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Antioxidant Activity of Water Extract of *Eruca sativa* Mill.

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This study examined the antioxidant activities of water extract of *Eruca sativa* Mill., which is a plant used as both herbal medicine and food in Turkey. The antioxidant activity of water extract of *Eruca sativa* Mill. was evaluated using different antioxidant test such as reductive potential, free radical scavenging, metal chelating activity, hydroxyl radical activities. In addition, total phenolic compounds in the extract of *Eruca sativa* Mill. were determined as pyrocatechol equivalent. Those various activities were compared to standard antioxidants such as, α-tocopherol, butylated hydroxyanisole and butylated hydroxytoluene. The results obtained in this study indicate that *Eruca sativa* Mill. is a potential source of natural antioxidant.

Key Words: *Eruca sativa* Mill., Antioxidant activity, Radical scavenging, Reducing power.

INTRODUCTION

Plants (fruits, vegetables, spices and medicinal herbs, *etc*.) constitute an important source of active natural products which differ widely in terms of structure and biological properties. They have had a remarkable role in the traditional medicine in different countries[1]. Fruits and vegetables in the diet have been found in epidemiological studies to be protective againts several chronic diseases associated with aging such as cancer, cardiovascular disease, cataract, brain and immune dysfunction. There is a great deal of evidence to suggest that a higher intake of such compounds is associated with a lower risk of mortality from these disease as well as from diabetes mellitus, acute hypertension and arteriosclerosis[2]. These pathologic conditions are the major causes of mortality in industrialied countries[3]. Antioxidants are widely used to protect oxidizable goods such as cosmetics, pharmaceuticals, processed food or plastics from damage caused by reactive oxygen species. Plant products are also known to possess potential for food preservation[4]. They play a major role in the food industry, to minimize rancidity, to delay the emergence of potentially toxic oxidative products, to protect and to stabilize colours, aroma and nutritional quality and to increase shelf life of food products[5]. In order to prolong the storage stability of foods, synthetic antioxidants are used for industrial processing commercially antioxidants used in food processing such as, butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propylgallate (PG) and *tert*-butylhydroquinone (TBHQ) potentially dangerous for human health. For example, these substances have suspected of being responsible for liver damage and carcinogenesis in living organisms[6]. They have shown to cause pulmonary damage in mice, liver necrosis, haemorrhagic death and neoplasia in rats. From this point of view, governmental authorities and consumers are concerned about the safety of food and about the potential effects of synthetic additivies on health[7].

Most of greens have been used traditionally for their health benefits including prevention of cancer, antiedema effects, diuretic effects, antidiabetic, antihepatotoxic, tonics and soporific drugs. *Eruca sativa* Mill. leaves from the family of cabbage family *i.e.*, cruciferous/Brassicaceae crops is used especially as a salad or a food ingredient and has a characteristic horseradish-like odor and biting taste[8]. Leaves of salad rocket (*Eruca sativa* L.) are increasingly eaten by humans either alone or as part of mixed salads and are also used in herbal remedies[9]. In Asia, the plant serves as an important source of oil seeds[9]. *Eruca sativa* Mill. is used in Turkish traditional medicine for alleviating coughs, giving strength, appetizing and stimulant[10]. *Eruca sativa* Mill. is used as diuretic drugs in traditional medicine[11]. Phytochemical screening of the dried powdered seeds of *Eruca sativa* Mill. revealed the presence of volatile oils[12], sterol and/or triterpenes carbohydrates and/or glysodies, tannins, flavonoids[11] and thiocyanates[13,14]. No studies have been conducted to investigate the antioxidant activity of *Eruca sativa* Mill. leaves.

In the present study, the antioxidant activities of water extracts of *Eruca sativa* Mill. were examined in the different antioxidant assays including total antioxidant activity, reductive potential, DPPH free radical scavenging, hydroxyl radical scavenging and metal chelating activity.

EXPERIMENTAL

Eruca sativa Mill. was collected during March 2004 from Rami-Istanbul in Turkey. Plant leaves were carefully washed with tap water and left to dry in the shade at room temperature. They were stored in well closed cellophane bags.

Preparation of the aqueous extract: 10 g dried leaves were extracted by boiling for 0.5 h in 100 mL distilled water. The extract was then filtrated and the filtrate was evaporated to dryness under reduced pressure by using a rotary evaporator. The water extract was subjected to preminilary phytochemical testing for the detection of major chemical groups (Table-1)[15]. The details of the test are as follows:

TABLE-1
PRELIMINARY PHYTOCHEMICAL SCREENNIG OF WATER EXTRACT OF *Eruca sativa* Mill.

Tested for	Presence/ absence*	Test performed
Phenols	+++	Phosphomolybdic acid test
Tannins	+++	Braemer's test
Steroids and Terpenoids	++	Liebermann-Burchardt test
Alkaloids	–	Dragendorff's test
Anthraquinones	+++	Bornträger test
Flavonoids	++	Shinoda's test

*– Absent; +++ Abundant

For phenols: The water extract was spotted on a filter paper. A drop phosphomolybdic acid reagent was added to the spot and was exposed to ammonia vapours (blue colouration of spot indicate the presence of phenols).

Braemer's test for tannins: To 2-3 mL of water extract, 10 % alcoholic ferric chloride solution was added (dark blue or greenish grey colouration of the solution indicate the presence of tannins in the drug).

Liebermann-Burchardt test for steroids and terpenoids: To 1 mL extract of drug, 1 mL of chloroform, 2-3 mL of acetic anhydride and 1 to 2 drops of concentrated sulfuric acid were added (dark green colouration of the solution indicate the presence of steroids and dark pink or red colouration of the solution indicate the presence of terpenoids).

Alkaloids: To 1 mL water extract Dragendorff's reagent was added (orange colouration of the spot indicate the presence of alkaloids).

Bornträger's test anthraquionones: About 50 mg of water extract was heated with 10 % ferric chloride solution and 1 mL of concentrated hydrochloric acid. The extract was cooled, filtered and filtrate was shaken with diethyl ether. The ether extract was further extracted with strong ammonia (pink or deep red colouration of aqueous layer indicate the prensece of anthraquinones).

Shinoda test for flavonoids: To 2-3 mL of water extract a piece of magnesium ribbon and 1 mL concentrated hydrochloric acid were added (pink red or red colouration of the solution indicate the presence of flavonoids).

Determination of antioxidant activity of the extract

Ferric thiocyanate (FTC) antioxidant activity method: The ferric thiocyanate (FTC) method was adapted from Osawa and Namiki[16]. Samples (4 mg or 4 mL) in 99.5 % ethanol were mixed with 2.51 % linoleic acid in 99.5 % ethanol (4.1 mL), 0.05 M phosphate buffer pH 7.0 (8 mL) and distilled water (3.9 mL) and kept in screw cap containers under dark conditions at 40 °C. To 0.1 mL of this solution was added 9.7 mL of 75 % ethanol and 0.1 mL of 30 % ammonium thiocyanate. Precisely 3 min after addition of 0.1 mL of 2×10^{-2} M ferrous chloride in 3.5 % hydrochloric acid to the reaction mixture, the absorbance of the red colour was measured at 500 nm every 24 h until absorbance of the control reached maximum. The control and standard were subjected to the same procedure as the sample expected for the control, where there was no addition of sample and for the standard, where 4 mg of sample were replaced with 4 mg of α-tocopherol.

Thiobarbituric acid test (TBA): The test was conducted according to the methods of Ottolenghi[17] and Kikuzaki and Nakatani[18]. The same samples as prepared for the ferric thiocyanate method were used. To 1 mL of sample solution, 20 % aq. trichloroacetic acid (2 mL) and of aq. thiobarbituric acid solution (2 mL) were added. This mixture was then placed in a boiling water bath for 10 min. After cooling, it was centrifuged at 3000 rpm for 20 min. Absorbance of supernatant was measured at 532 nm. Antioxidative activity was recorded, based on absorbance on the final day.

Determination of reducing power: The reducing power of plant extract was determined according to the method of Oyaizu[19]. Different amounts of extracts (20-100 µg) in 1 mL of distilled water were mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL potassium ferricyanide [$K_3Fe(CN)_6$] (1 %) and then the mixture was incubated at 50 °C for 0.5 h. Afterwards, 2.5 mL of trichloroacetic acid (10 %) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. Finally, 2.5 mL of upper layer solution was mixed with 2.5 mL distilled water and 0.5 mL $FeCl_3$ (0.1 %) and the absorbance was measured at 700 nm in a spectrophotometer, increased absorbance of the reaction mixture indicated increased reducing power.

Determination of the chelating activity on Fe^{2+}: The chelating activity of samples on the Fe^{2+} was measured according to Rival[20] and Duh *et al.*[21] 1 mL of samples (250-1000 µg/mL) was mixed with 3.7 mL deionized water. Briefly, each sample was incubated with 0.1 mL $FeCl_2$ (2.0 mM) for 5, 10, 30 and 60 min. After incubation, the reaction was initiated by addition of 0.2 mL ferrozine (5.0 mM) and the mixture was left to stand for 10 min at room temperature. The absorbance of the mixture (formation of the ferrous iron-ferrozine complex) was measured at 562 nm. The control was performed in the same way using $FeCl_2$ and water. The lower the absorbance of the reaction mixture, the higher the $FeCl_2$-chelating ability. The capability to chelate the ferrous iron was calculated using the following equation: chelating activity

$$\text{Chelating activity (\%)} = [1-(\text{absorbance of sample}/\text{absorbance of control})] \times 100$$

Hydroxyl radical scavenging: The effect of hydroxyl radical was assayed by using the 2-deoxyribose oxidation method[22]. 2-Deoxyribose is oxidized by hydroxyl radical that is formed by the Fenton reaction and degraded to malondialdehyde[23]. The reaction mixture contained 0.45 mL of 0.2 M sodium phosphate buffer (pH 7.4), 0.15 mL of 10 mM 2-deoxyribose, 0.15 mL of 10 mM $FeSO_4$-EDTA, 0.15 mL of 10 mM hydrogen peroxide, 0.525 mL of distilled water and 0.075 mL of extract solution in a tube. The reaction was started by the addition of hydrogen peroxide. After incubation at 37 ºC for 4 h, the reaction was stopped by adding 0.75 mL of 2.8 % (w/v) trichloroacetic acid and 0.75 mL 1.0 % (w/v) of thiobarbituric acid. The mixture was boiled for 10 min, cooled in ice and then measured at 520 nm. Hydroxyl radical-scavenging ability was evaluated as the inhibition rate of 2-deoxyribose oxidation by hydroxyl radical.

Inhibition (I) of deoxyribose degradation in per cent was calculated in the following way:

$$I = (A_0 - A_1/A_0) \times 100$$

where A_0 is the absorbance of the control reaction (containing all reagents except the test compound) and A_1 is the absorbance of the test compound.

Determination of scavenging effect on the DPPH radical: The effect of the plant extracts on the DPPH radical was estimated according to the procedure described by Brand-Williams *et al.*[5]. An appropriate dilution series 0.25-1.00 mg/mL) was prepared for each aqueous extract in methanol 0.1 mL of each dilution was added to 3.9 mL of a 6×10^{-5} M methanolic solution of $DPPH^{\bullet}$ (1,1-diphenyl-2-picrylhydrazyl) followed by vortexing. The reaction was allowed to take place in the dark at room temperature to reach a plateu (time at the steady state). For this absorbance at 517 nm was measured at different time intervals. The time at the steady state was different for every sample. Methanol was used to zero of the spectrophotometer. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The DPPH radical concentration was calculated using the following equation:

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

Determination of total phenolic compounds: Total soluble phenolic compounds in the *Eruca sativa* Mill. were determined with Folin-Ciocalteu according to the method of Slinkard and Singleton[24] using pyrocatecol as a standart phenolic compound. Briefly, 1 mL of the *Eruca sativa* solution (20-100 µg extract) in a volumetric flask diluted with distilled water (46 mL). 1 mL of Folin-Ciocalteu reagent was added and the content of the flask was mixed thoroughly. After 3 min 3 mL Na_2CO_3 (2 %) was added and then was allowed to stand for 2 h with intermittent shaking. The absorbance was measured at 760 nm in a spectrophotometer. The total phenolic compounds in the *Eruca sativa* Mill. determined as microgram of pyrocatechol equivalent by using an equation that was obtained from standart pyrocatechol graph.

$$\text{Absorbance} = 0.0022 \times \text{total phenols [pyrocatechol equivalent } (\mu g)] - 0.0464$$

RESULTS AND DISCUSSION

The antioxidant activity of the water extract of *Eruca sativa* leaves was evaluated in different *in vitro* models. Further, the same water extract was subjected to preliminary phytochemical screening for the presence of different chemical groups (Table-1). Phenolics and tannins were found to be major groups present along with flavonoids in the water extract. Various studies have shown that polyphenolic compounds are associated with antioxidant activity and play an important role in stabilizing lipid peroxidation[25]. In order to elucidate the causes for antioxidant characteristics of water extract *Eruca sativa*, it is essential to determine whether the antioxidant activity is related to phenolic compounds. Ultraviolet-Visible absorption spectroscopy is one of the most useful techniques avaible for structure analysis of phenolic compounds. The UV-Visible spectra of the diluted *Eruca sativa* Mill. extract is shown in Fig. 1. Absorption maxima at 210 and 274 nm may be due to the presence of flavone/flavonol derivatives indicating that *Eruca sativa* Mill. may contain phenolic compounds. Phenols are very important plant constituents because of their scavenging ability due to their hydroxyl groups[26].

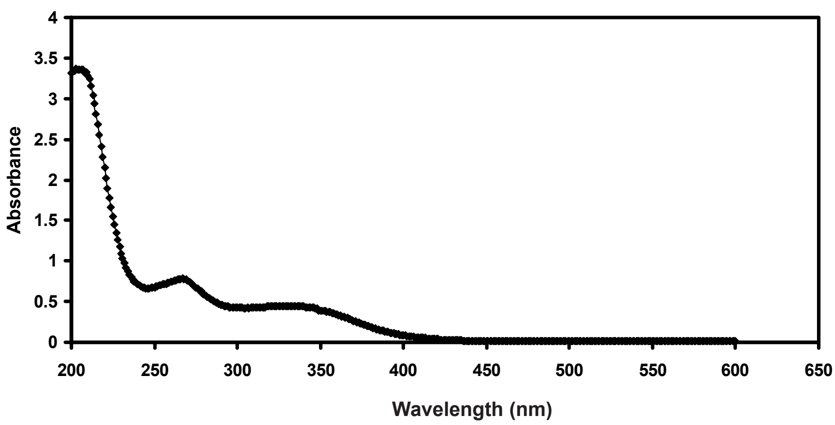

Fig. 1. UV-Vis spectra of water extract of *Eruca sativa* Mill.

Ferric thiocyanate (FTC) method: The FTC method used for the determination of total antioxidant activity is a reliable method. In the FTC test, which determines the amount of peroxide produced at the initial stage of lipid peroxidation, a lower absorbance indicates a higher level of antioxidant activity. The method depends on peroxide formation in the aqueous emulsion of linoleic acid. The autooxidation of linoleic acid without added was accompained by a rapid increase of peroxide value at 6 d of testing.The antioxidative activities of plant extracts were compared with commercial antioxidants such as DL-α-tocopherol. The effect of various amounts of water extracts of *Eruca sativa* Mill. (20-100 μg) on peroxidation of linoleic acid emulsion are shown in Fig. 2. The absorbance of the control increased in proportion to incubation time and the absorbance of the plant extracts also increased with increasing incubation time. Water extracts of *Eruca sativa* Mill. exhibited effective antioxidant activity at all concentrations.

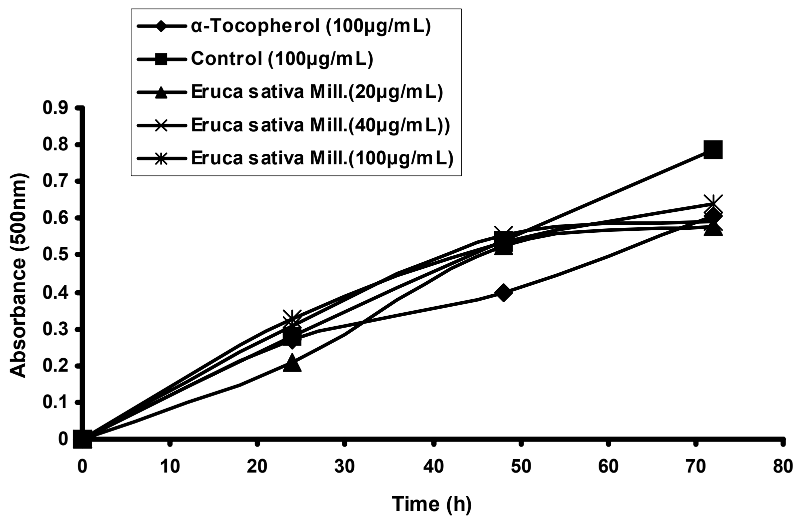

Fig. 2. Total antioxidant activity of α-tocopherol and different doses of *Eruca sativa* Mill. in the linoleic acid emulsion

Thiobarbituric acid test (TBA): Different from the FTC test, which is related to the peroxide formation in the initial stage of lipid oxidation, the TBA test measures the amount of malondialdehyde (MDA) produced after the decomposition of the lipid peroxide during the oxidation process. MDA is a very unstable compound causing mutagenic and cytotoxic events[27]. At a low pH and high temperature (100 °C), MDA binds TBA to form a red complex that can be measured at 532 nm after incubation for 72

h. Fig. 3 shows antioxidative activities of water extracts, measured on the sixth day, using the TBA method. The result from the TBA test strongly correlated with the FTC data and all of the extracts (20, 40 and 100 μg) showed a lower absorbance than the control (Fig. 3).

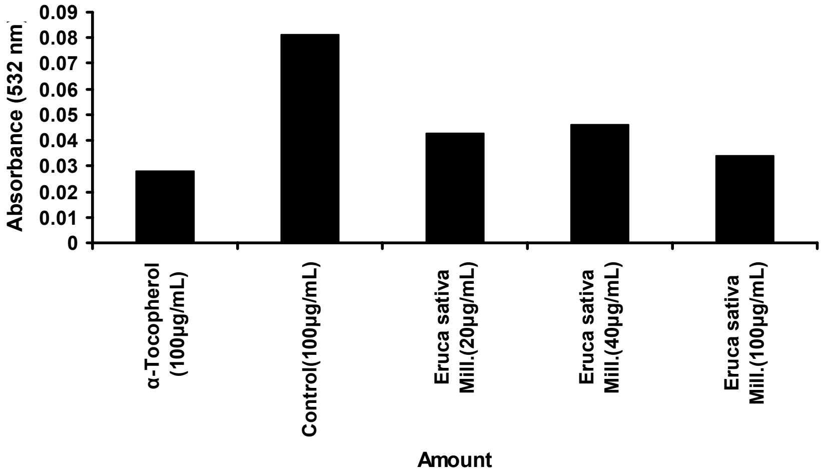

Fig. 3. Antioxidative activity of water extract of *Eruca sativa* Mill. as measured by the thiobarbituric acid method

Reducing power: Fig. 4 shows the reducing power of the extracts using the potassium ferricyanide reduction methods. For the measurements of the reductive ability, we investigated the Fe^{3+}-Fe^{2+} transformation in the presence of samples using the method of Oyaizu[19]. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity[28]. As shown in Fig. 4 the reducing power increased with increasing amounts of extracts. Reductive potential of water extract of *Eruca sativa* Mill. and standard compounds followed the order; butylated hydroxyanisole > ascorbic acid > α-tocopherol > water extract of *Eruca sativa* Mill.

Chelating effect of samples on Fe^{2+}: Iron is essential for life, for oxygen transport, respiration and the activity of many enzymes[21]. Metals are well-known initiators of unwanted oxidative reactions in lipids, proteins and other cellular components. In addition, iron is capable of generating free radicals from peroxides by Fenton reactions and minimization of the Fe^{2+} concentration in the Fenton reaction affords protection againts oxidative damage[29]. The chelating activity of samples increased with increasing incubation times with $FeCl_2$. *Eruca sativa* Mill. (1000 μg/mL) showed the strongest chelating activity Fe^{2+} ions. It displayed an 51.60 % chelating effect Fe^{2+} ions at an incubation time of 1 h (Fig. 5).

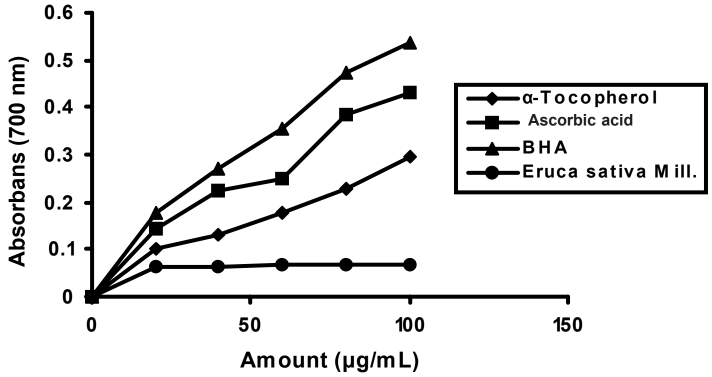

Fig. 4. Reducing power of *Eruca sativa* Mill.

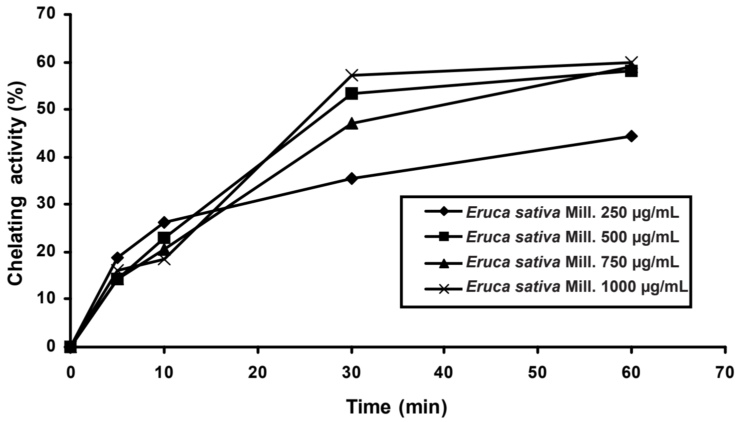

Fig. 5. Chelating effects different amount of *Eruca sativa* Mill. on Fe^{2+} ions at different incubation times with $FeCl_2$

Hydroxyl radical scavenging activity: Among the oxygen radicals, hydroxyl radical is the most reactive and induces severe damage to the adjacent biomolecules. The hydroxyl radical scavenging activity of *Eruca sativa* Mill. was investigated by using the Fenton reaction[30]. Fig. 6 shows the hydroxyl radical-scavenging effects by the 2-deoxyribose oxidation method. The results were indicated as the inhibition rate. Each concentration of *Eruca sativa* Mill. showed hydroxyl radical-scavenging activity and its activity was increased with increasing concentration of the extract sample. The activity of 1000 µg of extract was nearly equal to that of 1000 µg ascorbic acid. Hydroxy radicals are well known to abstract hydrogen atoms from membrane lipids and bring about lipid peroxidation. Apparently, the ability to quench the hydroxyl radical by the extract seems to relate directly to the prevention of propagation of the process of lipid peroxidation.

It is well-known that reactive oxygen species induce some oxidative damage to biomolecules like nucleic acid, lipids, proteins and carbohydrates and this damage causes ageing, cancer and other disease. According to the data presented, the extract of *Eruca sativa* Mill. remarkable scavenging effect on reactive oxygen species, suggesting that *Eruca sativa* Mill. eats may be benefical to health[31].

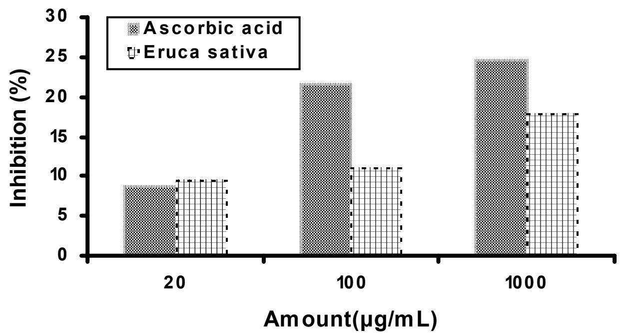

Fig. 6. Scavenging effect of different amounts of water extracts of *Eruca sativa* Mill. on hydroxyl radical

Free radical scavenging activity: The use of 1,1-diphenyl 2-picrylhydrazil (DPPH•) as a regent for screening the antioxidant activity of small molecules has been reported[1]. In the scavenging of DPPH radical is followed by monitoring the decrease in absorbance at 515 nm, which occurs due to reduction by the antioxidant[1]. Free radical are known to be a major factor in biological damages and DPPH• has been used to evaluate the free radical-scavenging activity of natural antioxidant[32]. DPPH•, which is a radical itself with a purple colour, changes into a stable compound with a yellow colour by reacting with an antioxidant and the extent of the reaction depends on the hydrogen donating ability of the antioxidant[33]. Free radical-scavenging capacities of the extract and synthetic antioxidants measured by DPPH• assay, are given in Table-2. DPPH• is usually used as a substrate to evaluate antioxidative activity of antioxidants. We used butylated hydroxyanisole, butylated hydroxytoluene and Trolox as standards. The scavenging effect of water extracts of *Eruca sativa* on the DPPH• radical decreased in the order of water extract > butylated hydroxytoluene > butylated hydroxyanisole > Trolox and 99.89 %, 85.42 %, 79.01 %, 72.59 % at the dose of 100 (μg/mL), respectively. In the present study, water extract showed a good antiradical activity by scavenging DPPH• radical.

$$\underset{\text{(purple colour)}}{DPPH^{\bullet}} + \text{Antioxidant} \longrightarrow DPPH\text{-}H + \underset{\text{(yellow colour)}}{\text{antioxidant}}$$

TABLE-2
in vitro SCAVENGING EFFECTS OF WATER EXTRACTS OF *Eruca sativa* Mill. AND STANDARDS ON DPPH

Extract (µg/mL)	DPPH• Inhibition (%)
Eruca sativa Mill.	99.89
Butylated hydroxytoluene (BHT)	85.42
Butylated hydroxyanisole (BHA)	79.01
Trolox	72.59

Determination of total phenolic compounds: Herbs, fruits, spices and vegetables are important natural antioxidant[34]. Their antioxidant activity has been attributed to the presence of polar phenolic compounds. Polyphenolic compounds in plants are powerful free radical-scavengers which can inhibit lipid peroxidation by neutralizing peroxy radicals generated during the oxidation of lipids[35]. According to the recent reports, a highly positive relation between total phenols and antioxidant activity was found in many plant species. Amount of total phenolic compounds are shown in Table-3. In the presence of 20-2000 µg/mL of *Eruca sativa* extract, the content of total phenolic compounds increased. An increase in total phenolic compounds was concentration dependent. In the aqueous extract of *Eruca sativa* (1 mg), 26.32 µg pyrocatechol equivalent of phenols was detected. Phenolic compounds may contribute directly to the antioxidative action[31]. It is suggested that polyphenolic compounds have inhibitor effects on carcinogenesis and mutagenesis in humans, when up to 1.0 g daily ingested from a diet rich in fruits and vegetables[36].

TABLE-3
AMOUNTS OF TOTAL PHENOLIC COMPOUNDS IN *Eruca sativa* Mill. EXTRACT

Extract (µg/mL)	Pyrocatechol equivalents (µg/mL)
Control	–
20	21.77
40	23.36
100	25.64
1000	26.32
1500	30.41
2000	35.41

Herbal and natural products have been used for the centuries, throughout the world, in every culture[37]. Nowadays the consumption of fruit and vegetables is regarded as important and good for health. Indeed, recent epidemiological studies have indicated that a high intake of fruit and vegetables is associated with reduced risk for a number of chronic disease[38]. In conclusion, extract from *Eruca sativa* have high levels antioxidant activity, reducing power, a metal chelating ability DPPH radical and hydroxyl radical scavenging activities. These finding strongly suggest that *Eruca sativa* Mill. has antioxidant activity. In several studies, performed with various plants with antioxidative effects, it was found that the active substances were polyphenolic compounds, tannins, isothiocyanates (glucosinolates), essential oil and flavonoids. The analysis of *Eruca sativa* leaves revealed a high amount of essential oils, tannins, flavonoids[11,12] and isothiocyanates[12,13]. The antioxidative activity of *Eruca sativa* Mill. leaves might be related to the flavonoid, isothiocyanates (glucosinolates) essential oil and tannins. Because of the side effects of the synthetic agents used as antioxidants today, there is increasing interest in the use of natural products both in the pharmaceutical and food processing fields. Therefore, the results presented here could be useful for such industries.

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