Phytochemical analysis and evaluation of antioxidant activities of hydro-ethanolic extract of\n*Moringa oleifera* Lam. Pods

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ABSTRACT

The aim of present study is to investigate the phytochemical profile and antioxidant activities of pods of *Moringa oleifera* against free radicals using specific *in vitro* standard procedures. The antioxidant activity of the Hydro-ethanolic extract of pods of *Moringa oleifera* was evaluated by free radical scavenging activity using 1,1-diphenyl-2-picryl-hydrazil (DPPH), FRAP assay, reducing power assay, FTC, TBA and Non specific assay. The results of the assay were then compared with a natural antioxidant Quercetin, rutin, BHA and BHT. On the basis of present investigation the extract showed high significance (p<0.001) as compared to standards. The extract of the pods of *Moringa oleifera* is a good source of compounds with antioxidant properties while the extract also exhibited significant free radical scavenging activity, reducing power activity and Total antioxidant activity.

Key words: *Moringa oleifera*; Antioxidants; Flavonols; Tannins; FRAP.

INTRODUCTION

*Moringa oleifera* commonly known as (family: Moringaceae) horse radish tree or drumstick tree is both nutritional and medicinal with some useful minerals, vitamins, amino acids, etc.1 A native of the sub-Himalayan regions of North West India *Moringa oleifera* is indigenous to many countries in Africa, Arabia, South East Asia, the Pacific, Caribbean Islands and South America. Almost all the parts of this plant: root, bark, gum, leaf, fruit (pods), flowers, seed and seed oil have been used for various ailments in the indigenous medicine of South Asia, including the treatment of inflammation and infectious diseases along with cardiovascular, gastrointestinal, hematological and hepatorenal disorders.2

Therefore, in recent years, considerable attention has been directed towards identification of plants with antioxidant ability that may be used for human consumption.3 Plants are potential sources of natural antioxidants. It produces various antioxidant compounds to counteract reactive oxygen species (ROS) in order to survive.4 The most frequently encountered free radicals are the hydroxyl radical (HO·), the superoxide radical (O²·), the nitric oxide radical (NO·) and the lipid peroxyl radical (LOO·) while non-free radical species principally being H₂O₂ and singlet oxygen (O¹).5 Natural antioxidants present in food of plant origin protect against these radicals and are therefore important tools in obtaining and preserving good health.6,7 Strong epidemiological evidence suggests that regular consumption of fruits and vegetables, which are a rich source of antioxidants, can reduce cancer and coronary heart diseases.8-10

The free radicals are the main agents in lipid per oxidation. Antioxidants thus play an important role of protecting the human body against damage by reactive oxygen species.11,12 The antioxidants may mediate their effect by directly reacting with ROS, quenching them and/or chelating the catalytic metal ions.13 Several synthetic antioxidants, e.g., BHA (butylated hydroxyl anisole) and BHT (butylated hydroxytoluene) are commercially available but are quite unsafe and their toxicity is a problem of concern.14 Natural antioxidants, especially phenolic and flavonoids are safe and also bioactive.

Searching for herbs that have therapeutic potential for the prevention and scientifically proven to be useful as an alternative treatment is needed. Thus, the aim of present study is to investigate the phytochemical profile and antioxidant activities of pods of *Moringa oleifera* against free radicals using specific *in vitro* standard procedures so as to assess the medicinal potential of the plant and justify its folklore use.

MATERIALS AND METHODS:

Sample:
The pods of *Moringa oleifera* (Gaertn) were collected from Krishi Vigyan Kendra, Banasthali University, Banasthali, Tonk district, Rajasthan, India. The plant material was taxonomically identified by Botanist of Krishi Vigyan Kendra, Banasthali, Tonk district. The collected pods were shade dried and milled into coarse powder with an electrical grinder and further passed through sieve-mesh 40 and stored in an air tight container at 25°C.

Chemicals and reagents:

DPPH (1,1-diphenyl-2-picryl-hydrazil), TPTZ (2,4,6-tripyridyl-s-triazine), Ferrozine, Deoxyribose were purchased from Sigma Chemical Co. Ltd USA. Trichloroacetic acid (TCA), thiobarbituric acid (TBA), butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), L-Ascorbic acid, ammonium molybdate, quercetin were purchased from HI Media, Mumbai. DMSO (Dimethyl sulfoxide) was purchased from Merck Co. (Germany), Mumbai. All other unlabelled chemicals and reagents were of analytical grade and used without further purification.

Extraction:

Dried powdered material was placed in the Soxhlet thimble with 80% ethanol in 500 ml flat bottom flask. Further refluxed for 18 h. at 80°C for two days. Collected solvent was cooled and poured in a glass plate. The marc was dried in hot air oven (MvTex, India) below 50°C for 48 h and kept in dissector for 2 days. The yield of the extract was 20.5% w/w of powdered plant material for further exploration. Collected dried extract was stored at 5°C in air tight containers.

Preliminary Phytochemical Screening:

Qualitative screening:
The freshly prepared ethanolic extract of plant (MOEE) was qualitatively tested for the presence of chemical constituents. Phytochemical screening of extract was carried out using the following reagents and chemicals according to the methods described.

Quantitative screening:

Determination of total Phenolics

The content of the total phenolic in plant extract is determined by Folin Ciocalteu method spectrometrically. To 1 ml of Folin-Ciocalteu’s reagent, previously diluted (1:20), was added to 1 ml of samples (250 µg/ml) and mixed thoroughly. To the mixture, 4 ml of sodium carbonate (75 g/l) and 10 ml of distilled water were added and mixed well. The mixture was allowed to stand for 2 h at room temperature. Contents were then centrifuged at 2000 g for 5 min and the absorbance of the supernatant was taken at 760 nm. A standard curve was obtained using various concentrations of gallic acid. Results were expressed as percentage of gallic acid equivalents (GAE).

Determination of Total proanthocyanidins

Proanthocyanidins content was determined according to the procedure reported. A volume of 0.5 ml of 0.1 mg/ml of extract solution was mixed with 3 ml of 4% vanillin-methanol solution and 1.5 ml hydrochloric acid; the mixture was allowed to stand for 15 min. The absorbance was measured at 500 nm. Extract samples were evaluated at a final concentration of 10 mg/ml. Total proanthocyanidin content were expressed as rutin equivalents (mg/g).

Determination of Tannins

Tannin content was determined by Vanillin hydrochloride method. Vanillin hydrochloride reagent was prepared by mixing equal volumes of 8% HCl in methanol and 4% vanillin in methanol. A volume of 1.0 ml of 0.1 mg/ml of extract solution was mixed with 5 ml vanillin hydrochloride reagent; the mixture was allowed to stand for 20 min. The absorbance was measured at 500 nm. Extract samples were evaluated at a final concentration of 1 mg/ml. Total tannin content were expressed as rutin equivalents (mg/g) using the following equation based on calibration curve: y = ax + b, where x was the absorbance and y was the rutin equivalent (mg/g).
Evaluation of Antioxidant assays:

The free-radical scavenging activity of MO pod extract was measured by decrease in the absorbance of methanol solution of DPPH. A stock solution of DPPH (33 mg in 1 L) was prepared in methanol, which gave initial absorbance of 0.493, and 5ml of this stock solution was added to 1 ml of MO pod extract solution at different concentrations (100-1000 µg/ml). After 30 min, absorbance was measured at 517 nm and compared with standards (100-1000 µg/ml). Scavenging activity was expressed as the percentage inhibition calculated using the following formula:

\[
\% \text{ Anti-radical activity} = \frac{\text{Control Abs} - \text{Sample Abs}}{\text{Control Abs}} \times 100
\]

Ferric reducing antioxidant power assay (FRAP).

A modified method\(^{19}\) was adopted for the FRAP assay. The stock solutions included 300 mM acetate buffer (3.1 g CH\(_3\)COONa and 16 ml CH\(_3\)COOH), pH 3.6, 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) solution in 40 mM HCl, and 20 mM FeCl\(_3\)-6H\(_2\)O solution. The fresh working solution was prepared by mixing 25 ml acetate buffer, 2.5 ml TPTZ, and 2.5 ml FeCl\(_3\)-6H\(_2\)O. The temperature of the solution was raised to 37°C before using. Plant extract (150µl) were allowed to react with 2850 µl of the FRAP solution for 30 min in the dark condition. Readings of the at colored product (ferrous tripyridyltriazine complex) were at 593 nm. The standard curve was linear between 200 and 1000 µM FeSO\(_4\).

Results are expressed in mM Fe (II)/g dry mass and compared with that of BHT, BHA and ascorbic acid.

Determining the reducing power:

The reducing power assay was determined according to the method\(^{20}\) with little modification. Different concentrations of pod extract (100-1000 µg/ml) in 1ml of methanol were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 ml, 1%). After the mixture was incubated at 50°C for 20 min. Trichloroacetic acid (2.5 ml, 10%) was added to each sample and centrifuged at 317 nm and compared with standards in the volume to 20.0 ml. BHT was used as a positive control while another bottle without sample was used as a negative control. The mixture was incubated at 40 – 45°C for 5 min. A 2.5-ml aliquot of the upper layer was mixed with distilled water (2.5 ml) and ferric chloride (0.5 ml, 0.1%) was added and then the absorbance was measured at 700 nm against a blank which consists of all the reagents without the tested sample and compared with standards. The increased absorbance indicated increased reducing power.

Ferric thiocyanate (FTC) method

The antioxidant potential of ethanolic extract (MOEE) of pods was determined according to the FTC method\(^{21}\) with slightly modification. Four milligrams of each extract samples were dissolved in 4 ml of methanol (99.5 %) and kept in dark bottle (d = 40.0 mm, t = 75.0 mm). Each mixture were mixed with 4 ml linoleic acid (2.5% in ethanol 99.5%), 8.0 ml phosphate buffer (0.02 M, pH 7.0) and 3.9 ml distilled water to make up the volume to 20.0 ml. BHT was used as a positive control while another bottle without sample was used as a negative control. The mixture was incubated at 40 – 45°C. After incubation, 9.7 ml ethanol (75 %) and 0.1 NH\(_4\)SCN (30%, as a colour reagent) was added to 0.1 ml of the mixture. Precisely 3 min after the addition of 0.1 ml of FeCl\(_3\) (0.002 M) in HCl 3.5% to the reaction mixture, the absorbance of the resulting red colour was measured at 500 nm using spectrophotometer (570455, Electronic corporation of India limited) every 24 h until a day after the absorbance of the control reached maximum value (day seven). The percentage inhibition of lipid peroxidation was calculated as follows:

\[
\% \text{ Inhibition} = 100 - \left[ \frac{A_o - A_i}{A_o} \right] \times 100
\]

Where A\(_o\) is the absorbance of the control reaction and A\(_i\) is the absorbance in the presence of the sample extracts\(^{22}\).

Thiobarbituric acid (TBA) test

The TBA test was conducted according to the combined method\(^{23}\). A millimole of sample from the previous FTC method was added with 2 ml of trichloroacetic acid and 2 ml of thiobarbituric acid solution. This mixture was then placed in a boiling water bath at 100°C for 10 min. After cooling, it was centrifuged at 3000 rpm for 20 min and absorbance of the supernatant was then measured at 532 nm using UV-Vis spectrophotometer (570455, Electronic corporation of India limited).

Metal chelating activity assay

The chelating activity of the extract for ferrous ions Fe\(^{2+}\) was measured according to the method of J Sabate (2003). To 0.5 ml of extract, 1.6 ml of deionized water and 0.05 ml of FeCl\(_3\) (2 mM) was added. After 30 s, 0.1 ml ferrozine (5 mM) was added. Ferrozine reacted with the divalent iron to form stable magenta complex species that were very soluble in water. After 10 min at room temperature, the absorbance of the Fe\(^{2+}\)-Ferrozine complex was measured at 562 nm. The chelating activity of the extract for Fe\(^{2+}\) was calculated as:

Chelating rate (%) = \[ \frac{[A_o - A_i]}{A_o} \] \times 100

where A\(_o\) was the absorbance of the control (blank, without extract) and A\(_i\) was the absorbance in the presence of the extract.

Statistical analysis

The experimental results were expressed as mean ± standard deviation (SD) of three replicates. The data were subjected to one way analysis of variance (ANOVA) and differences between samples were determined by Bonferroni’s multiple comparison test using the SPSS 16.0 (Statistical program for Social Sciences) program. Results with p<0.05 were regarded as statistically significant and considered p<0.001 as very significant.

RESULTS AND DISCUSSION:

Preliminary phytochemical screening

Phytochemical screening of the ethanolic extract of Moringa oleifera pods revealed the presence of various bioactive components of which alkaloid, phenolics, flavonoids, flavonol, proanthocyanidins, terpenoids, tannin, and cardiac glycosides are the most prominent. The result of phytochemical test is presented in Table 1. All these phytochemicals possess good antioxidant activities and has been reported to exhibit multiple biological effects including anti-inflammatory and antitumor activities.

Table 1. Qualitative analysis of the phytochemicals of ethanolic extract of Moringa oleifera pods.

<table>
<thead>
<tr>
<th>Phytochemicals</th>
<th>Ethanolic extract(MOEE)</th>
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<tbody>
<tr>
<td>Alkaloids</td>
<td>++</td>
</tr>
<tr>
<td>Saponins</td>
<td>+</td>
</tr>
<tr>
<td>Phytosteroids</td>
<td>+</td>
</tr>
<tr>
<td>Phenols</td>
<td>+++</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>+</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>++</td>
</tr>
<tr>
<td>Tannins</td>
<td>+</td>
</tr>
<tr>
<td>Hederagenin</td>
<td>++</td>
</tr>
<tr>
<td>Cardiac Glycosides</td>
<td>+</td>
</tr>
<tr>
<td>Proanthocyanidins</td>
<td>++</td>
</tr>
</tbody>
</table>

+++ Maximum Presence of the compound, ++ Moderate, + Least Presence; -Absence of the compound.

Total Phenolics, Proanthocyanidins and Tannin content

The observations made in the present investigation strongly suggest that phenolics are important constituent of this plant and some of their pharmacological effects could be attributed to the presence of these valuable constituents. The antioxidant activity of Moringa oleifera is probably due to its phenolic content. It is well known that phenolic compounds are constituents of many plants and they have attracted a great deal of public and scientific interest because of their health promoting effects as antioxidants. Table 2 shows total phenolic content of MOEE, determined in terms of gallic acid equivalents. Total phenolic content (Fig.1) was expressed as gallic acid equivalents (GAE) using the following equation based on the calibration curve: y = 0.151x, R\(^2\) = 0.869.

Table 2: Polyphenol contents of the ethanolic extract of the pods of Moringa oleifera.

<table>
<thead>
<tr>
<th>Polynol</th>
<th>MOEE</th>
</tr>
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<tbody>
<tr>
<td>Total phenolics</td>
<td>0.612 ± 0.244</td>
</tr>
<tr>
<td>Proanthocyanidins</td>
<td>1.893 ± 0.156</td>
</tr>
<tr>
<td>Tannins</td>
<td>0.355 ± 0.011</td>
</tr>
</tbody>
</table>

*Expressed as mg gallic acid/g of dry plant material.

**Expressed as mg rutin/g of dry plant material.

Data are presented as the mean ± SD of each triplicate test. All these values are expressed in terms of rutin equivalents.

Fig. 1. Total Phenolic content of MOEE and Gallic acid at various concentrations. Follow the equation based on the calibration curve: y = 0.151x, R\(^2\) = 0.869, for MOEE, where x was the absorbance and y was the gallic acid equivalent (mg/g). On the basis of the present investigation the extract showed high significance (p<0.001) as compared to standard. Table 2 shows total proanthocyanidin content of MOEE extract which were expressed in terms of rutin equivalents. Proanthocyanidins are a type of bioflavonoid that


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has been shown to have very potent antioxidant activity. There was a good correlation between the proanthocyanidin content and the DPPH assay (= 0.998) of MOEE. Total proanthocyanidin content (Fig. 2.) was expressed as rutin equivalents (mg/g) using the following equation based on the calibration curve: y = 7.181x, R² = 0.993 for MOEE and y = 0.826x, R² = 0.018, for MOAE, where x was the absorbance and y is the rutin equivalent (mg/g).

Total antioxidant activity (FRAP)
The ability of plant extract to reduce ferric ions was determined in FRAP assay. The change in absorbance at 593 nm owing to the formation of blue colored Fe⁺-tripyridyltriazine (TPTZ) compound from the colorless oxidized Fe³⁺ form by the action of electron donating antioxidants. The FRAP values of MOEE was significantly higher as compared to the standard i.e. quercetin and BHT (1mg/ml). The respective values were 147µmol/g, 43µmol/g, 333µmol/g as mentioned in table 3. Since FRAP assay is easily reproducible and linearly related to molar concentration of the antioxidant present it can be reported that MOEE may act as free radical scavenger, capable of transforming reactive free radical species into stable non radical products. The antioxidant potential of the ethanolic extract of the pods of *Moringa* was estimated from their ability to reduce TPTZ-Fe (III) complex to TPTZ-Fe (II) at 593 nm. Antioxidant activity increased proportionally with the polyphenol content.

Reducing Power Activity
Reducing power is to measure the reducing ability of antioxidant and it is evaluated by the transformation of Fe³⁺ to Fe²⁺ by donating an electron, in the presence of the ethanolic extract of *Moringa oleifera*. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The reducing capability of the extract was compared withascorbic acid and BHT. Increasing absorbance at 700nm indicated an increase in reducative ability (Table 3). The reducing power of the extract (MOEE) and the standards increased with the increase in their concentration (0.1-1mg/ml). The result shows that the extract consisted of hydrophilic poly phenolic compounds that cause the greater reducing power and therefore, can act as antioxidants. Our observations indicate that there is a good correlation between the reducing activity and MOEE of flavonol content (r = 0.932). From the results, it was evident that the extracts possess significant (p<0.001) reducing power as compared to standards.

**Ferric thiocyanate (FTC) activity**
The FTC method measures the amount of peroxide value in the beginning of the lipid per oxidation, 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-colored substance. The darker the color, the higher will be the absorbance. Results shows that the sample had been oxidized when stored for seven days at 40-45°C.

Initially, the absorbance of MOEE was the lowest (0.246). After seven days storage, extract exhibited good effect in inhibiting linoleic acid oxidation compared to control. The percentage of inhibition of linoleic acid of MOEE was 82.7%, with no significant difference compared to BHT (P >0.05). The antioxidant activities also increased with increasing the concentration of the MOEE (Figure 4). These phenolic compounds may donate hydrogen and can terminate the free radical reaction chain by changing it to the stable compounds.

Figure 4: FTC activity of MOEE extract during 7 days storage.

**Thioarbituric acid (TBA) test**
The TBA analysis of MOEE at seventh day storage is shown in Table 3. Absorbance at 532nm shows that the sample had been oxidized when stored for seven days at 40-45°C. Initially, the absorbance of MOEE was the lowest (0.246). After seven days storage, extract exhibited good effect in inhibiting linoleic acid oxidation compared to control. The percentage of inhibition of linoleic acid of MOEE was 82.7%, with no significant difference compared to BHT (P >0.05). The antioxidant activities also increased with increasing the concentration of the MOEE (Figure 4). These phenolic compounds may donate hydrogen and can terminate the free radical reaction chain by changing it to the stable compounds.
ml). The % of metal chelating capacity of MOEE and standard was found to be 41.46% and 54.87% respectively (Table 3). Metal chelating capacity was significant as they reduced the concentration of the catalyzing transition metal in lipid peroxidation. Though, the MOEE showed less significant value as comparison to standard used for this study, even then MOEE has a marked capacity for iron binding, suggesting the presence of polyphenol that has potent iron chelating capacity.

CONCLUSION
This research provides information, which could trigger further research in the direction of partial or full isolation and characterization of the constituents of pods extract of Moringa oleifera in order to decipher the specific phytochemical constituent(s) responsible for the free radical scavenging activity of the plant. The present data suggest that the extract could be a potential source of natural antioxidant that could have great importance as therapeutic agents in preventing or slowing the progress of ageing and age associated oxidative stress related degenerative diseases such as cancer. When this is done, extracts of Moringa oleifera could find important application in phytotherapy. Experiments confirming these activities of the extract of Moringa oleifera in an in vivo system would be necessary.

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