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Role of *Moringa oleifera* in regulation of diabetes–induced oxidative stressDolly Jaiswal¹, Prashant Kumar Rai¹, Shikha Mehta¹, Sanjukta Chatterji¹, Surekha Shukla¹, Devendra Kumar Rai⁴, Gaurav Sharma², Bechan Sharma³, Shahidul khair⁵, Geeta Watal^{1*}¹Alternative Therapeutics Unit, Drug Development Division, Medicinal Research Lab., Department of Chemistry, University of Allahabad, Allahabad–211 002, India²Department of NMR, All India Institute of Medical Sciences, New Delhi, 110029, India³Department of Biochemistry, University of Allahabad, Allahabad–211 002, India⁴Christopher Bond Life Sciences Center, Department of Molecular Microbiology & Immunology, University of Missouri School of Medicine, Columbia, MO65211, USA⁵National Medicinal Plants Boord, New Delhi, 110023, India

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ABSTRACT

Objective: To evaluate the antioxidant activity of aqueous extract of *Moringa oleifera* (*M. oleifera*) young leaves by *in vivo* as well as *in vitro* assays. **Methods:** *In vitro* study included estimation of total phenolic, total flavonol, total flavonoid and total antioxidant power (FRAP assay). In addition, *in vivo* study was done with the identified most effective dose of 200 mg/kg of its lyophilized powder on normal and diabetic rats. Its effect on different oxidative free radical scavenging enzymes, *viz.*, superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST), lipid peroxide (LPO) contents were measured. **Results:** Significant increase in activities of SOD, CAT, GST while, a decrease in LPO content was observed. Whereas, total phenolic, flavonoid and flavonol contents in the extract were found to be 120 mg/g of GAE, 40.5 mg/g of QE and 12.12 mg/g of QE, respectively. On the other hand, FRAP assay results of *M. oleifera* leaves was (85.00±5.00) μM of Fe²⁺/g of extract powder. **Conclusions:** The significant antioxidant activities of *M. oleifera* leaves from both *in vivo* as well as *in vitro* studies suggests that the regular intake of its leaves through diet can protect normal as well as diabetic patients against oxidative damage.

1. Introduction

Antioxidants are micronutrients that have gained importance in recent years due to their ability to neutralize free radicals and reactive oxygen species (ROS) or their actions. Under normal circumstances, the ROS are detoxified by the antioxidants present in the body and there is equilibrium between the generation and the antioxidants present (ROS). Sometimes, due to ROS overproduction or inadequate antioxidant defense, this equilibrium is hampered favoring the ROS upsurge that culminates in oxidative stress. ROS possess a strong oxidizing effect and induce damage to biological molecules, including proteins,

lipids and deoxy ribonucleic acid, with concomitant changes in their structure and function. This oxidative damage is a crucial etiological factor implicated in several chronic diseases such as diabetes mellitus, cancer, atherosclerosis, arthritis, neurodegenerative diseases and also in the ageing process[1].

Circulating levels of radical scavengers get impaired throughout the progression of diabetes. Therefore, antioxidants are needed in diabetes, with low levels of plasma antioxidants implicated as a risk factor for development of the disease. The etiology of the complications of diabetes involves oxidative stress perhaps as a result of hypoglycemia and together, they lead to protein oxidation and glycation. Antioxidants *i.e.* vitamins E and C have been considered as treatments to counter oxidative stress as vitamin E has been found to decrease the susceptibility of lipid peroxidation[2].

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Oxidative stress produced under diabetic condition is likely to be involved during progression of pancreatic β -cell dysfunction. Because of the relatively low expression of antioxidant enzymes such as catalase (CAT) and superoxide dismutase (SOD) in diabetes, pancreatic β -cells may be vulnerable to ROS attack when the system is under oxidative stress situation. Similarly, elevated levels of free radicals, due to inefficiency of the antioxidant defense system, may lead to disruption of cellular function by enhancing the susceptibility of membranes to lipid peroxidation^[3].

Diabetics usually exhibit high oxidative stress due to persistent and chronic hyperglycemia, which thereby depletes the activity of antioxidative defense system and thus promotes free radicals generation^[4]. Free radicals are formed disproportionately in diabetes by glucose oxidation, nonenzymatic glycation of proteins and the subsequent oxidative degradation of glycated proteins. These free radicals damage the pancreatic β -cells and induce insulin resistance. Oxygen free radicals could react with polyunsaturated fatty acids which lead to lipid peroxidation (LPO)^[5].

Plants often contain substantial amount of antioxidants, including tocopherols, carotenoids, ascorbic acid, flavonoids and tannins and it is suggested that, antioxidant action may be an important property of plant medicines associated with diabetes. Available synthetic antioxidants like butylated hydroxy anisole and butylated hydroxy toluene are associated with low margin of safety due to their toxicity. In recent years, natural antioxidants being safe and non-toxic are gaining much attention and therefore, the research is directed towards identification of plant foods with antioxidant ability that may be used for human consumption^[6].

Moringa oleifera Lam. (Family: Moringaceae) (*M. oleifera*) is a medium-sized tree, growing in Asia, Africa and tropical areas of the world as a valuable food source. Various parts of *M. oleifera* tree have been studied for several pharmacological actions. Many reports have described its leaves as antifungal, antimicrobial^[7], antiatherosclerotic^[8], antifertility, relieving pain, central nervous system depressant^[9], antiinflammatory, diuretic and regulating hyperthyroidism^[10].

Several carbamate and thiocarbamate glycosides have been isolated from its leaves. Ethanolic extract of drumstick leaves have been reported to contain saponin (5%) and phythate (3.1%) and its leaves are also a good source of proteins. Ethanolic extract of the leaves have been reported for its high antioxidant activity in both linoleic acid and peroxidation system and have been found to contain high poly unsaturated fatty acids oil lipid peroxidation contents^[11]. Leaves (in every 100 g) are rich in minerals such as Ca (440 mg), P (70 mg), Mg (42 mg), K (259 mg), S (137 mg) and Cl (43 mg). Moreover, vitamins such as Vitamin C (200 mg) and total carotene (42 000 μ g) have also been

abundantly found in the leaves of the plant^[12]. In the present study, the leaves of *M. oleifera* have been analyzed *in vivo* for their effect on different oxidative free radical scavenging enzymes, viz., SOD, CAT, glutathione-S-transferase (GST) as well as on LPO contents.

Since, our earlier studies have confirmed the antidiabetic and lipid lowering activities of aqueous extract of *M. oleifera* leaves in streptozotocin (STZ)-induced diabetic rats^[13] therefore, its *in vivo* antioxidant effect in diabetes-induced oxidative stress in experimental rats have been studied in the present work. The extract's antioxidant *in vitro* assessment was undertaken viz. total phenolic, total flavonol, total flavonoid and total antioxidant power (FRAP assay) to substantiate the above data. Thus, *M. oleifera* leaves may offer a new paradigm of healthcare as a natural antioxidant agent due to many beneficial health functionalities.

2. Materials and methods

2.1. Chemicals

1-chloro-2, 4-dinitrobenzene (CDNB), pyrogallol, thiobarbituric acid, H₂O₂, bovine serum albumin (BSA), GSH, folin-ciocalteu reagent, 2, 4, 6-tripyridyl-s-triazine, gallic acid, quercetin and sodium acetate were purchased from Sigma-Aldrich Chemicals Pvt. Ltd., St. Louis, USA and E. Merck, Darmstadt, Germany. All other chemicals and reagents used were of the highest commercially available purity.

2.2. Collection of plant material and extract preparation

Leaves of drumstick plant, *M. oleifera* Lam. (Family: Moringaceae) were collected from the Botanical Garden of our University and were authenticated by Prof. Satya Narayan, Taxonomist, Department of Botany, University of Allahabad, Allahabad. A voucher specimen (AD/428/07) was submitted to the University herbarium. The aqueous extract was prepared so as to simulate conditions of their extraction pertaining to their use for cooking purposes. The extract was prepared with 5 kg fresh leaves in distilled water at 40–60 °C for 48 h. The process was repeated twice for complete extraction. This aqueous extract was concentrated under reduced pressure and lyophilized to get a powder (yield 10.5% w/w). This powder was dissolved in distilled water and used for analyzing the antioxidant activity *in vivo* as well as *in vitro*.

2.3. Experimental animals and treatment protocol

Male albino Wistar rats weighing 180–220 g were used throughout the study. The rats were housed in an air-conditioned room and had free access to water and a

pellet diet (Pashu Aahar Kendra, Varanasi, India). All the experimental procedures were carried out in accordance with internationally accepted guidelines for the care and use of laboratory animals. All animal procedures used were in strict accordance with the Committee for the Purpose of Supervision on Animal Experiments under the Animal Welfare Departments and Ministry of Environment and Forest, Govt. of India, India. All experimental protocols were approved by the University of Allahabad Animal Experimentation Ethics Committee. Diabetes was induced by a single intraperitoneal injection of freshly prepared STZ at a dose of 55 mg/kg body weight in 0.1 M citrate buffer (pH 4.5) to overnight fasted rats. After 3 days of STZ administration, rats with marked hyperglycemia in terms of fasting blood glucose (FBG) and postprandial glucose (PPG) [FBG > 250 mg/dL and PPG > 350 mg/dL] were used in the study.

Our previous study had demonstrated 200 mg/kg dose of the aqueous extract as the most effective dose^[14]. Therefore, this dose was used in the present study to evaluate its antioxidant potential *in vivo* in diabetic rats. Rats were divided into 5 groups of six rats each. Group I consisting of normal rats, served as normal control, treated with vehicle (distilled water) only whereas, Group II comprised normal animals, treated with 200 mg/kg of the extract. Group III had diabetic animals, serving as diabetic control, received vehicle only. Diabetic animals of Group IV were treated with a known synthetic drug, Glipizide at a dose of 2.5 mg/kg. Diabetic animals of Group V received leaves aqueous extract at a dose of 200 mg/kg daily up to 21 days. After 21 days treatment, the rats were deprived of food overnight and sacrificed by cervical dislocation. Blood samples were collected from the heart. Key organs including heart, brain, liver, kidney, pancreas and spleen were quickly removed, washed immediately with ice-cold saline, dried and were used or stored at -80°C for further experiments. 10% (w/v) homogenates of the organs were prepared in phosphate buffer (100 mM, pH 7.4) containing 150 mM KCl. The homogenates were centrifuged at $9\,000 \times g$ for 30 min. Clear supernatants were used in the estimation of all the biochemical parameters.

2.4. Antioxidant *in vivo*

2.4.1. SOD assay

The activity of SOD was assayed by spectrophotometric measurement of pyrogallol auto oxidation at 412 nm for 3 min with or without enzyme protein^[15]. One unit of the enzyme activity was expressed as 50% inhibition of auto oxidation of pyrogallol/min.

2.4.2. CAT assay

The activity of CAT was determined by adopting the method of assaying the enzyme^[16]. Change in absorbance was recorded spectrophotometrically at 240 nm for 30 min.

One unit of Catalase activity was defined as micromoles of H_2O_2 decomposed/min, using molar extinction coefficient of H_2O_2 .

2.4.3. GST assay

The activity of GST was assayed using CDNB as a substrate^[17]. 3 mL of the assay mixture was prepared by adding 2.7 mL phosphate buffer (0.3M, pH 6.5), 0.1 mL of CDNB (30 mM) and 0.1 mL post mitochondrial supernatant. The reaction was initiated by the addition of 0.1 mL of 30 mM glutathione (GSH) and absorbance was recorded at 340 nm for 3 min at 30 sec intervals on Shimadzu UV-VIS spectrophotometer. Results were expressed as nanomoles of GSH-CDNB conjugate formed/min/mg protein, using molar extinction coefficient of conjugate ($9.6 \times 10^6 \text{ M}^{-1} \text{ cm}^{-1}$).

2.4.4. LPO assay

LPO was determined in cytosolic fraction of rat brain, liver and other tissue homogenates by colorimetric estimation of malonaldehyde (MDA)/thiobarbituric acid reactive substances (TBARS) formed^[18].

2.4.5. Protein estimation

The protein content in various tissues was determined by colorimetric method^[19], using BSA as a standard.

2.5. Antioxidant *in vitro*

2.5.1. Determination of total phenolics

Total phenolic contents in the aqueous extract were determined by the modified Folin-ciocalteu method^[20]. An aliquot (100 μL) of the extract of different concentration was mixed with 2.5 mL Folin-ciocalteu reagent (diluted with water 1:10 v/v) and 2 mL (75 g/L) of sodium carbonate. The tubes were vortexed for 15 s and allowed to stand for 30 min at 40°C for color development. Absorbance was recorded against reagent blank at 765 nm using Shimadzu UV-VIS spectrophotometer. The total phenolic contents were expressed as mg/g gallic acid equivalent (GAE). The calculation was based on the standard curve of gallic acid.

2.5.2. Determination of total flavonoids

Total flavonoid contents were determined using the method of Ordon *et al*, 2006^[21]. Solutions of different concentrations of the extract as well as quercetin were prepared in methanol. A volume of 0.5 mL of 2% AlCl_3 ethanol solution was added to 0.5 mL of varying concentration of sample solutions. After 1 h at room temperature, the absorbance was measured at 420 nm. A yellow color indicated the presence of flavonoids. The total flavonoid contents were expressed as mg/g quercetin equivalent (QE).

2.5.3. Determination of total flavonols

Total flavonols in the plant extract were estimated by

using the method of Kumaran and Karunakaran^[22]. The plant extract in varying concentrations as well as different concentrations of quercetin were dissolved in methanol separately. 2.0 mL of 2% AlCl₃ in ethanol and 3.0 mL (50 g/L) of sodium acetate solutions were added in 2.0 mL of extract solution. The absorption at 440 nm was read after 2.5 h at 20 °C. The analysis was performed in triplicate and total flavonoid content was calculated as quercetin equivalent (mg/g).

2.5.4. Total antioxidant power (FRAP assay)

FRAP (Ferric Reducing Antioxidant Power) was determined by the standardized method^[23]. The stock solutions included 300 mM acetate buffer (3.1 g CH₃COONa and 16 mL CH₃OOH), pH 3.6, 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) solution in 40 mM HCl, and 20 mM FeCl₃•6H₂O solution. The fresh working solution was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ, and 2.5 mL FeCl₃•6H₂O. The temperature of the solution was raised to 37 °C before using. Plant extract (150 μL) was allowed to react with 2850 μL of the FRAP solution for 30 min in dark condition. Readings of the colored product (ferrous tripyridyltriazine complex) were taken at 593 nm. The standard curve was linear between 200 and 1000 μM FeSO₄. Results were expressed in μM Fe (II)/g dry mass and compared with that of quercetin.

2.6. Statistical analysis

Data analysis and evaluation of statistical significance among different values determined were done using two-way ANOVA. The values were expressed as mean±SD. The individual values were compared with control values for the significance using Graph Pad Prism version 4 for Windows (Graph Pad Software, San Diego CA, USA) and considered significant at *P*<0.05.

3. Results

3.1. Estimation of total phenolic, total flavonol, total flavonoid and total antioxidant power (FRAP assay)

The total phenolic content of the extract powder was evaluated to be 120 mg/g in terms of GAE whereas, total flavonoid and total flavonol contents were found to be 40.50 and 12.25 mg/g in terms of QE, respectively. On the other hand, FRAP assay results of *M. oleifera* leaves was 85.00±5.00 in terms of micromoles of Ferric ions reduced by one gm of extract powder.

3.2. Effect of drumstick leaves on antioxidant defense system assays

In the present study, the protective ability of drumstick

leaves was assessed on diabetes-induced oxidative damage in brain (B), liver (L), kidney (K), pancreas (P) and spleen (S). Three of the major enzymes directly involved in the detoxification of ROS are SOD (scavenging superoxide anion), CAT (reducing H₂O₂ and organic hydroperoxide) and GST (reducing glutathione)^[24].

In order to explore the effect of the crude extract on the pancreatic function of the diabetic rats, lipid peroxidation and antioxidant defense system capabilities were evaluated according to appropriate methods reported in the Materials and Methods section. There was a significant elevation in MDA concentration, while the activity of SOD, CAT and GST increased in diabetic rats, when compared to normal control group.

Figure 1, 2, 3 and 4 show effect of *M. oleifera* leaves aqueous extract on the levels of SOD, GST, LPO and CAT, respectively in tissues of normal and diabetic rats. SOD content in B, L, K, P and S was significantly increased in extract treated rats by 42.3%, 86.3%, 54.2%, 52.6% and 46.7%, respectively compared to the corresponding diabetic control group. Whereas, in glipizide treated rats, it is increased by 34.6%, 37.8%, 24.1%, 46.6% and 5.9%, respectively. In diabetic rats, a significant increase in GST activities is evident in tissue samples. The levels of GST were significantly increased in B, L, K, P and S of extract-treated and glipizide-treated rats by 68.7%, 14.8%, 80.0%, 27.8% and 75% as well as 57.5%, 6.2%, 40.0%, 4.4% and 25.0%, respectively. Treatment of the rats with aqueous extract of drumstick leaves and glipizide significantly decreased the lipid peroxidation end product, MDA by 23.9%, 19.2%, 24.1%, 52.8%, 2.9% and 64.0%, 14.5%, 1.9%, 22.2%, 4.1% in B, L, K, P and S, respectively. In addition, Catalase contents were increased by 24.2%, 23.4%, 22.1%, 22.0%, 2.6% and 9.2%, 29.8%, 11.5%, 44.0%, 12.0% in B, L, K, P and S of extract-treated and glipizide-treated rats, respectively.

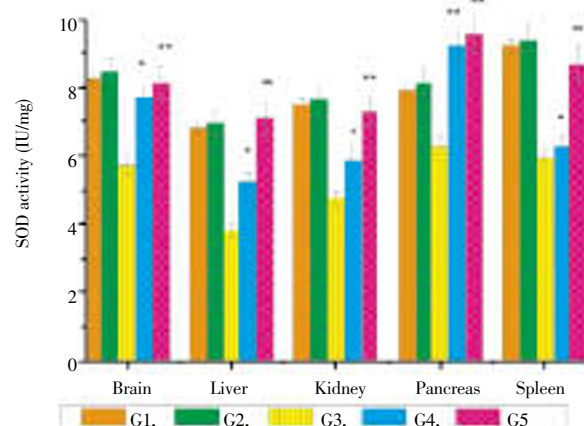


Figure 1. Effect of aqueous extract of *M. oleifera* leaves on levels of SOD in tissues of normal and STZ-diabetic rats.

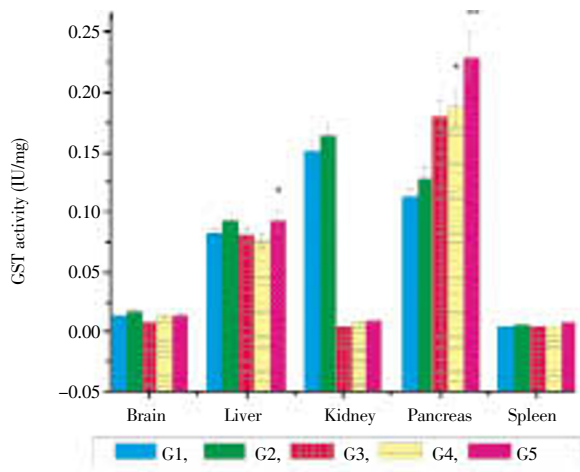


Figure 2. Effect of aqueous extract of *M. oleifera* leaves on GST content in tissues of normal and STZ-diabetic rats.

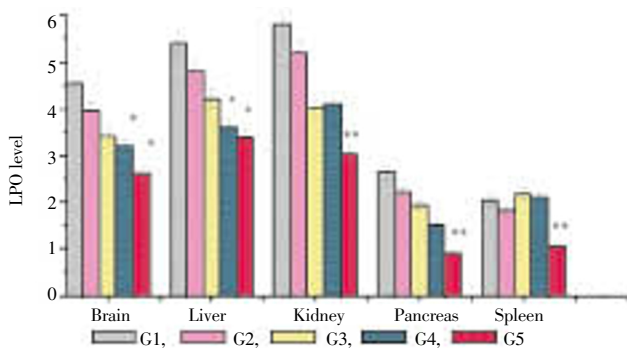


Figure 3. Effect of aqueous extract of *M. oleifera* leaves on LPO levels of normal and STZ-diabetic rats.

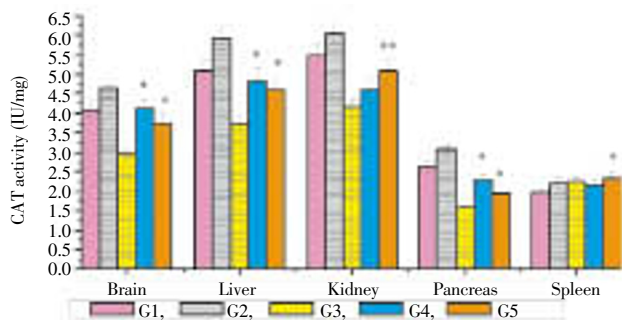


Figure 4. Effect of aqueous extract of *M. oleifera* leaves on levels of CAT in tissues of normal and STZ-diabetic rats.

4. Discussion

Oxidative damage in tissues due to the formation of ROS can be counter-balanced by the different oxidative system of the host. These defenses appear to be inducible by nutrients/non-nutrients in the diet. In addition to the protective effect of antioxidant enzymes, consumption of dietary antioxidants through the diet plays an important role against endogenous oxidative damage. Epidemiological studies show relationship

between fruit and vegetable intake and chronic diseases such as coronary heart diseases, cancer and diabetes[20]. Due to the prevalence of chronic degenerative diseases world wide, the availability of information on phytochemicals and antioxidant rich foods will help individuals to make informed choices in the consumption of food that could help and thus, protect them from such chronic diseases.

The major groups of phytochemicals contributing to the total antioxidant capacity of plant foods include polyphenols, carotenoids and antioxidant vitamins such as vitamins C and E. Leaves of *M. oleifera* have been reported to have sufficient amount of these antioxidants and are also used as green leafy vegetables of high total antioxidant capacity (260 mg/100 g). Leaves have also been found to be rich in flavonols such as total polyphenols (250 mg/100 g), quercetin (100 mg/100 g) kaempferol (34 mg/100 g) and β -carotene (34 mg/100 g)[25]. Dried powder of drumstick leaves is a rich source of Vitamin A, phenolics, glutathione, α -tocopherol and β -carotene. Its leaves extract has been reported to exhibit good antioxidant activity in the linoleic acid peroxidation system[26–31].

In the present study, the levels of both SOD and CAT activities of tissues of diabetic group were therefore, significantly increased on treatment with drumstick leaves for 21 days. The diminished SOD and CAT activities in the diabetic control group were regenerated. Decreased levels of SOD and CAT in diabetic state may be due to inactivation caused by ROS. In treated group, the increased CAT activity could be due to higher production of H_2O_2 . It is possible that CAT activity, which would protect SOD inactivation by H_2O_2 , causes an increase in SOD activity. Increase in SOD would protect CAT against inactivation by superoxide anion. Superoxides are important in the ischemic tissue injury commonly associated with diabetic microangiopathy[32] and as potential initiators of the chain reactions that can produce hydrogen peroxide.

In liver and other tissues, the glutathione system has a high capacity to deal with H_2O_2 . Some glutathione transferases can metabolize cytotoxic aldehydes produced during lipid peroxidation[33]. In the present study, it could be suggested that the increased levels of CAT activities may depend on the increased activity of GST.

LPO is the most extensively investigated process in antioxidant defense mechanism as, this is induced by extensive generation of free radicals. The abundant presence of lipids at sites where radicals in general and more specifically, ROS are formed, render them easily accessible as endogenous targets. Lipid peroxidation is one of the characteristic features of chronic diabetes[34]. Lipid peroxide mediated tissue damage has been observed in the development of both type 1 and 2 diabetes mellitus.

Some reports have shown that insulin secretion is closely associated with lipooxygenase derived peroxides. Hyperglycemia generates ROS, which causes lipid peroxidation and membrane damage. An increase in TBARS in B, L, K, P and S, as a marker of lipid peroxidation in diabetes has been observed in our study. However, oral administration of drumstick leaves extract decreases TBARS in above-mentioned tissues of diabetic rats. Hence, the present study confirms that the drumstick leaves possess an ability to inhibit the lipid peroxidation in diabetes.

Antioxidant activity of its leaves is very stable at different pH levels and also stable on storage in dark conditions^[26]. Extract prepared from *M. oleifera* leaves have shown more antioxidant activity than the synthetic antioxidants, BHA and BHT.

Aqueous extract of *M. oleifera* leaves has shown significant antioxidant activity which may be attributed to the presence of antioxidant compounds like Vitamins C, E and β -carotene in concentrated form. Aqueous extract of dried *M. oleifera* leaves have been analyzed recently for its free radical-scavenging activity in different systems of in vitro assays such as 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity, superoxide radical scavenging activity, hydroxyl radical scavenging activity and inhibition of lipid peroxidation and it was found to be a good scavenger. The aqueous extract also showed good phenol content (184 μ g/mg gallic acid equivalent) and flavonoids content (15.9 μ g/mg catechin equivalent)^[35]. Here, we have made an attempt to elucidate the mechanism of action of the antioxidant activity of the phenols, flavonoids, flavonols and total antioxidant power (FRAP) in the vitamins A, C, E rich aqueous extract of *M. oleifera* in the various tissues of STZ-induced diabetic rats.

Phenolics are major phytochemicals responsible for antioxidant activity of plant materials. The naturally occurring plant antioxidants are mostly phenolics including flavonoids, tannins, coumarins and lignins and a number of these compounds may act synergistically increasing the level of antioxidant activity within these plant products and thereby, creating their desired traditional therapeutic benefits. Plant-derived phenols in addition to their free radical scavenging and antioxidant activities have been extensively reviewed for their broad spectrum of biological activities. Drumstick leaves contain very high amount of total polyphenolics (gallic acid equivalents) and quercetin and kaempferol along with β -carotene and lutein^[36].

Overall, it could be concluded that the drumstick leaves (*M. oleifera*) bear a potent antioxidant activity which explain its ethnomedicinal use. *In vivo* antioxidant activity of the leaves in diabetes-induced oxidative stress is being reported in the present study for the first time. The extract's

constituents may thus, be assumed to scavenge free radicals and exert a protective effect against oxidative damage (induced damage) to cellular macromolecules. Drumstick leaves extract may also protect against oxidative injury induced by diabetes *in vivo* and could thus be capable of enhancing and maintaining the activities of hepatic enzymes implicated in combating ROS.

The *in vivo* as well as in vitro antioxidant studies of the aqueous extract thus, suggest that the presence of a number of polyphenols, may be responsible for the antioxidant activity. Isolation and chemical characterization of the leaves extract responsible for the antioxidant activity merits further study. Our studies on the antioxidant activity of the aforementioned plant remedy are in progress for the isolation of active constituents and to elucidate their mode of action.

Conflict of interest statement

The authors declare that there are no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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