In vitro Antioxidant Activity of *Moringa pterigosperma* (Gaertn) leaves.

Lobo Vijaya*, Phatak Anita, Varghese Jossy, Chandra Naresh.

Department of Botany, Birla College, Kalyan 421304, Maharastra. India.

ABSTRACT

The human body produces reactive oxygen species (ROS) as a result of normal metabolic process. These ROS are capable of oxidizing biomolecules that can damage DNA, cells and contribute to chronic disease. In treatment of these diseases, antioxidant therapy is gained an utmost importance. The important role of dietary antioxidants maintaining the integrity of the living organisms is gaining ever increasing recognition. The oxidative stress induced due to ROS can be attenuated or perhaps reversed by diets containing vegetables that have an ability to scavenge reactive oxygen species. *Moringa pterigosperma* (Gaertn) leaves has been used in Indian cooking and herbal remedies. Its possible mechanism of action was examined in terms of antioxidant availability. The study was designed to evaluate and compare the antioxidant activity, total phenolics, flavonoids content of aqueous (AEMP) and ethanolic extract (EEMP) extracts of leaves. The antioxidant activity was assessed by DPPH (1, 1–diphenyl–1,2–picryl hydrazyl), nitric oxide and superoxide radical scavenging assay, FRAP (Ferric Reducing Antioxidant Power), reducing power and TAC (Total antioxidant capacity). AEMP has shown higher antioxidant activity as compared to EEMP in DPPH radical scavenging assay with IC_{50} values 3649.63±1.81 in AEMP and 3048.78±1.23 in EEMP respectively. Like antioxidant activity the reducing power and FRAP values of AEMP are better as compared to EEMP. The amounts of total phenolic and flavonoid content were also determined. In conclusion, the studies reveal that *Moringa pterigosperma* Gaertn. can scavenge radicals and reduce iron complex may explain the possible mechanism by which it exhibits beneficial effects.

Keywords: *Moringa pterigosperma* , Antioxidant, DPPH, FRAP, *In vitro*.

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***Correspondent author:** E-mail address: vijayalobo@rediffmail.com

INTRODUCTION

Free radicals are fundamental to any biochemical process and represent an essential part of aerobic life and our metabolism. They are continuously produced by the body's normal use of oxygen such as respiration and some cell mediated immune functions. Naturally, there is a dynamic balance between the amount of free radicals generated in the body and antioxidants to quench and/or scavenge them and protect the body against their deleterious effects (1). The ROS inducing superoxide anionic radical ($O_2^{\cdot-}$), hydrogen peroxide (O_2^{-2}) and hydroxyl radicals (·OH) are implemented in oxidative damage to various cellular macromolecules. Increasing number of evidence suggested that oxidative stress induced biochemical changes are crucial etiological factors in several chronic human disease such as diabetes mellitus, cancer, atherosclerosis, arthritis, inflammation and neurodegenerative disease (2). There have been many studies undertaken on how to delay or prevent the onset of these diseases. The most likely and practical way to fight against degenerative diseases is to improve body antioxidant status, which could be achieved by higher consumption of vegetables and fruits. Foods from plant origin usually contain natural antioxidants that can scavenge free radicals. The antioxidants may mediate their effect by directly reacting with ROS, quenching them and / or chelating the catalytic metal ions. Several synthetic antioxidants, e.g. BHA and BHT are commercially available but they are suspected to cause or prompt negative health effects, and also show low solubility and moderate antioxidant activity. Natural antioxidants, especially phenolic and flavonoids are safe and also bioactive. Therefore, in recent years, considerable attention has been directed towards identification of plants with antioxidant ability that may be used for human consumption (3). *Moringa pterigosperma* Gaertn. (Moringaceae) is a small or medium sized tree, about 10m. high, found wild in sub Himalayan tract, from Chenab eastwards to Sarda, and

cultivated all over plains of India. All the parts of the tree are considered medicinal and used in treatment of ascites, rheumatism, venmous bites and as cardiac and circulatory stimulants (4). Leaves are anti inflammatory, anodyne, anthelmintic, ophthalmic and rich in vitamins A and C (5). Reports indicate that pharmacological activites of *Moringa pterigosperma* (Gaertn) leaves includes antitumour, radioprotective, antihypertensive, hypoglycemia, diuretic and hypocholestemia activities (6) and diabetes (7). *Moringa pterigosperma* Gaertn. has also been reported to possess antihepatotoxic, hypothermic and myocardial depressant activity (8). Therefore, the objective of the study was to investigate the phytochemical profile and *in vitro* antioxidant activity of *Moringa pterigosperma* (Gaertn) leaves.

MATERIALS AND METHODS

Chemicals

Trolox (6-hydroxy-2, 5, 7, S-tetramethylchromam-2-carboxylic acid) was purchased from Sigma Chemical Co. Ltd USA.. DPPH (1, 1 – diphenyl – 1, 2 – picryl hydrazyl), TPTZ (2, 4, 6,-tripyridy-s-triazine), potassium ferricyanide, trichloroacetic acid (TCA), $FeCl_3$, sodium nitroprusside, sulphanilamide, napthylethylenediamine dihydrochloride, TPTZ(2, 4, 6,-tripyridy-s-triazine), ascorbic acid, NBT (nitroblue tetrazolium), reduced NADH (nicotinamide adenine dinucleotide), PMS (phenazine methosulfate), sulphuric acid (H_2SO_4), ammonium molybdate, ascorbic acid/standard Vitamin C (Vit. C), quercetin and pyrocatechol was purchased from HiMedia, Mumbai. All other unlabelled chemicals and reagents were of analytical grade and used without further purification.

Plant material

The leaves of *Moringa pterigosperma* (Gaertn) were collected from Birla college campus, Kalyan, Thane district, Maharastra, India. The plant material was taxonomically identified by Blatter Herbarium St Xavier's College, Mumbai. A voucher specimen (No. 4891 of N.A Irani) has been preserved in a laboratory for further reference. The collected plant was dried under shade and powdered with a mechanical grinder and stored in an air tight container. The dried powder material of the leaves was soaked in distilled water and ethanol for 10hrs, to get an aqueous (AEMP) and ethanolic extract (EEMP) after filtration through Whatman paper No. 42.

Preliminary phytochemical screening

Qualitative phytochemical analysis of AEMP and EEMP was carried out as follows: Phenolics: 2ml of filtrate + 2ml $FeCl_3$, blue precipitate indicated presence of phenolics. Saponins (frothing test): 0.5 ml filtrate +5ml distilled water); frothing persistence indicated presence of saponins. Alkaloids: 2ml of filtrate +1%HCl+Dragendroff reagent, orange precipate indicate the presence of alkaloids. Flavonoids; 5ml dilute ammonia was added to a portion of filtrate +concentrated sulphuric acid; yellow colour indicates presence of flavonoids. Steroids (Liebermann-Burchard reaction: 2ml filtrate +2ml acetic anhydride +concentrated sulphuric acid; green color indicates the presence of steroids. Terpenoids: 4ml of filtrate +concentrated sulphuric acid 3ml was added to form a layer; reddish brown colouration interface indicates the presence of terpenoids. Cardiac glycosides (Keller-Kinliani test): 2ml filtrate + 1ml of glacial acetic acid + $FeCl_3$ +concentrated H_2SO_4; brown colour indicates the presence of cardiac glycosides (9).

Total phenolic content

The total phenolic content of different extracts was measured using colorimetric Folin –Ciocalteu method. The reaction mixture consisted 5ml of diluted sample to which 3 ml of distilled water and 0.5 ml Folin –Ciocalteu reagent was added. After 3minutes, add 2ml of 20% Na_2CO_3 solution and place the tubes in boiling water bath for one min, cooled and the absorbance was measured at 760 nm. Standard graph was prepared by using different concentration of pyrocatechol (10).

Total flavonoid content

The flavonoid content of different extracts was measured using a modified colorimetric method. 0.5ml of sample was mixed with 0.5 ml of 2% $AlCl_3$ and incubated for 10mins. and the absorbance was measured at 415 nm. The measurement was compared to a standard graph for quercetin (11).

Antioxidant Activity
Determination of reducing power (Fe^{3+} – Fe^{2+} transformation ability)

The reducing power of a compound serves as significant indicator of its potential antioxidant activity. Increased absorbance of the reaction mixture indicates increased reducing power.

Various conc. of the extracts in 1ml of water were mixed with phosphate buffer (2.5 ml, 0.2 M pH 6.6) and 1% potassium ferricyanide (2.5 ml). The mixture was incubated at 50M°C for 20 min. Aliquots of trichloroacetic acid (2.5 ml, 10%) were added to the mixture, which was then centrifuged al 3000g for 10 min. upper layer of

solution (2.5 ml) was mixed with distilled water (2.5 ml) and freshly prepared $FeCl_3$ solution (0.5ml, 0.1%). The absorbance was measured at 700nm (12).

Super oxide anion scavenging activity

1ml of NBT solution (144µM in 100mM phosphate buffer, pH 7.4), 1ml of reduced NADH (677µM in100mM phosphate buffer, pH 7.4) and 0.5 ml of sample extract was mixed and the reaction was started with adding 100µl of PMS solution (60µM PMS in100mM phosphate buffer, pH 7.4) . The reaction mixture was incubated at 25°C for 5 min, and the absorbance 560 was measured against blank (13).

$$\% \text{ scavenging} = \frac{A_{con} - A_{test}}{A_{con}} X\ 100$$

Nitric oxide radical scavenging activity

Sodium nitroprusside (5mM, 1ml) in phosphate buffer saline (PBS) (0.1 M, 7.4 pH) was mixed with 3 ml of different conc. of the extract and incubated at 25°C for 150 min. 0.5 ml of the samples was mixed with 0.5 ml of Griess reagent (1% sulphanilamide, 2% H_3PO_4 and 0.1% napthylethylenediamine dihydrochloride). Measure the absorbance at 546 nm (14).

$$\% \text{ scavenging} = \frac{A_{con} - A_{test}}{A_{con}} X\ 100$$

DPPH radical scavenging activity

The assay is based on the measurement of the scavenging ability of antioxidant towards the stable radical DPPH. DPPH radical react with suitable reagent, the electrons become paired off and the solution looses color stoichiometrically depending on number of electrons taken up.A volume of 2ml of sample was added to 2ml of phosphate buffer (0.02M, pH 6) and 2ml of 0.2mM DPPH in 95% ethanol. The mixture was shaken and left for 30 min. at R.T. and the absorbance was measured at 517 nm (15).

The capability to scavenge the DPPH radical was calculated using following equation:

$$\% \text{ scavenging} = \frac{A_{con} - A_{test}}{A_{con}} X\ 100$$

FRAP assay

The stock solutions of 10mM TPTZ in 40 mM HCl, 20mM $FeCl_3.6H_2O$ and 0.3 M acetate buffer (pH 3.6) were prepared. The FRAP reagent contained 2.5 ml TPTZ solution, 2.5 ml ferric chloride solution and 25 ml of acetate buffer . It was prepared freshly and warmed at 37°C. 900µl of FRAP reagent was mixed with 90 µl of distilled water and 30µl of sample solution. The reaction mixture was then incubated at 37°C for 30 min and absorbance was recorded at 595 nm. The concentration of $FeSO_4$ was in turn plotted against concentrations of the standard antioxidants (L-ascorbic acid and Trolox) (16).

Total antioxidant capacity

0.1ml of extract was combined in eppendorf tube with 1ml of reagent solution (0.6M sulphuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). The tubes were capped and incubated in thermal block at 95°C for 90 minutes. After cooling to room temperature; the absorbance of the aqueous solution of each was measured at 695 nm against blank (17).

Statistical analysis

Experimental results are expressed as means ± SD. All measurements were replicated three times. The data were analyzed by an analysis of variance i.e. one way ANOVA and student't' test using GraphPad QuickCalcs.. The two-tailed $P < 0.05$ the difference is considered to be statistically significant and if $P < 0.0001$ the difference is considered to be extremely statistically significant The IC_{50} values were calculated from linear regression analysis.

RESULT AND DISCUSSION

Preliminary phytochemical screening

Preliminary phytochemical screening of AEMP and EEMP revealed the presence of various bioactive components like phenolics, saponins, flavonoids, alkaloids, terpenoids, cardiac glycosides. The oil from the seeds of *Moringa oleifera* is applied externally for skin diseases and this is due to the presence of terpenoids, as terpenoids strengthen the skin, increase the concentration of antioxidants in wounds, and restore inflamed tissues by increasing blood supply (18).

Total phenolic and flavonoid content

Total phenolic compounds are reported as pyrocatechol equivalents. The total phenolic contents of phenolics content of AEMP and EEMP were 4.44 ± 0.04 and 3.84 ± 0.03 mg pyrocatechol equivalent/g of sample, respectively. The total flavonoid contents of AEMP and EEMP were 6.30 ± 0.03 and 4.31 ± 0.02 mg quercetin equivalent/g of sample. AEMP had higher total phenol

and flavonoids contents than EEMP. It has been reported that green leafy vegetables, soft fruits and medicinal plants exhibited higher levels of flavonoids (19). 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 (20).

Antioxidant activity

Antioxidant activity of the extracts of varying concentrations ranging form 10- 10000 µg/ml was evaluated by various in vitro models. It was observed that the test compounds scavenged free radicals in concentration dependent manner in all the models.

The result of FRAP and TAC assay were expressed as Trolox Equivalent Antioxidant Capacity (TEAC) and Ascorbic acid Equivalent Antioxidant Capacity as described. TEAC is the concentration of Trolox (µmol/L) required to give the same antioxidant capacity as 1% (w/v) test substance. AEAC is the same for Ascorbic acid (21).

Determination of reducing power (Fe^{3+} - Fe^{2+} transformation ability)

In the reducing power assay, the presence of antioxidants in the samples would result in the reducing of Fe^{3+} to Fe^{2+} by donating an electron. Amount of Fe^{2+} complex can be then be monitored by measuring the formation of Perl's Prussian blue at 700nm (22). Increasing absorbance at 700 nm indicates an increase in reductive ability. Table 1 shows that the reducing powers of all the extracts and standards also increased with the increase of their concentrations. There was a extremely significant difference ($p< 0.001$) among the AEMP and EEMP in reducing power. The activity of AEMP and EEMP was not comparable with Vit. C and trolox ($p< 0.05$) there were significant differences between them.

Table 1 Reducing power of AEMP, EEMP, Vit C and Trolox

conc. (µg/ml)	Absorbance at 700nm			
	AEMP	EEMP	Vit C	Trolox
5	0.07±0.01	0.04±0.01	0.20±0.01	0.11±0.01
10	0.11±0.01	0.08±0.01	0.23±0.02	0.13± 0.01
50	0.15±0.02	0.11±0.01	0.37±0.01	0.16± 0.02
100	0.19±0.01	0.15±0.02	0.48±0.02	0.2±0.001
500	0.21±0.01	0.17±0.01	0.88±0.03	0.58±0.02

Values are means ± S.D. (n=4)

Super oxide anion scavenging activity

Superoxides are produced from molecular oxygen due to oxidative enzymes (23) of body as well as via nonenzymatic reaction such as autoxidation by catecholamines (24). In the present study, superoxide radical reduces NBT to a blue colored formazan that is measured at 560 nm (25). Table 2 shows the superoxide scavenging effect of AEMP in comparison to EEMP on the PMS/NADH-NBT system. The increase of percentage scavenging activity thus indicates the consumption of superoxide anion in the reaction mixture by the plant extracts. Maximum percentage scavenging activity showed by EEMP is 16.17 ± 1.10 at 1000 µg/ml. Whereas AEMP showed 78.57± 3.10 at 10000 µg/ml. AEMP had strong superoxide radical scavenging activity as compared to EEMP which does not show IC_{50} value whereas AEMP gives IC_{50} value at 3875.96± 1.94 µg/ml. There were no significant differences ($p > 0.05$) between AEMP and EEMP. The IC_{50} value of Vit C is 66.31± 3.14, whereas Trolox shows at 2857.14± 1.01 µg/

Table 2 % Radical scavenging activity of AEMP and EEMP in different *In vitro* Assays at different concentrations

conc. (µg/ml)	% scavenging activity					
	Superoxide anion radical		Nitric Oxide radical		DPPH radical	
	AEMP	EEMP	AEMP	EEMP	AEMP	EEMP
10	0.90±0.01	7.17±0.16	3.05±0.71	7.07 ±0.78	10.15±2.14	2.10±0.70
50	7.12±0.71	9.19±1.28	10.11±1.08	18.17±0.91	15.18±1.17	3.18±1.28
100	12.01±1.78	7.17±1.00	28.27±0.91	50.01±2.78	17.18±2.28	20.17±2.17
500	28.17±1.01	13.18±2.16	31.31±2.01	75.04±0.46	20.15±2.18	28.98±0.18
1000	38.09 ±1.91	16.17±2.10	46.15±1.47	75.04±3.01	28.98±2.74	40.18±1.7
5000	57.14±0.21	14.17±1.10	81.07±0.71	75.04±2.01	77.78±1.11	60.61±1.10
10000	78.57±1.10	13.17±2.10	81.11±1.78	75.04±1.01	77.78±1.11	60.61±1.11

Values are means ± S.D. (n=4)

ml. Lower the IC_{50} value of better is the scavenging ability of the sample.

Nitric oxide radical scavenging activity

The extract effectively reduced the generation of nitric oxide from sodium nitroprusside (Table 3). In vitro inhibition of nitric oxide radical is a measure of antioxidant activity of plant drugs. Scavenging of nitric oxide radical is based on the generation of nitric oxide from sodium nitroprusside in buffered saline, which reacts with oxygen to produce nitrite ions that can be measured by using Griess reagent (26). Both AEMP and EEMP decreased the amount of nitrite generated from the decomposition of sodium nitroprusside in vitro which may be due to the presence of antioxidant principles in the extract.The percentage scavenging activity increased with increasing concentration of the extract. Lower the IC_{50} value of better is the scavenging abitity of the sample. There were no significant differences ($p > 0.05$) between AEMP and EEMP. The IC_{50} value of AEMP and EEMP was found to be 2793.29±1.63 µg/ml and 100.51±1.21µg/ml, However, Std Vit. C activity of was very more pronounced than that of our extracts (162±1.32µg/ml.)

DPPH radical scavenging activity

DPPH assay is one of the most widely used methods for screening of antioxidant activity of plant extracts (27). DPPH is a stable, nitrogen-centered free radical which produces violet colour in ethanol solution. It was reduced to a yellow coloured product, diphenyl picryl hydrazine, with the addition of all fractions in a concentration-dependent manner. All the concentraction of AEMP and EEMP demonstrated H-donor activity. Lower the IC_{50} value of better is the scavenging ability of the sample. The IC_{50} values of AEMP and EEMP were 3048.78±1.23µg/ml and 3649.63±1.18µg/ml respectively. These activities are less than that of the Std Vit C and Trolox i.e. 10.40±1.43µg/ml and 17.42±1.21µg/ml respectively (Table 2). There were no significant difference ($p > 0.05$) between AEMP and EEMP.

FRAP assay

In FRAP assay the ability of plant extract to reduce ferric ions was determined. FRAP assay measures the changes in absorbance at 593 nm owing to the formation of blue colored Fe^{+2}- tripyridyltriiazine compound from the colourless oxidized Fe^{+3} form by the action of electron donating antioxidants (28). The FRAP values of AEMP is higher as compared to EEMP (Table 3). Since FRAP assay is easily reproducible and linearly related to molar concentration of the antioxidant present it can be reported that AEMP and EEMP may act as free radical scavenger, capable of transforming reactive free radical species into stable nonradical products.

Table 3 FRAP and TAC value of AEMP and EEMP

1% (w/v) extracts used	FRAP Values (µmol/L)	TAC Values (µmol/L)
AEMP (AEAC)	0.57±0.02	0.59±0.01
EEMP (TEAC)	0.30±0.03	2.86±0.01

Values are mean ± S.D (n=4)

Total antioxidant capacity

Total Antioxidant capacity of AEMP and EEMP is shown in Table 3. The phosphomolybdenum method was based on reduction of MO (VI) to MO (V) by the antioxidant compound and the formation of green phosphate/ MO (V) complex at acidic pH (29). In this assay EEMP was found to have higher activity, AEMP showed lower activity. The extracts demonstrated electron donating capacity and thus they may act as radical chain terminators, transformating reactive free radical species into stable non reactive products (30).

CONCLUSION

The results of present study demonstrate that the radical scavenging of AEMP, indicate that the use of *Moringa pterygosperma* (*Gaertn*) for the treatment of diabetes, hypoglycaemia and hypertension treatment seems quite useful and reasonable. The percentage scavenging activity (with reference to IC_{50} value) in DPPH and super oxide anion radical scavenging assays shows that AEMP has better percentage scavenging activity as compared to EEMP. AEMP thus also give effective reducing power and FRAP values, But the TAC values AEMP are lower as compared to EEMP. The overall better antioxidant and free radical scavenging activities of AEMP might be due to the presence higher amounts of phenolic and flavonoid compounds in aqueous extract. Further studies are in progress in our laboratory to evaluate the in vivo antioxidant potential of this extract in various animal models and phytochemical studies are required to establish the types of compounds responsible for the bioactivity of this medicinal plant.

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