Asian Journal of Chemistry; Vol. 23, No. 7 (2011), 3172-3176

ASIAN JOURNAL OF CHEMISTRY

www.asianjournalofchemistry.co.in

Determination of Antioxidant Properties of Ethanol and Water Extracts of *Achillea millefolium* L. (Yarrow)

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(*Received*: 3 November 2010; *Accepted*: 18 March 2011) AJC-9756

Achillea millefolium L. is a member of the Asteraceae family that is commonly referred to as 'yarrow' and has been used in folk medicine against several disturbances including skin inflammations, spasmodic and gastrointestinal disorders, as well as hepato-biliary complaints. Ethanol and water extracts were prepared from powdered *A. millefolium* flowers, leaves and seeds. In this study, antioxidant properties were studied by 2,2-azino-*bis*(3-ethylbenzthiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) radical scavenging, 1,1-diphenyl-2-picryl-hydrazyl free radical ($DPPH^{\bullet}$) scavenging, superoxide anion ($O_2^{\bullet-}$) radical scavenging, reducing activity and ferrous metal chelating activity assays. In conclusion, *A. millefolium* flowers, leaves and seeds had effective $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ radical scavenging, superoxide anion radical scavenging, reducing power and metal chelating activities. Additionally, these various antioxidant activities were compared with BHA, BHT and α-tocopherol as reference antioxidants.

Key Words: *Achillea millefolium*, Yarrow, Antioxidant capacity, $DPPH^{\bullet}$, $ABTS^{\bullet+}$, Superoxide radical.

INTRODUCTION

Achillea millefolium L. is a member of the Asteraceae family that is commonly referred to as "yarrow". *Achillea millefolium* has been used in folk medicine against several disturbances including skin inflammations, spasmodic and gastrointestinal disorders and hepato-biliary complaints[1]. Phytochemical studies carried out with *Achillea millefolium* have identified several components, including essential oils, sesquiterpenes and phenolic compounds such as flavonoids and phenolcarbonic acids[2]. Moreover, the presence of sesquiterpene lactones, azulene, flavonoids such as apigenin, luteolin and rutin sustains the pharmacological activity of *Achillea millefolium*[3]. Different preparations of *Achillea millefolium* have demonstrated antiinflammatory, antitumor, antimicrobial, liver protective and antioxidant properties[4-9].

The aim of this study is to determine antioxidant properties of water and ethanol extracts of *Achillea millefolium* leaves, flowers and seeds.

EXPERIMENTAL

Plant materials and extraction procedures: *Achillea millefolium* leaves, flowers and seeds were obtained from Mus in Turkey. For ethanol extraction, 25 g powder of *A. millefolium* leaves, flowers and seeds ground into a fine powder in a mill and were mixed five times with 100 mL ethanol. Extraction continued until the extraction solvents became colourless (total solvent volume 400 mL). The obtained extracts were filtered over Whatman No.1 paper and the filtrate was collected, then ethanol was removed by a rotary evaporator at 50 °C.

For preparation of water extraction, 25 g of *A. millefolium* leaves, flowers and seeds ground into a fine powder in a mill and was mixed with 500 mL boiling water by magnetic stirrer during 10 min. Then the extract was filtered over Whatman No.1 paper and then water was removed by a rotary evaporator at 50 °C.

$ABTS^{\bullet+}$ radical scavenging capacity: ABTS also forms a relatively stable free radical, which decolourizes in its non-radical form Shirwaikar *et al.*[10]. The spectrophotometric analysis of $ABTS^{\bullet+}$ radical scavenging capacity was determined according to the method of Re *et al.*[11]. $ABTS^{\bullet+}$ was produced by reacting 2 mM ABTS in H_2O with 2.45 mM $K_2S_2O_8$, stored for 12 h at room temperature in the dark. The $ABTS^{\bullet+}$ solution was diluted to give an absorbance of 1.770 ± 0.025 at 734 nm in 0.1 M sodium phosphate buffer (pH 7.4). Then, 1 mL of $ABTS^{\bullet+}$ solution was added to 3 mL of *A. millefolium* extracts

in ethanol at 100 µg/mL concentrations. The absorbance was recorded for 0.5 h, after the mixing and percentage of radical scavenging were calculated for each concentration relative to a blank containing no scavenger. The extent of decolourization is calculated as percentage reduction of absorbance. The scavenging capability of test compounds was calculated by the following equation:

$$ABTS^{\bullet+} \text{ scavenging effect} = [(A_0 - A_1)/A_0] \times 100$$

where A_0 is the absorbance of control and A_1 is the absorbance in the presence of the sample of *A. millefolium* extracts or standards.

DPPH$^\bullet$ radical scavenging capacity: The free radical scavenging capacity of *A. millefolium* extracts was measured by 2,2-diphenyl-1-picryl-hydrazil (DPPH$^\bullet$) using the method of Shimada *et al.*[12]. Briefly, 0.1 mM solution of DPPH$^\bullet$ in ethanol was prepared and 1 mL of this solution was added to 3 mL of *A. millefolium* extracts solution in water at different concentrations (50, 100, 250 and 500 µg/mL). Absorbance at 517 nm was determined 0.5 h later against a blank solution containing the ethanol. Lower absorbance of the reaction mixture indicates the higher free radical scavenging activity. When a hydrogen atom or electron was transferred to the odd electron in DPPH$^\bullet$, the absorbance at 517 nm is decreased proportionally to the increase of non-radical forms of DPPH[13]. The capability to scavenge the DPPH$^\bullet$ radical was calculated by the following equation:

$$DPPH^\bullet \text{ scavenging effect } (\%) = [(A_0 - A_1)/A_0] \times 100$$

where A_0 is the absorbance of control reaction and A_1 is the absorbance in the presence of the sample of *A. millefolium* extracts or standards.

Superoxide anion scavenging capacity: Measurement of superoxide anion scavenging capacity of *A. millefolium* extracts was based on the method described by Liu *et al.*[14] with slight modification. 1 mL of nitroblue tetrazolium (NBT) solution (156 mmol/L NBT in 100 mmol/L phosphate buffer, pH 7.4), 1 mL NADH solution (468 mmol/L in 100 mmol/L phosphate buffer pH 7.4) and 100 µL of sample solution of *A. millefolium* extracts in water were mixed. The reaction was started by adding 100 µL of phenazine methosulphate (PMS) solution (60 mmol/L PMS in 100 mmol/L phosphate buffer, pH 7.4) to the mixture. The reaction mixture was incubated at 25 °C for 5 min and the absorbance at 560 nm was measured against blank samples. Decreased absorbance of the reaction mixture shows increase in superoxide anion scavenging capacity. The percentage inhibition of superoxide anion generation was calculated by the following formula:

$$\% \text{ Superoxide anion scavenging effect} = [(A_0 - A_1)/A_0] \times 100$$

where A_0 is the absorbance of the control and A_1 is the absorbance of *A. millefolium* extracts or standards[15].

Metal chelating activity: The chelating of ferrous ions by the *A. millefolium* extracts and standards was estimated by the method of Dinis *et al.*[16]. Briefly, extracts (50, 100, 250 and 500 µg/mL) were added to a solution of 2 mM $FeCl_2$ (0.05 mL). The reaction was started by addition of 5 mM ferrozine (0.2 mL) and the mixture was shaken vigorously and left standing at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 562 nm. All test and analyses were run in triplicate and averaged. The percentage of inhibition of ferrozine-Fe^{2+} complex formation was calculated by the following formula:

$$\text{Metal chelating } (\%) = [(A_0 - A_1)/A_0] \times 100$$

where A_0 is the absorbance of control and A_1 is the absorbance in the presence of the sample of *A. millefolium* extracts or standards. The control does not contain $FeCl_2$ and ferrozine, complex formation molecules.

Total reduction activity: The samples prepared for ferric thiocyanate method was used for the present and other antioxidant assays. The reducing activities of *A. millefolium* extracts were determined by the method of Oyaizu[17]. The capacity of *A. millefolium* extracts to reduce the ferric-ferricyanide complex to the ferrous-ferricyanide complex of prussian blue was determined by recording the absorbance at 700 nm after incubation. Simply, different concentrations of *A. millefolium* extracts (50-500 µg/mL) in 1 mL of distilled water were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide $K_3[Fe(CN)_6]$ (2.5 mL, 1 %). The mixture was incubated at 50 °C for 20 min. Aliquots (2.5 mL) of trichloroacetic acid (10 %) were added to the mixture, which was then centrifuged for 10 min at 1000×g. 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 in a spectrophotometer. In the control, the sample was replaced with an equal volume of ethanol. Increased absorbance of the reaction mixture indicates grater reduction capability[18].

RESULTS AND DISCUSSION

ABTS$^{\bullet+}$ radical scavenging capacity: The improved technique for the generation of ABTS$^{\bullet+}$ described here involves the direct production of the blue/green ABTS$^{\bullet+}$ chromophore through reaction between ABTS and potassium persulfate. As shown in Fig. 1 and Table-1, *A. millefolium* extracts had ABTS$^{\bullet+}$ radical scavenging activity. The scavenging effect of *A. millefolium* extracts and standards on the ABTS$^{\bullet+}$ decreased in the order: BHA > ethanol extract of *A. millefolium* flower > BHT > α-tocopherol > water extract of *A. millefolium* leaf > ethanol extract of *A. millefolium* seed > water extract of *A. millefolium* flower > ethanol extract of *A. millefolium* leaf >

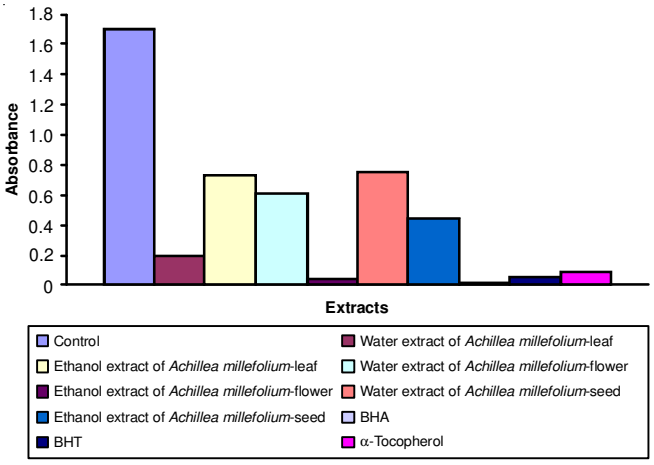

Fig. 1. ABTS assay results of *Achillea millefolium* and some standard antioxidants

TABLE-1
ABTS ASSAY RESULTS OF *A. millefolium* AND SOME STANDARD ANTIOXIDANTS (%)

Extracts (100 µg/mL)	ABTS scavenging activity (%)
Control	0
Water extract of *Achillea millefolium*-leaf	88.00
Ethanol extract of *Achillea millefolium*-leaf	56.90
Water extract of *Achillea millefolium*-flower	63.90
Ethanol extract of *Achillea millefolium*-flower	97.40
Water extract of *Achillea millefolium*-seed	55.76
Ethanol extract of *Achillea millefolium*-seed	74.30
BHA	98.70
BHT	96.41
α-Tocopherol	95.05

water extract of *A. millefolium* seed, which were 98.7, 97.4, 96.4, 95.05, 88, 74.3, 63.9, 56.9 and 55.8 %, respectively, at the 100 µg/mL concentration.

DPPH• radical scavenging capacity: The model of scavenging the stable DPPH• radical is a widely used method to evaluate antioxidant activities in a relatively short time compare with other methods. The effect of antioxidants on DPPH• radical scavenging was thought to be due to their hydrogen donating ability. DPPH• is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule[19].

The reduction capability of DPPH• radicals was determined by the decrease in its absorbance at 517 nm induced by antioxidants. The decrease in absorbance of DPPH• radical caused by antioxidants, because of the reaction between antioxidant molecules and radical progresses, which results in the scavenging of the radical by hydrogen donation. It is visually noticeable as a discolouration from purple to yellow. Hence, DPPH• is usually used as a substrate to evaluate antioxidative activity of antioxidants. The scavenging effect of *A. millefolium* extracts and standards on the DPPH• decreased in the order: Trolox > gallic acid > ethanol extract of *A. millefolium* flower > quercetin > water extract of *A. millefolium* flower > ethanol extract of *A. millefolium* leaf > BHT > water extract of *A. millefolium* seed > Pyrocatechol > water extract of *A. millefolium* leaf > ethanol extract of *A. millefolium* seed, which were 95.65, 93.84, 91.03, 90.48, 89.13, 88.31, 85.77, 85.41, 84.23, 80.79 and 79.94 %, respectively, at the 500 µg/mL concentration (Fig. 2, Table-2).

TABLE-2
DPPH RADICAL SCAVENGING RESULTS OF *A. millefolium* AND SOME STANDARD ANTIOXIDANTS (%)

Samples (500 µg/mL)	Scavenging activity DPPH• (%)
Control	0
Water extract of *Achillea millefolium*-leaf	80.79
Ethanol extract of *Achillea millefolium*-leaf	88.31
Water extract of *Achillea millefolium*-flower	89.13
Ethanol extract of *Achillea millefolium*-flower	91.03
Water extract of *Achillea millefolium*-seed	85.41
Ethanol extract of *Achillea millefolium*-seed	79.94
BHT	85.77
Trolox	95.65
Gallic acid	93.84
Quercetin	90.48
Pyrocatechol	84.23

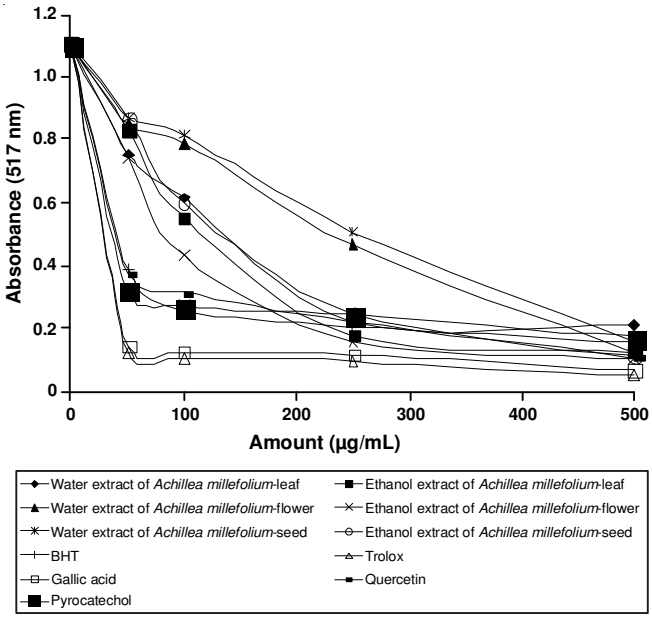

Fig. 2. DPPH radical scavenging results of *A. millefolium* extracts

Superoxide anion scavenging capacity: In this method, superoxide anion derived from dissolved oxygen by PMS-NADH coupling reaction reduces the yellow dye (NBT^{2+}) to produce the blue formazan, which is measured spectrophotometrically at 560 nm. Antioxidants are able to inhibit the blue NBT formation[20,21]. The decrease of absorbance at 560 nm with antioxidants indicates the consumption of superoxide anion in the reaction mixture. Fig. 3 and Table-3 shows that the percentage inhibition of superoxide radical generation by 100 µg/mL concentration of *A. millefolium* extracts and standards were found to be similar. As can be seen in Table-3, the scavenging effect of *A. millefolium* extracts and standards on the percentage inhibition of superoxide anion radical generation by 100 µg/mL concentration in the order: BHA > water extract of *A. millefolium* seed > water extract of *A. millefolium* flower > water extract of *A. millefolium* leaf > BHT > ethanol extract of *A. millefolium* leaf > α-tocopherol > ethanol extract of *A. millefolium* flower > ethanol extract of *A. millefolium* seed which were 94.83, 90.67, 89.66, 88.83, 82.67, 77.33, 59.67, 58.33 and 40 % superoxide anion radical scavenging activity, respectively.

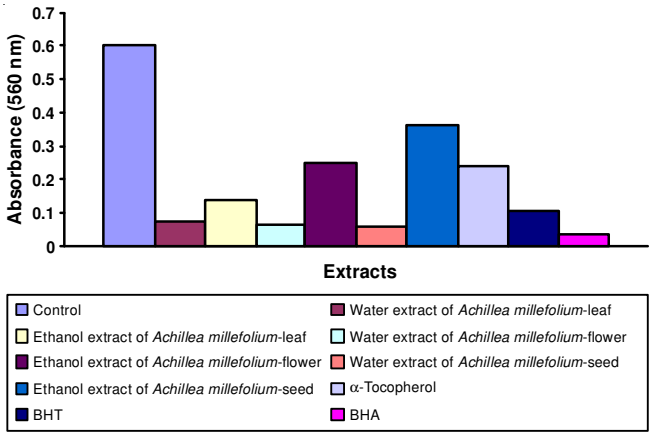

Fig. 3. Superoxide radical scavenging results of *A. millefolium* extracts and some antioxidants

TABLE-3
SUPEROXIDE RADICAL SCAVENGING RESULTS OF *A. millefolium* AND SOME STANDARD ANTIOXIDANTS (%)

Extracts (100 µg)	% Superoxide anion scavenging activity
Control	0
Water extract of *Achillea millefolium*-leaf	88.83
Ethanol extract of *Achillea millefolium*-leaf	77.33
Water extract of *Achillea millefolium*-flower	89.66
Ethanol extract of *Achillea millefolium*-flower	58.33
Water extract of *Achillea millefolium*-seed	90.67
Ethanol extract of *Achillea millefolium*-seed	40.00
α-Tocopherol	59.67
BHT	82.67
BHA	94.83

TABLE-4
METAL CHELATING ACTIVITY RESULTS OF *A. millefolium* AND SOME STANDARD ANTIOXIDANTS (%)

Extracts (100 µg)	% Metal chelating activities
Control	0
Water extract of *Achillea millefolium*-leaf	50.94
Ethanol extract of *Achillea millefolium*-leaf	36.00
Water extract of *Achillea millefolium*-flower	53.88
Ethanol extract of *Achillea millefolium*-flower	44.47
Water extract of *Achillea millefolium*-seed	65.76
Ethanol extract of *Achillea millefolium*-seed	22.94
α-Tocopherol	63.52
BHA	66.23
BHT	61.52

Metal chelating activity: Ferrous ion chelating activities of *A. millefolium* extracts, α-tocopherol, BHA and BHT are shown in Fig. 4 and Table-4. The chelating effect of ferrous ions by *A. millefolium* extracts and standards was determined according to the method of Dinis *et al.*[16]. Among the transition metals, iron is known as the most important lipid oxidation pro-oxidant due to its high reactivity. The ferrous state of iron accelerates lipid oxidation by breaking down hydrogen and lipid peroxides to reactive free radicals *via* the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\bullet}$). Fe^{3+} ion also produces radicals from peroxides, although the rate is tenfold less than that of Fe^{2+} ion[22]. Fe^{2+} ion is the most powerful pro-oxidant among various species of metal ions[23]. The chelating of ferrous ions by *A. millefolium* extracts was estimated by the ferrozine assay. Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of chelating agents, the complex formation is inhibited and the red colour of the complex fades. By measuring the colour reduction, therefore, it is possible to estimate the chelating activity of the co-existing chelator[24]. In this assay, the natural compound interfered with the formation of the ferrozine-Fe^{2+} complex, suggesting that it has chelating activity and captures ferrous ions before ferrozine.

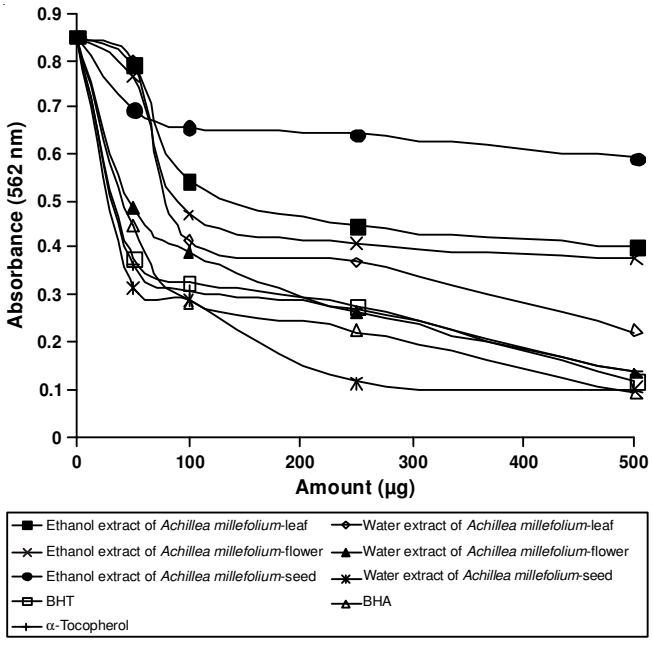

Fig. 4. Metal chelating activity results of *A. millefolium* extracts and some antioxidants

Fig. 4 shows that the chelation of ferrous ion by 0-500 µg/mL concentration of *A. millefolium* extracts and standards were found to be similar. As can be seen in Table-4, the chelating effect of *A. millefolium* extracts and standards on the percentage chelating of ferrous by 100 µg/mL concentration in the order: BHA > water extract of *A. millefolium* seed > α-tocopherol > BHT > water extract of *A. millefolium* flower > water extract of *A. millefolium* leaf > ethanol extract of *A. millefolium* flower > ethanol extract of *A. millefolium* leaf > ethanol extract of *A. millefolium* seed which were 66.23, 65.76, 63.52, 61.52, 53.88, 50.94, 44.47, 36.00 and 22.94 % metal chelating activity, respectively.

Total reduction activity: Fig. 5 depicts the reducing activity of the *A. millefolium* extracts and standards (BHA, BHT, α-tocopherol) using the potassium ferricyanide reduction method. For the measurements of the reductive activity, the Fe^{3+}-Fe^{2+} transformation was investigated in the presence of *A. millefolium* extracts using the method of Oyaizu[17]. The reducing activity of *A. millefolium* extracts, α-tocopherol, BHA and BHT increased with increasing concentration of samples. As can be seen in the Fig. 5, *A. millefolium* extracts showed more effective reducing activity than control at different concentrations. Reducing power of *A. millefolium* extracts and standard compounds are as follows: BHA > BHT > α-tocopherol > ethanol extract of *A. millefolium* flower > ethanol

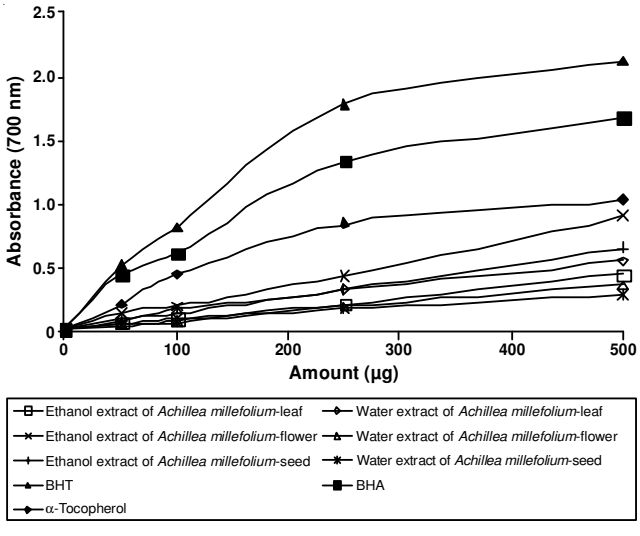

Fig. 5. Total reduction activity results of *A. millefolium* extracts and some antioxidants

extract of *A. millefolium* seed > water extract of *A. millefolium* leaf > ethanol extract of *A. millefolium* leaf > water extract of *A. millefolium* flower > water extract of *A. millefolium* seed.

Conclusion

This study demonstrated the potential antioxidant properties of the *A. millefolium* extracts, from Mus in Turkey. According to data of the present study, *A. millefolium* extracts were found to be effective antioxidants in different *in vitro* assays including reducing power, DPPH$^{\bullet}$ scavenging, $ABTS^{\bullet+}$ scavenging and superoxide anion radical scavenging and metal chelating activities when compared to standard antioxidant compounds, such as synthetic antioxidants (BHA, BHT), α-tocopherol, a natural antioxidant and trolox, which is a water-soluble analog of tocopherol.

ACKNOWLEDGEMENTS

This work was supported by TUBITAK, under Grant No. 107T898 and also supported by Firat University, under Grant Nos. FÜBAP 1936 and FÜBAP 1937.

REFERENCES

1. B. Benedek and B. Koop, *Wiener Medizinische Wochenschrift*, **157**, 312 (2007).
2. B. Benedek, B. Koop and M.F. Melzig, *J. Ethnopharmacol.*, **113**, 312 (2007).
3. N. Kocevar, I. Glavac, R. Injac and S. Kreft, *J. Pharm. Biomed. Anal.*, **46**, 609 (2008).
4. A.S. Goldberg, E.C. Mueller, E. Eigen and S.J. Desalva, *J. Pharm. Sci.*, **58**, 938 (1969).
5. T. Tozyo, Y. Yoshimura, K. Sakurai, N. Uchida, Y. Takeda, H. Nakai and H. Ishii, *Chem. Pharm. Bull.*, **42**, 1096 (1994).
6. C. Gagdoli and S.H. Mishra, *Fitoterapia*, **66**, 319 (1995).
7. H. Tunon, C. Olavsdotter and L. Bohlin, *J. Ethnopharmacol.*, **48**, 61 (1995).
8. L.T. Lin, L.T. Liu, L.C. Chiang and C.C. Lin, *Phytother. Res.*, **16**, 440 (2002).
9. F. Candan, M. Unlu, B. Tepe, D. Daferera, M. Polissiou, A. Sökmen and H.A. Akpulat, *J. Ethnopharmacol.*, **87**, 215 (2003).
10. A. Shirwaikar, A. Shirwaikar, K. Rajendran and I.S.R. Punitha, *Biol. Pharm. Bull.*, **29**, 1906 (2006).
11. R. Re, N. Pellegrini, A. Proteggente, A. Pannala, M. Yang and C. Rice-Evans, *Free Radicals Biol. Med.*, **26**, 1231 (1999).
12. K. Shimada, K. Fujikawa, K. Yahara and T. Nakamura, *J. Agric. Food Chem.*, **40**, 945 (1992).
13. I. Gulcin, M. Elmastas and H.Y. Aboul-Enein, *Phytother. Res.*, **21**, 354 (2007).
14. F. Liu, V.E.C. Ooi and S.T. Chang, *Life Sci.*, **60**, 763 (1997).
15. D. Ye, J.A. Pospisilik and D.A. Mathers, *Br. J. Pharm.*, **129**, 1035 (2000).
16. T.C.P. Dinis, V.M.C. Madeira and L.M. Almeida, *Arch. Biochem. Biophys.*, **315**, 161 (1994).
17. M. Oyaizu, *Jpn. J. Nutr.*, **44**, 307 (1986).
18. M. Elmastas, I. Gulcin, S. Beydemir, O.I. Kufrevioglu and H.Y. Aboul-Enein, *Anal. Lett.*, **39**, 47 (2006).
19. I. Gülçin, I.G. Sat, S. Beydemir, M. Elmastas, O.I. Küfrevioglu, *Food Chem.*, **87**, 393 (2004).
20. P. Cos, L.Y. Ying, M. Calomme, J.H. Hu, K. Cimanga, B. Van Poel, L. Pieters, A.J. Vlietinck and D.V. Berghe, *J. Nat. Prod.*, **61**, 71 (1998).
21. I. Parejo, F. Viladomat, J. Bastida, A. Rosas-Romero, N. Flerlage, J. Burillo and C. Codina, *J. Agric. Food Chem.*, **50**, 6882 (2002).
22. D.D. Miller, in ed.: O.R. Fennema, Mineral, In: Food Chemistry, Marcel Deckker, New York, USA (1996).
23. B. Halliwell and J.M.C. Gutteridge, *Biochem. J.*, **219**, 1 (1984).
24. F. Yamaguchi, T. Ariga, Y. Yoshimira and H. Nakazawa, *J. Agric. Food Chem.*, **48**, 180 (2000).