Moringa oleifera Lam. leaf extract prevents early liver injury and restores antioxidant status in mice fed with high-fat diet

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Consumption of high-fat diet (HFD) induces nonalcoholic fatty liver disease (NAFLD) and may lead to multiple complications affecting human health. In the present study, effect of Moringa oleifera leaf extract (MoLE) in alleviating HFD induced liver injury in mice has been reported. Liver histology and serum activity of hepatic marker enzymes i.e. aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) have been studied. Lipid peroxidation (LPO), ferric reducing antioxidant power (FRAP) and reduced glutathione (GSH) were also estimated using liver homogenate. Results of the study suggested that MoLE treatment protected HFD-induced liver damage as indicated by histopathology and liver enzyme activity compared to only-HFD fed group (\(P<0.05\)). Interestingly, early signs of HFD-induced fatty liver were also alleviated by MoLE. Moreover, significant increase in endogenous antioxidant parameters and lower lipid peroxidation were found in liver of all MoLE treated groups. Results of the study indicated that MoLE has both preventive as also curative hepatoprotective activity.

**Keywords:** Antioxidant, Hepatoprotection, High fat-diet, MoLE, Moringa oleifera leaf, NAFLD

Overconsumption of high-fat diet (HFD) promotes lipid metabolism, in particular mitochondrial \(\beta\)-oxidation of fatty acids\textsuperscript{1}. This increases formation of reducing equivalents (NADH and FADH\(_2\)), circulation of electrons within mitochondrial respiratory chain (MRC) and other mitochondrial components, leading to an overproduction of reactive oxygen species (ROS)\textsuperscript{1,2}. Higher ROS production within mitochondria may subsequently favour lipid peroxidation and lead to more ROS generation in a cyclical mode\textsuperscript{1}. Several components of MRC may be oxidatively damaged by ROS whereas impairment of MRC may further increase ROS production\textsuperscript{1,3}. Numerous investigations have shown that overconsumption of diet rich in fat and/or carbohydrates is leading to fatty liver, a liver lesion which can later progress in some patients to nonalcoholic steatohepatitis (NASH)\textsuperscript{1,4}. Besides mitochondrial ROS overproduction, different deleterious events are involved in the pathophysiology of NASH including lower antioxidant defences, lipotoxicity and overproduction of pro-inflammatory cytokines such as TNF\(_\alpha\)\textsuperscript{1,4,5}.

Several drugs are being currently tested for the treatment of nonalcoholic fatty liver disease (NAFLD)\textsuperscript{1}, and recently the use of plants emerged as a possible means to alleviate NAFLD\textsuperscript{6,7}. In the present study, leaf extract of Moringa oleifera (Family: Moringaceae) may alleviate liver injury in mice fed with HFD for 15 days was tested. Further, it was determined whether the Moringa oleifera leaf extract (MoLE) may reduce ROS overproduction and oxidative stress in this experimental model. Moringa oleifera leaves contain a large number of antioxidants such as quercetin, isoquercetin, kaempferol, zeatin, rutin, \(\beta\)-carotene and ascorbic acid\textsuperscript{8}. Moringa oleifera showed hepatoprotective activity in this sub-chronic treatment which may be due to protection against oxidative stress\textsuperscript{8-10}. Presence of high antioxidative compounds of MoLE complement the nutritive role by counteracting reactive species generated during liver injury in NAFLD.

**Materials and Methods**

**Chemicals**—2,4,6-Tris (2-pyridyl)-s-triazine (TP-TZ), naphthyl ethylenediamine dihydrochloride (NED), 5, 5′-dithio-bis-2-nitrobenzoic acid (DTNB),...
butylated hydroxyanisole (BHA), dimethyl sulfoxide (DMSO), thiobarbituric acid (TBA), trichloroacetic acid (TCA) and sulfanilamide were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA). Rest of the chemicals were of analytical grade and purchased from Merck (Darmstadt, Germany).

Preparation of Moringa oleifera leaf extract (MoLE)—Leaf extract was prepared according to a method previously described by Babu et al., with some modifications. Leaves of Moringa oleifera were collected from a specific tree of Salt Lake area, Kolkata in the month of March and authenticated at Botanical Survey of India (BSI), Howrah, India (voucher no. CNH/I-I(310)/2009/Tech.II/ 352). Leaves were thoroughly washed in distilled water and dried in a vacuum oven at 50 °C for 10 h. Clean, dry leaves were crushed and 5 g of the powder was mixed with 50 mL of 80% ethanol. The mixture was stirred using magnetic stirrer in air-tight container for about 1 h and filtered afterward. Resulting filtrate was evaporated in a rotary evaporator to remove alcohol (adjustment bath: 40-45 °C, rotation: 50 rpm, pressure: ~15 psi, condenser: 4 °C). The alcohol free residue of sample was weighed (~500 mg) and dissolved in 100 mL distilled water to constitute the final extract solution (5 mg/mL).

Antioxidant activity screening of MoLE

Estimation of total polyphenol content—Total polyphenol content of MoLE was estimated according to Taga et al., with modifications. Test samples were mixed with 2% Na₂CO₃ and allowed to stand at room temperature for 2 min. At this time 50% Folin-Ciocalteu’s phenol reagent was added and reaction tube was allowed to stand for another 30 min at room temperature prior to read the absorbance at 720 nm. Gallic acid was used as standard for a calibration curve. Polyphenol content of extract was expressed in gallic acid equivalent.

Free radical scavenging activity—Free radical scavenging activity of MoLE was measured by DPPH as described by Oktay et al., with modification. Briefly, about 0.2 mM of ethanolic DPPH solution was added to the extract in separate tubes to make final concentration of the samples 1, 2, 5, 10 and 20 μg/mL. Final concentration of MoLE for the experiment was set regarding its polyphenol content (polyphenol content 0.25 μg/μL for MoLE). After 30 min of incubation at room temperature, absorbance was measured at 517 nm. BHA was used as positive control and radical scavenging activity was expressed as % inhibition and was calculated using the formula:

\[
\text{% Radical scavenging activity} = \left[ \frac{1-(\text{O.D of sample/ O.D of blank})}{\text{control O.D}} \right] \times 100
\]

Hydroxyl radical scavenging activity—It was determined according to the method described by Singh et al., with modifications. Increasing amounts of MoLE were taken in different tubes and 1 mL of iron-EDTA (0.1% ferrous ammonium sulfate and 0.26% EDTA) solution and 1 mL of DMSO (0.85% v/v in 0.1 M phosphate buffer, pH 7.4) were added to the tubes. Reaction was initiated by adding 0.5 mL of 0.22% ascorbic acid. Test tubes were capped tightly and heated on a water bath at 80-90°C for 15 min. The reaction was terminated by the addition of 1 mL of ice cold TCA (17.5% w/v). Then 3 mL of Nash reagent (75 g of ammonium acetate, 3 mL of glacial acetic acid and 2 mL of acetyl acetone were mixed and raised to 1 L with distilled water) was added to all of the tubes and left at room temperature for 15 min for color development. The absorbance was measured at 412 nm. Activity of MoLE was estimated for the concentrations of 1, 2, 5, 10, 20 μg/mL. BHA was used as a positive control. The % of hydroxyl radical scavenging activity was calculated by the following formula:

\[
\text{% Hydroxyl radical scavenging activity} = \left[ 1 - \left( \frac{\text{O.D of sample/ O.D of blank}}{\text{control O.D}} \right) \right] \times 100
\]

Metal-chelating ability—Metal-chelating activity of MoLE was assessed using the method of Decker & Welch. MoLE was first mixed with 3.7 mL of distilled water. Then it was reacted with a solution containing 0.1 mL 2 mM FeCl₃ and 0.2 mL of 5 mM ferrozine. After 10 min, the absorbance of the reaction mixture was measured at 562 nm. Metal-chelating ability of MoLE was estimated for the concentrations of 1, 2, 5, 10, and 20 μg/mL and the activity was calculated by the following equation:

\[
\text{% Metal-chelating ability} = \left[ 1 - \left( \frac{\text{O.D of the sample/ O.D of the control}}{\text{control O.D}} \right) \right] \times 100
\]

Animals—Swiss strain male albino mice (initial weight 20 ± 2 g) were used in the experiment. Animals were housed in individual cages and maintained at a 12/12 h light/dark cycle in a special room at constant temperature (18 ± 2°C) and humidity (60 ± 5%). During the 7 days of acclimatization
animals were given *ad libitum* a standard laboratory diet containing (for 100 g) 13.9 g protein, 61.8 g carbohydrate, 3.9 g fat while the remaining constituents were vitamins and minerals. All the animal experiments were performed under the guideline and supervision of Institutional Animal Ethics Committee (IAEC).

**Determination of optimum dose of MoLE**—Mice were divided into 7 groups of 6 animals each. Five groups were administered MoLE by gavage (50, 100, 150, 200 and 300 mg/kg body wt) for 15 days along with HFD feeding (simultaneous treatment for 15 consecutive days). Two other groups were control and only HFD fed group. Control group was supplied with standard laboratory diet. The high fat diet contains (for 100 g) 11.1 g proteins, 32.8 g carbohydrate and fat 23.9 g. After 15 days animals were fasted overnight and body weight of the animal for each group was observed. Liver toxicity markers aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were estimated from serum after collecting blood from the retro-orbital sinus of the animal. All MoLE treated animals showed reduction in excess body weight when compared with only HFD fed group. MoLE treated groups also displayed reduction of HFD induced increase in serum activity of AST, ALT and ALP when compared to only HFD fed group. The dose of 150 mg/kg body wt was selected as optimum dose in view of amelioration of hepatoprotection (data not shown). This optimum dose (150 mg/kg) obtained was used for the experiment.

**Development of HFD induced animal model and treatment with MoLE**—After initial acclimatization period mice were divided into 5 groups of 6 animals in each group (n=6 per group) and submitted to distinct experimental protocols for 15 consecutive days.

- **Group I (Control)**—Mice fed with a standard diet.
- **Group II (LE group)**—Mice fed with the standard diet and treated with the leaf extract.
- **Group III (HFD group)**—Mice fed with HFD.
- **Group IV (H+L group)**—Mice fed with HFD for 15 days and treated with leaf extract only for the last 5 days of the schedule to observe curative effect of MoLE.
- **Group V (HL group)**—Mice fed with HFD and treated with the leaf extract simultaneously for 15 days. For MoLE treatment, the leaf extract was administered by gavage (150 mg/kg body weight of animal).

At the end of the treatment, animals were fasted overnight. Blood was then collected in the morning from the retro-orbital sinus and serum was separated for assessment of AST, ALT and ALP. Treated animals were then sacrificed by cervical dislocation. For histology, a small section of liver tissue was preserved immediately in 10 % buffered formaldehyde fixative. The rest of the liver was stored at -20 °C until further analyses.

**Antioxidant status and lipid peroxidation**—Ferric Reducing Antioxidant Power (FRAP) and reduced glutathione (GSH) were determined to assess *in vivo* antioxidant status. Lipid peroxidation was also estimated to indirectly evaluate the generation of pro-oxidants. All these parameters were measured using liver homogenates prepared in a TRIS-EDTA-HCl buffer (pH 7.4). Protein content in liver homogenates was determined by the Lowry’s method.

The FRAP assay was carried out by using a reagent containing 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) and 20 mM ferric chloride. An aliquot of each liver homogenate was mixed with 1 mL of FRAP reagent and absorbance was measured at 593 nm by using a Smartspec spectrophotometer (Biorad, CA). FRAP was subsequently calculated from a standard curve prepared by serial dilution of a 1 mM FeSO₄ solution, and values were expressed in FRAP Units.

Liver GSH was determined by using 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB) and glutathione reductase enzyme. Briefly, liver homogenate samples and glutathione reductase were added to a cocktail containing 100 mM phosphate buffer (pH 7.5) and 100 mM NADPH. After addition of 50 mM DTNB, the resulting solutions were subsequently incubated for 30 min at room temperature. Finally, absorbance was measured spectrophotometrically at 412 nm and GSH levels were calculated from a standard curve prepared by serial dilution of a 1 mM GSH solution.

Lipid peroxidation was assessed using the thiobarbituric acid (TBA) assay, which allows measuring malondialdehyde (MDA) generated from the oxidative break-down of polyunsaturated fatty acids. Briefly, liver homogenate samples were mixed with a solution containing 20% trichloroacetic acid, 0.68% TBA and 32 mM EDTA. Mixtures were heated at 80°C for 20 min and absorbance was then measured spectrophotometrically at 535 nm. Finally, MDA levels were calculated by taking into account the extinction coefficient of MDA (1.56×10³ /M/cm).
Markers of liver injury—Activities of AST, ALT and ALP were measured from serum using enzymatic kits purchased from Span diagnostic Ltd. (Surat, India).

Liver histology—Small portions of liver were cleaned and immediately preserved in 10% buffered formaldehyde fixative. The liver slices were then processed, embedded in paraffin wax and 5 µm sections were cut and stained with hematoxylin and eosin (H&E) for histopathological examination using an Olympus 207444 light microscope (Tokyo, Japan) at 100x and 400x magnification. Photomicrographs were taken with a Canon Power Shot S70 camera.

Statistical analysis—Data was expressed as mean ± standard error of mean (SEM). One-way ANOVA performed with the Origin software (version 7.0) was used for statistical analysis between groups. Differences were considered significant at \( P < 0.05 \).

Results

In vitro antioxidant activity screening of Moringa oleifera leaf extract—MoLE is rich in polyphenols. 1µL of MoLE contains 0.25 µg of polyphenols in terms of gallic acid equivalence (graph not shown here). MoLE showed 17%, 22%, 20%, 22% and 25% higher DPPH radical scavenging activity compared to BHA for respective concentrations of 1, 2, 5, 10 and 20 µg/mL (Fig. 1A). MoLE showed 15%, 31%, 44%, 55% and 66% hydroxyl radical scavenging activity while BHA showed 14%, 29%, 39%, 48% and 57% hydroxyl radical scavenging activity for the respective concentrations of 1, 2, 5, 10 and 20 µg/mL (Fig. 1B). MoLE exhibited 33%, 30%, 33%, 40%, 33% higher metal chelating activity compared to BHA for the respective concentrations of 1, 2, 5, 10 and 20 µg/mL (Fig. 1C).

Body weight of mice—At the end of the experiment, the mean body weight of mice in different groups was 24 ± 2 g, 25 ± 2 g, 45 ± 3 g, 30 ± 2 g and 36 ± 2 g in the control, LE, HFD, HL and H+L groups, respectively. Thus, HFD significantly increased body weight and treatment with MoLE reduced diet-induced obesity.

MoLE maintains antioxidant status and diminishes lipid peroxidation—Hepatic FRAP and GSH levels were measured in different groups of mice to assess the antioxidant status. FRAP represents the total antioxidant activity excluding GSH and other thiol-containing molecules. Hence, high FRAP value indicates a greater strength to scavenge ROS and vice versa. In this study, FRAP was significantly decreased (\( P < 0.05 \)) by 66% in the HFD group compared to control indicating that HFD strongly lowered the hepatic antioxidant status (Fig. 2A). Treatment with MoLE prevented the reduction of FRAP in the H+L group and in case of HL group the rise of FRAP value was significant (\( P < 0.05 \)) when compared to HFD alone treated animals (Fig 2a).
addition, FRAP was increased in the LE group indicating that MoLE ameliorated the hepatic antioxidant status whatever the diet. Interestingly, hepatic GSH levels almost paralleled the FRAP values. Indeed, GSH content was significantly reduced ($P<0.05$) by 77% in the HFD group when compared to the control and treatment with MoLE prevented GSH depletion in both the H+L and HL groups (Fig. 2B).

Lipid peroxidation was assessed from liver homogenate using thiobarbituric acid reactive substance (TBARS) assay which allows the measurement of MDA levels. In the present study, MDA levels were significantly increased ($P<0.05$) in the HFD group when compared to the control indicating that HFD strongly favored hepatic lipid peroxidation. Interestingly, treatment with MoLE significantly prevented lipid peroxidation in both the H+L and HL groups (Fig. 2C).

MoLE reduces the markers of liver injury—Liver injury was evaluated by measuring the serum activity of AST, ALT and ALP as these enzymes are released secondarily to liver cell damage. In the present study, serum activities of these 3 enzymes were greatly enhanced in HFD group ($P<0.05$) when compared to the control group thereby suggesting HFD induced significant liver damage. Interestingly, treatment with MoLE significantly prevented ($P<0.05$) HFD-induced increase in serum activity of AST, ALT and ALP. This preventive effect was prominent in the HL group than the HFD alone treated animals. The activities of liver function marker enzymes were reduced significantly in HL group; though the activities of these enzymes were also regulated in H+L group but not to the extent of HL group in comparison to the HFD alone group (Fig. 3).

MoLE prevents and cures HFD induced liver damage—Histological examination was carried out in liver sections stained with hematoxylin and eosin. Normal hepatic architecture was observed in mice from both the control and LE groups, with intact hepatocytes radiating from the central vein (Fig. 4). In contrast, liver injury was observed in the only HFD fed group with abnormal lobular architecture and the presence of some enlarged (deformed) hepatocytes. Moreover, some hepatocytes showed the presence of lipid accumulation in liver of only HFD fed group (Fig. 5) which is prominent in higher magnification. This indicates very early stage of fully developed fatty liver. Liver damage was almost prevented by MoLE in the HL group, though this protective effect was only partial in the H+L group as some alteration of the lobular architecture could still be observed (Figs 4, 5).

Discussion

Consumption of diet rich in fat and carbohydrates promotes obesity, type 2 diabetes and nonalcoholic fatty liver disease (NAFLD) and is a serious threat for human health worldwide. Indeed, NAFLD and in particular NASH can progress to cirrhosis and
hepatocellular carcinoma\textsuperscript{21,22}. Thus, it is important to find therapies able to prevent or cure NAFLD in its earliest state. Besides modifications of life style including exercise and reduction of calorie intake, several drugs are being tested for the treatment of NAFLD\textsuperscript{1,23}. In addition, some plants could be of interest in order to treat NAFLD although only a few clinical studies have been performed\textsuperscript{24-26}. In present study, effect of leaf extract of \textit{Moringa oleifera} (MoLE) on liver injury in mice fed with HFD for 15 days has been reported. Indeed, \textit{Moringa oleifera} leaves contain a large number of antioxidants\textsuperscript{8,10} which may be beneficial in NAFLD since oxidative stress plays a key pathophysiological role in this disease\textsuperscript{1,5,27}. In the present study, potent antioxidant activity of MoLE in terms of free radical scavenging activity, hydroxyl radical scavenging power and metal chelating ability has been found (Fig. 1). Results indicated that MoLE significantly improved the status of liver injury in mice fed with HFD and this was associated with improved hepatic antioxidant status (FRAP and total GSH), lower lipid peroxidation, lower serum activity of liver function marker enzymes AST, ALT and ALP along with improved hepatocellular structure.

The possible mechanism may be due to the direct protective action of natural antioxidants present in the leaf extract. Several studies reported hepatoprotective effects of some of these antioxidants such as quercetin, rutin, and ascorbic acid\textsuperscript{28-30}. However, another mechanism may be the induction of hepatic enzymes and/or proteins involved in the protection of oxidative stress. Hence, further investigations are required to study the hepatic expression of key antioxidant enzymes and/or proteins and to determine which antioxidants are specifically involved in hepatoprotective action of MoLE in murine model of HFD-induced liver injury.

\textit{Moringa oleifera} leaves are able to protect the liver against different hepatotoxic drugs and toxins such as acetaminophen, antitubercular drugs and carbon tetrachloride\textsuperscript{9,10,31}. Moreover, extracts of \textit{Moringa oleifera} leaves, seeds and root are effective against cyclophosphamide induced toxicity, ulceration, fluoride toxicity and in the improvement of hepato-renal function\textsuperscript{32-35}. \textit{Moringa oleifera} leaf extract also ameliorates ionizing radiation induced lipid peroxidation in mice liver\textsuperscript{36}. The present study reports that MoLE showed hepatoprotection in NAFLD at its earliest possibility. In addition, the hepatoprotective action of MoLE was both preventive and curative since a beneficial effect was observed when MoLE was given only during the last 5 days of experimental protocol. Importantly, MoLE partially prevented diet-induced obesity and early signs of fatty liver. Further investigations are required to determine the mechanism(s) whereby MoLE is able to limit fat accumulation.
Fig. 4—Histopathology of mice liver, H&E; 100×. (A)-control: normal hepatic architecture; (B)-LE: normal lobular architecture same as in control group; (C)-HFD: abnormal lobular architecture, deformities in hepatocytes; (D)-H+L: protective effect of MoLE was only partial in this group as some alterations of the lobular architecture are still be observed; (E)-HL: liver damage was almost fully prevented by MoLE in this group.

Fig. 5—Histopathology of mice liver, H&E; 400×. (A)-control: normal hepatic architecture was observed in control group with intact hepatocytes radiating from central vein; (B)-HFD: abnormal lobular architecture and presence of some enlarged (deformed) hepatocytes was found in liver of this group. Some hepatocytes showed the presence of lipid accumulation in liver of HFD treated group; (C)- H+L: This group showed persistence of some alteration in lobular structure; (D)- HL: Liver damage was almost fully prevented by MoLE in HL group.
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