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Research Article

Evaluating Antiproliferative and Antioxidant Activity of *Marrubium crassidens*

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Abstract

Purpose: Naturally occurring substances as novel drugs in cancer therapy, at all times, represent a challenge to science since medicinal plants are proving to be brilliant sources of new chemopreventive agents.

Methods: In the present study, methanol extract from aerial parts of *Marrubium crassidens* was assessed for its antiproliferative activity in the breast cancer cell line MCF-7 through MTT bioassay using cell viability and cytotoxicity indices. The antioxidant property of *M. crassidens* extract together with its phenolic and flavonoids content were evaluated, as well.

Results: According to data obtained in the study, *M. crassidens* exhibited antiproliferative activity with a gradual rise in cytotoxicty effect setting out on 240µg/mL concentration of the extract. Moreover, the RC_{50} value for antioxidant activity of the extract was determined as 40µg/mL and values for the total phenolic and flavonoids were calculated as 512.64mg gallic acid equivalent and 212.73mg quercetin equivalent per 100g of dry plant material.

Conclusion: Generally, the observed antiproliferative and antioxidant properties of *M. crassidens* could be certified to the high amounts of phenolic and flavonoid content detected in the extract.

Introduction

Not surprisingly, the upward desire in capturing the wisdom of traditional healing systems in management of different sorts of diseases among the nations, has led to a renewal of interest in herbal medicines. In this regard, plants with a wide range of biologically active constituents, at all times, have been providing scientists with innovative visions both in their natural forms and also by templates for novel molecular prototypes of drugs. Followed by a variety of investigations on a fundamentally unregulated cell growth, cancer has remained a major health problem worldwide with a choice of different expressions and pathologies. Seeing as the deadly nature of cancer, there exists severe scientific challenge among the researchers to understand the disease processes headed for discovery of potential fresh therapies from natural products. It has been accepted that an imbalance between the production of oxidants and frequency of antioxidant defenses namely oxidative stress could be one of the ensuing factors in DNA and protein damage, cancer, ageing, lipid peroxidation and inflammatory activities.[1] Following free radicals that are generated during oxidative stress with unpaired electrons seek for stability through electron pairing with biological macromolecules like proteins, lipids, and DNA of healthy human cells, bringing in about serious consequences of the oxidative stress conditions. In this issue, breast cancer developing from the metastatic progress of primary stage of cell tumors, has been considered as the prevalent malignancy among women the foremost cause of cancer related death.[2] Even so, phyto medicines confirming to be appealing sources of new compounds with new applications in clinical stages seem to have much to offer in treatment of cancers bringing about rational opportunities in this filed.[3-5]

In spite of the fact that herbal preparations from different parts of plants belonging to various families have been regarded as valuable medicinal plants, abundant members of the family Lamiaceae (particularly the genus *Marrubium*) have prominent medicinal properties. *Marrubium* (horehound) is a genus of about 40 species native to temperate regions of Europe and Asia, that are characterized with some potential therapeutic activities supported by a choice of reports demonstrating cytotoxicity, immunomodulating, vasorelaxant, antispasmodic, hypolipidemic, hypoglycemic, and analgesic properties of this genus in vitro and in vivo.[6-14] Further studies on the composition, antimicrobial and antioxidant activities of essential oils extracted from genus *Marrubium* have also been reported.[15-26] Moreover, plants from this genus are customarily famous for producing several classes of compounds including diterpenes, polyphenols, steroids, phenylpropanoids and flavonoids, some of which have important biological

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properties.[27-31] Nonetheless, in order to provide an efficient herbal remedy it would be of utmost importance to associate between chemical constituents of a natural product with its biological properties. Accordingly, in this study *M. crassidens* endemic to Armenia, Azarbaijan, Turkey and Iran was selected to search for its possible anti proliferative activity against MCF-7 human breast cancer cell line along with its antioxidant activity in relation to the phenolic and flavonoid contents of the herbal extract.

Materials and Methods

Materials

In this study, 3(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT), 2,2-diphenyl-1-picrylhydrazyl (DPPH), quercetin, 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

Marrubium crassidens was collected during the flowering stage from Chichaklou in East Azarbaijan province, Iran, in June 2011. A voucher specimen of the plant (Tbz-Fph-719) representing this collection has been deposited at the Herbarium of the Faculty of Pharmacy, Tabriz University of Medical science, Iran. The air-dried and well grounded aerial parts of *M. crassidens* (0.5kg) were extracted with solvents of increasing polarity, petroleum ether (40–60°C), dichloromethane and methanol (5L of each solvent, thrice every 48h) by maceration at room temperature. Afterwards, for further analysis upon methanol extract, concentration was qualified under reduced pressure via a rotary evaporator at 40°C, up to obtain a dried powdered extract.

MTT bioassay

Cytotoxic effect of *M. crassidens* methanol extract was assessed by MTT bioassay in MCF-7 human breast cancer cell line. In the MTT assay reduction of mitochondrial succinate dehydrogenase converts yellow dye to a blue formazan product, which shows the normal activity of mitochondria and thus the cell viability.[32] The MCF-7 cell line was established from National cell bank of Iran (Pasteur institute, Iran), and cultivated in RPMI 1640 medium (Sigma Aldrich Co. Germany) supplemented with 10% fetal bovine serum (FBS), 100 mg/ml streptomycin and 100 units/ml penicillin G. The MCF-7 cells were cultivated at 37 °C in a 5% CO_2 incubator. Then, cells with ~90% confluency were detached by 0.05% trypsin/EDTA. Cell suspension was distributed into 96- well microtitre plate (200µl/well) with concentration of 15×10^3 cells/well. After 24 hours the cultivated cells were treated with different amount of methanolic extract (1, 0.75, 0.5, 0.25, 0.1, 0.075, 0.05, 0.025, 0.01mg/ml) dissolved in 1% dimethyl sulfoxide (DMSO) and were incubated for 24, 48 and 72 hours. Control groups received the same amounts of DMSO with four wells remained untreated. Moreover, the MTT reagent was prepared at 2mg/ml in PBS. The normal culture medium was replaced with 150µl fresh media plus 50µl of MTT reagent (2mg/ml in PBS), excluding the cell-free blank control wells. Cells were incubated in 37°C, 5% CO_2 and full humidity for 4h. Consequently, the MTT solution was exchanged by 200µl of DMSO and 25µl sorenson buffer (0.1M NaCl, 0.1M glycine regulated to pH: 10.5 with 1M NaOH). The plate was shaken for 15 min at 37 °C, later optical density (OD) of the wells were determined at 570 nm using a spectrophotometric plate reader (SUNRISE TECAN, Austria). Eventually, the viability and growth of tumoral cells were calculated via the below formula:

$$Viability\ \% = \frac{optical\ density\ of\ sample}{optical\ density\ of\ control} \times 100$$

Additionally, cytotoxicity of the *M. crassidens* extract was defined 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. crassidens*.

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 of the *M. crassidens* methanol extract (1mg/mL) was prepared followed by dilution to reach for concentrations 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 of the extract. The acquired concentrations in the same volumes of 2mL were added to 2mL of a 0.004% of DPPH solution. Later than a 30 min of incubation at 30 ºC, the absorbance of each solution was read against a blank sample at 517 nm. The average absorption value was noted for each sample after the test was carried out in triplicate. Moreover, as the positive control the same procedure was gone over with quercetin. The inhibition percentage of DPPH free radicals of by the methanol extract was calculated as follows:

$$R\ (\%) = 100 \times \frac{A\ blank - A\ sample}{A\ blank}$$

Herein, "A blank" stands for the absorbance value of the control reaction and "A sample" is the absorbance value for each sample. In addition, RC_{50} value, the concentration of the extract reducing 50% of the DPPH free radicals, was calculated from the graph of inhibition percentages versus concentrations of *M. crassidens* extract in mg/mL.

Assay for total phenolics content

Total phenolic constituents of the *M. crassidens* methanol extract was verified by assigning Folin-Ciocalteu reagent and Gallic acid as the standard compound for phenolics, the same procedure as given in the literature.[33-35] Briefly, 0.5mL of methanol solution of the extract was mixed with 5mL of folin Ciocalteu

reagent (a 10% v/v in distilled water) with 4mL 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 by the reaction was measured using 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). Eventually, the value for total phenol content of the *M. crassidens* extract was represented in terms of gallic acid, equivalent (mg/100g of powdered dry plant material) which is a common reference compound.

Assay for total flavonoids content

Determination of the total flavonoid content of the *M. crassidens* methanol extract was carried out according to the colorimetric aluminum chloride method.[33-35] Concisely, 0.5mL solution of the extract was mixed with 1.5mL of methanol, 0.1mL of 10% aluminum chloride, 0.1mL of 1M potassium acetate and 2.8mL of distilled water, which were left at room temperature for 30 min. Next, the absorbance of the reaction mixture was measured at 415 nm, spectrophotometrically. After all, the total flavonoids content of the *M. crassidens* methanol extract was calculated as equivalent of quercetin as the standard compound for flavonoids from a calibration curve (mg/100g of powdered dry plant material). The standard curve was managed and evaluated by different concentrations of quercetin in methanol 31.25-250 µg/mL, as well.

Results

Antiproliferative activity

Antiproliferative activity of *M. crassidens* extract on MCF-7 cell line was quantified by MTT method showing the time and dose dependent effects through plots of viability and cytotoxicity index percentages versus different concentrations of the extract in Figure 1. According to the findings, the highest descent in cells viability reached to 21.7% by 1mg/mL of the extract after incubation of 48 h compared to the control group of untreated cells. Moreover, in the viewpoint for cytotoxicity indices, a gradual increase in cytotoxicty activity had been detected setting out on concentrations of 240µg/mL plant extract, reaching up to 70% of cytotoxicity index at 1mg/mL for the MCF-7 cell lines.

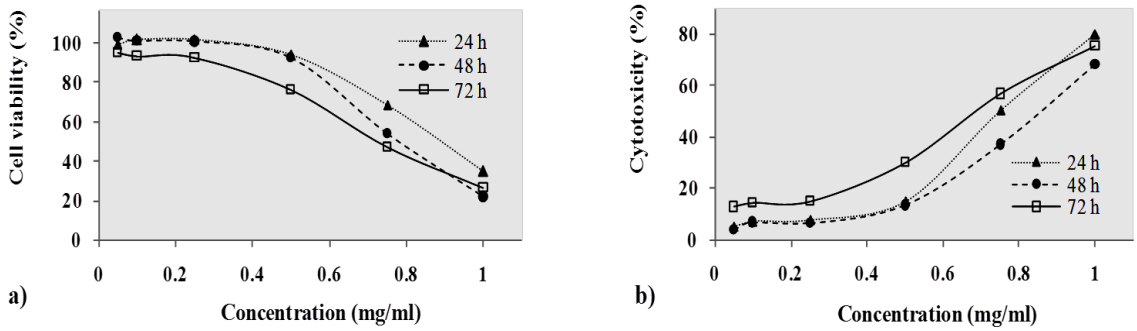

Figure 1. Effect of *M. crassidens* methanol extract on cell proliferation of MCF-7 cell lines presented as percentage of, a) cell viability and b) cytotoxicity index, versus concentration of the extract.

Antioxidant activity of the M. crassidens extract

Assay for the free radical scavenging activity of the extract was accomplished via the DPPH method, which has been generally developed for detection of the antioxidative properties of the materials in a fairly short time.[36-38] Regarding the results for antioxidant assay, *M. crassidens* extract exhibited pleasant antioxidant activity with RC_{50} values of 40µg/mL for the extract and 3µg/mL for the control quercetin.

Total phenols content

The amount of total phenolic compounds present in the *M. crassidens* methanol extract was calculated as gallic acid equivalents ascertained by Folin Ciocalteu method. The following equation obtained from the standard gallic acid graph was applied in calculation of the phenolic compounds concentration.

Sample absorbance = 0.0067 × gallic acid (µg) + 0.0132, (R^2: 0.987)

Correspondingly, the content for *M. crassidens* total phenolics showed the value 512.64mg of gallic acid equivalent in 100g of dry plant material.

Total flavonoids content

The total flavonoids content in *M. crassidens* methanol extract via aluminum chloride as the shift reagent was determined according to the equation obtained from the standard quercetin graph:

Absorbance = 0.008 × Quercetin (µg) - 0.0683 (R^2: 0.9999).

With reference to the relative standard curve, amounts for the flavonoid contents was calculated as 212.73mg quercetin equivalent in 100g of powdered plant material, comparing the absorbance values for methanolic extract solution with the standard solutions of quercetin.

Discussion

As far as we know, plants had been used for various medicinal purposes long before recorded history. Even so, advances in clinical studies is evidence for the implication of herbal medicine in the treatment and management of different sorts of diseases so far as improvements in analysis and quality control of medicinal plants come about. In this regard, the obtained data in our study strongly suggest the methanolic extract

of *M. crassidens* is moderately cytotoxic to MCF-7 cells in a dose and time dependent manner. In consistence to our lately published paper on other species of genus *Marrubium*, *M. persicum*, disclosing its anti proliferative activity,[39] the present study strengthen the concept that incorporation of this plant in herbal remedy as a potential novel cancer chemopreventive agent might help prevent or downgrade the chance of breast cancer or other oxidative stress related diseases, as well.

Surveys in this filed, such as that conducted by Alkhatib et al. have shown a common natural source for the hemi-synthesis of future ladanein-derived flavones was found to be *Marrubium vulgare* which possessed moderate antileukemic activity on K562, K562R (imatinib-resistant), and 697 human leukemia cell lines.[30] Elsewhere, some phenylethanoid derivatives isolated from aerial parts of *M. deserti de Noé* were undertaken through antigenotoxic analyses by Zaabat et al. The findings of their study showed that the isolated compounds were able to significantly inhibit β-galactosidase induction caused by the mutagen agent nitrofurantoin along with potent antioxidant capacity even more than positive control trolox.[31] It is of note to mention the diversity of compounds present in different species of genus *Marrubium* like diterpenoid contents that could be responsible for the chemopreventive effects of the extract, according to the available reports describing the protective role of these compounds in a range of oxidative stress related diseases.

The imperative role of exogenous antioxidants such as natural herbal antioxidant compounds like phenolic acids, polyphenols and flavonoids, plus endogenous antioxidants produced by the human body in scavenging peroxide, hydroperoxide or lipid peroxyl free radicals leading degenerative diseases could not be disclaimed.[40,41] Concerning the results of the study, significant antioxidant activity with regard to the potential radical scavenging ability was detected by the methanol extract of *M. crassidens.* Although the potential antioxidant activity of this plant could be linked to the complex mixtures of different compounds that are present in most botanicals and herbs, the conventional role of flavonoids and other phenolics present in the methanolic extract of *M. crassidens* donating a hydrogen atom for scavenging the stable DPPH radical is inevitable. Many researchers believe there is a linear relation between antioxidant activity and phenolic contents such as polyphenols, flavonoids and catechins that had been recognized to be connected with anticancer activity of various plants.[42-44] On the whole, it was established that the increase in the phenolics and flavonoids content of *M. crassidens* methanol extract brought about higher radical scavenging activity in line with enhanced cytotoxicity activity of the extract in MCF-7 cells.

Conclusion

The present study puts forward the antioxidant activity of *M. crassidens* extract together with its cytotoxic activity could be helpful in prevention of some serious diseases like breast cancer. It is of value to indicate the potential role of some known phenolics in inhibition of the transformed or malignant cells growth via initiation of programmed cell death or apoptosis. Therefore, it seems requisite to search for the distinctive mechanism of action in the relative antiprolifreative activity of *M. crassidens* methanolic extract through investigation on cell cycle analysis. Although many phytomedicines from herbal products exert their beneficial effects through the additive or synergistic action of various chemical compounds, additional bioassay-guided fractionation approaches on *M. crassidens* extract might be worthy in purifying and identifying the foremost active constituents in charge of proliferation inhibition of MCF-7 cells, since it is the very first report in this regard.

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Conflict of interest

The authors report no conflict of interests.

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