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Antioxidant properties of *Helichrysum pseudoplicatum* Nab

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Abstract

The genus *Helichrysum* (Asteraceae) is comprised of approximately 500-600 species and used for the treatment of a variety of pathological conditions in folk medicine in many countries. In this study, antioxidant activities of aerial parts of *H. pseudoplicatum* were investigated employing various *in vitro* assay systems, i.e. 2,2-diphenyl-1-picrylhydrazyl (DPPH) and nitric oxide radical scavenging, reducing power and hydrogen peroxide scavenging. IC_{50} for DPPH radical-scavenging activity was 438.9 ± 15.6 µg/ml. The extract exhibited good reducing power at 25 - 400 µg/ml but was not comparable with that of vitamin C. The extract showed good nitric oxide-scavenging activity. IC50 was 474.3 ± 11.8 µg/ml. It was capable of scavenging hydrogen peroxide in a concentration dependent manner. It showed good activity. Its IC50 was 159.8 ± 8.2 µg/ml. The IC50 values for ascorbic acid and BHA were 21.4 and 52.0 µg/ml, respectively. The total amount of phenolic compounds in the extract was determined as gallic acid equivalents (22.7 ± 3.1 mg/g of extract) and total flavonoid contents were calculated as quercetin equivalents (9.6 ± 1.3 mg/g of extract) from a calibration curve. This plant contained low amount of total flavonoids and phenolic compounds. Its moderate antioxidant activities may be attributed to its low levels of phenols and flavonoids.

Keywords: Antioxidant activity, *Helichrysum pseudoplicatum*, radical scavenging, reducing power.

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Introduction

Free radicals and reactive oxygen species (ROS) are well known inducers of cellular and tissue pathogenesis leading to more than 100 diseased including inflammatory disorders, malaria, AIDS, heart disease, stroke, arteriosclerosis, diabetes and cancer, Parkinson´s and Alzheimer`s diseases, and aging processes (1,2). Minimizing oxidative damage may well be one of the most important approaches to the primary prevention of these diseases and health problems. Antioxidants provide protection to living organisms from this damage caused by uncontrolled production of ROS and the concomitant lipid peroxidation, protein damage and DNA strand breaking (3). Antioxidants have been detected in a large number of foods and plant extracts. Among the various medicinal plants, some endemic species are of particular interest because they may be used for producing raw materials or preparations containing phytochemicals with significant antioxidant capacities and high content of mineral with health benefits (4, 5). Although there are some synthetic antioxidant compounds, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), which are commonly used in processed foods, it has been reported that these compounds may have side effects (6, 7). So the use of traditional medicine is widespread, and plants still present a large source of natural antioxidants that might serve as leads for the development of novel drugs (8).

Helichrysum Mill., belonging to the family Asteraceae, includes about 500-600 species, widespread throughout the world (9, 10). These species popularly are known

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as immortal flower. Aerial parts of the plants widely used as tea. They are widely used for the treatment of kidney stone (11). Effects of *Helichrysum* species are due to their flavonoid contents (9, 12). These plants have been used in folk medicine for thousands of years to treat gall bladder disorders, due to their regulation of bile and diuretic effects. They are used for stomach pain and wound dressings; for their antiinfective, hepatoprotective, detoxifying, cholagogic, and choleretic effects; to stimulate the secretion of gastric juices; and in the treatment of coughs, jaundice, diarrhea and asthma, erythema, and diabetes mellitus (11, 13-16). *Helichrysum* species are commonly used in Turkey and other parts of the world for their various biological properties, including anti-inflammatory, antioxidant, and antimicrobial activities (9). Although the biological activities of many *Helichrysum* species have been investigated in different countries, there is no report about the *Helichrysum* species belonging to Iranian flora. According to the Flora Iranica (10), 19 *Helichrysum* species have been reported in Iran. There are few reports on systematic study of *Helichrysum* species from Iran (17). There is a very limited report about the activity of *Helichrysum* species belonging to Iranian flora. Antibacterial and antioxidant activities of *H. oligocephalum* have been reported recently (18). This work attempts to contribute to this lack of knowledge about the antioxidant effects of Iranian *H. pseudoplicatum.* In this study, the antioxidant activity of aerial parts of *H. pseudoplicatum* were examined employing four various *in vitro* assay systems, i.e. DPPH and nitric oxide radical scavenging, reducing power and scavenging of hydrogen peroxide, in order to understand the usefulness of this plant as a foodstuff as well as in medicine.

Materials and methods

Plant material and preparation of freeze-dried extract

Aerial parts of H. pseudoplicatum Nab were collected from Sari in north of Iran, in summer 2011 and identified by Dr. Bahman Eslami. A voucher (No. 870) has been deposited in herbarium of Islamic Azad University, Qaemshahr branch. The materials were dried at room temperature and coarsely ground (2-3 mm) before extraction. 100 g of powder was extracted at room temperature by maceration using methanol for 24 h at room temperature. The extract was then separated from the sample residue by filtration through Whatman No.1 filter paper, and repeated three times. The resultant extracts were concentrated in a rotary evaporator until a crude solid extracts were obtained, which was then freeze-dried for complete solvent removal (yield, 16.5%, w/w).

Determination of total phenolic compounds and flavonoid content

The total phenolic content of the extract was determined colorimetrically by the Folin-Ciocalteu method, using gallic acid as the reference compound (19). The extract sample (0.5 mL) was mixed with 2.5 ml of 0.2 N Folin-Ciocalteau reagent for 5 min and 2.0 ml of 75 g/l sodium carbonate were then added. The absorbance of reaction was measured at 760 nm after 2 h of incubation at room temperature. Results were expressed as gallic acid equivalents. Total flavonoids were estimated using $AlCl_3$ colorimetric method (19). To 0.5 ml of sample, 0.5 mL of 2% $AlCl_3$ ethanol solution was added. After 1 h at room temperature, the absorbance was measured at 420 nm. Total flavonoid contents were calculated as quercetin from a calibration curve.

DPPH radical-scavenging activity

The ability of the extract to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals was assessed spectrophotometrically (19). Different concentrations of extract were added, at an equal volume, to methanolic solution of DPPH (100 M) at the dark. After 15 min at room temperature, the absorbance was recorded at 517 nm. The experiment was repeated for three times. BHA was used as standard control. IC50 values denote the concentration of sample, which is required to scavenge 50% of DPPH free radicals.

Determination of reducing power

The reducing power of sample was determined according to the method of Yen

and Chen (20). 2.5 mL of extract (25-400 g ml^{-1}) in water were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 mL) of trichloroacetic acid (10%) was added to the mixture to stop the reaction, which was then centrifuged at 3000 rpm for 10 min. The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and FeCl3 (0.5 mL, 0.1%), and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Vitamin C was used as positive control.

Assay of nitric oxide-scavenging activity:
In this experiment, 1 mL of sodium nitroprusside (10 mM) in phosphate-buffered saline was mixed with 1 mL of extract (at different concentrations) dissolved in water and incubated at room temperature for 150 min. The same reaction mixture, without the extract but with an equivalent volume of water, served as control. Following the incubation period, 0.5 mL of Griess reagent was added. The absorbance was measured at 546 nm. Quercetin was served as positive control (21).

Scavenging of hydrogen peroxide
The ability of the extract to scavenge hydrogen peroxide was determined according to the method of Ruch (22, 20). A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). 1.4 ml of extract (at different concentrations) in distilled water was added to a H2O2 solution (0.6 mL, 40 mM). The absorbance of H_2O_2 at 230 nm was determined after ten minutes against a blank solution containing phosphate buffer without H_2O_2. The percentage of hydrogen peroxide scavenging by the extract and standard compounds was calculated as follows: % Scavenged [H2O2] = [(Ao − A1)/Ao] × 100 where Ao was the absorbance of the control and A1 was the absorbance in the presence of the sample of extract and standard.

Statistical analysis
Experimental results were expressed as mean ± SD. The data were analysed by Analysis of Variance and the means separated by Duncan's multiple range test. Data were considered significant at $p < 0.05$. The IC_{50} values were calculated from linear regression analysis.

Results
The total phenol content was measured by Folin Ciocalteu reagent in terms of gallic acid equivalent by reference to standard curve ($y = 0.005x + 0.062$, $r^2 = 1$). The total phenolic content of aerial parts of H. pseudoplicatum was 22.7 ± 3.1 mg gallic acid equivalent/g of extract. The total flavonoid content was 9.6 ± 1.3 mg quercetin equivalent/g of extract, by reference to standard curve ($y = 0.006x + 0.014$, $r^2 = 0.998$). IC_{50} for DPPH radical-scavenging activity was 438.9 ± 15.6 µg/ml. The IC_{50} value for BHA was 92.9 ± 4.5 µg/ml (Fig. 1).

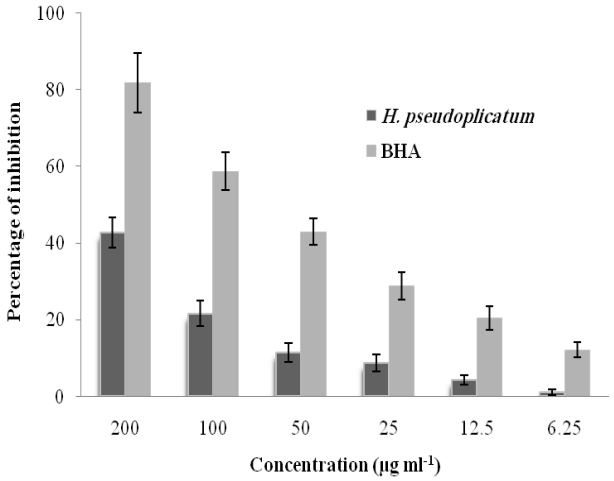

Figure 1 DPPH radical-scavenging activity of *H. pseudoplicatum*. BHA used as standard. There were statistically significant difference between samples and control groups ($p < 0.001$).

Figure 2 shows the dose-dependent curves for the reducing powers of the extract from H. pseudoplicatum.

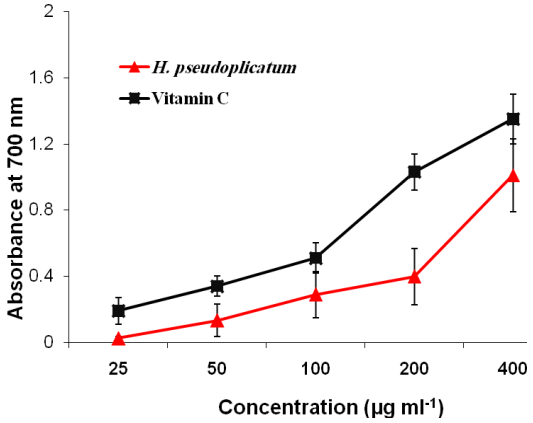

Figure 2 Reducing power of *H. pseudoplicatum*. Vitamin C used as standard. There were statistically significant difference between samples and control groups ($p < 0.001$).

It was found that the reducing power of extract augmented with the increase of its concentration. The extract exhibited good reducing power at 25 - 400 µg/ml but its activity was not comparable with that of vitamin C ($P > 0.05$). The extracts showed good nitric oxide-scavenging. IC_{50} was 474.3 ± 11.8 µg/ml. IC_{50} for quercetin was 155.0 ± 6.4 µg/ml. The extract showed weaker potency than quercetin in this study. Extract was capable of scavenging hydrogen peroxide in a concentration dependent manner. It showed good activity. Its IC_{50} was 159.8 ± 8.2 µg/ml. The IC_{50} values for ascorbic acid and BHA were 21.4 and 52.0 µg/ml, respectively.

Discussion

Plants have been used traditionally for the treatment and prophylaxis of different disorders. The protection has been attributed to plant antioxidants such as polyphenols and vitamins C, E and β-carotene (23). *Helichrysum* has long been used medicinally, proving to be beneficial in treatment of acne, asthma, bronchitis and circulatory problems, and lymphatic system diseases (18). The objective of this research was to study the antioxidant activities of *H. pseudoplicatum* Nab. The total phenol content was measured by Folin Ciocalteu reagent in terms of gallic acid equivalent by reference to standard curve. This plant is not a good source of phenols and contains low amount of total phenolics. Many plants have higher total phenolic contents (24). The total flavonoid content was reported as mg quercetin equivalent/g of extract, by reference to standard curve. It has been recognized that flavonoids show antioxidant activity and their effects on human nutrition and health are considerable. The mechanisms of the actions of flavonoids are through scavenging or chelating processes (25). The compounds, such as flavonoids, which contain hydroxyl groups, are responsible for the radical scavenging effect in the plants (20). Studies have shown that increasing levels of flavonoids in the diet could decrease certain human diseases (23). It seems other components, except polyphenolics, have a major role in antioxidant activity of this plant. The stable 1,1-diphenyl-2-picryl hydrazyl radical DPPH method is an easy, rapid, and sensitive way to survey the antioxidant activity of a specific compound or plant extracts (21). The model of scavenging the stable DPPH radical is a widely used method to evaluate the free radical scavenging ability of various samples. DPPH is a stable nitrogen-centered free radical the color of which changes from violet to yellow upon reduction by either the process of hydrogen- or electron- donation. Substances which are able to perform this reaction can be considered as antioxidants and therefore radical scavengers (21). The amount of reduced DPPH could be quantified by measuring the decrease in absorbance at 517 nm. The capacity of extract to scavenge DPPH was measured and the results are shown in Figure 1. It was found that the radical- scavenging activities of extract augmented with increasing concentration. There are at least three reports about essential oils composition of *Helichrysum spp.* in Iran. These studies identified the primary components of the essential oil of *H. oocephalum* as β-caryophyllene and menthone. Those of *H. oligocephalum* were thymol and carvacrol. Rosifoliol and β-caryophyllene were the main constituents found in *H. leucocephalum*, and hexadecanoic acid and β-caryophyllene were the primary

components identified in the oil of *H. artemisioides* (18). Stem and root oils of *H. oligocephalum* demonstrated antibacterial activity, particularly in relation to Gram-positive bacteria. In a β-carotene/linoleic acid bleaching assay, the root oil of *H. oligocephalum* demonstrated an antioxidant effect. Antioxidant capacity measured with DPPH assay was 1205.0 for the stem oil and 722.8 µg ml^{-1} for the root oil (18). Our species in this study was about three times more potent.

Fe (III) reduction is often used as an indicator of electron-donating activity, which is an important mechanism of phenolic antioxidant action. In the reducing power assay, the presence of antioxidants in the samples would result in the reducing of Fe^{3+} to Fe^{2+} by donating an electron. Amount of Fe^{2+} complex can be monitored by measuring the formation of Perl's Prussian blue at 700 nm. Increasing absorbance at 700 nm indicates an increase in reductive ability. Fig. 2 shows the dose-dependent curves for the reducing powers of the extract from *H. pseudoplicatum*. It was found that the reducing power of extract augmented with the increase of their concentration. The extract exhibited good reducing power at 25-400 µg ml^{-1} but this activity was not comparable with that of vitamin C ($P>0.05$).

Assay of nitric oxide-scavenging activity is based on the principle that, sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions (26). The extract showed good nitric oxide-scavenging. The percentage of inhibition was augmented with increasing concentration of the extract. In addition to reactive oxygen species, nitric oxide is also implicated in inflammation, cancer and other pathological conditions (26). Natural products may have the property to counteract the effect of NO formation and in turn may be of considerable interest in preventing the ill effects of excessive NO generation in the human body. Further, the scavenging activity may also help to arrest the chain of reactions initiated by excess generation of NO that are detrimental to human health. Scavenging of H_2O_2 by *H. pseudoplicatum* extract may be attributed to its phenolics, which can donate electrons to H_2O_2, thus neutralizing it to water. Extract was capable of scavenging hydrogen peroxide in a concentration dependent manner. It showed good activity. Although hydrogen peroxide itself is not very reactive, it can sometimes cause cytotoxicity by giving rise to hydroxyl radicals in the cell. Thus, removing H_2O_2 is very important throughout food systems (21).

Different compounds such as phenolics e.g. flavonoids and chalcones, phthalides, α-pyron derivatives, terpenoids, essential oils, volatiles and fatty acids have been found in the Helichrysum genus (27). The aqueous methanolic extract of *H. stoechas* has been investigated for its antioxidant activity. Extract showed interesting antioxidant and radical scavenging activity. High contents in phenolics, tannins, and total antioxidant capacity as gallic acid equivalents of 97.5 ± 0.33 g^{-1} was obtained for the flowers of this plant (28). Total phenolic contents and antioxidant activities of the methanolic extracts of *H. plicatum* have been reported. 17 different phenolic constituents were measured by HPLC in the three parts of the plant. All parts of the plant showed high antioxidant activity containing large amounts of antioxidant compounds. Chlorogenic acid, quercetin and rutin found in the three parts of the samples as main phenolic components. The methanolic extract of the plant proved to be a good source of phenolic compounds and antioxidants agents (29). *H. longifolium* has shown potential as a source of chemotherapeutic compounds (30). Phytochemical studies have revealed that this plant is rich in flavonoids and other water soluble polyphenolic compounds (31). Antibacterial potentials of *H. longifolium* extracts have been studied (32). Phytochemical compositions and the *in vitro* antioxidant and free radical scavenging potential of crude aqueous leaf extract of *H. longifolium* have been investigated. Phytochemical analyses revealed the presence of tannins, flavonoids, steroids and saponins. The total phenolic content was 0.499 mg GA equivalent g^{-1} of extract. The total flavonoid content was 0.705 mg GA

equivalent g^{-1} of extract. The percentage inhibition of lipid peroxide at the initial stage of oxidation showed antioxidant activity of 87% compared to those of BHT (84.6%) and gallic acid (96%). Also, the percentage inhibition of malondialdehyde by the extract showed percentage inhibition of 78% comparable to those of BHT (72.24%) and gallic acid (94.82%) (33). The good antiinflammatory and antioxidant activities of the aerial part of *H. italicum* (34) and high antioxidative properties of methanol extract of *H. Foetidum* have been reported, recently (35).

Conclusion

The aerial part extract of *H. pseudoplicatum* exhibited good but different levels of antioxidant activity in all the models studied. Further investigations of individual compounds for their *in vitro* or *in vivo* antioxidant activities, are needed. It is promising for further phyto-pharmacological studies, which will be focused on evaluating other biological effects or improving mechanism of these effects.

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Conflict of interest

The authors declared no potential conflict of interest with respect to the authorship, and/or publication of this study.

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