# DETERMINATION OF ANTIOXIDANT ACTIVITY, PHENOL AND FLAVONOID CONTENT OF *PARROTIA PERSICA MEY*

Nabavi S. M., Ebrahimzadeh M. A.*, Nabavi S. F., Hamidinia A. and Bekhradnia A. R.

Pharmaceutical Sciences Research Center, School of Pharmacy, Mazandaran University of Medical Sciences, 48189, Sari, Iran. Tel: +98 151 3543081-3; Fax: +98 151 3543084. zadeh20@yahoo.com.

## Summary

Antioxidant activity of bark and leaves of *Parrotia persica* was investigated employing various in vitro assay systems, i.e. DPPH and nitric oxide radical scavenging, reducing power, iron ion chelating power, linoleic acid and $H_2O_2$ scavenging. $IC_{50}$ for DPPH radical-scavenging activity was 5.21 ± 0.04 for leaves and 1.84 ± 0.02 μg $ml^{-1}$ for bark, respectively. Extracts showed weak nitric oxide-scavenging activity between 0.8 and 3.2 mg $ml^{-1}$. The leaves showed better activity than bark. It was found that antioxidant activity was dose dependent i.e. activity was increased with the increase of their concentrations. The leaves extract showed good $Fe^{2+}$ chelating ability with $IC_{50}$= 162 μg $ml^{-1}$. The extracts exhibited a moderate activity in linoleic acid model. Leaves extract had potent scavenging ability in $H_2O_2$ scavenging assay model.

**Key words:** Antioxidant activity, DPPH, Iron chelation, *Parrotia persica* **,** Reducing power,

## 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). So the use of traditional medicine is widespread, and plants still present a large source of natural antioxidants that might serve as leads for the development of novel drugs (5). *Parrotia persica* (DC) CA. Mey (Hamamelidaceae) (Persian Ironwood) is a deciduous tree and has one species in Iran. It is native to northern Iran and endemic to the Alborz Mountains (6, 7). Little information is available about its biological activity. Many herbalists use this plant in the treatment various fevers and respiratory infections (8).

It is also used for food coloring and food flavoring (6). *P. persica* has significant antibacterial activity (8). In this study, we examined the antioxidant activity of P. persica Mey bark and leaves, employing various in vitro assay systems, i.e. DPPH and nitric oxide radical scavenging, reducing power, linoleic acid, Scavenging of $H_2O_2$ and iron ion chelating power.

## 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). 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:** Bark and fresh leaves of P. persica were collected from Sari forest, Iran, in spring 2006. After identification of the plant by Dr. Bahman Eslami Jadidi a voucher (No. 270 and 271) has been deposited in the Faculty of Pharmacy herbarium. Leaves and bark 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. The bark extracts yielded 5 and leaves 23% respectively.

**Determination of total phenolic compounds and flavonoid content:** Total phenolic compound contents were determined by the Folin-Ciocalteau method (9). The extract samples (0.5 ml of different dilutions) were mixed with 2.5 ml of 0.2 N Folin-Ciocalteau reagent (Sigma–Aldrich) 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. Results were expressed as gallic acid equivalents. Total flavonoids were estimated using the method of Ordonez et al., (9). To 0.5 ml of sample, 0.5 ml of 2% $AlCl_3$ ethanol solution was added. After 1 h at room temperature, the absorbance was measured at 420 nm. Total flavonoid contents were 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 the extracts (10, 11). 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. Vitamine 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 (12). The reducing power of P. persica was determined according to the method of Yen and Chen (13). 2.5 ml of each extracts (25-800 μg $ml^{-1}$) in water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (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. Vitamine 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 (14).

**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 (15). These processes can be delayed by iron chelation and deactivation. The chelating of ferrous ions by P. persica was estimated by the method of Dinis et al., (16). 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.

**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 (17). 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. Hydroxyl radicals eliminate hydrogen atoms from the membrane lipids, which results in lipid peroxidation. The inhibitory capacity of P. persica extracts against oxidation of linoleic acid by FTC method was tested. This method was adopted from Osawa and Namiki (18). 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 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 the method of Ruch (19). 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 $[H_2O_2] = [(A_o - A_1)/A_o] \times 100$ where $A_o$ was the absorbance of the control and $A_1$ was the absorbance in the presence of the sample of extract and standard (19).

**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 $EC_{50}$ 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 ($y = 0.0063x$, $r^2 = 0.987$). The total phenolic contents of leaves and bark were 139.2 ± 5.6 and 200.4 ± 2.2 mg gallic acid equivalent/g of extract powder, respectively. The total flavonoid contents of leaves and bark were 28.7 ± 0.9, 4.7 ± 0.1 mg quercetin equivalent/g of extract powder, respectively, 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 (20). Studies have shown that increasing levels of flavonoids in the diet could decrease certain human diseases (21).

**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 (22). 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 (23). It was found that the radical- scavenging activities of extracts increased with increasing concentration. $IC_{50}$ for DPPH radical-scavenging activity was in the order: 5.21 ± 0.04 for leaves and 1.84 ± 0.02 μg $ml^{-1}$ for bark. The $IC_{50}$ values for Ascorbic acid, quercetin and BHA were 1.26 ± 0.11, 1.32 ± 0.07 and 13.49 ± 1.04 μg $ml^{-1}$, respectively.

**Reducing power of Parrotia persica extracts:** In the reducing power assay, the presence of reductants (antioxidants) in the samples 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. 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 P. persica. It was found that the reducing powers of extracts also increased with the increase of their concentrations. There were no significant differences ($p > 0.001$) among the extracts in reducing power that was comparable with Vit C ($p > 0.01$). Because the reductive ability of extracts, it was evident, that P. persica did show reductive potential and could serve as electron donors, terminating the radical chain reaction.

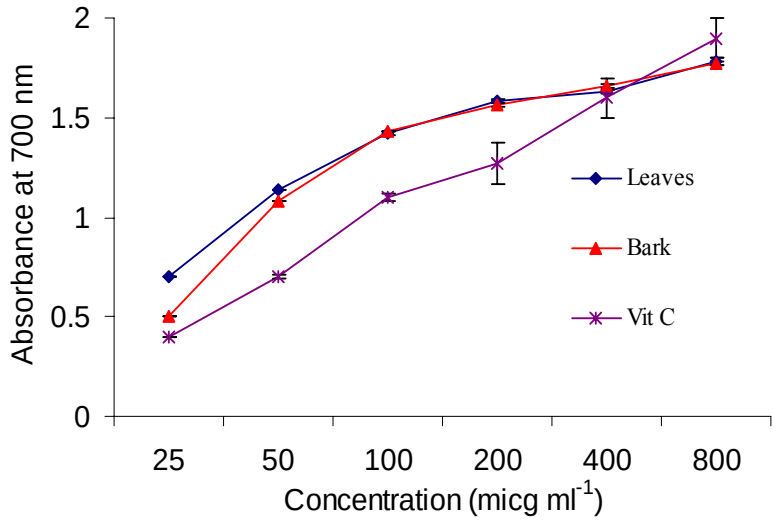


**Fig.1. Reducing power of methanolic extract of *P. persica* Mey leaves and bark.**

**Assay of nitric oxide-scavenging activity:** The extracts showed weak nitric oxide-scavenging activity between 0.2 and 3.2 mg $ml^{-1}$. The % inhibition was increased with increasing concentration of the extract. The leaves extract had shown slightly better reducing power than bark extract ($IC_{50}$ were 46.6 and 36.6% for leaves and bark, respectively. At 3.2 mg $ml^{-1}$. $IC_{50}$ for quercetin was 17.01 ± 0.03 µg $ml^{-1}$. In addition to reactive oxygen species, nitric oxide is also implicated in inflammation, cancer and other pathological conditions (24). 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 Parrotia persica extracts :** The transition metal, iron, is capable of generating free radicals from peroxides by Fenton reactions and may be implicated in human cardiovascular disease (25). 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. (16). 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 color 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 (26). 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 (27). P. persica leaves extract showed good $Fe^{2+}$ chelating ability. $IC_{50}$ were 162 ± 4.1 µg $ml^{-1}$ for leaves and only 23% at 0.8 mg $ml^{-1}$ for bark. EDTA showed very strong activity ($IC_{50}$ = 18 µg $ml^{-1}$).

**Scavenging $H_2O_2$:** Scavenging of $H_2O_2$ by P. persica leaves and bark extracts may be attributed to their phenolics, which can donate electrons to $H_2O_2$, thus neutralizing it to water (25). The differences in $H_2O_2$ scavenging capacities between the two extracts may be attributed to the structural features of their active components, which determine their electron donating abilities (25). The P. persica extracts were capable of scavenging hydrogen peroxide in a concentration-dependent manner. $IC_{50}$ for $H_2O_2$ scavenging activity was in the order: leaves 94 and bark 210 µg $ml^{-1}$. The $IC_{50}$ values for Ascorbic acid and BHA were 21.4 and 52.0 µg $ml^{-1}$, 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 $H_2O_2$ is very important throughout food systems

**FTC Method:** Figure 2 shows the time-course plots for the antioxidative activity of the two extracts of P. persica using the FTC method. The peroxidation inhibition (antioxidant activity) of extracts exhibited values from 93 to 97% (at 24$^{th}$ hrs) and from 81 to 91% (at 72$^{nd}$ hrs). Two extracts exhibited weak antioxidant activity. There were significant differences among antioxidative activity of extracts and two controls (Vit C and BHA) at different incubation times ($p< 0.01$). The leaves and bark extracts of P. persica exhibited good but different levels of antioxidant activity in some models studied. Further investigation of individual compounds, their in vivo antioxidant activities and in different antioxidant mechanisms is needed.

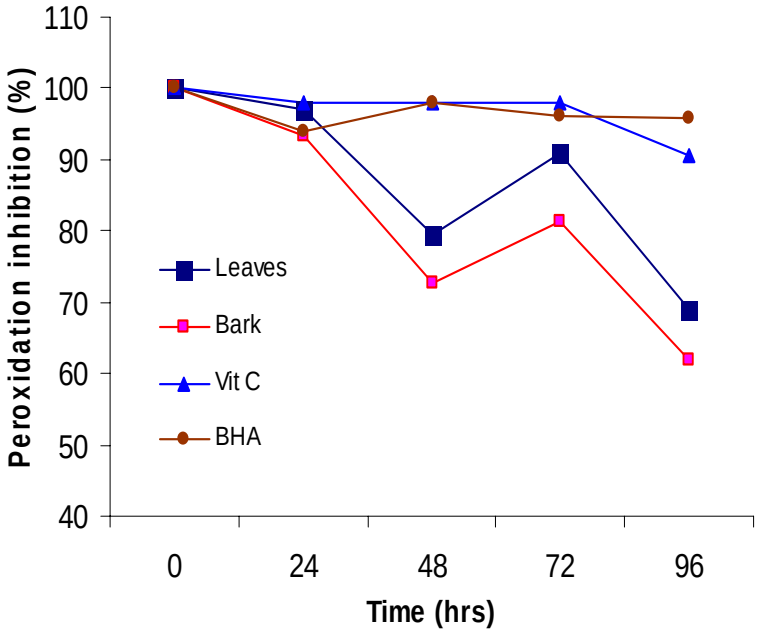


**Fig. 2. Antioxidant activity of methanolic extract of *P. persica* Mey leaves and bark in FTC method at different incubation times. *P. persica* (0.2 mg $ml^{-1}$), Vit C and BHA (0.1 mg $ml^{-1}$).**

**Acknowledgements**

The authors wish to thank Pharmaceutical sciences research center of mazandaran university of medical sciences (Sari, Iran) for the sanction of research grants to conduct the present study. The authors are indebted to Dr. B. Eslami of the Islamic Azad University of Qhaemshahr, Iran, for authenticating plant scientific name.

## References

1. Halliwell B. Free radicals, antioxidants and human diseases; curiosity, cause, or consequence? Lancet 1994; 334: 721-724.
2. Ghosal S, Tripathi VK, Chauhan S. Active constituents of *Emblica officinalis*. Part I. The chemistry and antioxidant effects of two new hydrolysable tannins, emblicanin A and B. Indian Journal of Chemistry 1996; 35B: 941- 948.
3. Lin CC, Huang PC. Antioxidant and hepatoprotective effects of Acathopanax senticosus. Phytotheraphy Research 2000; 14: 489- 494.
4. Sokmen A, Gulluce M, Akpulat HA, Daferera D, Tepe B, Polissiou M, Sokmen M, Sahin F. The in vitro antimicrobial and antioxidant activities of the essential oils and methanol extracts of endemic Thymus spathulifolius. Food Control 2004; 15: 627- 634.
5. Perry EK, Pickering AT, Wang WW, Houghton PJ, Perry NS. Medicinal plants and Alzheimer's disease: from ethnobotany to phytotherapy. The Journal of Pharmacy and Pharmacology 1999; 51: 527-534.
6. Rechinger KH. editor. In: Flora Iranica. Graz, Austria. Akademische Druk-u.Verlagsanstalt. 1999.
7. Mozaffarian V. editor. In: A dictionary of Iranian plant names. Tehran. Farhang Moaser. 2006.
8. Ahanjan M, Mohana DC, Raveesha KA, Azadbakht M. Antibacterial potential of extracts of leaves of *Parrotia persica,* African Journal of Biotechnology 2007; 6 (22): 2526-2528.
9. Ordon˜ez AAL, Gomez JD, Vattuone MA, lsla MI. Antioxidant activities of Sechium edule (Jacq.) Swartz extracts Food Chemistry 2006; 97: 452-458.
10. 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.
11. Ebrahimzadeh MA, Pourmorad F, Hafezi S. Antioxidant Activities of Iranian Corn Silk. Turkish Journal of Biology 2008; 32: 43-49.
12. 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.
13. Yen GC, Chen HY. Antioxidant activity of various tea extracts in relation to their antimutagenicity. Journal of Agricultural and Food Chemistry 1995; 43(1): 27-32.
14. Sreejayan N, Rao MNA. Nitric oxide scavenging by curcuminoids. Journal of Pharmacy and Pharmacology 1997; 49: 105-107.
15. Halliwell B. Antioxidants: the basics – what they are and how to evaluate them. Advances in Pharmacology 1997; 38: 3-20.
16. Dinis TCP, Madeira VMC, Almeida LM. Action of phenolic derivates (acetoaminophen, salycilate and 5-aminosalycilate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. Archives of Biochemistry and Biophysics 1994; 315: 161-169.
17. Yu LL. Free radical scavenging properties of conjugated linoleic acids. Journal of Agricultural and Food Chemistry. 2001; 49(7): 3452–3456.
18. Osawa T, Namiki M. A novel type of antioxidant isolated from leaf wax of Eucalyptus leaves. Agric Biol Chem. 1981; 45(3): 735-739.
19. Elmastaş M, Gülçinb İ, Işildaka Ö, Küfrevioğlub Öİ, İbaoğlua K, Aboul-Enein HY. Radical acavenging activity and antioxidant capacity of Bay leaf extracts. Journal of the Iranian Chemical Society 2006; 3(3): 258-266.
20. Van Acker SABE, van Den Berg DJ, Tromp MNJL, Griffioen DH, Van Bennekom WP, vader Vijgh WJF, Bast A. Structural aspects of antioxidant activity of flavanoids. Free Radical Biol Med 1996; 20(3): 331-342.
21. Hertog MLG, Feskens EJM, Hollman PHC, Katan MB, Kromhout D. Dietary antioxidants flavonoids and the risk of coronary heart disease: the zutphen elderly study. Lancet 1993; 342: 1007-1011.

22. Lee SE, Hwang HJ, Ha JS, Jeong HS, Kim JH. creening of medicinal plant extracts for antioxidant activity. Life Sciences 2003; 73: 167-179.
23. Brand-Williams W, Cuvelier M, Berset C. Use of a free radical method to evaluate antioxidant activity. Lebensmittel- Wissenschaft und -Technologie 1995; 28: 25-30.
24. Moncada A, Palmer RMJ, Higgs EA. Nitric oxide: physiology, pathophysiology and pharmacology. Pharmacological Reviews 1991; 43: 109-142.
25. Halliwell B, Gutteridge JMC. Role of free radicals and catalytic metal ions in human disease: an overview. Methods in Enzymology 1990; 186: 1-85.
26. Duh PD, Tu YY, Yen GC. Antioxidant activity of water extract of harng Jyur (Chrysanthemum morifolium Ramat). Lebensmittel-Wissenschaft Und-Technologie 1999; 32: 269-277.
27. Hudson BJF, editor. The mechanism of antioxidant action in vitro. In: Food antioxidants. London. Elsevier Applied Science.1990: 1-18.