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# Investigation of Antioxidant Properties of Spearmint (*Mentha spicata* L.)

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The antioxidant properties of ethanol extract of spearmint (*Mentha spicata* L.) (MS) were studied and evaluated using different antioxidant tests, including reducing power, free radical scavenging, superoxide anion radical scavenging and metal chelating activities. The concentration of 100 and 250 μg/mL of ethanol extract of spearmint (MS) showed 97 and 99% inhibition on peroxidation of linoleic acid emulsion, respectively. On the other hand, 100 and 250 μg/mL of standard antioxidant such as BHA, BHT and α-tocopherol exhibited 85, 87% (BHA), 97, 99% (BHT) and 51, 77% (α-tocopherol) inhibition on peroxidation of linoleic acid emulsion, respectively. The ethanol extract of MS had effective reducing power, free radical scavenging, superoxide anion radical scavenging, hydrogen peroxide scavenging and metal chelating activities at same concentration (50, 100 and 250 μg/mL). These various antioxidant activities were compared to standard antioxidants such as butylated hydroxyianisole (BHA), butylated hydroxytoluene (BHT) and α-tocopherol. In addition, total phenolic compounds in the ethanol extract were also determined as gallic acid equivalent.

**Key Words: Antioxidant activity, Spearmint, *Mentha spicata* L., Antioxidant.**

## INTRODUCTION

The family of Labiatae, which have commercial and medical values, are widespread throughout the world as well as Turkey. *Mentha spicata* L. (MS) usually grows in wet sites of ditch banks and streamsides[1]. *Mentha spicata* L. (MS), commonly known as spearmint, is used as a herb with spices to give the food a special flavour and fragrance. It is also used for flavouring chewing gums, toothpaste, confectionery and pharmaceutical preparations. It has been in use for centuries by country doctors for treatment of different diseases, particularly in digestive system ailments. This herb is considered stimulative, carminative and

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antispasmodic[2]. It has been found to possess antidote properties for poisons and it has also been reported as a remedy for inflammation, fevers, bronchitis, infantile troubles, vomiting in pregnancy and hysteria[3].

Many species of fruits, vegetables, herbs, cereals, sprouts and seeds have been investigated for antioxidant activity during the last decade[4–6]. Natural antioxidants are extensively studied for their capacity to protect organisms and cells from damage that are induced by oxidative stress, the latter being considered a cause of ageing, degenerative diseases and also cancer[7]. Herbs and spices are harmless sources for obtaining natural antioxidants. Plant tissue antioxidant capacity is clearly associated with activity of free radical scavenging enzymes (superoxide dismutase, catalase, peroxidase) and with the contents of antioxidant substances, mainly phenolic compounds, carotenoids, tocopherol and ascorbic acid. Obviously, there has been an increasing demand to evaluate the antioxidant properties of direct plant extracts or isolated products from plant origin rather than looking for synthetic ones[8]. It is an established fact that polyphenolic compounds, such as flavonoids, anthraquinones, anthocyanids and xanthones, possess remarkable antioxidant activities which are present quite commonly in plants[9].

Free radicals can be described as species, which had an unpaired electron. The reactivity of free radicals varies from relatively low, as in the case of the oxygen molecule itself, to very high, as in the case of the short-lived and highly reactive hydroxyl radical ($OH^-$).[10] Oxygen and reactive oxygen species (ROS) are among the major sources of primary catalysts that initiate oxidation *in vivo* and *in vitro*. The reaction of oxygen with ground state molecules of singled multiplicity such as poly-unsaturated fatty acids (PUFA) is spin-forbidden. However, this barrier does not apply to reactions, which involve single electrons, hydrogen atoms, and molecules containing unpaired electrons, such as transition metal complexes and free radicals. Therefore, the triplet state oxygen can react with other molecules to yield ROS such as hydrogen peroxide ($H_2O_2$), superoxide ($O_2\cdot^-$) and hydroxyl radical ($OH\cdot$).[11–13] Superoxide radical ($O_2\cdot^-$) is generated by four-electron reduction of molecular oxygen into water. This radical is also formed in aerobic cells due to electron leakage from the electron transport chain. Superoxide radicals ($O_2\cdot^-$) are also formed by activated phagocytes such as monocytes, macrophages, eosinophils and neutrophils and the production of $O_2\cdot^-$ is an important factor in the killing of bacteria by phagocytes. In living organisms, $O_2\cdot^-$ is removed by the enzymes called superoxide dismutases (SOD)[11, 13, 14].

Exogenous chemical and endogenous metabolic processes in the human body or in food system might produce highly reactive free radicals, especially oxygen-derived radicals, which are capable of oxidizing biomolecules, resulting in cell death and tissue damage. Oxidative damages play a significantly pathological role in human diseases. Cancer emphysema, cirrhosis, arteriosclerosis and arthritis have all been correlated with oxidative damage[11]. Also, excessive generation of ROS induced by various stimuli and which exceeds the antioxidant capacity of the organism leads to a variety of pathophysiological processes such as inflammation, diabetes, genotoxicity and cancer[8, 15]. However, antioxidant supplements or foods containing antioxidants may be used to help the human body reduce oxidative damage[11, 16].

Several studies have been reported on the chemical composition of *Mentha* species in literature[17-19]. The modulating effect of spearmint on benzyl peroxide-induced responses of tumour promotion in marine skin was investigated by Saleem *et al.* The result of this study suggests that spearmint is an effective chemopreventive agent that may suppress benzyl peroxide-induced cutaneous oxidative stress, toxicity and hyperproliferative effects *in vivo*[17]. In addition to this, there are some medicinal and pharmaceutical studies about spearmint. The effect of the essential oil of spearmint (MS) on the proliferation of *Helicobacter pylori, Salmonella enteritidis, Escherichia coli and Staphylococcus aureus* was studied. The essential oil of spearmint (MS) inhibited the proliferation of each strain in liquid culture in a dose-dependent manner. Thus, the essential oils and their constituents may be useful as potential antibacterial agents for inhibition of the growth of pathogens[20]. Spearmint also is capable of curing inflammatory disorders[3]. The nature of the spearmint has not been elucidated exactly so far.

However, there is no information about *in vitro* antioxidant activity of spearmint (MS). In the current investigation, the antioxidant effects of *Mentha spicata* L. have been reported and compared their antioxidant effects with those commonly used as food antioxidants, such as butylated hydroxyl toluene (BHT), butylated hydroxyl anisole (BHA) and α-tocopherol.

## EXPERIMENTAL

Ammonium thiocyanate and butylated hydroxyl toluene (BHT) were purchased from E. Merck. Ferrous chloride, polyoxyethylenesorbitan monolaurate (Tween-20), α-tocopherol, 1,1-diphenyl-2-picryl-hydrazyl ($DPPH^{\cdot}$), 3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine (ferrozine), nicotinamide adenine dinucleotide (NADH), butylated hydroxyanisole (BHA) and trichloracetic acid (TCA) were purchased from Sigma (Sigma-Aldrich GmbH, Sternheim, Germany). Muller Hinton agar was also obtained from Oxoid Ltd. (Basingstok, Hampshire, England, Lot/Ch-B: 241603, CM337). All other chemicals used were of analytical grade and were obtained from Sigma (Sigma-Aldrich GmbH, Sternheim, Germany).

Spearmint (*Mentha spicata* L.) was harvested from the Black Sea region of Turkey. For ethanol extraction 25 g sample was ground into a fine powder in a mill and was mixed with 500 mL ethanol. The residue was re-extracted under same conditions until extraction solvents became colourless. The obtained extract was filtered over Whatmann No. 1 paper and the filtrate was collected in a rotary evaporator at 40°C to obtain ethanol-free dry extract. The extract was placed in a plastic bottle, and then stored at 20°C until being used.

### Total antioxidant activity determination

The antioxidant activity of spearmint was determined according to the thiocyanate method[21]. 10 mg ethanol extract of spearmint was dissolved in 10 mL ethanol. 100, 250 μg/mL of spearmint extract or standard samples in 2.5 mL of potassium phosphate buffer (0.04 M, pH 7.0) were added to 2.5 mL linoleic acid emulsion in potassium phosphate buffer (0.04 M, pH 7.0), 50 mL linoleic

acid emulsion consisting of 175 μg Tween-20, 155 μL linoleic acid and 0.04 M potassium phosphate buffer (pH 7.0). On the other hand, 50 mL control consisting of 25 mL linoleic acid emulsion and 25 mL potassium phosphate buffer (0.04 M, pH 7.0) was taken. The mixed solution was incubated at 37°C in a glass flask and in the dark. After the mixture was stirred for 3 min, the peroxide value was determined by reading the absorbance at 500 nm in a spectrophotometer (Jasco V-530 UV/Vis spectrophotometer, Japan Servo Co. Ltd., Japan, Serial No. B099560512), after reaction with $FeCl_2$ and thiocyanate at intervals during incubation. During the linoleic acid oxidation, peroxides were formed and these compounds oxidized $Fe^{2+}$ to $Fe^{3+}$. The latter $Fe^{3+}$ ions form complex with $SCN^-$ and this complex has maximum absorbance at 500 nm. Therefore high absorbance indicates high linoleic acid oxidation. The solutions without added extract were used as blank samples. All data about total antioxidant activity are the average of duplicate analyses. The inhibition of lipid peroxidation in % was calculated by the following equation:

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

where $A_0$ is the absorbance of the control reaction and $A_1$ is the absorbance in the presence of the sample of spearmint extract[22].

**Reducing power**

The reducing power of spearmint (*Mentha spicata* L.) extract was determined according to the method of Oyaizu[23]. Different concentrations of spearmint extract (50, 100, 250 μg/mL) in 1 mL of ethanol 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, which was then centrifuged for 10 min at 1000 × g (MSE Mistral 2000, UK, Serial No. S693/02/444). 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. Higher absorbance of the reaction mixture indicated greater reducing power.

**Superoxide anion scavenging activity**

Measurement of superoxide anion scavenging activity of *Mentha spicata* L. extract was based on the method described by Liu *et al.*[24]. Superoxide radicals are generated in PMS-NADH systems by oxidation of NADH and assayed by the reduction of nitroblue tetrazolium (NBT). In this experiment, the superoxide radicals 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 spearmint (MS) extract (from 20 to 60 μg/mL) in ethanol. The reaction started by adding 1 mL of phenazine methosulphate (PMS) solution (10 μM) to the mixture. The reaction mixture was incubated at 25°C for 5 min, and the absorbance at 560 nm in a spectrophotometer was measured against blank samples. L-Ascorbic acid was used as a control. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The percentage inhibition of superoxide anion generation was calculated using the following formula:

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

where $A_0$ is the absorbance of the control and $A_1$ is the absorbance of spearmint (MS) extract and standards.

**Free radical scavenging activity**

The free radical scavenging activity of spearmint (MS) extract was measured by 1,1-diphenyl-2-picryl-hydrazil (DPPH·) using the method of Blois[25]. Briefly, 0.1 mM solution of DPPH· in ethanol was prepared and 1 mL of this solution was added to 3 mL of spearmint extract solution in ethanol at different concentrations (60, 120, 180 5g/mL). The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. Then the absorbance was measured at 517 nm in a spectrophometer. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The DPPH· concentration (mM) in the reaction medium was calculated from the following calibration curve, determined by linear regression ($R^2$: 0.997):

$$\text{Absorbance} = 0.0003 \times [\text{DPPH}\cdot] - 0.0174$$

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

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

where $A_0$ is the absorbance of the control reaction and $A_1$ is the absorbance in the presence of the sample of spearmint (MS) extract.

**Metal chelating activity**

The chelating of ferrous ions by the spearmint extract and standards was estimated by the method of Dinis *et al.*[26]. Briefly, extracts (50, 100, 150 μg/mL) 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 mixture was shaken vigorously and left standing at room temperature for 10 min. After the mixture had reached equilibrium, the absorbance of the solution was then measured spectrophotometrically at 562 nm in a spectrophotometer. All test and analyses were run in triplicate and averaged. The percentage of inhibition of ferrozine-$Fe^{2+}$ complex formation was given by the formula:

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

where $A_0$ is the absorbance of the control, and $A_1$ is the absorbance in the presence of the sample of spearmint (MS) extract and standards. The control did not contain $FeCl_2$ and ferrozine, complex formation molecules.

**Determination of total phenolic compounds**

Total soluble phenolic compounds in the *Mentha spicata* L. extract were determined with Folin-Ciocalteu reagent according to the method of Slinkard and Singleton[27] using gallic acid as a standard phenolic compound. Briefly, 1 mL of extract solution (containing 1000 μg extract) was taken in a volumetric flask diluted with distilled water (46 mL). 1 ml of Folin-Ciocalteu reagent was added and the content of the flask mixed thoroughly. After 3 min 3 mL of $Na_2CO_3$ (2%)

concentration of α-tocopherol. The spearmint (MS) percentage inhibition of peroxidation in linoleic acid system was 97 and 99% respectively, and greater than the 100 and 250 μg/mL of α-tocopherol (50, 77%), BHA (85, 87%). On the other hand, percentage inhibition of 100 and 250 μg/mL concentration of BHT was found 97 and 99%.

## Reducing power

Fig. 2 shows the reductive capabilities of spearmint (MS) extract compared to BHA, BHT and α-tocopherol. For the measurements of the reductive ability, we investigated the $Fe^{3+}$–$Fe^{2+}$ transformation in the presence of the spearmint (MS) extract samples using the method of Oyaizu[23]. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant

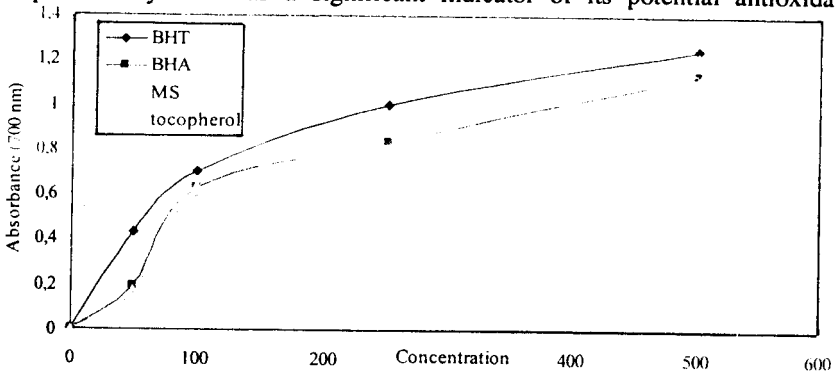


Fig. 2. Reducing power of ethanol extracts of MS, BHA, BHT, and α-tocopherol (Spectrophotometric deduction of $Fe^{3+}$-$Fe^{2+}$ transformation, MS: *Mentha spicata*, BHA: Butylated hydroxyanisole BHT: Butylated hydroxytoluene).

activity[30]. The antioxidant activity of putative antioxidants has been attributed to various mechanisms, among which are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging[31]. Like the antioxidant activity, the reducing power of the spearmint (MS) extract increased with increasing concentration. All of the amounts of the spearmint (MS) extract showed higher activities than control and these differences were statistically very significant ($p < 0.01$). Reducing power of ethanol extract of MS and standard compounds followed the order: BHT > ethanol extract (MS) > BHA > α-tocopherol.

## Superoxide anion scavenging activity

In the PMS-NADH-NBT system, superoxide anion derived from dissolved oxygen by PMS-NADH coupling reaction reduces NBT. The decrease of absorbance at 560 nm with antioxidants indicates the consumption of superoxide anion in the reaction mixture. Fig. 3 shows the per cent inhibition of superoxide radical generation by 100 μg/mL of ethanol extract of spearmint and comparison with same doses of BHA, BHT and α-tocopherol. Extracts of *Mentha spicata* L. have strong superoxide radical scavenging activity and exhibit higher superoxide

radical scavenging activity than BHT and α-tocopherol. The results were found statistically significant ($p < 0.05$). The percentage inhibition of superoxide generation by 100 μg/mL concentration of BHA and ethanol extract of the spearmint was found as 89 and 8% and greater than the same doses of BHT and α-tocopherol (16 and 45%), respectively. Superoxide radical scavenging activity of those samples followed the order: BHA > ethanol extract of MS > α-tocopherol > BHT.

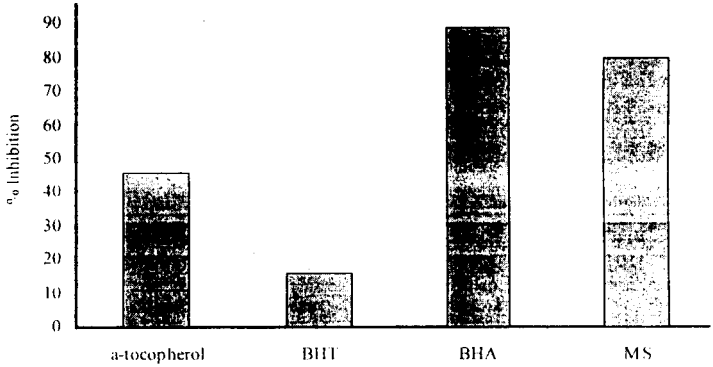


Fig. 3. Superoxide anion radical scavenging activity of and ethanol extract of MS, BHA, BHT, and α-tocopherol by the PMS-NADH-NBT method. (MS: *Mentha spicata*, BHA: Butylated hydroxyanisole, BHT: Butylated hydroxytoluene).

**Free radical scavenging activity**

The model of scavenging the stable DPPH radical is a widely used method to evaluate antioxidant activities in a relatively shorter time compared with other methods. The effect of antioxidants on DPPH radical scavenging was thought to be due to their hydrogen donating ability[32].

DPPH· is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule[33]. The reduction capability of DPPH radicals was determined by the decrease in its absorbance at 517 nm induced by antioxidants. The absorption maximum of a stable DPPH radical in ethanol was at 517 nm. 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 discoloration from purple to yellow. Hence, DPPH· is usually used as a substrate to evaluate antioxidative activity of antioxidants[22, 29]. Fig. 4 illustrates a significant ($p < 0.01$) decrease the concentration of DPPH radical due to the scavenging ability of the spearmint (MS) extract and standards. Ethanol extract of spearmint (MS) showed strong DPPH scavenging activity. We used BHA, BHT and α-tocopherol as standards. The scavenging effect of ethanol extract of the spearmint (MS) and standards on the DPPH radical decreased in the order of BHA > α-tocopherol > ethanol extract > BHT and were 96, 95, 92, and 61 at the concentration of 180 μg/mL, respectively. These results indicate that the spearmint extract has a noticeable effect on scavenging free radical. Free radical scavenging activity also increases with increasing concentration.

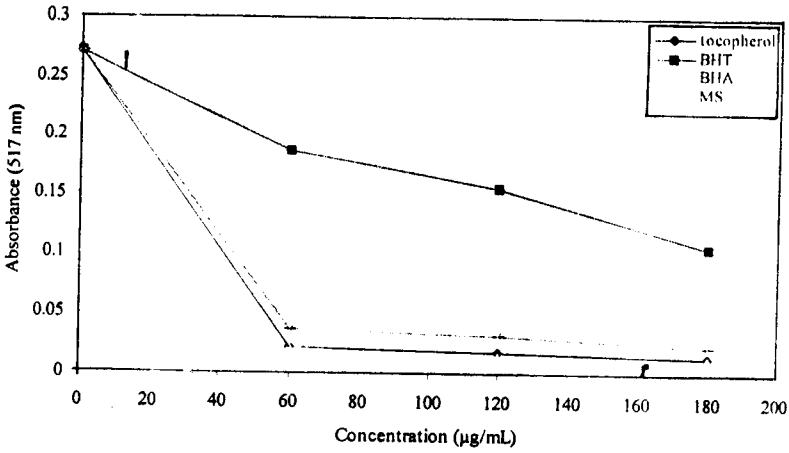


Fig. 4. Free radical scavenging activity of and ethanol extract of MS, BHA, BHT, and α-tocopherol by 1,1-diphenyl-2-picrylhydrazyl radicals. (MS: *Mentha spicata*, BHA: Butylated hydroxyanisole, BHT: Butylated hydroxytoluene).

## Metal chelating activity

Ferrozine can quantitatively form complexes with $Fe^{2+}$. In the presence of chelating agents, the complex formation is disrupted with the result that the red colour of the complex is decreased. Measurement of colour reduction therefore allows estimation of the chelating activity of the coexisting chelator[34].

Iron can stimulate lipid peroxidation by the Fenton reaction and also accelerates peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals that can themselves abstract hydrogen and perpetuate the chain reaction of lipid peroxidation[12, 29].

$$H_2O_2 + Fe^{2+} \longrightarrow Fe^{3+} + OH^- + OH\cdot \quad \text{(Fenton Reaction)}$$

As shown in Fig. 5, the formation of the $Fe^{2+}$-ferrozine complex is not complete in the presence of ethanol extract of spearmint (MS), indicating that the extract of *Mentha spicata* L. chelates with iron. The absorbance of $Fe^{2+}$-ferrozine complex linearly decreased dose-dependently (from 50 to 150 µg/mL). The difference between *Mentha spicata* L. and the control was statistically significant ($p < 0.01$). The percentage of metal chelating capacity of 50 µg/mL concentration of ethanol extract of spearmint (MS), BHA, α-tocopherol and BHT was found as 54, 53, 52 and 46%, respectively. However, there was a statistically significant difference between 50 µg/mL of ethanol extract and 50 µg/mL of BHA, α-tocopherol and BHT ($p < 0.05$). The metal scavenging effect of the extract of spearmint (MS) and standards decreased in the order of ethanol extract of the spearmint (MS) > BHA > α-tocopherol > BHT.

Metal chelating capacity was significant since it reduced the concentration of the catalyzing transition metal in lipid peroxidation[22]. It was reported that chelating agents, which form σ-bonds with a metal, are effective as secondary

antioxidants because they reduce the redox potential thereby stabilizing the oxidised form of the metal ion[35]. The data obtained from Fig. 5 reveal that the *Mentha spicata* L. extract demonstrates a marked capacity for iron binding, suggesting that their action as peroxidation protector may be related to its iron binding capacity.

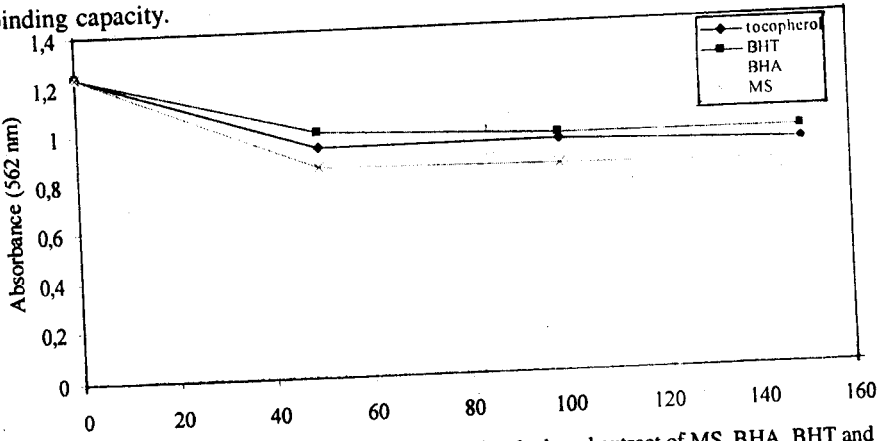


Fig. 5. Metal chelating effect of different amount of and ethanol extract of MS, BHA, BHT and α-tocopherol on ferrous ions. (MS: *Mentha spicata*, BHA: Butylated hydroxyanisole, BHT: Butylated hydroxytoluene).

## Determination of total phenolic compounds

Phenols are very important plant constituents because of their scavenging ability due to their hydroxyl groups[36]. In the ethanol extract of *Mentha spicata* L. (1 mg), 84.5 μg gallic acid equivalent of phenols was detected. The phenolic compounds may contribute directly to the antioxidative action[22]. It is suggested that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans, when up to 1.0 g daily ingested from a diet rich in fruits and vegetables. In addition, it was reported that phenolic compounds were associated with antioxidant activity and play an important role in stabilizing lipid peroxidation[9].

## Conclusion

On the basis of the results of this study, it is clearly indicated that the *Mentha spicata* L. extract has a significant antioxidant activity against various antioxidant systems *in vitro*; moreover, *Mentha spicata* L. can be used as an easily accessible source of natural antioxidants and as a possible food supplement or in pharmaceutical industry. The various antioxidant mechanisms of the *Mentha spicata* L. extract may be attributed to strong hydrogen donating ability, a metal chelating ability, and their effectiveness as good scavengers of superoxide and free radicals. In addition, phenolic compounds appear to be responsible for the antioxidant activity of both the spearmint (MS) extract. However, the components responsible for the antioxidative activity of the extract of *Mentha spicata* L. are currently

unclear. Therefore, it is suggested that further work should be performed on the isolation and identification of the antioxidant content of *Mentha spicata* L.

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