Asian Journal of Chemistry Vol. 22, No. 6 (2010), 4524-4532

Antioxidant and Free Radical Scavenging Potential of *Otostegia limbata*

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Otostegia limbata is a medicinal herb of family *Lamiaceae* and is known for its medicinal activities for various ailments. In view of the possibility that some of these activities may be related to the antioxidant compounds found in the plant, we carried out comprehensive antioxidant studies on extracts of the leaves of the plant in water, *n*-hexane, chloroform, ethyl acetate, *n*-butanol and methanol. Some extracts showed strong antioxidant activity. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity was determined and EC_{50} (µg) values were found to be in between 61 (ethyl acetate fraction) and 96 (*n*-butanol fraction). The total phenolic content varied from 489-1273 mg GAE/100 g, total flavonoid content was 198-3018 mg QE/100 g, TEAC (trolox equivalent antioxidant capacity) values ranged from 30-139 (µmol/g) using ABTS assay, while FRAP (ferric reducing antioxidant power) values ranged from 5-41 (mmol/g). The results of these studies suggest that *Otostegia limbata* could serve as a source of natural antioxidants with potential applications in food and pharmaceutical industries.

Key Words: *Otostegia limbata*, Antioxidant activity, Scavenging effects, Polyphenols.

INTRODUCTION

Free radicals are reactive species having odd electrons. The free radicals produced in the human body exist in different forms, including superoxide, hydroxyl, hydroperoxyl, peroxyl and alkoxyl radicals. Production of free radicals and other reactive species in cells and body tissues has been linked to aging and more than 100 disease states[1]. Oxygen free radicals play a significant role as causative species for oxidative damage of membranes and tissues, eventually resulting in a variety of diseases, cancer, aging *etc.*[2,3]. When an imbalance occurs between the generation of reactive oxygen species (ROS) and antioxidants, oxidative damage may spread over all the cell targets (DNA, lipids, proteins)[4]. Antioxidants are compounds that inhibit the oxidation process by blocking the initiation or propagation of oxidizing chain reactions. Natural antioxidants are known to have wide range of biochemical

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activities, including scavenging of free radicals, alteration of intracellular redox potential and inhibition of ROS generation. These antioxidants with desired physico-chemical properties are, therefore, in high demand for their application as nutraceuticals as well as food additives[5]. In the recent years a growing number of plants have been shown to have antioxidant and/or antiradical scavenging mechanism as part of their activity[6-8].

Presently various methods are used to evaluate antioxidant activities of natural compounds in foods or biological systems with varying results. They include determination of total phenolic and flavonoid contents, DPPH scavenging, $ABTS^+$ decolourization and FRAP assays and lipid peroxidation value determination.

Otostegia limbata BTH. (*Lamiaceae*) is a medicinal herb found in the hilly areas of the north-west of Pakistan. The genus *Otostegia* consists of about 33 species mainly found in the Mediterranean region[9]. These are widely used by the traditional practitioners against various diseases. *Otostegia larendana* and *Otostegia nigra* have potent antidepressant activity, while *O. larendana* also possesses antiulcer, antispasmodic, antidepressant, anxiolytic and sedative activities[10]. Methanolic extract of *Ototestiga persica* has been found to exhibit antioxidant activity[11]. The leaves of *Otostegia limbata*, locally called "Bui" or "Phut kandu"[12], are used by the local people in the treatment of children gum diseases and for ophthalmia in man and for curing wounds[13]. The herb has yielded diterpenoids upon chemical investigations[14].

Literature survey showed that studies have not yet been conducted on antioxidant and free radical scavenging activity of *O. limbata*, which are important in rationalizing its medicinal applications. We therefore carried out a comprehensive evaluation of antioxidant activity of extracts of the leaves of the herb in various solvents (*n*-hexane, chloroform, ethyl acetate, *n*-butanol, methanol and distilled water) using different methods.

EXPERIMENTAL

Sodium acetate, 2,4,6-tri(2-pyridyl)-S-triazine (TPTZ), Folin-Ciocalteu reagent, 1,1-diphenyl-2-picrylhydrazyl (DPPH), ABTS [2,2'-azinobis(3-ethylbenzothiazoline -6-sulphonic acid)], quercitin, trolox, $FeCl_3 \cdot 6H_2O$, potassium dihydrogen phosphate (KH_2PO_4), dipotassium hydrogen phosphate (K_2HPO_4), gallic acid, linoleic acid, tween-20 and ammonium thiocyanate were purchased from Aldrich Chemicals Co. (Gill Ingham, Dorset, UK). All reagents and solvents were of analytical grade.

Solvent extraction: The aerial parts of *Otostegia limbata* (*Lamiaceae*) were collected in October 2007 from hills near Abbottabad, Pakistan and identified by the taxonomist of Hazara University, Mansehra. After drying the herb under shade for 20 days, leaves were separated with care and were ground to fine powder. Extraction was carried out by soaking 50 g of powdered leaves in 200 mL of methanol for 15 days at room temperature. The extract was filtered and the solvent was evaporated from the filtrate under reduced pressure by using rotary evaporator. The amount of the extract obtained was 5.25 g. The methanolic extract was suspended in water

(50 mL) and after obtaining 10 mL of this aqueous fraction, the rest of the extract was partitioned successively with equal volumes of *n*-hexane, chloroform, ethyl acetate and *n*-butanol, leaving a residual water soluble fraction. Each fraction was concentrated by rotary evaporator and then dried. The dry extract obtained with each solvent was weighed and the percentage yield in terms of air dried weight of plant material was calculated. The extracts thus obtained were stored at 4 ºC until they were used for various assays.

Determination of total phenolic content: The total phenolic content (TPC) in various extracts was determined according to the method employed by Singleton and Rossi[15]. In the method, from each extract 40 µL aliquot was taken and mixed with 0.2 mL of 0.2 N Folin-Ciocalteu reagent and added to 3.16 mL of distilled water. The mixture was kept at room temperature for 8 min. Then 0.6 mL saturated solution of sodium carbonate (20 g/100 mL) was added. After incubation at 40 ºC for 0.5 h, with intermittent shaking, the absorbance was measured at 765 nm. The concentration of total phenolics was expressed as gallic acid equivalent (GAE) in mg/100 g of dry sample.

Determination of total flavonoid content: The total flavonoid content in various extracts of leaves parts of the herb was estimated according to the method reported by Dewanto *et al.*[16]. In our test, 150 µL of 5 % $NaNO_2$ solution was added to a mixture of an extract (0.05 mL) in distilled water (2.5 mL) in a test tube. After 6 min, 300 µL of 10 % $AlCl_3 \cdot 6H_2O$ solution was added and the mixture was further allowed to stand for 5 min and then 1 mL of NaOH aqueous solution (1 M) was added. Distilled water was added to raise the volume to 5 mL. The mixture was shaken well to mix up the contents. Absorbance was measured against blank at 510 nm. A calibration curve was prepared with quercitin and the results were expressed as quercitin equivalent (QE) in mg/100 g of the sample.

$ABTS^+$ decolorization assay: The total antioxidant activity was determined by using the method described by Re *et al.*[17]. A solution of ABTS radical cation ($ABTS^+$) was prepared by reacting 7.5 mL of 7 mM ABTS with 0.25 mL of 2.5 mM potassium thiosulphate. The reaction mixture was then incubated at room temperature in the dark for about 18 h. The intensely coloured $ABTS^+$ solution so obtained was diluted with 0.01 M PBS (phosphate buffer saline, pH 7.4) to give an absorbance of 0.700 ± 0.02 at 734 nm. The mixture was allowed to equilibrate at 30 ºC. Then, to 2.99 mL of the ($ABTS^+$) solution, 10 µL of the sample solution was mixed, and the absorbance was measured for each fraction. The same experiment was repeated with standard antioxidant (trolox) and a calibration curve was drawn. The results of the assay were expressed relative to trolox in terms of TEAC (trolox equivalent antioxidant capacity) values[17].

DPPH free radical scavenging activity: The free radical scavenging activity of the extracts was determined by using DPPH according to the method described by Sanchez-Moreno *et al.*[18]. In a test, 3.9 mL DPPH solution (25 mg/L) in MeOH

was mixed with 0.1 mL of a sample solution and the reaction progress was monitored at 517 nm for about 0.5 h. The percentage of DPPH remaining ($DPPH_{rem}$ %) was calculated using the following formula:

$$DPPH_{rem}(\%) = 100 \times [DPPH]_T = t/[DPPH]_{T=0}$$

where $[DPPH]_{T=0}$ and $[DPPH]_{T=t}$ are the absorbances of DPPH solution at the beginning of its reaction with a sample (*i.e.*, T = 0) and at time T = t (*i.e.*, until the absorbance becomes zero), respectively. The EC_{50} (the concentration required for 50 % scavenging activity) values for each fraction was also calculated as a function of time.

FRAP Assay: The FRAP (ferric reducing antioxidant power) of various extracts was determined by the method described by Benzie and Strain[19]. FRAP reagent was prepared by mixing 25 mL of 300 mM sodium acetate buffer (pH 3.6), 2.5 mL of 10 mM TPTZ solution in 20 mL HCl solution (40 mM) and 2.5 mL of 20 mM iron(II) chloride solution. The reagent was kept at 37 ºC through out the experiment. After 10 min of incubation, absorbance of the blank reagent was measured at 593 nm. Then, 3 µL of the FRAP reagent were added to 100 µL of sample and 300 µL of distilled water. Absorbance of this solution was measured at 593 nm after every minute for 4 min. The FRAP value of each extract of the herb was determined using the equation obtained by the calibration curve of the standard $FeSO_4$ solution.

Lipid peroxidation value in linoleic acid emulsion system: The lipid peroxidation value of various extracts was determined according to the method described by Mitsuda *et al.*[20]. An emulsion of linoleic acid was prepared by mixing 175 µg Tween-20 and 155 µL linoleic acid and the volume was made 50 mL by adding enough potassium phosphate buffer (0.04 M, pH 7). Then, 100 µL of a plant extract was mixed with 2.4 mL of potassium phosphate buffer (0.04 M, pH 7) and 2.5 mL linoleic acid emulsion. The mixture was incubated at 37 ºC. 100 µL of this solution was regularly taken at 24 h intervals (for 5 days) and reacted with $FeCl_2$ (100 µL, 20 mM) and potassium thiocyanate (30 %, 100 µL) and absorbance was measured at 500 nm. A 5 mL solution consisting of linoleic acid emulsion (2.5 mL) and potassium phosphate buffer (2.5 mL) was used as blank. BHA and Trolox were used as standard antioxidants.

Statistical analyses: All measurements were run in triplicates (n = 3), unless specified and the values were averaged. Various statistical techniques such as analysis of variance (ANOVA), Duncan's multiple range method and regression analysis were used for analyzing the data. A difference was considered statistically significant when $p < 0.05$.

RESULTS AND DISCUSSION

Total phenolic content: As plant phenolics constitute one of the major groups of compounds acting as primary antioxidants or free radical terminators, it was reasonable to determine their total amount in the extracts[21]. The phenolic compounds may contribute directly to the antioxidant action due to the presence of hydroxyl

groups that are potent hydrogen donors[22]. The total phenolic content, determined as gallic acid equivalent (mg GAE/100 g of dry extract) ranged from 489-1273 mg/100 g (Table-1). The highest total phenolic levels were detected in *n*-butanol extract (1273 mg/100 g) and the lowest in aqueous after partition extract (489 mg/100 g). *n*-Hexane, chloroform and methanolic extracts show comparable values (Table-1). The results are in accordance with the suggestion that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans when up to 1 g is daily ingested from a diet rich in fruits and vegetables[23].

TABLE-1
YIELD (%), TOTAL PHENOLIC CONTENT (TPC), TOTAL FLAVONOID CONTENT (TFC) AND EC_{50} VALUES OF LEAVES OF *Otostegia limbata*

Fractions	Yield (%)	TPC (mg/100 g of dry extract)	TFC (mg/100 g of dry extract)	EC_{50} (µg)
n-Hexane	0.1238	889.6	3017.9	226.1
Chloroform	1.4759	882.9	661.9	109.5
Ethyl acetate	7.1594	736.4	383.5	60.9
1-Butanol	2.8812	1272.7	1764.1	96.3
Methanol	0.9363	926.9	1374.3	238
Aq. (b.p.)	1.9815	815.1	954.8	220.8
Aq. (a.p.)	85.4165	488.7	198.1	348.8

Total flavonoid content: Flavonoids as one of the most diverse and widespread group of natural compounds are probably the most important natural phenolics. The polyphenolic flavonoids have the diphenylpropane ($C_6C_3C_6$) skeleton. The family includes flavanols, flavones, anthocyanidins and flavonols[24]. The total flavonoid content was determined as quercitin equivalent (mg/100 g). *n*-Hexane fraction showed the highest value (3018 mg/100 g) while aqueous fraction after partition showed the lowest values. This is supported by earlier study[25]. The methanolic extract and butanolic extract also gave considerable values (Table-1) which shows that both of these are effective solvents for most phenolics and flavonoids. Flavonoids such as phenolic acids have repeatedly been implicated as natural antioxidants in fruits, vegetables and other plants. For example, caffeic acid, ferulic acid and vanillic acid are widely distributed in the plant kingdom[26].

$ABTS^+$ decolorization assay: This method is based on reduction of ABTS [2,2'-azinobis (3-ethylbenzothiazoline-6-sulphonic acid)] radical. The $ABTS^+$ radical which shows maximum absorbance at 734 nm and reacts with free radical scavengers present in the given plant extract which results in the decrease in the absorbance. The extent of inhibition of the absorbance of $ABTS^+$ radical is plotted as a function of concentration and compared with standard Trolox curve in order to calculate TEAC (trolox equivalent antioxidant capacity) values. The TEAC values of various extracts of the leaves of *O. limbata* are given in Fig. 1. *n*-Butanol fraction showed the highest TEAC value (139.31 µmol/g) while *n*-hexane extract showed minimum (30 µmol/g); ethyl acetate, aqueous before partition and aqueous after partition

fractions also showed considerable TEAC values (54.92, 59.69 and 78.33 µmol/g, respectively). In general, extracts with high phenolic content showed high radical scavenging and antioxidant activity and in present study an important relation was found among them. *n*-Butanol fraction showed maximum results in both $ABTS^+$ decolourization assay and total phenolic content. Small and high molecular mass phenolics, including flavonoids, phenolic acids and tannins have been shown to be good quencher of free radicals[27,28].

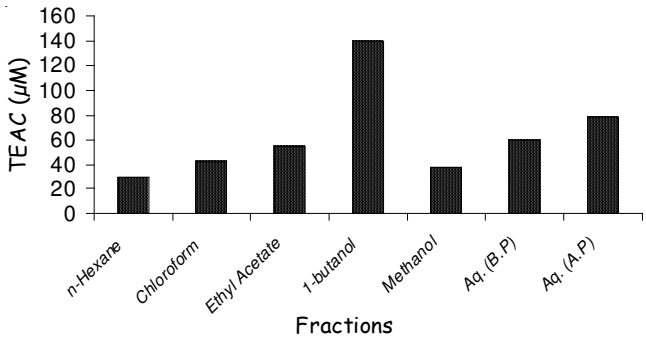

Fig. 1. Comparison of TEAC values of different fractions of *Otostegia limbata* using $ABTS^+$ decolourization assay

2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity: The free radical scavenging activity of various extracts was measured using DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) is stable at room temperature and accepts an electron or hydrogen free radical to form a stable diamagnetic molecule[29] . This ability of DPPH to undergo reduction by an antioxidant is measured in terms of decrease in its absorbance at 517 nm as a function of time[30]. As a DPPH radical reacts with a suitable reducing agent, the odd electron becomes paired and the solution loses colour stoichiometrically depending on the number of DPPH radicals undergone reduction[31]. The colour of DPPH changes during the reaction from purple to yellow. A plot of % DPPH remaining for various extracts *versus* time is shown in Fig. 2. There is a sharp fall in the absorbance of DPPH (a decrease in its concentration) in the first 5 min after addition of extracts. Then the change becomes gradual during the next 0.5 h. The ethyl acetate and *n*-butanol extracts showed very strong DPPH scavenging activity. Chloroform fraction also showed good result. These three extracts showed better free radical scavenging effect than the standard BHA. The results of DPPH method have also been expressed as EC_{50} values. The EC_{50} values were calculated by the linear regression of plots of the per cent of antiradical activity against the concentration of the tested sample (Table-1). A lower value indicates a higher antioxidant activity. A dose-dependent scavenging activity of DPPH free radical was observed. Ethyl acetate (61 µg) and 1-butanol (96 µg) fractions showed promising values. The results indicate that *O. limbata* extracts have significant free radical scavenging potential.

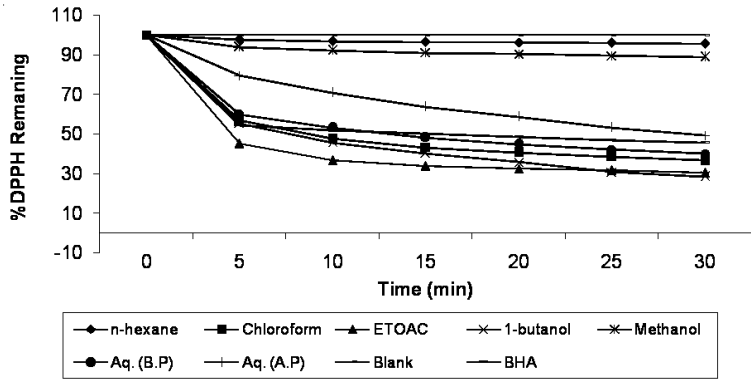

Fig. 2. DPPH free radical scavenging activity of different fractions of *Otostegia limbata*. Data are mean ± SD (n = 3)

FRAP assay: The FRAP (ferric reducing antioxidant power) method was developed to measure the ferric reducing ability of plasma at low pH[32]. It involves a single electron transfer between Fe^{3+}-TPTZ and an electron donating species. When Fe^{3+} is reduced to Fe^{2+}, an intense blue colour is produced. In present studies, the FRAP values varied over a wide range between 4.843 mmol/g for chloroform to 41.269 mmol/g for 1-butanol extracts (Fig. 3). Methanolic and aqueous extracts before partition fraction also showed good results. Extracts in polar solvents, thus, showed better activity than those of less or non-polar solvents, the trend which has been supported by other studies[24,32]. There is evidence that the antioxidant activity of many compounds of botanical origin is proportional to the TPC, suggesting a causative relationship between TPC and antioxidant activity[33]. Present study confirmed the observation and showed excellent correlation between FRAP and TPC values of different extracts of *O. limbata*.

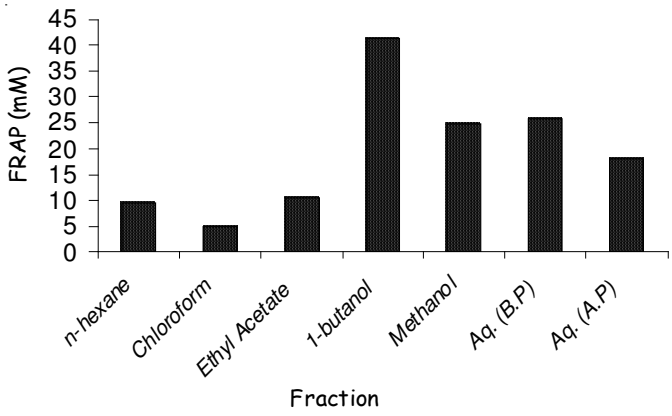

Fig. 3. Comparison of FRAP values of different fractions of *Otostegia limbata*

Lipid peroxidation value in linoleic acid emulsion system: Linoleic acid is allowed to undergo oxidation to produce peroxyl radicals which are scavenged by the antioxidants present in the sample. The unscavenged peroxyl radicals are allowed to oxidize Fe^{2+}-Fe^{3+}, which are estimated spectrophotometrically as a coloured complex with SCN^- ion. A low absorbance value shows high concentrations of antioxidant components in a given simple. All the extracts of *O. limbata* showed inhibition to peroxidation of linoleic acid. However, ethyl acetate and *n*-butanol extracts exhibited antioxidant potential comparable with BHA and Trolox (Fig. 4) and both of these extracts were also found to have high phenolic contents. Polyphenolic compounds have an important role in stabilizing lipid oxidation and are associated with anti-oxidant activity[34]. *Otostegia limbata* extracts, thus, can be taken as an alternative source of natural antioxidants.

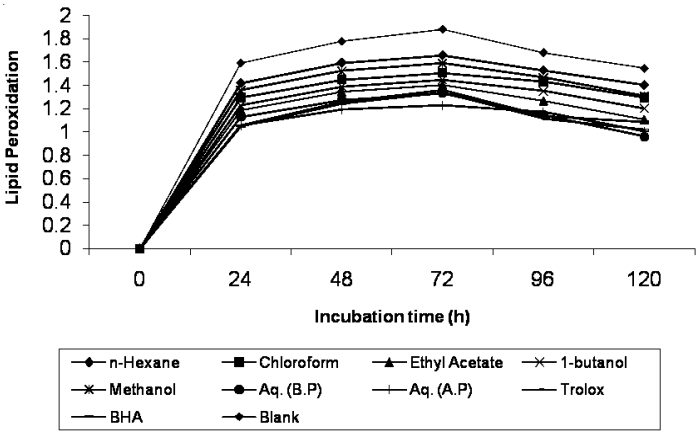

Fig. 4. Antioxidant activity in terms of lipid/oxidation values for different fractions of *Otostegia limbata*. Data are mean + SD (n = 3)

ACKNOWLEDGEMENT

The authors wish to thank Dr. Islamullah Khan, Department of Chemistry, Government College University, Lahore, for his cooperation to carry some work in his laboratory.

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(*Received*: 5 September 2009; *Accepted*: 15 February 2010) AJC-8439