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ANTIOXIDANT ACTIVITY AND PHENOLIC CONTENTS OF *ACHILLEA WILHEMSII*

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Summary

Achillea wilhelmsii C. Koch (Asteraceae) is widely found in different parts of Iran. This plant is full of flavonoids and sesquiterpene lactones, which have been shown to have many biological activies. *Achillea* genius is widely used in traditional medicine for gastrointestinal disorders. The aim of this study was to investigate the antioxidant activity of methanol extract of *Achillea wilhelmsii* by different in vitro tests. IC_{50} for DPPH radical-scavenging activity was 58.9 ± 2.7 µg/ml. The extract showed potent nitric oxide-scavenging activity. The extract showed weak Fe^{2+} chelating ability. IC_{50} was 393.4 ± 14.9 µg/ ml. The extract was capable of scavenging hydrogen peroxide in a concentration dependent manner. The total amount of phenolic compounds in each extract was determined as gallic acid equivalents and total flavonoid contents were calculated as quercetin equivalents from a calibration curve. Biological effects may be attributed, at least in part, to the presence of phenols and flavonoids in the extract. It is promising for further phytopharmacological studies, which will be focused on evaluating other biological effects or improving mechanism of these effects.

Key words: Antioxidant activity, Antihemolytic activity, *Mentha piperita*.

Introduction

The aerial parts of different species of the genus *Achillea* L. (Asteraceae , Compositae) are widely used in folk medicine due to numerous pharmacological properties, such as anti-inflammatory(1), antioxidant (2), antispasmodic, antihemorrhoidal, stomatiche, antiseptic and emmenagogue (3,4). Also several effects such as antibacterial (2,5), antihypertensive and anti-hyperlipidemia (6), and antitumoral (7, 8) have been reported for *Achillea*. The genus was named for the Greek mythological character Achilles. According to the Iliad, Achilles' soldiers used yarrow to treat wounds, hence some of its common names such as allheal and bloodwort. It is

widely used in traditional medicine for gastrointestinal disorders (9) and there are some reports of its effects on gastrointestinal tract such as antispasmotic (10-12), choleretic (13), antiulcer (14), antibacterial (*Helicobacter pylori*) (15) and hepatoprotective (11). It has chemical components including flavonoids, alkaloids (achilleine), cineol, borneol, α and β pinen, camphor, caryophllene, thujene, rutin, sesquiterpenoids and monoterpenoids (16-18). Essential oil composition isolated from *A. clavennae, A. lingulata* and *A. holosericea* have been reported (19-21). *Achillea* comprises more than 115 species (22). *A. wilhelmsii* is the major species which is grown in Iran and widely used in Iranian traditional medicine for gastrointestinal disorders. *A. wilhemsii* leaves use for its antihemorrhoids effect and for decreasing cholesterol in Turkey (23). Antihypertensive and antihyperlipidemic effects of this plant have been reported (24). A good linoleic acid peroxidation inhibition has been reported from *A. wilhelmsii* (25). To date, very limited data exists on phenolic compounds reported in *A. wilhemsii* arial parts as well as its antioxidant effects to support their traditional claims. Therefore, we aimed to undertake this task in the present study as *A. wilhemsii* is widely-used. If the presence of phenolic and flavonoid compounds present in *A. wilhemsii* can be confirmed, the plant can be used as a good possible source of antioxidant. This study aimed to investigate the in vitro antioxidant activities of extract from *A. wilhemsii* using different antioxidant tests included 1,1-diphenyl-1-picrylhydrazyl (DPPH), hydrogen peroxide and nitric oxide radical scavenging activities, iron chelatory capacity and reducing power. The total phenolic and total flavonoid contents also were investigated. This study is an attempt to establish a scientific basis for the use of this plant in Iranian traditional medicine and possibly mechanism involvement.

Figure 1. Arial parts of *Achillea wilhemsii* C. Koch.

Materials and methods

Plant materials and preparation of freeze-dried extract: Aerial parts of *Achillea wilhemsii* C. Koch was collected from Sari, Iran, in spring 2011. Sample identified by Dr. Bahman Eslami. Sample was dried at room temperature and coarsely ground before extraction. A known amount of sample was extracted at room temperature by maceration using methanol. The resulting extract was concentrated over a rotary vacuum until a crude extract was obtained, which was then freeze-dried for complete solvent removal.

Chemicals: Trichloroacetic acid (TCA), 1,1-Diphenyl-2-picryl hydrazyl (DPPH), Potassium ferricyanide and Hydrogen peroxide H_2O_2 were purchased from Sigma Chemicals Co. (USA). Butylated hydroxyanisole (BHA), Vitamin C, Sulfanilamide, N-(1-naphthyl) ethylenediamine dihydrochloride, Ethylenediaminetetraacetic acid (EDTA) and Ferric chloride were purchased from Merck (Germany). All other chemicals were of analytical grade or purer.

Determination of total phenolic compounds and flavonoid content: Total phenolic compound contents were determined by the Folin-Ciocalteau method (26, 27). The extract samples (0.5 ml) were 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 as previously described (26, 27). Briefly, 0.5 mL solution of extract in methanol were separately mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate, and 2.8 ml of distilled water and left at room temperature for 30 minutes. The absorbance of the reaction mixture was measured at 415 nm with a double beam spectrophotometer (Perkin Elmer). Total flavonoid contents were calculated as quercetin from a calibration curve.

Antioxidant activity

DPPH radical-scavenging activity: The stable 1,1-diphenyl-2-picryl hydrazyl radical (DPPH) was used for determination of free radical scavenging activity of the extract (28). Different concentrations of extract were added, at an equal volume, to methanolic solution of DPPH (100 µM). After 15 min at room temperature, the absorbance was recorded at 517 nm. The experiment was repeated for three times. Vitamin C, BHA and Quercetin were used as standard controls. IC_{50} values denote the concentration of sample, which is required to scavenge 50% of DPPH free radicals.

Determination of metal chelating activity: The ability of the extract to chelate ferrous ions was estimated by our recently published papers (29,30). Briefly, different concentrations of extract were added to a solution of 2 mM $FeCl_2$ (0.05 ml). The reaction was initiated by the addition of 5 mM ferrozine (0.2 ml) and the mixtures was then shaken vigorously and left to stand at room temperature for 10 min. The absorbance of the solutions was measured spectrophotometrically at 562 nm. The percentage inhibition of ferrozine-Fe^{2+} complex formation was calculated as $[(A_0 - A_1)/A_0] \times 100$, where A_0 was the absorbance of the control, and A_1 of the mixture containing the extract or the absorbance of a standard solution. EDTA was used as a standard.

Assay of nitric oxide-scavenging activity: The procedure 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.
For the experiment, 1 ml of sodium nitroprusside (10 mM), in phosphate-buffered saline, was mixed with different concentrations of extract dissolved in water and incubated at room temperature for 150 min. The same reaction mixture, without the extract but with an equivalent amount of water, served as control. After the incubation period, 0.5 ml of Griess reagent (1% sulfanilamide, 2% H_3PO_4 and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride) was added. The absorbance of the chromophore formed was read at 546 nm. Quercetin was used as positive control (31,32).

Scavenging of hydrogen peroxide: The ability of extract to scavenge H_2O_2 was determined according to our recently published paper (33). A solution of H_2O_2 (40 mM) was prepared in phosphate buffer (pH 7.4). The concentration of H_2O_2 was determined by absorption at 230 nm using a spectrophotometer. The Extract (0.1-3.2 mg/ ml) in distilled water were added to a H_2O_2 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 H_2O_2 scavenging by the extract and standard compounds was calculated as follows: % Scavenged $[H_2O_2] = [(A_o - A_1)/A_o] \times 100$ where A_o was the absorbance of the control and A1 was the absorbance in the presence of the sample of extract and standard.

Reducing power determination: The reducing power of extract was determined according to our recently published paper (34). 2.5 ml of extract (25-800 μg/ml) in water were mixed with phosphate buffer (2.5 ml, 0.2M, pH 6.6) and potassium ferricyanide $[K_3Fe(CN)_6]$ (2.5 ml, 1%). The mixture was incubated at 50^oC 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 $FeCl_3$ (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.

Statistical analysis: Experimental results are expressed as means ± SD. All measurements were replicated three times. The IC_{50} values were calculated from linear regression analysis.

Results and discussion

Total phenol and flavonoid contents: Total phenol compounds, as determined by Folin Ciocalteu method, are reported as gallic acid equivalents by reference to standard curve ($y = 0.0054x + 0.0628$, $r^2 = 0.987$). The total phenolic content of extract was 37.4 ± 0.3 mg gallic acid equivalent/g of extract. The total flavonoid content was 2.5 ± 0.1 mg quercetin equivalent/g of extract, by reference to standard curve ($y = 0.0063x$, $r^2 = 0.999$). Phenols and polyphenolic compounds, such as flavonoids, are widely found in food products derived from plant sources, and they have been shown to possess significant antioxidant activities. Studies have shown that increasing levels of flavonoids in the diet could decrease certain human diseases (27).

DPPH radical-scavenging activity: The model of scavenging the stable DPPH radical is a widely used method to evaluate the free radical scavenging ability of various samples (31,32). 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 (26). It was found that the radical-scavenging activity of extract increased with increasing concentrations. IC_{50} for DPPH radical-scavenging activity was 58.9 ± 2.7 µg/ml. The IC_{50} values for ascorbic acid, quercetin and BHA were 3.7 ± 0.1, 3.9 ± 0.2 and 29.3 ± 5.9 µg ml^{-1}, respectively.

Metal chelating activity: Iron chelators mobilize tissue iron and cause its excretion therefore can reduces iron-related complications in human and improves quality of life in some diseases such as Thalassemia major and Alzheimer's disease (AD) pathology and so iron chelation could be considered a rational therapeutic strategy for AD (35). The transition metal, iron, is capable of generating free radicals from peroxides by Fenton reactions and may be implicated in human cardiovascular disease (36). Because Fe^{2+} also has been shown to cause the production of oxyradicals and lipid peroxidation, minimizing Fe^{2+} concentration in Fenton reactions affords protection against oxidative damage. In this assay, both extract and EDTA interfered with the formation of ferrous and ferrozine complex, suggesting that it has chelating activity and captures ferrous ion before ferrozine. The absorbance of Fe^{2+}-ferrozine complex was decreased dose dependently, i.e. the activity was increased on increasing concentrations from 0.4 to 1.6 mg ml^{-1}. Metal chelating capacity was significant since the extract reduced the concentrations of the catalyzing transition metal in lipid peroxidation (21). Extract showed weak Fe^{2+} chelating ability. IC_{50} were 393.4 ± 14.9 µg/ ml. EDTA showed very strong activity (IC_{50} = 4.5 µg ml^{-1}).

Nitric oxide-scavenging activity: The extract showed potent nitric oxide-scavenging activity between 25 and 400 µg ml^{-1}. The % inhibition was increased with increasing concentrations of the extract. IC_{50} was 69.7 ± 4.3 µg/ ml. IC_{50} for quercetin was 6.8 ± 0.25 µg/ ml. In addition to reactive oxygen species, nitric oxide is also implicated in inflammation, cancer and other pathological conditions (31, 32). The plant/plant 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 hydrogen peroxide:
The H2O2-scavenging of the extract may be attributed to its phenolic contents as well as other active components such as anthocyanins, tannins and flavonoids which can donate electrons to H2O2, thus neutralizing it to water (34). The extract was capable of scavenging hydrogen peroxide in a concentration-dependent manner. IC_{50} was 290.5 ± 21.1 µg/ml. The IC_{50} values for ascorbic acid and BHA were 14.9 and 36.4 µg ml^{-1}, respectively. 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 (33).

Reducing power: In this 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 then be monitored by measuring the formation of Perl's Prussian blue at 700 nm (34). Increasing absorbance at 700 nm indicates an increase in reductive ability. Fig. 1 shows the dose-response curves for the reducing power of the extract. Reducing power of extract increased with the increase of its concentrations (Fig 1).

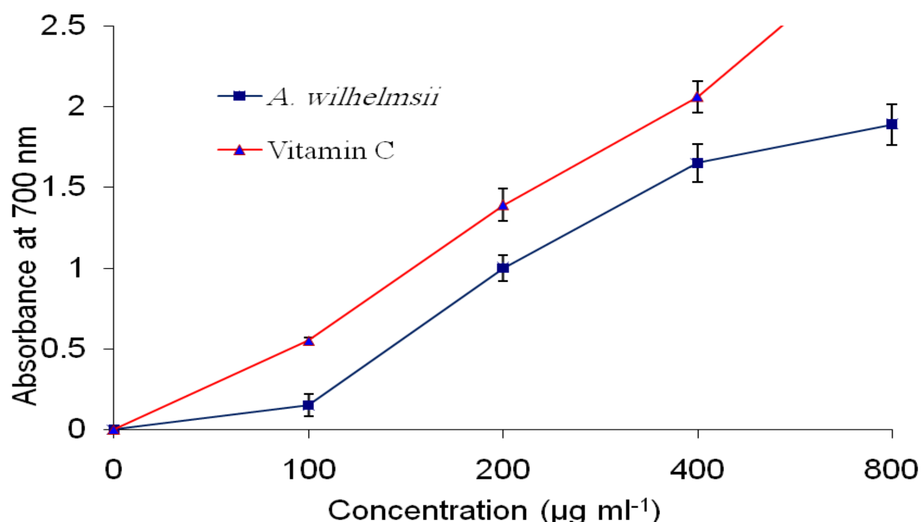

Fig 1. Reducing power of *A. wilhelmsii* extract.

Conclusions

Our studies indicate that the methanolic extract of *A. wilhelmsii* has good antioxidant that maybe result of its good phenol and flavonoid contents. It is promising for further phytopharmacological studies, which will be focused on evaluating other biological effects or improving mechanism of these effects.

Acknowledgements

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