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INVESTIGATION OF ANTIOXIDANT PROPERTIES OF *NASTURTIUM OFFICINALE* (WATERCRESS) LEAF EXTRACTS

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Abstract : The objective of this study was to examine the *in vitro* and *in vivo* antioxidative properties of aqueous and ethanolic extracts of the leaf of *Nasturtium officinale* R. Br. (watercress). Extracts were evaluated for total antioxidant activity by ferric thiocyanate method, total reducing power by potassium ferricyanide reduction method, 1,1-diphenyl-2-picrylhydrazyl (DPPH˙) radical scavenging activities, superoxide anion radical scavenging activities *in vitro* and lipid peroxidation *in vivo*. Those various antioxidant activities were compared to standards such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and α-tocopherol. The ethanolic extract was found as the most active in total antioxidant activity, reducing power, DPPH˙ radicals and superoxide anion radicals scavenging activities. Administration of the ethanol extract to rats decreased lipid peroxidation in liver, brain and kidney. These results lead to the conclusion that *N. officinale* extracts show relevant antioxidant activity by means of reducing cellular lipid peroxidation and increasing antioxidant activity, reducing power, free radiacal and superoxide anion radical scavenging activities. In addition, total phenolic compounds in the aqueous and ethanolic extract of *N. officinale* were determined as pyrocatechol.

Keywords: *Nasturtium officinale* (watercress), antioxidant activity, lipid peroxidation

Reactive oxygen species (ROS) induce in many diseases, such as cancer, DNA damage and cellular degeneration related to aging. Their sources are derivatives of oxygen and they show an important role in mediating ROS-related effects (1). Most living species have efficient defense systems to save from harm themselves against the oxidative stress cause by ROS (2). Recent investigations have shown that the antioxidant properties of plants could be correlated with oxidative stress defense and different human diseases including cancer, atherosclerosis, and the aging processes (3). It has far been known that substances synthesized naturally in higher plants have antioxidant properties. Accordingly, the use of natural antioxidants has been effective in the prevention and protection of these diseases such as polyphenols, flavonoids and terpenes (4).

Nasturtium officinale R. Br. (watercress) is used to cure abdominal pain in Turkish folk medicine and is eaten as a vegetable and in salads in Turkey (5). This herb is used to treat diabetes, bronchitis, and diuresis (6), as anti-ulcerogenic (7), in treatment of scurvy, tuberculosis, influenza, asthma, (8), nutritional supplement and digestive aid (9) and also seems to have antimicrobial (10), anticarcinogenic, and antiestrogenic activity (11). The effects of watercress also decrease breast cancer risk (12), treatment of lung and other cancers in humans (13) and the spreading of pancreas, gastric, and lung cancer (14).

A literature survey showed that no study has been done on antioxidative activity of watercress. Therefore, this investigation was intended to study antioxidant properties of different extracts (water and etanol) of this plant by *in vitro* tests. The studies included *in vitro* total antioxidant activity, reducing power, DPPH˙ free radical and superoxide anion scavenging activity and lipid peroxidation in liver, brain and kidney homogenate.

MATERIAL AND METHODS

Plant material

Watercress leaves were collected from the Arhavi-Artvin, Turkey, in June-July (2006), during the early hours of the day. They were authenticated by Dr. Hamdi Güray Kutbay and deposited at Ondokuz Mayýs University Arts and Sciences Faculty Herbarium (No.: OMUB 4020).

Extraction of plant materials

Watercress leaves were shade-dried and sliced. For preparing water extract, the dried aerial parts of watercress (20 g of leaves) were powdered in a mill

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and mixed with 500 mL of boiling water. The aquatic extract was filtered. Then, the filtrates were frozen and lyophilized in a lyophilizator. For preparing ethanol extract, 20 g of watercress leaves was pound into a fine power in a mill. Then, the sample was extracted in a Soxhlet apparatus until becoming colorless and filtered. The filtrate was evaporated by rotary evaporator at 50°C. The final residues of aqeous and ethanolic extract were used for the assays and stored at +4°C until using.

Chemicals

Ammonium thiocaynate, ferrous chloride and 1,1-diphenyl-2-picryl-hydrazyl (DPPH$^{\cdot}$) were purchased from E. Merck. Nicotine adenine dinucleotide (NADH), BHA, BHT, α-tocopherol, tricholoracetic acid (TCA), nitroblue tetrazolium (NBT), phenazine methosulfate (PMS), potassium ferricyanide and linoleic acid were purchased from Sigma Chemical Co. All other chemicals and reagents were of analytical grade and/or obtained from other commercial sources.

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

The ferric thiocyanate method (FTC) was adapted from the model of Mitsuda (15). The solution which contains the same concentration of watercress and standard antioxidant (100 µg/mL) in 2.5 mL of potassium phosphate buffer (0.04 M, pH 7.0) was added to linoleic acid emulsion in potassium phosphate buffer (2.5 mL, 0.04 mM, pH 7.0). In addition to these solutions, 5 mL control solution was prepared with linoleic acid emulsion (2.5 mL) and potassium phosphate buffer (2.5 mL, 0.04 M, pH 7.0). The solutions were shaken and periodical reaction mixture was incubated at 37°C in dark. The peroxide values were determined by reading the absorbance and investigated on every two days until the control reached its maximum absorbance value. The inhibition of lipid peroxidation in percent was calculated by the following the equation:

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

where A_0 was the absorbance of the control incubated with linoleic acid but without the samples and A_1 was the absorbance of watercress extracts or α-tocopherol (16).

Reducing power

The reducing power of extracts was determined according to the method of Oyaizu (17). Watercress and standard antioxidant (100 µg/mL) in 1 mL of distilled water were mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and potassium ferricyanide (2.5 mL; 10 g/L). The mixtures were incubated at 50°C for 20 min. Then, TCA (10%; 2.5 mL) was added to each mixture and centrifuged. Finally, the supernatants were mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL; 0.1%). The absorbance of the solution was measured at 700 nm. Higher absorbance of the reaction mixture indicated that the reducing power is increased.

Free radical scavenging activity

The effect of watercress on DPPH$^{\cdot}$ radical was estimated according to the method of Blois (18)

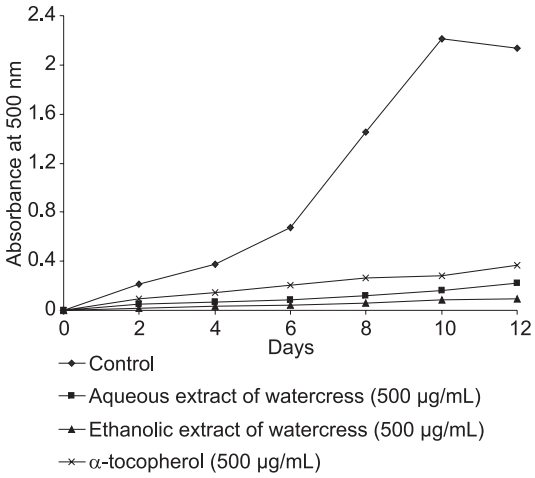

Figure 1. Determination of the antioxidant activity of watercress (*Nasturtium officinale*) by ferric thiocyanate method.

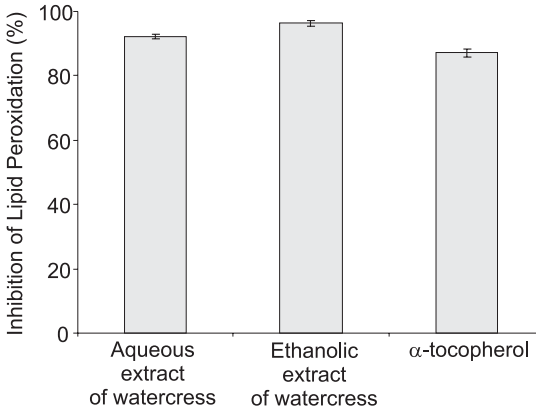

Figure 2. The inhibition percentage of peroxides formation in the presence of watercress (*Nasturtium officinale*) as compared to α-tocopherol after 10 days.

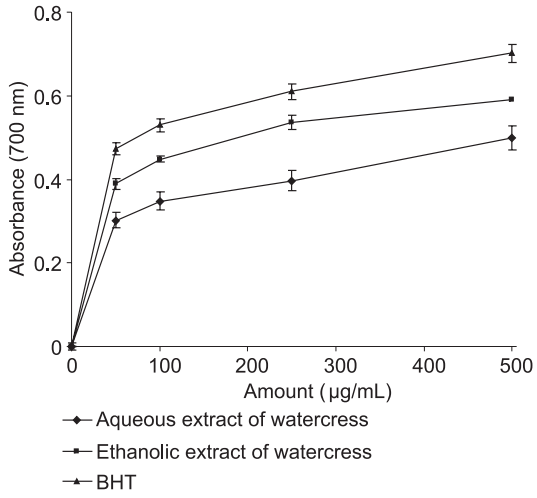

Figure 3. Reducing power of aqueous and ethanolic extracts of watercress (*Nasturtium officinale*) and BHT. (Spectrophotometric detection of the Fe^{3+}-Fe^{2+} transformation).

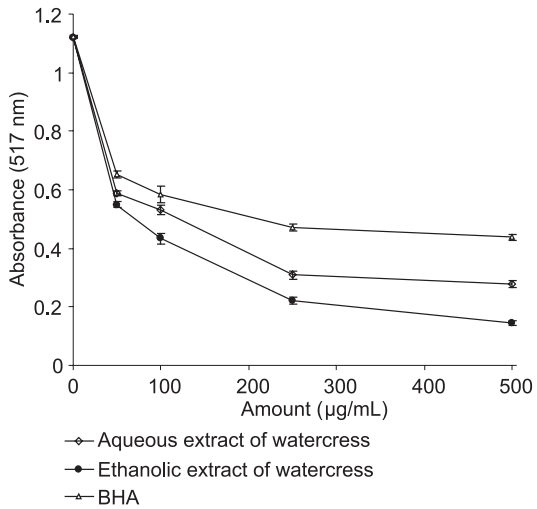

Figure 4. DPPH free radical scavenging activity of watercress (*Nasturtium officinale*). Various concentrations of extracts (50, 100, 250, and 500 µg/mL) were assayed in 10 min at 517 nm.

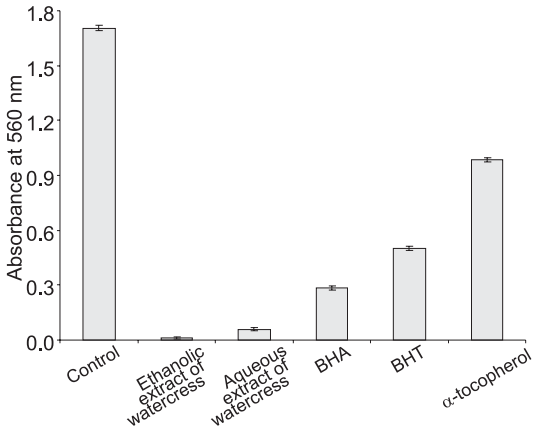

Figure 5. Superoxide radicals scavenging activity of watercress (*Nasturtium officinale*). 100 µg/mL of extracts, BHA, BHT and α-tocopherol were assayed at 517 nm.

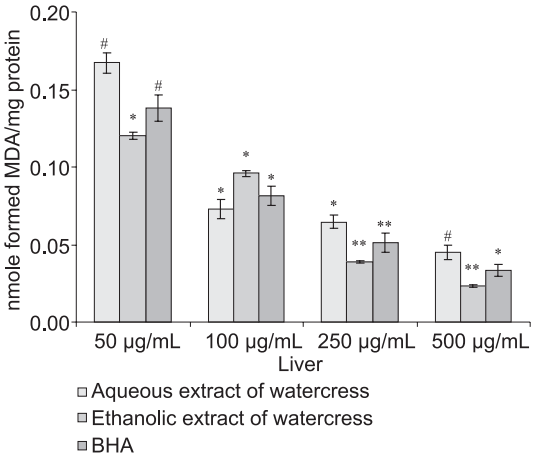

Figure 6. Changes in rat liver lipid peroxidation at 532 nm. *p < 0.05, **p < 0.01, #not significant, when compared with the control

wherein the bleaching rate of a stable free radical, $DPPH^{\bullet}$ is monitored at a characteristic wavelength in the presence of samples. An amount of 0.5 mL of 0.1 mM ethanolic solution of $DPPH^{\bullet}$ was added to 3.0 mL of watercress extracts or standard antioxidant solution (100 µg/mL). The mixture was mixed and after 10 min the absorbance was measured at 517 nm. The $DPPH^{\bullet}$ concentration (mM) in the reaction medium was calculated from the calibration curve (R^2: 0.9998).

Absorbance = 6.5781 × [$DPPH^{\bullet}$ mM] + 0.0058

This activity was calculated by the equation:

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

where A_0 was the absorbance of the control and A_1 was the absorbance of watercress or BHT (19).

Superoxide anion scavenging activity

The determination of superoxide anion scavenging activity of the extracts of watercress was measured according to slightly modified Nishimiki method (20). Superoxide radicals were generated in phenazine methosulfate (PMS)-nicotinamide adenine dinucleotide (NADH) system by oxidation of NADH and assayed by the reduction of nitroblue tetrazolium (NBT). One milliliter of watercress extract and standard antioxidants (100 µg/mL) in

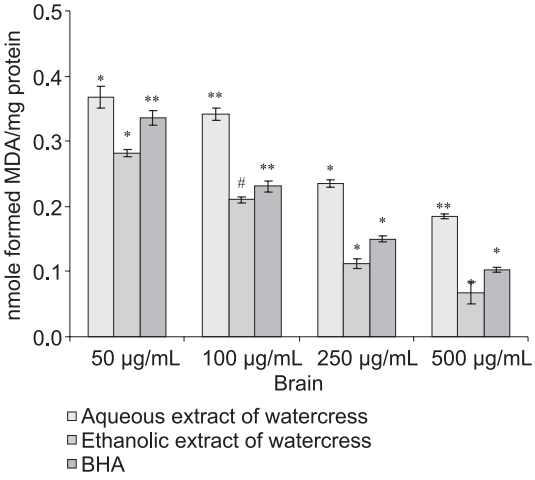

Figure 7. Changes in rat brain lipid peroxidation at 532 nm. *p < 0.05, **p < 0.01, #not significant, when compared with the control.

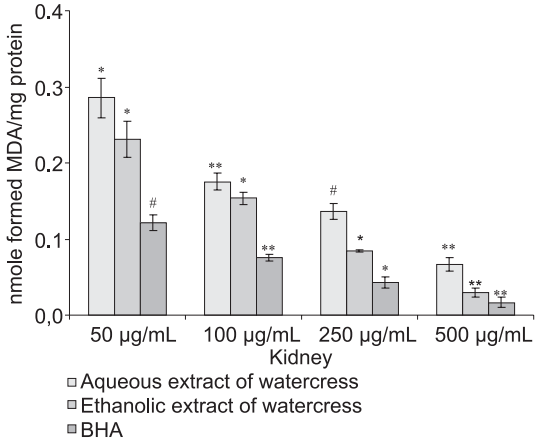

Figure 8. Changes in rat kidney lipid peroxidation at 532 nm. *p < 0.05, **p < 0.01, #not significant, when compared with the control.

water, 1.0 mL NBT solution (156 µM NBT in 100 mM phosphate buffer, pH 7.4) and 1.0 mL NADH solution (468 µM in 100 mM phosphate buffer, pH 7.4) were mixed. The reaction was started by adding 100 µL of PMS solution (60 µM PMS in 100 mM phosphate buffer, pH 7.4) to the mixture. The mixture was incubated at 25°C for 5 min, and its absorbance was measured at 560 nm wavelength against blank samples. L-ascorbic acid was used as a control sample. The percentage inhibition of superoxide anion generation was calculated using the following formula:

Inhibition of superoxide anion generation (%) = $[(A_0-A_1)/A_0 \times 100]$

where A_0 was the absorbance of the control, and A_1 was the absorbance of watercress or standards (19).

Amount of total phenolic compounds

The total phenolic compounds presented in extracts of watercress were determined using pyrocatechol equivalent as a standard phenolic compound (21). 1 mL of extract (containing 1000 mg of extract) was mixed with water (46 mL). 1 mL of Folin-Ciocalteu reagent was added and mixed thoroughly. 3 mL of Na_2CO_3 (2 %) was added and incubated in shaking waterbath for 2 h at 25°C. The absorbance was measured at 760 nm. The concentration of total phenolic compounds in both extracts was determined as mg of pyrocatechol equivalent using an equation obtained from the standard pyrocatechol graph as:

Absorbance = 0.0058 mg pyrocatechol – 0.0072 (r^2 = 0.9988)

Determination of lipid peroxidation level in rat liver, brain, and kidney homogenate

Male Wistar rats (6-8 weeks old, 180-200 g), were used. The animals were maintained under normal conditions (12 h light/dark cycle) and were fed with a standard laboratory diet and had access to tap water *ad libitum*. Liver, brain, and kidney tissues of rats were removed after animals were sacrificed (22).

The effect of crude watercress extracts induced with anti-$FeCl_2$-ascorbic acid and stimulated lipid peroxidation was determined by the modified method of Kimuya et al. (23) on the liver homogenate. Parts of the liver sections were cut into approximately 500 to 1000 mg portions on ice and stored separately at -70°C in plastic tubes. The frozen liver samples were homogenized in Tris-HCl buffer (0.15 M, pH 7.2) to give a 20% homogenate. In measurement of lipid peroxidation levels, homogenates were centrifuged at 17000 rpm and +4°C for 20 min. The reaction mixture contains 0.5 mL of liver homogenate, 0.1 mL of Tris-HCl buffer (0.15 M, pH 7.2), 0.05 mL ascorbic acid (0.1 mM), 0.05 mL $FeCl_2$ (4 mM) and different concentrations of the extract or standard (0.30 mL) in a total volume of 1.0 mL. After incubated for 1 hour at 37°C, 0.9 mL of distilled water and 2 mL of TBA was added to the incubation solution and shaken vigorously and then heated for 30 min in a boiling water bath. The n-butanol layer, separated by centrifugation at 1000 × g for 10 min, was determined at 532 nm (24).

The lipid peroxidation in the brain and kidney was measured by the modified method of Liu and

Ng (25). The brains and kidneys of normal rats were dissected and homogenized in ice-cold Tris–HCl buffer (20 mM, pH 7.4). The homogenate was centrifuged at 14000 rpm for 15 min. Aliquots of the supernatant were incubated with the watercress extract in the presence of $FeSO_4$ (10 μM) and ascorbic acid (0.1 mM) at 37°C for 1 hour. The reaction was stopped by addition of 1.0 mL TBA and the solution was then heated at 100°C for 15 min. After centrifugation, the color of the malondialdehyde–TBA complex was detected at 532 nm (26).

Cytosolic proteins were determined using the method of Lowry with slight modifications; bovine serum albumin (BSA) was used as a standard at 660 nm (27). A standard curve of 0 to 100 μg/mL BSA was also constructed and was used for the calculation of cytosolic protein in the samples.

Statistical analysis

The experimental results are presented as the mean ± S.D. of five parallel measurements and the values were evaluated by using one-way analysis of variance. p values < 0.05 were regarded as significant and p values < 0.01 as very significant.

RESULTS AND DISCUSSION

Figure 1 demonstrates the antioxidant activity of aqueous and ethanolic extracts of watercress and α-tocopherol. The extracts of watercress exhibited effective antioxidant activity. The data of peroxidation of linoleic acid emulsion was determined by FTC at 37°C, after addition of different concentrations of aqueous and ethanolic extracts of watercress (500 mg/mL). The percentage of inhibition on lipid peroxidation of linoleic acid system from 500 mg ethanolic extract (96.34%) showed higher antioxidant activity than 500 mg of aqueous extract (92.21%) and α-tocopherol (87.09%) (Figure 2). These results reveal that the ethanolic extracts of watercress has strong antioxidant activity in inhibition of linoleic acid oxidation. The difference between both extracts of watercress and the control was statistically significant ($p < 0.01$). Linoleic acid was considered not to reflect completely the lipid peroxidation, because of its only physical properties in aqueous micelles (28). Phospholipid is generally thought to be a major fraction responsible for the oxidative deterioration of foods, because of its greater degree of unsaturation (29). Therefore, phospholipids may be regarded as a more relevant substrate for evaluating antioxidant activity in food systems.

The reducing power capacities of samples of watercress were compared to BHT in Figure 3. It has been reported that the reducing power is associated with the antioxidant activity and this relationship was also established for the compounds some of which have the potential of serving as a significant indicator (30). However, the antioxidant activity has been attributed to different mechanisms and reactions such as prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, and radical scavenging (31). All amounts of aqueous and ethanolic extracts of watercress showed higher activities than the control and these differences were statistically very significant ($p < 0.01$). Reducing power of aqueous and ethanolic extracts of watercress, and standard compound showed the following order: BHT > ethanolic extract > aqueous extract.

The effect of antioxidants on DPPH• radical scavenging is 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. The model of scavenging the stable DPPH• is a widely used method to evaluate antioxidant activities compared to other methods (32). Table 1 shows the scavenging effect of watercress extracts on the DPPH• radical, where the scavenging effect, as the absorbance difference from the control, was calculated. The radical scavenging activity using a DPPH• generated radical, was tested with water and ethanol extracts of watercress, and standards. The change in absorbance of DPPH• radical caused by antioxidants is due to the reaction between the antioxidant molecules and the radical, which results in the scavenging of the radical by hydrogen donation. It is visually noticeable as a discoloration from purple to yellow (16). All of amounts of watercress extracts exhibited significantly higher activities than the control ($p < 0.05$). The scavenging effect of water and ethanol extracts of watercress and standards on the DPPH• radical decreased in order of ethanol extract > water extract > BHA which were 87.08, 75.15, and 61.08% at the dose of 500 mg/mL, respectively. These results show that watercress is a free radical inhibitor, as well as antioxidant that reacts with free radicals.

Superoxide radicals are generated in a NADH-PMS system by oxidation of NADH and are determined by the reduction of NBT. The lower absorbance at 560 nm with antioxidants thus indicates the consumption of superoxide anion in the reaction mixture. The initial rate of NBT reduction was initiated by the extract (19). Figure 5 shows the

percentage inhibition of superoxide radicals generation by 100 mg of ethanol and water extract of watercress in comparison with the same doses of BHA, BHT and α-tocopherol. These results were found statistically significant ($p < 0.05$). The initial rate of NBT reduction was inhibited by the extracts of watercress in an amount-dependent manner, and each sample at 100 mg/mL exhibited 95.70, 93.92, 97.64, 88.99 and 80.23% for ethanol extract, water extract, BHA, BHT and α-tocopherol, respectively. The scavenging effect of the extracts and standards followed the order: BHA > ethanol extract > water extract > BHT > α-tocopherol. Superoxide indirectly initiated lipid peroxidation serving as precursors of singlet oxygen hydroxyl radicals (1). These results denote that water and ethanol extracts of watercress are superoxide scavengers and their capacity to scavenge superoxide may contribute to their antioxidant activity.

Phenols are very important plant constituents because of scavenging of radicals and containing hydroxyl groups (33). It was detected that there were 88.60 ± 2.41 and 74.18 ± 1.72 µg pyrocatechol equivalent of phenolic compounds in the 1000 mg of water and ethanol extracts, respectively. The phenolic compounds can contribute directly to evaluate antioxidative action (16) and stabilize lipid peroxidation (34). These results demonstrated that there is a correlation between phenolic content of watercress and antioxidant activities.

The lipid peroxide effects of water and ethanol extracts of watercress were presented in Figures 6-8. Quantification of MDA, one of the products of lipid peroxidation, with MDA-TBA is the most common assay used for determination of the rate extent of lipid peroxidation. This experiment proved that incubation of the rat liver, brain and kidney homogenate with Fe^{2+}-ascorbate at pH 7.4 caused lipid peroxidation, detectable by the TBA method as compared to the control at 532 nm. There is a significant increase of MDA level compared with that of control without Fe^{2+}-ascorbate and BHA ($p < 0.05$). To gather more information on the formation and behavior of lipid peroxides in biological system, the rat liver, kidney and brain homogenates were used as substrates for evaluation of antioxidant activity. At concentrations of 50-500 mg, all extracts displayed an antilipid peroxidation activity, with an inhibition rate varied from 88.78 to 97.41% for liver, from 64.66 to 92.69% for brain and from 71.80 to 97.06% for kidney. At 500 mg/mL, the formation of MDA was inhibited effectively by water and ethanol extracts of watercress. It is interesting to note that the water and ethanol extracts of watercress were the most effective extracts on the MDA formation. The ability of water and ethanol extracts of watercress to inhibit lipid peroxidation in the liver correlated closely with a similar action in the brain and kidney. These results indicate that the extracts of the watercress may prevent the formation of toxic carbonyl compounds such as MDA *in vitro*, and may play an important role in protecting against effects of membrane function.

CONCLUSION

On the basis of the results of this study, it is clearly demonstrated that water and ethanol extracts of watercress are novel natural antioxidants against lipid peroxidation in linoleic acid, liver, brain and kidney homogenate model systems. According to the results, water and ethanol extracts may act as: a) reducing agents, b) scavengers of superoxide, and c) free radical scavengers *in vitro*. This also underlines the importance of watercress as a possible food supplement and the very promising of indigenous drugs.

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