

## ANTIOXIDANT ACTIVITY AND FREE RADICAL SCAVENGING ACTIVITY OF *SALVIA GLUTINOSA* GROWING IN IRAN

Esmaeili A.<sup>1</sup>, Tavassoli A.<sup>1</sup>, Ebrahimzadeh M. A.<sup>2\*</sup>

<sup>1</sup>Department of Chemistry, North Tehran Branch, Islamic Azad University, Tehran, Iran.

<sup>2</sup>Pharmaceutical Sciences Research Center, School of Pharmacy, Medical Sciences University of Mazandaran, Sari, Iran. zadeh20@yahoo.com  
Ghaemshahr

### Summary

Antioxidant activity of *Salvia glutinosa* aerial parts was investigated employing six in vitro assay systems. IC<sub>50</sub> for DPPH radical-scavenging activity was  $36.1 \pm 1.1 \mu\text{g ml}^{-1}$ . The extracts also showed weak nitric oxide-scavenging activity between 0.1 and 0.8 mg ml<sup>-1</sup> and Fe<sup>2+</sup> chelating ability. IC<sub>50</sub> for Fe<sup>2+</sup> chelating ability was  $757 \pm 3.1 \mu\text{g ml}^{-1}$ . The peroxidation inhibition of extract exhibited values from 83 to 91% (at 72<sup>nd</sup> hrs). Extract had shown good reducing power that was comparable with Vit C (p > 0.05). Extract also showed weak scavenging activity of H<sub>2</sub>O<sub>2</sub>. IC<sub>50</sub> was  $416 \pm 2.7 \mu\text{g ml}^{-1}$ . The extract has contained high total phenolic and flavonoid contents.

**Key words:** Antioxidant activity, DPPH, Iron Ion chelating, *Salvia glutinosa*, phenol contents, flavonoids.

### Introduction

The role of free radicals in many disease conditions has been well established. Several biochemical reactions in our body generate reactive oxygen species. If they are not effectively scavenged by cellular constituents, they lead to disease conditions (1). Current research into free radicals has confirmed that foods rich in antioxidants play an essential role in the prevention of cardiovascular diseases and cancers (2,3) and neurodegenerative diseases, including Parkinson's and Alzheimer's diseases (4), as well as inflammation and problems caused by cell and cutaneous aging (5). *Salvia* is a fascinating plant genus and one of the wide-spread members of the labiatae family, which comprises about 900 herbs and shrubs (6). The therapeutic effects of many traditional drugs are attributed to this group of compounds because of their inhibitory effects on certain enzymes and antioxidative activity (7). They have been shown to possess antibacterial (8), antifungal (9), antiviral (10) and anti-inflammatory (11) activities. Their antiallergic (8) antioxidative (12) and antimutagen (13)

activities have also been published. Reduced risk of breast, prostate and colon cancers is related to its isoflavonoid activity (14). Twenty-three volatile constituents were identified in the oil of *Salvia glutinosa*, representing 86.5% of the total oil. Sesquiterpene hydrocarbon compounds constituted 32.2% (15). In this study, we examined the antioxidant activity of *S. glutinosa* aerial parts, employing various six in vitro assay systems, i.e. DPPH and nitric oxide radical scavenging, reducing power, linoleic acid, iron ion chelating power and scavenging of hydrogen peroxide in order to understand the usefulness of this plant in medicine.

### Materials and methods

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

**Plant Material and Preparation of Extract:** *S. glutinosa* aerial parts were collected during blossoming from natural populations. It collected in August 2007 altitude ca. 1750 m near the Amol city in Mazandaran province, Iran. Voucher 90-205 deposited in the Herbarium of the Department of Botany Shahid Beheshti University, Eeven, Tehran. Aerial parts 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 Contents:** Colorimetric aluminum chloride method was used for flavonoid determination (16). *S. glutinosa* extract (0.5 ml of 1:10 g ml<sup>-1</sup>) in methanol was separately 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<sup>-1</sup> in methanol. Total phenol content was determined by Folin Ciocalteu reagent (17). A dilute solution of *S. glutinosa* extract (0.5 ml of 1:10 g ml<sup>-1</sup>) or gallic acid (standard phenolic compound) was mixed with Folin Ciocalteu reagent (5 ml, 1:10 diluted with distilled water) and aqueous Na<sub>2</sub>CO<sub>3</sub> (4 ml, 1 M). The mixture was allowed to stand for 15 min and the phenols were determined by colorimetry at 765 nm. The standard curve was prepared by 0, 50, 100, 150, 200, and 250 mg ml<sup>-1</sup> solutions of gallic acid in methanol:water (50:50, v/v). Total phenol values are expressed in terms of gallic acid equivalent (mg g<sup>-1</sup> of dry mass), which is a common reference compound.

**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 extracts (18, 19). 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<sub>50</sub> 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 (20). The reducing power of *S. glutinosa* was determined according to our recently published paper (21). Different amounts of each extracts (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  $\text{FeCl}_3$  (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. After the incubation period, 0.5 ml of Griess reagent was added. The absorbance of the chromophore formed was read at 546 nm. Quercetin was used as positive control (22).

**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 (23). The chelating of ferrous ions by *S. glutinosa* was estimated by our recently published paper (24). Briefly, the extract (0.2–3.2 mg/ml) was added to a solution of 2 mM  $\text{FeCl}_2$  (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- $\text{Fe}^{2+}$  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.  $\text{Na}_2\text{EDTA}$  was used as positive control.

**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 (25). The inhibitory capacity of *S. glutinosa* extracts was tested against oxidation of linoleic acid by FTC method. This method was adopted from Osawa and Namiki (26). Twenty mg/mL of samples dissolved in 4 mL of 95% (w/v) ethanol were 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 =  $100 - [(\text{absorbance increase of the sample}/\text{absorbance increase of the control}) \times 100]$ . 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 extracts to scavenge hydrogen peroxide was determined according to our recently published paper (27). A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). Extract (0.1-1 mg ml<sup>-1</sup>) 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 [H<sub>2</sub>O<sub>2</sub>] = [(A<sub>0</sub> - A<sub>1</sub>)/A<sub>0</sub>] × 100 where A<sub>0</sub> was the absorbance of the control and A<sub>1</sub> was the absorbance in the presence of the sample of extract and standard (27).

**Statistical Analysis:** Experimental results are expressed as means ± 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 EC<sub>50</sub> 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 = 0.0063x, r<sup>2</sup> = 0.987). The total phenolic content extract was 187.8 ± 1.23 mg gallic acid equivalent/g of extract powder. The total flavonoid content was 44.6 ± 0.78 mg quercetin equivalent/g of extract powder, respectively, by reference to standard curve (y = 0.0067x + 0.0132, r<sup>2</sup> = 0.999). The plant had good total phenol contents and they may cause the antioxidative activities of the *S. glutinosa*. 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 (28).

**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 (29). It was found that the radical- scavenging activity of all the extract increased with increasing concentration. IC<sub>50</sub> for DPPH radical-scavenging activity was 36.1 ± 0.18 µg ml<sup>-1</sup>. The IC<sub>50</sub> values for Ascorbic acid, quercetin and BHA were 5.05 ± 0.12, 5.28 ± 0.43 and 53.96 ± 2.13 µg ml<sup>-1</sup>, respectively.

**Reducing Power:** In the reducing power assay, the presence of reductants (antioxidants) in the samples would result in the reducing of Fe<sup>3+</sup> to Fe<sup>2+</sup> by donating an electron. Amount of Fe<sup>2+</sup> 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 extracts from *S. glutinosa*. It was found that the reducing power of extract also increased with the increase of its concentration. It is not a significant difference (p > 0.05) among the extract and control (Vit C) in reducing power.

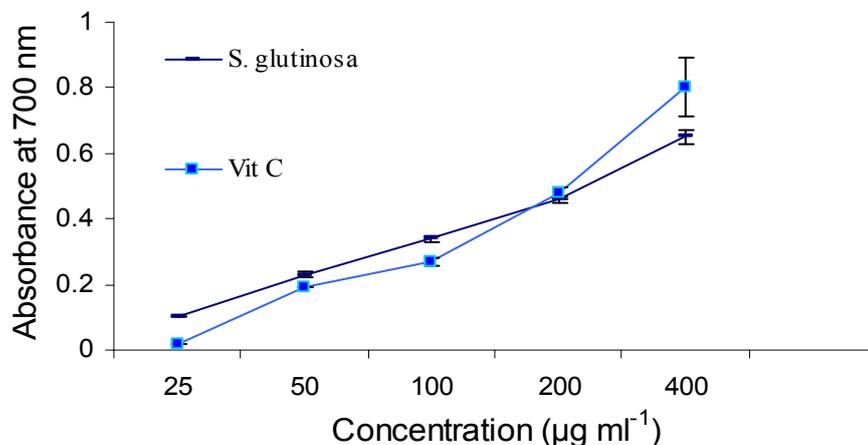


Fig.1. Reducing power of methanolic extract of *S. glutinosa* aerial parts. Vitamin C used as control.

**Assay of Nitric Oxide-Scavenging Activity:** The extracts also showed weak nitric oxide-scavenging activity between 0.1 and 0.8 mg ml<sup>-1</sup>. The percentage of inhibition was increased with increasing concentration of the extract. Extract showed only 33% inhibition at 0.8 mg ml<sup>-1</sup>. However, activity of quercetin was very more pronounced than that of extract (IC<sub>50</sub> = 17 µg ml<sup>-1</sup>). In addition to reactive oxygen species, nitric oxide is also implicated in inflammation, cancer and other pathological conditions (30).

**Fe<sup>2+</sup> Chelating Ability:** The transition metal, iron, is capable of generating free radicals from peroxides by Fenton reactions and may be implicated in human cardiovascular disease (31). Because Fe<sup>2+</sup> causes the production of oxyradicals and lipid peroxidation, minimizing its concentration affords protection against oxidative damage. In Fe<sup>2+</sup> chelating ability test, in the presence of other chelating agents, the ferrozine complex formation is disrupted with the result that the red color of the complexes decreases. The absorbance of Fe<sup>2+</sup>-ferrozine complex was decreased dose-dependently, i.e. the activity was increased on increasing concentration from 0.2 to 3.2 mg ml<sup>-1</sup>. 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 (32). Results reveal that *S. glutinosa* extract have weak capacity for iron binding. IC<sub>50</sub> for Fe<sup>2+</sup> chelating ability was 757 ± 3.1 µg ml<sup>-1</sup>. EDTA showed very strong activity (IC<sub>50</sub> = 18 µg ml<sup>-1</sup>).

**FTC Method:** Figure 2 shows the time-course plots for the antioxidative activity of the different extracts of *S. glutinosa* using the FTC method. The peroxidation inhibition of extract exhibited values from 91 to 95% at 24<sup>th</sup> - 96<sup>th</sup> hrs. The extract exhibited high antioxidant activity. Extract manifested almost the same pattern of activity as Vit C and BHA at different incubation times (p > 0.05).

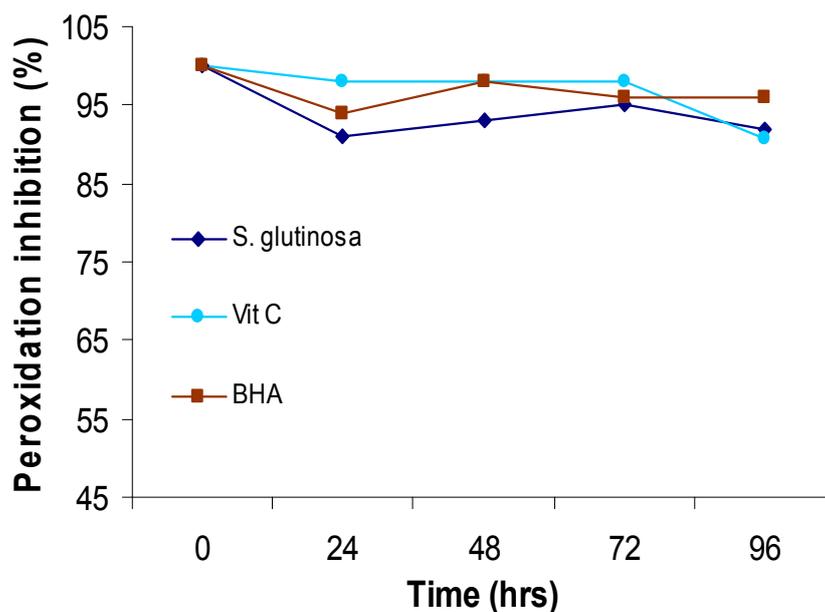


Fig. 2. Antioxidant activity of *S. glutinosa* in FTC method at different incubation times. *S. glutinosa* ( $0.2 \text{ mg ml}^{-1}$ ), Vit C and BHA ( $0.1 \text{ mg ml}^{-1}$ ).

**Hydrogen Peroxide Scavenging:** Scavenging of  $\text{H}_2\text{O}_2$  by extracts may be attributed to their phenolics, which can donate electrons to  $\text{H}_2\text{O}_2$ , thus neutralizing it to water (27). The ability of the extracts to effectively scavenge hydrogen peroxide, determined according to the method of Ruch (27), where they are compared with that of Quercetin as standard. The extracts were capable of scavenging hydrogen peroxide in a concentration-dependent manner. The extract showed moderately good scavenging activity.  $\text{IC}_{50}$  for scavenging of  $\text{H}_2\text{O}_2$  was  $416 \pm 2.7 \mu\text{g ml}^{-1}$ . The  $\text{IC}_{50}$  values for Ascorbic acid and quercetin were  $21.4 \pm 0.12$  and  $52.0 \pm 3.11 \mu\text{g ml}^{-1}$ , 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  $\text{H}_2\text{O}_2$  is very important throughout food systems (27).

The extract of *S. glutinosa* aerial parts exhibited different levels of antioxidant activity in all the models studied. The effect was significant in some tests but further investigation of individual compounds, their in vivo antioxidant activities and in different antioxidant mechanisms is needed.

#### Acknowledgements

This research was partially supported by a grant from the research council of Mazandaran University of Medical Sciences, Iran.

#### References

- Halliwell B, Gutteridge JMC, Cross CE. Free radicals, antioxidants and human disease: where are we now? *Journal of Laboratory and Clinical Medicine*, 1992; 119: 598-620.
- Gerber M, Boutron-Ruault MC, Herberg S, Riboli E, Scalbert A, Siess MH. Food and cancer: state of the art about the protective effect of fruits and vegetables. *Bulletin du Cancer*, 2002; 89 (3): 293-312.

3. Kris-Etherton PM, Hecker KD, Bonanome A, Coval SM, Binkoski AE, Hilpert KF, Griel AE, Etherton TD. Bioactive compounds in foods: their role in the prevention of cardiovascular disease and cancer. *American Journal of Medicine*, 2002; 113 (Suppl. 9B): 71S-88S.
4. Di Matteo V, Esposito E. Biochemical and therapeutic effects of antioxidants in the treatment of Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis. *Current Drug Target CNS Neurological Disorders*, 2003; 2: 95-107.
5. Ames SN, Shigenaga MK, Hagen TM. Oxidants, antioxidants and degenerative diseases of aging. *Proceedings of the National Academy of Sciences of the United States of America*, 1993; 90: 7915-7922.
6. Rustaiyan A, Masoudi SH, Tabatabaei-Anaraki M. Terpenoids from Iranian *Salvia* species. *Natural Product Communications*, 2007; 2(10): 1031-1042.
7. Velickovic DT, Nikolova MT, Ivancheva SV, Stojanovic JB, Veljkovic VB. Extraction of flavonoids from garden (*Salvia officinalis* L.) and (*Salvia glutinosa* L.) sage by ultrasonic and classical maceration. *J. Serb. Chem. Soc.* 2007; 72 (1): 73-80.
8. Tomas-Barberan FA. Flavonoid compounds from the Labiatae. *Fitoterapia*, 1986; 57(2): 67-95.
9. Weidenbornen M, Yha HC. Structure-activity relationships among isoflavonoids with regard to their antifungal properties. *Mycol. Res.* 1994; 98(12): 1376-1378.
10. Abad MJ, Bermejo P, Villar A, Sanchez Palomino S, Carrasco L. Antiviral activity of medicinal plant extracts. *Phytother. Res.* 1997; 11: 198-202.
11. Recio MC, Giner RM, Manez S, Rios JL, Marston A, Hostettmann K. Screening of tropical medicinal plants for antiinflammatory activity *Phytother. Res.* 1995; 9(8): 571-574.
12. Marinova EM, Yanishlieva NV. Antioxidative activity of extracts from selected species of the family lamiaceae in sunflower oil. *Food Chemistry* 1997; 58: 245-248.
13. Kootstra A. Protection from UV-B-induced DNA damage by flavonoids. *Plant Molecular Biology* 1994; 26: 771-774.
14. Messina MJ. Legumes and soybeans: overview of their nutritional profiles and health effects. *American Society for Clinical Nutrition*. 1999; 70 (3): 439S-450S.
15. Pitarokili D, Tzakou O, Loukis A. Essential oil composition of *Salvia verticillata* S.verbenaca, *S.glutinosa* and *S.candidissima* growing wild in Greece. *Flavour and Fragrance Journal* 2006; 21: 670-673.
16. Nabavi SM, Ebrahimzadeh MA, Nabavi SF, Fazelian M, Eslami B. In vitro Antioxidant and Free Radical Scavenging Activity of *Diospyros lotus* and *Pyrus boissieriana* growing in Iran. *Pharmacognosy Magazine* 2009; 4(18): 123-127.
17. Nabavi SM, Ebrahimzadeh MA, Nabavi SF, Hamidinia A, Bekhradnia AR. Determination of antioxidant activity, phenol and flavonoids content of *Parrotia persica* Mey. *Pharmacologyonline* 2008; 2: 560-567.
18. Ebrahimzadeh MA, Pourmorad F, Hafezi S. Antioxidant Activities of Iranian Corn Silk. *Turkish Journal of Biology* 2008; 32: 43-49.
19. Ebrahimzadeh MA, Hosseinimehr SJ, Hamidinia A, Jafari M. Antioxidant and free radical scavenging activity of *Feijoa sallowiana* fruits peel and leaves. *Pharmacologyonline* 2008; 1: 7-14.
20. Yildirim A, Mavi A, Kara A. Determination of antioxidant and antimicrobial activities of *Rumex crispus* L. extracts. *Journal of Agricultural and Food Chemistry* 2001; 49: 4083-4089.
21. Dehpour AA, Ebrahimzadeh MA, Nabavi SF, Nabavi SM. Antioxidant activity of the methanol extract of *Ferula assafoetida* and its essential oil composition. *Grasas Y Aceites* 2009; 60 (4): 405-412.
22. Ebrahimzadeh MA, Bahramian F. Antioxidant activity of *Crataegus pentagina* subsp. *elbursis* Fruits extracts used in traditional medicine in Iran, *Pakistan journal of biological sciences* 2009; 12(5), 413-419.

23. Halliwell B. Antioxidants: the basics- what they are and how to evaluate them. *Advances in pharmacology* 1997; 38: 3-20.
24. Ebrahimzadeh MA, Pourmorad F, Bekhradnia AR. Iron chelating activity screening, phenol and flavonoid content of some medicinal plants from Iran. *African Journal of Biotechnology* 2008; 7 (18): 3188-3192.
25. Yu LL. Free radical scavenging properties of conjugated linoleic acids. *Journal of Agricultural and Food Chemistry* 2001; 49(7): 3452-3456.
26. Nabavi SM, Ebrahimzadeh MA, Nabavi SF, Bahramian F. In Vitro Antioxidant activity of *Phytolacca Americana* Berries *Pharmacologyonline* 2009; 1: 81-88.
27. Ebrahimzadeh MA, Nabavi SF, Nabavi SM. Antioxidant activities of methanol extract of *sambucus ebulus L.* flower. *Pakistan journal of biological sciences* 2009; 12(5): 447-450.
28. Van Acker SABE, Van Den Berg DJ, Tromp MNJL, Griffioen DH, Van Bennekom, WP, van der Vijgh WJF et al., Structural aspects of antioxidant activity of flavanoids. *Free Radical Biol Med* 1996; 20(3): 331-342.
29. Nabavi SM, Ebrahimzadeh MA, Nabavi SF, Jafari M. Free Radical Scavenging Activity and Antioxidant Capacity of *Eryngium caucasicum Trautv* and *Froriepia subpinnata*. *Pharmacologyonline*, 2008; 3: 19-25.
30. Moncada A, Palmer RMJ, Higgs EA. Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmacological Reviews* 1991; 43: 109-142.
31. Halliwell B, Gutteridge JMC. Role of free radicals and catalytic metal ions in human disease: an overview. *Methods in enzymology* 1990; 186: 1-85.
32. Hudson BJB, editor. The mechanism of antioxidant action in vitro. In: *Food antioxidants*. London. Elsevier Applied Science.1990: 1-18.