# Correlation of Anticancer Effects of 12 Iranian Herbs on Human breast Adenocarcinoma cells with antioxidant Properties

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## ABSTRACT

**Introduction:** Many of Medicinal plants have been investigated for possible anticancer properties. The correlation cytotoxic effect of herbs with antioxidant activity is unclear, yet. The aim of the present study was to examine the antioxidant contents and anticancer effects of 12 Iranian medicinal plants on proliferation of breast cancer, MCF-7, and epithelial normal, MCf-10A, cells. **Methods:** Free radical scavenging and total antioxidant capacities of herbal waters assessed by DPPH and FRAP methods, respectively. Their total phenolic contents and cell viability determined by Folin–Ciocalteu and MTT assays, respectively. **Results:** Among the herbs, group A which contained relatively highest total phenolics content, antioxidant and free radical scavenging activities inhibited significantly greater growth of breast cancer cells in a dose- and time-dependent manner as compared to other groups. These medicinal plants were not cytotoxic components against normal cells. **Conclusion**: Therefore our results indicated that anti proliferative effects of 12 selected Iranian herbs showed a direct correlation with antioxidant properties. The herbs as natural antioxidants with fewer side effects could potentially improve the outcome of human breast cancer therapy. These findings endorse further investigations on these plants to determine their active metabolites and mode of action.

**Key words:** Anticancer, Antioxidant, MCF-7 cells, MCF-10A cells, Herbs.

**Key Messages:** In this study *in vitro* antioxidant and anticancer activities of 12 Iranian herbs evaluated. Our data indicated that herbs with high antioxidant and phenolics contents and DPPH radical scavenging effect have more anti proliferative effects against human breast cancer cells compared to other plants.

## INTRODUCTION

Nowadays, many patients are faced with different types of cancers for instance breast cancer which is the most frequently malignancy among women worldwide.[1] Cancer is a complex disease characterized by an uncontrolled growth of cells in the body.[2] The oxidative stress promote or develop cancer through damage to DNA, genes mutations, alteration in cell signal transduction pathways and leading to severe cellular/tissue damages.[2,3] Many of epidemiological studies indicated that peoples with a low consumption of antioxidant substances are more susceptible to cancer.[4]

Numerous medicinal plants have been used for many pharmacological and clinical applications including antioxidative, anticarcinogenic, antiatherosclerosis, antimutagenic and antiangiogenesis.[5-7] These herbs have a

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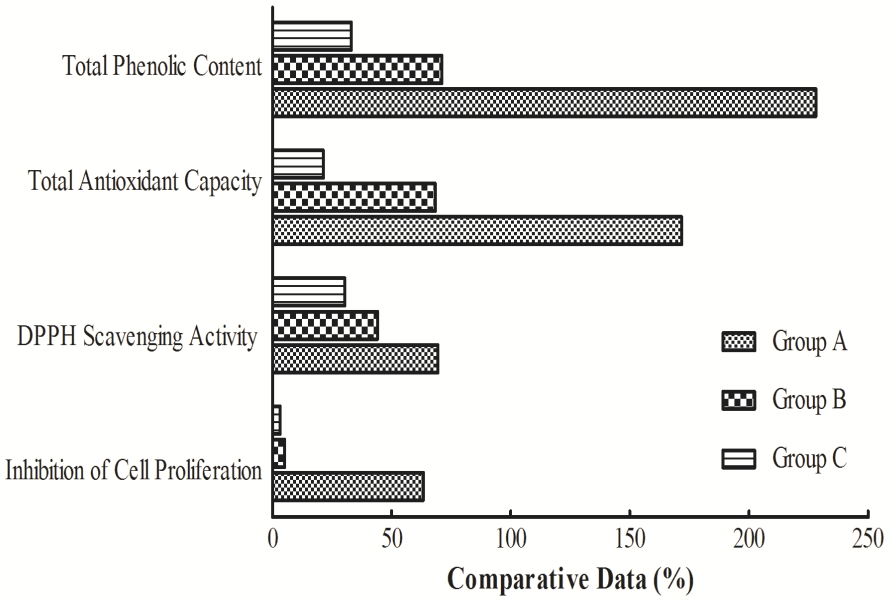


**Graphical Abstract**

wide variety of antioxidants such as phenolics and nitrogen compounds, vitamins, terpenoids.[8,9] Phenolics components are very noticeable antioxidant agents because they act as free radical-scavenger, oxygen radical absorbent and metal ions chelator.[10] In addition, they can interfere with the enzymes called the cytochrome P450 mixed–function oxidases which contribute to reactive oxygen species producing in the body.[11] The herbs with natural antioxidants including phenolics reduce cellular damages of oxidative stress and commonly used for cancer treatment and prevention.[12,13]

In this study, among 50 herbs which received herbarium codes by Mohsen Pouyan in Herbarium of Agriculture Faculty, Birjand University, Iran we selected 12 medicinal plants with various phenolics and antioxidant contents then investigated relationship between their cytotoxic effects on proliferation of breast cancer cells and antioxidant capacity and radical scavenging activity under the same experimental conditions (Table 1).

## OBJECTIVES OF RESEARCH

Cancer is a major public health worldwide. Botanicals have long been used traditionally in treatment of various types of cancers and often less associated with the side effects like the modern chemotherapy has. Recently medicinal plants constitute a main alternative for cancer therapy in

**Table 1: The names, parts used and herbarium codes of 12 selected Iranian medicinal plants in this study**

| Scientific Name | Family | Parts used | Herbarium code |
|---|---|---|---|
| ***Rosmarinus officinalis*** | Lamiacea | Leaves & flowers | 28 |
| ***Ephedra sarcocarpa*** | Ephedraceae | Branches & leaves | 493 |
| ***Hymenocrater platystegius*** | Lamiaceae | Leaves & flowers | 105 |
| ***Nepeta bracteata*** | Lamiaceae | Flowering shoot | 163 |
| ***Tribulus terrestris*** | Zygophylaceae | Leaves & seeds | 682 |
| ***Avena fatua*** | Poaceae | Seeds | 477 |
| ***Cichorium intybus*** | Asteraceae | Leaves & roots | 2291 |
| ***Ziziphora tenuir*** | Lamiaceae | Flowers & leaves | 657 |
| ***Solanum nigrum*** | Solanaceae | Fruits & Leaves | 574 |
| ***Foeniculum vulgare*** | Apiaceae | Fruits | - |
| ***Achillea wilhelmsii*** | Asteraceae | Flowers | 327 |
| ***Cardaria drabal*** | Brassicaceae | Flowering shoot | 64 |

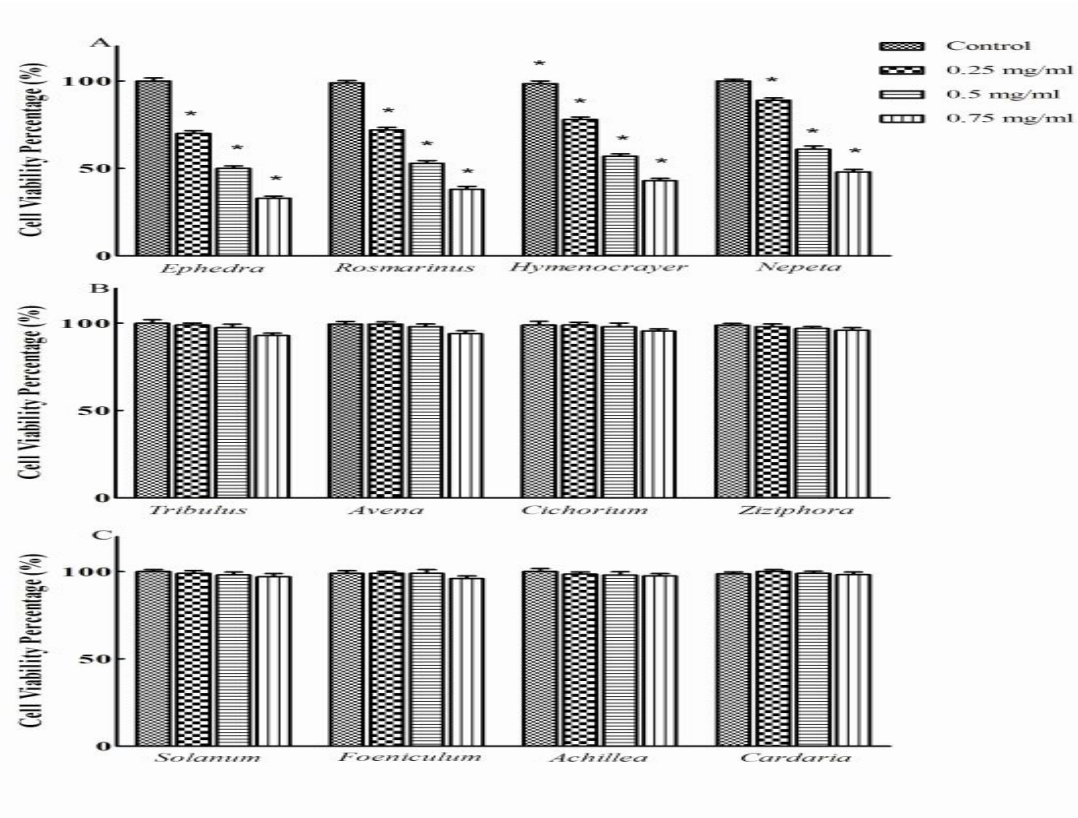


**Figure 1: Cytotoxicity of various concentrations of herbal extracts (0-0.75 mg/ml) against MCF-7 cells after 72 h incubation. All tested were taken in triplicate and results are reported as the mean ± SEM. *P<0.05, compared with untreated cells**

Iran. Although, there is an important local ethno botanical biography describing the most frequently used plants in treatment of various cancers such as breast however, very few have been studied scientifically for action mechanisms of the herbs to identify the active components. The vast majority is still unexplored phytochemically and their anticancer properties have not yet validated. To realize if the cytotoxic activities correlated with phytochemical contents of the plant extracts we selected about 12 Iranian medicinal plants and screened their extracts for antioxidant and anticancer activities. Herbs with highest antioxidant contents like polyphenolics recognized as the potential therapeutic agents targeting breast cancer. It is strongly suggested to carry out more cellular and molecular studies to clear mechanisms in anticancer actions of these local herbs.

## MATERIALS AND METHODS

To investigate the relationship between cytotoxic and antioxidant properties of several herbs we selected 12 herbs from South Khorasan, Iran among 50 common plants according to their antioxidant activities. Then the *in vitro* antiproliferative effects of herbs against MCF-7 and MCF-10A cells evaluated by MTT method.

- *In vitro cytotoxicity assay*
- *Cell culture*

Human breast adenocarcinoma (MCF-7) and human normal breast epithelial (MCF10A) cell lines was provided from Iranian Biological Resource Center (IBRC) (Tehran, Iran). The cells were cultured in Dulbecco's Modified Eagle's (DMEM): Ham's F-12 medium containing 10% heat in activated fetal bovine serum, 100 units/ml penicillin and 100 mg/ml streptomycin. We should use20 μg/ml EGF (Epithelial growth factor) in culture medium for MCF10-A cells. Cells were grown in a humidified atmosphere in 5% $CO_2$ at 37°C. Then, cells were plated and treated with various concentrations of herbal extracts (0-3 mg/ml) for different times (0-72h).[14]

### Cell viability (MIT assay)

The methyl thiazolyldiphenyl-tetrazolium bromide (MTT) assay was applied to evaluation cytotoxic effect of herbal extracts on breast cancer cells. The cells were seeded in 96-well plates (20×103 cells/well). The synchronized cells were treated with various concentrations of herbal extracts (0-3 mg/ml) for different time intervals (0-72 h). After aspirating the medium, MTT solution (5 mg/ml) was added to each well and was incubated for 4 h. Then, formazan precipitate

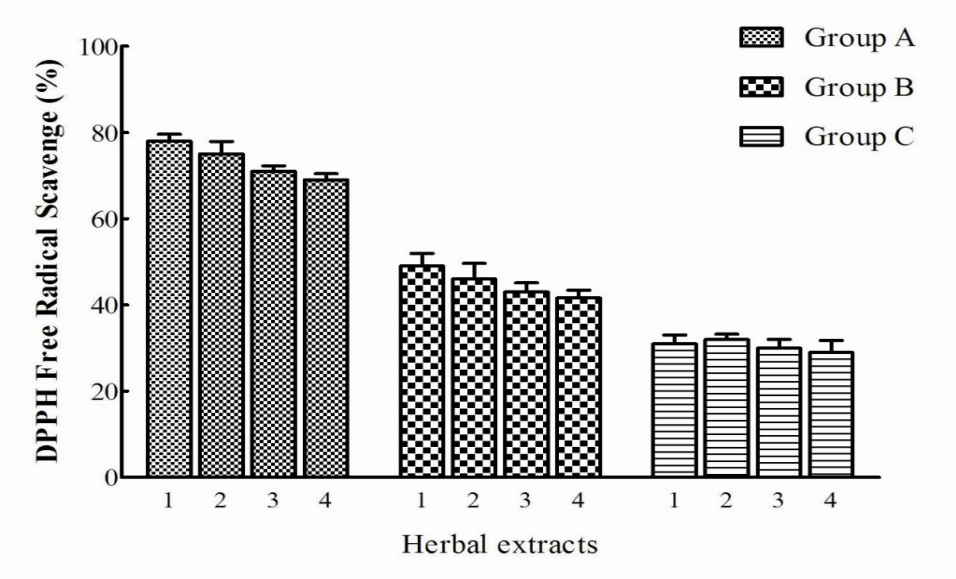


**Figure 2: Percentage of DPPH radical quenching activity of similar concentrations of herbal extracts (0.5 g/l). Data are presented as means ± SEM (n = 3), and histograms marked with * are significantly different at P<0.05. Group A) 1.*Rosmarinus officinalis* 2.*Ephedra sarcocarpa* 3.*Hymenocrater platystegius* 4.*Nepeta bracteata*. Group B) 1.*Tribulus terrestris* 2.*Avena fatus* 3.*Cichorium intybus* 4. *Ziziphora tenuir*. Group C) 1.*Solanum nigrum* 2.*Foeniculum vulgare* 3.*Achillea wilhelmsii* 4.*Cardaria drabal***

was solubilized with 200 µl of dimethyl sulfoxide (DMSO). The absorbance was recorded at 540 nm by an ELISA plate reader (Awareness).[15] For analysis of the cytotoxic efficiency, the $IC_{50}$ values (drug concentration that reduced the absorbance of treated cells by 50% compared to untreated cells) of all of herbal extracts were calculated using the dose- and time-dependent curves by linear interpolation. In addition, the cell viability was calculated by the formula:

*Viability (%) = (OD treated / OD untreated) * 100*

### Selection of herbs and preparation of their extract

We selected 12 herbs from South Khorasan, Iran between 50 common plants based on theirantioxidant activities. According to our *in vitro* antioxidant results, we sorted herbs intothree differentgroups including: A. high antioxidant (*1. Rosmarinus officinalis, 2. Ephedra sarcocarpa, 3. Hymenocrater platystegius*and *4. Nepeta bracteata*), B. medium antioxidant (*1. Tribulus terrestris, 2. Avena fatus, 3. Cichorium intybus*and *4. Ziziphoratenuir*) and C. low antioxidant agents (*1. Solanumnigrum, 2. Foeniculum vulgare, 3. Achillea wilhelmsii* and *4. Cardaria drabal*).

To prepare aqueous extract, the plants were washed and dried at 50°C.Then; the samples were grounded into powder by grinder. Five grams of each plant was mixed with 100 ml of boiling water and infused for 30 min. The obtained solutions of each herb were centrifuged at 7,000 *g* for 30 min. The supernatants were filtered using Whatman No.1. Eventually, the samples lyophilized by firstfreezing at−80°C for 2 h.

### *In vitro* antioxidant assays

#### Free radicals-scavenging ability (DPPH assay)

The free radical-scavenging ability of plant extracts were measured using the DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) method.[16] The percentage scavenging of herbal extracts as its antioxidant activity was calculated.

### FERRIC REDUCING ANTIOXDANT POWER (FRAP ASSAY)

FRAP assay was used to determine the antioxidant capacity of plant extracts.[17] Our results expressed as mol Fe (II)/g dry weight of plant extract.

#### *Total phenolic contents (Folin-Ciocalteu assay)*

The Folin-Ciocalteu method was used to determine the total phenolic content.[18] The results expressed as Gallic acid equivalents (GAE)/g dry weight of plant extract.

### Statistical Analysis

All experiments were accomplished in triplicate and the data expressed as mean ± standard error of the mean (SEM).

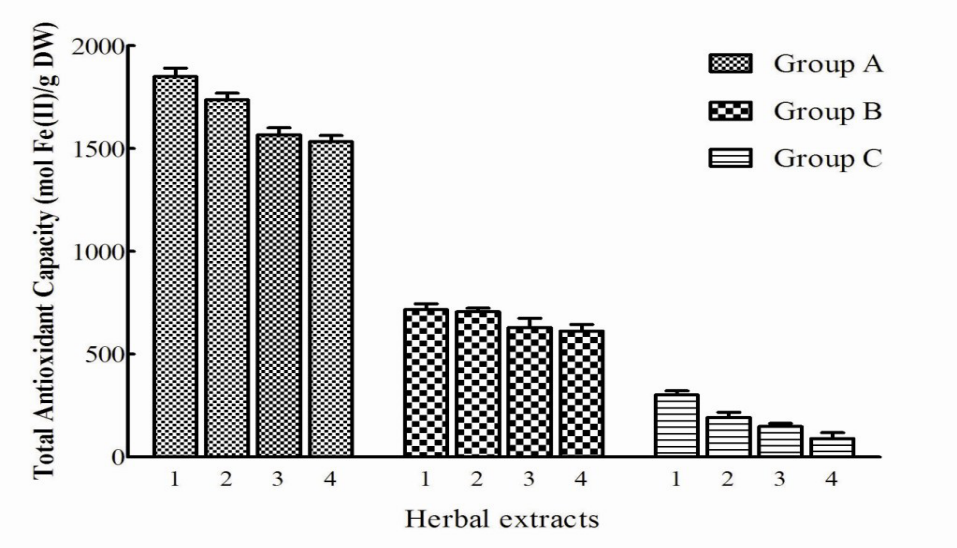


**Figure 3: FRAP (Ferric Reducing Antioxidant Power) activity or total antioxidant capacity (TAC) of selected herbs that expressed as mol Fe (II)/g dry weight (DW). Data are presented as means ± SEM (n=3), and histograms marked with * are significantly different at P<0.05. Group A) 1. *Rosmarinus officinalis* 2. *Ephedra sarcocarpa* 3. *Hymenocrater platystegius* 4. Nepeta bracteata. Group B) 1. Tribulus terrestris 2. Avena fatus 3. Cichorium intybus 4. Ziziphora tenuir. Group C) 1. *Solanum nigrum* 2. *Foeniculum vulgare* 3. *Achillea wilhelmsii* 4. *Cardaria drabal***

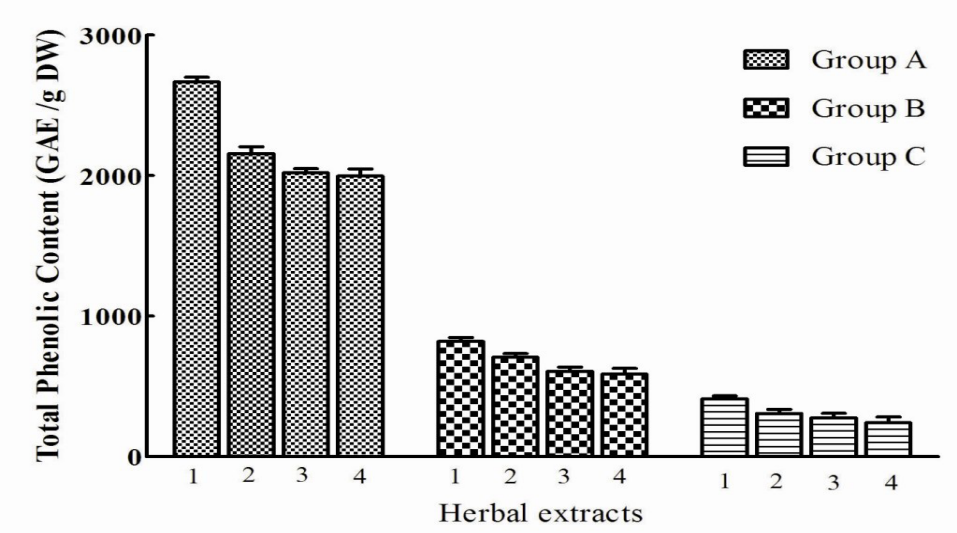


**Figure 4: The total phenolics content of selected herbs that expressed Total phenolic content expressed as Gallic acid equivalents (GAE)/g dry weight (DW). Data are shown as means ± SEM (n = 3), and histograms marked with * are significantly different at P<0.05. Group A) 1. *Rosmarinus officinalis* 2. *Ephedra sarcocarpa* 3. *Hymenocrater platystegius* 4. *Nepeta bracteata.* Group B) 1. *Tribulus terrestris* 2. *Avena fatus* 3. *Cichorium intybus* 4. *Ziziphora tenuir.* Group C) 1. *Solanum nigrum* 2. *Foeniculum vulgare* 3. *Achillea wilhelmsii* 4. *Cardaria drabal***

Results were analyzed using one-way ANOVA followed by Tukey's post hoc test using SPSS version 16 software. Differences at $p<0.05$ were considered to be significant.

## RESULTS

### *Effect of various herbs on cells viability of MCF-7 and MCF10-A cells*

To evaluate the effect of 12 herbal aqueous extracts on cell proliferation, we investigated the cytotoxic effects of their various concentrations (0-3 mg/ml) on the growth of human breast cancer (MCF-7) and normal mammalian (MCF10-A) cells after different time incubation (0-72 h) MTT assay (Bathaie *et al.,* 2013). Our results indicated that the treatment of MCF-7cells with the plant extracts of group A (0.25, 0.5 and 0.75 mg/ml) significantly decreased the cell viability and increased cell death percentage by increasing of extract concentration after 72 h. Two other groups of

herbal extracts have shown low toxicity on growth and proliferation of cancer cells compared to group A (Figure 1). Parallel treatments of the normal cells with these extracts indicted a much less inhibitory effect on the viability of MCF10-A cells (data not shown). The mean of $IC_{50}$ values of four herbs at group A were 2.5, 1.2 and 0.5 mg/ml for MCF-7 cells at 24, 48 and 72 h, respectively. Therefore, cell viability in the breast cancer cells was markedly decreased after exposure to group A in a dose- and time- dependent manner.

## ANTIOXIDANT ACTIVITY

### DPPH scavenging

We have used the DPPH assay to determine free radical scavenging ability of herbal extracts. The results (Figure 2) indicated that the group A showed high antioxidant activity (65%), demonstrating they have good potential as free radical scavenger. Although the group B illustrated medium activity of free radicals scavenging (40-45%) the group C represented low DPPH activity (27-32%) in comparison with the other groups.

### Total antioxidant capacity

We used FRAP test to determine the antioxidant contents of aqueous herbal extracts. The results of FRAP assay showed that *Rosmarinus, Ephedra, Hymenocrater* and *Nepeta* belonging to group A have highest level of total antioxidant activity (between 1566.6 to 1850 mol Fe(II)/ g DW) compared to other groups (Figure 3). *Tribulus, Avena, Cichorium* and *Ziziphora* exerted the medium of total antioxidant capacity (630 to 716.6 mol Fe(II)/ g DW) and the herbs of group C showed the lowest level of total antioxidant activity (1438.3 to 301.6 mol Fe(II)/ g DW).

### Total phenolics content

The Folin-Ciocalteu assay used to determine the total phenolic content of aqueous herbal extracts. As shown in Figure 4, group A has the highest level of phenolics, while group C has the lowest level. Group B showed medium phenolic content compared with other groups.

## DISCUSSION

Breast Cancer is a one of main leading causes of death among women worldwide.[19] Current therapies for breast cancer consist of surgery, radiation therapy and chemo-hormone therapy. However, these therapies are often ineffective. Therefore, the design of more effective drugs with fewer side effects or complementary treatment to enhance the quality of life of these patients is essential. In the last two decades, many studies have been illustrated various anti-proliferative properties of Iranian herbs; however, no collective study on their comparative anticancer and antioxidant properties has been reported.[20]

The reactive oxygen species (ROS) produced by internal and external sources in the body may be causing various diseases such as cancer. Thus, reduced ROS by natural antioxidants for instance herbal extracts should improve the disorders and their side effects. Different medicinal plants which have anticancer property scavenge free radicals[21] and decrease the amount of ROS in the body. On the other hand some antioxidants have pro-oxidant activity. Flavonoids and other polyphenolic compounds have powerful antioxidant effects *in vitro* in many test systems, but can act as pro-oxidant in some others.[22] Studies suggested that at higher doses or under certain conditions antioxidant-type functional ingredients such as carotenoids, and polyphenols like flavonoids may exert toxic prooxidant activities.[23] Therefore antioxidant-rich herbs (group A) can improve side effects of cancer while their high doses can be considered to pro-oxidant and cause to cell death.

The results (Figure 2) illustrated that the herbs in group A have good potential as free radical scavengers. Although the group B demonstrated medium activity of free radicals scavenging the group C represented low DPPH activity in comparison with the other groups. The free radical-scavenging of *Rosmarinus* has been studied by Erkan *et al* (54.0 ± 1.4 mM trolox/100 g DW)[24] and Wojdylo *et al* (513 ± 5.99 mM trolox/100 g DW).[25] The FRAP data indicated that group A has highest level of total antioxidant activity (between 1566.6 to 1850 mol Fe(II)/ g DW) compared to other groups (Figure 3). Group B exerted the medium of total antioxidant capacity and the herbs of group C showed the lowest 23 level of total antioxidant activity. Already the total antioxidant capacity of *Ephedra* (6.7 ± 0.16 mM/g DW) demonstrated with Rustaiyan *et al*.[23]

Medicinal plants have shown effective antioxidant properties because of their phyto-constituents such as phenolic and carotenoid compounds. The phenolics, sometimes called phenols, including flavonoids and phenolic acids.[27] The antioxidant mechanisms of phenolics include scavenging the free radicals to terminate the radical chain reaction, absorbance of oxygen radicals (ROS), chelating transition metals, interfering with the enzymes ROS producing and stimulating the anti-oxidative enzyme activities, thus they decreased the incidence of cancer.[10] In addition, they have been identified as anti-proliferative agents due to their ability to cell cycle arrest, induce

apoptosis, destruction mitotic spindle formation and inhibit angiogenesis.[28,29] On the other hand, the flavonoids effectively inhibited functional enzymes in inflammatory and cancer pathologies including 5-lipoxygenase, cyclo oxygenase, mono oxygenase and xanthine oxidase.[28,30] The flavonoids also modulated the several proteins that involved in cancer promotion for instance protein kinases, epidermal growth factor receptors, platelet derived growth factor receptors, vascular endothelial growth factor receptors and cyclin-dependent kinases.[28,31] As shown in Figure 4, group A has the highest level of phenolics, while group Chas the lowest level. Group B showed medium phenolic content compared with other groups. In addition, *Ephedra* and *Solanum* posed the highest (2666.6 ± 33.99 GAE/g DW) and lowest (277 ± 4.24 GAE/g DW) phenolic contents, respectively. Other researchers also use this assay to present phenolic content of herbs for instance Rustaiyan *et al*.[26] have previously reported phenolic content of *Ephedra* extract (709.18 ± 14.08 mg catechin/g). Wojdylo *et al*[25] have demonstrated total phenolics content of *Rosemarinu* is 171 ± 0.02 GAE/ g DW. An interesting point is that the relative levels of total phenolics and total antioxidant capacity showed relatively the same pattern. Several studies have reported on the strong relationship between the total phenolic content and antioxidant activity in vegetables, grains and fruits.[32] We found a higher correlation between total antioxidant activity and total phenolics.

As shown in Figure 1 the herbs of group that inhibit significantly proliferation of cancer cells contained the highest antioxidant capacity, especially phenolic contents, compared to other groups. Therefore, the antioxidant effects of herbs on cancer cell viability can be explained by direct scavenging ROS. The nontoxic effects of them on the growth of normal cells also indicated.

## CONCLUSION

In conclusion, among 12 selected herbs from South Khorasan, Iran, *Rosmarinus, Ephedra, Hymenocrater* and *Nepeta* (group A) that posses highest total antioxidant activity, total phenolic content and DPPH radical scavenging activity dramatically decreased the cancer cell viability while other plants were relatively low. The great interest is that morphological observation and the number of communicating normal cells treated with these herbs were very similar to those of the control cells (untreated with herbs). Finally, we can suggest group A herbs with great antioxidant properties may be anticancer agents against breast cancer in humans.

## RESEARCH HIGHLIGHTS

More cytotoxic effects on MCF-7 cells for herbs with high phenolics and antioxidant contents.

The direct correlation between free radical scavenging and anticancer activities of herbs.

No anti-proliferative property of herbs against normal MCF-10A cells.

## AUTHORS' CONTRIBUTION AND COMPETING INTERESTS

Hoshyar and Zarban designed the experiments. Partovfari carried out the antioxidant assays. Mostafavinia and Taheri performed the MTT method. Hoshyar participated in cytotoxic study on the plants. Hoshyar, Hassanpour and Pouyan analyzed the data and interpreted the results. Hoshyar and Mostafavinia drafted the manuscript. All the authors read and approved the final manuscript. The authors declare that they have no competing interests.

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## ABBREVIATION

MCF-7 : Michigan Cancer Foundation-7
MCF-10A : normal mammary epithelial cells
MTT : methyl thiazolyldiphenyl-tetrazolium bromide
DMSO : dimethyl sulfoxide
DPPH : 2,2-diphenyl-1-picryl-hydrazyl-hydrate
FRAP : ferric reducing antioxidant power
GAE : gallic acid equivalent
ROS : reactive oxygen species
IC50 : half maximal inhibitory concentration

## Highlights of the paper

- More cytotoxic effects on MCF-7 cells for herbs with high phenolics and antioxidant contents.
- The direct correlation between free radical scavenging and anticancer activities of herbs.
- No anti-proliferative property of herbs against normal MCF-10A cells.

## About Authors

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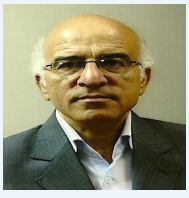

*Mr Mohsen Pouyan (born 1961, Iran) medicinal herbs scholar with more than 32 years of research on medicinal herbs of South Khorasan. He is an author of 35 books and 22 scientific papers as well as performing 31 research projects about medicinal herbs of Iran.*

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