease. At the dose of 15 mg/mL, the lowest concentration of IL-4 was in the culture supernatant incubated with Jordanian zhourat extract (217.00 ± 4.30) pg/mL and this was significantly lower than that of the control group (300.00 ± 4.10) pg/mL. Lemon verbena extract and Syrian zhourat decreased IL-4 concentration to 267 pg/mL, while ginger extract decreased IL-4 concentration to (275.00 ± 3.00) pg/mL. Whereas the lowest decrease was in the extract of hibiscus (292.00 ± 3.50) pg/ mL. The secretion of IL-10 was down-regulated by the plants extract at the dose of 15 mg/mL. Compared to the control (288.00 ± 2.50) pg/mL, the IL-10 concentration has significantly decreased similarly by all the extracts, which were (260.00 ± 1.40), (259.00 ± 4.20), (262.00 ± 2.50), (261.00 ± 2.60), (263.00 ± 4.30) pg/mL in ginger, lemon verbena, Syrian zhourat, hibiscus and Jordanian zhourat extract respectively.

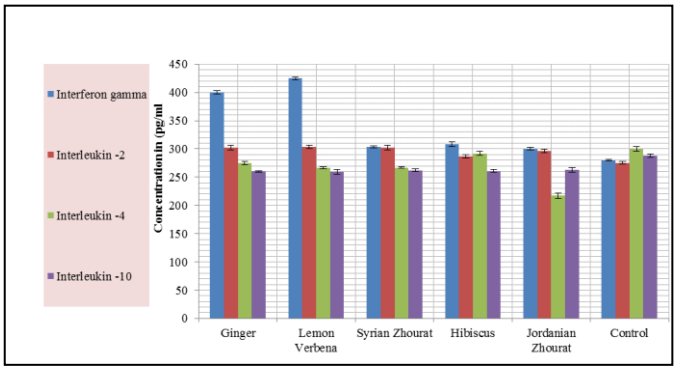


**Fig 6:** The effect of aqueous extract of analyzed plants on the expression pattern of various cytokines, where pg/ml= picogram per milliliter

### 3.8 Effect on the proliferation of splenic lymphocytes

The immunomodulatory effect in the cell proliferation model has been a target of study in the search for new therapeutic agents of natural origin, in this study the water extracts of assessed plants induced an increase in cell proliferation. (Fig.7) displays the results of the splenic lymphocyte proliferation after treatment with increasing concentrations of aqueous extracts. Increased metabolic activity of the cells in a dose-dependent manner was found as evidenced from the cell proliferation. At the concentration of 80 mg/mL, the extract of Jordanian zhourat showed the highest stimulation percentage of cell viability *in vitro* culture which was (1290.900%±3.10). Followed by lemon verbena, Syrian zhourat and hibiscus with cell viability of (1075.716%±03.5), (1048.17%±5.30) and (961.086%±0.4.10) respectively, whereas the lowest splenic lymphocytes proliferation was in extract of ginger of (270.689%±03.08) at the same dose. The average of splenic lymphocytes proliferation has a range of (1290.900%-270.689%) with the highest concentration of the extracts that has been used. Details about the effect of each extract on the proliferation of splenic lymphocytes at gradual decreasing concentrations illustrated in (Fig.8.9.10.11.12).

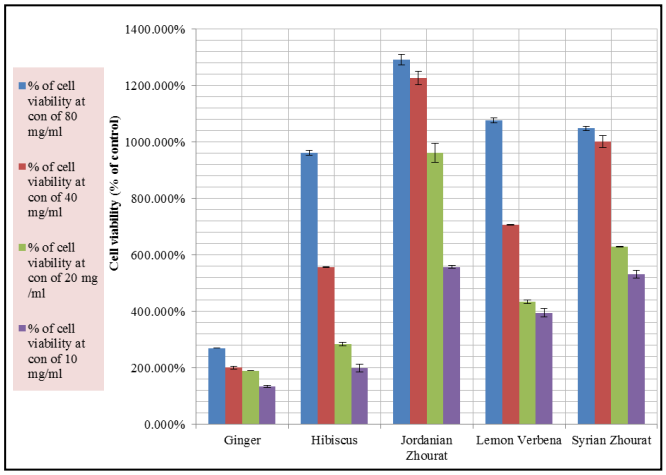


**Fig 7:** The effect of aqueous extract of the analyzed plants at different concentrations on the proliferation of splenic lymphocytes

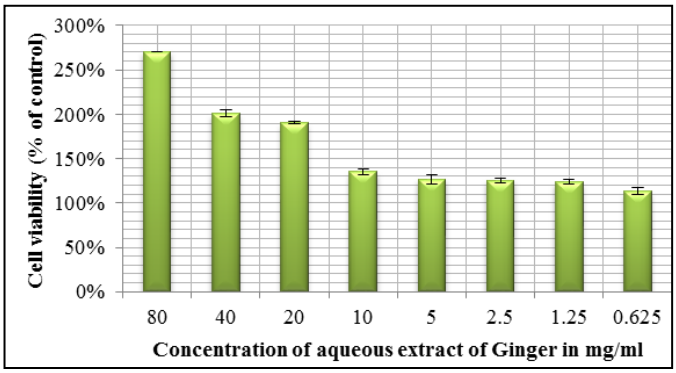


**Fig 8:** The effect of aqueous extract of Ginger on the proliferation of splenic lymphocytes.

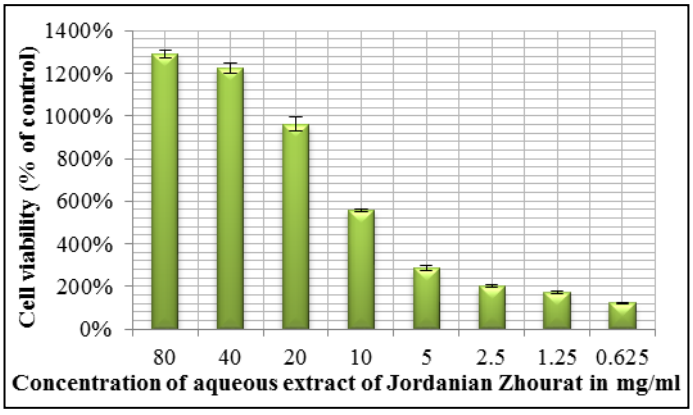


**Fig 9**: The effect of aqueous extract of Jordanian zhourat on the proliferation of splenic lymphocytes

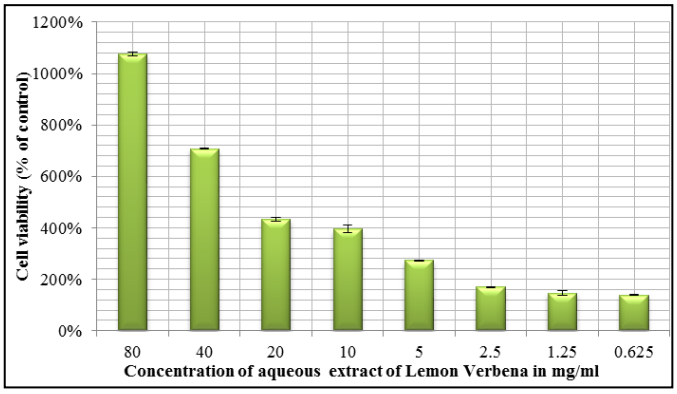


**Fig 10**: The effect of aqueous extract of Lemon verbena on the proliferation of splenic lymphocytes.

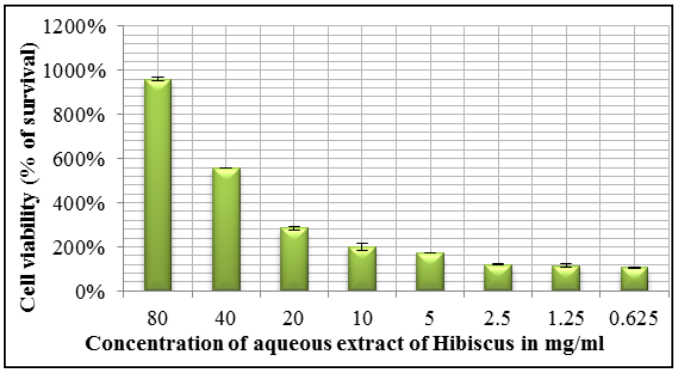


**Fig 11**: The effect of aqueous extract of Hibiscus on the proliferation of splenic lymphocytes

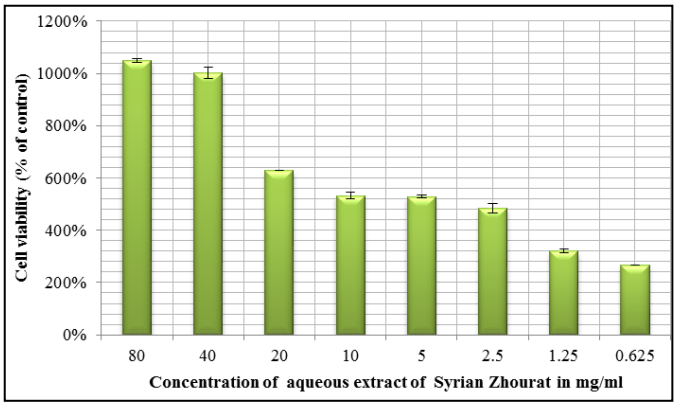


**Fig 12**: The effect of aqueous extract of Syrian zhourat on the proliferation of splenic lymphocytes

### 3.9 Extract effects on phagocytosis

Phagocytic activity of peritoneal macrophages was determined by measuring of NBT reduction ability after treatment with different extracts. After 60 min incubation with yeast cells and NBT, the effects of the aqueous extracts of analyzed plants on phagocytic indices of the cells were evaluated. The results showed that following exposure to the aqueous extracts peritoneal phagocytic activity was increased significantly as the dose levels increased by using doses from 2.5,5 to10 mg/ml (Fig.13). Extract of Jordanian zhourat showed the highest stimulation of peritoneal phagocytic activity *in vitro* culture at dose of 10 mg/ml which was (411.381% ± 5), followed by lemon verbena extract by phagocytic activity of (281.847% ±3) at the same dose. Similar results have been reported with extracts from ginger and hibiscus by phagocytic activity of (214.949% ± 4) and (214.407% ± 4.7), whereas the lowest phagocytic activity was in extract of Syrian zhourat of (136.406% ± 4.7) at the same dose. The average phagocytic activity has a range of (136.406% - 411.381) with the highest concentration of the extracts that has been used.

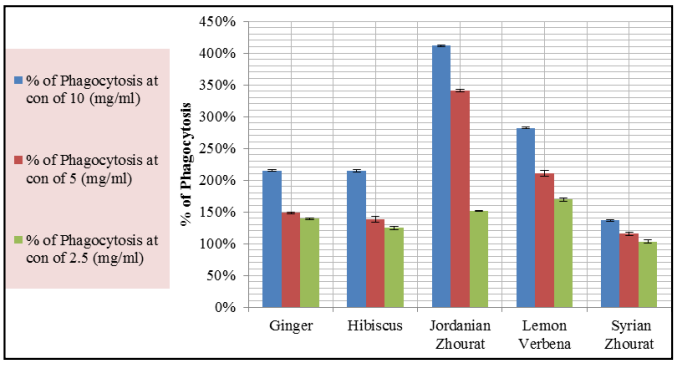


**Fig 13:** *In vitro* phagocytic assay on nitro blue tetrazolium (NBT reduction test of peritoneal macrophage treated with various concentrations of aqueous extracts of analyzed plants on for 48 h. Values shown are mean ±SD

## 4. Discussion

In this study the anticancer and immune modulation activity of selected herbal teas were evaluated and they were tested for various phytochemical constituents that giving them a great value in improving the health status of the consumers. The aqueous extracts were used to target different cell lines (MCF-7, T47D and Vero). These extracts inhibited the growth of cancer cells (MCF-7 and T47D) *in vitro* in dose dependent manner. Ginger and lemon verbena aqueous extracts were the most effective; that caused the highest response against T47D and MCF-7, whereas Jordanian zhourat, hibiscus and Syrian zhourat extracts were effective only against T47D cell line. MCF-7 was the least sensitive to these extracts. Ginger and lemon verbena target cancer cells through the induction of apoptosis and suppression of breast cancer angiogenesis. An increase in Th1 cytokines (IFN- γ, IL-2) level and decrease inTh2 cytokine (IL-4) level were evident after lymphocytes stimulation by herbal drinks. Jordanian zhourat is a potent stimulator of innate and acquired immunity.

The high antiproliferative activity of ginger is consistent with those from previous studies that reported similar behavior of ginger [6]-gingerol against MDA-MB-231 human breast cancer cell lines, which inhibits cell adhesion, invasion and motility [10]. Cancer preventive properties of ginger is also explained by the presence of flavonoid and polyphenolic components especially quercetin [11]. This agrees with our preliminary test which gave positive result for the presence of flavonoid in ginger extract. Hibiscus extract has antiproliferative activity against T47D cell line and was less effective against MCF-7 cell line. The effectiveness of Hibiscus extract has been shown previously, it was able to possess anti-proliferation on HeLa, Caov-3, MCF-7 and MDA-MB-231 cells [12]. Dried flower of *Hibiscus sabdariffa* extract has shown high levels of polyphenol, flavonoid, and anthocyanin which was observed to be associated with anticancer effects [13]. These data are consistent with phytochemical tests where Hibiscus extract gave positive result for the presence of flavonoid. Lemon verbena aqueous extract was the most potent against the two breast cancer cell lines. Its extracts gave positive result for all preliminary phytochemical tests which reflect the presence of diverse phytochemical groups of this plant. Lemon verbena is rich in Citral a terpenoid that is supposed as a new inducer of caspase-3 in tumor cell lines [14] and luteolin a flavone bioflavonoid that has anticancer properties and inhibits tumor

angiogenesis [15]. Our research found that Jordanian zhourat extract had the best antiproliferative effect against T47D cell line but was least effective against MCF-7 cell line. Its extract gave positive results for all preliminary phytochemical tests. This was expected as this herbal drink consists of a combination of medicinal plants. Previous studies showed divers activities of plant components of this herbal drink. For example, *Salvia officinalis* hydroalcoholic extract decreased the tumor cell viability [16]. Furthermore, many diterpenes, isolated from the genus of Salvia have demonstrated antitumor activity [17]. Review of literatures indicated that cinnamomum showed various cytotoxic activities in different cancer cell lines, namely basal cell carcinoma, human epithelioid cervix carcinoma (HeLa) human cancer promyelocytic leukemia (HL-60) human colorectal carcinoma (HCT 116, HT 29, and SW 480). *Cinnamomum malabatrum* stem bark both alcoholic and aqueous extracts possess a protective effect against Dalton's Ascitic lymphoma induced cancer in mice. Such activity is due to the presence of flavonoids, fixed oil, amino acids, tannins and phytosterols [18]. Cardamom can inhibit the viability and proliferation of MM (Multiple Myeloma). A recent study showed that indole-3-carbinol and diindolylmethane of cardamom have anti-cancer effects against breast, ovarian and prostate cancers [19]. Phytochemical screening showed that cardamom aqueous extracts have medium levels of antioxidant phenolics and flavonoids [20]. Our research found that Syrian zhourat extract was the least effective against T47D cell line but had better effect than Jordanian zhourat and hibiscus against MCF-7 cell line. Its extracts gave positive result for all preliminary phytochemical tests. The antiproliferative effect of this herbal drink is due to the presence of active extracts in its composition. Chamomile extracts induced apoptosis and cause reductions in cell viability in many human cancer cell lines including: T-47D (breast carcinoma) HeLa (cervical adenocarcinoma) HT 1080 (fibrosarcoma) [21]. The presence of apigenin and several naturally occurring glycosides like apigenin-7-*O*-glucoside in chamomile aqueous extracts gave significant reduction in the viability of various human cancer cell lines [22]. Green tea is rich in polyphenols that increase the inhibitory effect of tamoxifen on the proliferation MCF-7, ZR75 and T47D cell lines *in vitro* [23].

In order to gain a comprehension understanding of action of our extracts, the most potent extracts (ginger and lemon verbena) were further tested for their abilities to inhibit angiogenesis. Vascular permeability factor (VPF) or VEGF is a protein that stimulates angiogenesis. VEGF up-regulation is well known mechanism in many types of tumor and blocking or inhibition of this pathway is a target in cancer therapy. In our study, Ginger showed high ability to suppress breast cancer angiogenesis. This result agrees with a previous study that showed 6-Gingerol has high degree of VEGF- and bFGF inhibition and caused cell cycle arrest in the G1 phase [24]. Ginger 6-shogaol modulates secretion of angiogenic factors in ovarian cancer cells [25]. Lemon verbena herbal extract exhibited high ability to suppress breast cancer angiogenesis by inhibiting VEGF expression but less than ginger. Previous studies showed that lemon verbena extracts were able to slow the degradation of the extracellular matrix which is essential features in the pathogenesis of a number of chronic diseases. The degradation of glycosaminoglycanes which are components of the extracellular matrix is of high importance for the induction of angiogenesis [26]. The main class of compounds of lemon verbena aqueous preparations were phenylpropanoids, verbascoside, verbenalin together with flavonoids, mono- and di-glucuronidic derivatives of luteolin and apigenin [27].

Another mechanism responsible for the observed anticancer activity is induction of apoptosis, which is defined as the process of programmed cell death, it is highly organized and controlled process and help the body in maintaining its homeostasis [28]. In cancer this process is inoperative due to the up-regulation of anti-apoptotic genes and down-regulation of apoptotic genes, thus cells will continue in division and proliferation [29]. One of the targeted pathways in cancer treatment is the activation of apoptosis. In our study, ginger has an ability to induce apoptosis inT47D cell line, this result agree with a previous study that revealed the activity of 6-Shogaol to induces apoptosis in human hepatoma cells [30]. Lemon verbena also has an ability to induce apoptosis in T47D cell line, a previous study showed that citral the active constituents of lemon verbena has the ability to inhibit MCF-7 cell growth with a cycle arrest in G2/M phase and apoptosis induction [31]. Other studies showed that citral induces apoptosis in hematopoietic cancer cells [32, 33]. Induction of apoptosis by ginger and lemon verbena aqueous extract is consistent with positive results of phytochemical test regarding to flavonoid and terpenoide.

The immune system is a sophisticated defense system in vertebrates, having the role to protect them from numerous types of infectious agents during their lifetime. It uses a variety of cells, tissues and organs and is capable of recognizing and eliminating invading pathogens [34]. The immune system can be modulated and this involves induction or inhibition of any part or phase of the immune response [35]. In the present study, changes in immune system due to the exposure to aqueous herbal extracts of analyzed plants were also explored through measuring levels of IFN-γ, IL-4, IL-2 and IL-10. The results indicate that there is significant up-regulation in IFN- γ and IL-2 in lymphocytes treated with herbal extracts. The increase in the production of IFN-γ and IL-2 which is signature cytokines in Th1 antitumor immune response and decrease in IL-4 which is a key cytokine in Th2 was reported. Moreover they decrease levels of IL-10. Our result showed the capacity of these herbal drinks to shift the immune response toward Th1 response which is the response that stimulates anticancer immune mechanisms.

IFN- γ concentration was the highest in the supernatants of splenocytes that were cultured with lemon verbena extract followed by ginger extract, whereas hibiscus, Syrian zhourat and Jordanian zhourat induced similar increasing in IFN- γ concentration. Lemon verbena extract, ginger and Syrian zhourat increased IL-2 concentration by the same range followed by Jordanian zhourat. The lowest increase was in the extract of hibiscus. All of the extracts considerably down-regulated IL- 4 secretion, the lowest concentration of IL-4 was in the culture supernatant incubated with Jordanian zhourat extract. Lemon verbena extract and Syrian zhourat decreased IL-4 concentration by the same range, followed by ginger extract, whereas the lowest decrease was in the extract of hibiscus. IL-10 concentration has significantly decreased similarly by all the extracts. These results agree with reports which showed that Zerumbone a sesquiterpene compound of ginger reduced the production of keratinocyte-derived chemokine interleukin IL-4, IL-5, IL-10, and IL-13, and promoted Th1 cytokine interferon (IFN)-γ production [36]. The presence of terpenoid in phytochemical test of ginger may further support our results and it seems that the immunomodulatory effect of ginger is due to the presence of several phytochemicals including terpenoids like Zerumbone.

On the other hand, our study disagree with previous study showed that both the aqueous and alcoholic extracts of *Hibiscus sabdariffa* calyx possess a profound increase in production of IL-10 and a great reduction in production of tissue necrosis factor - alpha [37]. Although our study found that IFN- γ concentration was the highest in the supernatants of splenocytes that were cultured with lemon verbena, also this extract increased IL-2 concentration by the same range as ginger and Syrian zhourat but this finding disagreed with a previous study that showed the *Lippia citriadora* (lemon verbena) extracts have been proved to be effective in the prevention of inflammatory diseases and affect cytokine profiles in multiple sclerosis. IFN-γ levels decreased, whereas IL-10 increased in secondary multiple sclerosis patients [38]. Infusion of lemon verbena contains polyphenols, mainly verbascoside and flavone glycosides such as luteolin-7-diglucuronide, which has a high antioxidant activity. Verbascoside decreased proinflammatory cytokine levels [39]. The difference in our results compared with previous studies could be due to the difference in plant samples used. Plant growing in different regions may have different concentrations of specific phytochemicals. Our research found that Jordanian zhourat, hibiscus and Syrian zhourat induced similar increasing for IFN- γ concentration and also increased IL-2 concentration. Jordanian zhourat extract, lemon verbena and Syrian zhourat decreased IL-4 concentration by the same range while IL-10 concentration has significantly decreased similarly by all the extracts. Our results agree with previous findings regarding the immunomodulatory effects of some plants used to prepare these herbal drinks. Water extract of *Salvia officinalis* can inhibit IL-4 production and suppress IgE production from B cells [40]. This attributed to their triterpene [41] or flavonoid contents [42]. Oral administration of water extract of cinnamon bark decreased systemic levels of IFN-γ *in vitro* [43]. Oral administration of cinnamon extract also inhibited development of intestinal colitis by inhibiting expression of COX-2 and (IL-1β, IFN-γ and TNF-α) while enhancing IL-10 levels [44]. Cinnamon bark contains benzaldehyde, cuminaldehyde, cinnamaldehyde, carbohydrates, tannin and terpenes [45]. Cinnamaldehyde is reported to inhibit lymphocyte proliferation [46]. Cardamom aqueous extracts have medium levels of antioxidant phenolics and flavonoids; significantly suppress T helper (Th) 1 cytokine release by splenocytes. While Th2 cytokine is significantly enhanced [47]. Syrian zhourat induced IFN- γ in concentration similar to hibiscus and Jordanian zhourat but less than lemon verbena and ginger, while induced IL-2 secretion similar to lemon verbena and ginger extract, and greater than ginger and Jordanian zhourat. Syrian zhourat decreased IL-4 similar to lemon verbena but greater than hibiscus and ginger while less than Jordanian zhourat. IL-10 concentration has significantly decreased similarly by all the extracts. Herbal teas prepared from chamomile flowers mainly contain flavonoids and their glycosides [48]. A previous study showed that German chamomile down regulates of IL-4 production from Th2 cells and following by suppression of IgE [49]. Chamomile flavone (apigenin) exhibits anti-inflammatory activity via inhibition of proinflammatory cytokines like interleukin IL 1β, 6 and TNF-α [50]. Polyphenolic compounds from green tea (PGT) possess antiarthritic activity via suppression both of the proinflammatory cytokine IL-17 combined with an increase in the anti-inflammatory cytokine IL-10 [51]. Another study also showed that tea extracts decreased production of the T lymphocyte growth factor IL-2 by leukemic cells while increasing its production by normal cells [52]. A previous study showed that aqueous extracts of the of marshmallow (*Althaea officinal*) displaying anti- inflammatory activity by induction the release of interleukin-6 and tumor necrosis factor *in vitro* [53]. A water-soluble polysaccharide of petals *rosa damascena* induce intestinal immune system activity through Peyer's patch cells and IL-6 producing activity from macrophages [54]. So as mentioned above some components of Syrian zhourat down regulated IL-4, IL-17 others induced the release of IL-10 and IL-6.

Phagocytic cells are key components of the innate immune response like neutrophil, monocytes and macrophages. However, Macrophages play a most important role in phagocytosis which is responsible for the intracellular killing of antigen and other apoptotic cells. Therefore, the modulation of macrophage activity seems to be chief role in regulation of innate immunity [55]. Our study on herbal drinks showed that they enhance the phagocytic activity. Extract of Jordanian zhourat showed the highest stimulation of peritoneal phagocytic activity *in vitro* culture followed by lemon verbena extract. Similar results have been reported with extracts from ginger and hibiscus, whereas the lowest phagocytic activity was in extract of Syrian zhourat of at the same dose. Such results are consistent with previous findings reporting that Polyphenol compounds (6-gingerol and its derivatives) of *Zingiber officinale* extracts increased proliferation of macrophages and enhanced phagocytic activities [55]. Water-soluble polysaccharide from *Hibiscus sabdariffa*, HSP41 up-regulated the immune response by stimulating RAW264.7 (macrophage cell line) cell activity [57]. Extract of Jordanian zhourat was the highest stimulator of peritoneal phagocytic activity *in vitro* culture. Previous findings reporting that water-soluble polysaccharides of *Salvia officinalis* stimulate the macrophage phagocytosis [58]. Sage aqueous extracts make augmentations of humoral and cellular immune responses involve mainly four immune cells (neurophils, macrophages and T- and B-lymphocytes). Chemical analysis of sage aqueous extract revealed some of these constituents' steroids, tanins, glycosides, flavonoids, saponines and terpens [59]. Cardamom aqueous extract has medium levels of antioxidant phenolic and flavonoids and is capable of promoting proliferation in macrophages *in vitro* [60]. Cinnamon at low dose shows effect only on humoral immunity and at high dose increases both cell-mediated and humoral immunity [61]. Cinnamon bark contains benzaldehyde, cinnamaldehyde, cuminaldehyde and terpenes [45]. Cinnamaldehyde is reported to inhibit lymphocyte proliferation [46]; an immune system booster could be to the presence of terpenes. All of contents of Jordanian zhourat had stimulated phagocytic activity *in vitro* culture due to the presence of various phytochemicals as mentioned above this explains why Jordanian zhourat given the highest stimulation of peritoneal phagocytic activity, it gave a positive result for all phytochemicals that were tested in our experiment. The lowest phagocytic activity was in extract of Syrian zhourat, the presence of polyphenols in the hydroalcoholic extract of chamomile could have an important implication in the activation of the immune cells such as macrophages also strengthen the ability to react to external agents. Chamomile extract induces sensibility in phagocytosis to bacteria in mouse macrophages [62]. Herbal teas prepared from chamomile flowers mainly contain flavonoids and their glycosides [48]. Aqueous extracts of *Althaea officinalis* stimulated phagocytosis and the release of oxygen radicals from human neutrophils *in vitro*. Intraperitoneal

administration of marshmallow polysaccharides to mice produced an increase in phagocytic activity of macrophages [63]. Administration of epigallocatechin gallate polyphenol of green tea extract (EGTE) to mice increased peritoneal cell phagocytosis [64]. Presence of carbohydrates, flavonoids, and glycosides by phytochemical tests on the Syrian zhourat may confirm the phagocytic activity of this extract.

Our research showed that the degree of stimulation of splenocytes by aqueous extracts of the analyzed plants increased in a dose-dependent manner. At dose of 80 mg/mL, the extract of Jordanian zhourat showed the highest stimulation percentage of cell viability in *vitro* culture followed by lemon verbena, Syrian zhourat and hibiscus whereas the lowest splenic lymphocytes proliferation was in extract of ginger at the same dose. *Zingiber officinale* aqueous extract improves the symptoms of radiation sickness and mortality induced by the gamma-irradiation in mice; it enhanced the cellular immune response by higher macrophage, splenocyte survival and splenocyte proliferation due to the presence of polyphenol compounds [65]. Thujone a naturally occurring monoterpene of *Salvia officinalis* enhance splenocytes proliferation in Balb/C mice [66]. Moreover, cardamom aqueous extracts were verified *in vivo* that significantly enhanced splenocyte proliferation [67]. Cinnamon at high dose increases both cell-mediated and humoral immunity and at low dose shows effect only on humoral immunity [61]. An immune system booster could be to the presence of terpenes in aqueous extract since cinnamaldehyde is reported to inhibit lymphocyte proliferation [46]. All of combination of plant s in Jordanian zhourat that stimulated splenic lymphocyte, this explain why the extract of Jordanian zhourat caused the highest stimulation percentage of cell viability. Syrian zhourat stimulated splenic lymphocyte at percentage nearly similar to lemon verbena and hibiscus. *Matricaria chamomilla* extract has immunomodulatory properties on lymphocyte proliferation [68]. Herbal teas prepared from chamomile flowers mainly contain flavonoids and their glycosides [48]. Moreover; administration of epigallocatechingallate (EGTE) of green tea extract to mice increased natural killer cell splenocyte proliferation [64]. Scopoletin coumarin is a potential anti-tumoral of *Althaea officinalis* induced proliferation of normal T lymphocytes [69]. Three of components of Syrian zhourat, chamomile, green tea and marshmallow officinal stimulated splenic lymphocyte while there was no a previous study on *Rosa damascene***.**

## 5. Conclusion

In conclusion, the growth of breast cancer cell lines (MCF-7, and T47D) was inhibited by the extracts in dose dependent manner; five extracts were most effective toward T47D cell lines rather than MCF-cell lines. Ginger and lemon verbena was most potent against both cell lines. Ginger and lemon verbena augment the anticancer activity through the induction of apoptosis and suppression of breast cancer angiogenesis. Jordanian zhourat is a potent stimulator of innate and acquired immunity. People should encourage consuming such herbal drinks since they have wide benefits, available and relatively safe. However, further *in vivo* studies are required to explore their anti-tumor effect and more testing is needed to identify the exact composition of each herbal drink.

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