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Evaluation of Total Phenol, Flavonoid and *in vitro* Antioxidant Activity of Methanolic Extract of Leaves of *Melastoma malabathricum* Linn.

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Plant containing polyphenols and flavonoids has been reported to possess strong antioxidant properties. Antioxidants are important in protective against various diseases like hypertensive, diabetes, cardiovascular disease and cancer. In present study the antioxidant activity of the methanolic extract of the leaves of *Melastoma malabathricum* L. was carried out using standard *in vitro* models. The amount of total phenolic compounds and total flavonoids were also determined by Folin Ciocalteu reagent and aluminium chloride method, respectively. The antioxidant activity was evaluated *in vitro* by using six different assays *viz.*, reducing power assay, DPPH scavenging activity, hydrogen peroxide scavenging activity, metal chelating assay, nitric oxide scavenging activity and super oxide anion scavenging activity. The result of the study reveals that methanolic extract of the leaves of *M. malabathricum* possesses antioxidant activity. The activity was found to be concentration dependent. This study will provide ample opportunities for further investigation to verify these activities *in vivo*.

Key Words: Antioxidant activity, DPPH, Phenol, Flavonoids, Reducing power.

INTRODUCTION

Oxygen free radicals or more commonly, reactive oxygen species (ROS) as well as reactive nitrogen species (RNS) are result of normal cellular metabolism. ROS and RNS are well known for possessing a dual character as both harmful and beneficial species, since they can be either harmful or beneficial to living systems[1]. The harmful consequence of free radicals causing potential biological damage is termed oxidative stress and nitrosative stress. We are protected from oxidative stress induced by ROS by efficient defense system. However, capacity of the defensive system is affected by age, diet, health status of individual[2]. The use of synthetic antioxidant like butylated hydroxy anisole (BHA) or butylated hydroxy toluene (BHT) has negative health effects, so there has been a general effort to replace synthetic food additives with natural alternatives[3,4]. Many antioxidant compounds obtained from plant sources have been identified as free radical or active oxygen scavengers[5]. In recent times, therefore interest has increased significantly in finding naturally occurring antioxidants for use in food or medicinal substances to replace the synthetic antioxidant[6]. Plant constituents like flavonoid and phenolic compounds are broadly distributed and have been reported to exert multiple biological effects including antioxidant, antiinflammatory or anticarcinogenic *etc.*[7].

The aim of the present investigation is to evaluate *in vitro* antioxidant and free radical scavenging activity of the *Melastoma malabathricam* L. leaves extract.

EXPERIMENTAL

Ferrozine and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich. Sodium nitroprusside, potassium ferric cyanide, ferric chloride, sodium carbonate, naphthyl ethylene diamine dihydrochloride (NEDD) and ferrous sulphate were purchased from Rankem fine chemicals Ltd. India. L-ascorbic acid, gallic acid and quercetin were purchased from S.D. fine chemicals Ltd. India. Aluminium chloride, trichloroacetic acid and sulphanilic acid were purchased from Merck Ltd., Mumbai, India. Nitroblue tetrazolium chloride (NBT), phenazine methosulphate (PMS) and nicotinamide adenine dinucleotide (NADH) were purchased from Sisco Research Laboratories, India. All the reagents were of analytical grade and used without further purification.

Plant materials and extraction procedures: *Melastoma malabathricam* L. leaves were collected during January 2008 from Dibrugarh University campus and was authenticated by Botanical Survey of India Eastern circle (BSI) Shillong, India and a voucher specimen No. BSI/EC/2008/Tel.103 has been deposited at the herbarium of the Department of Pharmaceutical Sciences, Dibrugarh University. About 500 g of powdered

leaves of the plant was first defatted with petroleum ether and then extracted with methanol using soxhlet apparatus for 48 h. The extract was concentrated under reduced pressure using rotary evaporator until a semi solid stick mass was obtained. Methanolic extract of the leaves was directly used for the determination of total phenol and flavonoid content.

Evaluation of antioxidant activities

Total phenolic content: The total phenolic content of the leaf extract was determined separately using the method of Macdonald with little modifications[8]. The solution for calibration curve was prepared by mixing methanolic solution of gallic acid (1 mL; 50-0.250 μg mL^{-1}) with 5 mL Folin Ciocalteu reagent (diluted ten fold) and sodium carbonate (4 mL, 0.7 M). The absorbance measured at 765 nm and the calibration curve was prepeared and 1 mL of methanolic plant extract (10 g L^{-1}) was also mixed with the reagents above and after 2 h the absorbance was measured to determine total phenolic content. All determinations were carried out in triplicate. The total content of phenolic compounds in the extract expressed in Gallic acid equivalents (GAE) was calculated by the following formula:

$$T = C \times \frac{V}{M}$$

where, T = total phenolic contents, mg g^{-1} plant extract, in GAE; C = concentration (mg mL^{-1}) of gallic acid obtained from the calibration curve; V = volume of extract (mL); M = weight (g) of methanolic plant extract.

Total flavonoid content: Aluminium chloride colorimetric method[9] was used for flavonoids determination. Quercetin was used to prepare the calibration curve. 10 mg of quercitin was dissolved in 10 mL of methanol and then was diluted to 20, 40, 60, 80 and 100 μg mL^{-1}. The diluted standard solution (0.5 mL) were separately mixed with 1.5 mL of methanol, 10 % aluminium chloride, 0.1 mL of 1 M potassium acetate and 2.8 mL of distilled water. After incubating at room temperature for 0.5 h, the absorbance of the reaction mixture was measured at 415 nm with a Shimadzu UV-1700 (Japan) spectrophotometer. The amount of 10 % aluminium chloride was substituted by the same amount of distilled water. Similarly, 0.5 mL of methanolic extract of the leaves (1 mg/mL) was reacted with aluminium chloride for determination of flavonoids content as described above.

Reducing power assay: The reducing power of leaves extract was determined by the method of Yen and Chen with some modifications[10]. Sample with different concentrations was mixed with 2.5 mL of phosphate buffer (200 mM, pH 6.6) and 2.5 mL of 1 % potassium ferricyanide. The mixture was incubated for 20 min at 50 ºC. After incubation, 2.5 mL of 10 % trichloroacetic acid was added to the mixture, followed by centrifugation at 3000 rpm for 10 min. The upper layer (5 mL) was mixed with 5 mL of distilled water and 1 mL of 0.1 % ferric chloride and the absorbance of the resultant solutions were measured at 700 nm. Ascorbic acid, quercetin and gallic acid were used as reference compounds.

DPPH scavenging activity: The free radical scavenging activity was determined by the DPPH assay described by Blois[11]. In its radical form, maximum absorption of DPPH at 517 nm, but upon reduction by an antioxidant or a radical species its absorption decreases. Briefly, 0.1 mM solution of DPPH in methanol was prepared and 4 mL of this solution was added to 1 mL of sample solution in methanol at different concentrations. After 0.5 h, the absorbance was measured at 517 nm. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity.

Hydrogen peroxide scavenging activity: Hydrogen peroxide scavenging activity was determined by the method described by Jayaprakasha *et al.*[12]. A solution of hydrogen peroxide (20 mM) was prepared in phosphate-buffer saline (PBS at pH 7.4). Different concentrations of the extract and standard compound in methanol (1 mL) were added to 2 mL of hydrogen peroxide solution in PBS. After 10 min the absorbance was measured at 230 nm. All the data presented are average of triplicate analysis.

Metal chelating assay: The metal chelating assay of leaf extract was determined by the method of Singh and Rajini[13] with some modifications. Solutions of 2 mM $FeSO_4$ and 5 mM ferrozine were diluted 20 times. $FeSO_4$ (1 mL) was mixed with different dilutions of extract (1 mL), followed by ferrozine (1 mL). Absorbance was measured at 562 nm after 10 min. The ability of extracts to chelate ferrous ions was calculated as follows:

$$\text{Chelating effect}\,(\%) = \frac{(A_0 - A_1)}{A_0} \times 100$$

where A_0 is the absorbance of control and A_1 is the absorbance in the presence of extract.

Nitric oxide scavenging activity: Nitric oxide scavenging activity of methanolic extract was carried out based on method described by Marcocci with slight modification[14]. The reaction mixture (6 mL) containing sodium nitroprusside (4 mL, 10 mM), phosphate buffer saline (1 mL) and the extracts, the compound and standard solutions (1 mL) were incubated at 25 ºC for 2.5 h. After incubation, 0.5 mL of the reaction mixture was removed and 1 mL of sulphanilic acid reagent (0.33 % in 20 % glacial acetic acid) was mixed and allowed to stand for 5 min for completion of diazotization reaction and then 1 mL of 0.1 % solution of naphthyl ethylene diamine dihydrochloride (NEDD) was added, mixed and allowed to stand for another 0.5 h in diffused light. The absorbance was measured at 540 nm against the corresponding blank solutions.

Superoxide anion scavenging activity: Measurement of superoxide anion scavenging activity of methanolic extract was carried out based on the method described by Nishikimi with slight modification[15]. One mL of nitroblue tetrazolium (NBT) solution (156 μM NBT in 100 mM phosphate buffer, pH 7.4), 1 mL NADH solution (468 μM in 100 mM phosphate buffer, pH 7.4) and 0.1 mL of sample solutions of leaves extract in methanol were mixed. The reaction was initiated by adding 1 mL of a phenazine methosulphate (PMS) solution (60 μM PMS in 100 mM phosphate buffer, pH 7.4) to the mixture. The reaction mixture was incubated at 25 ºC for 5 min and the absorbance at 560 nm was measured against blank samples. Decreased absorbance of the reaction mixture indicated increased super oxide anion scavenging activity.

Statistical analysis: All values are expressed as means ± SD. Statistical analysis was performed by one-way ANOVA.

Differences were considered significant at $p < 0.05$. The values for IC_{50} were estimated graphically by linear regression analysis.

RESULTS AND DISCUSSION

Total phenolic content: The total phenolic content of the *Melastoma malabathricum* L. leaves extract measured by Folin Ciocalteu reagent in terms of gallic acid equivalent (GAE) was 210 ± 2.99 mg g^{-1}. The Folin Ciocalteu method is a rapid and widely used assay to study the total phenolic content but it is known that different phenolic compounds have different responses in the Folin-Ciacaltu method. Phytochemicals especially plant phenolics constitute a major group of compound that act as primary antioxidant[16]. The results show that leaves of the plant have rich source of phenolic compounds which is responsible for antioxidant activity.

Total flavonoid content: Flavonoids are phenolic compounds, which are very effective antioxidant. It is a group of naturally occurring benzo-γ-pyrone derivatives possessing numerous biological properties as well as hepatoprotective, antithrombotic, antiinflammatory and antiviral activity which may be associated to their antioxidant and free radical scavenging capability[17,18]. The total flavonoid content of the *Melastoma malabathricum* L. leaves extract measured by $AlCl_3$ method in terms of quercetin equivalent (QUE) was found to be 25.27 ± 0.219 mg g^{-1}. The results indicate that leave of the plant have a low concentration of flavonoids as compared to phenolic compounds.

Reducing power assay: The measurement of reducing assay was studied for Fe^{3+}-Fe^{2+} transformation with the presence of extract. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity[19]. Table-1 shows the extent of the reduction in terms of absorption value at 700 nm. There was a direct association between antioxidant activity and reducing capacity. Reducing power of extract and standard compound exhibited the following order: gallic acid > quercetin > ascorbic acid > extract. Reducing power will increase accordingly to the increase in absorbance. The results demonstrate that the reducing power of extract is significance in comparison to that of standards.

DPPH scavenging activity: The extract showed maximum hydrogen donating ability in the presence of DPPH free radical at high concentration. As shown in Table-2, the extract showed a strong hydrogen donating capacity and can powerfully scavenge DPPH radical. The extract showed antioxidant activity with IC_{50} value of 21.86 ± 0.625 μg mL^{-1}. However, the known antioxidants such as ascorbic acid and quercitin exhibited IC_{50} values of 13.81 ± 0.549 and 8.02 ± 0.246 μg mL^{-1}, respectively. The loss of the DPPH radical based on the absorbance at 517 nm wavelength can be monitored by decreased optical density[13]. The result obtained in this investigation reveals that the DPPH radical scavenging ability of methanolic extract of leaves may be attributed to the hydrogen donating ability.

Hydrogen peroxide scavenging activity: Hydrogen peroxide scavenging activity of extract is compared with ascorbic acid, quercetin and gallic acid. Result has been summarized in Table-3. The ability of extract to scavenge hydrogen peroxide is compared with standard compounds that are ascorbic acid, quercetin and gallic acid. IC_{50} values of extract, ascorbic acid, quercetin and gallic acid were found to be 314 ± 0.494, 192.61 ± 1.631, 38.34 ± 2.133 and 50.49 ± 0.167 μg mL^{-1}, respectively. These results showed that extract was effective in scavenging hydrogen peroxide. Hydrogen peroxide scavenging activity of extract and standard comp-ounds exhibited the following order: quercetin > gallic acid > ascorbic acid > extract.

TABLE-1
REDUCING POWER ASSAY OF EXTRACT AND STANDARD

Concentration (μg mL^{-1})	Absorbance (mean ± SD)			
	Extract	Ascorbic acid	Quercetin	Gallic acid
20	0.025 ± 0.007	0.183 ± 0.023	0.175 ± 0.011	0.304 ± 0.008
40	0.168 ± 0.024	0.365 ± 0.014	0.457 ± 0.042	0.538 ± 0.017
60	0.233 ± 0.019	0.591 ± 0.021	0.675 ± 0.047	0.750 ± 0.045
80	0.355 ± 0.037	0.775 ± 0.020	0.890 ± 0.079	0.936 ± 0.035
100	0.483 ± 0.019	0.978 ± 0.031	1.060 ± 0.070	1.216 ± 0.066

Where n = 3.

TABLE-3
HYDROGEN PEROXIDE SCAVENGING ACTIVITY OF EXTRACT AND STANDARD

Concentration (μg mL^{-1})	Percentage inhibition (mean ± SD)			
	Extract	Ascorbic acid	Quercetin	Gallic acid
20	*	*	37.36 ± 2.137	31.00 ± 1.805
40	*	*	49.11 ± 2.213	40.82 ± 1.212
60	*	*	66.79 ± 2.100	57.09 ± 1.717
80	*	*	81.22 ± 0.674	68.94 ± 1.623
100	7.07 ± 1.563	36.29 ± 0.826	91.94 ± 0.652	83.95 ± 0.936
200	17.12 ± 1.381	52.55 ± 1.135	*	*
300	43.00 ± 1.406	65.18 ± 1.582	*	*
400	76.23 ± 1.474	77.22 ± 1.812	*	*
500	90.06 ± 0.663	94.92 ± 0.903	*	*
IC_{50} Values in μg mL^{-1} (mean ± SD)	314.64 ± 0.494	192.61 ± 1.631	38.34 ± 2.133	50.49 ± 0.167

Where n = 3, *not determined.

TABLE-2
DPPH SCAVENGING ACTIVITY OF EXTRACT AND STANDARD

Concentration ($\mu g\ mL^{-1}$)	Percentage inhibition (mean ± SD)		
	Extract	Ascorbic acid	Quercetin
5	*	32.28 ± 2.343	38.21 ± 0.204
10	37.46 ± 0.947	40.71 ± 1.120	56.73 ± 0.182
20	47.17 ± 1.439	62.29 ± 0.985	83.60 ± 1.006
30	58.66 ± 0.652	87.62 ± 1.080	90.79 ± 0.333
40	70.39 ± 0.931	92.55 ± 0.415	*
50	80.47 ± 2.130	*	*

Where n = 3, *not determined.

Metal chelating assay: The method of metal chelating activity is based on chelation of Fe^{2+} ions by ferrozine, which quantitatively forms complex with Fe^{2+} ions[20]. In the presence of other chelating agents, the complex formation is disrupted and as a result, intensity of red colour of the complex also decreases. The chelating effect of the test samples on Fe^{2+} ions are shown in Table-4. It was observed that, chelating ability of tested samples increased as a function of concentration. Metal chelating activity is claimed as one of the antioxidant mechanisms, since it reduced the concentration of the catalyzing transition metal in lipid per-oxidation. So the results of present studies suggest that methanolic extract of leaves exhibited significant chelating activity on Fe^{2+} ions.

TABLE-4
METAL CHELATING ACTIVITY OF EXTRACT

Concentration ($\mu g\ mL^{-1}$)	Metal chelating effect % (mean ± SD)
200	42.81 ± 0.570
400	51.30 ± 0.660
600	58.42 ± 0.669
800	64.83 ± 0.213
1000	73.11 ± 2.381

Where n = 3.

Nitric oxide scavenging activity: Nitric oxide (free radical) has an important role in various types of inflammatory and other physiological conditions[21]. 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. In presence of extract, production of nitrite ions was reduced due to scavenging of nitric oxide, which interacts with oxygen to produce nitrite ions. IC_{50} value of extract was found to be 661.20 ± 11.82 $\mu g\ mL^{-1}$; however gallic acid exhibited 71.31 ± 0.546 % inhibition at a concentration of 20 $\mu g\ mL^{-1}$ as show in Table-5, which indicates that the methanolic extract of leaves posses the nitric oxide scavenging activity.

TABLE-5
NITRIC OXIDE SCAVENGING ACTIVITY OF EXTRACT AND STANDARD

Concentration ($\mu g\ mL^{-1}$)	Percentage inhibition (mean ± SD)
200	14.61 ± 3.036
400	36.76 ± 1.099
600	44.50 ± 1.920
800	57.55 ± 1.679
1000	75.05 ± 1.638
Gallic acid (20 $\mu g\ mL^{-1}$)	71.31 ± 0.546

Where n = 3.

Super oxide anion scavenging activity: Super oxide radical ($O_2^{\cdot -}$) is highly toxic species, which is generated during several biological and photochemical reactions. It can be decomposed to form stronger oxidative species such as singlet oxygen and hydroxyl radicals and it is very injurious to the cellular components in the biological system[22,23]. In the PMS-NADH-NBT system, super oxide anion resulting from the dissolved oxygen by PMS/NADH coupling reaction reduces NBT. The extract inhibited the blue NBT formation. The decrease of absorbance at 560 nm indicates the consumption of super oxide anion in reaction mixture. The extract shows 61.80 ± 1.860 inhibition at 100 $\mu g\ mL^{-1}$ and the standard gallic acid shows 84.10 ± 0.495 inhibition at 20 $\mu g\ mL^{-1}$. The results (Table-6) indicate that the extract has significant antioxidant activity. IC_{50} value of the extract was found to be 70.09 ± 1.850 $\mu g\ mL^{-1}$.

TABLE-6
SUPER OXIDE ANION SCAVENGING ACTIVITY OF EXTRACT AND STANDARD

Extract concentration ($\mu g\ mL^{-1}$)	Percentage inhibition (mean ± SD)
20	26.38 ± 1.145
40	37.13 ± 0.873
60	47.85 ± 1.201
80	54.00 ± 0.771
100	61.80 ± 1.860
Gallic acid (20 $\mu g\ mL^{-1}$)	84.10 ± 0.495

Where n = 3.

Conclusion

The results obtained in the present study reveals that the methanolic extract of the leaves of *Melastoma malabathricum* L. can successfully scavenge various reactive oxygen species/ free radicals under *in vitro* conditions. The presence of phenolics and flavonoids further suggest the possibility of its antioxidant activity. Further purification of the extract is required for the identification of active principles which may be valuable in a number of free radical mediated disease processes.

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