RESEARCH ARTICLE

Antiproliferative Activity of *Marrubium persicum* Extract in the MCF-7 Human Breast Cancer Cell Line

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Abstract

Aim: Developing antitumor drugs from natural products is receiving increasing interest worldwide due to limitations and side effects of therapy strategies for the second leading cause of disease related mortality, cancer. Methods: The antiproliferative activity of a methanolic extract from the aerial parts of *Marrubium persicum* extract was assessed with the MCF-7 breast cancer cell line using the MTT test for cell viability and cytotoxicity indices. In addition, antioxidant properties of the extract were evaluated by measuring its ability to scavenge free DPPH radicals. Moreover, the total phenolic and flavonoid content of the extract was determined based on Folin-Ciocalteu and colorimetric aluminum chloride methods. Results: The findings of the study for the antiproliferative activity of the methanolic extract of *M. persicum* showed that growth of MCF-7 cells was inhibited by the extract in a dose and time dependent manner, where a gradual increase of cytotoxicity effect has been achieved setting out on 200 µg/mL concentration of the plant extract. The antioxidant assay revealed that the extract was a strong scavenger of DPPH radicals with an RC_{50} value of 52 µg/mL. The total phenolic and flavonoids content of the plant extract was 409.3 mg gallic acid equivalent and 168.9 mg quercetin equivalent per 100g of dry plant material. Conclusion: Overall, *M. persicum* possesses potential antiproliferative and antioxidant activities on the malignant MCF-7 cell line that could be attributed to the high content of phenolics and flavonoids, and therefore warrants further exploration.

Keywords: *Marrubium persicum* - lamiaceae - MTT assay - MCF-7 cell line - DPPH assay - phenolics - flavonoids

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Introduction

Of ancient times, plants are being applied with a wide spectrum of biologically active compounds in the treatment of abundant diseases which are providing the researchers new visions both in their natural forms and also as templates for synthetic modifications of novel drugs. Drug discovery from natural products for confronting cancer has bring in the rational opportunity to attain most new clinical applications of plant secondary metabolites and their derivatives (Balunas and Kinghorn, 2005). Ever since an imbalance between the production of oxidants and occurrence of antioxidant defenses namely oxidative stress faces the worldwide, confronting this oxidative stress, as a process with implications for many pathophysiological states, seems prerequisite for ensuring healthy aging. Free radicals that are generated during oxidative stress are instable species since they have unpaired electrons that search for stability through electron pairing with biological macromolecules like proteins, lipids, and DNA of healthy human cells. Accordingly, DNA and protein damage, lipid peroxidation, cancer, ageing, and inflammatory activities are the major consequences of the oxidative stress conditions (Sharhar et al., 2008). Breast cancer, the most frequent malignancies among women is the leading cause of cancer related death, resulting from the metastatic development of primary stage of cell tumors (Jemal et al., 2006). Application of plants in treatment of different diseases including cancers is inevitable and it seems to be the basis for modern medical science, as they are considered to be great sources of new drugs (Jones et al., 2006; Kim, 2008; Aune et al., 2009; Hasan et al., 2011; Woo and Kim, 2011).

Lamiaceae family has been holding a place of value for hundreds of years due to the infusions and tinctures of numerous aromatic species used as components of herbal treatments for a variety of ailments (Naghibi et al., 2005). *Marrubium* (horehound) a genus of about 40 species of flowering plants in the family Lamiaceae is native to temperate regions of Europe and Asia which

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has a high reputation in traditional herbal medicine and different cultures with by several known healing attributes (Meyre-Silva and Cechinel-Filho, 2010). Species of this genus are characterized through having potential therapeutic activities, which have been supported by various studies demonstrating cytotoxicity, immunomodulating, vasorelaxant, antispasmodic, hypolipidemic, hypoglycemic, and analgesic properties of this genus (El Bardai et al., 2001; 2004; Meyre-Silva et al., 2005; Berrougui et al., 2006; Karioti et al., 2007; Rigano et al., 2009; Boudjelal et al., 2011). Additionally, studies dealing with the composition, antimicrobial and antioxidant activities of essential oils extracted from genus *Marrubium* have been previously reported (Nagy and Svajdlenka, 1998; Demirci et al., 2004; Javidnia et al., 2007; Morteza-Semnani et al., 2008; Sarikurkcu et al., 2008; Tajbakhsh et al., 2008; Laouer et al., 2009; Petrovic et al., 2009; Argyropoulou and Skaltsa, 2011; Zarai et al., 2011). Regarding the phytochemical analysis of the plants of this genus, they mostly produce diterpenes, polyphenols, steroids, phenylpropanoids and flavonoids, some of which have important biological properties (Calis et al., 1992; Karioti et al., 2003; Rigano et al., 2006; Alkhatib et al., 2010; Zaabat et al., 2011).

Developing an efficient herbal remedy is reliant to a superior notion of the relation between chemical constituents and biological properties of a natural product. In view of these facts, *M. persicum*, endemic to the countries; Armenia, Azarbaijan, Turkey and Iran which is considered to be an unknown source of varied chemical constituents, was selected to seek for its potential anti proliferative activity against human breast cancer cell line along with its antioxidant activity with its phenolic and flavonoid contents.

Materials and Methods

Materials

3(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT), 2,2-diphenyl-1-picrylhydrazyl (DPPH), rutin, gallic acid, and Folin-Ciocalteu reagent, aluminum chloride, streptomycin, penicillin G, all from Sigma Aldrich chemical company Germany and fetal bovine serum (FBS) from Gibco, UK were used. All other reagents and chemicals were of analytical grade.

Plant material, extraction and preparation

Aerial parts of *Marrubium persicum* were collected during the flowering stage from Varzeghan in East Azarbaijan province, Iran (38° 30' 33.9' 'N latitude, 46° 30' 41" E longitude, and 1940 m above sea level), in June 2011. A voucher specimen of the plant representing this collection has been deposited at the Herbarium of the Faculty of Pharmacy, Tabriz University of Medical science, Iran. The air-dried and finely grounded aerial parts of *M. persicum* (0.5 kg) were successively extracted at room temperature with solvents of increasing polarity, petroleum ether (40-60°C), dichloromethane and methanol (5L of each solvent, thrice every 48h). Subsequently, the methanol solution was concentrated under reduced pressure using a rotary evaporator at 30°C, to obtain a dried powdered methanolic extract.

MTT bioassay

The cytotoxic effect of *M. persicum* methanolic extract against MCF-7 (human breast carcinoma) was evaluated using MTT bioassay. The following assay detects the reduction of yellow dye MTT by mitochondrial succinate dehydrogenase to a blue formazan product, which reflects the normal functioning of mitochondria and hence the cell viability (Mosmann, 1983). The human breast cancer MCF-7 cell line was obtained from National cell bank of Iran (Pasteur institute, Iran), and cultured in RPMI 1,640 medium (Gibco, UK) complemented with 10% fetal bovine serum (FBS), 100 mg/mL streptomycin and 100 units/mL penicillin G. MCF-7 cells were cultured at 37°C in a 5%CO_2 incubator. The cells were detached with 0.05% trypsin/EDTA when they reached ~90% confluency. Afterwards, the cells were seeded in 96-well microtitre plate (200 µl/well) with concentration of 4×10^4 cells/cm^2. At 40-50% confluency, the cultivated cells were exposed to various concentration of the methanolic extract (1, 0.75, 0.5, 0.25, 0.1, 0.075, 0.05, 0.025, 0.01 mg/mL) prepared in 1% dimethyl sulfoxide (DMSO) and were incubated for different periods of time (24, 48 and 72 h). Control groups received the same amounts of DMSO with four wells remained untreated as control. After the treatment, normal culture medium was substituted with 200 µl fresh media and 50 µl MTT reagent (2 mg/mL in PBS), except the cell-free blank control wells. Cells were maintained in 37°C with 95% air, 5%CO_2 and complete humidity for 4h. Subsequently, the MTT solution was replaced with 200 µl of DMSO and 25 µl sorenson buffer (0.1M NaCl, 0.1M glycine regulated to pH: 10.5 with 1M NaOH), incubated for 15 min at 37°C. Eventually, the optical density of the wells was measured at 570 nm by means of a spectrophotometric plate reader (Sunrise Tecan, Austria). The growth of tumoral cells and viability of the cells was determined using the formula: *Viability%=(optical density of sample/optical density of control)×100*

Furthermore, the cytotoxicity of the extract was determined by plotting of the percent cytotoxicity index, *CI%=[1-(optical density of sample/optical density of control)]×100*, versus concentrations of the methanolic extract of *M. persicum*.

Assay for antioxidant activity

The free radical scavenging capacity of the extract was measured from the bleaching of the purple-colored methanolic solution of DPPH. The stock concentration 1 mg/mL of the methanolic extract of the *M. persicum* was prepared followed by dilution in order to obtain concentrations of 5×10^{-1}, 2.5×10^{-1}, 1.25×10^{-1}, 6.25×10^{-2}, 3.13×10^{-2} and 1.56×10^{-2} mg/mL. The obtained concentrations in equal volumes of 2 mL were added to 2 mL of a 0.004% of DPPH solution. After a 30 min incubation period at 30ºC, the absorbance was read against a blank at 517 nm. Tests were carried out in triplicate where the average absorption was noted for each concentration. Additionally, the same procedure was repeated with quercetin as the positive control. Inhibition of free radical by DPPH in percent was calculated as

follows: *R(%)=100×[(A blank-A sample)/A blank]*

Where A blank is the absorbance of the control reaction (containing all reagents except the plant extract) and A sample is the absorbance of the sample. Moreover, the RC_{50} value, which is the concentration of the plant extract reducing 50% of the free radical concentration, was calculated from the graph of inhibition percentages against *M. persicum* extract concentrations in mg/mL.

Assay for total phenolics content

Total phenolic constituents of the *M. persicum* methanol extract was determined by involving Folin-Ciocalteu reagent and Gallic acid as standard, the same way as given in the literature (Ebrahimzadeh et al., 2009; 2011; Zakizadeh et al., 2011). Briefly, 0.5 mL solution of the plant extract in methanol was mixed with 5mL of Folin Ciocalteu reagent (a 10% v/v in distilled water) with 4 mL of 1M aqueous Na_2CO_3 after 5min and the mixture was allowed to stand for 15 min with intermittent shaking. The absorbance of the blue color produced was measured with a UV/ Visible spectrophotometer (Shimadzu, 2100 - Japan) at 765 nm. The standard curve was prepared using 25-300 μg/mL solutions of Gallic acid in methanol: water (50:50). After all, the total phenol values of the *M. persicum* extract was expressed in terms of Gallic acid, equivalent (mg/100g of powdered dry plant material) which is a common reference compound.

Assay for total flavonoids content

In order to determine the total flavonoid content of the *M. persicum* methanolic extract sample the colorimetric aluminum chloride method was employed (Ghasemi et al., 2009; Ebrahimzadeh et al., 2011). Concisely, 0.5 mL solution of methanolic extract were mixed with 1.5 mL of methanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1M potassium acetate and 2.8 mL of distilled water, and were left at room temperature for 30 min. The absorbance of the reaction mixture was measured spectrophotometrically at 415 nm. Total flavonoids contents were calculated as quercetin from a calibration curve. The standard curve was prepared using 31.25-250 μg/mL solutions of quercetin in methanol.

Results

Cytotoxic activity

The time and dose dependent effects of *M. persicum* methanolic extract on proliferation of MCF-7 cell line assessed using MTT method, has been shown in Figure 1 via plots of viability and cytotoxicity index percentages versus plant extract concentrations. Comparing the results of the control group of untreated cells with treated cells, treated cells exhibited a dose and time dependent decline in viability, so that the highest decline in viability was rendered to 16.2% by 1mg/mL of the extract after 72 h incubation. In terms of the cytotoxicity indexes for the plant extract, a gradual decrease of cytotoxicty effect had been achieved setting out on 200μg/mL concentration, reaching up to 62, 65 and 70% of cytotoxicity indexes at 1mg/mL of the *M. persicum* methanolic extract for the MCF-7 cell lines.

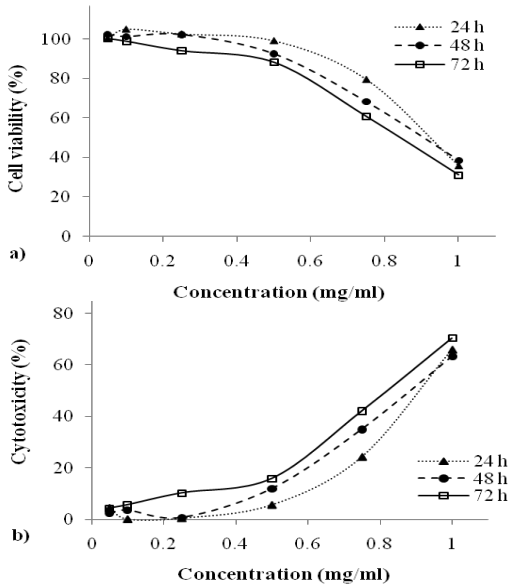

Figure 1. Effect of Methanolic Extract of *M. persicum* on Cell Proliferation of MCF-7 Cell Line Presented as Percentage of, a) Cell Viability and b) Cytotoxicity Index, Versus Concentration of the Plant Extract

Antioxidant property of the M. persicum extract

The free radical scavenging activity of the extract was evaluated using the DPPH method, mentioned above. This method of scavenging stable DPPH free radicals has been widely developed to evaluate the antioxidative activities in a relatively short time (Nazemiyeh et al., 2008; 2010). Reduction of the DPPH radical in the test medium was determined spectrophotometrically in its absorbance at wavelength 517 nm as a result of a color change from purple to yellow since free radicals are scavenged by antioxidants through donation of hydrogen to form the stable DPPH-H molecule. According to the findings of the free radical scavenging capacity of the extract, although *M. persicum* revealed satisfactory antioxidant activity, the concentration of *M. persicum* methanolic extract resulting in a 50% reduction of the free radical were lower when compared to the control quercetin with RC_{50} values of 52 μg/mL for the former and 3 μg/mL for the latter. It has been suggested that the observed scavenging activity of DPPH and OH radicals could be assigned to the hydrogen-donating capacity of the phenolic components, present in significant concentrations in the methanolic extract of *M. persicum*. All the same, the antioxidant activities of a plant extract are not only attributed to the active compounds present in high percentages, but also to the presence of other constituents in small quantities as well as the synergism among them.

Total phenols content

The total phenolic compounds of the methanolic extract of *M. persicum*, as determined by Folin Ciocalteu method, have been reported as gallic acid equivalents. The concentrations of phenolic compounds were calculated according to the following equation obtained from the standard gallic acid graph: *Sample absorbance=0.0067×Gallic acid (μg)+0.0132, (R^2: 0.987)*

Accordingly, the total phenolic content of *M. persicum* extract investigated by the method revealed the value of was 409.28 mg Gallic acid equivalent of 100 g dry plant material.

Total flavonoids content

Quantification of the total flavonoids content in *M. persicum* extract via aluminum chloride as the shift reagent has proven to be extremely a practical guide. The flavonoids content of the *M. persicum* methanolic extract was calculated according to the following equation obtained from the standard quercetin graph: *Absorbance=0.008×Quercetin (µg)-0.0683 (R^2: 0.9999)*.

Comparing the absorbance values for methanolic extract solution, reacted with aluminum chloride reagent with the standard solutions of quercetin equivalents, the total flavonoid contents of *M. persicum* extract was calculated 168.87 mg quercetin equivalent in 100 g of powdered plant material, by reference to the relative standard curve.

Discussion

The MTT assay on the cytotoxic activity of the methanolic extract of the plant on MCF-7 cell line suggested the extract was moderately cytotoxic to MCF-7 cells in a dose and time dependent manner. Our findings provided insight into a new implication of the traditional usage of *M. persicum* found in Armenia, Azarbaijan, Turkey and Iran as a potential novel cancer chemopreventive agent, where incorporation of this plant in herbal remedy may help prevent or reduce the risk of breast cancer and other oxidative stress associated diseases. Several studies in this filed have shown that genus *Marrubium* is of potential value for detecting antiproliferative agents, as Yamaguchi et al. determined the anti-inflammatory and antiproliferative activity of *M. vulgare* leaves in human colorectal cancer cells via suppression of cell growth as well as induction of apoptosis (Yamaguchi et al., 2006). Elsewhere, diterpenoids from *M. cylleneum* and *M. velutinum* were evaluated for their cytotoxic effects against various cancer cell line and immunomodulating potential in human peripheral blood mononuclear cells in vitro assays by Karioti et al, revealing strong tumor regression in a broad range of tumor cells (Karioti et al., 2007). The genus *Marrubium* is famous for its diterpenoid contents including marrubiin and marrubenol that might be responsible for the chemopreventive effects of the extract, in consistence to the previously published papers describing the protective role of these compounds in various oxidative stress related diseases.

Not only different endogenous antioxidants produced by the human body have potential health benefits against oxidative stress and related diseases, but also natural herbal antioxidant compounds such as phenolic acids, polyphenols and flavonoids scavenge free radicals like peroxide, hydroperoxide or lipid peroxyl that inhibit the oxidative mechanisms leading degenerative diseases (Kandaswami et al., 2005; Wang et al., 2011). In addition, this study has clearly shown that *M. persicum* has relatively sizeable antioxidant activity with regard to the potential radical scavenging ability of its methanolic extract. Since, botanicals and herbs are normally complex mixtures of different compounds searching for the flavonoids and other phenolic compounds present in the methanolic extract of *M. persicum* which donates a hydrogen atom for scavenging the stable DPPH radical, would be beneficial in quantifying the putative role of them as antioxidant and anti-cancer agents.

Owing to the fact that anticancer activity of plants has been verified to be connected with a variety of phytochemicals, such as polyphenols, flavonoids and catechins (Uddin et al., 2009), many researchers had also confirmed higher phenolic content of plant materials increases the antioxidant activity of the plant since there is a linear correlation between phenolic contents and antioxidant activity (Rietjens et al., 2005; Ravikumar et al., 2008; Rahman et al., 2011). Although plant phenolics are good source of antioxidants, they differ in their abilities to quench various free radicals, so as verifying the exact role of these phytochemicals in certain kind of an oxidative stress related diseases is of substantial importance. In this survey the correlation between total phenolic and flavonoids contents as well as radical scavenging activity along with the antiprolifreative activity of *M. persicum* methanolic extract in breast cancer was analyzed. Considering the results, cytotoxicity of the extract in MCF-7 cells enhanced with increase in the phenolics and flavonoids content of the plant extract and higher radical scavenging activity in higher concentrations.

On the whole, this study suggests the antioxidant and cytotoxic activity of *M. persicum* extract might be helpful in preventing or slowing the progress of various oxidative stress-related diseases such as breast cancer. Evidently, further bioassay-guided fractionation approaches on this species would be of value to purify and identify the foremost active constituents of the extract inhibiting the proliferation of MCF-7 cells. Likewise, as some phenolic antioxidants have role in suppressing the growth and proliferation of transformed or malignant cells through induction of programmed cell death or apoptosis, it appears to warrant the need for additional investigation on cell cycle analysis and determination of the distinctive mechanism of action for providing antiprolifreative activity of *M. persicum* methanolic extract. The authors believe this study could furnish the background for detailed anticancer examination of this plant, since it is the very first report on the analysis of the antiproliferative and antioxidant activity of *Marrubium persicum*.

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