

## FREE RADICAL SCAVENGING ABILITY OF METHANOLIC EXTRACT OF HYOSCYAMUS *SQUARROSUS* LEAVES

Ebrahimzadeh M.A.\*<sup>a</sup>, Nabavi S.M.<sup>a</sup>, Nabavi S.F.<sup>a</sup>, Eslami B.<sup>b</sup>

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

<sup>b</sup>Department of Biology, Islamic Azad University of Ghaemshahr, Iran

### Summary

The antioxidant activity and free radical scavenging activity of methanol extract of *Hyoscyamus squarrosus* leave was investigated employing various in vitro assay systems. IC<sub>50</sub> for DPPH radical-scavenging activity was 215± 5.6 µg ml<sup>-1</sup>. The extracts showed weak nitric oxide-scavenging activity (IC<sub>50</sub> was 0.75 ± 0.02 mg ml<sup>-1</sup>) and Fe<sup>2+</sup> chelating ability (IC<sub>50</sub> was 0.86 ± 0.02 mg ml<sup>-1</sup>). The extract had show good reducing power that was comparable with Vitamin C (p> 0.05). It exhibited no antioxidant activity in FTC method. It showed good H<sub>2</sub>O<sub>2</sub> scavenging activity. Total phenol compounds, as determined by the Folin Ciocalteu method, were 98.95 ± 3.16 mg gallic acid equivalent/g of extract powder and the total flavonoid content, by AlCl<sub>3</sub> method, was 44.94 ± 2.02 mg quercetin equivalent/g of extract powder. The results improved that the leaves extract can serve as an electron donor for terminating the radical chain reaction.

**Key words:** Antioxidant activity, *Hyoscyamus squarrosus*, DPPH, phenols.

### Introduction

Free radicals are usually short-lived species but they possess a single unpaired electron, rendering them highly reactive against biologically important macromolecules including DNA, proteins and membrane lipids. To counteract this threat to their integrity, cells have evolved a variety of defense systems based on both water-soluble and lipid-soluble antioxidant species, and on antioxidant enzymes. A high proportion of the antioxidant systems of the human body are dependent on dietary constituents (1). Consequently, the need to identify alternative natural and safe sources of antioxidant arose and the search for safe and natural antioxidants, especially of plant origin, has notably increased in recent years (2). Additionally, it has been determined that the antioxidant effect of plant products is mainly attributed to phenolic compounds, such as flavonoids, phenolic acids, tannins, and phenolic diterpenes (3). *Hyoscyamus* genus is member of *solanaceae* family and has 18 species in Iran (4). This genus known to possess medicinal properties related to high content of tropane alkaloids such as Hyoscyamine, scopolamine and to lesser extent atropine (5).

*Hyoscyamus squarrosus* grow naturally in Iran, Pakistan and Afghanistan (4) and to the best of the author's Knowledge just antimicrobial activity of this species previously has been reported (6). In this study, we examined the antioxidant activity of methanol extract of *H. squarrosus* leave, 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 freeze-dried extract:** *Hyoscyamus squarrosus* leaves (summer 2007) was collected from Golestanak area (Central Elburz, Iran) and identified by Dr. Bahman Eslami A voucher (No.1143) has been deposited in the Sari School of Pharmacy herbarium. Materials dried at room temperature and coarsely ground before extraction. Then, it was extracted 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:** Total phenolic compound content was determined by the Folin-Ciocalteu reagent according to our recently published method (7,8). The extract sample (0.5 ml) was mixed with 2.5 ml of 0.2 N Folin-Ciocalteu reagent for 5 min and 2.0 ml of 75 g/l sodium carbonate were then added. The absorbance of reaction was measured at 760 nm after 2 h of incubation at room temperature. Result was expressed as gallic acid equivalents. Total flavonoids were estimated using our published method (7,8). Briefly, 0.5 ml solution of extract 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 and left at room temperature for 30 minutes. The absorbance of the reaction mixture was measured at 415 nm with a double beam spectrophotometer (Perkin Elmer). Total flavonoid content was calculated as quercetin from a calibration curve.

**DPPH Radical-Scavenging Activity:** The stable 1,1-diphenyl-2-picryl hydrazyl radical (DPPH) was used for determination of free radical-scavenging activity of extract (9). Different concentrations of extract were added, at an equal volume, to methanolic solution of DPPH (100  $\mu$ M). 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> value denotes the concentration of sample, which is required to scavenge 50% of DPPH free radicals.

**Determination of Reducing Power:** Fe (III) reduction is often used as an indicator of electron-donating activity, which is an important mechanism of phenolic antioxidant action (10). The reducing power of extract was determined according to our recently publish paper (11). Different amounts of extract (50-1600  $\mu$ g ml<sup>-1</sup>) in water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [K<sub>3</sub>Fe(CN)<sub>6</sub>] (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<sub>3</sub> (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 extract 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 (12).

**Metal Chelating Activity:** 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 (13). The chelating of ferrous ions by extract was estimated by our recently published paper (14). Briefly, extract (0.2-3.2 mg/ml) was added to a solution of 2 mM FeCl<sub>2</sub> (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<sup>2+</sup> complex formation was calculated as  $[(A_0 - A_s)/A_s] \times 100$ , where A<sub>0</sub> was the absorbance of the control, and A<sub>s</sub> was the absorbance of the extract/ standard. Na<sub>2</sub>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 (15). The inhibitory capacity of extract was tested against oxidation of linoleic acid by FTC method. This method was adopted from our recently published paper (16). 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 – [(absorbance increase of the sample/absorbance increase of the control) × 100]. All tests were run in duplicate, and analyses of sample was run in triplicate and averaged. Vitamin 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 of Ruch (16). 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 was 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 extract and standard compounds was calculated as follows: % Scavenged [H<sub>2</sub>O<sub>2</sub>] =  $[(A_0 - A_1)/A_0] \times 100$  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 (17).

**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  $EC_{50}$  values were calculated from linear regression analysis.

### Results and discussion

**Total Phenol and Flavonoid Contents:** Total phenolic compounds are reported as gallic acid equivalents by reference to standard curve ( $y = 0.0063x$ ,  $r^2 = 0.987$ ). The total phenolic contents of *H. squarrosus* leaves were  $98.95 \pm 3.16$  mg gallic acid equivalent/g of extract powder. The total flavonoid contents of *H. squarrosus* was  $44.94 \pm 2.02$  mg quercetin equivalent/g of extract powder, by reference to standard curve ( $y = 0.0067x + 0.0132$ ,  $r^2 = 0.999$ ). 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 (18).

**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 (19). It was found that the radical-scavenging activity of extract increased with increasing concentration.  $IC_{50}$  for DPPH radical-scavenging activity was  $215 \pm 5.6 \mu\text{g ml}^{-1}$ . The  $IC_{50}$  values for Ascorbic acid, Quercetin and BHA were  $5.05 \pm 0.12$ ,  $5.28 \pm 0.43$  and  $53.96 \pm 2.13 \mu\text{g ml}^{-1}$ , respectively.

**Reducing Power:** In the reducing power assay, the presence of antioxidants in the sample would result in the reducing of  $Fe^{3+}$  to  $Fe^{2+}$  by donating an electron. Amount of  $Fe^{2+}$  complex can be then be monitored by measuring the formation of Perl's Prussian blue at 700 nm (19). Increasing absorbance at 700 nm indicates an increase in reductive ability. Fig. 1 shows the dose-response curves for the reducing powers of the extract. The extract had show good reducing power that was comparable with Vitamin C ( $p > 0.05$ ). The reductive abilities of the leaves extract shows it can serve as an electron donor for terminating the radical chain reaction.

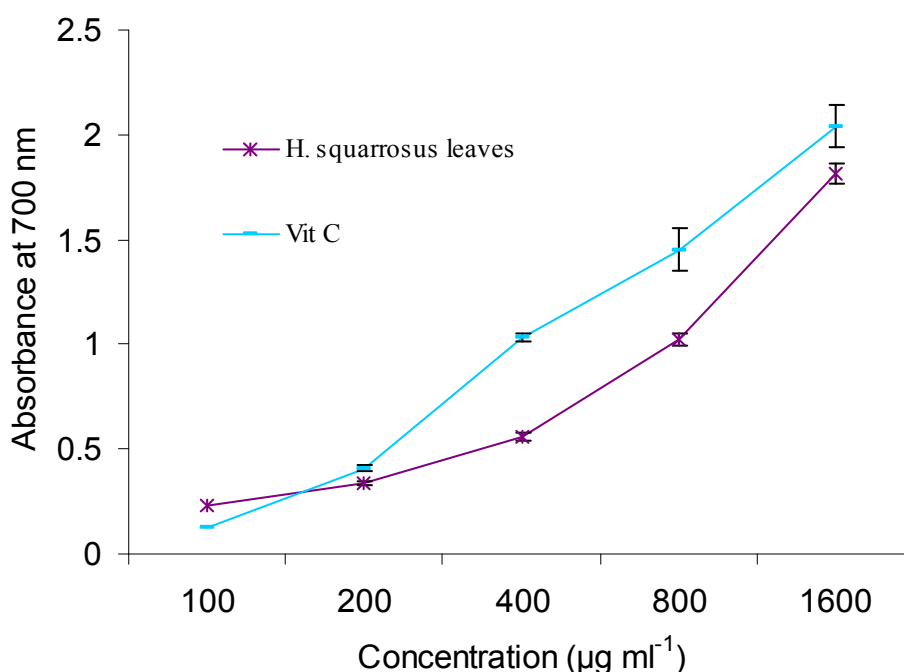


Fig.1. Reducing power of methanol extract of *H. squarrosus* leaves.

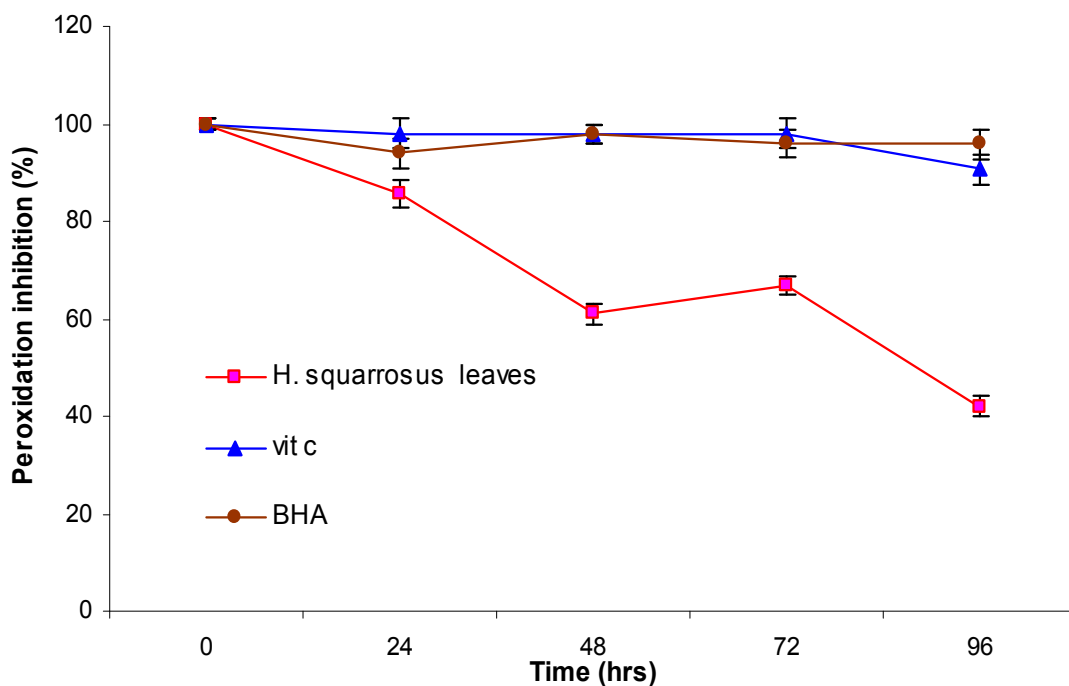
**Assay of nitric oxide-scavenging activity:** The % inhibition was increased with increasing concentration of the extract. The extract had show weak nitric oxide scavenging ( $749 \pm 22.7$  vs.  $20 \pm 0.8 \mu\text{g ml}^{-1}$  for quercetin). In addition to reactive oxygen species, nitric oxide is also implicated in inflammation, cancer and other pathological conditions (17). 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<sup>2+</sup> chelating activity of *H. squarrosus* 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 (14, 15). Because Fe<sup>2+</sup> also has been shown to cause the production of oxyradicals and lipid peroxidation, minimizing Fe<sup>2+</sup> concentration in Fenton reactions affords protection against oxidative damage. Ferrozine can quantitatively form complexes with Fe<sup>2+</sup>. 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<sup>2+</sup>-ferrozine complex was decreased dose-dependently, i.e. the activity was increased on increasing concentration from 50 to 800  $\mu\text{g ml}^{-1}$ . 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 (20, 21). *H. squarrosus* leaves extract showed weak Fe<sup>2+</sup> chelating ability ( $\text{IC}_{50} = 866 \pm 25 \mu\text{g ml}^{-1}$ ). EDTA showed very strong activity ( $\text{IC}_{50} = 18 \mu\text{g ml}^{-1}$ ).

**Scavenging H<sub>2</sub>O<sub>2</sub>:** Scavenging of H<sub>2</sub>O<sub>2</sub> by *H. squarrosus* extract may be attributed to their phenolics, which can donate electrons to H<sub>2</sub>O<sub>2</sub>, thus neutralizing it to water (22). The differences in H<sub>2</sub>O<sub>2</sub> scavenging capacities between the extract may be attributed to the structural features of their active components, which determine their electron donating abilities (9). The *H. squarrosus* leaves extract was capable of scavenging hydrogen peroxide in a concentration dependent manner ( $\text{IC}_{50} = 334.5 \pm 13.7 \mu\text{g ml}^{-1}$ ). The  $\text{IC}_{50}$  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 H<sub>2</sub>O<sub>2</sub> is very important throughout food systems

**FTC Method:** The extract exhibited no antioxidant activity in the FTC method. The peroxidation inhibition (antioxidant activity) of extract exhibited values from 85.7% (at 24<sup>th</sup> hrs) to 42.1% (at 72<sup>nd</sup> hrs). Vitamin C and BHA showed very good activity at different incubation times (Fig. 2).

The extract of *H. squarrosus* exhibited different levels of antioxidant activity in various in vitro assay systems. Highest activity has been showed in reducing power that was comparable with Vitamin C ( $p > 0.05$ ). It shows the extract can serve as an electron donor for terminating the radical chain reaction. Further investigation of individual compounds, their in vivo antioxidant activities and in different antioxidant mechanisms is needed.



**Fig. 2.** Antioxidant activity of methanolic extract of *H. squarrosus* leaves in FTC method at different incubation times. *H. squarrosus* ( $0.2 \text{ mg ml}^{-1}$ ), Vitamin C and BHA ( $0.1 \text{ mg ml}^{-1}$ ).

#### Acknowledgements

This research was partially supported by a grant from the research council of Mazandaran University of Medical Sciences, Iran. This paper dedicated to Mrs. Maryam Nabavi and Fatemeh soghra noruzi nejad.

#### References

1. Nehir EIS, Karakaya S. Radical scavenging and iron-chelating activities of some greens used as traditional dishes in Mediterranean diet. *International Journal of Food Sciences and Nutrition* 2004; 55(1): 67 -74.
2. Hou WC, Lin RD, Cheng KT, Hung YT, Cho CH, Chen CH, Hwang SY, Lee MH. Free radical-scavenging activity of Taiwanese native plants. *Phytomedicine* 2003; 10:170–175.
3. Pietta PG. Flavonoids as antioxidants. *Journal of natural products* 2000; 63:1035-1042.
4. Mozaffarian V. editor. In: A dictionary of Iranian plant names, Farhang moaser, Tehran Iran 2006: 283.
5. Sharifi G, Kouhsari SM, Ebrahimzadeh H, Khatamsaz M. Isozyme analysis of seedling samples in some species of *Hyoscyamus* from Iran. *Pakistan journal of biological sciences* 2006; 9(9): 1685-1692.
6. Bazzaz BS, Haririzadeh G. Screening of Iranian plants for antimicrobial activity, *Pharmaceutical Biology* 2003; 41(8): 573-583.
7. Ebrahimzadeh MA, Pourmorad F, Hafezi S. Antioxidant Activities of Iranian Corn Silk. *Turkish Journal of Biology* 2008; 32: 43-49.
8. Ghasemi K, Ghasemi Y, Ebrahimzadeh MA. Antioxidant activity, phenol and flavonoid contents of 13 Citrus specis peels and tissues. *Pakistan journal of pharmaceutical sciences* 2009; 22 (3): 277-281.

9. Dehpour AA, Ebrahimzadeh MA, Nabavi SF, Nabavi SM. Antioxidant activity of methanol extract of *Ferula assafoetida* and its Essential oil composition. *Grasas y Aceites* 2009; 60 (4): 405-412.
10. 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.
11. 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.
12. Ebrahimzadeh MA, Ehsanifar S, Eslami B. *Sambucus ebulus elburensis* fruits: A good source for antioxidants. *Pharmacognosy magazine* 2009; 4(19): 213-218.
13. Halliwell B. Antioxidants and human diseases: a general introduction. *Nutrition reviews* 1997; 55 : S44–S52.
14. 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.
15. Ebrahimzadeh MA, Nabavi SM, Nabavi SF, Eslami B. Antioxidant Activity of Aqueous Extract Of *Pyrus boissieriana* Fruit. *Pharmacologyonline* 2009; 1: 1318-1323.
16. 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.
17. Nabavi SM, Ebrahimzadeh MA, Nabavi SF, Bahramian F. In Vitro Antioxidant activity of *Phytolacca Americana* Berries. *Pharmacologyonline* 2009; 1: 81-88.
18. Halliwell B, Gutteridge JMC. Role of free radicals and catalytic metal ions in human disease: an overview. *Methods in enzymology* 1990; 186:1-85.
19. 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.
20. 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.
21. 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.
22. Ebrahimzadeh MA, Nabavi SF, Nabavi SM, Eslami B. Antihypoxic and antioxidant activity of *Hibiscus esculentus* seeds. *Grasas Y Aceites impress*, DOI: 10.3989/gya.053809.