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Full Length Research Paper

In vitro antioxidant and free radical scavenging activity of *Leonurus cardiaca* subsp*. Persicus, Grammosciadium platycarpum* and *Onosma demawendicum*

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Antioxidant activity of the aerial parts of *Leonurus cardiaca* subsp*. Persicus*, *Grammosciadium platycarpum* and *Onosma demawendicum* were investigated employing six *in vitro* assay systems. IC_{50} for 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical-scavenging activity was in the order: *O. demawendicum* (221 ± 11.7) > *L. cardiaca* (144 ± 12.1) > *G. platycarpum* (45 ± 2.1) μgml^{-1}, respectively. The extracts showed very good nitric oxide-scavenging and Fe^{2+} chelating ability activity. The *L. cardiaca* subsp*. Persicus* showed Fe^{2+} chelating ability activity as ethylenediaminetetraacetic acid (EDTA) (IC_{50} were 20 versus 18 μgml^{-1}). None of the extracts exhibited good antioxidant activity in linoleic acid model. The extracts showed good reducing power that was better than vitamin C ($p < 0.05$). *G. platycarpum* and *L. cardiaca* subsp*. Persicus* had higher total phenolic and flavonoid contents than *O. demawendicum*. The total phenolic compounds in the extracts were determined as gallic acid equivalents, and total flavonoid contents were calculated as quercetin equivalents from a calibration curve.

Key words: Antioxidant activity, Fe^{2+} chelating, 1,1-diphenyl-2-picryl hydrazyl (DPPH), phenol, *Leonurus cardiaca, Grammosciadium platycarpum*, *Onosma demawendicum.*

INTRODUCTION

Plants are rich sources of natural antioxidants. Among the various medicinal and culinary plants, some endemic species are of particular interest because they may be used for producing raw materials or preparations containing phytochemicals with significant antioxidant capacities and health benefits (Exarchou et al., 2002). Reactive oxygen species (ROS) have been found to play an important role in the initiation and/or progression of various diseases such as atherosclerosis, inflammatory injury, cancer and cardiovascular disease (Halliwell, 1997). Thus, recent studies have investigated the potential of plant products as antioxidants against various diseases induced by free radicals (Hou et al., 2003). 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 (Pietta, 2000). Three species of the genus *Grammosciadium* (family *Umbelliferae*), *Grammosciadium pterocarpum*, *Grammosciadium platycarpum* and *Grammosciadium scabridum* occur in Iran (Mozaffarian,

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Abbreviations: DPPH, 1,1-Diphenyl-2-picryl hydrazyl radical; **ROS,** reactive oxygen species; **TBARS,** thiobarbituric acid reactive substances; **TCA,** trichloroacetic acid; **BHA,** butylated hydroxyanisole; **FTC,** ferric thiocyanate method; **AD,** Alzheimer's disease.

2006). Generally, they occur in temperate or temperate cold pasturelands and are considered to be attractive animal foods. These species occur also in Iraq, Anatoly, the Caucasus and Armenia (Rechinger, 1982). *G. platycarpum* which grows naturally in the mountainous regions of the country is a perennial plant with a very strong fragrance (Nickavar et al., 2006). Only the essential oil composition (Nickavar et al., 2006) and anti bacterial activity (Sonboli et al., 2005) of this plant has been reported so far. *Leonurus cardiaca L.* (*Lamiaceae*) has been used in traditional medicine against nervous and functional cardiac disorders since the 15th century and now is described in pharmaco-poeias (Russian Pharmacopoeia, 1968; BHP, 1992) for producing sedative, hypotensive and cardiotonic pharmacological effects. Indications are for the treatment of neuropathic and functional cardiac disorders. Many chemical constituents of the plant (alkaloids, iridoids, saponins, flavornoids, cardenolidlike glycosides and diterpenoids) have been detected and isolated, but their relevance for medical use has not been investigated completely (Milkowska-Leyck et al., 2002). Antioxidative activity has been reported by thiobarbituric acid reactive substances (TBARS), phosphomolybdenum method and 1,1-diphenyl-2-picryl hydrazyl (DPPH) in *L. cardiaca* L. (Matkowski and iotrowska, 2006). *L. cardiaca* subsp. *Persicus* is native to Iran and endemic to Elburz Mountains (Mozaffarian, 2006). To the best of our knowledge, there is no biological activity reported from this subspecies. The genus *Onosma* (*Boraginaceae*) comprises of about 85 species, occurring mainly in Iran and westward to Syria, Turkey and Europe (Ahmad et al., 2003). A literature survey revealed that very little phytochemical work has been carried out on the genus *Onosma* and only some naphthaquinones, alkaloids and phenolic compounds have been reported so far (Ahmad et al., 2003). The genus has several utilizations in traditional medicines worldwide. It is noted that the roots of *Onosma* species are used for the treatment of various disorders such as bronchitis, tonsillitis, hemorrhoids as well as alleviating pains in folk medicine in Turkey (Tosun et al., 2008; Ozgen et al., 2006). The root has important usage regarding recovery of wounds and burns. The graded roots or barks from *Onosma* species are prepared as an ointment and kept as a home remedy to treat wounds, burns and hemorrhoids, externally (Sezik et al., 1997; Ozgen et al., 2006). In addition, *Onosma hispida* can be used for laxative and anthelmintic purposes and is also used to treat eye diseases, blood disorders, bronchitis, abdominal pains, as well as against thirst, itching, fever, wounds, piles, dysuria and urinary calculi. The plant flowers are prescribed as a cardiac stimulant on heart as well as against rheumatism (Ahmad et al., 2005). The root extract of *Onosma argentatum* (containing deoxy-shikonin, acetyl shikonin, 3-hydroxy-isovaleryl shikonin and 5,8-O-dimethyl acetyl shikonin, (Ozgen et al., 2004)) has been found to be an effective *in vitro* antioxidant and antimicrobial agent against *Staphylococcus aureus, Bacillus subtilis* and *Escherichia coli* (Ozgen et al., 2003). *Onosma demawendicum* is native and endemic to Elburz Mountains. There are no scientific reports about *O. demawendicum.* In this study, we examined the antioxidant activity of the aerial parts of *L. cardiaca* subsp. *Persicus, G. platycarpum* and *O. demawendicum*, employing various *in vitro* assay systems, that is, DPPH and nitric oxide radical scavenging, reducing power, linoleic acid, iron ion chelating power and hydrogen peroxide scavenging 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), DPPH and potassium ferricyanide were purchased from Sigma Chemicals Co. (USA). Gallic acid, quercetin, 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 materials and preparation of extract

Aerial parts of *L. cardiaca* subsp. *Persicus*, *G. platycarpum* and *O. demawendicum* were collected at flowering stage from Gaduk area, north of Firuzkooh, Iran, in summer 2008. After identification of the plant by Dr. Bahman Eslami, a voucher (No. 472-4) was deposited in the school of Pharmacy herbarium. The 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 flavonoid content

Colorimetric aluminum chloride method was used for flavonoid determination (Ghasemi et al., 2009; Nabavi et al., 2008a). Briefly, 0.5 ml solution of each plant extracts in methanol were 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 min. The absorbance of the reaction mixture was measured at 415 nm with a double beam Perkin Elmer UV/Visible spectrophotometer (USA). Total flavonoid contents were calculated as quercetin from a calibration curve. The calibration curve was prepared by preparing quercetin solutions at concentrations of 12.5 to 100 mg ml^{-1} in methanol.

Determination of total phenol content

Total phenolic compound contents were determined by the Folin-Ciocalteau method (Ghasemi et al., 2009; Nabavi et al., 2009a). The extract samples (0.5 ml of different dilutions) were mixed with Folin Ciocalteu reagent (5 ml, 1:10 diluted with distilled water) for 5 min and aqueous Na_2CO_3 (4 ml, 1 M) were then added. 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^{-1} solutions of gallic acid in methanol : water (50:50, v/v). Total phenol values are expressed in terms of gallic acid equivalent (mg g^{-1} of dry mass), which is a common reference compound.

DPPH radical-scavenging activity

The stable DPPH was used for the determination of free radical-scavenging activity of the extracts (Dehpour et al., 2009, Nabavi et al., 2009b). 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 (Ebrahimzadeh et al., 2009d, Nabavi et al., 2008b). The reducing power of extracts was determined according to the method of Yen and Chen (1995). Different amounts of each extracts (25 - 800 µgml^{-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. 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 (Ebrahimzadeh et al., 2009a).

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 (Halliwell, 1997). The chelating of ferrous ions by extracts was estimated Ebrahimzadeh et al. (2008 and 2009c). 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 to stand 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 ferric thiocyanate method (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 (Yu, 2001). The inhibitory capacity of extracts was tested against oxidation of linoleic acid by FTC method (Ebrahimzadeh et al., 2008b; Nabavi et al., 2008b). 20 mg ml^{-1} 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. Vitamin C and BHA were used as positive control.

Scavenging of hydrogen peroxide

The ability of the extracts to scavenge hydrogen peroxide was determined according to Ebrahimzadeh et al. (2009b, e). A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). The concentration of hydrogen peroxide was determined by absorption at 230 nm using a spectrophotometer. Extracts (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.

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 was separated by Duncan's multiple range test. The IC_{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 *L. cardiaca*, *G. platycarpum* and *O. demawendicum* are 54.3 ± 1.8, 66.9 ± 4.3 and

Table 1. Antioxidant activities of *L. cardiaca*, *G. platycarpum* and *O. demawendicum* aerial parts methanol extracts.

Sample	DPPH IC_{50} (mg ml^{-1})[a]	Nitric oxide IC_{50} (mg ml^{-1})[b]	H_2O_2 activity, IC_{50} (µg ml^{-1})[c]	Fe^{2+} chelating ability, IC_{50} (µg ml^{-1})[d]
L. cardiaca	144 ± 12.1	0.15 ± 0.01	438.2 ± 21.8	20 ± 1
G. platycarpum	45 ± 2.1	1.19 ± 0.09	293 ± 14	79 ± 3
O. demawendicum	221 ± 11.7	0.63 ± 0.02	638 ± 27	268 ± 11

[a]IC_{50} of BHA was 53.96 ± 3.1; vitamin C, 5.05 ± 0.1 and quercetin 5.28 ± 0.2 µg ml^{-1}.
[b]IC_{50} of quercetin was 5.28 ± 0.2 µg ml^{-1}.
[c]IC_{50} for vitamin C and quercetin were 21.4 ± 1.1 and 52 ± 2.6 µg ml^{-1}, respectively.
[d]IC_{50} for EDTA was 18 ± 1.5 µg ml^{-1}.

47.2 ± 2.2 mg gallic acid equivalent/g of extract, respectively. The total flavonoid contents of *L. cardiaca, G. platycarpum* and *O. demawendicum* are 35.2 ± 0.9, 32.8 ± 1.5 and 13.7 ± 0.8 mg quercetin equivalent/g of extract, respectively, by reference to standard curve ($y = 0.0067x + 0.0132$, $r^2 = 0.999$). *G. platycarpum* extract had higher total phenol and *L. cardiaca* had higher flavonoid contents than others. 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 (van Acker et al., 1996). Studies have shown that increasing levels of flavonoids in the diet could decrease certain human diseases (Hertog et al., 1993).

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 (Ebrahimzadeh et al., 2009f, g). DPPH is a stable nitrogen-centered free radical whose color 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 (Brand-Williams et al., 1995). It was found that the radical-scavenging activities of all the extracts increased with increasing concentration. IC_{50} for DPPH radical-scavenging activity was in the order: *O. demawendicum* (221 ± 11.7) > *L. cardiaca* (144 ± 12.1) > *G. platycarpum* (45 ± 2.1) µgml^{-1}, respectively (Table 1). The IC_{50} values for ascorbic acid, quercetin and BHA were 5.05 ± 0.12, 5.28 ± 0.43 and 53.96 ± 2.13 □gml^{-1}, respectively. *G. platycarpum* was more active than our positive control, BHA ($p < 0.05$).

Reducing power of extracts

In the reducing power assay, the presence of reductants (antioxidants) in the samples would result in the reduction of Fe^{3+} to Fe^{2+} by donating an electron. Amount of Fe^{2+} complex can 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 (Ebrahimzadeh et al., 2010b). Figure 1 shows the dose-response curves for the reducing powers of the extracts from *L. cardiaca, G. platycarpum* and *O. demawendicum*. It was found that the reducing powers of extracts also increased with increase in their concentrations. There were significant differences ($p < 0.001$) among the different extracts in reducing power. The aerial parts of *G. platycarpum* and *O. demawendicum* extracts showed better reducing power than *L. cardiaca* extract ($p < 0.01$). Both of them exhibited a high reducing power at 25 - 400 µgml^{-1} that was significantly better than vitamin C ($p < 0.05$). Because the reductive abilities of these extracts were significantly higher than vitamin C, it was evident that these extracts did show reductive potential and could serve as electron donors, terminating the radical chain reaction.

Assay of nitric oxide-scavenging activity

The extracts also showed weak nitric oxide-scavenging activity between 0.05 and 1.6 mg ml^{-1}. The percentage of inhibition was increased with increasing concentration of the extract. The *L. cardiaca* extract showed better scavenging activity than others (IC_{50} were 0.15 ± 0.01 for *L. cardiaca*, 0.63 ± 0.02 for *O. demawendicum* and 1.19 ± 0.09 mg ml^{-1} for *G. platycarpum* vs 17 µgml^{-1} for quercetin) (Table 1). In addition to reactive oxygen species, nitric oxide is also implicated in inflammation, cancer and other pathological conditions (Moncada et al., 1991). 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 extracts

Iron chelators mobilize tissue iron by forming soluble and

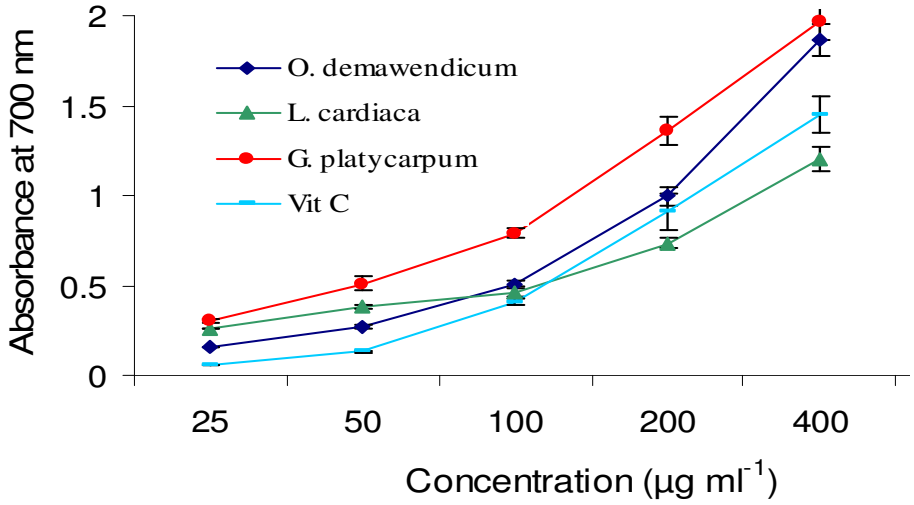

Figure 1. Reducing power of *L. cardiaca, G. platycarpum* and *O. demawendicum* methanol aerial parts. Vitamin C was used as control.

stable complexes that are then excreted in the feces and/or urine. Chelation therapy reduces iron-related complications in human and thereby improves quality of life and overall survival in some diseases such as thalassemia major (Shinar and Rachmilewitz, 1990). In addition, brain iron dysregulation and its association with amyloid precursor protein plaque formation are implicated in Alzheimer's disease (AD) pathology and so iron chelation could be considered as a rational therapeutic strategy for AD (Reznichenko et al., 2006). The transition metal, iron, is capable of generating free radicals from peroxides by Fenton reactions and may be implicated in human cardiovascular disease (Halliwell and Gutteridge, 1990). 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 Ebrahimzadeh et al. (2008, 2009c). 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, that is, 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 (Ebrahimzadeh et al., 2010b). *L. cardiaca* extracts showed very good Fe^{2+} chelating ability. IC_{50} were 20 ± 1 for *L. cardiaca*, 79 ± 3 for *G. platycarpum* and 268 ± 11 µg ml^{-1} for *O. demawendicum*. EDTA showed very strong activity (IC_{50} = 18 µg ml^{-1}) (Table 1).

FTC method

None of our tested extracts exhibited good activity using the FTC method. The peroxidation inhibition (antioxidant activity) of extracts exhibited values from 80.8 to 86% (at 24th h) only. At other incubation times (48th and 72nd hour), extracts showed only 13 to 30% inhibition. There were significant differences ($p < 0.001$) among plants extracts and vitamin C or BHA at different incubation times. It suggests that peroxidation inhibition have no role in antioxidant activity of our extracts and other mechanism maybe involve.

Hydrogen peroxide scavenging

Scavenging of H_2O_2 by extracts may be attributed to their phenolics, which can donate electrons to H_2O_2, thus neutralizing it to water (Ebrahimzadeh et al., 2010a). The ability of the extracts to effectively scavenge hydrogen peroxide was determined according to the method of Mahmoudi et al. (2009), where they are compared with that of quercetin as standard. The extracts were capable of scavenging hydrogen peroxide in a concentration-dependent manner. IC_{50} for scavenging of H_2O_2 were 293 ± 14 for *G. platycarpum*, 438.2 ± 21.8 for *L. cardiaca* and 638 ± 27 µg ml^{-1} for *O. demawendicum*, respectively (Table 1). The IC_{50} values for ascorbic acid and quercetin were 21.4 ± 0.12 and 52.0 ± 3.11 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 (Nabavi et al., 2010).

Conclusions

The aerial parts extracts of *L. cardiaca* subsp. *Persicus*, *G. platycarpum* and *O. demawendicum* exhibited very good but different levels of antioxidant activity in all the models studied. The extracts had good reducing power activity, Fe^{2+} chelating, nitric oxide and DPPH radical-scavenging activity. Further investigation of individual compounds, their *in vivo* antioxidant activities and different antioxidant mechanisms is needed.

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