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Full Length Research Paper

In vitro antioxidant analysis of *Achillea tenuifolia*

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Achillea tenuifolia **(AT) is one of the most herbs are being used by people as a traditional medicinal remedy. Antioxidant activity of AT different extracts and total flavonoid and phenol levels in the extracts were investigated in this study. Plant extracts were prepared by maceration method using ethyl acetate, methanol and methanol-water (1:1). Folin Ciocalteu reagent in terms of gallic acid equivalent achieved the total phenol's content. $AlCl_3$ was used as a reagent for flavonoid determination. Flavonoid content of the plant extracts obtained in terms of quercetin equivalent. DPPH radical scavenging effect of the extracts was determined by UV spectroscopy. Also in order to determine lipid peroxidation inhibition of the extracts of *A. tenuifolia,* ferric thiocyanate method with BHT, a synthetic reference standard, was carried out in this study. Phenol contents were 43.97 ± 0.034, 74.16 ± 0.55 and 106 ± 0.693 mg g^{-1} in the ethyl acetate, methanol and methanol-water extracts, respectively. Flavonoid amount obtained in the ethyl acetate, methanol and methanol-water extracts were 10.6 ± 1.85, 23.1 ± 0.5 and 190 ± 1.3 mg g^{-1}, respectively. The percentage of DPPH radical scavenged by the most active extract (methanol-water) of *A. tenuifolia* was 92% at a concentration of 1 $mgml^{-1}$ greater than 94% of BHT at 2 $mgml^{-1}$. IC_{50} of methanol-water extract and BHT were 0.015 and 0.053 $mgml^{-1}$, respectively. Lipid peroxidation inhibition was observed by the most polar extract of AT about 91.84%. Phenol and flavonoids content confirm the existence of more polar hydroxyl containing chemical structures in the plant. The potency of radical scavenging effect of methanol-water extract was about 3.5 times greater than synthetic antioxidant BHT. The inhibitory activity of the extracts on the lipid peroxidation of linoleic acid in ferric thiocyanate test was also significant (> 90%). In this study we concluded that there is a direct relation between phenol and flavonoid content of plant extracts and the antioxidant activity. So that the greater amount of phenolic compounds leads to more potent radical scavenging and lipid peroxidation inhibition activities as it was observed in *A. tenuifolia* polar extract in the present study.**

Key words: Flavonoids, phenols, *Achillea tenuifolia*, DPPH, lipid peroxidation.

INTRODUCTION

As a consequences of exposure to exogenous chemicals, the reactive derivatives of oxygen, ascribed as ROS are continuously generated inside the human body. Normally the ROS generated are detoxified by the antioxidants present in the body and there is equilibrium between the ROS generated and the antioxidants present. However, owing to ROS overproduction and/or inadequate anti-oxidant defense, this equilibrium is hampered favoring the ROS upsurge that culminates in oxidative stress (Farber, 1994). This oxidative damage is a crucial etiological factor implicated in several chronic human diseases. Epidemiological studies have found that the in-take of antioxidants reduces the risk of the diseases (Marchioli et al., 2001). The antioxidants may mediate their effect by directly reacting with ROS, quenching them and/or chelating the catalytic metal ions (Robak and Marcinkiewicz, 1995). Several synthetic antioxidants, e.g., butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are commercially available but are quite unsafe and their toxicity is a problem of concern.

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Abbreviations: DPPH, 1, 1-diphenyl-2-picryl hydrazyl radical; BHA, BHT, FTC, AT

Natural antioxidants, especially phenolics and flavonoids, are safe and also bioactive. Recently focus has been concentrated on identification of plants with antioxidant ability that may be used for human consumption (Madhavi and Salunkhe, 1995; Kaur et al., 2006; Pourmorad et al., 2006; Hosseinimehr et al., 2007). Besides, reports concerning the local uses and effects of the essential oils and extracts of several *Achillea* species have been cited in the literature (Candan et al., 2003; Konyalioglu and Karamenderes, 2005; Rezaeipoor et al., 1999). Nineteen species of the genus *Achillea* (Family: Asteraceae, Section: Santolinoidea) are distributed in Iran (Mozaffarian, 1996). As far as ethnopharmacologic background is concerned, *Achillea* used in folk remedies as an appetizer, wound healer, diuretic, carminative or menstrual regulator, anti fever, anti-inflammatory, asthma, bronchitis and cough reliever. We previously reported immunosuppressive activity of *Achillea talagonica* (Rezaeipoor et al., 1999). To the best of our knowledge, information concerning the *in vitro* antioxidant features of *Achillea tenuifolia* has not been found in the literature. As a part of our continuing research project on *Achillea* species, an *in vitro* antioxidant activity of three various extracts with different polarity of was carried out in this study.

MATERIALS AND METHODS

Chemicals

Gallic acid, DPPH, quercetin, BHA and BHT were purchased from Merck and Fluka. All other chemicals and reagents used were of the highest commercially available purity.

Plant material

A. tenuifolia aerial parts was collected in January 2007 and authenticated by the University botanist Dr. I. Mehregan. A voucher specimen, number 239, was deposited at the herbarium section of the faculty. It was dried at room temperature and a successive extraction was performed by maceration method in ethyl acetate, methanol and methanol-water (1:1). The extracts were collected after removing the solvent and lyophilization.

Total flavonoids determination

Aluminum chloride colorimetric method was used for flavonoid determination (Chang et al., 2002). Each plant extracts (0.5 ml of 1, 2, 4 $mgml^{-1}$ methanol and methanol-water and 2.5, 5, 10 $mgml^{-1}$ ethyl acetate 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. It remained at room temperature for 30 min; the absorbance of the reaction mixture was measured at 415 nm with a double beam Perkin Elmer UV/Visible spectrophotometer (USA). The calibration curve was prepared by preparing quercetin solutions at concentrations of 12.5 to 100 μgml^{-1} in methanol.

Total phenols determination

Total phenols were determined by Folin Ciocalteu reagent (McDonald et al., 2001). A dilute solution of each plant extract (0.5 ml of 1, 2, 4 $mgml^{-1}$ methanol and methanol-water and 2.5, 5, 10 $mgml^{-1}$ ethyl acetate extract) or gallic acid was mixed with Folin Ciocalteu reagent (5 ml, 1:10 diluted with distilled water) and aqueous Na_2CO_3 (4 ml, 1 M). The mixtures were allowed to stand for 15 min and the total phenols were determined by colorimetric method at 765 nm. The standard curve was prepared using 50 to 250 $mgml^{-1}$ solutions of gallic acid in methanol-water (1:1, v/v). Total phenol values are expressed in terms of gallic acid equivalent (mg g^{-1} of dry mass), which is a common reference phenolic compound.

DPPH assay

The stable 1,1-diphenyl-2-picryl hydrazyl radical (DPPH) was used for determination of free radical-scavenging activity of the extracts (Koleva et al., 2002). 1 ml of different concentrations of each herbal extract (0.01, 0.5, 1, 2 $mgml^{-1}$ of methanol and methanol-water extracts and 0.1, 0.5, 1, 2, 4 $mgml^{-1}$of ethyl acetate extract) were added, at an equal volume, to methanolic solution of DPPH (0.15 mM). After 15 min at room temperature, the absorbance was recorded at 517 nm. The experiment was repeated for three times. BHT was used as standard controls in 0.01, 0.1, 0.5, 2 mg ml^{-1}. Inhibition percent was calculated by following formula. IC50 values denote the concentration of sample, which is required to scavenge 50% of DPPH free radicals.

[Control (containing all reagent without extract) absorption – test absorbance / control absorption] x 100

Antioxidant activity by ferric thiocyanate (FTC) method

The FTC method was adopted from Kikuzaki et al. (Kikuzaki and Nakatani, 1993). To 2 ml of different concentrations of the extracts and BHT (20, 40, 60 μgml^{-1}) 2 ml of linoleic acid (2.51%, w/v in ethanol), 4 ml of phosphate buffer (pH = 7) and 2 ml distilled water were added and incubated at 40°C for 96 h . To 0.1 ml of this solution was then added 9.7 ml of 75% (v/v) ethanol and 0.1 ml of 30% (w/v) ammonium thiocyanate. 3 min after the addition of 0.1 ml of 20 mM ferrous chloride in 3.5% (v/v) hydrochloric acid to the reaction mixture, the absorbance was measured at 500 nm every 24 h until 1 day after absorbance of the control reached its maximum value.

Statistical analyses

All values are expressed as mean ± S.E. Statistical analyses were performed by Student's t-test. The values of p lower than 0.05 were considered significant (p is probability).

RESULTS AND DISCUSSION

Flavonoid and total phenol contents of the extracts

It has been recognized that flavonoids show antioxidant activity and their effects on human nutrition and health are considerable. The mechanisms of action of flavonoids are through scavenging or chelating process (Kessler et. al., 2003; Cook and Samman, 1996). Phenolic compounds are a class of antioxidant compounds which act as free radical terminators (Shahidi and Wanasundara,

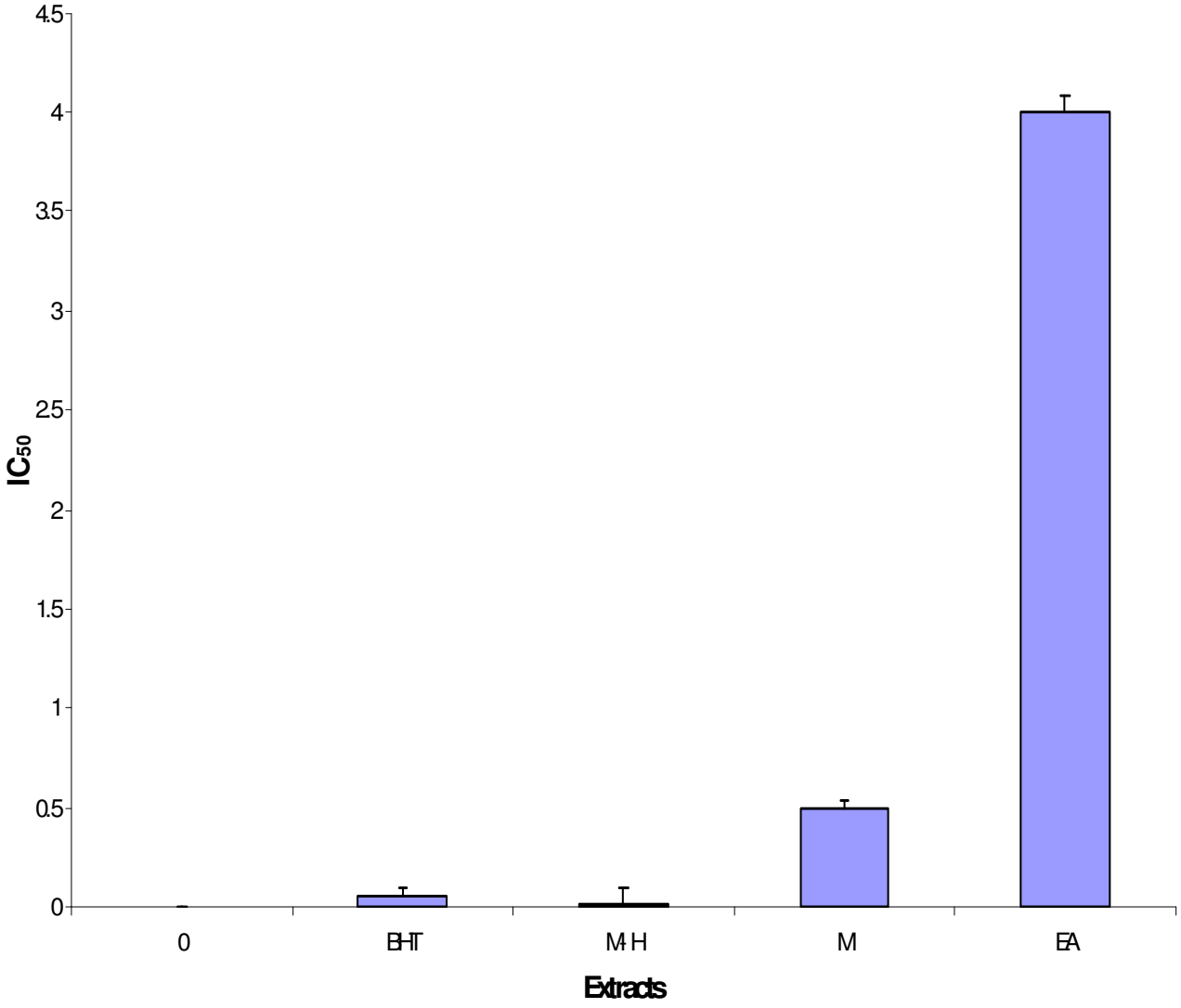

Figure 1. IC_{50} of ethyl acetate (EA), methanol (M) and methanol- water (1:1) (M- H) extracts of *Achillea tenuifolia* compared with BHT as a reference standard in DPPH radical scavenging test. Each value is presented as mean ± S.E. (n = 3).

1992). The flavonoid content of the ethyl acetate, methanol and methanol-water (1:1) extracts in terms of quercetin equivalent were 10.6 ± 1.85, 23.1 ± 0.5 and 190 ± 1.3 mg g^{-1}respectively. Total phenols measured by Folin Ciocalteu reagent in terms of gallic acid equivalent. The total phenol obtained 43.97 ± 0.034, 74.16 ± 0.55 and 106 ± 0.693 mg g^{-1}in ethyl acetate, methanol and methanol-water extracts. The compounds such as flavonoids, which contain hydroxyl functional groups, are responsible for the radical scavenging effect in the plants (Das and Pereira, 1990; Younes, 1981). According to our study, the contents of these phytochemical compounds in AT extracts can explain its antioxidant activity. So in this study there is a direct relation between total phenol and flavonoid content and polarity of the extraction solvent. Methanol-water fraction showed the highest activity according to its active compounds content compared with ethyl acetate and methanol extracts ($p < 0.05$).

Antioxidant activity

Free radicals are involved in many disorders like neurodegenerative diseases, cancer and AIDS (Halliwell, 1994). Antioxidants through their scavenging power are useful for the management of those diseases. DPPH stable free radical method is an easy, rapid and sensitive way to survey the antioxidant activity of a specific compound or plant extracts (Koleva et al., 2002). The capacity of different extracts of AT to scavenge DPPH was measured and the results are shown in Figure 1. The antioxidants react with DPPH, a purple colored stable free radical and convert it into a colorless α-α-diphenyl-β-picryl hydrazine. The amount of DPPH reduced could be

Table 1. Lipid peroxidation inhibition (LPI %*, capacity to inhibit the peroxide formation in linoleic acid) of ethyl acetate (EA, 40 µgml^{-1}), methanol (M, 40 µgml^{-1}) and methanol- water (M- H, 20 µgml^{-1}) extracts of *Achillea tenuifolia* and BHT(20 µgml^{-1}) in ferric thiocyanate method.

Time (h)	EA LPI%	M LPI%	M-H LPI%	BHT LPI%
0	4 ± 0.01**	3 ± 0.02	5 ± 0.012	10 ± 0.054
12	53 ± 0.013	50 ± 0.03	58 ± 0.098	54 ± 0.02
24	68 ± 0.02	67 ± 0.034	67 ± 0.087	63 ± 0.2
36	81 ± 0.021	78 ± 0.076	83 ± 0.1	82 ± 0.032
48	84 ± 0.07	81 ± 0.035	83 ± 0.088	84 ± 0.06
60	87 ± 0.04	85 ± 0.079	89 ± 0.03	85 ± 0.13
72	95 ± 0.06	91 ± 0.032	95 ± 0.054	93 ± 0.08
84	94 ± 0.12	90 ± 0.09	95 ± 0.076	90 ± 0.16

* LPI% = (extract absorbance - control absorbance/ control absorbance) x 100.
** Results are presented as mean ± S.E (n = 5).

quantified by measuring a decrease in absorbance at 517 nm. AT extracts reduced DPPH radicals in a dose dependent manner. IC_{50} of the standard compound, BHA and the extracts were 0.053, 4, 0.5 and 0.015 mgml^{-1} respectively (Figure 1). Ethyl acetate, methanol, methanol-water extracts and BHT at concentrations of 4, 2, 1, 2mgml^{-1} scavenged about 50, 93, 92 and 94% of DPPH radicals. The extracts showed comparable activity to the control in this study. So the DPPH scavenging ability of the extracts may be attributed to its hydrogen donating ability that probably shows the role of phenols and flavonoids existing in the extracts.

FTC Method

Membrane lipids are rich in unsaturated fatty acids that are most susceptible to oxidative processes. Specially, linoleic acid and arachidonic acid are targets of lipid peroxidation (Yu, 2001). The inhibition of lipid peroxidation by AT maybe due to their free radical-scavenging activities as its significant effect in scavenging of DPPH has shown in this study. Table 1 shows lipid peroxidation inhibition percent obtained in FTC method. Methanol-water extract, the most polar extract of AT, showed a higher potency than ethyl acetate and methanol extracts at 20 µgml^{-1}. Methanol- water extract exhibited about 95% inhibition like BHT but ethyl acetate and methanol extracts' inhibitory effects were about 3.5 and 1.1% at 20 µgml^{-1}. Figure 2 also illustrates antioxidant activity of 40, 40, 20 and 20 µg/ml of AT extracts and BHT, determined according to ferric thiocyanate method. All extracts showed significant antioxidant activity in this test in a proper concentration.

Conclusion

Plants showing potent antioxidant activity may be used as a safer source for inhibition of oxidative reactions which are being under investigation (Ebrahimzadeh et al., 2008a, b). Free radicals are often generated as by products of biological reactions or from exogenous factors and cause damage on biological molecules like membrane lipids. The involvements of free radicals in the pathogenesis of a large number of diseases are well documented. A potent scavenger of free radicals may serve as a possible preventative intervention for the diseases (Gyamfi et al., 1999; Aboul- Enein et al., 2003). In the present study *A. tenuifolia* extracts has shown potent antioxidant activity. Its constituents scavenge free radicals, may exert a protective effect against oxidative damage induced to cellular macromolecules. It was mentioned that it has been used as an effective remedy in folk medicine. The preliminary chemical examination of the extracts has demonstrated the presence of phenols and flavonoids, which may be responsible of the anti-oxidant and lipid peroxidation inhibition activities. The high scavenging property of AT may be due to hydroxyl groups existing in the phenolic compounds' chemical structure that can provide the necessary component as a radical scavenger. Further studies on the isolation of active compounds of AT are in progress.

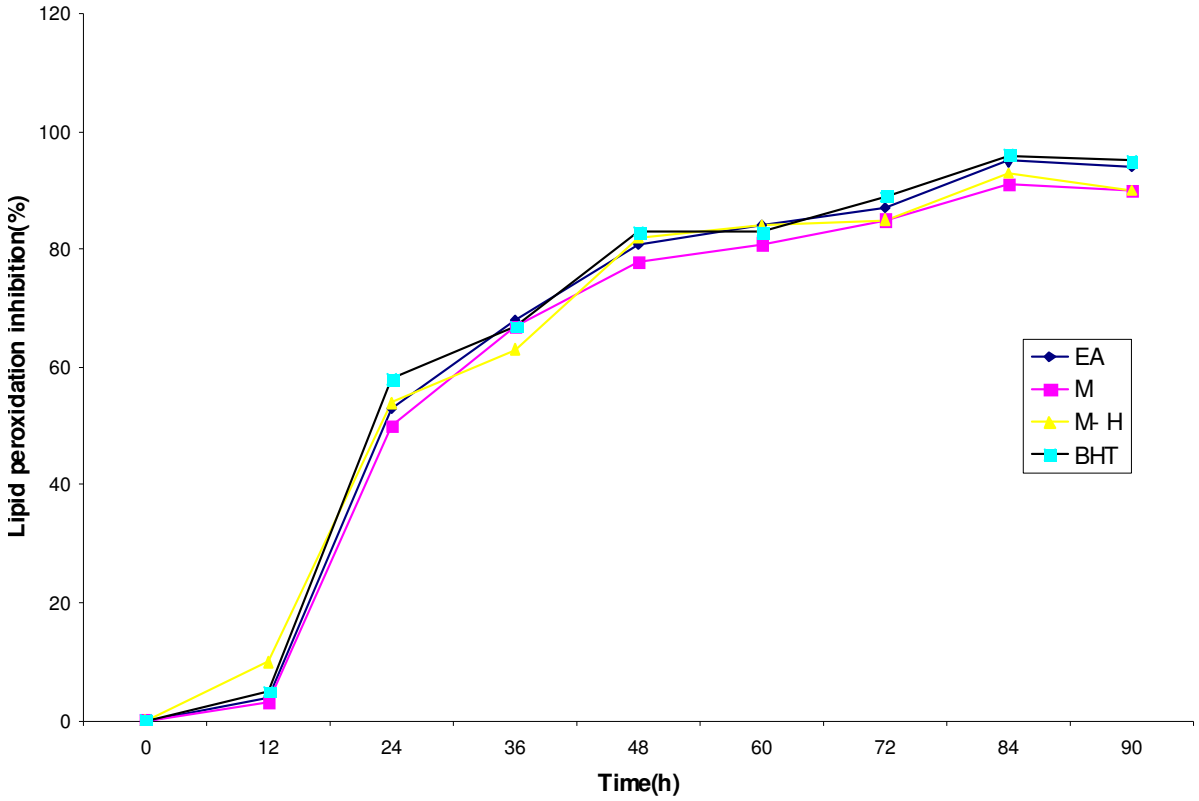

Figure 2. Antioxidant activity of ethyl acetate (EA, 40 µgml^{-1}), methanol (M, 40 µgml^{-1}) and methanol-water (M-H, 20 µgml^{-1}) extracts of *Achillea tenuifolia* and BHT (20 µg ml^{-1}) in FTC method.

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