

Assessment of Relief to Oxidative Stress by different Fractions of Mazus

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Abstract:

The purpose of this study was to evaluate the antioxidant potential of *Mazus pumilus* (Burm. f.) Steenis. Methanolic extract of the whole powdered plant was obtained on soxhlet apparatus, dissolved in distilled water and partitioned with *n*-hexane, chloroform, ethyl acetate and *n*-butanol sequentially. The antioxidant potential of all these fractions and remaining aqueous fraction was evaluated by four methods: 1,1-Diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity, total antioxidant activity, Ferric reducing antioxidant power (FRAP) assay and ferric thiocyanate assay along with determination of their total phenolics. The results revealed that *n*-butanol fraction exhibited highest percent inhibition of DPPH radical as compared to other fractions. It showed 80.42±1.68% inhibition of DPPH radical at a concentration of 60 µg/ml. The IC_{50} value of this fraction was 28.79±1.35 µg/ml, relative to ascorbic acid, having IC_{50} 58.84± 0.89 µg/mL. It also showed highest total antioxidant activity (1.01±0.08) as well as highest FRAP value (626.83±0.39 TE µM/mL), highest total phenolic contents (88.30±1.09 GAE mg/g) and highest value of inhibition of lipid peroxidation (53.91±1.21%) as compared to the other studied fractions. Ethyl acetate soluble fraction also showed good results.

Keywords: *Mazus pumilus*, Phytochemical screening, DPPH assay, total antioxidant activity, FRAP value, total phenolics, inhibition of lipid peroxidation.

INTRODUCTION

In response to the increased popularity and greater demand for medicinal plants, a number of conservation groups are recommending that wild medicinal plants be brought into cultivation. A large number of medicinal plants and their purified constituents have shown beneficial therapeutic potentials. Various herbs and spices have been reported to exhibit antioxidant activity. The majority of the antioxidant activity is due to the flavones, isoflavones, flavonoids, anthocyanin, coumarin, lignans, catechins and isocatechins.¹ Antioxidant-based drug formulations are used for the prevention and treatment of complex diseases like atherosclerosis, stroke, diabetes, Alzheimer's disease and cancer.² In living systems, free radicals are generated as part of the body's normal metabolic process, and the free radical chain reactions are usually produced in the mitochondrial respiratory chain, liver mixed function oxidases, through xanthene oxidase activity, atmospheric pollutants and from transitional metal catalysts, drugs and

xenobiotics. In addition, chemical mobilization of fat stores under various conditions such as lactation, exercise, fever, infection and even fasting, can result in increased radical activity and damage. Free radicals or oxidative injury now appears the fundamental mechanism underlying a number of human neurologic and other disorders. Oxygen free radical can initiate peroxidation of lipids, which in turn stimulates glycation of protein, inactivation of enzymes and alteration in the structure and function of collagen basement and other membranes, and play a role in long term complication of diabetes.³ Number of synthetic antioxidants such as BHA, BHT and TBHQ, have been added to foodstuffs. Although these synthetic antioxidants are efficient and relatively cheap, there are some disadvantages, because they are suspected of having some toxic properties. Therefore search for natural antioxidants has received much attention and efforts have been made to identify natural compounds that can act as suitable antioxidants to replace synthetic ones.⁴

Currently, there is growing interest towards natural antioxidants of herbal resources. Epidemiological and *in vitro* studies on medicinal plants and vegetables strongly supported this idea that plant constituents with antioxidant activity are capable of exerting protective effects against oxidative stress in biological systems.⁵ *Mazus pumilus* (Burm. f.) Steenis belongs to the family Scrophulariaceae, is an important medicinal plant.⁶ Its flowering period is from May to October. It is an annual herb having height upto 15 cm. It grows in wet grassland, along streams, trailsides, waste fields, wet places and the edges of forests. The plant is aperient, emmenagogue, febrifuge and tonic. The juice of the plant is used in the treatment of typhoid.⁷ According to our knowledge, no work has been done on the comparative antioxidant potential of various fractions of *Mazus pumilus* so, it was considered worthwhile to study the antioxidant potential of different fractions of *Mazus pumilus* whole plant for future investigations towards the finding of new, potent and safe antioxidant compounds.

EXPERIMENTAL

Plant Material

The plant *Mazus pumilus* (Burm. f.) Steenis was collected from Jallo Park, Lahore, Pakistan in November 2011, and identified by Mr. Muhammad Ajaib (Taxonomist), Department of Botany, GC University, Lahore.

Extraction and Fractionation of Antioxidants

The shade-dried ground whole plant (0.4 kg) was exhaustively extracted with methanol (20L × 4) on the soxhlet apparatus. The extract was evaporated to yield the residue (94 g), which was dissolved in distilled water (1 L) and partitioned with *n*-hexane (1 L × 4), chloroform (1 L × 4), ethyl acetate (1 L × 4) and *n*-butanol (1 L × 4) respectively. These organic fractions and remaining water fraction was concentrated separately on rotary evaporator. The residues thus obtained were used to evaluate their *in vitro* antioxidant potential.

Chemicals and Standards

DPPH[•] (1,1-Diphenyl-2-picrylhydrazyl radical), TPTZ (2,4,6-Tripyridyl-s-triazine), Trolox, Gallic acid, Follin Ciocalteu reagent and BHT (Butylated hydroxytoluene) were obtained from Sigma Chemical Company Ltd. (USA) and organic solvents (*n*-hexane, chloroform, ethyl acetate, *n*-butanol), sulphuric acid, sodium phosphate, ammonium molybdate, linoleic acid, tween-20, ferric chloride and ferrous chloride from Merck (Pvt.) Ltd. (Germany).

DPPH Radical Scavenging Activity

The DPPH radical scavenging activities of various fractions of plant were examined by comparison with that of known antioxidant, butylated hydroxytoluene (BHT) using the method of Lee and Shibamoto.⁸ Briefly, various amounts of the samples (1000 µg/mL, 500 µg/mL, 250 µg/mL, 125 µg/mL, 60 µg/mL, 30 µg/mL, 15 µg/mL, 8 µg/mL) were mixed with 3 ml of methanolic solution of DPPH (0.1mM). The mixture was shaken vigorously and allowed to stand at room temperature for one an hour. Then absorbance was measured at 517 nm against methanol as a blank in the spectrophotometer. Lower absorbance of spectrophotometer indicated higher free radical scavenging activity.

The percent of DPPH decoloration of the samples was calculated according to the formula:
$$\text{Antiradical activity} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100$$

Each sample was assayed in triplicate and mean values were calculated.

Total Antioxidant Activity

The total antioxidant activities of various fractions of plant were evaluated by phosphomolybdenum complex formation method.⁹ Briefly, 500 µg/mL of each sample was mixed with 4 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) in sample vials. The blank solution contained 4 mL of reagent solution. The vials were capped and incubated in water bath at 95°C for 90 minutes. After the samples had been cooled to room temperature, the absorbance of mixture was measured at 695 nm against blank. The antioxidant activity was expressed relative to that of butylated hydroxytoluene (BHT). All determinations were assayed in triplicate and mean values were calculated.

Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP assay was done according to Benzie and Strain¹⁰ with some modifications. The stock solutions included 300 mM acetate buffer (3.1 g CH₃COONa.3H₂O and 16 mL CH₃COOH), pH 3.6, 10 mM TPTZ (2, 4, 6-Tripyridyl-s-triazine) solution in 40 mM HCl, and 20 mM FeCl₃.6H₂O solution. The fresh working solution was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ solution and 2.5 mL FeCl₃.6H₂O solution and then warmed at 37°C before using. The solutions of plant samples and that of trolox were formed in methanol (500 µg/mL). 10 µL of each of sample solution and BHT solution were taken in separate test tubes and 2990 µL of FRAP solution was added in each to make total volume up to 3 mL. The plant samples were allowed to react with FRAP solution in the dark for 30 minutes. Readings of the coloured product [ferrous tripyridyltriazine complex] were then taken at 593 nm. The FRAP values were determined as micromoles of trolox equivalents per mL of sample by computing with standard calibration curve constructed for different concentrations of trolox. Results were expressed in TE µM/mL.

Total Phenolic Contents

Total phenolics of various fractions of plant were determined by the method of Makkar et al.¹¹ The 0.1 mL (0.5 mg/mL) of sample was combined with 2.8 mL of 10% Na₂CO₃ and 0.1 mL of 2N Folin-Ciocalteu reagent. After 40 minutes absorbance at 725 nm was measured by UV-visible spectrophotometer. Total phenolics were determined as milligrams of gallic acid equivalents per gram of sample by computing with standard calibration curve constructed for different concentrations of gallic acid. Results were expressed in GAE mg/g.

Ferric Thiocyanate (FTC) Assay

The antioxidant activities of various fractions of plant on inhibition of linoleic acid peroxidation were assayed by thiocyanate method.¹² The 0.1 mL of each of sample solution (0.5 mg/ mL) was mixed with 2.5 mL of linoleic acid emulsion (0.02 M, pH 7.0) and 2.0 mL of phosphate buffer (0.02 M, pH 7.0). The linoleic emulsion was prepared by mixing 0.28 g of linoleic acid, 0.28 g of Tween-20 as emulsifier and 50.0 mL of phosphate buffer. The reaction mixture was incubated for 5 days at 40°C. The mixture without extract was used as control. The 0.1 mL of the mixture was taken and mixed with 5.0 mL of 75% ethanol, 0.1 mL of 30% ammonium thiocyanate and 0.1 mL of 20 mM ferrous chloride in 3.5% HCl and allowed to stand at room temperature. Precisely 3 min. after addition of ferrous chloride to the reaction mixture, absorbance was recorded at 500 nm. The antioxidant activity was expressed as percentage inhibition of peroxidation (IP%) [IP% = {1-(abs. of sample) / (abs. of control)} × 100]. The antioxidant activity of BHT was assayed for comparison as reference standard.

Statistical Analysis

All the measurements were done in triplicate and statistical analysis was performed by Statistical software. All the data were expressed as ± S.E.M. Statistical analysis were determined using one way analysis of variance (ANNOVA) followed by post-hoc Tukey's test.

RESULTS AND DISCUSSION

DPPH Radical Scavenging Activity

DPPH radical scavenging activity is widely used to evaluate antioxidant activities in a relatively short time. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The effects of phenolic compounds on DPPH radical

scavenging are thought to be due to their hydrogen donating ability¹³. It is reported that the decrease in the absorbance of DPPH radical caused by phenolic compound is due to the reaction between antioxidant molecules and radicals, resulting in the scavenging of the radical by hydrogen donation and is visualized as a discoloration from purple to yellow¹⁴. DPPH is a preformed stable radical used to measure radical scavenging activity of antioxidant samples. This method is based on the reaction of DPPH radical that is characterized as a stable free radical with deep violet colour and any substance that can donate hydrogen atom to DPPH thus reduces it to become stable diamagnetic molecule⁸. Reduction of DPPH radical was observed by the decrease in absorbance at 517 nm where as colour changes from purple to yellow. The various fractions of *Mazus pumilus* significantly reduced DPPH radicals. It was observed from the results (table 1) that activity was increased by increasing the concentration of the fractions in the assay. The various concentrations of *n*-butanol soluble fraction exhibited highest percent inhibition of DPPH radical as compared to other fractions. It showed 80.42 ± 1.6 % inhibition of DPPH radical at a concentration of 60 $\mu\text{g/ml}$. The various concentrations of the fractions which showed percent inhibition greater than 50% were found to be significant ($p < 0.05$) when compared with negative control i.e. blank. IC_{50} value is defined as the concentration of substrate that causes 50% loss of the DPPH activity and was calculated by linear regression mentioned of plots of the percentage of antiradical activity against the concentration of the tested compounds. IC_{50} is a measure of the effectiveness of a compound in inhibiting biological or biochemical function. This quantitative measure indicates how much of a particular drug or other substance (inhibitor) is needed to inhibit a given biological process. A lower value would reflect greater antioxidant activity of the fraction.¹⁵ The IC_{50} values of the studied fractions were calculated and the results have been shown in table 2. *n*-Butanol soluble fraction exhibited lowest IC_{50} value i.e. $28.79 \pm 1.35 \mu\text{g/ml}$ as compared to other studied fractions, relative to ascorbic acid, a standard reference antioxidant, having IC_{50} value 58.84 ± 0.89 . Ethyl acetate fraction also showed good IC_{50} value ($64.76 \pm 0.98 \mu\text{g/ml}$), while chloroform soluble fraction showed moderate value ($272.35 \pm 1.16 \mu\text{g/ml}$). Very poor IC_{50} values were found for *n*-hexane soluble fraction and remaining aqueous fraction. The results for chloroform soluble fraction, ethyl acetate soluble fraction and *n*-butanol soluble fraction were found to be significant ($p < 0.05$) while that of *n*-hexane soluble fraction and remaining aqueous fraction were found to be non significant ($p > 0.05$) when compared with ascorbic acid, a reference standard.

Total Antioxidant Activity

The total antioxidant activity of the studied fractions was measured spectrophotometrically by phosphomolybdenum method, which is based on the reduction of Mo (VI) to Mo (V) by the sample analyte and the subsequent formation of green phosphate / Mo (V) compounds with a maximum absorption at 695 nm. The reaction takes place at acidic pH. The phosphomolybdenum method usually detects antioxidants such as ascorbic acid, some phenolics, tocopherols and carotenoids⁹. From results (table 2), it was observed that *n*-butanol soluble fraction showed highest total antioxidant activity i.e. 1.01 ± 0.08 as compared to other fractions. Ethyl acetate fraction also showed good total antioxidant activity (0.92 ± 0.04). Chloroform soluble fraction showed moderate activity (0.53 ± 0.04) while *n*-hexane soluble fraction (0.25 ± 0.02) and remaining aqueous fraction (0.38 ± 0.05) didn't show good activity. The results were compared with butylated hydroxytoluene (BHT), a reference standard whose total antioxidant activity was found to be 1.15 ± 0.07 . The results for chloroform soluble fraction, ethyl acetate soluble fraction and *n*-butanol soluble fraction were found to be significant ($p < 0.05$) while that of *n*-hexane soluble fraction and remaining aqueous fraction were found to be non significant ($p > 0.05$) when compared with BHT.

Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP assay measures the reducing ability of antioxidants against oxidative effects of reactive oxygen species. Electron donating anti-oxidants can be described as reductants and inactivation of oxidants by reductants can be described as redox reactions. This assay is based on the ability of antioxidants to reduce Fe^{3+} to Fe^{2+} in the presence of tripyridyltriazine [TPTZ] forming an intense blue Fe^{2+} -TPTZ complex with an absorbance maximum at 593 nm.¹⁰ Increasing absorbance indicates an increase in reductive ability. The FRAP values of the studied fractions were calculated and results have been shown in table 2. Among all the fractions the *n*-butanol fraction showed highest FRAP value (626.83 ± 0.39 TE $\mu\text{M}/\text{mL}$). FRAP values exhibited by chloroform soluble fraction and ethyl acetate soluble fraction were 116.76 ± 0.34 TE $\mu\text{M}/\text{mL}$ and 225.58 ± 1.30 TE $\mu\text{M}/\text{mL}$ respectively while that of aqueous fraction and *n*-hexane fraction were found to be poor. High FRAP values obtained for more polar fractions may be ascribed partially to the presence of phenolic and flavonoid contents. The FRAP values of chloroform soluble fraction, ethyl acetate soluble fraction and *n*-butanol soluble fraction were found to be

significant ($p < 0.05$) while that of *n*-hexane soluble fraction and aqueous fraction were found to be non significant ($p > 0.05$) when compared with blank.

Total Phenolic Contents

Phenolic compounds and flavonoids have been reported to be associated with antioxidative action in biological systems, acting as scavengers of singlet oxygen and free radicals.¹⁶ In this assay Folin-Ciocalteu reagent, a mixture of phosphotungstic ($H_3PW_{12}O_{40}$) and phosphomolybdic ($H_3PMo_{12}O_{40}$) acids, is reduced to blue oxides of tungstene (W_8O_{23}) and molybdene (Mo_8O_{23}) during phenol oxidation. This reaction occurs under alkaline condition provided by sodium carbonate. The intensity of blue colour reflects the quantity of phenolic compounds, which can be measured using spectrophotometer.¹⁷ Table 2 shows the phenolic concentration in the different fractions, expressed as milligram of gallic acid equivalents (GAEs) per gram of fraction. Among these five fractions *n*-butanol soluble fraction possessed the highest amount of total phenolics compounds i.e. 88.30 ± 1.09 GAE mg/g) followed by the ethyl acetate soluble fraction (66.76 ± 0.98 GAE mg/g), chloroform soluble fraction (38.50 ± 1.35 GAE mg/g), remaining aqueous fraction (7.81 ± 1.10 GAE mg/g), while *n*-hexane soluble fraction exhibited the lowest total phenolic content (4.42 ± 1.55 GAE mg/g) respectively. The values of total phenolic contents of chloroform soluble fraction, ethyl acetate soluble fraction and *n*-butanol soluble fraction were found to be significant ($p < 0.05$) while that of *n*-hexane soluble fraction and aqueous fraction were found to be non significant ($p > 0.05$) when compared with blank.

Ferric Thiocyanate (FTC) Assay

Membrane lipids are rich in unsaturated fatty acids that are most susceptible to oxidative processes. Especially, linoleic and arachidonic acid are targets of lipid peroxidation. The inhibition of lipid peroxidation by antioxidants may be due to their free radical-scavenging activities.¹⁸ The FTC assay measures the amount of peroxide value in the beginning of the lipid peroxidation, where ferric ion was formed upon reaction of peroxide with ferrous chloride. The ferric ion will then unite with ammonium thiocyanate producing ferric thiocyanate, a red-

coloured substance. The darker the colour, the higher will be the absorbance.¹⁹ The inhibition of lipid peroxidation was checked for all the fractions and results have been shown in table 2. The results showed that *n*-butanol soluble fraction showed highest percent inhibition of lipid peroxidation i.e. 53.91±1.21%. Ethyl acetate soluble fraction also exhibited good value (46.81±1.03%) while *n*-hexane soluble fraction (5.31±0.38%), chloroform soluble fraction (18.09±0.56%) and remaining aqueous fraction (8.24±0.65%) didn't show good results. The results were compared with BHT having percent inhibition 62.35±1.52 %. The values of lipid peroxidation inhibition shown by ethyl acetate soluble fraction and *n*-butanol soluble fraction were found to be significant ($p < 0.05$) while that of *n*-hexane soluble fraction, chloroform soluble fraction and remaining aqueous fraction were found to be non significant ($p > 0.05$) when compared with BHT.

Conclusion

It was observed from the results that ethyl acetate soluble fraction and *n*-butanol soluble fraction showed good antioxidant activity. Chloroform soluble fraction showed moderate activity while *n*-hexane soluble fraction and remaining aqueous fraction showed no activity. *n*-Butanol fraction exhibited highest percent inhibition of DPPH radical as compared to other fractions. It showed 80.42±1.68% inhibition of DPPH radical at a concentration of 60 µg/ml. The IC_{50} of this fraction was 28.79±1.35 µg/ml, relative to ascorbic acid, having IC_{50} 58.84± 0.89 µg/mL. It also showed highest total antioxidant activity (1.01±0.08) as well as highest FRAP value (626.83±0.39 TE µM/mL), highest total phenolic contents (88.30±1.09 GAE mg/g) and highest value of inhibition of lipid peroxidation (53.91±1.21%) as compared to the other studied fractions. Ethyl acetate soluble fraction also showed good results. So it was concluded from the present study that ethyl acetate fraction and *n*-butanol fraction are rich in strong antioxidants, so these fractions are potentially valuable sources of natural antioxidants and bioactive materials and further phytochemical investigations on this plant may bring new natural antioxidants into the food industry that might provide good protection against the oxidative damage which occurs both in the body and our daily foods.

Table 1: DPPH radical scavenging activity of *Mazus pumilus* (Burm. f.) Steenis.

Sr.No.	Sample	Conc.($\mu\text{g} / \text{mL}$)	% Scavening of DPPH radical \pm S.E.M ^{a)}
1	<i>n</i> -Hexane soluble fraction	1000	55.07 \pm 1.31*
		500	34.16 \pm 1.53
		250	18.80 \pm 1.05
2	Chloroform soluble fraction	500	76.31 \pm 0.34*
		250	48.80 \pm 1.44
		120	31.59 \pm 0.35
3	Ethyl acetate soluble fraction	120	76.23 \pm 1.30*
		60	52.16 \pm 1.27*
		30	33.34 \pm 1.16
		15	21.18 \pm 1.04
4	<i>n</i> -Butanol soluble fraction	60	80.42 \pm 1.68*
		30	57.12 \pm 0.33*
		15	31.84 \pm 0.23
5	Remaining aqueous fraction	1000	53.37 \pm 0.91*
		500	36.43 \pm 1.74
		250	25.47 \pm 0.33
		120	23.29 \pm 1.42
6	Ascorbic acid ^{b)}	125	79.14 \pm 0.93
		60	58.09 \pm 0.86
		30	29.97 \pm 0.55

^{a)} standard mean error of three assays.

^{b)} a reference standard antioxidant

Table 2: IC_{50} , total antioxidant activity, FRAP values, total phenolics and lipid peroxidation of different fractions of *Mazus pumilus*

Sr. No	Sample	IC ₅₀ (µg/mL)	Total antioxidant activity	FRAP value TE (µM/ml)	Total phenolics (GAE mg/g)	Inhibition of Lipid Peroxidation (%)
1	<i>n</i> -Hexane soluble fraction	878.61±1.74	0.25±0.02	22.51±1.27	4.42±1.55	5.31±0.38
2	Chloroform soluble fraction	272.35±1.16**	0.53±0.04**	116.76±0.34*	38.50±1.35*	18.09±0.56
3	Ethyl acetate soluble fraction	64.76±0.98**	0.92±0.04**	225.58±1.30*	66.76±0.98*	46.81±1.03**
4	<i>n</i> -Butanol soluble fraction	28.79±1.35**	1.01±0.08**	626.83±0.39*	88.30±1.09*	53.91±1.21**
5	Remaining aqueous fraction	911.16±1.23	0.38±0.05	42.36±1.01	7.81±1.10	8.24±0.65
6	Ascorbic acid ^{a)}	58.84± 0.89	-	-	-	-
6	BHT ^{a)}	-	0.91±0.07	-	-	62.35±1.52
7	Blank	-	-	15.34	3.57	-

All results are presented as mean ± standard mean error of three assays.

^{a)} Standard antioxidant.

*p < 0.05 when compared with negative control i.e. blank/solvent (p < 0.05 is taken as significant).

**p < 0.05 when compared with reference standards (BHT/Ascorbic acid).

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