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Antioxidative studies in *Moringa oleifera* Lam.

J. Sravanthi and S. Gangadhar Rao

Department of Botany, University College of Science, Osmania University, Hyderabad - 500007, Telangana State, India

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Abstract

Moringa oleifera Lam. (Moringa) is a medicinal plant found in most parts of the world. Various parts of this plant such as leaves, roots, seeds, barks, fruits, flowers and immature pods are reported to possess various therapeutic properties including antitumour, 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 activitivies 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

1. Introduction

Moringa oleifera Lam. (Moringaceae) is a plant that is native to the sub-Himalayan areas of India, Pakistan, Bangladesh, and Afghanistan. It is also grown in tropics. The leaves, barks, flowers fruits, seeds and roots are used to make medicines. *Moringa* contains proteins, vitamins, and minerals. As an antioxidant, it helps to protect cells from damage.

Moringa oleifera Lam. is a small, fast growing ornamental plant, which grows up to the height of 10 m in length. It is commonly known as Drumstick in English, Munaga in Telugu, Sahijan in Hindi. It is known as a natural nutrient of tropics (Anwar and Rashid, 2007; Gupta *et al*., 2010). The leaves of *Moringa oleifera* possess pharmacological properties such as antidiabetic, antispasmodic, anti-inflammatory, antiparasitic, antianaemic, antiscorbutic, antifertility, anticancer and antiulcer (Singh and Sharma, 2009). It is used for the treatment of infectious diseases, cardiovascular and gastrointestinal problems, hematological and hepatorenal disorders, treatment of mucous membrane, curing diarrhoea, fever, eye and ear infections, bronchitis, diuretic and abortifacient (Nadkarni, 2009; Sabale *et al.*, 2008). In addition to this, it is also used as an appetizer, cardio tonic and stimulates the body immune system (Gilan, 2012) and is used in folk medicine too (Fahay, 2005). Most of the diseases and disorders are caused due to oxidative stress releasing free radicals. Medicinal plants having various phytochemicals and bioactive components such as trace metal ions, vitamins, alkaloids, carotenoids, polyphenols, fats, carbohydrates, and proteins (Coppin, 2008; Amaglo *et al.*, 2010) are involved in enhancing long-term health benefits (Scalbert and Williamson, 2000). Antioxidant activity of plants have been monitored, using FRAP, ABTS and DPPH. They quench, scavenge and suppress the formation of reactive oxygen species (ROS) and oppose their actions (Adesegun *et al*., 2008; Sofidiya *et al*., 2006; Patel *et al*., 2012).

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 - 2 - picryl hydrazyl (DPPH), Folin ciocalteau, TPTZ (2,4,6 - Tri (2-pyridyl)-*s*-triazine), NADP(Nicotinamide adenine dinucleotide phosphate), ABTS (2, 22- azinobis3-ethylbenzthiazoline-sulphonic acid), aluminium chloride was obtained from Sigma - Aldrich Co., St. Louis, USA. All chemicals used were of GR grade only.

Author for correspondence: Mrs. J. Sravanthi
Department of Botany, University College of Science, Osmania University, Hyderabad - 500007, Telangana State, India
E-mail: janapatlasravanthi@gmail.com
Tel.: + 09492438438; 040-64517726.

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 the sample was weighed and extracted twice with a volume of 100 ml of 70% aqueous methanol. The mixture was shaken on an orbital shaker for 75 min at 300 rpm and then filtered through Whatman No.4 filter paper. The combined methanol extract was then evaporated at 40°C using rotary evaporator to dryness and then dissolved in an absolute methanol for analysis (Oljair and Azeez, 2011).

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

Flavonols: 2.0 ml of the sample, 2.0 ml of 2% $AlCl_2$, 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 (CH*f* COONa.3H, O) buffer solution at pH 4.5. The absorbance was determined at 510 nm in a Shimadzu 160A UV-Visible double beam spectrophotometer.

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-dichlorophenol indophenol dye (Ranganna, 1997) which was already standardized against standard ascorbic acid. The principle of the method is that with 2-6-dichlorophenol 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. (Smitha *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 H_2SO_4. After 5 min. of incubation at 20°C, the residual H_2O_2 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 H_2O, (0.05M) and 1ml enzyme, 1ml of 0.05M H_2O_2 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 H_2SO_4. The absorbance was measured at 425 nm (Kar and Mishra, 1976).

Polyphenol oxidase: The reaction mixture consists of 2ml of Tris -HCl buffer 0.1M (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 H_2S0_4 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 (Fez ³) (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: (%) = [(Initial reading-final reading)/Initial reading] × 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 ±1.0. 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 seavenging 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 (Aqil *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, flavonols and proanthocyanins which are known to be the most potent classes of phytochemical antioxidants. Total phenolic content was estimated by Folin 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 ($AlCl_3$). The Al_3^+ 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, antithrombiotic 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; Sreelatha 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 oliefera* 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 (Tammana, 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 indispensable 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, 22 -azinobis 3-ethylbenzthiazoline-sulphonic acid) activity (5.0 ± 0.3 %) as compared to DPPH assay (2, 2 - Diphenyl-2-picryl 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.)

Table 1: Extraction and determination of antioxidant compounds

S.No.	Plant parts	Antioxidant compounds	Antioxidant content (mg/g dry wt.)
1	Leaves	FRAP	510±10.1
2	Leaves	Phenol	260±0.58
3	Leaves	Flavonoid	232±0.6
4	Leaves	Flavonol	110±0.58
5	Leaves	Proanthocyanin	66±1.5
6	Leaves	Anthocyanin	1.34±0.01
7	Leaves	Beta carotene	3.2±0.1
8	Leaves	Total Carotenoid	5.3±0.5
9	Leaves	Total Chlorophyll	12.6±1.6
10	Leaves	Ascorbic acid	10.5±1.0
11	Leaves	ABTS	5.0±0.3
12	Leaves	DPPH	0.63±0.005

Mean ± SE, statistically significant at $p<0.05$

Table 2: Extraction and determination of antioxidant enzymes

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

Mean ± SE, statistically significant at $p<0.05$

Enzymatic activities in catalase and peroxidase have been shown to increase when subjected to stress condition. They reduce H_20_2 to water while oxidizing a variety of substrates, oxidoreducases use H_20_2 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; polyphenoloxidase 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.

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Conflict of interest

We declare that we have no conflict of interest.

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