1. Introduction

*Moringa oleifera* Lam. (Moringaceae) is a medicinal plant found in most parts of the world. Various parts of this plant such as leaves, roots, seeds, barks, fruits and immature pods are reported to possess various therapeutic properties including antitumor, anti-inflammatory, antihypertensive, cholesterol lowering, antidiabetic, and antimicrobial properties. The aim of the study was to evaluate antioxidants in *Moringa oleifera* Lam. by spectrophotometric method (phenols, flavonoids, flavonols, total chlorophyll, total carotenoid, β-carotene, radical scavenging activity by FRAP, ABTS, DPPH assays and enzymatic activitivities of catalase, peroxides, polyphenol oxidase, glutathione reductase-a). Results showed higher phenol content (260 ± 0.58 mg/g dry. wt.) in *Moringa oleifera* leaves than other antioxidant compounds and FRAP activity was recorded maximum (510 ± 10.1mg/g dry. wt.) than the other scavenging assays. Study concludes that using leaves of *Moringa oleifera* in health promotive purposes may be a better option than other parts of the plant.

**Key words:** *Moringa oleifera* Lam., phytochemical, antioxidant activity (FRAP, ABTS, DPPH), spectrophotometer method

Plants have their own chemical substances which are present in various tissues with specific physiological action in human body. Many of the plant species that provide medicinal compounds have been scientifically evaluated for their possible use in human health. India recognizes more than 2500 plant species which have medicinal values, and synthesize secondary plant products that are considered as the most important sources of chemical compounds (Kritikar and Basu,1995). In the last few decades, the experimental growth in the field of herbal medicine is getting popularized in developing and developed countries, owing to its natural origin with lesser side effects (Brahmachari, 2010). Herbal drugs constitute a major share of all the officially recognized systems of health in India, viz., Ayurveda, Yoga, Unani, Siddha, Homeopathy and Naturopathy (Vaidya and Devasagayam, 2007). Herbal medicines have also become more widely available commercially.

2. Material and Methods

2.1 Chemicals used

Ferric reducing antioxidant property (FRAP), (2,2'-Diphenyl-1'H-benzofuran, TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine), NADP (Nicotinamide adenine dinucleotide phosphate), ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid), aluminium chloride was obtained from Sigma - Aldrich Co., St. Louis, USA. All chemicals used were of GR grade only.
2.2 Preparation of plant material

The *Moringa oleifera* Lam. (Variety : PKM-1) plant material was collected from the Agricultural Research Station, Rajendra Nagar, Hyderabad, Telangana State, India.

The leaves were ground and made into a paste in an electric mixer. The paste was used for analysis. Samples were prepared by drying the leaves in an electric oven at 70°C until two consecutive weights were the same and the samples were used for further analysis.

2.3 Determination of antioxidant compounds

**Phenols:** One gram of each plant extracts (0.5ml) in methanol were separately mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminium chloride, and 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water. The absorbance was recorded at 415 nm (Chang et al., 2002).

**Flavonoids:** 2.0 ml of the sample, 2.0 ml of 2% AlCl₃ ethanol and 3.0 ml (50g/l) sodium acetate solutions were added. The absorption was read at 440 nm (Kumaran and Karunakaran, 2007).

**Proanthocyanidins:** A volume of 0.5 ml of 0.1mg /ml of the extract solution was mixed with 3 ml of 4% vanillin-methanol solution and 1.5 ml of HCl. The mixture was allowed to stand for 15 min. The absorbance was measured at 500 nm (Sun et al., 1998).

**Anthocyanin:** Each one gram sample was dissolved in potassium chloride-hydrochloride acid buffer solution at pH 1.0 and sodium acetate trihydrate (CH₂COONa.3H₂O) buffer solution at pH 4.5. The absorbance was determined at 510 nm (Kar and Mishra, 1976).

**β-Carotene:** Ten grams of the sample leaf was washed and ground to fine pulp using mortar and pestle. The operation was carried out under dim light to reduce the rate of carotene oxidation contained in them (Mustapha and Bahura, 2009).

**Total carotenoid:** Ten grams of the fruit or leaf was dehydrated at 60°C to constant moisture content. Moisture content was determined in dried samples according to Ambreen et al. (2005). One gram of the sample was dissolved in 20 ml petroleum ether and 3 ml chloroform mixture, and filtered and made to various concentrations. The absorbance was measured at 452 nm.

**Chloroplast pigments:** Ten grams of the sample (leaf or fruit) was taken into a clean mortar and was ground to a fine pulp with the addition of 20 ml of 80% acetone. Centrifugation was carried out at 5000 rpm for 5 min and the supernatant was transferred to a 100 ml volumetric flask. The residues were ground again with 20 ml of acetone. After centrifugation, the supernatant was transferred to the same volumetric flask. The procedure was repeated twice until the residue became colorless. The mortar and the pestle were thoroughly washed with 80% acetone and the washings were collected in the volumetric flask. The volume was made up to 100 ml with 80% acetone, optical density recorded at 645 nm, 663 nm, 652 nm.

**Ascorbic acid:** One ml of the sample was titrated against standard 2-6-dichlorphenol indophenol dye (Ranganna, 1997) which was already standardized against standard ascorbic acid. The principle of the method is that with 2-6-dichlorphenol indophenols dye, the end point appears pink in color.

2.4 Extraction and determination of antioxidant enzymes

**Extraction:** One gram of fresh plant material was taken and placed in a pre-cooled mortar and ground with 10 ml of cold 0.05M Tris HCl buffer (pH7.0). The extract was passed through cheese cloth and centrifuged at 1000 rpm for 20 min. (Smittha et al., 2011).

**Determination of catalase:** The reaction mixture consists of 1ml of enzyme, 2ml of hydrogen peroxide and 3ml of 0.05M Tris-HCl buffer (pH7.0). The reaction was stopped by 1ml of 2.5N H₂SO₄. After 5 min. of incubation at 20°C, the residual H₂O₂ was titrated with 0.01 KMN (Barber et al., 1980).

**Peroxidase activity:** The reaction mixture consists of 2ml of Tris - HCl buffer 0.1 M(pH 7.0), 1ml of pyrogallol (0.01M), 1ml of H₂O₂, (0.05M) and 1ml enzyme, 1ml of 0.05M H₂O₂ and 1ml of enzyme extract. The reaction mixture was incubated at 25°C for 5 min. The reaction was stopped by adding 1ml of 2.5N H₂SO₄. The absorbance was measured at 425 nm (Kar and Mishra, 1976).

**Polyphenol oxidase:** The reaction mixture consists of 2ml of Tris -HCl buffer 0.1 M(pH7.0), 1ml of pyrogallol (0.01M) and 1ml of enzyme extract. The assay mixture was incubated for 5 min. at 25°C. The reaction was stopped by adding 1ml of 2.5 N H₂SO₄ and the absorbance was recorded at 425 nm (Kar and Mishra, 1976).

**Glutathione reductase:** 0.2ml of sample, 1.5ml of 0.3 M phosphate buffer (pH 6.8), 0.5ml of 25 mM EDTA, 0.2ml of 12.5 mM oxidized glutathione and 0.1ml of 3 mM NADPH were added. Decrease in absorbance was measured against that of blank at 340 nm (Beutler, 1984).

2.5 Extraction and determination of FRAP, DPPH and ABTS assay

**Extraction:** The leaf powered was extracted by Soxhlet extraction method for 6 h. The extract was evaporated, dried and stored at 4°C.

**Determination of ferric reducing antioxidant power (FRAP) assay:** The FRAP reagent was prepared from sodium acetate buffer (300 mM, and pH 3.6), 10 mM TPTZ solution (40 mM HCl as a solvent ) and 20 mM iron (Fe²⁺) (Gacche et al.,2010), chloride solution in a volume ratio of 10:1:1. The absorbance was recorded at 593 nm.

**Determination of DPPH radical scavenging activity:** Five ml of DPPH solution (3.3 mg of DPPH in 100 ml methanol) was added to 1ml of each plant extract. Incubated for 30 min in the dark and the absorbance was read at 517 nm (Brand-Williams et al.,1995).
Determination of ABTS radical scavenging activity: 0.98 ml of ABTS solution was mixed with 0.02 ml of the plant extracts. The decrease in absorbance was recorded at 0 min. and after 6 min. ABTS•• radical scavenging activity has been found from the formula: 
\[ \% = \frac{[\text{Initial reading-final reading}/\text{Initial reading}] \times 100.} \]

2.6 Statistical analysis

All the results are expressed as Mean± Standard error. The data were correlated using Pearson correlation coefficient at p<0.05. Correlations, among data obtained were calculated using Pearson’s correlation coefficient (r) and p < 0.05 was considered significantly different. SPSS 15 Version was used for the statistical analysis.

3. Results and Discussion

Over the years, exploration of natural plant products has been increased, leading to the identification and improvement of plant products beneficial to mankind. *Moringa oleifera* Lam. has a multifunctional properties with enormous economic, nutritional and health benefits. It is well known that plant polyphenols are widely distributed in plant kingdom and are present in high concentrations (Harborne., 1993). The increase in phytochemical antioxidant compounds, obtained in the present study revealed that the maximum FRAP followed by phenol activity as compared to the other phytochemical antioxidant compounds. The decreasing trend recorded as FRAP scavenging activity 510 ± 10.1 mg/g dry wt., phenol 260 ± 1.58 mg/g dry wt., flavonoids, 232 ±10. mg/g dry wt., flavonols 110 ± 0.6 mg/g dry wt., proanthocyanin 66 ± 0.06. mg/g dry wt., which were significantly different at 5% level of probability. Similar results of FRAP scavenging activity have also been reported by Suaib and Kumar (2012); Patel et al. (2012). FRAP activity in many plant extracts have been reported to have multiple biological effects and antioxidant properties due to their phytoconstituents. The antioxidant activity of phenols is mainly due to their redox properties which played an important role in neutralizing free radicals, quenching singlet and triplet oxygen molecule. The previous reports on *Moringa oleifera* have shown maximum scavenging activity (Aquil et al., 2006; Pourmorad et al., 2006; Ratnesh et al., 2009; Jaiswal et al., 2009; Singh and Sharma, 2009; Rumit Shah et al., 2010) (Table 1).

The polyphenolic compound contents were determined in terms of total phenols, flavonoids, flavonol and proanthocyanins which are known to be the most potent classes of phytochemical antioxidants. Total phenolic content was estimated by Folins Ciocalteau which is one of the important constituents of plants responsible for higher antioxidant property (Gupta et al., 2010). Total flavonoid content was performed by precipitating the crude extract with aluminium chloride (AlCl3). The Al3+ will bind with the ketone and hydroxyl group of the flavonoids through electron transfer reaction and give intense yellow colour when observed under the spectrophotometer at the maximum absorbance of 510 nm and reported to interfere with the biochemical pathways involved in the generation of reactive oxygen species (ROS), in quenching free radicals, and in chelating transition metals (Heim et al., 2002). Polyphenolic plant secondary metabolites played an important role in biological activities such as antibacterial, antiviral, anti-inflammatory, antiallergic, antithrombosis vasodilatory actions and also exhibited free radical scavenging properties. Therapeutic actions of *M. oleifera* were relatively high in leaves, flowers, and seeds (Chumark et al., 2008; Sreeethala and Padma, 2009; Verma et al., 2009; Atawodi et al., 2010). Among the major classes of phytochemicals found in the plant, flavonoids appear to carry most of this activity, through scavenging or chelating process (Shazia, 2013; Mohammad et al., 2010; Saraswati et al., 2013) (Table 1).

Total carotenoid content in *Moringa oleifera* Lam. leaf extract was recorded maximum (5.3 ± 0.5 mg/g Fr. wt.) followed by β-carotene content (3.2 ± 0.1 mg/g Fr. wt.), but anthocyanin content (1.34 ± 0.01 mg/g Fr. wt.) was recorded as the least. Carotenoid exhibited a central role against cancers, cardiovascular diseases and HIV infection and other age-related disorders (Gerster, 1997; Verma et al., 2009.) (Table 1).

Chlorophyll is the most indispensible compound and the only substance that captures sunlight and make it available to plant system. *Moringa* leaf extract had very dark green colour because of high chlorophyll content (12.6 ± 1.6 mg/g Fr. wt.) and results also coincide with the similar findings (Tammama, 2011; Mustapha and Bahura, 2008). The values are significant at 5% level of probability. In the present investigation, the leaves of *M. oleifera* varied in phytochemical composition (Savita et al., 2010) and chlorophyll which is the most indispensible class of primary compounds (Table 1).

Ascorbic acid (Vitamin C) is an important antioxidant (Nicolas, 1996 ; Mapson, 1958) and scavenges the harmful free radicals, produced in the body and also enhances the antioxidant defense mechanism in the body (Nagel and Bertels, 1997). There is no correlation between total ascorbic acid and total antioxidant activities in phenol as reported by Bahorun et al. (2004). It was normal when total ascorbic acid did not correlate with the total antioxidant activities (Table 1).

The leaf extracts of *M.oleifera* Lam. showed the highest ABTS (2, 2'-azinobis 3-ethylbenzthiazoline-sulphonic acid) activity (5.0 ± 0.3 %) as compared to DPPH assay (2, 2'- Diphenyl-2-picyril hydrazyl), (0.63 ± 0.06 %). Similar findings were reported by Rajeshwari et al. (2013) in coriander seeds (Table 1).

DPPH (2, 2-diphenyl-2-picryl hydrazyl) activity showed less (0.63 ± 0.005 %) percentage of inhibition. It did not respond to that of the ascorbic acid standard. Similar studies on DPPH activity were conducted by Divya et al. (2012) and Rajeshwari et al. (2013). This assay widely used as it was relatively quick and a precise method for the evaluation of free radical scavenging activity. It is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The reduction capacity of DPPH radical determined by the decrease in its absorbance at 517 nm induced by antioxidants. The decrease in absorbance of DPPH radical caused by antioxidants because of the reaction between antioxidant molecules and radical which resulted in the scavenging of the radical by hydrogen donation. It was visually noticeable as a change in colour from purple to yellow, hence, DPPH antioxidant activity showed radical scavenging activity and was a sensitive way to survey the antioxidant activity (Singh et al., 2012) which was dependent on solvent type, pH and temperature of the system (Settharaksa et al., 2012) (Table 1.)
Extraction and determination of antioxidant compounds

Plant parts have been shown to vary significantly among different scavenging assays, and the results showed a promising source of natural antioxidant, which can prevent diseases.

Acknowledgement

I sincerely thank the Department of Botany, Osmania University, Hyderabad, Telangana State, for sponsoring RFSMS (UGC) fellowship.

Conflict of interest

We declare that we have no conflict of interest.

References


Table 1: Extraction and determination of antioxidant compounds

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Plant parts</th>
<th>Antioxidant compounds</th>
<th>Antioxidant content (mg/g dry wt.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Leaves</td>
<td>FRAP</td>
<td>510±10.1</td>
</tr>
<tr>
<td>2</td>
<td>Phenol</td>
<td></td>
<td>260±0.5</td>
</tr>
<tr>
<td>3</td>
<td>Flavonoid</td>
<td></td>
<td>232±0.6</td>
</tr>
<tr>
<td>4</td>
<td>Flavonol</td>
<td></td>
<td>110±0.5</td>
</tr>
<tr>
<td>5</td>
<td>Proanthocyanin</td>
<td></td>
<td>66±1.5</td>
</tr>
<tr>
<td>6</td>
<td>Anthocyanin</td>
<td></td>
<td>1.34±0.01</td>
</tr>
<tr>
<td>7</td>
<td>Beta carotene</td>
<td></td>
<td>3.2±0.1</td>
</tr>
<tr>
<td>8</td>
<td>Total Carotenoid</td>
<td></td>
<td>5.3±0.5</td>
</tr>
<tr>
<td>9</td>
<td>Total Chlorophyll</td>
<td></td>
<td>12.6±1.6</td>
</tr>
<tr>
<td>10</td>
<td>Ascorbic acid</td>
<td></td>
<td>10.5±1.0</td>
</tr>
<tr>
<td>11</td>
<td>ABTS</td>
<td></td>
<td>5.0±0.3</td>
</tr>
<tr>
<td>12</td>
<td>DPPH</td>
<td></td>
<td>0.63±0.005</td>
</tr>
</tbody>
</table>

Table 2: Extraction and determination of antioxidant enzymes

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Plant parts</th>
<th>Antioxidant enzymes</th>
<th>Antioxidant enzyme activity (units/g Fr. wt.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Leaves</td>
<td>Catalase</td>
<td>1.06±0.05</td>
</tr>
<tr>
<td>2</td>
<td>Leaves</td>
<td>Peroxidase</td>
<td>2.51±0.05</td>
</tr>
<tr>
<td>3</td>
<td>Leaves</td>
<td>Polyphenol oxidase</td>
<td>0.04±0.006</td>
</tr>
<tr>
<td>4</td>
<td>Leaves</td>
<td>Glutathione reductase</td>
<td>0.5±0.01</td>
</tr>
</tbody>
</table>

Enzymatic activities in catalase and peroxidase have been shown to increase when subjected to stress condition. They reduce H2O2 to water while oxidizing a variety of substrates, oxidoreductases use H2O2 as electron acceptor for catalyzing different oxidative reactions. The enzymatic activity was high in peroxidase when compared with the other enzyme activities (peroxidase 2.51 ± 0.05 units/g Fr. wt.; catalase 1.06 ± 0.05; polyphenol oxidase 0.04 ± 0.006; glutathione reductase 0.5 ± 0.01). Similar studies on enzyme activity have been conducted by Garima Mishra et al. (2011) (Table 2).

4. Conclusion

Moringa oleifera Lam. leaves have exhibited high phenol content, rich polyphenol profile and strong antioxidant capacity. The antioxidant potential and radical scavenging activities vary considerably among different scavenging assays, and the results showed a promising source of natural antioxidant, which can prevent and protect from various diseases. The phytochemical composition of M. oleifera parts have been shown to vary significantly among regions and seasons (Iqbal and Bhanger, 2006; Juliani et al., 2009).

Phytochemicals are a rich source of phenols and medicinally important for curing and treating diseases. It has been suggested that the Moringa is a wonderful plant with antioxidative properties and, hence, for radical scavenging activity and the phenols contribute maximum to the antioxidant activity. Thus, Moringa oleifera Lam. leaves are cheap and a good source of antioxidants that can help us in many ways.