

**ANTIHEMOLYTIC AND ANTIOXIDANT ACTIVITY OF
HIBISCUS ESCULENTUS LEAVES**

Ebrahimzadeh Mohammad Ali*, Nabavi Seyed Fazel, Nabavi Seyed Mohammad

Pharmaceutical Sciences Research Center, School of Pharmacy, Mazandaran University of Medical Sciences, Sari, Iran. Tel: +98 151 3543081-3; fax: +98 151 3543084. E-mail: zadeh20@yahoo.com.

Summary

Antioxidant activity of leaves of *Hibiscus esculentus* was investigated employing different models in vitro assay systems. Extract showed good antioxidant activity. IC₅₀ for DPPH radical-scavenging activity was 245 ± 12.2 µg ml⁻¹. Extract showed weak nitric oxide-scavenging activity between 0.1 and 1.6 mg ml⁻¹. The leaves extract had show good reducing power that was comparable with Vitamin C (p < 0.01). Also, extract showed weak Fe²⁺ chelating ability. IC₅₀ was 1.24 ± 0.09 mg ml⁻¹. *H. esculentus* leaves Extract exhibited low antioxidant activity in linoleic acid model but were capable of scavenging hydrogen peroxide in a concentration dependent manner. Also, extract showed potent activity in inhibition of rat erythrocyte hemolysis. IC₅₀ was 274.3 ± 11 µg ml⁻¹. The total amount of phenolic compounds in extract was determined as gallic acid equivalents and total flavonoid contents were calculated as quercetin equivalents from a calibration curve.

Key words: Antioxidant activity, *Hibiscus esculentus*, DPPH, phenol

Introduction

Free radicals and reactive oxygen species (ROS) are well known inducers of cellular and tissue pathogenesis leading to several human diseases such as cancer, inflammatory disorders, as well as in aging processes [1]. Antioxidants provide protection to living organisms from damage caused by uncontrolled production of ROS and the concomitant lipid peroxidation, protein damage and DNA strand breaking [2]. Several anti-inflammatory, digestive, antinecrotic, neuroprotective and hepatoprotective drugs have recently been shown to have antioxidant and/or radical scavenging mechanism as part of their activity [3]. Synthetic antioxidants such as butylhydroxyanisole (BHA) or butylhydroxytoluene (BHT) are used to decelerate these processes. However, due to their unstable and highly volatile nature, they have frequently brought some questions about their safety and efficiency ever since their first introduction to the food industry [4]. Consequently, the need to identify alternative natural and safe sources of food antioxidant arose and the search for natural antioxidants, especially of plant origin, has notably increased in recent years [5, 6]. Okra (*Hibiscus esculentus* L.), a tropical to subtropical plant that is widely distributed from Africa to Asia, Southern European and America [7] belongs to the family of Malvaceae. Its Persian name is known as “bamyeh”. It is common vegetable in most regions of Greece, Turkey and Iran, especially in the northern. It is available all year-round, with a peak season during the summer months [8]. Okra is primarily a southern vegetable garden plant, grown for its immature pods, which are consumed when cooked either alone or in combination with other foods. The seeds of mature okra are roasted and ground and used as a coffee substitute in Turkey [9].

The quality of the seed protein is high. The seeds of mature pods are sometimes used for chicken feed and have been used on a small scale for the production of oil [8]. Studies have confirmed okra seeds as a good source of oil and protein [10]. Okra seed oil is rich in unsaturated fatty acids such as linoleic acid [11] and its fruit is rich source of α -Tocopherol [12] which is essential for human nutrition. In addition Okra seed has high content of minerals include of Ca, Cu, Fe, Mg, Zn, P [13]. previously we reported antioxidant and antihypoxic activity of methanolic extract of okra seed [14]. In this study, we examined the antioxidant activity of *H. esculentus* leaves, employing various in vitro assay systems, such as DPPH and nitric oxide radical scavenging, reducing power, linoleic acid and iron ion chelating power and its antihemolytic activity with employing hydrogen peroxide induced oxidative damage in rat erythrocytes model, in order to understand the usefulness of this plant as a foodstuff as well as in medicine.

Materials and methods

Chemicals: Ferrozine, Linoleic acid, trichloroacetic acid (TCA), 1,1-diphenyl-2-picryl hydrazyl (DPPH), potassium ferricyanide, H₂O₂ were purchased from Sigma Chemicals Co. (USA). Butylated hydroxyanisole (BHA), ascorbic acid, sulfanilamide, N-(1-naphthyl) ethylenediamine dihydrochloride, ethylenediaminetetraacetic acid (EDTA) and ferric chloride were purchased from Merck (Germany). All other chemicals were of analytical grade or purer.

Plant material and preparation of freeze-dried extract: Leaves of *H. esculentus* were collected from dashte naz area (Panbe Choole village) north of Sari, Iran, in summer 2007. After identification of the plant by Dr. Bahman Eslami (assistance professor of plants systematic, Islamic azad university of ghaemshahr, Iran) a voucher (No. 263) has been deposited in the Faculty of Pharmacy herbarium. Leaves were dried at room temperature and coarsely ground before extraction. A known amount of each part was extracted at room temperature by percolation method using methanol. The resulting extract was concentrated over a rotary vacuum until a crude solid extract was obtained, which was then freeze-dried for complete solvent removal.

Determination of total phenolic compounds and flavonoid content: Total phenol contents were determined by Folin Ciocalteu reagent [15]. The extract sample (0.5 ml of different dilutions) or gallic acid (standard phenolic compound) was mixed with 2.5 ml of 0.2 N Folin-Ciocalteu reagent (Sigma-Aldrich) for 5 min and 2.0 ml of 75 g l⁻¹ sodium carbonate were then added. The absorbance of reaction was measured at 760 nm after 2 h of incubation at room temperature. The standard curve was prepared by 0, 50, 100, 150, 200, and 250 mg ml⁻¹ solutions of gallic acid in methanol: water (50:50, v/v). Results were expressed in terms of gallic acid equivalents which is a common reference compound. Colorimetric aluminum chloride method was used for flavonoid determination [15]. 0.5 ml of sample in methanol was mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate, and 2.8 ml of distilled water. The extract remained at room temperature for 30 min; the absorbance of the reaction mixture was measured at 415 nm with a double beam Perkin Elmer UV/Visible spectrophotometer (USA). The calibration curve was prepared by preparing quercetin solutions at concentrations 12.5 to 100 mg ml⁻¹ in methanol.

DPPH radical-scavenging activity: The stable 1,1-diphenyl-2-picryl hydrazyl radical (DPPH) was used for determination of free radical-scavenging activity of the extract [16]. Different concentrations of each extracts were added, at an equal volume, to methanolic solution of DPPH (100 μM). After 15 min at room temperature, the absorbance was recorded at 517 nm. The experiment was repeated for three times. Vitamin C, BHA and quercetin were used as standard controls. IC_{50} values denote the concentration of sample, which is required to scavenge 50% of DPPH free radicals.

Reducing power determination: Fe (III) reduction is often used as an indicator of electron- donating activity, which is an important mechanism of phenolic antioxidant action [17]. The reducing power of leave and seed of *H. esculentus* was determined according to the method of Yen and Chen [4]. 2.5 ml of leaves extract (25-800 $\mu\text{g ml}^{-1}$) in water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [$\text{K}_3\text{Fe}(\text{CN})_6$] (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 to stop the reaction, which was then centrifuged at 3000 rpm for 10 min. 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. Increased absorbance of the reaction mixture indicated increased reducing power. Vitamin C was used as positive control.

Assay of nitric oxide-scavenging activity: The procedure is based on the principle that, sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions. For the experiment, sodium nitroprusside (10 mM), in phosphate-buffered saline, was mixed with different concentrations of each extracts dissolved in water and incubated at room temperature for 150 min. The same reaction mixture, without extract but with an equivalent amount of water, served as control. After the incubation period, 0.5 ml of Griess reagent (1% sulfanilamide, 2% H_3PO_4 and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride) was added. The absorbance of the chromophore formed was read at 546 nm. Quercetin was used as positive control [18].

Metal chelating activity: Foods are often contaminated with transition metal ions which may be introduced by processing methods. Bivalent transition metal ions play an important role as catalysts of oxidative processes, leading to the formation of hydroxyl radicals and hydroperoxide decomposition reactions via Fenton chemistry [19]. These processes can be delayed by iron chelation and deactivation. The chelating of ferrous ions by *H. esculentus* leaves Extract was estimated by the method of Dinis *et al.* [20]. Briefly, the extract (0.2–3.2 mg/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), the mixture was shaken vigorously and left standing at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 562 nm. The percentage inhibition of ferrozine– Fe^{2+} complex formation was calculated as $[(A_0 - A_s)/A_s] \times 100$, where A_0 was the absorbance of the control, and A_s was the absorbance of the extract/ standard. Na_2EDTA was used as positive control [17].

Determination of Antioxidant Activity by the FTC Method: Membrane lipids are rich in unsaturated fatty acids that are most susceptible to oxidative processes. Specially, linoleic acid and arachidonic acid are targets of lipid peroxidation [21].

The inhibition of lipid peroxidation by antioxidants may be due to their free radical-scavenging activities. Superoxide indirectly initiates lipid peroxidation because Superoxide anion acts as a precursor of singlet oxygen and hydroxyl radical [22]. Hydroxyl radicals eliminate hydrogen atoms from the membrane lipids, which results in lipid peroxidation. The inhibitory capacity of *H. esculentus* extracts against oxidation of linoleic acid by FTC method was tested. This method was adopted from our recently published paper [17]. Twenty mg/mL of sample dissolved in 4 mL of 95% (w/v) ethanol was mixed with linoleic acid (2.51%, v/v) in 99.5% (w/v) ethanol (4.1 mL), 0.05 M phosphate buffer pH 7.0 (8 mL), and distilled water (3.9 mL) and kept in screwcap containers at 40°C in the dark. To 0.1 mL of this solution was then added 9.7 mL of 75% (v/v) ethanol and 0.1 mL of 30% (w/v) ammonium thiocyanate. Precisely 3 min after the addition of 0.1 mL of 20 mM ferrous chloride in 3.5% (v/v) hydrochloric acid to the reaction mixture, the absorbance at 500 nm of the resulting red solution was measured, and it was measured again every 24 h until the day when the absorbance of the control reached the maximum value. The percent inhibition of linoleic acid peroxidation was calculated as: (%) inhibition = 100 - [(absorbance increase of the sample/absorbance increase of the control) × 100]. All tests were run in duplicate, and analyses of all samples were run in triplicate and averaged. Vit C and BHA used as positive control.

Scavenging of hydrogen peroxide: The ability of the extract to scavenge hydrogen peroxide was determined according to the method adapted from our recently published paper [18]. A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). Extract (0.1-1 mg ml⁻¹) in distilled water were added to a hydrogen peroxide solution (0.6 ml, 40 mM). The absorbance of hydrogen peroxide at 230 nm was determined after ten minutes against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenging by the extracts and standard compounds was calculated as follows: % Scavenged [H₂O₂] = [(A₀ - A₁)/A₀] × 100 where A₀ was the absorbance of the control and A₁ was the absorbance in the presence of the sample of extract and standard [18].

Preparation of rat erythrocytes: All the animal experiments were carried out with the approval of institutional animal ethical committee. Male Wistar rats in the body weight range of 180–220g were housed in individual polypropylene cages and had free access to food and water. The animals were fed with standard diet. The animals were sacrificed under anesthesia and blood was collected by heart puncture in heparinized tubes. Erythrocytes were isolated and stored according to the method described by Yuan *et al.* [23] and Yang *et al.* [24]. Briefly blood samples collected were centrifuged (1500×g, 10 min) at 4 °C, erythrocytes were separated from the plasma and buffy coat and were washed three times by centrifugation (1500×g, 5 min) in 10 volumes of 10 mM phosphate buffered saline (pH 7.4; PBS). The supernatant and buffy coats of white cells were carefully removed with each wash. Washed erythrocytes stored at 4 °C and used within 6 h for further studies.

In vitro assay inhibition of rat erythrocyte hemolysis: The inhibition of rat erythrocyte hemolysis by the extract was evaluated according to the procedure described by Tedesco *et al.* [25]. The rat erythrocyte hemolysis was performed with H₂O₂ as free radical initiator. To 100 µl of 5% (v/v) suspension of erythrocytes in PBS, 50 µl of extract with different concentrations (5–25 µg in PBS pH 7.4), which corresponds to 100– 3200 µg of extract, was added.

To this, 100 μ l of 100 mM H₂O₂ (in PBS pH7.4) was added. The reaction mixture was shaken gently while being incubated at 37 °C for 3 h. The reaction mixture was diluted with 8 ml of PBS and centrifuged at 2000 \times g for 10 min. The absorbance of the resulting supernatant was measured at 540 nm by spectrophotometer to determine the hemolysis. Likewise, the erythrocytes were treated with 100 μ M H₂O₂ and without inhibitors (*H. esculentus* leaves extract) to obtain a complete hemolysis. The absorbance of the supernatant was measured at the same condition. The inhibitory effect of the extract was compared with standard antioxidant Vitamin C. To evaluate the hemolysis induced by leaves extract, erythrocytes were preincubated with 50 μ l of peel extract corresponding to 25 μ g extract for 1 h and the hemolysis was determined. Percentage of hemolysis was calculated by taking hemolysis caused by 100 μ M H₂O₂ as 100%. The IC₅₀ values were calculated from the plots as the antioxidant concentration required for the inhibition of 50% hemolysis.

Statistical analysis: Experimental results are expressed as means \pm SD. All measurements were replicated three times. The data were analyzed by an analysis of variance ($p < 0.05$) and the means separated by Duncan's multiple range test. The EC₅₀ values were calculated from linear regression analysis.

Results and Discussion

Total phenol and flavonoid contents: Total phenol compounds, as determined by Folin Ciocalteu method, are reported as gallic acid equivalents by reference to standard curve ($y = 0.0054x + 0.0628$, $r^2 = 0.987$). The total phenolic content of leaves was 68.81 ± 3.9 mg gallic acid equivalent/g of extract. The total flavonoid contents of leaves was 49.3 ± 2.2 mg quercetin equivalent/g of extract powder, by reference to standard curve ($y = 0.0063x$, $r^2 = 0.999$). Phenols and polyphenolic compounds, such as flavonoids, are widely found in food products derived from plant sources, and they have been shown to possess significant antioxidant activities [26]. Studies have shown that increasing levels of flavonoids in the diet could decrease certain human diseases [27].

DPPH radical-scavenging activity: The model of scavenging the stable DPPH radical is a widely used method to evaluate the free radical scavenging ability of various samples [28]. DPPH is a stable nitrogen-centered free radical the color of which changes from violet to yellow upon reduction by either the process of hydrogen- or electron- donation. Substances which are able to perform this reaction can be considered as antioxidants and therefore radical scavengers [29]. IC₅₀ for DPPH radical-scavenging activity was 245 ± 12.2 μ g ml⁻¹. The IC₅₀ values for Ascorbic acid, quercetin and BHA were 1.26 ± 0.11 , 1.32 ± 0.07 and 13.49 ± 1.04 μ g ml⁻¹, respectively.

Reducing power of *H. esculentus* leaves extract: In the reducing power assay, the presence of reductants (antioxidants) in the samples would result in the reducing of Fe³⁺ to Fe²⁺ by donating an electron. Amount of Fe²⁺ complex can be then be monitored by measuring the formation of Perl's Prussian blue at 700 nm. Increasing absorbance at 700 nm indicates an increase in reductive ability. Fig. 1 shows the dose– response curves for the reducing powers of the leaves extract from *H. esculentus*. It was found that the reducing powers of extracts also increased with the increase of their concentrations.

The leaves extract show good reducing power that was comparable with Vit C ($p < 0.01$). Because the reductive abilities of the leaves extract of *H. esculentus* were significantly comparable to Vit C, it was evident that *H. esculentus* did show reductive potential and could serve as electron donors, terminating the radical chain reaction.

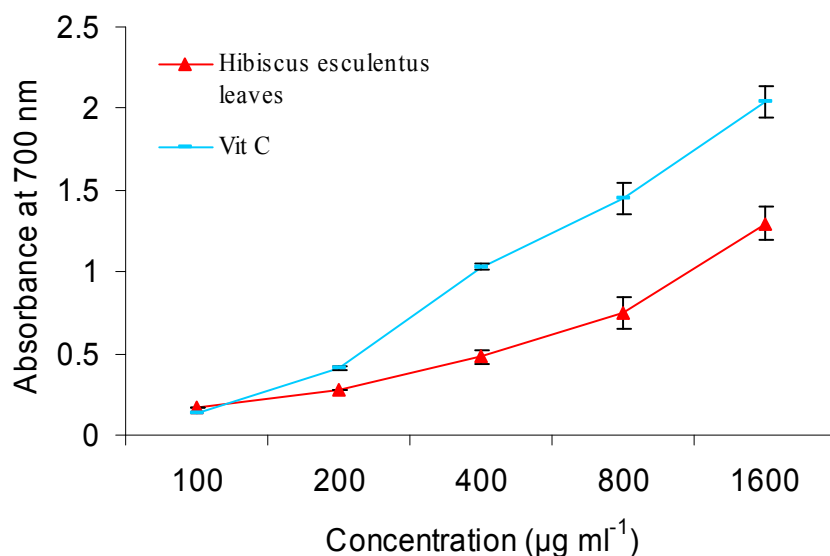


Fig.1. Reducing power of methanolic extract of *H. esculentus*.

Assay of nitric oxide-scavenging activity: The extracts also showed weak nitric oxide-scavenging activity between 0.1 and 1.6 mg ml^{-1} . The % inhibition was increased with increasing concentration of the extract. The leaves extract show good nitric oxide scavenging (IC_{50} were $0.63 \pm 0.02 \text{ mg ml}^{-1}$ vs $0.20 \pm 0.01 \text{ mg ml}^{-1}$ for quercetin). In addition to reactive oxygen species, nitric oxide is also implicated in inflammation, cancer and other pathological conditions [30]. The plant/plant products may have the property to counteract the effect of NO formation and in turn may be of considerable interest in preventing the ill effects of excessive NO generation in the human body. Further, the scavenging activity may also help to arrest the chain of reactions initiated by excess generation of NO that are detrimental to human health.

Fe^{2+} chelating activity of *H. esculentus* leaves extract: The transition metal, iron, is capable of generating free radicals from peroxides by Fenton reactions and may be implicated in human cardiovascular disease [31,32]. Because Fe^{2+} also has been shown to cause the production of oxyradicals and lipid peroxidation, minimizing Fe^{2+} concentration in Fenton reactions affords protection against oxidative damage. The chelating of ferrous ions by the extract was estimated by the method of Dinis et al. [20]. Ferrozine can quantitatively form complexes with Fe^{2+} . In the presence of other chelating agents, the complex formation is disrupted with the result that the red colour of the complexes decreases.

In this assay, both extract and EDTA interfered with the formation of ferrous and ferrozine complex, suggesting that it has chelating activity and captures ferrous ion before ferrozine. The absorbance of Fe^{2+} -ferrozine complex was decreased dose-dependently, i.e. the activity was increased on increasing concentration from 0.2 to 3.2 mg ml⁻¹. Metal chelating capacity was significant since the extract reduced the concentration of the catalyzing 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 [33]. *H. esculentus* leaves extract showed weak Fe^{2+} chelating ability (IC_{50} was 1.24 ± 0.09 mg ml⁻¹). EDTA showed very strong activity ($\text{IC}_{50} = 18$ $\mu\text{g ml}^{-1}$).

FTC Method: Both tested extracts exhibited low antioxidant activity in the FTC method. The peroxidation inhibition (antioxidant activity) of leaves exhibited values from 79 % (at 24th hrs) and from 83 % (at 48nd hrs), respectively.

Scavenging H₂O₂: Scavenging of H₂O₂ by *H. esculentus* extract may be attributed to their phenolics, which can donate electrons to H₂O₂, thus neutralizing it to water [14]. The differences in H₂O₂ scavenging capacities between the extracts may be attributed to the structural features of their active components, which determine their electron donating abilities [18]. The *H. esculentus* extract was capable of scavenging hydrogen peroxide in a concentration dependent manner (IC_{50} 288.5 ± 14.7 $\mu\text{g ml}^{-1}$). The IC_{50} values for Ascorbic acid and BHA were 21.4 and 52.0 $\mu\text{g ml}^{-1}$, respectively. Although hydrogen peroxide itself is not very reactive, it can sometimes cause cytotoxicity by giving rise to hydroxyl radicals in the cell. Thus, removing H₂O₂ is very important throughout food systems.

Inhibition of rat erythrocyte hemolysis: Initially, the effect of peel extracts was tested and found that it did not show any harmful effect on erythrocytes. The extract inhibited the hemolysis of rat erythrocytes in a dose dependent manner with 85.2 % as maximum inhibition of erythrocyte hemolysis at 3.2 mg ml⁻¹. The peel extract showed 50% hemolysis inhibition at concentrations ranging from 100 to 3200 μg of *H. esculentus* extract ($\text{IC}_{50} = 274.3 \pm 11$). The Vitamin C exhibited with an IC_{50} value of 235 ± 9 μg that is comparable to that of peel extracts.

The leaves extract of *H. esculentus* exhibited good but different levels of antioxidant activity in all the models studied. The extracts had good reducing power and nitric oxide scavenging activity. Further investigation of individual compounds, their in vivo antioxidant activities and in different antioxidant mechanisms is needed.

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References

1. Halliwell B., Free radicals, antioxidants and human diseases; curiosity, cause, or consequence? *Lancet*, 334, 721-724, 1994.

2. Ghosal S., V.K. Tripathi, S. Chauhan, Active constituents of *Emblca officinalis*. Part I. The chemistry and antioxidant effects of two new hydrolysable tannins, emblicanin A and B. *Ind. J. Chem.*, 35B, 941-948, 1996.
3. Ebrahimzadeh MA, Nabavi SM, Nabavi SF. Correlation between the *in vitro* iron chelating activity and poly phenol and flavonoid contents of some medicinal plants. *Pak J Biol Sci* 2009, in press.
4. Nabavi S.M. , M.A. Ebrahimzadeh, S.F. Nabavi, A. Hamidinia and A.R. Bekhradnia, Determination of Antioxidant Activity , Phenol And Flavonoid Content of *Parrotia persica MEY* . *Pharmacologyonline*, 2, 560-567, 2008.
5. Ebrahimzadeh MA, Nabavi SF, Nabavi SM. Essential oil composition and antioxidant activity of *Pterocarya fraxinifolia* *Pak J Biol Sci* 2009, in press.
6. Ghasemi K., Y. Ghasemi, and M.A. Ebrahimzadeh. Antioxidant activity, phenol and flavonoid contents of 13 Citrus species peels and tissues. *Pak J Pharm Sci* 22 (3): 277-281, 2009.
7. Oyelade O.J., B.I.O. Ade-Omowaye & V.F. Adeomi, Influence of variety on protein, fat contents and some physical characteristics of okra seeds. *J. Food Eng*, 57, 111-114, 2003.
8. Purewal S.S. & G.E. Rhandhawa, Studies in Hibiscus esculentus (Ladyfinger) (okra) I. Chromosome and pollen studies. *Ind. J. Agric Sci*, 17, 129-136, 1947.
9. Calısır S., M.Ozcan, H.Hacıseferogulları, M. Ugur Yıldız , A study on some physico-chemical properties of Turkey okra (*Hibiscus esculenta* L.) seeds. *J. Food Eng*, 68, 73-78, 2005.
10. Karakoltsidis P.A. & S.M. Constantinides, Okra seeds: A new protein source. *J. Agric Food Chem.*, 23(6), 1204-1207, 1975.
11. Savello P.A., F. Martins & W. Hull, Nutrition composition of okra seed meals. *J. Agric Food Chem.*, 28(6),1163-1166, 1980.
12. Ching L.S. and S. Mohamed, Alpha-Tocopherol Content in 62 Edible Tropical Plants. *J Agric Food Chem.*, 49, 3101-3105, 2001.
13. Akpanabiatu M.I., N.B. Basse, E.O. Udosen and E.U. Eyong, Evaluation of Some Minerals and Toxicants in Some Nigerian Soup Meals. *J. food Compos anal*, 11, 292-297, 1998.
14. Ebrahimzadeh M. A., Nabavi S. F., Nabavi S. M., Eslami B. Antihypoxic and antioxidant activity of *Hibiscus esculentus* seeds, *Grasas y Aceites*. in press. DOI: 10.3989/gya.053809, 2010
15. Nabavi S.M., M.A. Ebrahimzadeh, S.F. Nabavi and M. Jafari, Free radical scavenging activity and antioxidant capacity of *Eryngium caucasicum Trautv* and *Froripia subpinata*. *Pharmacologyonline* , 3, 19-25, 2008.
16. Nabavi S.M. , M.A. Ebrahimzadeh , S.F. Nabavi and F. Bahramian , In Vitro Antioxidant activity of *Phytolacca Americana* Berries. *Pharmacologyonline* , 1, 81-88, 2009.
17. Nabavi S.M., M.A. Ebrahimzadeh, S.F. Nabavi, M. Fazelian, B. Eslami, In vitro Antioxidant and Free Radical Scavenging Activity of *Diospyros lotus* and *Pyrus boissieriana* growing in Iran. *Pharmacog Mag* ,4(18), 123-127, 2009.
18. Dehpour A.A., M.A. Ebrahimzadeh, S.F. Nabavi and S.M. Nabavi, Antioxidant activity of the methanol extract of *Ferula assafoetida* and its essential oil composition. *Grasas Aceites* ,60(4), 405-412, 2009.
19. Ebrahimzadeh M.A., F. Pourmorad, A.R. Bekhradnia, Iron chelating activity, phenol and flavonoid content of some medicinal plants from Iran. *Afr J Biotech* ,7 (18), 17, 3188-3192, 2008.

20. Dinis T.C.P., V.M.C. Madeira, L.M., Almeida Action of phenolic derivates (acetoaminophen, salicylate and 5-aminosalicylate) as inhibitors of membrane lipid peroxidation and as peroxy radical scavengers. *Arch Biochem Biophys* , 315, 161-169, 1994.
21. Yu L.L., Free radical scavenging properties of conjugated linoleic acids. *J. Agric Food Chem*, 49(7), 3452-3456, 2001.
22. Ordoñez A.A.L., J.D. Gomez, M.A. Vattuone, M.I. Isla, Antioxidant activities of *Sechium edule* (Jacq.) Swartz extracts. *Food Chem.*, 97, 452-458, 2006.
23. Yuan X., J. Wang, H. Yao, F. Chen, Free radical-scavenging capacity and inhibitory activity on rat erythrocyte hemolysis of feruloyl oligosaccharides from wheat bran insoluble dietary fiber. *LWT-Food Sci Technol*, 38, 877-883, 2005.
24. Yang H.L., S.C. Chen, N.W. Chang, J.M. Chang, M.L. Lee, P.E. Tasi, H.H. Fu, W.W. Kao, H.C. Chiang, H.H. Wang, Y.C. Hseu , Protection from oxidative damage using *Bidens pilosa* extracts in normal human erythrocytes. *Food Chem Toxicol* 44,1513-1521, 2006.
25. C.M. Ajila, U.J.S., Prasada Rao, Protection against hydrogen peroxide induced oxidative damage in rat erythrocytes by *Mangifera indica* L. peel extract. *Food Chem Toxicol*, 46, 303-309, 2008.
26. Ebrahimzadeh M.A., S. Ehsanifar, B. Eslami, *Sambucus ebulus elburensis* fruits: A good source for antioxidants. *Pharmacog mag* , 4(19),213-218, 2009.
27. Ebrahimzadeh M.A. and F. Bahramian , Antioxidant activity of *Crataegus pentagyna subsp . elbursis* Fruits extracts used in traditional medicine in Iran. *Pak. J. oil. Sci.*,12(5),413-419, 2009.
28. Ebrahimzadeh M.A., S.M. Nabavi, S.F. Nabavi, B. Eslami, Antioxidant Activity of Aqueous Extract Of *Pyrus boissieriana* Fruit. *Pharmacologyonline*, 1, 1318-1323, 2009.
29. Brand-Williams W., M. Cuvelier, C. Berset, Use of a free radical method to evaluate antioxidant activity. *LWT-Food Sci Technol* , 28, 25-30, 1995.
30. Moncada A., R.M.J. Palmer, E.A. Higgs, Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmacol ,Rev* , 43, 109-142, 1991.
31. Halliwell B., J.M.C. Gutteridge , Role of free radicals and catalytic metal ions in human disease: an overview. *Methods Enzymol.*, 186, 1-85,1990.
32. Ebrahimzadeh M.A., S.F. Nabavi, S.M. Nabavi , Antioxidant activities of methanol extract of *sambucus ebulus* L. flower. *Pak j. boil. Sci.*, 12(5), 447-450, 2009.
33. Ebrahimzadeh M.A., F. Pourmorad & S. Hafezi, Antioxidant Activities of Iranian Corn Silk. *Turk. J. Biol.*,32,43-49, 2008.