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THE ANTIOXIDANT POTENTIAL OF PHENOLIC COMPOUNDS FROM THE LEAVES OF *MORINGA OLEIFERA* COLLECTED FROM JABALPUR (INDIA)

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ABSTRACT
The present study investigated the potential of antioxidant activity of leaves of *Moringa oleifera* from Central Indian region of Jabalpur. The dried leaves were extracted sequentially with the solvents of decreasing polarity. The DPPH scavenging was shown by ethyl acetate extract of the leaf. Upon purification through gel filtration, thin layer chromatography and subsequent LC ESI MS, phenolic compounds, i.e. catechin and related compounds were identified. Leaves of *Moringa oleifera* could be the potential source of antioxidants.

KEYWORDS: free radicals, antioxidants, DPPH, *Moringa oleifera*, catechin.

INTRODUCTION
Free radicals play an important role in the pathogenesis of several human diseases, including cancer and Alzheimer's disease.[1] Natural antioxidants present in food of plant origin protect against these radicals and are therefore important tools in obtaining and preserving good health.[2] Strong epidemiological evidence suggests that regular consumption of fruits and vegetables, which are a rich source of the antioxidants, can reduce cancer and coronary heart diseases.[3]

Flavonoids and phenolic compounds widely distributed in plants which have been reported to exert multiple biological effect, including antioxidant, free radical scavenging abilities, anti-inflammatory, anticarcinogenic etc.[4] Recently there has been an increased interest in the therapeutic potentials of medicinal plants as antioxidants in reducing such free radical induced tissue injury.

Moringa oleifera Lam. (drumstick tree, horseradish tree) is an indigenous tree from northwestern India and is often cultivated in hedges and home yards. The tree is valued mainly for the tender pods, which are esteemed as a vegetable.[5] Flowers and young leaves are also eaten as vegetables. The present study is an attempt to identify the antioxidant potential of the leaves of *Moringa oleifera* from the Central Indian town of Jabalpur.

MATERIALS AND METHODS
Collection of plant material
The young leaves of *Moringa oleifera* were obtained from the local nursery of Jabalpur from young plants (approx. length of 1 to 2 meter) during the summer of 2012. For the study, the leaves were dried completely under shade for one week or longer till a constant weight was achieved. The dried leaves were ground in a mixer grinder. The powder was passed through a test sieve having 100 µM pore size (Sonar, India) to obtain a particle size that is less than 100 µM. The remaining course powder was again ground and sieved. The process was continued four to five times or till the material could not be ground further. The fine powder of less than 100 µM was immediately stored in an airtight container for further use.

Extraction of plant parts
For the extraction of phytochemicals, the powdered leaves were extracted with solvents of decreasing polarity. For this, 10 g of leaves was extracted sequentially with water, ethanol, ethyl acetate and petroleum ether (40-60 °C). The aqueous extract was prepared using the cold percolation method, while the extracts with organic solvents were prepared using the Soxhlet extraction method. All the extracts thus prepared were dried under vacuum and redissolved in 20 ml of their respective solvent.

Estimation of phenols by Folin-Ciocalteau method

The total phenolic content was estimated using the Folin Ciocalteau reagent.[6] For this, 0.3 ml of plant extract was added to 1.5 ml of Folin-Ciocalteau reagent and 1.2 ml of 75% (w/v) sodium carbonate solution in test tubes. The tubes were vortexed for 15 s and allowed to stand for 30 min at room temperature. Absorbance was measured at 765 nm with the spectrophotometer (EI, India) against blank which received no plant extract. Amount of phenolic contents was calculated using a standard curve of tannic acid prepared using the same method (R^2=0.976). Results were expressed as milligram of tannic acid equivalent per gram of extract weight.

DPPH radical scavenging activity

The antioxidant activity was measured as the plant's extracts' ability to scavenge 2,2, diphenyl-1-picrylhydrazyl (DPPH) free radical modified method of Kumar et al. [7]

DPPH solution

2.366 mg of 2,2-diphenyl-1-picrylhydrazyl (Sigma, USA) was dissolved in 100 ml of absolute ethanol to obtain 60 µM DPPH.

Sample preparation

25 mg of dried extract was dissolved in 25 ml of absolute ethanol and then was further diluted to obtain 200, 400, 600, 800 and 1000 µg equivalent dry matter.

Procedure

The scavenging effect of plant samples as well as ascorbic acid (Vitamin C) corresponding to quenching intensity of 2,2-diphenyl-1-picrylhydrazyl (DPPH) was carried out. The sample solution of each tested material (500µl) was mixed with the same volume of DPPH solution and allowed to stand for 1 h at room temperature in dark (or until stable absorption values were obtained). The absorbance was then measured at 517 nm using a spectrophotometer against blank that received 500 µl of absolute alcohol instead of plant sample. The result was shown as the mean of three measured values for each sample. The ascorbic acid in the same concentration was used as positive control. The percentage scavenging effect of ascorbic acid was also measured against the blank.

Calculation

% antioxidant activity for DPPH = $(A-A_x)/A \times 100$

Where

A- Absorbance of DPPH solution with ethanol.

A_x- Absorbance of DPPH solution with test solution.

Purification of potent antioxidant compounds

Gel Filtration Chromatography

Since, the ethyl acetate fraction of *M. oleifera* leaves showed prominent antioxidant activity during DPPH assay, this fraction was selected for gel filtration chromatography.

For purification, 150 g of dried leaves powder was extracted with ethyl acetate in a Soxhlet extractor. The extract was concentrated to 5 ml before proceeding and filtered. A small portion of the extract (20 µl) was used to confirm the antioxidant activity using DPPH free radical method. The extract showed antioxidant activity against DPPH free radical.

The gel filtration column (3 x 75 cm) was prepared with silica gel 60-120 mesh (SRL, India) in ethyl acetate. The sample was applied using a 2 cm sand bed prepared on top of the column. The election was made with 500 ml of ethyl acetate and 100 fractions, each of 5 ml were collected. From each fraction 20 µl aliquots were taken for antioxidant assay using DPPH free radical. In this way, active fractions were identified as having antioxidant activity. Adjacent fractions with antioxidant activities were pooled and were dried under vacuum. This pooled fraction was sent for further analysis.

Thin Layer Chromatography

The active fraction obtained after gel filtration chromatography was further purified using preparative thin layer chromatography. The vacuum dried fraction (100 µl on each plate) was applied onto silica gel coated glass plates of 5 x 20 cm. The plates were chromatographed using a solvent mixture of methanol and chloroform (85:15 v/v). After the chromatographic run,, the plates were air dried. One plate was developed with 0.04% DPPH solution in methanol using a spray gun for 5 sec. The image was observed under visible light at exactly 2 min after spraying using a white light illuminator. The area of bright yellow bands against the purple background determined DPPH radical scavenging activity. The silica gel material with sample was scratched off from the other plates from the same relative front (R_f) area and re-dissloved in ethyl acetate. The purified sample was sent for LC ESI MS analysis to Indian Institute of Technology, Bombay (IITB), Mumbai (India).

Briefly, the sample was run through a ODS column (250 x 4.6 mm, 5µM, Thermo Fisher, India) using a binary gradient of methanol and acetonitrile. All the peaks at the LC were subsequently subjected to electro spray ionization mass spectrum (ESI MS) in positive ion mode using the capillary voltage of 80.0 Volts. For ionization, the source voltage was set to 5.3 KV and the current was set to 80.0 UA. The ions were scanned in the positive ESI mode in the mass range of 50-1000 *m/z*.

RESULTS

The leaves of *M. oleifera* showed high phenolic content in methanol (2.33 mg equivalent tannic acid) followed by ethyl acetate extracts (1.15 mg equivalent tannic acid). The aqueous and petroleum ether extracts also showed considerable amounts of phenols (fig 1). When compared to the DPPH scavenging activity of ascorbic acid, only ethyl acetate extract showed a comparable antioxidant

activity (48.6% in comparison to 66.1% by ascorbic acid) (fig 2).

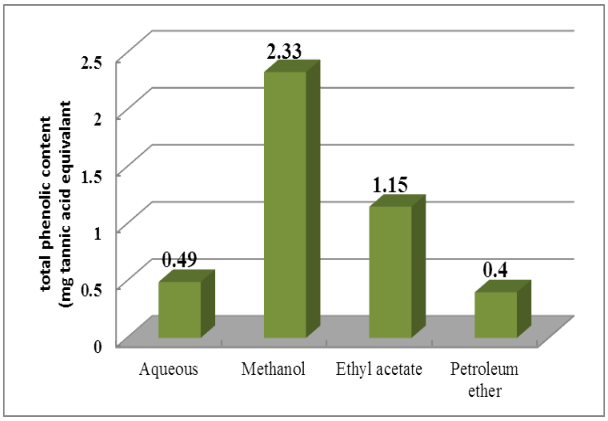

Fig 1: Total phenolic content in different solvent extract of leaves of *Moringa oleifera* from Central India. The phenolic contents are presented as mg equivalent of tannic acid.

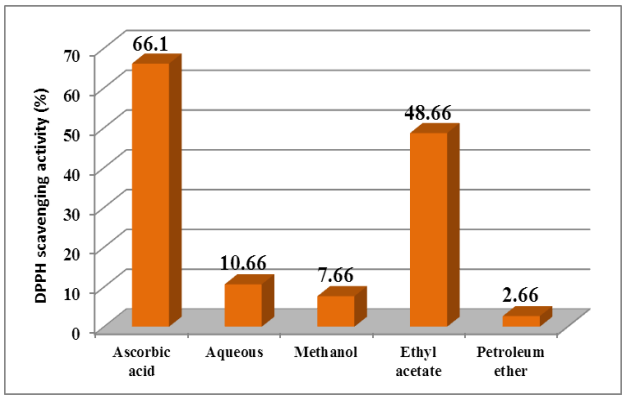

Fig 2: DPPH scavenging (antioxidant) activity of different solvent extract of leaves of *Moringa oleifera* from Central India. The antioxidant activity was shown as percent of activity compared with negative control (ethanol). Ascorbic acid was used as a positive control.

Upon fractionation by gel filtration chromatography, the potent antioxidant appeared in fraction no. 10-12. These fractions were pooled and when loaded on the preparative TLC plates, a single band with Rf value of 8.5 was obtained (Fig 3). The material from this region from other undeveloped plates was scratched off, checked for DPPH scavenging activity, and sent for identification of potent antioxidant compound. This purified fraction showed 98% DPPH scavenging activity in *in vitro* system as compared to ascorbic acid.

Fig 3: Thin layer chromatograph of potent antioxidant fraction obtained from gel filtration chromatography. The material around the R_f value of 8.5 was scratched off and sent for LC MS analysis.

The LC ESI^+ MS revealed base peak with *m/z* value of 281.4, which on fragmentation produced peak at 212.5 (Fig 4). This peak was identified as (+) catechin based on the LC MS profiles of the major antioxidants reported till date using scientific literature and online databases. The other peak with *m/z* value of 277.4 could not be identified further.

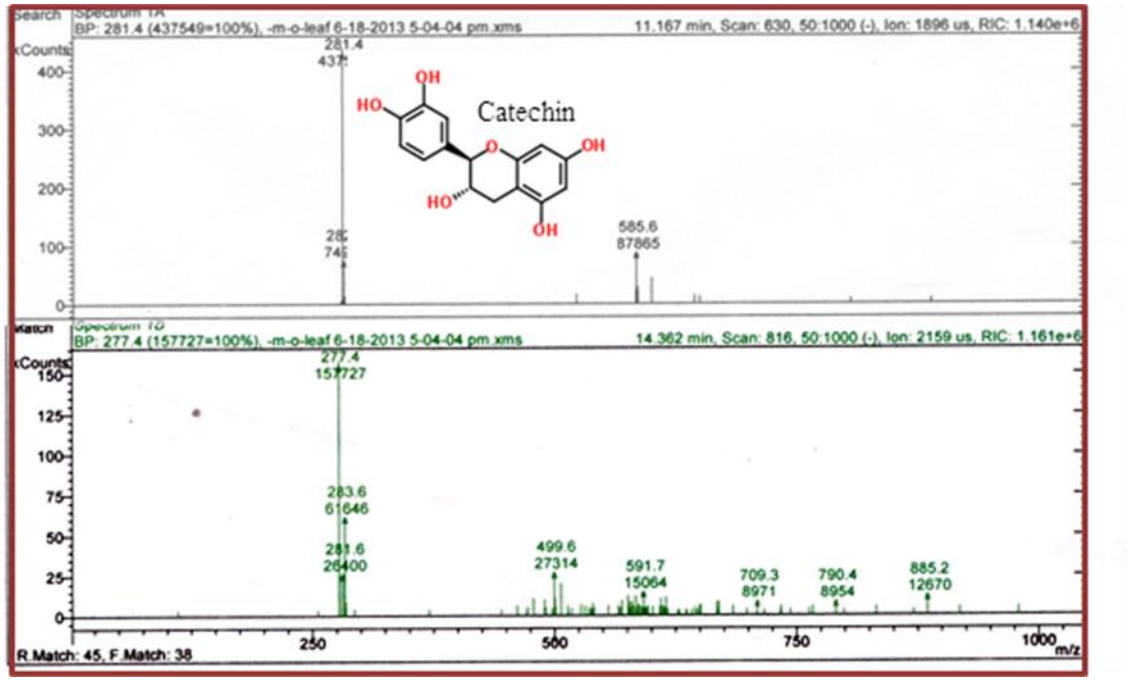

Fig 4: Mass spectrum profile of two major peaks after liquid chromatography of purified antioxidant fraction through gel filtration followed by thin layer chromatography of ethyl acetate extract of leaves of *Moringa oleifera.*

DISCUSSION

Reactive oxygen species or the free oxygen radicals play an important role in the pathogenesis of several human diseases.[8] Natural antioxidants present in food of plant origin protect against these radicals and are therefore important tools in obtaining and preserving good health.[9]

Free radicals, especially peroxides are also responsible for the rancidity of food items, especially oils and fats.[10] Currently available synthetic antioxidants like butylated hydroxy anisole (BHA), butylated hydroxyl toluene (BHT), tertiary butylated hydroquinon and gallic acid esters, have been suspected to cause or prompt negative health effects. Hence, strong restrictions are being placed on their application with a trend to substitute them with naturally occurring antioxidants. Moreover, these synthetic antioxidants also show low solubility and moderate antioxidant activity.[11] Hence there arise need to discover new potential natural sources of antioxidants. Since, in most part of India, *M. oleifera* is used as a common tree and high food value, and the young leaves are used for culinary purposes in various parts of India and Africa, we focused to identify the antioxidant properties of leaves from the plants collected in Central India.

The present study came up with the leaves of *Moringa oleifera* as a potent source of antioxidants. Leaves of *Moringa oleifera* were earlier shown to contain kaempferol, which is a known phenolic group phytochemical showing antioxidant properties.[12] The presence of phenolic acids, i.e. gallic acid and ferulic acid have been reported earlier in *M. oleifera*.[13] (-)-Epicatechin and (+)-catechin are among the main natural phenols in argan oil.[14]

During the study, the aqueous, methanolic and ethyl acatate extracts of leaves showed good amounts of phenolic content. The results indicate the presence of different polyphenols which can be separated on the basis of their polarity. Sultana et al. showed that higher extract yields, phenolic contents and antioxidant activity could be obtained using aqueous organic solvents, as compared to the respective absolute organic solvents.[15]

The study highlights the use of *M. oleifera* leaves as a source of antioxidants apart from the usual candidates i.e. seeds and fruit. The results are in line with the study of Sidduraju and Becker, who have examined water, aqueous methanol, and aqueous ethanol extracts of freeze-dried leaves of *Moringa oleifera* Lam. and showed that all leaf extracts were capable of scavenging peroxyl and superoxyl radicals.[16]

CONFLICTS OF INTERESTS

Author has no conflict of interests.

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