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Phytochemical analysis and *in vitro* anti-oxidant activity of *Moringa oleifera* leaves extract

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ABSTRACT

The phytochemical contents such as, total phenolic, flavonoid and tannin content of ethanol extract of tender and matured leaves of *Moringa oleifera* were investigated. On the other hand, antioxidant activity test (total antioxidant activity, ABTS free radical scavenging activity, reducing power and ferrous ion chelating ability) were also studied. Total Phenolic content (203.80 ± 0.0014 and 105.00 ± 0.0045) mg/g gallic acid equivalent, total flavonoid content (26.02 ± 0.0031 and 18.20 ± 0.0025) mg/g quercetin equivalent and the total tannin content (12.90 ± 0.0021 and 10.50 ± 0.0012) mg/g of tannic acid equivalent were found in the ethanol extracts of tender and matured leaves of *Moringa oleifera*, respectively. The total antioxidant capacity of both leaves of the ethanol extracts were 217.43 ± 0.0025 mg/g and 210.65 ± 0.0019 mg/g respectively of ascorbic acid equivalent, respectively. Firstly, the IC_{50} value of tender and matured leaves of free radical scavenging activity test for ABTS (0.57 ± 0.0135 and 0.61 ± 0.0258) mg/mL were obtained using ascorbic acid as positive control (0.50 ± 0.0265 mg/mL). Secondly, reducing power ability was increased with higher concentration of all samples and standard. Finally, the IC_{50} value for ferrous ion chelating ability test was observed 5.10 ± 0.0121 and 9.20 ± 0.0212 mg/mL for ethanol extracts of tender and matured leaves respectively, whereas standard Na_2EDTA was 4.0±0.0135 mg/mL. The result revealed that the ethanol extract of tender leaves showed more *in-vitro* antioxidant properties that of matured leaves.

Keywords: *Moringa oleifera* leaves; Antioxidant activity; Phenolic; Flavonoid; Tannin

Introduction

Moringa is a member of the Moringaceae family, which consists of a single genus and 13 species. The most widely used and well-known species is *Moringa oleifera* Lamk. The sub-Himalayan regions of northwestern India, Pakistan, Bangladesh, and Afghanistan are the original location of *M. oleifera.* Additionally, it is native to numerous other nations in Southeast Asia, Africa, Arabia, the Caribbean, and South America (Fahey, 2005). *M. oleifera* is known as the "drum stick tree" or the "horse radish tree" in some regions of the world, and the "kelor, marango, mlonge, moonga, mulangay, nebeday, saijhan, sajna, or Ben oil tree" in others. (Anwar and Bhanger, 2003; Prabhu *et al.* 2011). *M. oleifera* is used as an antidiabetic (Gupta and Mishra, 2002), antipyretic (Singh and Kumar, 1999) anthelmintic (Bondya *et al*. 2002) and anti-herpes simplex virus type 1 (HSV-1) (Lipipun *et al*. 2003) agent. The root has laxative, expectorant, and diuretic properties, and it is beneficial for inflammations, piles, cures stomatitis, urine discharges, and stubborn asthma.(Kirtikar and Basu, 1975). The root bark is beneficial for conditions such as heart problems, problems with the eyes, all tridosha fevers, inflammation, dyspepsia, and spleen enlargement (Satyavati and Gupta, 1987).

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The most commonly used component of *M. oleifera* is its leaves. This part have good sources of vitamin C and minerals (Ahmed *et al*. 2016, 2018). Several bioactive compounds were recognized in the leaves of *M. oleifera.* The bioactive compounds found in *M. oleifera* leaves such as Catechin hydrate, 3,4-Dihydroxybenzoic acid, p-Coumaric acid, Catechol, (-) Epicatechin, Rutin hydrate, Rosmarinic acid, trans-Ferulic acid, Quercetin, and trans-Cinnamic acid (Ahmed *et al*. 2021). The leaves are used to treat asthma, hiccoughs, dry tumors, and hallucinations (Nath *et al*. 1992). Muscle illnesses and inflammations are treated by the flowers of *M. oleifera*. The fruit is effective in treating tumors, leucoderma, biliousness, and discomfort. The seeds gives good antioxidant activity (Jahan *et al*. 2018) and it cures eye diseases and head complaints. Oil is helpful for rheumatism and leprous ulcers when applied externally (Kirtikar and Basu, 1975).

The human body constantly produces the potentially reactive oxygen derivatives known as ROS (reactive oxygen molecule), including O_2^-, H_2O_2 and OH,as a result of exposure to numerous exogenous chemicals in the environment and/or various endogenous metabolic processes involving redox enzymes and bioenergetic electron transfer (Chitra and Pillai, 2002). Antioxidants in the body detoxify the ROS produced in a normal environment, and there is an equilibrium between the amount of ROS produced and the amount of antioxidants in the body. This equilibrium is hindered, favoring an increase in ROS that leads to oxidative stress, as a result of excessive ROS generation and/or insufficient antioxidant defense (Kohen and Gati, 2000). Antioxidants are crucial in reducing stress, which can lead to a number of degenerative disorders. Antioxidants are chemicals that can stop free radicals from attacking cells by stabilizing or inactivating them (Kaliora *et al*. 2006). Food antioxidants have significance for human nutrition because they reduce the oxidative damage caused by free radicals to lipids, proteins, and nucleic acids (Soler-Rivas *et al*. 2000). Due to the presence of phytochemicals and antioxidants in food, such as flavonoids and anthocyanins, regular eating of fruits and vegetables has been linked to a decreased risk of cancer, heart disease, hypertension, and stroke (Lako *et al*. 2007). A biological property of plans materials varies due to geographical, environmental cultivation method etc. It also depends on the age or maturity of the plant or plant parts. The present research was carried out to make a comparative study of phytochemical content and antioxidant activity of tender and matured leaves grown in Bangladesh.

Materials and methods

Collection of raw materials

M. oleifera leaves (tender and matured) were collected from BCSIR Campus Dhaka, Bangladesh. The leaves were cleaned from dirt and other impurities and dried under the shade. The dried leaves were powded by pulverizes. The powdered samples were kept in airtight containers and stored in a cool place until use.

Reagents and chemicals

Ethanol, Folin-ciocalteu's reagent, Na_2CO_3, Gallic acid, $AlCl_3$ solution ($AlCl_3$ and Sodium acetate), Quercetin, Ascorbic acid, H_2SO_4, Na_3PO_4, Ammonium Molybdate, ABTS, Potassium persulfate, Phosphate buffer, Potassium fcrricyanide, Trichloro acetic acid, $FeCl_3.6H_2O$, $FeCl_2.4H_2O$, Ferrozine solution, Na_2EDTA, and Tannic acid.

Instrumentation

Cintra-6, double beam UV-visible Spectrophotometer, GBC Scientific Equipment PTY. Ltd. was used during this research.

Preparation of sample extracts

Dry sample *M. oleifera* tender and matured leaves 50 to 1000 mg were weighted separately. 50 mL of ethanol were then added separately and allowed to stand for 24 hours with continuous stirring. The ratios of the sample weight to solvent volumes were 1, 2, 4, 8, 12, 16 and 20 mg/mL. The extracts were then vacuum filtered and used as stock solutions for the following tests.

Phytochemical content determination

Phenolic compounds are well known to have antioxidant properties. So total phenolic, flavonoids and tannin content were determined during this study.

Determination of total phenolic content

The total phenolics were determined by the modified folin-ciocalteu method (Hemayet *et al*. 2013). 1 mL of the ethanolic extracts of tender and maturate leaves of *M. oleifera* was taken separately in different test tube, then added 5 mL of Folin-ciocalteu's reagent (1: 10 v/v distilled water) and 4 mL (75 g/L) of sodium carbonate. The solutions were then vortex for 15 seconds for proper mixing and allowed to stand for 30 min at 40°C for color development. After 30 minutes of reaction absorbance was measured against the blank in a double beam UV/Visible

Spectrophotometer (cintar-6, double beam UV-visible Spectrophotometer) at absorption maxima 765 nm. Three readings were taken per each experimental sample to get reproducible results. The total phenolic content was determined and expressed as mg gallic acid equivalents per gram of dry extract using the equation obtained from a standard gallic acid calibration curve.

Determination of total flavonoid content

Aluminium chloride colorimetric method was used for the determination or total flavonoid content of the *M. oleifera* extracts (Chang *et al.* 2002). 5 mL of each o the extracts were individually mixed with 2.5 mL of aluminium trichloride ($AlCl_3$) solution. They were allowed to stand for 30 min at room temperature and the absorbance of the reaction mixture was measured at 430 nm with a double beam spectrophotometer. The total flavonoid content was determined as mg of quercetin equivalent per gram using the equation obtained from a standard quercetin calibration curve.

Determination of total tannin content

The total tannin content was determined by the modified folin-ciocalteu phenol reagent method (Meghashri *et al.* 2010). 1 mL of the ethanolic and water extracts was collected in 10 mL testube separately. To each testube 7.5 mL of de-ionized water and 0.5 mL Folin-ciocalteu reagent (no dilution) added. Finally 1 mL of 35% sodium carbonate was added. The solutions were held in a vortex for proper mixing before allowed to stand for 30 min for color development. The absorbance was measured against the blank in a spectrophotometer at absorption maxim a 725 nm. Three readings were taken per solution to get reproducible results. The total tannin content was determined and expressed as mg tannic acid equivalents per gram using the equation obtained from a standard tannic acid calibration curve.

Antioxidant activity

The antioxidant activity of *M. oleifera* samples (tender and matured leaves) were determined following four complimentary methods such as total antioxidant capacity, ABTS radical scavenging, reducing power and ferrous ion chelating ability tests.

Determination of total antioxidant capacity

The total antioxidant capacity of the *M. oleifera* sample extracts were evaluated by the phosphomolybdenum assay method (Prieto *et al.* 1999) which is based on the reduction of Mo (VI) to Mo (V) and the subsequent formation of a green phosphate-Mo (V) complex in acidic condition. The 0.3 mL of each extracts were allowed to mix with 3.0 mL of the reagent solution (0.6 M H_2SO_4, 28 mM Na_3PO_4, 4 mM ammonium molybdate). This reaction mixture was incubated at 95°C for 90 min. After letting the solution cool back to room temperature, the absorbance was measured at 695 nm using a spectrophotometer against a blank solution. The total antioxidant capacity was determined and expressed as mg ascorbic acid equivalents per gram of dry extract using the equation obtained from a standard ascorbic acid calibration curve.

ABTS radical scavenging activity

ABTS radical scavenging activity *M. oleifera* samples ware determined by the Fan YJ and Coworkers method (Fan *et al.* 2009). The ABTS solution was prepared by reacting 7 mM ABTS solution with 2.45 mM potassium persulfate and the mixture was allowed to stand in the dark at room temperature for 16 hour. The ABTS solution was diluted with ethanol to an absorbance of 0.70 ± 0.02 at 734 nm. 1 mL of each extract sample at different concentrations (1 to 20 mg/ml) was added to 1 mL of the ABTS solution and mixed vigorously. The reaction mixture was allowed to stand at room temperature for 6 min and absorbance at 734 nm was recorded. The ABTS scavenging effect was calculated as per the equation:

ABTS scavenging effect = $(A_{blank} – A_{sample} / A_{blank})$ x 100

Where, A_{blank} is the absorbance of the control (containing all the reagents except the testing compound), and A_{sample} is the absorbance of the experimental sample with all reagents.

Reducing power assay

The reducing power of *M. oleifera* samples was determined according to Dehpour and Nabavi method (Dehpour *et al.* 2009). Different concentrations of the plant extracts (1 to 20 mg/mL) in 1 mL of sample were mixed with 2.5 mL phosphate buffer (0.2 M, pH 6.6) and 2.5 mL potassium ferricyanide $[K_3Fe(CN)_6]$ (1%). The mixture was incubated at 50°C in water bath for 20 min. After 20 min. cool the solution and 2.5 mL solution of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. 2.5 mL of the upper layer of the solution was mixed with 2.5 mL of distilled water and 0.5 mL $FeCl_3$ (0.1%). After 10 minutes of reaction the absorbance of the mixture was measured at 700 nm with spectrophotometer. Increased absorbance of the reaction mixture indicated increased reducing power. All the tests were carried out in triplicate and average of the absorptions was recorded. Ascorbic acid was used as the standard reference compounds.

Ferrous ion chelating ability

The ferrous ion chelating ability of *M. oleifera* samples were determined by Siraj *et al.* (2016) method. 5 mL of each extract sample was added to 0.1 mL solution of 2 mM ferrous chloride ($FeCl_2$). The reaction was initiated by the addition of 0.2 mL of 5 mM Ferrozine. The mixture was shaken vigorously and left standing at room temperature for 10 min. After the mixture had reached equilibrium, the absorbance of the solution was measured at 562 nm by spectrophotometer. The percentage of inhibition of ferrozine-Fe^{+2} complex formations was calculated according to the following equation:

Ferrous ion chelating ability (%) = $[(A_{blank} - A_{sample}) / A_{blank}] \times 100$

Where, A_{blank} is the absorbance of the control solution (containing all reagents except extract); A_{sample} is the absorbance in the presence of the sample of plant extracts. All the tests were carried out in triplicate for more accurate results. Na_2EDTA was used as the standard.

Table I. Total phenolic content, total flavonoid content and total tannin content in ethanol extract of *M. oleifer*a leaves

Extracts of M. oleifera leaves	Total phenolic content (mg GAE/g of dry extract)	Total flavonoid content (mg QCE/g of dry extract)	Total tannin content (mg TAE/g of dry extract)
TLEE	230.80 ± 0.0014	26.02 ± 0.0031	12.90 ± 0.0021
MLEE	105.00 ± 0.0045	18.20 ± 0.0025	10.50 ± 0.0012

Values are expressed as meant ± SD (mean = 3); TLEE= Tender leaves of ethanol extract; MLEE= Maturate leaves of ethanol extract.

Table II. Total antioxidant capacity at in ethanol extract of *M. oleifera* leaves

Extracts of *M. oleifera* leaves	Total antioxidant capacity (mg AAE/g of dry extract)
TLEE	217.43 ± 0.0025
MLEE	210.65 ± 0.0019

Values are expressed as mean ± SD (mean = 3); TLEE= Tender leaves of ethanol extract; MLEE= Maturate leaves of ethanol extract.

Resul and discussion

The present research was carried out to make a comparative study on tender and matured leaves of *M. oleifera* from Bangladesh. The study was carried out to determine the phytochemical content e.g. total phenolic, total flavonoid and total tannin contents in tender and matured leaves of *M. oleifera*, and the antioxidant activity e.g. total antioxidant capacity, ABTS free radical scavenging, reducing power and ferrous ion chelating ability of ethanol extracts of the leaves (tender and matured).

Total phenolic content

The total phenolic content of the *M. oleifera* dry tender leaves (ethanol extract) was found to be 203.80 ± 0.0014 mg/g of gallic acid equivalent, while that of the matured leaves was found to be 105.00 ± 0.0045 mg/g of gallic acid equivalent shown in table I. So the phenolic content was found to be higher in tender leaves than that of maturate leaves. Qadir *et al.* (2022) also reported that the total phenolic content of the tender leaves was higher than that of matured leaves.

Total flavonoid content

The total flavonoid content of the *M. oleifera* tender leaves (ethanol extract) was 26.02 ± 0.0031 mg/g of QC equivalent, while that of the matured leaves was 18.20 ± 0.0025 mg/g of QC equivalent shown in table I. So the flavonoid content was found to be higher in tender leaves than maturet leaves. The antioxidant activity of flavonoids depends on the presence of both aromatic OH groups and their number per molecule.

Total tannin Content

The total tannin content of the *M. oleifera* tender leaves (ethanol extract) was 12.90 ± 0.0021 mg/g of tannic acid equivalent, while that of the *M. oleifera* matured leaves was

10.50 ± 0.0012 mg/g of tannic acid equivalent shown in table I. The total tannin content was found to be higher in the tender leaves than matured leaves. This result comply with Du Toit *et al*. (2020). They said the total tannin content of the tender leaves was higher than matured leaves.

Total antioxidant capacity

The total antioxidant capacity of the *M oleifera* tender leaves (ethanol extract) was 217.43 ± 0.0025 mg/g of ascorbic acid equivalent, while that of the matured leaves was 210.65 ± 0.0019 mg/g of ascorbic acid equivalent shown in table II. This study reveals that when the concentration of the plant extract increased, the extract's antioxidant activity showed an increasing trend. As a result, the extract showed the ability to donate electrons, which suggests that it could serve as radical chain terminators, converting reactive free radical species into stable nonreactive products (Prieto *et al*. 1999).

ABTS free radical scavenging activity

In ABTS freeradical scavenging activities of the *M. oleifera* leaves (tender and matured leaves, 1 to 20 mg/mL) of ethanol extract varied from 86.08 ± 0.0251% to 93.66 ± 0.0152% and 82.50 ± 0.0150% to 93.19 ± 0.0337% inhibition, respectively. Standard ascorbic acid (AA) showed 98.25 ± 0.0443% to 99.95 ± 0.0265% inhibitionas shown in fig. 1. From the fig. 1 we observed that ABTS radical scavenging activity of the two extract showed almost same activity at high concentration (20 mg/mL), but at low concentration the ethanol extract of tender and matured leaves showed significant activity. The activity was found to be increased with increase of concentration of the extract. From the above study we say that the ABTS free radical scavenging activity are higher in tender leaves than matured leaves of ethanol extract. The IC_{50} value for tender and matured leaves of free radical scavenging activity test of ABTS (0.57 ± 0.0135 and 0.61 ± 0.0258) mg/mL were obtained using ascorbic acid as positive control (0.50 ± 0.0265 mg/mL) shown in fig. 2. The ability of an antioxidant species to donate an electron and a hydrogen atom to these inactive radical species is reflected by the ABTS radical scavenging characteristic (Roy *et al*. 2006; Preethi *et al*. 2006).

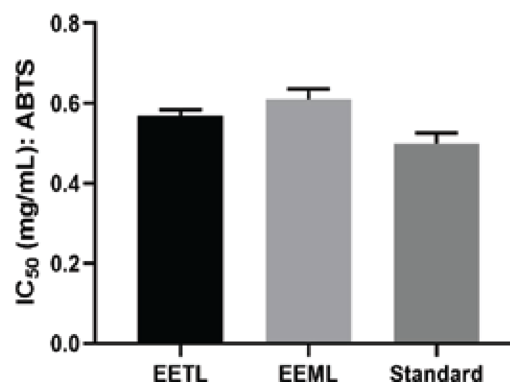

Fig. 2. IC_{50} of ABTS free radical scavenging activity

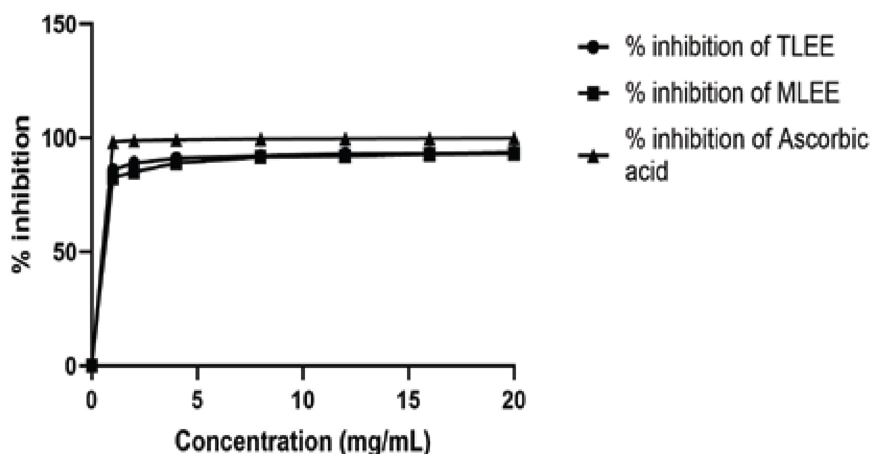

Fig. 1. ABTS free radical scavenging activity of TLEE and MLEE

Reducing power assay

In reducing power the absorbance of the *M. oleifera* leaves (tender and matured leaves, 1 to 20 mg/mL) of ethanol extract varied from 0.23 ± 0.0132 to 1.43 ± 0.0354 and 0.18 ± 0.0123 to 1.16 ± 0.0120, respectively. Standard ascorbic acid (AA) showed 1.42 ± 0.0443 to 2.75 ± 0.0232 as shown in fig. 3.

The reducing power activity of all the extracts of *M. oleifera* leaves were showing at similar absorbance except the standard ascorbic acid. At high concentrations the reducing power activity of all the extracts showed comparable activity to the standard ascorbic acid.

Ferrous ion chelating ability

In ferrous ion chelating ability test of the ethanol extract of *M. oleifera* tender leaves showed significant activity. The activity was found to increase with increase of concentration of the extract. At 1 mg/mL concentration inhibition was 18.19 ± 0.0124% and 20 mg/mL concentration the inhibition was 87.85 ± 0.0240% which is very comparable to the activity of the standard compounds Na_2EDTA (88.96 ±

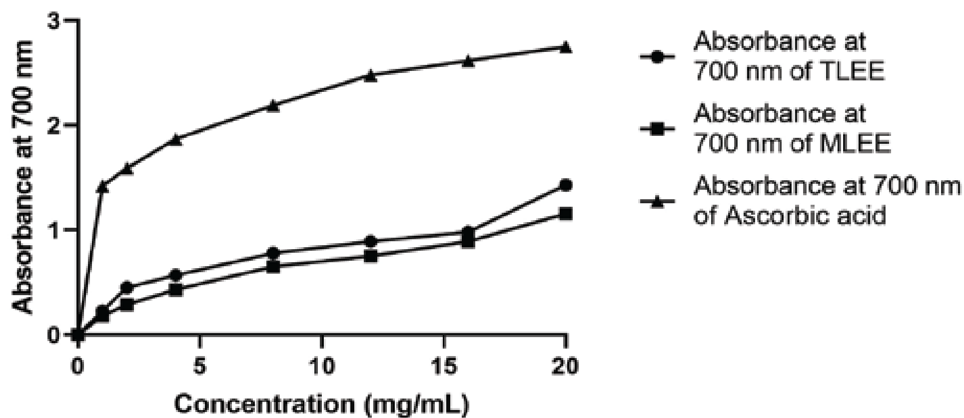

Fig. 3. Reducing power assay of TLEE and MLEE

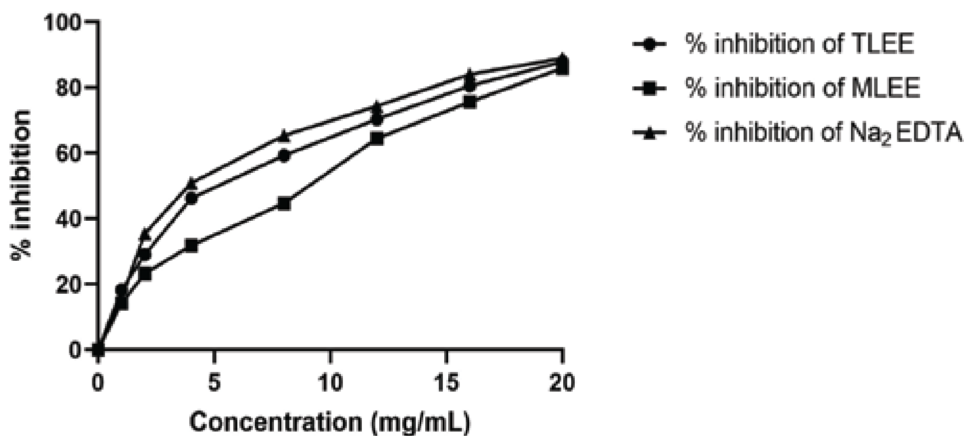

Fig. 4. Ferrous ion chelating ability of TLEE and MLEE

0.0142%) as shown in fig. 4. IC_{50} values of *M. oleifera* ethanol extract of tender leaves and matured leaves were 5.1 ± 0.0121 mg/mL and 9.2 ± 0.0212 mg/mL where as standard Na_2EDTA 4.0 ± 0.0135 mg/mL illustrate in fig. 5. The IC_{50} value of the ethanol extract of tender leaves is very close to that of standard Na_2EDTA.

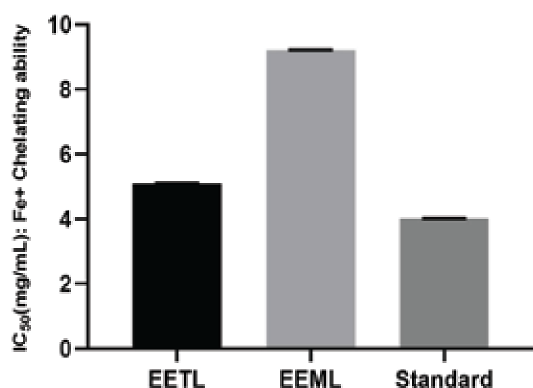

Fig. 5. IC_{50} of ferrous ion chelating ability

Conclusion

Based on different concentrations it was observed that the ethanol extract of tender leaves showed more activity than that of matured leaves for ABTS, reducing power and ferrous ion chelating activity test. It might be due to the presence of higher amount of total phenolic, total flavonoid content and antioxidant activity in the ethanolic extract of tender leaves. Several parts of this plant can be used more effectively in the human diet because to the antioxidant properties of *M. oleifera* leaves. These leaves provide with the added benefits of nutritional antioxidants. These can be consumed as tea, vegetable, side-dishes like chutney, soups, etc and the findings of the present study suggest that *M. oleifera* leaves are better sources of antioxidants.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

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