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Full Length Research Paper

A comparative study on the antioxidant activity of fringe tree (*Chionanthus virginicus* L.) extracts

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Fringe tree (*Chionanthus virginicus* L.) is used as a raw material by pharmaceutical industries for the preparation of homeopathy tinctures. In this study, antioxidant activity of methanol extract (MEFT) and ethylacetate extract (EEFT) from root bark of fringe tree (*C. virginicus* L.) were evaluated. 2,2-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical scavenging, 1,1-diphenyl-2-picryl-hydrazyl free radical (DPPH·) scavenging, superoxide anion ($O_2^{\bullet-}$) radical scavenging, total antioxidant activity, reducing activity, hydrogen peroxide (H_2O_2) scavenging and ferrous metal chelating activities were for used antioxidant evaluation of MEFT and EEFT. The both extracts neutralized the activities of radicals and inhibited the peroxidation reactions of linoleic acid emulsion. Total antioxidant activity was measured according to ferric thiocyanate method. Butylated hydroxyanisole (BHA), butylated hydroxy-toluene (BHT), α-tocopherol and trolox, a water-soluble analogue of tocopherol, were used as the reference antioxidant compounds. MEFT and EEFT showed 69.4, 79.3, 72.3 and 83.7% inhibition on lipid peroxidation of linoleic acid emulsion, respectively, at the 10 and 20 µg/mL concentrations. On the other hand, 20 µg/mL of α-tocopherol, trolox, BHA and BHT exhibited 54.7, 20.1, 74.4 and 71.2% inhibition on peroxidation of linoleic acid emulsion, respectively. Also, MEFT and EEFT have effective DPPH•, $ABTS^{\bullet+}$ and superoxide anion radicals scavenging, hydrogen peroxide scavenging, total reducing power and metal chelating on ferrous ions activities.

Key words: *Chionanthus virginicus*; oleaceae; antioxidant activity; fringe tree; radical scavenging, metal chelating.

INTRODUCTION

Oxidation processes are very important for living organism. The uncontrolled production of reactive oxygen species (ROS) and the unbalanced mechanism of antioxidant protection result in the onset of many diseases and accelerate ageing. Oxidative stress occurs, when an imbalance occurs, leading to potentially serious health consequences. ROS include free radicals such as superoxide anion radicals ($O_2^{\bullet-}$), hydroxyl radicals (OH•) and non free-radical species such as H_2O_2 and singlet oxygen ($^1O_2$) (Halliwell and Gutteridge, 1989; Gülçin et al., 2002a; Gülçin et al., 2002b). There is a balance between the generation of ROS and inactivation of ROS by the antioxidant system in organisms. In case of the imbalance between ROS and antioxidant defence mechanisms, ROS leads to oxidative modification in cellular membrane or intracellular molecules (Duh et al., 1999; Büyükokuroğlu et al., 2001; Gülçin et al., 2003). In addition, under pathological conditions or oxidative stress, ROS are overproduced and result peroxidation of membrane lipids, leading to the accumulation of lipid peroxides. However, they are removed by antioxidant defence mechanisms. Antioxidants are considered as possible protection agents reducing oxidative damage of human body from ROS and retard the progress of many chronic diseases as well as lipid peroxidation (Pryor, 1991; Kinsella et al., 1993; Lai et al., 2001; Gülçin et al., 2003a). Therefore, there is a growing

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interest in the substances exhibiting antioxidant properties that are supplied to human and animal organisms as food components or as specific pharmaceutics. Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate and tert-butylhydroquinone are the most commonly used antioxidants at the present time. However, they have suspected of being responsible for liver damage and carcinogenesis (Wichi, 1988; Sherwin, 1990). Hence, in recent years, the restriction in the use of synthetic antioxidants, such as BHA and BHT, has caused an increased interest towards natural antioxidant substances (Baardseth, 1989; Gülçin et al., 2005a).

Novadays, natural antioxidants have become one of the major areas of scientific research (Demo et al., 1998; Sanchez-Moreno et al., 1999). Therefore the importance of searching for and exploiting natural antioxidants, especially of plant origin, has increased greatly in recent years. There is a growing interest in natural additives as potential antioxidants (Grice, 1986; Moure et al., 2001; Oktay et al., 2003; Gülçin et al., 2005b).

Chionanthus virginicus L., fringe tree, is a shrub of the eastern of America. This Oleaceae is used in folk medicine as cholagogue, diuretic and tonic (Duke and Wain, 1981). The leaves contain flavonoids, as rutin, kaempferol-3-glucoside, kaempferol-3-rutinoside, quercetin triglycosides (Harborne and Green, 1980) and triterpenoid compound as ursolic acid (Pourra et al., 1954). Stem and root barks contain a lignin, phillyrin (Steinegger and Jacober, 1959). Nowadays, root bark is used in homeopathy for hepatitis and icterus (Guermonprez and Pinkas, 1997). There is no found any information about *in vitro* antioxidant and antiradical activities of MEFT and EEFT. The main objectives of the present study were to assess the antioxidant potential of MEFT and EEFT in different *in vitro* antioxidant assays including 1, 1-diphenyl-2-picrylhydrazyl free radical scavenging, ABTS radical scavenging, total antioxidant activity by ferric thiocyanate method, reducing power, superoxide anion radical scavenging, hydrogen peroxide scavenging and metal chelating activities.

MATERIALS and METHODS

Chemicals

2,2´-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), nicotinamide adenine dinucleotide (NADH), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), nitroblue tetrazolium (NBT), phenazine methosulphate (PMS), the stable free radical 1,1-diphenyl-2-picryl-hydrazyl (DPPH•), linoleic acid, 3-(2-Pyridyl)-5,6-bis (4-phenyl-sulfonic acid)-1,2,4-triazine (Ferrozine), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), ethylenediaminetetraacetice acide (EDTA), α-tocopherol, polyoxyethylene-sorbitan monolaurate (Tween-20), and trichloroacetic acid (TCA) were obtained from Sigma (Sigma-Aldrich GmbH, Sternheim, Germany). Ammonium thiocyanate was purchased from Merck. All other chemicals used were analytical grade and obtained from either Sigma-Aldrich or Merck.

Plant materials

Samples of dried root bark of *C. virginicus* were provided by laboratories BOIRON (No: 97070214). They were collected in south-east of USA and dried naturally on site. Root bark was powdered and stored in a dry place, protected from light until used.

Extraction procedure

Powdered root bark of *C. virginicus* (100 g) were extracted with 300 ml of MeOH twice under reflux during 30 min. The extract was concentrated in rotary evaporator. The residue was dissolved in 300 mL of distilled water and was successively extracted with 4x100 mL of AcOEt. Organic phase was evaporated in rotary evaporator to give residues of 4.4 g.

Total antioxidant activity-ferric thiocyanate method

The antioxidant activity of MEFT and EEFT was determined according to the ferric thiocyanate method in linoleic acid emulsion (Mitsuda et al., 1996). For stock solutions, 10 mg of MEFT and EEFT was dissolved in 10 mL deionized water. Then, the solution, which contains different concentration of stock MEFT and EEFT solution samples (10-20 µg/mL) in 2.5 mL of potassium phosphate buffer (0.04 M, pH 7.0), was added to 2.5 mL of linoleic acid emulsion in potassium phosphate buffer (0.04 M, pH 7.0). The mixed solution (5 mL) was incubated at 37°C in glass flask. At regular intervals during incubation, a 0.1 ml aliquot of the mixture was diluted with 3.7 ml of ethanol, followed by the addition of 0.1 ml of 30% ammonium thiocyanate and 0.1 ml of 20 mM ferrous chloride in 3.5% hydrochloric acid. The peroxide level was determined by reading the absorbance at 500 nm in a spectrophotometer (CHEBIOS s.r.l. UV-VIS Spectrophotometer). During the linoleic acid oxidation, peroxides are formed, which oxidize Fe^{+2} to Fe^{+3}. The latter ions form a complex with thiocyanate and this complex has a maximum absorbance at 500 nm. These steps were repeated every 12 h until the control reached its maximum absorbance value. Therefore, high absorbance indicates high linoleic acid emulsion oxidation. Solutions without added samples were used as blanks. All data on total antioxidant activity are the average of duplicate experimetns. The percentage inhibition of lipid peroxidation in linoleleic acid emulsion was calculated by following equation:

$$\text{Inhibition of lipid peroxidation (\%)} = 100 - [(A_{Sample}/A_{Control}) \times 100$$

where $A_{Control}$ is the absorbance of the control reaction and A_{Sample} is the absorbance in the presence of the sample of MEFT, EEFT or standard compounds (Gülçin et al., 2004a).

Total reduction ability by Fe^{3+}- Fe^{2+} transformation

The samples prepared for ferric thiocyanate method was used for this and the other antioxidant assays. The reducing activity of MEFT and EEFT was determined by the method of Oyaizu (1986). The capacity of MEFT and EEFT to reduce the ferric-ferricyanide complex to the ferrous-ferricyande complex of Prussian blue was determined by recording the absorbance at 700 nm after incubation. For this purose, different concentrations of MEFT and EEFT (10-20 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 $[K_3Fe(CN)_6]$ (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. The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%). The absorbance

was measured at 700 nm in a spectrophotometer. Increased absorbance of the reaction mixture indicates increased reduction capability.

Metal chelating activity on ferrous ions (Fe^{2+})

Ferrous ion (Fe^{2+}) chelation by MEFT and EEFT was estimated by the Ferrozine assay (Dinis et al., 1994). Briefly, MEFT and EEFT (10 µg/mL) in 0.4 mL was 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 total volume was adjusted to 4 mL ethanol. Then, the mixture was shaken vigorously and left standing at room temperature for ten minutes. After the mixture had reached equilibrium, the absorbance of the solution was then measured spectrophotometrically at 562 nm. The results were expressed as percentage of inhibition of the Ferrozine-Fe^{2+}complex formation. The percentage of inhibition of ferrozine-Fe^{2+} complex formation was calculated using the formula given below:

$$\text{Bound } Fe^{2+}(\%) = [(A_{Control} - A_{Sample})/A_{Control}] \times 100$$

where $A_{Control}$ is the absorbance of the ferrozine–Fe^{2+} complex and A_{Sample} is the absorbance in the presence of the sample of MEFT and EEFT (Gülçin et al., 2004b).

H_2O_2 scavenging activity

H_2O_2 scavenging ability of MEFT and EEFT was determined according to the method of Ruch et al. (1989). A solution of H_2O_2 (40 mM) was prepared in phosphate buffer (pH 7.4). MEFT and EEFT at the 30 µg/mL concentration in 3.4 mL phosphate buffer were added to a H_2O_2 solution (0.6 mL, 40 mM). The absorbance value of the reaction mixture was recorded at 230 nm. Blank solution was containing the phosphate buffer without H_2O_2. The percentage of H_2O_2 scavenging of MEFT and EEFT and standard compounds was calculated as:

$$\%\ \text{Scavenged } [H_2O_2] = [(A_{Control} - A_{Sample})/A_{Control}] \times 100$$

where $A_{Control}$ is the absorbance of the control, and A_{Sample} is the absorbance in the presence of the sample of MEFT and EEFT (Elmastaş et al., 2005; Gülçin, 2005c).

ABTS radical cation decolorization assay

The spectrophotometric analysis of $ABTS^{\bullet+}$ radical scavenging activity was determined according to Re et al. (1999). The $ABTS^{\bullet+}$ cation radical was produced by the reaction between 7 mM ABTS in H_2O and 2.45 mM potassium persulfate, stored in the dark at room temperature for 12 h. Before usage, the $ABTS^{\bullet+}$ solution was diluted to get an absorbance of 0.700 ± 0.025 at 734 nm with phosphate buffer (0.1 M, pH 7.4). For stock solutions of 10 mg of MEFT and EEFT was dissolved in 10 ml distillated water. Then, 1 ml of $ABTS^{\bullet+}$ solution was added 3 mL of MEFT and EEFT solution in ethanol at different concentrations (10-20 µg/mL). After thirty minutes later, the percentage inhibition of absorbance at 734 nm was calculated for each concentration relative to a blank absorbance (ethanol). All determinations were carried out at least three times, and in triplicate. The $ABTS^{\bullet+}$ concentration (mM) in the reaction medium was calculated from the following calibration curve, determined by linear regression (R^2: 0.9922):

$$\text{Absorbance} = 0.0116 \times [ABTS^{\bullet+}] + 0.0479$$

The capability to scavenge the $ABTS^{\bullet+}$ radical was calculated using the following equation:

$$ABTS^{\bullet+}\ \text{scavenging effect } (\%) = [(A_{Control} - A_{Sample} / A_{Control}) \times 100]$$

where in $A_{Control}$ is the initial concentration of the $ABTS^{\bullet+}$ and A_{Sample} is ansorbence of the remaining concentration of $ABTS^{\bullet+}$ in the presence of ligustroside and oleuropein.

1,1-Diphenyl-2-picryl-hydrazil (DPPH) free radical scavenging activity

The hydrogen atoms or electrons donation ability of MEFT and EEFT was measured from the bleaching of purple coloured methanol solution of DPPH, following the methodology described by Blois (1958). The capacity of MEFT and EEFT to scavenge the lipid-soluble DPPH radical, which results in the bleaching of the purple colour exhibited by the stable DPPH radical, is monitored at an absorbance of 517 nm. Basically, a 0.1 mM ethanolic solution of DPPH· was prepared daily. Then, 1 mL of this solution was added 3 mL of MEFT and EEFT solution in ethanol at different concentrations (10-20 µg/mL). Thirty minutes later, the absorbance was measured at 517 nm. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The DPPH· concentration (mM) in the reaction medium was calculated from the following calibration curve, determined by linear regression (R^2:0.9974):

$$\text{Absorbance} = 0.5869 \times 10^{-4}[DPPH\cdot] + 0.0134$$

The capability to scavenge the DPPH· radical was calculated using the following equation:

$$DPPH\cdot\ \text{Scavenging effect } (\%) = [(A_{Control} - A_{Sample} / A_{Control}) \times 100]$$

where in $A_{Control}$ is the initial concentration of the stable DPPH radical without the test compound and A_{Sample} is ansorbence of the remaining concentration of DPPH· in the presence of MEFT and EEFT (Gülçin et al., 2004c; Cristiane de Souza et al., 2004).

Superoxide anion radical ($O_2^{\bullet-}$) scavenging activity in PMS-NADH/ NBT systems

Measurement of $O_2^{\bullet-}$ scavenging activity of MEFT and EEFT was based on the method described by Liu (1991). $O_2^{\bullet-}$ are generated in PMS-NADH systems by oxidation of NADH and assayed by the reduction of NBT. In these experiments, the $O_2^{\bullet-}$ were generated in 3 mL of Tris-HCl buffer (16 mM, pH 8.0) containing 1 mL of NBT (50 µM) solution, 1 mL NADH (78 µM) solution and sample solution of MEFT and EEFT (30 µg/mL) in water. The reaction was started by adding 1 mL of PMS solution (10 µM) 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. L-Ascorbic acid was used as a positive control. Decreased absorbance of the reaction mixture indicates increased $O_2^{\bullet-}$ scavenging activity. The percentage inhibition of $O_2^{\bullet-}$ generation was calculated using the following formula:

$$\%\ \text{Inhibition} = 100 - [(A_{Sample} / A_{Control}) \times 100]$$

where $A_{Control}$ is the absorbance of the L-ascorbic acid, and A_{Sample} is the absorbance of MEFT and EEFT (Gülçin et al., 2004d).

Statistical analysis

All the analyses on total antioxidant activity were done in duplicate sets. The other analyses were performed in triplicate. The data

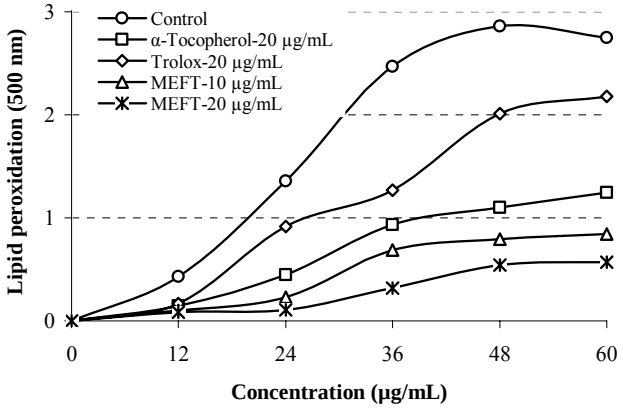

Figure 1. Total antioxidant activities of MEFT at different concentrations (10-20 μg/mL), α-tocopherol and trolox (20 μg/mL) by ferric thiocyanate methods [MEFT: Methanol extract from root bark of fringe tree (*Chionanthus virginicus L.*)].

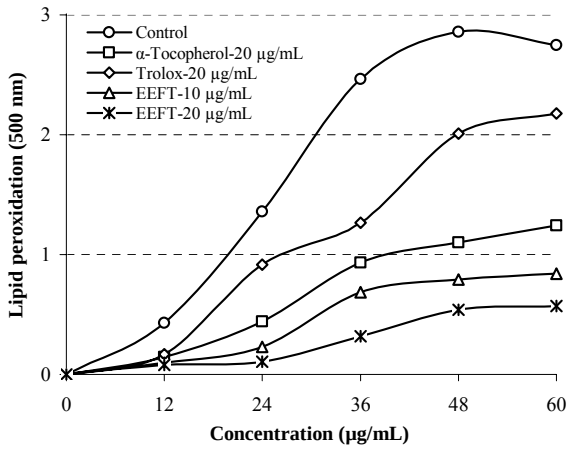

Figure 2. Total antioxidant activities of EEFT at different concentrations (10-20 μg/mL), α-tocopherol and trolox (20 μg/mL) by ferric thiocyanate methods [EEFT: Ethyl acetate extract from root bark of fringe tree (*Chionanthus virginicus* L.)].

were recorded as mean ± standard deviation and analysed by SPSS (version 11.5 for Windows 98, SPSS Inc.). One-way analysis of variance was performed by ANOVA procedures. Significant differences between means were determined by LSD tests. *P* values < 0.05 were regarded as significant and *p* values < 0.01 very significant.

RESULTS AND DISCUSSION

Antioxidants are a chemical that reduces the rate of particular oxidation reactions in a specific context, where oxidation reactions are chemical reactions that involve the transfer of electrons from a substance to an oxidising agent. Antioxidants are particularly important in the context of organic chemistry and biology, all living cells contain complex systems of antioxidant chemicals and/or enzymes to prevent chemical damage to the cells' components by oxidation. Also, they can interfere with the oxidation process by reacting with the free radicals, chelating free catalytic metals and also by acting as oxygen scavengers (Larrauri et al., 1999). The antioxidant properties of plants could be correlated with oxidative stress defense in different human diseases including cancer, atherosclerosis, Alzheimer's and the ageing processes (Stajner et al., 1998; Malencic et al., 2000).

Natural antioxidants have biofunctionalities such as the reduction of chronic diseases like DNA damage, mutagenesis, carcinogenesis, etc. and inhibitions of pathogenic bacteria growth, which are often associated with the termination of free radical propagation in biological systems (Covacci et al., 2001; Zhu et al., 2002). In this study, the antioxidant activity of the MEFT and EEFT were compared to BHA, BHT, α-tocopherol and its water-soluble analogue trolox. The antioxidant activity of the MEFT, EEFT, α-tocopherol, trolox, BHA and BHT has been evaluated in a series of *in vitro* tests including DPPH free radical, ABTS radical and superoxide anion radicals scavenging, total antioxidant activity by ferric thiocyanate method, reducing activity, hydrogen peroxide scavenging activity and metal chelating activitiy.

Total antioxidant activity determination in linoleic acid emulsion by ferric thiocyanate method

The ferric thiocyanate method measures the amount of peroxide produced during the initial stages of oxidation which are the primary products of oxidation. Total antioxidant activity of MEFT, EEFT and standard compounds was determined by the ferric thiocyanate method in the linoleic acid system. MEFT and EEFT had effective antioxidant activity. The effects of various concentrations of MEFT and EEFT (from 10-20 μg/mL) on lipid peroxidation of linoleic acid emulsion are shown in Figure 1 and 2, were found to be 69.4, 79.3, 72.3 and 83.7% respecttively. On the other hand, α-tocopherol, trolox, BHA and BHT exhibited 54.7, 20.1, 74.4 and 71.2% inhibition on peroxidation of linoleic acid emulsion, respectively at the 20 μg/mL concentration. The results clearly showed that MEFT, especially EEFT had stronger total antioxidant activity than α-tocopherol, trolox, BHA and BHT at the same concentration (20 μg/mL).

Total reductive capability using the potassium ferricyanide reduction method

Reductants provide electrons to the reduced species. The presence of reductants such as antioxidant substances in the antioxidant samples causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. The yellow colour of the test solution changes to various shades of green and blue depending on the reducing

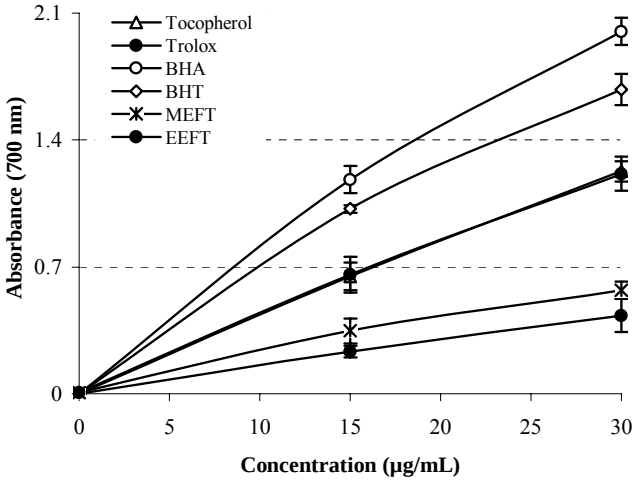

Figure 3. Total reductive potential of different concentrations (10-20 µg/mL) of MEFT, EEFT, α-tocopherol and trolox by Fe+3-Fe+2 transformation [MEFT: Methanol extract from root bark of fringe tree (*Chionanthus virginicus* L.), EEFT: Ethyl acetate extract from root bark of fringe tree (*C. virginicus* L.)].

power of antioxidant samples. Therefore, Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm (Chung et al., 2002).

Figure 3 depicts the reducing activity of the MEFT, EEFT and standards (BHA, BHT, α-tocopherol and trolox) 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 MEFT and EEFT by the method of Oyaizu (1986). The reducing activity of MEFT, EEFT, BHA, BHT, α-tocopherol and trolox increased with increasing concentration of samples. As can be seen in Figure 3, MEFT and EEFT demonstrate an effective reducing activity than control, at different concentrations. These differences were statistically significant (p<0.01). Reducing power of MEFT, EEFT and standard compo-unds exhibited the following order: α-tocopherol ≅EEFT> MEFT > trolox. MEFT and α-tocopherol statiscally have similar reductive ability (p< 0.05).

Ferrous ions chelating capacity

Transition metal ions have a great importance in the generation of oxygen free radicals in living organisms. Iron exists in two distinct oxidation states; ferrous ion (Fe^{2+}) and ferric ion (Fe^{3+}). The ferric ion (Fe^{3+}) is the relatively biologically inactive form of iron. However, it can be reduced to the active Fe^{2+}, depending on the conditions, particularly pH (Strlic et al., 2002), and oxidized back through Fenton type reactions, with production of hydroxyl radicals; or Haber-Weiss reactions with superoxide anions (Kehrer, 2000; Wong and Kitts, 2001). The production of these radicals can lead to lipid peroxidation, protein modification and DNA damage. Chelating agents may inactivate metal ions and potentially inhibit the metal -dependent processes (Finefrock et al., 2003).

Also, the production of highly ROS such as superoxide anion radicals, hydrogen peroxide, and hydroxyl radicals is also catalysed by free iron through Haber-Weiss reaction ($O_2^{\bullet-} + H_2O_2 \rightarrow O_2 + OH^- + OH\cdot$) (Haber and Weiss, 1934). Free iron is known to have low solubility and a chelated iron (i.e., iron-ligand) complex, such as EDTA–Fe, has greater solubility in solution, which can be contributed solely from the ligand. Furthermore, chelated iron, such as EDTA-Fe, is also known to be active, since it can participate in iron-catalyzed reactions (Wong and Kitts, 2001). 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 ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH\cdot$). Fe^{3+} ion also produces radicals from peroxides, although the rate is tenfold less than that of Fe^{2+} ion (Miller, 1996). Fe^{2+} ion is the most powerful pro-oxidant among various species of metal ions (Halliwell and Gutteridge, 1984).

Ferrous ion chelating activities of MEFT, EEFT, BHA, BHT, α-toco-pherol and trolox are shown in Figure 4. The chelating of ferrous ions by MEFT and EEFT was estimated by the ferrozine assay. Ferrozine can quantitatively form com-plexes with Fe^{2+}. In the presence of chelating agents, the complex formation is inhibited and the red colour of the complex fades. Measuring of the colour reduction, there-fore, it is possible to estimate of the chelating activity of the co-existing chelator (Yamaguchi et al., 2000). 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.

In fact, as shown in Figure 4, MEFT and EEFT disrupted the Fe^{2+}-ferrozine complex at 10 µg/mL concentration. The difference amoung all MEFT and EEFT concentrations and the control was statistically significant (p<0.01). In additon, MEFT and EEFT exhibited 94.5 and 88.6% chelation of ferrous ion at the above concentration. On the other hand, the percentages of metal chelating capacity of same concentration of BHA, BHT, α-tocopherol and trolox were found as 72.1, 64.3 and 21.6 and 48.5%, respectively. The metal scavenging effect of those samples decreased in the order of MEFT > EEFT > BHA > BHT > trolox > α-tocopherol.

Metal chelating capacity was significant, since it reduced the concentration of the catalysing transition metal in lipid peroxidation. It was reported that chelating agents are effective as secondary antioxidants because they reduce the redox potential thereby stabilizing the oxidized form of the metal ion. The data obtained from Figure 4 reveal that MEFT and EEFT demonstrates a marked capacity for iron binding, suggesting that their main action as peroxidation protector may be related to its iron binding capacity.

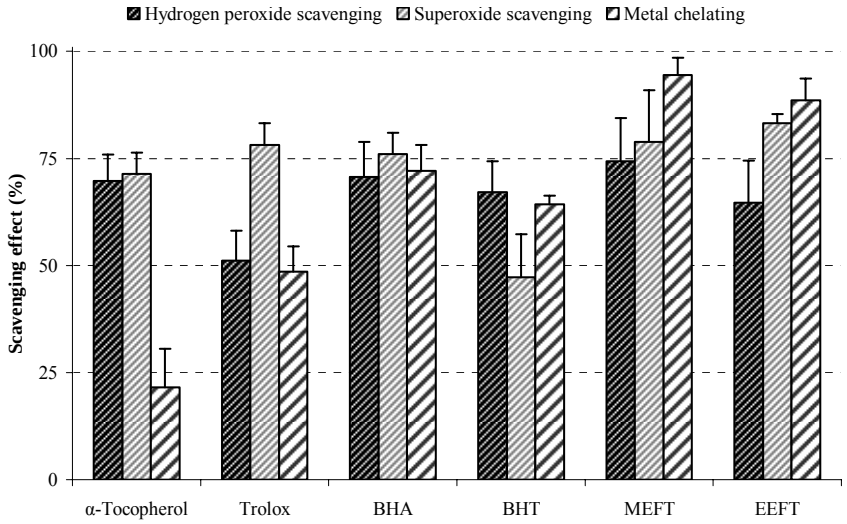

Figure 4. Comparison of hydrogen peroxide scavenging, superoxide anion radical scavenging and ferrous ions chelating activity of MEFT, EEFT, BHA, BHT, α-tocopherol and trolox at the same concentration (10 µg/mL) (MEFT: Methanol extract from root bark of fringe tree (*Chionanthus virginicus* L.), EEFT: Ethyl acetate extract from root bark of fringe tree (C. *virginicus* L.), BHA: Butylated hydroxyanisole, BHT: butylated hydroxytoluene).

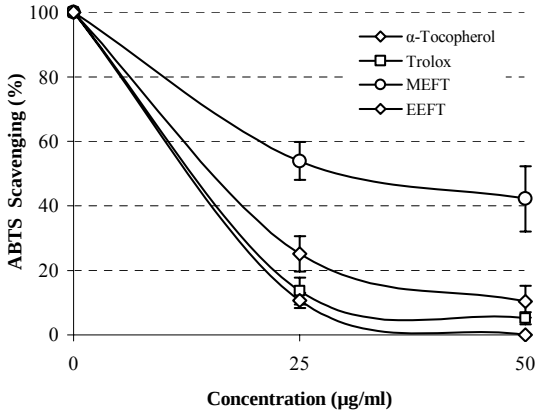

Figure 5. Scavenging effect of MEFT, EEFT, α-tocopherol and trolox on the $ABTS^{\bullet+}$ at different concentrations (10-20 µg/ml) [MEFT: Methanol extract from root bark of fringe tree (*Chionanthus virginicus* L.), EEFT: Ethyl acetate extract from root bark of fringe tree (C. *virginicus* L.), ABTS•+: 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonate) radicals).

H_2O_2 scavenging activity

H_2O_2 can cross membranes and may slowly oxidize a number of compounds. It can be formed in vivo by many oxidizing enzymes such as superoxide dismutase. The ability of MEFT and EEFT to scavenge H_2O_2 was determined according to the method of Ruch et al. (1989) was shown in Figure 4 and compared with that of BHA, BHT, α-tocopherol and trolox as standards. MEFT and EEFT exhibited 74.3 and 64.6% scavenging effect of hydrogen peroxide, at the 10 µg/mL concentration. On the other hand, BHA, BHT, α-tocopherol and trolox exhibited 70.7, 67.1, 69.8 and 51.1% hydrogen peroxide scavenging activity at the same concentration. These results showed that MEFT and EEFT have an effective hydrogen peroxide scaven-ging activity. At the above concentration, the hydrogen peroxide scavenging effect of MEFT and EEFT and four standards decreased in the order of MEFT > BHA > α-tocopherol>BHT>EEHT>trolox. H_2O_2 itself is not very rea-ctive; however it can sometimes be toxic to cell because it may give rise to hydroxyl radical in the cells. Addition of H_2O_2 to cells in culture can lead to transition metal ion-dependent OH radicals mediated oxidative DNA damage. Levels of H_2O_2 at or below about 20-50 mg seem to have limited cytotoxicity to many cell types. Thus, removing H_2O_2 as well as superoxide anion is very important for protection of farmaceutical and food systems.

$ABTS^{\bullet+}$ Radical scavenging activity

Excessive formation of free radicals accelerates the oxidation of lipids in foods and decreases food quality and consumer acceptance (Min, 1998). Hence, radical scavenging activities are very important due to the deleterious role of free radicals in foods and in biological systems.

Generation of the ABTS radical cation forms the basis of one of the spectrophotometric methods that have been applied to the measurement of the total antioxidant activity of solutions of pure substances, aqueous mixtures and beverages (Wolfenden and Willson 1982; Miller et al., 1996). A more appropriate format for the assay is a decolorization technique in that the radical is generated directly in a stable form prior to reaction with putative

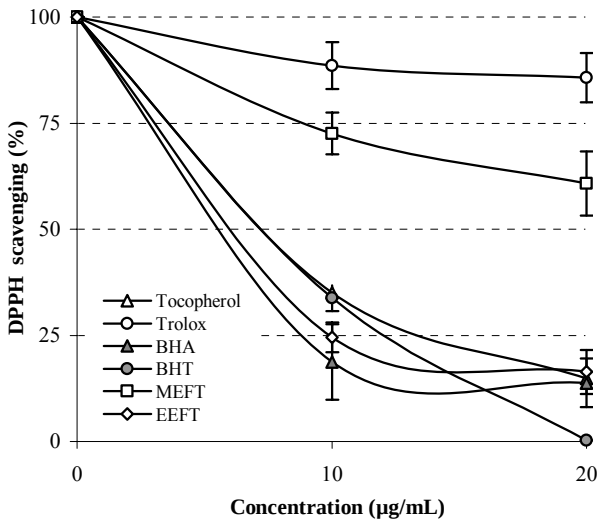

Figure 6. Scavenging effect of MEFT, EEFT, BHA, BHT, α-tocopherol and trolox on the stable DPPH· at different concentrations (10-20 µg/ml) [MEFT: Methanol extract from root bark of fringe tree (*Chionanthus virginicus* L.), EEFT: Ethyl acetate extract from root bark of fringe tree (C. *virginicus* L.), DPPH·:1, 1-Diphenyl-2-picrylhydrazyl radicals, BHA: Butylated hydroxyanisole, BHT: Butylated hydroxytoluene).

antioxidants. The improved technique for the generation of $ABTS^{\bullet+}$ described here involves the direct production of the blue/green $ABTS^{\bullet+}$ chromophore through the reaction between ABTS and potassium persulfate.

As seen in Figure 5, MEFT and EEFT had $ABTS^{\bullet+}$ radical scavenging activity in a concentration-dependent manner (10-20 µg/mL). There is a significant decrease ($p<0.01$) in the concentration of $ABTS^{\bullet+}$ due to the scavenging capacity of MEFT, EEFT and standards. In addition, the scavenging effect of MEFT, EEFT and standards on the $ABTS^{\bullet+}$ decreased in that order: EEFT > trolox > α-tocopherol >MEFT, which were 99.9, 95.2, 93.3 and 57.8%, at the 20 µg/mL concentration, respectively.

DPPH• radical scavenging activity

Antioxidants react with DPPH•, which is a stable free radical, and convert it to 1,1-diphenyl-2-picryl hydrazine. The degree of discolouration indicates the radical-scavenging potential of the antioxidant (Singh et al., 2002). The stable free radical DPPH has been widely used to test the free radical-scavenging ability of various dietary antioxidants (Brand-Williams et al., 1995). In this study, antioxidant activities of MEFT, EEFT and standard antioxidants such as α-tocopherol and trolox were determined using a DPPH• method. Since the DPPH• assay can accomodate a large number of samples in a short period and is sensitive enough to detect natural compounds at low concentrations, it was used in the present study for a primary screening of the MEFT and EEFT free radical-scavenging activity. This assay provides information on the reactivity of test compounds with a stable free radical. Because of its odd electron, DPPH• gives a strong absorption band at 517 nm in visible spectroscopy. As this electron becomes paired off in the presence of a free radical scavenger, the absorp-tion vanishes, and the resulting decolorization is stoichiometric with respect to the number of electrons taken up. MEFT and EEFT exhibited marked DPPH free radical scavenging activity in a concentration-dependent manner. Figure 6 illustrates a significant decrease ($p<0.05$) in the concentration of DPPH radical due to the scavenging ability of MEFT, EEFT and standards. The scavenging effect of MEFT, EEFT and standards on the DPPH radical decreased in that order: BHT > BHA > α-tocopherol > EEFT >MEFT > trolox, which were 99.7, 86.2, 85.2, 83.6, 39.2 and 14.3%, at the 20 µg/mL concentration, respectively.

$O_2^{\bullet-}$ radical scavenging activity

Superoxide ion, had one unpaired electron, is a free radical and therefore paramagnetic. $O_2^{\bullet-}$ is formed in almost all aerobic cells and is a major agent in the mechanism of oxygen toxicity (Sun et al., 2004). Also, they$^-$ are produced *in vivo* by electron leakage from the mitochondrial electron transport chain, by activated phagocytes (Halliwell, 1991). $O_2^{\bullet-}$ are a precursor to active free radicals that have potential of reacting with biological macromolecules and thereby inducing tissue damage (Halliwell and Gutteridge, 1984). It related closely to the biological course of apolexis, tumor, and inflammation etc. Compared with other oxygen radicals, $O_2^{\bullet-}$ has a longer lifetime, can move to an aim at a longer distance, and thus has more dangerous. Therefore, it is very important to study the scavenging of superoxide anion (Sun et al., 2004). Also, it has been implicated in several pathophysiological processes, due to its transformation into more reactive species such as hydroxyl radical that initiate lipid peroxidation. $O_2^{\bullet-}$ has also been observed to directly initiate lipid peroxidation (Wickens, 2001). It has also been reported that antioxidant properties of some flavonoids are effective mainly via scavenging of $O_2^{\bullet-}$ (Yen and Duh, 1994). Also, $O_2^{\bullet-}$ plays an important role in formation of other ROS such as H_2O_2, OH·, and singlet oxygen, which induce oxidative damage in lipids, proteins, and DNA (Pietta, 2000). Also, $O_2^{\bullet-}$ is an oxygen-centred radical with selective reactivity. This species is produced by a number of enzyme systems in autooxidation reactions and by nonenzymatic electron transfers that univalently reduce molecular oxygen. It can also reduce certain iron complex such as cytochrome C.

In this method, $O_2^{\bullet-}$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 (Cos et al., 1998; Parejo

et al., 2002). The decrease of absorbance at 560 nm with antioxidants indicates the consumption of $O_2^{\bullet-}$ in the reaction mixture. Figure 4 shows the percentage inhibition of $O_2^{\bullet-}$ generation by 10 μg/mL concentration of MEFT, EEFT and standards were found similar statistically. As can see in Figure 4, the percentage inhibition of $O_2^{\bullet-}$ generation by 10 μg/mL concentration of MEFT and EEFT was found as 78.9 and 83.2%. On the other hand, at the same concentration, BHA, BHT, α-tocophe-rol and trolox exhibited 76.0, 47.3, 71.4 and 78.2% superoxide anion radical scavenging activity, respectively.

Conclusion

According to data obtained from the present study, MEFT and EEFT were found to be an effective antioxidant in different in vitro assay including ferric thiocyanate method, reducing power, DPPH• scavenging, $ABTS^{\bullet+}$ scavenging and superoxide anion radical scavenging, hydrogen peroxide scavenging and metal chelating activities when compared to standard antioxidant compounds such as seynthetic antioxidants (BHA, BHT), α-tocopherol, a natural antioxidant, and trolox which is a water-soluble analogue of tocopherol.

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