Investigation of Phytochemical and Antioxidant Properties of Methanol Extract and Fractions of *Ballota limbata* (Lamiaceae)

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Ballota limbata **(Lamiaceae) has been used for its antispasmodic, antiulcer, diuretic, vermifuge and sedative effects in folk medicine. However, little is known about how does it work to produce these therapeutic actions. Present research investigated phytochemical components and antioxidant properties of methanol extract and different fractions of *Ballota limbata*. In this study, phytochemical investigation was done by performing different chemical tests. Here, antioxidant property of the extract and fractions was investigated by using 1,1-diphenyl-2-picryl hydrazyl radical scavenging activity, total antioxidant activity by the phosphomolybdenum method, linoleic acid peroxidation, ferric thiocyanate analysis and ferric-reducing antioxidant power. Methanol extract and fractions showed presence of numerous chemical principles including alkaloids, cardiac glycosides, tannins and flavonoids. The ethyl acetate fraction exhibited higher scavenging activity compared to the other fractions under investigation. This fraction displayed 84.16±1.02% 1,1-diphenyl-2-picryl hydrazyl radical inhibition at a dose of 60 µg/ml. IC_{50} for 1,1-diphenyl-2-picryl hydrazylradical-scavenging activity was 13.53±0.22 µg/ml, relative to the standard, butylatedhydroxytoluene, having IC_{50} of 12.33±0.88 µg/ml. Thus, *Ballota limbata* showed significant antioxidant activity, which may contribute in the mechanism of above pharmacological actions.**

Key words: Antioxidant, *Ballota limbata*, DPPH, ethyl acetate, phytochemical

Some of the plants are considered good sources of antioxidant substances, as they use these antioxidants for their own protection[1]. Oxidation may lead to free radicals production in biological systems[2-5], which most likely be responsible for a variety of disorders in animal and human species[4,6]. The enzyme catalase acts as a natural antioxidant in humans. Free radicals from environmental and other sources may weaken the immune system and ease the way for different infections to invade human biological system. Hence, antioxidants may be more helpful as free radical scavengers[2-5].

Antioxidants from artificial source are not recommended for therapeutic use, as they may cause severe toxicity. Therefore, various plants are intensively investigated for antioxidant activity[7,8], to overcome the hazards associated with the synthetic compounds. Presence of phenolic compounds in plants may be related to their antioxidant activity[6]. These include flavonoids, which scavenge the free radicals[9]. Present study screens phytochemical constituents of *Ballota limbata (B. limbata)* and further investigates antioxidant activity of its extract and various fractions.

B. limbata was collected from Kotli, Pakistan. The plant was identified at the Sultan Herbarium of Department of Botany, GC University, Lahore, Pakistan. A voucher specimen GC-Herb-Bot.1490 was kept in the herbarium. It was dried under shade for about 15 days. The whole weed was crushed into powder and stored in polythene bags.

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DPPH (1,1-diphenyl-2-picrylhydrazyl), TPTZ (2,4,6-tripyridyl-s-triazine), BHT (butylatedhy droxytoluene), Folin–Ciocalteu phenol reagent, gallic acid and Troloxwere purchased from Sigma-Aldrich Company (St. Louis, MO, USA). Chloroform, ethyl acetate, *n*-butanol, methanol, sulphuric acid, sodium phosphate, ammonium molybdate, ferric chloride all were obtained from Merck Pvt. Ltd.(Germany). All the chemicals used were of analytical grade. For measurements of absorbance, a Shimadzu UV/Vis 1700 spectrophotometer was used.

Five kilograms of dried powdered plant material was soaked in methanol (15 l×3 times) at room temperature for a few weeks for the preparation of methanol extract in order to get alkaloids, terpenoids and other organic constituents extracted from the plant material. The methanol extract was allowed to concentrate using a rotary evaporator to yield a residue of about 400 g. One hundred grams of crude methanol extract was used for evaluating biological activities and the remaining 300 g was subjected to fractionation. Fractionation was performed by dissolving 300 g of crude methanol extract in distilled water (1 l) and partitioned with chloroform (1.5 l×3 times), ethyl acetate (1.5 l×3 times) and pre-saturated *n*-butanol (2 l×3 times), respectively. The fractions were subjected individually to evaporation using rotary evaporator. The extract with methanol and its fractions were used to investigate *in vitro* antioxidant activity.

Standard procedures for qualitative phytochemical screening were undertaken to characterize phytochemical constituents in methanol extract and fractions, i.e., alkaloids, terpenoids, saponins, tannins, sugars, phenols, flavonoids and cardiac glycosides[10,11].

Flavonoids concentration of plant extract was determined using spectrophotometric method[12]. Initially 1 ml of methanol solution was prepared from the extract and the fractions at 1 mg/ml of concentration. Quercetin was employed as standard drug in 1 mg/ml and further dilutions of 10, 20, 40, 80, and 120 µg/ml. All the plant material samples and quercetin dilutions (200 µl each) were mixed with 100 µl aluminium chloride. 1M potassium acetate (100 µl) and 4.6 ml of distilled water. Samples stayed for 45 min at 25°. The absorbance of all samples in triplicate was determined at λ_{max}=415 nm by spectrophotometer.

The radical scavenging activity of crude methanol extract of *B. limbata* and its different fractions were estimated by comparing with butylatedhydroxytoluene (BHT) as standard reference[13]. For the preparation of sample solutions, the extract/fractions (0.02 g) were dissolved in 20ml of the methanol (1000 µg/ml). Samples of different concentrations, such as 500, 250, 120, 60, 30 and 15 µg/ml were mixed with 3 ml methanol solution of DPPH (0.1 mM). After vigorous shaking, solution was kept for 1 h at 25°. Regression equation applied to calculate IC_{50} values.

For investigation of total antioxidant activity of all the plant samples (each in triplicate number), method involving phosphomolybdenum complex formation was employed[14]. Each sample (500 µg/ml) was mixed with 4 ml of reagent solution containing sulphuric acid (0.6 M), ammonium molybdate (4 mM) and sodium phosphate (28 mM), whereas 4 ml of reagent solution used as a blank. Samples were incubated in water bath at 95° for one and a half hour. Samples were cooled down at 20-25°. Absorbance of each testing sample was measured at 695 nm when compared to blank.

Ferric reducing antioxidant power (FRAP) assay of Benzie and Strain was used with a little modification[15]. Stock solutions for above assay were prepared as follows; 300 mM acetate buffer (3.1 g $CH_3COONa.3H_2O$ plus 16 ml CH_3COOH, pH 3.6), 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) solution in 40 mM hydrochloric acid, and 20 mM ferric chloride hexahydrate solution. For an experimental protocol, fresh solution made by using acetate buffer (25 ml), 2.5 ml TPTZ solution and 2.5 ml ferric chloride hexahydrate solution. This solution was heated to 37° before the experimental protocol was followed. The plant extract, fractions and trolox were prepared in methanol (500 µg/ml). 2990 µl of FRAP solution was added to all sample solutions including BHT (10 µl each), thus making a 3 ml of total volume. The sample solutions were kept in dark for half an hour to react properly. The absorbance of the product thus formed was calculated at 593 nm.

Total phenol contents of methanol extract and its fractions were investigated by Folin-Ciocalteu reagent method[16]. In this method, 0.1 ml (0.5 mg/ml) sample solution was mixed with 0.1ml of 2 N Folin-Ciocalteu reagent and 2.8 ml of 10% sodium carbonate. The

mixture kept for 40 min at 20-25° before recording the absorbance at 725 nm.

To determine antioxidant activity of the extract and various fractions of the plant, ferric thiocyanate method was used, which involved inhibition of linoleic acid peroxidation[17]. Each sample solution (0.1 ml; 0.5 mg/ml) was used by mixing 2.5 ml linoleic acid emulsion with 2 ml of phosphate buffer (0.02 M each, pH 7.0). For preparation of linoleic acid emulsion, linoleic acid, Tween-20 (0.28 g each) and 50 ml of phosphate buffer were mixed. Above mixture incubated at 40° for 5 d. Mixture devoid of the extract or fractions was considered as the control. Thereafter, to the mixture (0.1 ml), 75% ethanol (5 ml), 30% ammonium thiocyanate (0.1 ml) and 20 mM ferrous chloride (0.1 ml) were added in 3.5% HCl. The mixture was then kept at 20-25° for an hour. After adding ferrous chloride to the mixture, absorbance was calculated at 500 nm. The antioxidant activity was expressed as a percentage inhibition of lipid peroxidation. Percent inhibition of lipid peroxidation (IP %) = $(1–A_{sample}/A_{control}) \times 100$. Here, BHT was used as a reference standard.

The data were statistically analyzed using SPSS version 20. Student's t-test and one way analysis of variance (ANOVA) followed by post-hoc Tukey's test were employed as appropriate. All the data were expressed as mean±SEM.

Phytochemical investigation carried out using methanol extract and its various fractions (Table 1) showed presence of myriad of chemical constituents including alkaloids, cardiac glycosides, flavonoids, phenols, saponins, carbohydrates, terpenoids and tannins. Among all the chemical compounds tested, alkaloids, flavonoids, phenols, carbohydrates and terpenoids showed strong presence in methanol extract and its various fractions. However, cardiac glycosides in all the tested samples were virtually absent, indicating that *B. limbata* may not have any cardiogenic activity. In addition tannins also showed very weak presence in the methanol extract and its fractions. Compounds containing phenols act as free radical scavengers[18].

The flavonoid contents detected in the extract and fractions equivalent to quercetin[19] (Standard curve equation: $y=0.0029x+0.0129$, $R^2=0.9985$) were between 23.06±0.58 and 221±8.66 (Table 2). The flavonoid contents in ethyl acetate (221±8.66 mg/g) and *n*-butanol (133.78±0.72 mg/g) were higher when compared to the remaining fractions.

At 60 µg/ml concentration, ethyl acetate showed 84.16±1.02% inhibition of DPPH radical (Table 3). Various concentrations of the fractions showed 50% or more inhibition of DPPH when compared to BHT ($p<0.05$). IC_{50} values of all the fractions are shown in Table 4. Our results exhibit that smallerthe IC_{50} value higher the scavenging activity. Ethyl acetate fraction exhibited smaller IC_{50} value (13.53±0.22 µg/ml) when compared to the other fractions. Methanol extract showed comparatively higher IC_{50} value (85.98±0.64 µg/ml) to the ethyl acetate and *n*-butanol (35.84±0.44 µg/ml) fractions; However chloroform (113.4±0.82 µg/ml) and aqueous (129.52±1.20 µg/ml) fractions showed relatively the highest values of all tested samples. BHT a reference standard showed IC_{50} value of 12.33±0.88 µg/ml in our experimental settings. The IC_{50} values of methanol extract, ethyl acetate and *n*-butanol fractions showed significant results ($p<0.05$), while chloroform and aqueous fractions were not significant when compared with BHT.

The results (Table 4) revealed that methanol extract, ethyl acetate and *n*-butanol fractions

TABLE 1: PHYTOCHEMICAL CONSTITUENTS OF METHANOL EXTRACT AND VARIOUS FRACTIONS OF *B. LIMBATA*

Test	Methanol extract	Chloroform fraction	Ethyl acetate fraction	*n*-butanol fraction	Aqueous fraction
Alkaloids	++	+	++	++	+
Cardiac glycosides	-	-	+	-	-
Flavonoids	+++	++	+++	+++	+
Phenolics	++	+	+++	++	-
Saponins	-	-	+	+	-
Carbohydrates	+++	-	++	++	+++
Terpenoids	+++	++	+++	++	+
Tannins	+	-	+	+	-

"+" represents weak presence, "++" moderate presence, "+++" strong presence and "-" represents absence

TABLE 2: FLAVONOID CONTENTS IN THE *B. LIMBATA*

Extract/fraction	Flavonoid content (mg/g)
Methanol extract	66.12±1.05
Chloroform fraction	53.61±0.98
Ethyl acetate fraction	221±8.66
***n*-butanol fraction**	133.8±0.72
Aqueous fraction	23.06±0.58

Flavonoid content values are expressed as mean±SEM of three values

showed maximum total antioxidant activity i.e; 1.16±0.04 µg/ml and 0.88±0.02 µg/ml, respectively as compared to other fractions. Methanol extract also exhibited significant activity i.e; 0.76±0.01 µg/ml. The chloroform and aqueous fraction exhibited very less activity 0.67±0.06 µg/ml and 0.39±0.19 µg/ml, respectively. The results so found were further evaluated by comparing with BHT which has total 1.47±0.04 µg/ml antioxidant activity. The total antioxidant activity showed by the methanol extract, ethyl acetate and *n*-butanol fractions showed significant results ($p<0.05$), while chloroform and aqueous fractions were found to be non-significant when compared with BHT.

TABLE 3: DPPH RADICAL SCAVENGING ACTIVITY *B. LIMBATA*

Sample	Concentration in assay (µg/ml)	% scavenging of DPPH±SEM[a]
Methanol extract	250	69.65±0.58*
	120	44.24±1.14
	60	34.04±1.31
	30	29.12±0.87
	15	25.23±0.88
Chloroform fraction	250	51.69±0.61
	120	38.16±1.83
	60	30.72±0.92
	30	28.62±0.99
	15	25.44±1.20
Ethyl acetate fraction	60	84.16±1.02*
	30	62.75±1.11*
	15	49.60±0.99
n-butanol fraction	250	74.40±0.62*
	120	62.18±1.12*
	60	53.42±0.34
	30	44.48±0.26
	15	40.18±0.60
Aqueous fraction	500	79.12±0.42*
	250	61.40±1.44*
	120	42.29±0.41
BHT[b]	60	90.35±0.12
	30	74.46±0.10
	15	40.57±0.06

[a]All results are expressed as mean±SEM of three values, [b]standard antioxidant, *$P<0.05$

The ethyl acetate fraction exhibited highest FRAP value 334.27±1.22 TE µM/ml. Methanol extract and *n*-butanol fractions have also shown to possess good FRAP values 181.21±0.42 TE µM/ml and 198.62±0.88 TE µM/ml, while chloroform fraction showed moderate value 164.8±0.92 TE µM/ml. Aqueous fraction showed very less FRAP value 88.36±1.24 TE µM/ml. Chloroform and aqueous fractions did not show significant activity against the blank.

The fractions prepared in ethyl acetate and *n*-butanol exhibited the maximum content of the total phenolic compounds i.e; 208.78±2.27 GAE mg/g and 84.22±0.38 GAE mg/g, respectively. Methanol extract showed moderate amount of total phenolic contents i.e; 62.26±0.42 GAE mg/g. The total phenolic contents of fractions obtained through chloroform and aqueous were 56.53±1.34 GAE mg/g and 39.87±0.27 GAE mg/g, respectively. Total phenolic contents of methanol extract, ethyl acetate and *n*-butanol fractions showed significant results ($p<0.05$), while total phenolic contents of aqueous and chloroform fractions were found to be non-significant when compared with blank.

Similarly, it was also observed that ethyl acetate and *n*-butanol fractions exhibited maximum percentage of inhibition of lipid peroxidation i.e; 61.76±0.29% and 53.27±0.57%. Methanol extract, chloroform and aqueous fractions were found to possess the percent inhibition of lipid peroxidation as 45.26±0.36%, 38.47±0.58% and 12.67±0.48%, respectively which were found non-significant. The obtained results were put in comparison to a reference standard i.e; BHT, which showed 63.84±0.63% inhibition of lipid

TABLE 4: IC_{50}, TOTAL ANTIOXIDANT ACTIVITY, FRAP VALUES, TOTAL PHENOLICS AND LIPID PEROXIDATION INHIBITION VALUES OF *B. LIMBATA*

Sample	DPPH-radical scavenging activity IC_{50} (µg/ml)	Total antioxidant activity (abs. at 695 nm)	FRAP value (TEµM/ml)	Total phenolics (GAE mg/g)	Inhibition of lipid peroxidation (%)[c]
Methanol extract	85.98±0.64	0.76±0.01*	181.21±0.42*	62.26±0.42*	45.26±0.36
Chloroform fraction	113.4±0.82	0.67±0.06	164.8±0.92	56.53±1.34	38.47±0.58
Ethyl acetate fraction	13.53±0.22*	1.16±0.04*	334.27±1.22*	208.78±2.27*	61.76±0.29*
n-butanol fraction	35.84±0.44*	0.88±0.02*	198.62±0.88*	84.22±0.38*	53.27±0.57*
Aqueous fraction	129.52±1.20	0.39±0.19	88.36±1.24	39.87±0.27	12.67±0.48
BHT[a]	12.33±0.88	1.47±0.040	-	-	63.84±0.63
Blank[b]	-	-	18.62	16.49	-

All results are expressed as mean±SEM of three values; [a]standard antioxidant, [b]solvent (methanol) taken as blank, [c]Tested concentration at 500 µg/ml, *$P<0.05$ when compared with blank and when compared with BHT, FRAP: Ferric reducing antioxidant power, GAE: galic acid equivalents

peroxidation. The results for percent inhibition of lipid peroxidation of ethyl acetate and *n*-butanol fractions were found to be significant ($p<0.05$) while methanol extract as well as chloroform and aqueous fractions were found to be insignificant when compared with BHT.

It was witnessed that increasing the concentration of the test samples (fractions of *B. limbata*) lead to increase in antioxidant activity. Various concentrations of methanol extract, ethyl acetate and *n*- butanol fractions displayed greatest percent inhibition of DPPH radical in comparison to other fractions. DPPH is *N*-centered stable radical and is considered to be the best for evaluation of antioxidant activity. Reaction of any compound with DPPH indicates its H donating ability. Since stable radical DPPH accepts "e" and H-radical for its conversion into a diamagnetic molecule[20]. It is documented that decrease in DPPH radical absorbance at 517 nm by compounds containing phenols is responsible for the reaction of antioxidant molecules with radicals. Results are indicated by appearance of yellow colour due to the scavenging activity of radicals by donation of hydrogen[21]. Radical scavenging activity of antioxidant sample is measured by DPPH which is a preformed stable free radical with deep violet colour. Visible deep purple colour is due to an odd electron[13]. All plant fractions were subjected to this assay. The significant values of percent scavenging of DPPH radical of different fractions are believed to be due to the phenol contents (Table 3).

The total antioxidant potential was measured by employing phosphomolybdenum complex formation method. In this assay, the conversion of Mo (V) from Mo (VI) by reduction occurred by various fractions of plant which was detected at 695 nm by spectrophotometer[22] Being simple and independent, the assay was successfully used to quantify vitamin E in seeds; and further its applications were extended to plant polyphenols. High antioxidant activity is due to higher absorbance.

FRAP assay was employed to determined the antioxidant activity of samples. The colour of test sample changes from yellow to blue and green in proportionate to the reducing potential of various samples shown. In a redox- linked colorimetric method antioxidants are used as reductants in FRAP assay and in stoichiometric excess it provides an easy reduced oxidant system. Ferric form in a ferric tripyridyltriazine complex changes to ferrous form displaying intense blue colour. This change was observed by measuring the absorption at 593 nm. In the reaction mixture the absorption change was linked directly with the total reducing power of electron donating antioxidants which reduced the ferric form to ferrous form[11]. It is well accepted that phenol and flavonoid contents exhibited important antioxidant value on human well-being and strength. These compounds showed their activity by scavenging or chelating process[6,19]. The high potential of phenols to scavenge free radicals is supposed to be due to many phenolic hydroxyl groups[23]. Table 4 shows the concentration of phenols in the extract and fractions of *B. limbata*. Moreover, phytochemical results of extract and fractions of *B. limbata* except aqueous fraction have shown positive results for phenolic components, which supported the antioxidant activity of the test fractions.

Ferric thiocyanate assay includes the quantification of peroxide value in the start of the lipid peroxidation[24]. Free radical-scavenging activities of the antioxidants determined the inhibition of lipid peroxidation[25]. High antioxidant activity measured by this method was due to low absorbance values[26]. Results of percent inhibition of lipid peroxidation were shown in Table 4.

The results showed that methanol extract, ethyl acetate and *n*-butanol fractions contained more phenols and flavonoids. Good antioxidant activity of these extract/fractions was due to the presence of such compounds. Chloroform fraction showed moderate activity due to fewer amounts of such compounds. Due to the absence of phenols and flavonoids in aqueous fraction, this fraction showed no antioxidant activity. Ethyl acetate fraction exhibited highest concentration of polyphenols which showed maximum percent inhibition of DPPH radical as compared to remaining extract/fractions. Therefore, from this investigation it is concluded that *B. limbata* is rich in strong antioxidants, so its methanol extract, ethyl acetate and *n*-butanol fractions are proved to be potentially valuable sources of natural antioxidants and bioactive materials. Further phytochemical investigations may possibly bring new natural antioxidants in foodstuff that might contribute excellent defence against the oxidative damage occurring in human body system.

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