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Antioxidative Effect and DNA Protecting Property of *Moringa oleifera* Root Extracts

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Moringa oleifera *root (dehydrated) powder, extracts - aqueous (WE), acetone (AE) and methanol (ME) were analyzed for phytochemical and antioxidant components by various* in vitro *assays. The root was found to be a good source of phytochemicals viz, glutathione, a-Tocopherol polyphenols, tannins, alkaloids, saponins. All three solvent extracts exhibited a dose dependent antioxidant activity. The antioxidant properties were established on free radicals; its ability to reduce iron and $FeSO_4$-induced microsomal lipid peroxidation. All three extracts were able to protect DNA from oxidative damage. The suggestive antioxidant mechanism of action of* Moringa oleifera *is by scavenging reactive oxygen species.*

KEYWORDS Radical scavenging activity, reducing power assay, lipid peroxidation, microsomes, calf thymus DNA

INTRODUCTION

Diseases that remain most challenging in today's health care system tend to be complex, involving multiple mechanisms, targets, and drugs for effective

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disease management. In contrast to current combination therapies, plant-based drugs contain a mixture of multiple components, thereby saving considerable time and expense (17).

Plant secondary metabolites is a generic term used for more than 30,000 different substances that are exclusively produced by plants that can boost the immune system, protect the body from free radicals, kill pathogenic germs, and much more. A diet rich in plant foods contains a variety of secondary metabolites and contributes to protecting the body against cancer and cardiovascular illnesses (1). Recent studies in humans have shown that supplementation with antioxidant compounds such as vitamins E and C, lycopene and β carotene can prevent free-radical damage. This lends support to the hypothesis that dietary antioxidants exert a protective effect against degenerative disorders by decreasing DNA damage. Medicinal plants are currently being explored as nutraceuticals and health care supplements in maintaining good health and combating diseases (22).

Moringa oleifera is a tree of the Moringaceae family widely cultivated throughout the tropics and subtropics. The leaves, seed pods, seeds, seed oil, roots, bark, flowers, and sap are commonly consumed as food and used in traditional medicines. The antibiotic property of the roots has been exploited to treat a variety of conditions including asthma, circulatory/endocrine disorders, digestive disorders, inflammation, nervous disorders, reproductive health, and skin disorders (13). The roots of *Moringa Oleifera* seedlings are rich in fiber, protein, vitamins, and minerals and are used to prepare a sauce recommended in treating malnutrition. The leaves have been reported to have excellent antioxidant activity and have been used in food systems (7,8,28). *Moringa oleifera* leaf and root are reported to possess protease activity against blood coagulation cascade (24). However, no reports are available on the use of roots as a source of antioxidant. Hence, the present study aims at investigating the antioxidant activity of *Moringa oleifera* root and its protective role against pro-oxidant induced calf thymus DNA damage.

MATERIALS AND METHODS

Agarose and Ethidium bromide were purchased from Sigma Aldrich, Bangalore, India. Calf thymus DNA was purchased from Sisco Research Laboratory, India. All other chemicals and reagents used in the study were of analytical grade.

Collection of the Sample

The roots of *Moringa oleifera* were collected in the month of September from Mysore district, subsequently identified by Dr. Shivamurthy, Professor,

Department of Studies in Botany, University of Mysore. The root was cleaned, cut into small pieces, dried in the oven at 50°C overnight, powdered, passed through 60 mesh, and stored at 4°C until further use.

Phytochemical Screening by Sequential Extraction

The sample was subjected to sequential extraction using a series of solvents increasing polarity (petroleum ether→benzene→chloroform→ acetone→methanol→water) and screened for various phytochemicals such as alkaloids, tannins, saponins, steroids, triterpenoids, phenols, and flavonoids (20).

Quantitative Estimation of Phytochemicals

In the dehydrated sample, different phytochemical components were estimated by using standard methods (27). α- Tocopherol was extracted by direct saponification of dried sample and estimated based on formation of a red complex from reaction of α, α′ –bipyridyl with ferrous ion due to reduction of ferric ion by tocopherol (11). Reduced glutathione was determined based on the development of a yellow compound due to reaction of 5,5-Dithio (bis) nitrobenzoic acid with compounds containing sulphydryll groups (3).Total phenols were analyzed by Folin-Ciocalteu micro-method as Gallic acid equivalent (25). Flavonoid content was determined by a pharmacopoeia method using Rutin as a reference compound (19), alkaloids by the method based on the reaction with bromocresol green (BCG) using atropine has a standard (9), saponins by using vanillin/sulfuric acid reagents and Diosgenin as standard (15), and tannins by gravimetric method (18).

Preparation of Extract

Sample (15 g) was extracted with 50-ml solvent, (methanol- ME and Acetone - AE separately) for 6 h in a mechanical shaker. The extracts were filtered, and filtrates were evaporated at 40°C under reduced pressure to dryness in a rotary evaporator (Superfit, India). The residue of each extract was stored in an airtight container at 4°C until used. In case of hot water extract (WE), the sample was added to boiling water and extracted for 15 min and filtered. The filtrate was freeze-dried and stored at 4°C until further use.

Determination of Antioxidant Activity

The antioxidant activity of the three solvent extracts was evaluated by the following assays:

Radical Scavenging Assay

The ability of extracts to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals was determined by the RSA method (4). Briefly, 1 ml of 0.1 mM DPPH solution was mixed with 3 ml of extract (containing 300–500 μg) in methanol. The mixture was then vortex-mixed vigorously and left for 30 min at room temperature in the dark. The absorbance was measured at 517 nm and activity expressed as % radical scavenging (RSA) relative to control using the following equation:

$$\text{RSA}\,\% = \frac{\text{Absorbance of control-Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Reducing Power Assay

The ability of extracts to reduce iron (III) to iron (II) was determined in this method. The dried extract (300−500 μg) in 1 ml of the corresponding solvent was mixed with 2.5 ml of phosphate buffer (0.2 mol, pH 6.6) and 2.5 ml of the potassium ferricyanide, ($K_3Fe(CN)_6$: 10 $g1^{-1}$); then the mixture was incubated at 50°C for 30 min. followed by addition of 2.5 ml of trichloroacetic acid (100 g 1^{-1}) and centrifuged at 6,500 rpm for 10 min. Finally 2.5 ml of the supernatant solution was mixed with 2.5 ml of the distilled water and 0.5 ml of $Fecl_3$ ($1g1^{-1}$), and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power (30).

Ferric-Reducing Antioxidant Power Assay

The ferric-reducing antioxidant power (FRAP) assay was carried out according to the procedure of Benzie and Strain (2). The FRAP reagent was prepared by mixing acetate buffer (25 mL, 300 mM/L, pH 3.6), 10 mM/L TPTZ solution (2.5 mL) in 40 mM/L HCl and 20 mM/L $FeCl_3$ solution (2.5 ml) in proportions of 10:1:1 (v/v), respectively. The FRAP reagent was prepared fresh and warmed to 37°C in a water bath prior to use. The extracts were added 150 μg, to the FRAP reagent (4.5 mL). The absorbance of the reaction mixture was recorded at 593 nm after 4 min; the assay was carried out in triplicates. The standard curve was constructed using $FeSO_4$ solution (0.5–10 mg/mL). The results were expressed as μmol Fe (II)/g dry weight of plant material.

Inhibition of Microsomal Lipid Peroxidation

Adult male Wistar rats (150–175 g) were obtained after taking the Animal Ethical Committee clearance from the Central Animal House of the University of Mysore (MGZ/1041/08-09. dated 25/08/08). Animals were excised, and

livers were homogenized (20% w/v) in 0.02 M tris buffer pH 7 (13). Microsomes were isolated according to calcium aggregation method (5,16). One hundred microliters (0.5 mg of protein) of liver microsomal suspension was mixed with $FeSO_4$ (1 mM) and ascorbic acid with or without the isolated compound in a total volume of 1 ml of 0.1 m phosphate buffer (pH 7.4) and incubated at 37°C for 60 min.

The degree of oxidation of microsomes was measured by the 2- thiobarbituric acid (TBA) assay: The reacted solution (1 ml) mentioned above was mixed with 0.2 % (w/v) TBA (3 ml) and 0.05 mol sulfuric acid (2.5 ml), and the mixture was heated for 30 min in a 95°C water bath. After the solution was then cooled in ice for 5 min. the colored substances were extracted by 4.0 ml of 1-butanol. The absorbance of 1-butanol layer was measured at 532 nm. A calibration curve was constructed by using malonaldehyde-bis-diethyl-acetal as a standard, and results were expressed as malonaldehyde equivalents. Antioxidant activity (AOA) was expressed as percentage inhibition of lipid peroxidation relative to the control using the following equation:

$$\text{AOA}\,\% = \frac{\text{Absorbance of control- Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Protective Effect on DNA Damage Induced by Hydroxyl Radical

The reaction mixture contained 50 ng/μlcalf thymus DNA in TAE (Tris acetate EDTA) buffer, 8 μM of Fe(II), 25 μM of H_2O_2 (Fenton reaction), and 100 μg of ME, AE, and WE were added. After incubation at 37°C for 60 min, 3 μl of electrophoresis loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF and 30% glycol) was added to the reaction mixture; then an aliquot (10 μl) was loaded to a 1% agarose gel (8 mm) in 1 × TAE buffer, and electrophoresis was carried out at 100 V for 90 min. Following electrophoresis, gels were stained with ethidium bromide for 30 min. The DNA was visualized and photographed using an Alpha Digidoc (RT2) digital gel documentation system (6).

STATISTICAL ANALYSIS

Results are mean of triplicate determinations and, wherever applicable, the data were analyzed by analysis of variation followed by Tukey's multiple comparisons test for significant difference using SPSS 16.0 software. The IC_{50} values were calculated by Probit analysis (Finney method) by using Statplus 5.0 software. The values were considered significant at $p \leq 0.05$.

RESULTS

Phytochemical Screening and Quantification

The results of phytochemical screening in the sequential extracts revealed the presence of various bioactive components such as alkaloids, phenols, flavonoids, tannins, saponins, and triterpenoids (Table 1). *Morniga oleifera* roots contained (per 100 g dry weight): glutathione (178 mM), α-Tocopherol (61.5 mg), total polyphenols (1.01 g), saponins (33.26 mg), tannins (1.58 g) and alkaloids (28 mg) and flavonoids (176 μg/g).

Antioxidant Activity

The RSA and RPA of the three extracts analyzed at different concentration (100–500 μg) is given in Figure 1a and b where it is shown that, among all the extracts, ME was more effective in scavenging the DPPH and reducing the ferrous significantly ($p \leq 0.05$) in a dose-dependent manner followed by WE and AE. The IC_{50} values were 220,370 and 490 μg for methanol, acetone, and aqueous extracts, respectively.

Further, the IC_{50} concentrations of the three extracts were subjected to FRAP and the ability to reduce ferric (III) iron to ferrous (II) iron. The standard curve was generated in the range of 0.1 mM to 1 M of ferrous sulphate, and the results were expressed as mM ferrous ion equivalent per gram of sample dry weight by constructing a linear regression ($y = 0.2453 x + 0.078$, $r^2 = 0.949$) for standard (absorbance against concentration). Even in case of FRAP, ME was found to be most effective in chelating the ferric metal ion (see Figure 1c).

All extracts inhibited lipid peroxidation (LPO) in the microsomal membrane; however, AE and ME were most effective in at 400 μg (see Figure 1d). AE and WE both exhibited a potent LPO-inhibitory activity with an IC_{50} of 156 μg and 172 μg, whereas, the IC_{50} of ME was 178 μg. These results suggest that extracts may have a mixture of biomolecules with hydroxyl groups that prevent the abstraction of hydrogen atom from the double bond of lipid bilayer thereby avoiding the damage to lipid membrane (Fig 1d).

TABLE 1 Phytochemical Screening of *Moringa oleifera* Root by Sequential Extraction

Extracts	Alkaloids	Flavonoids	Saponins	Tannins	Terpenoid
Petroleum ether	−	−	−	++	+
Benzene	−	−	−	+	++
Chloroform	−	−	−	++	++
Acetone	+	+	+	++	+
Methanol	++	++	++	++	++
Water	+	−	++	++	+

−, not found; +, traces; ++, average.

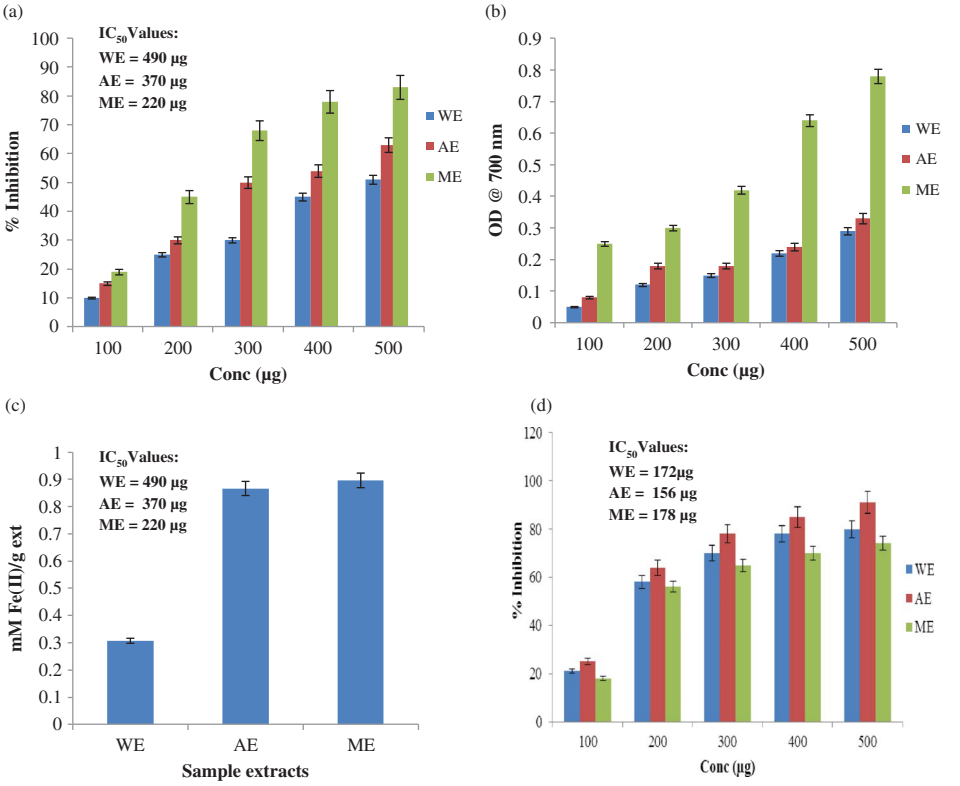

FIGURE 1 Antioxidant activity assays for different solvent extracts of *Moringa oleifera* root. WE, water extract; AE, acetone extract; ME, methanol extract: (a) Radical scavenging activity (RSA); (b) Reducing power assay (RPA); (c) Ferrous reducing antioxidant power (FRAP); (d) Inhibition of microsomal lipid peroxidation (LPO). All the analyses were carried out in triplicates.

Protective Effect on DNA Damage Induced by Hydroxyl Radical

The extracts were further studied for their ability to inhibit calf thymus DNA damage induced by hydroxyl radical at a fixed dose of 200 µg of all three extracts (WE, AE, and ME). All the extracts were able to protect DNA from oxidative damage (Figure 2).

DISCUSSION

Medicinal plants are good sources of phytochemicals, especially phenolic groups such as polyphenols, flavonoids, tannins, and the like, which exhibit excellent radical scavenging activity. Apart from phenolic compounds, other phytochemicals like saponins, steroids, terpenoids, and

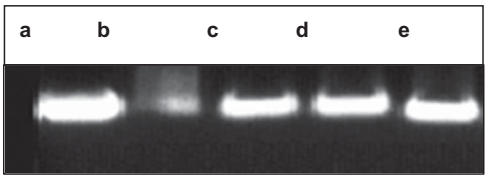

FIGURE 2 Electrophoresis picture of the calf thymus DNA. The sample was treated by 8 μM Fe(II) and 25 μM H_2O_2 (fenton reaction) for 60 min with the three solvent extracts of MOR (WE, AE, and ME) at fixed dose (200 μg): (a) DNA (control) without Fe(II) and H_2O_2 (fenton reaction); (b) DNA with fenton reaction; (c) DNA with fenton reaction + WE; (d) DNA with fenton reaction + AE; (e) DNA with fenton reaction + ME.

alkaloids are well-explored antioxidants that function as peroxide decomposers, enzyme inhibitors, and oxygen quenchers. These phenolics and other phytochemicals act synergistically and prevent the generation of reactive oxygen species, superoxide anions, H_2O_2, and OH radicals that cause oxidative damage to biomolecules (lipids, proteins and nucleic acids).

Antioxidants protection from the free radicals is vital for the integrity of cellular structures and macromolecules. Antioxidant such as vitamin C, carotenoids, and phytochemicals act as a defense system and protect the human body against oxidative stress. At the physiological level, aging, diabetic mellitus, atherosclerosis, and other degenerative diseases demand for the antioxidant due to less biological activity and more oxidative stress.

In the present study, MO root (dehydrated) was analyzed for phytochemicals and antioxidants activity of different extracts by different methods. And also an attempt was made to check the DNA protective property of the MO root extracts. MO root is found to be good source of phenolic compounds like polyphenols, flavonoids, and tannins. In another study conducted in our laboratory, a positive correlation between the phenolic compounds and antioxidant activity of *Moringa oleifera, Aegle marmelos*, and *Psidium guajava* leaf extracts was reported (29).

Here in the root powder, phytochemicals screening was done in different solvent extracts where maximum phytochemicals were identified in water, acetone, and methanol. Further in the same extracts, antioxidant activity was measured by radical scavenging activity, reducing power , FRAP, and inhibition of oxidation in liver microsomes was done. The radical scavenging and reducing potency of ME was significantly higher followed by AE > WE. Similarly in the phytochemical screening, maximum phytochemicals were identified in ME extract. These results also support the previous reported studies on MO leaves (28).

The presence of antioxidant components such as α-tocopherol, glutathione, total polyphenols, flavonoid, and tannins always contribute to medicinal value of the sample. Tocopherol and glutathione as primary and secondary antioxidants protect cell membrane by reacting with lipid radicals

produced in lipid peroxidation chain reaction and also participate directly in the neutralization of free radicals and reactive oxygen compounds.

The different enzymatic and non-enzymatic reactions in cell membrane lead to an increase in the free radical generation. Due to the high polyunsaturated fatty acid (PUFA) content, the membrane lipids are more susceptible to oxidation. Microsomes are a heterogeneous mixtures of vesicles derived from both endoplasmic reticulum and plasma membrane, which are used as an *in vitro* test system to assess the lipid peroxidation effect of a wide range of drugs. Also, many medicinal plant extracts were tested for their potency in preventing lipid oxidation. LPO has been used as a reliable marker of oxidative stress, both *in vitro* and *in vivo*. Several plant extracts have been shown to inhibit lipid per oxidation by acting as chain-breaking peroxyl-radical scavengers and can protect LDL from oxidation as measured by the levels of thiobarbituric acid reactive substances (TBARS) (21).

Polyphenols other than vitamin E have been known to exert a powerful antioxidant effect *in vitro*. Figure 1 shows the inhibition of lipid oxidation by MO root extracts in a dose-dependent manner in microsomes, WE shows higher activity than ME and AE. However, few studies on MO have reported a positive correlation between phenolics and its antioxidant activity whereas in case of MO root, ME extracts showed significantly less activity than WE and AE. Inhibition of the lipid peroxidation by *Moringa* extracts in the liver microsomal membrane suggesst the presence of a mixture of biomolecules with hydroxyl groups like tocopherols that prevent the abstraction of hydrogen atom from the double bond of lipid bilayer. Similar results were found in case of MO mature and tender leaves, which were good sources of antioxidants like tocopherols and ascorbic acid (26).

Excessive stress can induce DNA damage in the form of oxidized nucleosides, strand breaks, or DNA cross-links. DNA contains reactive group in its bases that are highly susceptible to free radical attack. The membrane damage and subsequent mitochondrial dysfunction by reactive oxygen species can lead to modifications in mitochondrial DNA. The hydroxyl radicals generated by H_2O_2 plays an important role in inducing DNA damage and initiates cancer and mutations (10). The activity of these radicals and reactive oxygen species (ROS) can be reduced by natural antioxidants found in plants including herbs and may serve as a possible prevention against free radical–mediated diseases. Many studies have reported the herbal extracts and formulations protection against oxidative DNA damage. Among the natural antioxidants, polyphenolic compounds such as flavonoids, flavonols, terpenoids, and so on, from plant origin have appeared as favored choice. By virtue of being electron-rich, these molecules can donate electrons to ROS and neutralize these chemical species(12,23). A reduction in the basal levels of DNA oxidative damage upon treatment with 4-coumaric and protocatechic acids was reported (14).The results of the present study are also in agreement with the above reports. These findings support the use of the *Moringa*

oleifera root to protect against oxidative DNA damage. These activities of MOR would provide one of pharmacological backgrounds for its use in folk medicine.

CONCLUSION

In conclusion, the presence of various bioactive components in MOR is confirmed by the activity of the different extracts in scavenging the free radical and reducing the hydroxyl radical and also inhibition of microsomal LPO activities. The study also demonstrates protection of DNA against hydroxyl radical-induced damage. The analysis revealed differences in the antioxidant activity of different solvent extracts and the most suitable solvent for the extraction of the antioxidants and bioactive components. However, further studies are needed to elucidate the relationships between the observed activity and pharmacological efficacy of *Moringa oleifera* root.

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