Comparative analysis of phenolic contents and total antioxidant capacity of *Moringa oleifera* Lam.

Swati Vyas[1], Sumita Kachhwaha[1]and S.L.Kothari[1,2*]

[1]*Department of Botany, University of Rajasthan, Jaipur, Rajasthan, India–302004*
[2]*Institute of Biotechnology, Amity University, Rajasthan, Jaipur, Rajasthan, India-302019*

ABSTRACT

Introduction: Accumulation of reactive species higher than permissible limits in biological systems may lead to various degenerative disorders due to oxidative damage. **Materials And Methods:** Oxidation is a serious concern faced by the food industry causing deterioration of shelved-food quality. Antioxidant compounds like polyphenolics scavenge such free radicals and thus protect against oxidative stress. Consumption of polyphenol-rich plants as dietary component confers protection against such cellular damage. **Results:** Present study explores antioxidant capacity, total phenolic content (TPC) and total flavonoid content (TFC)of different extracts prepared from various parts of *Moringa oleifera* Lam. Higher TPC, TFC and antioxidant activity was shown by methanolic extracts followed by aqueous, petroleum benzene and chloroform extracts.The present study suggests that all the extracts might act as radical scavengers to certain extent possibly due to presence of polyphenolic compounds. **Conclusion:** *M. oleifera* exhibits strong antioxidant activity and could serve as prospective source of natural antioxidants to food and health industries.

Key words: Antioxidant activity, total phenolic content, total flavonoid content, sequential extract, oxidation.

INTRODUCTION

Oxidative stress results due to imbalanced homeostasis between the systematic manifestation of reactive oxygen species i.e. pro-oxidants and biological system's ability to readily detoxify the reactive intermediates i.e. anti-oxidants, which leads to disruption of cellular redox signaling pathways, nucleic acids, proteins, lipids, and membrane integrity.[1] Crucial etiological relation has been suggested among oxidative stress and a number of degenerative disorders such as diabetes mellitus, cancer, atherosclerosis, arthritis, neurodegenerative diseases and also in the ageing process.[2] The brain is more vulnerable to oxidative damage because of its elevated oxygen consumption resulting into generation of large number of reactive intermediates, despite of intricate network of defense mechanisms against oxidative stress.[3] Shelved-food may undergo oxidation with passage of time. Antioxidants (synthetic or natural; endogenous or exogenous) are directly added to such food products for combating lipid peroxidation which is major reason for deterioration of food. An antioxidant can be defined as "any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate".[4]

Antioxidants significantly extend the shelf life of foods containing lipids. Phenolic antioxidants form a radical with low reactivity, due to delocalization of the unpaired electron over the aromatic ring, and exhibit no further potential to react with lipids after hydrogen abstraction.[5] Consumption of food items rich in antioxidant is recommended by several studies to augment array of free radical scavengers inside the body. To combat excess of free radicals, nutraceutical antioxidants as dietary supplements exerting positive pharmacological effects on specific human diseases must be explored. However, a concern about the safety of synthetic antioxidants has increased interest in their replacement by natural antioxidants. The traditional medicinal system identifies plants as great source of natural antioxidants. They are likely to be promising, safe and effective with least side effects and therefore, serve as leads for the development

***Corresponding author:**
Dr. Prof. S.L. Kothari,
Department of Botany, University of Rajasthan, Jaipur, Rajasthan, India–302004. Institute of Biotechnology Amity University, Rajasthan, Jaipur, Rajasthan, India-302019.
Phone: +91-9829179692
E-mail: slkothari28@gmail.com

DOI: *10.5530/pj.2015.7.5*

of novel drugs to prevent and/or cure chronic human diseases.[6] Also, natural antioxidants may be utilized as alternative to prevent deterioration of stored food products.

To determine the total antioxidant activity many protocols either using commercially obtainable radicals (DPPH, ABTS, ORAC, TEAC, etc.) or metal ions (like FRAP, LDL, etc.) are available.[7] The antioxidant activity measured depends substantially on various aspects such as test system used, solvent system etc. Therefore, it is recommended to base any conclusions on at least two different test systems.[8] Thus, the present study employed two assays namely, DPPH and TEAC inhibition assays.

Moringa oleifera Lam., popularly known as drum stick tree or horse radish tree or *Saijna*, is a member of a monogeneric family, the Moringaceae along with 12 other species. It is believed to be an aboriginal of Indian subcontinent that has become naturalized in the tropical and subtropical areas around the world. It is a rapidly-growing perennial soft wood tree whose all parts are edible and has long been consumed by humans.[9] Almost all the parts of this plant: root, bark, gum, leaf, fruit (pods), flowers, seed and seed oil are medicinally as well as functionally important food thereby giving the name "Miracle Vegetable".[10] *M. oleifera* is an outstanding source of nutritional components. Leaves are highly rich in vitamin A, vitamin C, protein, calcium, magnesium, potassium, iron, zinc, sodium, copper, manganese and phosphorus. This plant is known to have vast range of medicinally important properties such as antimicrobial, antiasthmatic, antioxidant, hepatoprotective, anti-cancerous, spasmolytic, bradycardiac, hypotensive as well as cholesterol lowering and hypoglycemic effects.[11] It also has numerous non-food product uses such as lumber, charcoal, textile industry, fencing, water clarification, fine-machine lubricating oil, biogas, fertilizer, animal forage, perfumery, hair-care products, etc.[9]

To the best of our knowledge, there are only a few detailed data available on free radical scavenging and/or antioxidant properties of *M. oleifera*.[10,12] The present work is a comprehensive study that evaluates antioxidant capacity, total phenolic and flavonoid content of successive extracts of various parts of *M. oleifera* at different concentrations using spectrophotometric assays. Also, correlation between various study parameters was determined to find out relationship between free radical scavenging assays and phenolic compounds.

MATERIALS AND METHODS

Plant samples

Different parts, namely, flowers (MoF), seeds (MoS), leaves (MoL), roots (MoR), bark (MoB) and gum (MoG) of *M. oleifera* (Voucher No. - RUBL 21189) were collected from Jaipur, Rajasthan, India during full bloom and were authenticated from Herbarium, Department of Botany, University of Rajasthan, Jaipur, Rajasthan, India.

Processing of plant samples

All collected plant parts (except MoG) were rinsed thrice using distilled water and excess water was dripped off. Then plant samples were weighed and shade dried in a room with active ventilation and ambient temperature for 10 days. Dried samples were weighed and powdered using a grinder. Finely grinded samples and MoG were stored at -20°C in dark air-tight containers till further use.

Chemicals

2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), aluminum chloride hydrate, Folin & Ciocalteu's phenol reagent (FCPR), gallic acid, potassium acetate, potassium persulfate, quercetin, sodium carbonate, petroleum benzene, chloroform, methanol were purchased from Sigma-Aldrich, India.

Preparation of the extracts

The successive extracts from powdered samples (50g) packed in a Whatman® cellulose extraction thimbles (Sigma-Aldrich) were prepared using different solvents namely, petroleum benzene (PE), chloroform (C), methanol (M) and water (AQ) via a Soxhlet apparatus (Borosil). MoG (10g) was dissolved by constant stirring using a magnetic stirrer (Digital Spinot, Tarsons) at 250 rpm for 6 hours at 40 °C in the 20% M (100 mL) and filtered using metal sieve. Then centrifugation (Eppendorf) was done at 1000 rpm for 10 minutes and the supernatant was transferred to a flask. The residue was washed several times with 20% M and the washings were added to the separated supernatant. Finally, the solvents were evaporated with the help of vacuum desiccator (Tarsons) to get a solid residue. The solid residue was stored in dark colored glass bottles at -20 °C till further use.

Assay for total phenolics (TPC)

The content of total phenolic compounds in plant extracts

was determined by Folin Ciocalteu's method[13] with slight modifications. One mL of plant extract (10 g/L) was mixed with 3 mL of 10% FCPR (w/v) and 2 mL of 20% sodium carbonate (w/v). This mixture was incubated for 30 minutes on water bath (40 °C) then cooled to room temperature and absorption was read at 765 nm. Gallic acid was taken as standard and calibration curve was prepared using its various aliquots. TPC was calculated by the following formula:

$$P = c * V/e$$

where; P—total content of phenolic compounds, mg/g plant extract, in gallic acid equivalents (GAE); c—the concentration of gallic acid established from the calibration curve, mg/mL; V— the volume of extract, mL; e—the weight of pure plant extract, g.

Assay for total flavonoids (TFC)

For determining the total flavonoid content pharmacopeia method[14] was followed with minor modifications. One mL of plant extract (10 g/L) was mixed with 1 mL of 10% aluminium trichloride (w/v) and 1mL of 1M potassium acetate, then volume was raised up to 10 mL with the corresponding solvent. This mixture was then shaken vigorously and incubated for 30 minutes at room temperature. The absorption was read at 415 nm. Quercetin was used as positive control. The absorption of quercetin solutions was measured under the same conditions to plot calibration curve.

$$F = a * V/e$$

where; F — total content of flavonoid compounds, mg/g plant extract, in Quercetin equivalent (QE); a —the concentration of gallic acid established from the calibration curve, mg/mL; V— the volume of extract, mL; e—the weight of pure plant extract, g.

DPPH radical scavenging assay

The radical scavenging activity[15] of the plant extracts so prepared was measured spectrophotometrically using stable DPPH. One mL of different concentrations of the extracts was added to a 3 mL of a 0.004% DPPH solution (w/v). After shaking vigorously, the mixture was allowed to stand undisturbed for 30 minutes in complete dark. The absorbance of the resulting solution was measured at 517 nm with a UV/visible light spectrophotometer (Shimadzu UV 1700, Kyoto, Japan). Blank sample was prepared following same steps but test sample was replaced with same amount of corresponding solvent with each set of experiment. Inhibition activity was calculated in following way:

$$\% \text{ Inhibition} = [(AB - AS) / AB] \times 100$$

where, AB - absorption of blank sample; AS - absorption of tested extract solution.

Trolox equivalent antioxidant capacity (TEAC) assay

ABTS radical cation decolorization test[16] is another spectrophotometric method widely used for the assessment of antioxidant activity.

Four mL of 2 mM ABTS cation solution were mixed with 100 μL test extract and the decrease of absorption was measured spectrophotometrically at 734 nm during 6 min.

The degree of decolorization as percentage inhibition of the ABTS radical cation is determined as a function of concentration and time and calculated relative to the reactivity of trolox as a standard, under the same conditions. Therefore, this assay is often referred to as the Trolox equivalent antioxidant capacity (TEAC) assay.

Statistical analysis

Analyses were run in triplicates and the results were expressed as mean values with standard error mean. Correlation coefficients (R) to determine the relationship among different antioxidant assays, TPC and TFC were calculated using MS Excel software (CORREL statistical function).

RESULTS AND DISCUSSION

Extraction method and solvent selection

In this study, Soxhlet apparatus was used to achieve sequential extraction of the plant material to assure recovery of even compounds with limited solubility in a solvent. Four different solvents (PE, C, M and AQ) in order of increasing polarity were employed for this purpose. This proved to be advantageous method due to two reasons; firstly, this provided a comparative data to analyze effectiveness of extraction solvent having active principles; secondly, this abridges identification of the active compounds in the crude extracts so obtained via their further fractionation.

Content of phenolic and flavonoid compounds

Diversity of secondary metabolites is produced by plants

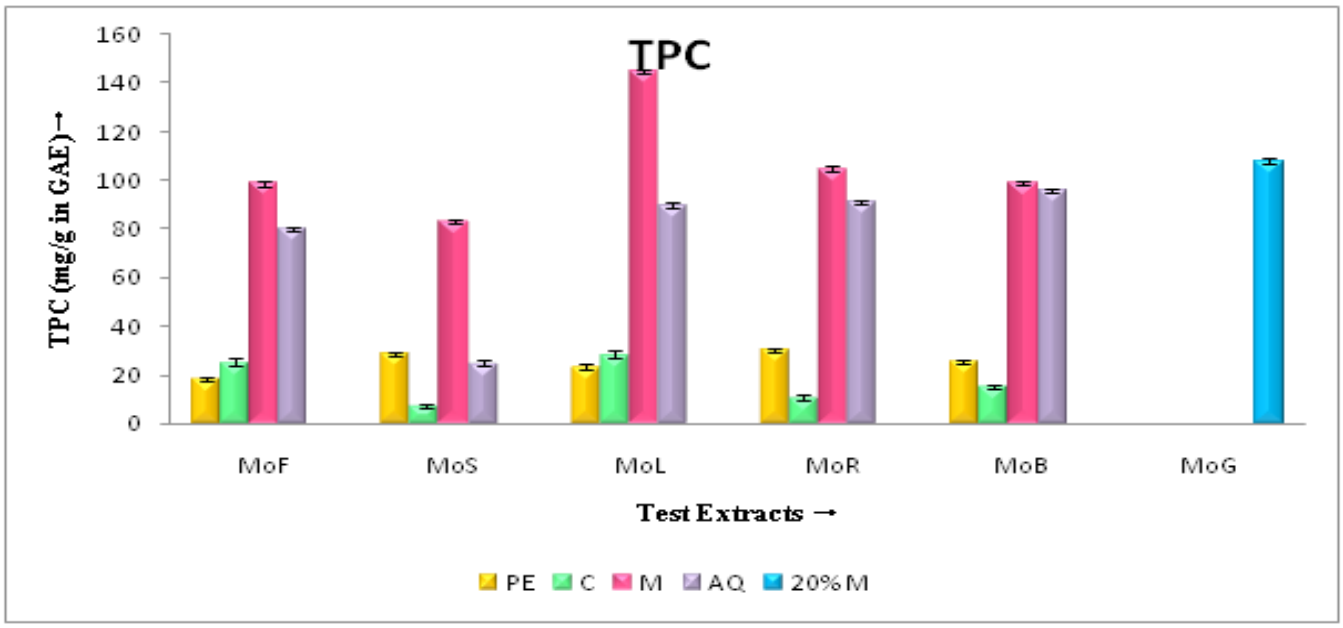


Figure 1: Total phenolic content of different test extracts. Each value is expressed as mean ± standard error. {Petroleum ether (PE), chloroform (C), methanol (M), water (AQ) extracts; *Moringa oleifera* flowers (MoF), seeds (MoS), leaves (MoL), roots (MoR), bark (MoB) and gum (MoG)); Gallic acid equivalent (GAE), Total phenolic content (TPC)}.

and phenolic compounds are one of its most important groups. Phenolics possessing at least one aromatic ring (C6) bearing one or more hydroxyl groups posses' ideal structural chemistry to scavenge free radicals. In general, phenolic compounds act as potential metal chelators as well as inhibit lipid per-oxidation by quenching free radicals via formation of resonance-stabilized phenoxyl radicals. Flavonoids are probably the most important class of natural phenolics and have ability to donate electrons or hydrogen atoms readily, so they can directly scavenge reactive oxygen species.[17] Therefore, TPC and TFC were investigated (Figure 1 and 2).

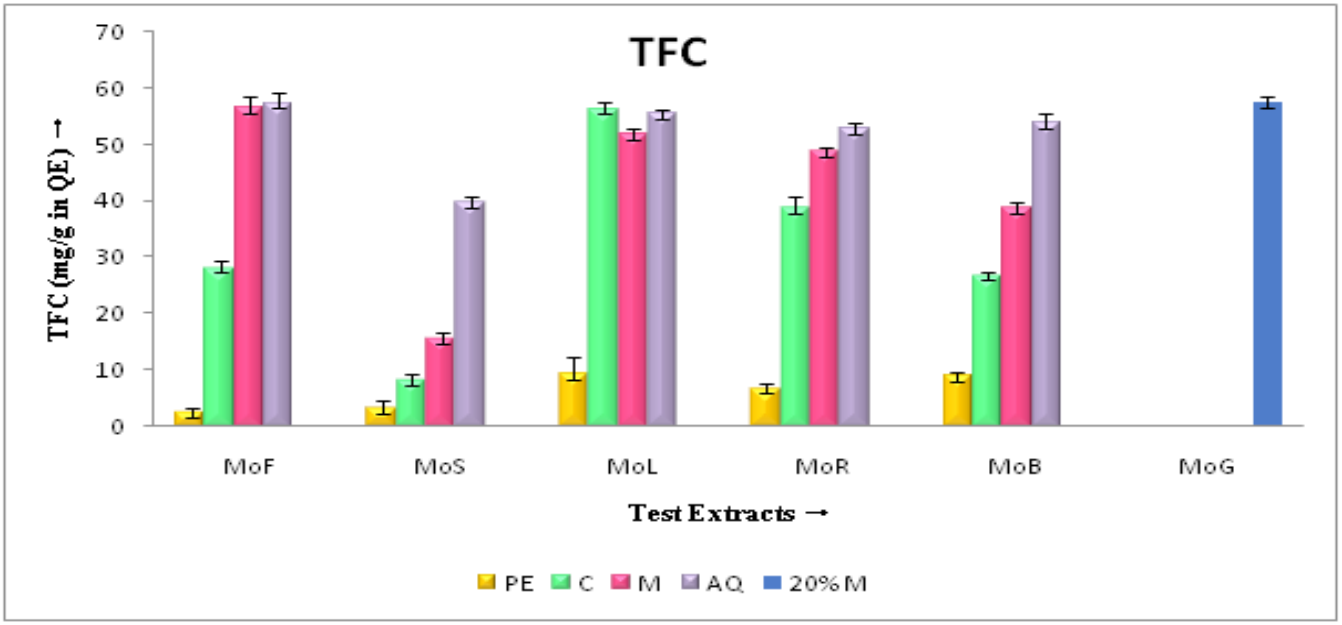


Figure 2: Total flavonoid content of different test extracts. Each value is expressed as mean ± standard error. {Petroleum ether (PE), chloroform (C), methanol (M), water (AQ) extracts; *Moringa oleifera* flowers (MoF), seeds (MoS), leaves (MoL), roots (MoR), bark (MoB) and gum (MoG); Quercetin equivalent (QE), Total flavonoid content (TFC)}.

The TPC (mg/g), determined from regression equation of calibration curve (y = 0.0018x - 0.0469, R^2 = 0.99) and expressed in GAE, varied between 7.44 and 144.77. TPC was in order M> AQ> PE> C which was well correlated with previous studies about higher recovery of phenolic compounds in M followed by AQ, PE and C.[18] M extract of MoL showed the highest value (144.77 mg/g in GAE) whereas C extract of MoS showed the lowest value (7.44 mg/g in GAE). 20 % M extract of MoG possessed high TPC (108.11mg/g in GAE). It was observed that M and AQ extracts were richer in TPC which suggests presence of polar phenolic compounds. Also, TPC was relatively higher in PE extracts than C extracts which indicates presence of some non-polar phenolic compounds.

The TFC (mg/g), determined from regression equation of calibration curve (y = 0.6942x - 0.0042, R^2 = 0.99) and expressed in QE, varied between 2.51 and 57.64. Significantly higher results were found in M and AQ extracts followed by C and PE extracts. Highest concentration of flavonoids was observed in 20% M extract of MoG whereas C extract of MoS revealed lowest TFC. C extracts of MoL exhibited high TFC (56.42 mg/g in QE) followed by MoR (38.812 mg/g in QE) which suggests presence of less polar compounds in *M. oleifera*.

Antioxidant activity

Results of TEAC and DPPH assays were revealed (Figure 3 and 4). Both of them are spectrophotometric electron transfer-based assays which were employed to measure the capacity of an antioxidant in the reduction of an oxidant, which changes color when reduced. The degree of color change (either an increase or decrease of absorbance of the probe at a given wavelength) is correlated to the concentration of antioxidants in the sample.

The present study reveals that all extracts exhibit radical scavenging activity to certain extent and their distinct activities are probably derived from diversity of phytochemicals reacting uniquely with various free radicals.[19] Also, different solvents with different polarities were used in our work that likely extracts different classes of compounds. The highest radical scavenging activity was exhibited by M extracts followed by AQ, PE and C in all samples. Previous studies have suggested that extracts obtained from polar solvents were likely to show higher antioxidant activity.[20] It can be noted that flower extract displayed highest antioxidant activity followed by leaf, root, gum, bark, and seed. A possible explanation might be that flowers contain variety of compounds (such as anthocyanins, reductones, etc.) which exert higher antioxidant activities. On the other hand, leaf having longer life span in comparison with other plant parts and being site of energy production faces high magnitude of oxidative damage therefore, requires greater production of antioxidants and their capacity to act as shield.[21]

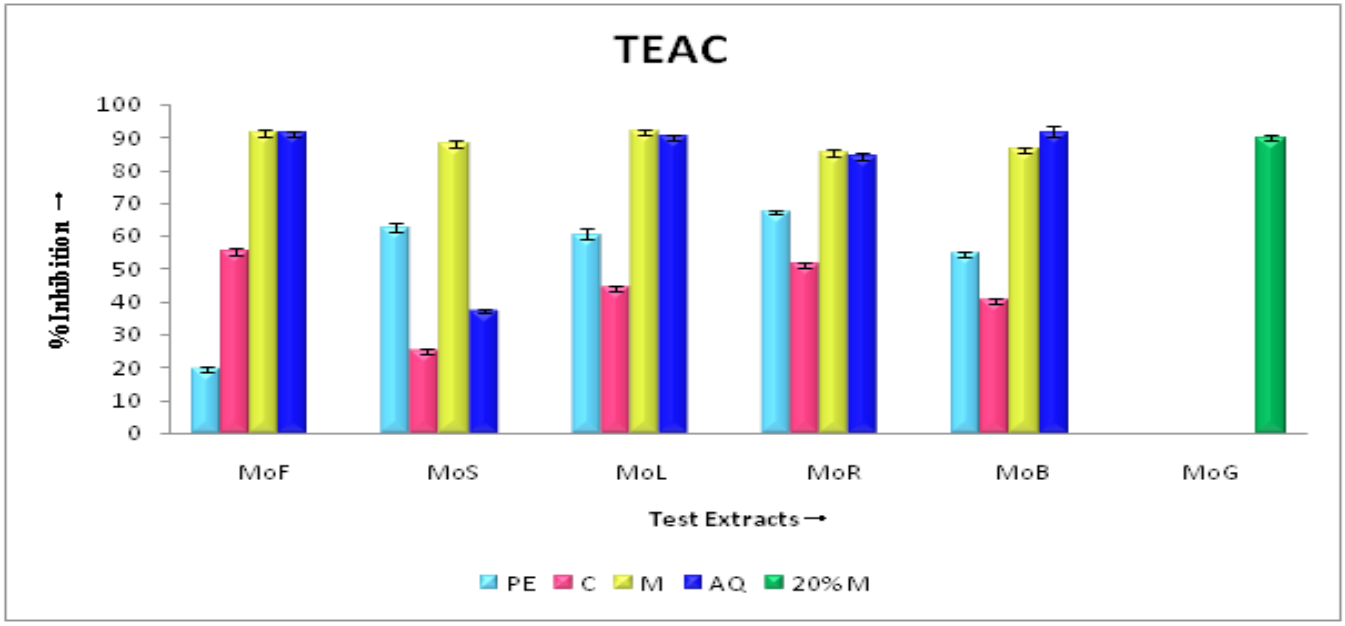
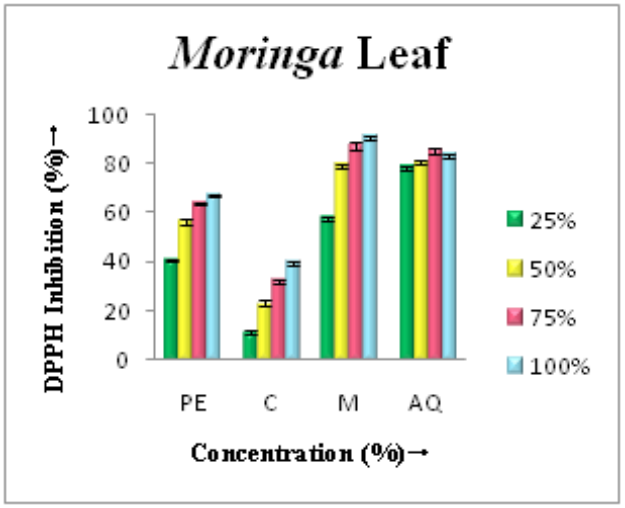
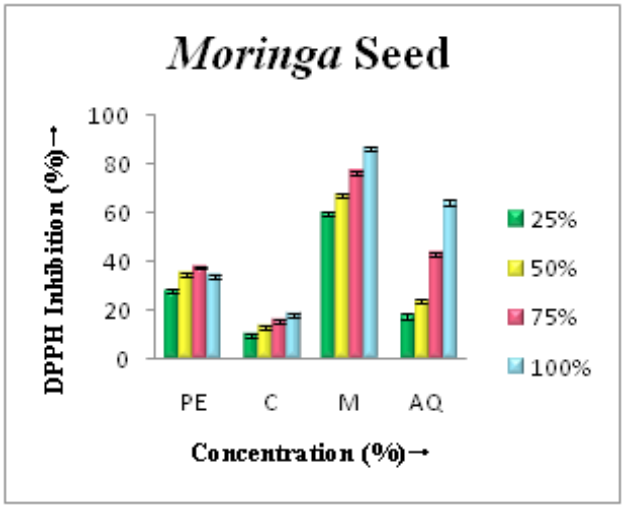
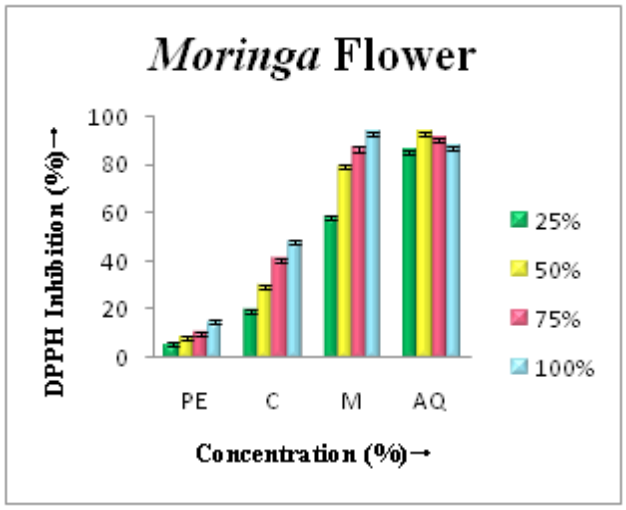
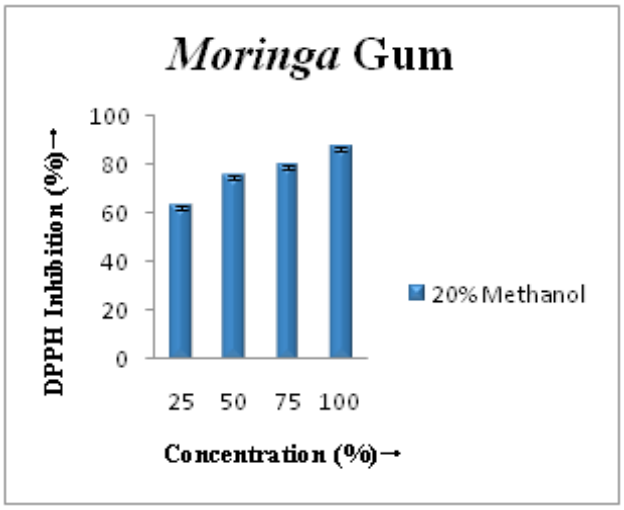


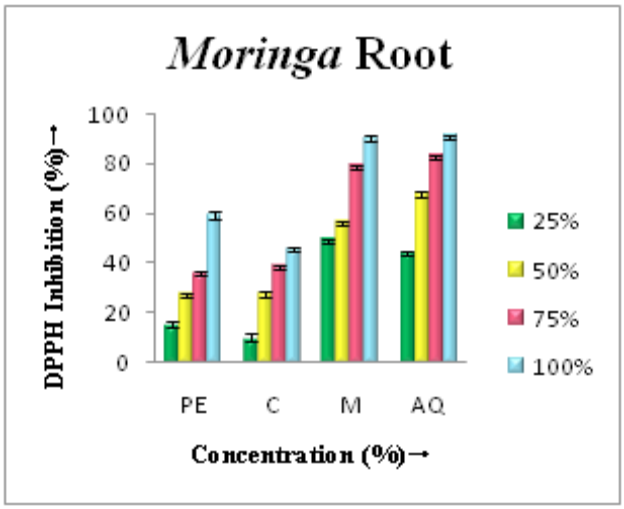
Figure 3: The effects of different test extracts on the inhibition of the ABTS cation. Each value is expressed as mean ± standard error. {Petroleum ether (PE), chloroform (C), methanol (M), water (AQ) extracts; *Moringa oleifera* Leaves (MoL), seeds (MoS), flowers (MoF) and gum MoG); Trolox equivalent antioxidant capacity (TEAC) assay}.











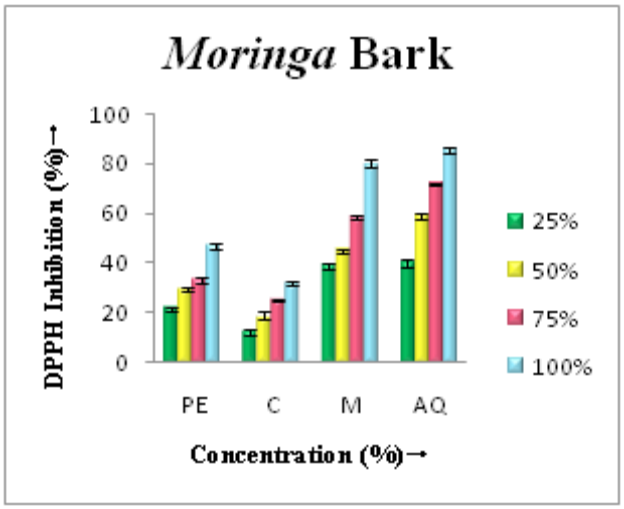


Figure 4: The effects of different test extracts of *Moringa oleifera* on the inhibition of the DPPH. Each value is expressed as mean ± standard error. {Petroleum ether (PE), chloroform (C), methanol (M), water (AQ) extracts; 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay}.

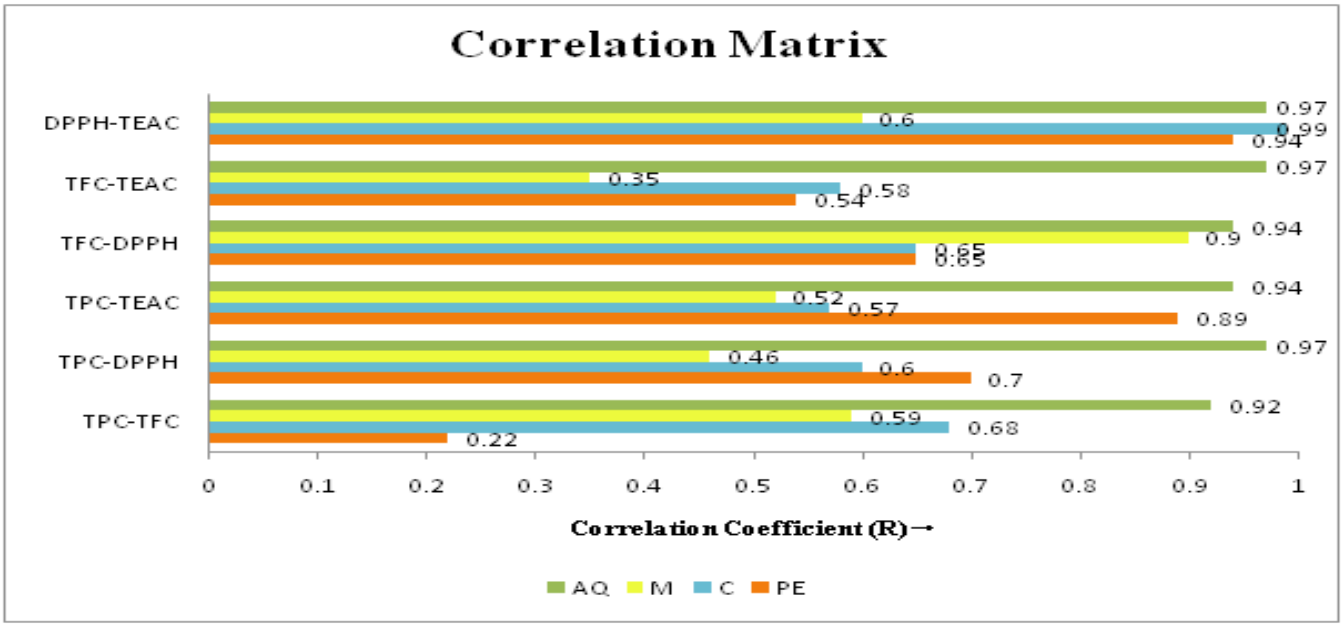


Figure 5: Correlation matrix representing relationship between study variables {Petroleum ether (PE), chloroform (C), methanol (M), water (AQ) extracts; Total phenolic content (TPC); Total flavonoid content (TFC); 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay; Trolox equivalent antioxidant capacity (TEAC) assay}.

On comparing various parameters of the study, good correlation was observed (Figure 5). Data so obtained revealed good correlation between polyphenolic content with their antioxidant activity, implying plausible contribution of phenolics and flavonoids to the radical scavenging activity of these plants extracts.[15, 20, 22, 23] Strong correlation was observed on comparing results of two radical scavenging tests. This can be explained due to fact that both the assays are based on similar underlying mechanism i.e. electron donating ability.[7]

CONCLUSION

Numerous studies are going on globally to identify pharmacologically potent antioxidant compounds with low profile of side effects for food and health industry. Antioxidants may be derived from various sources like plants, animals and synthetic chemical preparations. However, synthetic antioxidants cause safety issues whereas animal-derived antioxidants face ethical issues. Ethnobotany along with traditional medical systems provides lots of prospects to find active and therapeutically useful compounds from plants. *M. oleifera* is a medicinally important and commonly consumed plant. Therefore, antioxidant capacities of its various parts were compared in the present study. In vitro assessment of polyphenolic compounds and various antioxidant activities showed positive correlation, indicating that polyphenols may be due to their hydroxyl groups, were the major contributors to free radical scavenging ability of the extracts.The overall result of the present study certainly provides promising baseline information to ascertain the potency of the crude extracts of *M. oleifera* as a potential source of natural antioxidants. This suggests that these extracts possess reasonable prospect as a source of natural antioxidants to be used by food industry. They have great potential in health-related area via preventing or treating diseases caused by the oxidative stress and might be extensively used for the treatment of degenerative diseases. However, further investigation is suggested to identify and isolate individual components forming antioxidative system and utilize such agents with high efficacy and activity to develop their application for pharmaceutical and food industries. In addition, *in vivo* pharmacological studies should also be conducted.

ACKNOWLEDGEMENTS

The authors wish to express their gratitude to the Department of Science and Technology, Indiathrough the "Innovation in Science Pursuit for Inspired Research (INSPIRE)-Fellowship" to Ms. Swati Vyas for financial support. Special thanks to Dr. Rohit Jain and Dr. Pooja Sharma for their suggestions and improvisations. Ms. Swati Vyas heartily thank her family and friends for their constant support and encouragement.

CONFLICT OF INTEREST

Authors declare no conflict of interest

REFERENCES

1. Issabeagloo E, Taghizadieh M, Asadi J. Sage leaves (*Salvia miltiorrhiza*) extract attenuates hepatic injury in Isoniazid induced hepatotoxicity in rats. Life Sci. J. 2013; 10: 495-500.
2. Gutteridge J. Lipid peroxidation and antioxidants as biomarkers of tissue damage. Free Radical Res Com. 1995; 19: 14(12)1819-28.
3. Cui K, Luo X, Xu K, Ven Murthy M. Title Role of oxidative stress in Neurodegeneration: Recent developments in assay methods for oxidative stress and nutraceutical antioxidants Progress in Neuro-Psychoph. 2004; 28: 771-99.
4. Halliwell B. How to characterize a biological antioxidant. Free Radical Res. 1990; 9: 1-32.
5. Shahidi F, Zhong Y. Lipid oxidation: measurement methods. John Wiley & Sons, Inc.;2005.
6. Perry EK, Pickering AT, Wang WW, Houghton PJ, Perry NS. Medicinal Plants and Alzheimer's Disease: from Ethnobotany to Phytotherapy. J Pharm Pharmacol.1999; 51: 527-34.
7. Apak R, Gorinstein S, Böhm V, Schaich KM, Özyürek M, Güçlü K. Methods of measurement and evaluation of natural antioxidant capacity/activity (IUPAC Technical Report). Pure Appl Chem. 2013; 85(5): 957-98.
8. Tirzitis G, Bartosz G. Determination of antiradical and antioxidant activity: basic principles and new insights. Determination of antiradical and antioxidant activity: basic principles and new insights. Acta Biochim Pol. 2010; 57: 139-42.
9. Fahey JW. *Moringa oleifera*: A Review of the Medical Evidence for Its Nutritional, Therapeutic, and Prophylactic Properties. Part 1. Phytochemistry. 2005; 47: 123-57.
10. Sreelatha S, Padma P. Antioxidant activity and total phenolic content of *Moringa oleifera* leaves in two stages of maturity. Plant Food Hum Nutr. 2009; 64(4): 303-11.
11. Farooq F, Rai M, Tiwari A, Khan AA, Farooq S. Medicinal properties of *Moringa oleifera*: An overview of promising healer. J Med Plant Res. 2012; 6: 4368-74.
12. Siddhuraju P, Becker K. Antioxidant properties of various solvent extracts of total phenolic constituents from three different agroclimatic origins of drumstick tree (*Moringa oleifera* Lam.) leaves. J Agric Food Chem. 2003; 51(8): 2144-55.
13. Folin O, Ciocalteu V. On tyrosine and tryptophane determinations in proteins. J BiolChem. 1927; 73: 627-50.
14. M. State Pharmacopoeia of USSR, Medicina. 1989; 2: 324-34.
15. Miliauskas G, Venskutonis P, Van Beek T. Screening of radical scavenging activity of some medicinal and aromatic plant extracts. Food Chem. 2004; 85(2): 231-7.
16. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M. Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Biol Med. 1999; 26: 1231-17.
17. Michalak A. Phenolic compounds and their antioxidant activity in plants growing under heavy metal stress. Pol J Environ Stud. 2006; 15(4); 523-30.
18. Srinivasan R. Chandrasekar M, Nanjan M. Suresh B. Antioxidant activity of *Caesalpinia digyna* root. J Ethnopharmacol. 2007; 113: 284-91.
19. Chun OK, Kim DO, Lee CY. Quantification of polyphenolics and their antioxidant capacity in fresh plums. J Agric Food Chem. 2003; 51(22): 8067-72.
20. Custódio L, Patarra J, Alberício F, Neng NR, Nogueira JMF, Romano A. Extracts from Quercus sp. acorns exhibit in vitro neuroprotective features through inhibition of cholinesterase and protection of the human dopaminergic cell line SH-SY5Y from hydrogen peroxide-induced cytotoxicity.Ind Crop Prod. 2013; 45: 114-20.
21. Kalaivani T Mathew L. Phytochemistry and free radical scavenging activities of Oroxylum indicum. Food Chem Toxicol. 2010; 48: 298-305.
22. Tabart J, Kevers C, Pincemail J, Defraigne JO, Dommes J. Comparative antioxidant capacities of phenolic compounds measured by various tests. Food Chem. 2009; 113(4): 1226-33.
23. Chew YL, Goh JK, Lim YY. Assessment of in vitro antioxidant capacity and polyphenolic composition of selected medicinal herbs from Leguminosae family in Peninsular Malaysia. Food Chem. 2009; 116: 13-8.