Indian Journal of Experimental Biology
Vol. 53, December 2015, pp. 794-802

Moringa oleifera Lam. seed extract prevents fat diet induced oxidative stress in mice and protects liver cell-nuclei from hydroxyl radical mediated damage

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Received 12 October 2014; *Revised 08 November 2015*

High fat diet (HFD) prompts metabolic pattern inducing reactive oxygen species (ROS) production in mitochondria thereby triggering multitude of chronic disorders in human. Antioxidants from plant sources may be an imperative remedy against this disorder. However, it requires scientific validation. In this study, we explored if (i) *Moringa oleifera* seed extract (MoSE) can neutralize ROS generated in HFD fed mice; (ii) protect cell-nuclei damage developed by Fenton reaction *in vitro*. Swiss mice were fed with HFD to develop oxidative stress model (HFD group). Other groups were control, seed extract alone treated, and MoSE simultaneously (HS) treated. Treatment period was of 15 days. Antioxidant enzymes with tissue nitrite content (TNC) and lipid peroxidation (LPO) were estimated from liver homogenate. HS group showed significantly higher (P <0.05) superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), reduced glutathione (GSH) activity, and ferric reducing antioxidant power (FRAP) compared to only HFD fed group. Further, TNC and LPO decreased significantly (P <0.05) in HS group compared to HFD fed group. MoSE also protected hepatocytes nuclei from the hydroxyl radicals generated by Fenton reaction. MoSE was found to be polyphenol rich with potent reducing power, free radicals and hydroxyl radicals scavenging activity. Thus, MoSE exhibited robust antioxidant prospective to neutralize ROS developed in HFD fed mice and also protected the nuclei damage from hydroxyl radicals. Hence, it can be used as herbal medication against HFD induced ROS mediated disorders.

Keywords: Antioxidants, Drumstick tree, High fat diet (HFD), Lipid peroxidation, Obesity, ROS

Consumption of high fat diet (HFD), physical inactivity and stressful life-style are now a common problem in every household. HFD induces Alzheimer's disease, atherosclerosis, cancer, diabetes, dyslipidemia, obesity, oxidative stress, pancreatitis, renal failure, and even steatosis[1-7]. The interrelation between overconsumption of fat and development of multifarious chronic disorders lie in metabolic route of fat. HFD accelerates lipid metabolism and induces excess ROS production through mitochondrial electron transport[6,8]. Moreover, HFD induced ROS triggers a number of cell signaling cascades[6] that ultimately lead to multitude of chronic disorders even cancer[9].

Commonly used vegetables are now claimed beneficial against a range of pathophysiological states. People of Indian subcontinent use varieties of vegetables and medicinal plant parts for their general health benefit either knowingly or against specific ailments from age old beliefs[10-16]. Among them, phytoextracts of *Moringa oleifera* Lam. (family Moringaceae) has been extensively used in Ayurveda and Unani medicine. It has been thoroughly characterized and its biological roles are elucidated in animal models[16-21]. Seeds and seed oil of this plant are reported to contain a large number of bioactive components (polyphenols, niazinin, niazimicin, β-sitosterol, etc.), which individually or synergistically render protection against various stress situations[16,18]. This plant is a rich source of number nutritive

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Abbreviations: $ABTS^+$, 2,2′ Azino-bis (3-ethyl-benzthiazoline-6-sulfonic acid) diammonium salt; BHA, butylated hydroxyl anisole; CAT, catalase; DMSO, dimethyl sulfoxide; DPPH, 1,1-Diphenyl-2-picrylhydrazyl; DTNB, 5,5′-Dithio-bis-2-nitrobenzoic acid; EDTA, Ethylenediaminetetraacetic acid; FFA, Free fatty acid; FRAP, Ferric reducing antioxidant power; GPx, Glutathione peroxidase; GSH, Reduced glutathione; HFD, high fat diet; LPO, Lipid peroxidation; MDA, Malondealdehyde; MoSE, *Moringa oleifera* seed extract; NED, Naphthyl ethylenediamine dihydrochloride; RNS, Reactive nitrogen species; ROS, Reactive oxygen species; SOD, Superoxide dismutase; TBA, Thiobarbituric acid; TBARS, Thiobarbituric acid reactive substances; TCA, Trichloroacetic acid; TNC, Tissue nitrite content; TPTZ, 2,4,6-tris (2-pyridyl)-s-triazine.

elements like amino acids, minerals, vitamins and other non nutritional anti-inflammatory and antioxidant components. We have earlier demonstrated that leaf extract of *Moringa oleifera* (MoLE) prevents early liver injury in HFD fed mice even after a short term treatment[19]; and also it attenuates the radiation induced lipid peroxidation[22]. Further, we observed that ferulic acid, a major active compound of Moringa leaf, alone and in combination with lipid lowering drug atorvastatin ameliorates fat diet induced stress in mice[23]. Moreover, we have shown that quercetin, a vital component of both Moringa leaf as well as seed, ameliorates the HFD-induced dyslipidemia hepatotoxicity and inflammatory situation[2,24].

In the present study, we explored whether the alkaloids, phytosterols and polyphenols rich *Moringa oleifera* seed extract (MoSE) is capable of inhibiting HFD induced ROS generation *in vivo*. The antioxidant properties of this semi purified MoSE were identified and characterized by an array of biochemical analysis. The ability of MoSE to protect the cell-nuclei against hydroxyl radical mediated challenge was also tested *in vitro*.

Methods and Materials

Chemicals— The following chemicals such as 1,1-Diphenyl-2-picrylhydrazyl (DPPH), 2,2′ azino-bis (3-ethyl-benzthiazoline-6-sulfonic acid) diammonium salt ($ABTS^+$), butylated hydroxyanisole (BHA), 2,4,6-tris (2-pyridyl)-*s*-triazine (TPTZ), Naphthyl ethylenediamine dihydrochloride (NED), 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB) were purchased from Sigma (St. Louis, MO, USA). Rest of the chemicals were of analytical grade and purchased from Merck (Darmstadt, Germany).

Preparation of M. oleifera seed extract (*MoSE*)— MoSE was prepared as per Babu *et al.*[25], with some modifications. Drumsticks of *M. oleifera* were collected from a specific tree of Salt Lake area, Kolkata and authenticated at Botanical Survey of India (BSI), Howrah, India. Seeds were collected from the drumsticks, thoroughly washed in distilled water and dried in vacuum oven at 50°C for 10 h. Clean, dry seeds were then crushed and 5 g of it was poured in 50 mL of 80% ethanol. Mixture was stirred in air-tight container and filtered. Filtrate was then evaporated using Rotary Evaporator (adjustment bath: 40-45°C, rotation: 50 × *g*, pressure: ~15psi, condenser: 4°C) to remove alcohol. Alcohol free residue of sample was weighed (500 mg) and dissolved in 100 mL distilled water to make final extract solution (5 mg/mL).

Antioxidant activity screening of MoSE

Estimation of total polyphenol content— Total polyphenol content of MoSE was quantified using the method of Taga *et al.*[26], with modifications. Test samples were mixed with 2% Na_2CO_3 and allowed to stand at room temperature (37 °C) for 2 min. Then, 50% Folin-Ciocalteu's phenol reagent was added to mixture and allowed to stand for another 30 min at room temperature prior to read absorbance at 720 nm. Gallic acid was used as standard for calibration curve. Polyphenol content of MoSE was expressed in terms of gallic acid equivalence.

Free radical scavenging activity— Free radical scavenging activity of MoSE was measured by DPPH using the method of Oktay *et al.*[27] with modifications. Ethanolic DPPH solution (0.2 mM) was added to the extract in separate tubes to make final concentration of samples 1, 2, 5, 10 and 20 µg/mL. After 30 min of incubation at room temperature, absorbance was measured at 517 nm. DPPH scavenging activity was expressed as percentage inhibition using the formula:

$$\% \text{ Radical scavenging activity} = \frac{(\text{control O.D} - \text{sample O.D})}{\text{control O.D}} \times 100$$

$ABTS^+$ scavenging activity— $ABTS^+$ scavenging activity of MoSE was estimated according to Re *et al.*[28], with some modifications. $ABTS^+$ (7 mM) was reacted with 140 mM potassium persulfate overnight in dark to yield $ABTS^+$ radical cation. Prior to use in assay, $ABTS^+$ radical cation was diluted with 50% ethanol for an initial absorbance at 734 nm, with temperature control set at 30°C. Free radical scavenging activity was assessed by mixing 1 mL diluted $ABTS^+$ with a series of test samples (MoSE) and monitoring the change in absorbance at 734 nm at 0, 1, 2, 3, 4 and 5 min intervals along with immediate reading until a steady state was achieved. Antioxidant capacity of MoSE was expressed as EC_{50}

Reducing activity— Reducing activity of MoSE was estimated by the method of Oyaizu, 1986 with some modifications[29]. Different concentrations of extract (1, 2, 5, 10 and 20 µg/mL) were mixed with 0.2 M phosphate buffer (pH 6.6) and 1% potassium ferricyanide. Mixture was incubated at 50°C for 20 min. Then 10% TCA was added and centrifuged at 1000 *g* for 10 min. Upper layer of the solution was mixed with distilled water and 0.1% $FeCl_3$ and absorbance was measured at 700 nm.

Hydroxyl radical scavenging activity— It was determined as described by Singh *et al*. [30], with some modifications. Different concentrations (1, 2, 5, 10, 20 µg/mL) of MoSE were taken in separate tubes and 1 mL of iron-EDTA (0.1% ferrous ammonium sulfate and 0.26% EDTA) and 1 mL of DMSO (0.85% v/v in 0.1 M phosphate buffer, pH7.4) were added. Reaction was initiated by adding 0.5 mL of 0.22% ascorbic acid. Reaction tubes were capped tightly and heated on a water bath at 80-90ºC for 15 min. Reaction was terminated by adding 1 mL 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 the tubes and left at room temperature for 15 min for colour development. Absorbance was measured at 412 nm. Percentage of hydroxyl radical scavenging activity was calculated using the formula:

% Hydroxyl radical scavenging activity = 1− (O.D of sample/O.D of blank) × 100

$$\%\ \text{Hydroxyl radical scavenging activity} = 1 - \frac{\text{O.D of sample}}{\text{O.D of blank}} \times 100$$

Antioxidant activity in linoleic acid emulsion system— Antioxidant activity of MoSE against lipid peroxidation in linoleic acid emulsion system was estimated by ferric thiocyanate method[31]. A reaction mixture containing MoSE (for three different concentrations 25, 50 and 100 µg/mL), 0.02 M linoleic acid emulsion and 0.2 M phosphate buffer (pH 7) was incubated at 37ºC overnight in dark. An aliquot of 0.1 mL of reaction solution was then added to 75% ethanol and 30% ammonium thiocyanate. After 3 min, 0.02 M $FeCl_2$ in 3.5 % (w/v) HCl was added to reaction mixture. Absorbance was measured at 500 nm. For all the four assays above and antioxidant activity BHA was used for comparison.

Fluorescence microscopic study of hepatocytes using Fenton reaction— Hydroxyl radical was generated using the principle of Fenton reaction. Fe−EDTA reagent was prepared immediately before the reaction by mixing equal volume of 0.6 mM Fe ammonium sulfate and 1.2 mM EDTA. A small liver tissue slice was macerated using frosted glass slide and using iron EDTA reagent. The macerated liver was taken in three microfuge tubes in equal volume of 100 µL and tubes were marked as control, H_2O_2 treated, H_2O_2 and MoSE simultaneously treated. H_2O_2 (100 µL 0.3%) and 500 µL (5 mg/mL) MoSE were added to relevant tubes. Samples were incubated at room temperature for 45 s and the reaction was stopped by adding 100 µL of 100 mM thiourea in 0.2 M EDTA. Then 50 µL of mixture from each tube was taken in three different glass slides and stained with ethidium bromide and observed under fluorescence microscope (400X). Concentration of reagents and time of incubation was appropriately standardized.

Animal treatment— Animal experiment was performed following the guidelines of Institutional Animal Ethics Committee. Twenty four Swiss male albino mice (20±2 g) were housed in individual cages and maintained at a 12/12 h light-dark cycle, 16-20°C. They were divided equally into 4 groups (n=6 in each group): Mice fed with a standard diet (control group, C); fed with standard diet and treated every day with seed extract (SE group); fed only with high fat diet (HFD group); fed with HFD + seed extract (HS group) during 15 days of treatment schedule. Animals of control and SE group were supplemented with a standard laboratory diet containing (for 100 g) 13.9 g protein, 61.8 g carbohydrate, 3.9 g fat (remaining constituents were vitamins and minerals) while HFD and HS group were supplemented with HFD containing (for 100 g) 11.1 g proteins, 32.8 g carbohydrate and 23.9 g fat[32]. The detailed composition of the control and high fat diet is given in the table 1.

Table 1— High fat diet composition compared with control laboratory diet. The standard and laboratory and high fat diet were evaluated biochemically for essential nutrient components.

Control Diet	(100 g)	Energy	HFD (100 g)	Energy
Carbohydrate	61.8	63.4	32.8	33.7
Fat				
Total Fat	3.9g	9%	23.9	54.9%
Saturated :	1.85g	-	17.42g	
Palmitic acid	1.06	-	5.32g	
Stearic acid	0.39	-	2.01g	
Lauric acid	-	-	4.80g	
Myristic acid	0.33g	-	3.41g	
Caprylic acid	-	-	0.79g	
Capric acid	-	-	0.68g	
Caproic acid	0.07g	-	0.40g	
Archidic acid	-		0.01g	
Unsaturarted:	1.24g	-	5.3g	
MUFA:	0.09g	-	4.23g	
Oleic acid	0.09g	-	4.23g	
Polyunsaturated:	1.15g	-	1.07g	
Linoleic acid	0.78g	-	0.93g	
α-linolenic acid	0.37g	-	0.14g	
Protein	13.9	14.2	11.1	11.4
Dietary fibre:	17.6	-	9.9	

Seed extract treated animals were fed through oral gavages with (150 mg/kg body wt.) seed extract. Treatment started after 7 days of acclimatization and lasted for 15 consecutive days. Thereafter, animals were sacrificed by cervical dislocation. Tissues (liver) were collected and stored in −20°C until analysis. Dose of MoSE used in the experiment was properly standardized (data not shown) and toxicity of MoSE was also validated. No lethality was observed up to 1500 mg/Kg body wt.

Determination of antioxidant profile, lipid peroxidation and tissue nitrite content from liver homogenate— Liver homogenate was prepared using TRIS-EDTA-HCl buffer (pH 7.4) and used for the estimation of Ferric reducing antioxidant power (FRAP), reduced glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), lipid peroxidation (LPO) and tissue nitrite content (TNC). Protein content of the liver homogenate was estimated by Lowry's method[33].

Estimation of FRAP— FRAP assay was done from liver homogenate using FRAP reagent[34], containing 300 mM acetate buffer (pH 3.6), 10 mM TPTZ and 20 mM ferric chloride. An aliquot (10 µL) of each sample was mixed with 1 mL FRAP reagent and reading was taken at 593 nm using spectrophotometer (Shimadzu, Tokyo, Japan). Values were calculated from standard curve prepared by 1 mM $FeSO_4$ solution.

Estimation of GSH— For GSH assessment[35], a cocktail was prepared by mixing 100 mM phosphate buffer (pH 7.5) and 100 mM NADPH. Then 5 µL of liver homogenate and 3 µL of 25 times diluted glutathione reductase (100 units/mL) were added to mixture. Finally, 50 mM DTNB was added. After 30 min of incubation at room temperature, reading was taken at 412 nm and GSH value was calculated from standard curve prepared by using 1 mM GSH.

Evaluation of SOD activity— SOD activity was determined using the involvement of superoxide anion radical and autoxidation of pyrogallol[36]. Reading was taken at 420 nm.

Evaluation of CAT activity— CAT activity was assessed by its ability to decompose 1 µmol of H_2O_2 per min and taken as 1 enzyme unit activity using the ε for H_2O_2 at 240 nm, i.e., 43.6 $M^{-1}cm^{-1}$ as done in Yumoto *et al.*[37]. For both, SOD and CAT activities, the enzyme activity was expressed in U/mg of tissue protein.

Evaluation of GPx activity— To estimate GPx activity[38], a reaction mixture (6 mL) was prepared using 50 mM phosphate buffer with 0.4 mM EDTA (pH 7), 10 µL of glutathione reductase (100 units/mL), 200 mM GSH and 100 mM β-NADPH. Reaction mixture was then diluted to 10 times with buffer. For each reaction 3 mL of this reaction cocktail and 10 µL of liver homogenate was used. GPx activity was expressed in U/mL/mg of protein using millimolar extinction coefficient of β-NADPH at 340 nm, i.e., 6.22.

Assessment of LPO— LPO was determined from tissue by thiobarbituric acid (TBA) assay[39] using TBA and TCA reagents. Liver homogenate was mixed with 20% TCA, 0.68% TBA and 32 mM EDTA. Mixture was then heated at 80°C for 20 min. Absorbance was measured at 535 nm and LPO was calculated using extinction coefficient of MDA to be 1.56×10^5 M^{-1} cm^{-1}.

Determination of TNC— TNC was estimated using Griess reagent; containing 0.1% NED, 1% sulfanilamide and 2.5% H_3PO_4[40]. Reading was taken at 540 nm and value was calculated from standard curve prepared by using 1 mM $NaNO_2$ solution. Results were expressed in terms of 'n'moles/mg of tissue protein.

Statistical analysis— Data were expressed as mean ± standard error of mean. One-way ANOVA was used for statistical analysis between groups. The *F* ratio of one-way ANOVA was considered significant when $P < 0.05$. Statistical analysis was done using Origin software (version 7.0).

Results

In vitro antioxidant activity screening of Moringa oleifera seed extract— MoSE is rich in polyphenols. In the present study, polyphenol content of MoSE was 0.125 µg/µL in terms of gallic acid equivalence (graph not shown). MoSE showed potent free radical scavenging activity by quenching DPPH. Fig. 1A depicts that DPPH radical scavenging activity of MoSE as 22, 21, 15, 22 and 20% higher than that of BHA for respective concentrations of 1, 2, 5, 10 and 20 µg/mL. Fig. 1B represents $EC_{50,}$ i.e., the concentration necessary for 50% reduction of $ABTS^+$ to be 13.98 µg/mL for MoSE and 14.16 µg/ml for BHA.

The dose response curve evidently exhibits the electron donating capacity of MoSE in terms of reducing powerfrom (Fig. 1C). MoSE exhibited 25, 29, 33, 26 and 29% higher reducing activity compared to BHA for the respective concentrations of 1, 2, 5, 10 and 20 µg/mL.

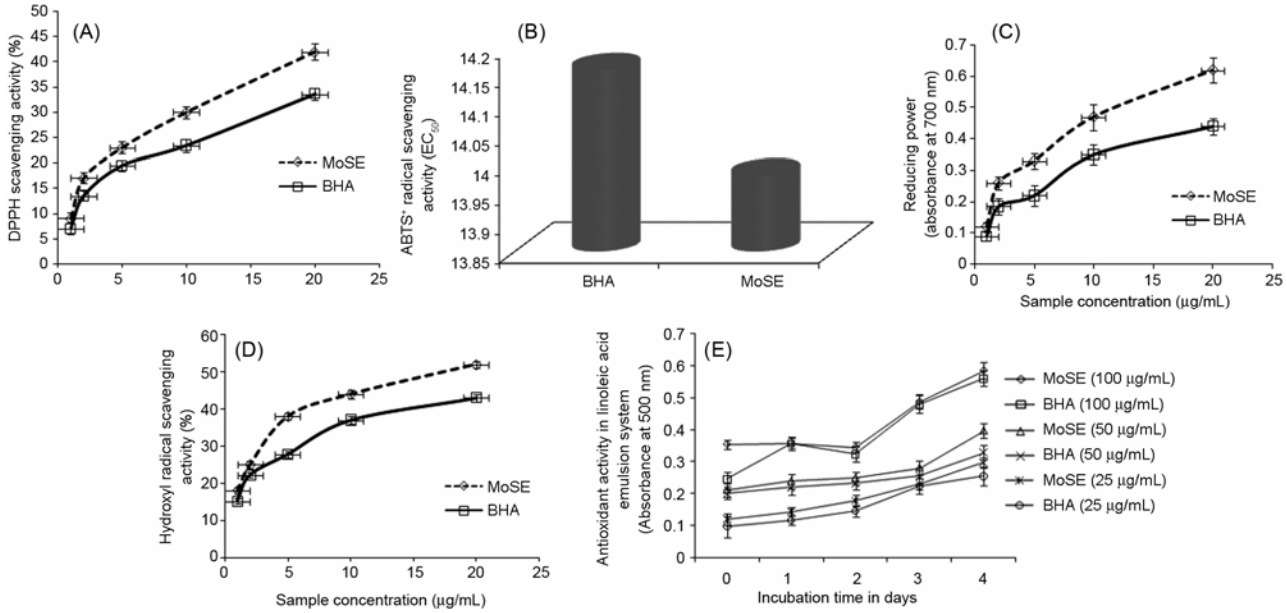

Fig. 1— Antioxidant activity of MoSE. (A) DPPH stable free radical scavenging activity; (B) $ABTS^{+}$ radical scavenging activity; (C) Reducing activity; (D): Hydroxyl radical scavenging effect; and (E): Antioxidant activity in linoleic acid emulsion. [Values are mean ± SEM of three parallel determinations. BHA represents the positive control]

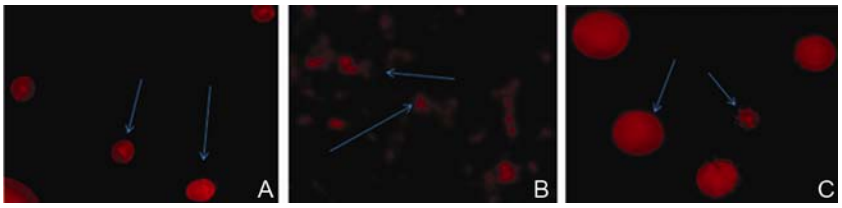

Fig. 2— MoSE prevents hepatocytes nuclei from damaging effect of hydroxyl radicals developed by Fenton reaction. Photomicrograph represents (A) Hepatocytes nuclei without any treatment (control); (B) Hydroxyl radical treated i.e., the hepatocytes went through the burden of hydroxyl radical developed by Fenton reaction; and (C) Hydroxyl radical and seed extract simultaneously treated. [Hepatocytes were treated for 45s, stained with ethidium bromide and observed under fluorescence microscope (400X)]

Moreover, MoSE showed 16, 11, 27, 16 and 17% higher hydroxyl radical scavenging activity than BHA for the respective concentrations of 1, 2, 5, 10 and 20 µg/mL (Fig. 1D).

Fig. 1E represents the antioxidant activity of MoSE on peroxidation of linoleic acid. The effect of MoSE was found overlapping on 1^{st}, 2^{nd}, 3^{rd} and 4^{th} day with BHA for 100 µg/mL. For 25 µg/mL concentration, activity of MoSE and BHA was almost similar on day 3; and for 50 µg/mL concentration, it was on day 0.However, distinguishable increased activity in MoSE was observed on day 4 for both concentrations (Fig 1E).

Fluorescence microscopic study of hydroxyl radical scavenging activity of MoSE— The damaging effect of hydroxyl radical developed by Fenton reaction and its protection by MoSE in hepatocyte nuclei is shown in Fig. 2. No damage was observed in untreated hepatocyte nuclei (Fig. 2A). Fig. 2B with damaged and ruptured cell nuclei and diffused nucleic acid revealed that the hydroxyl radical developed by Fenton reaction, damaged the membranes and even the nuclei in hepatocytes. Fig. 2C demonstrates the ameliorative action of MoSE. Here, the nuclei were either undamaged or comparatively less damaged revealing that the swelling and peripheral damage by hydroxyl radical was prevented by MoSE components.

MoSE restored endogenous antioxidant status in HFD fed mice liver— Liver was targeted for *in vivo* experimentation as liver is the major metabolic hub amongst the organs. FRAP value represents total antioxidant activity excepting GSH (thiol containing ones). Higher FRAP value depicts greater strength of sample to scavenge ROS. It is evident from Table 2 that mean FRAP value of HFD group decreased

Table 2— Effect of MoSE on antioxidant status, lipid peroxidation and tissue nitrite content of HFD fed mice liver (n=6)

Parameters	Groups			
	C	SE	HFD	HS
FRAP (FU)	2.21±0.15	3.00±0.19*	0.76±0.17**	3.10±0.16***
GSH (μmol/mg of protein)	1.23±0.06	1.62±0.27	0.38±0.13**	1.42±0.16***
SOD activity (U/mg of protein)	1.62±0.33	1.65±0.16	0.78±0.32**	2.14±0.26***
Catalase activity (U/μg of protein)	2.91±0.42	3.50±0.35	1.15±0.18**	4.10±0.29***
GPx activity (U/ml of enzyme/mg of protein)	1.34±0.09	1.42±0.16	0.74±0.09**	1.81±0.12***
TBARS (nmoles of MDA/mg of protein)	0.80±0.14	0.76±0.12	2.43±0.22**	0.76±0.15***
Tissue nitrite(nmoles/mg of protein)	42.0±3.4	44.0±2.6	126.0±4.2**	68.0±3.4***

[C (Control): Mice fed with a standard diet; SE: Mice fed with standard diet and treated with the seed extract; HFD: Mice fed with HFD; HS: Mice fed simultaneously with HFD and seed extract for continuous 15 days. All the values were mean ± SEM. *P <0.05 was considered significant and indicated by * when values were compared between C and SE group; **P <0.05 when compared between C and HFD group; ***P <0.05 when compared between HFD and HS group.]

significantly by 65% from control (P <0.05), whereas that of HS group increased significantly compared to HFD group (P <0.05).

GSH, the thiol containing antioxidant whose metabolism is highly associated with modulation of redox sensitive components of signal transduction cascade, showed 68% decrease from control (P<0.05) in HFD fed group . In contrast, HS group showed significant increase (P <0.05) in GSH content compared to HFD fed group (Table 2).

Similarly, SOD, CAT and GPx activity in HFD group also decreased significantly (P <0.05) by 5162 and 43%, respectively compared to control. Whereas HS group showed significantly increased SOD, CAT and GPx activity (P <0.05) compared to only HFD fed group (Table 2).

Malondealdehyde (MDA) content is defined as a marker of lipid peroxidation. In present study, mean MDA content of HFD group increased significantly from control (P <0.05) whereas MDA content of HS group decreased significantly (P <0.05, 68%) from HFD group (Table 2).

In present study, significantly elevated (P <0.05) TNC in HFD fed group from control has been found. In contrast, significantly lower TNC in MoSE treated group [46% decrease in HS group from HFD group (P <0.05)] has also been found (Table 2). These results thus ensure the efficacy of MoSE as a true aid to *in vivo* antioxidant status.

Discussion

HFD feeding, both for short or long term, triggers ROS generation and shifts the homeostasis towards more free radical rich oxidizing stae *in vivo*[6]. The present model of high fat diet induced stress is an acute metabolic stress replica with substantial development of systemic as well as cellular trauma, where free radicals impart a major role at all levels. The free radicals, if remains unchallenged, prove to be fatal. Consumption of staple foods, fruits and vegetable is thus important to prevent or cure this pro-oxidative status. Otherwise, it might induce several long term harmful effects in individual cells, tissues and eventually in the whole system. The phytocompounds can bring harmony against any derangement of metabolism. Bioactive phytocomponents of *Moringa oleifera* seeds are efficient radical scavengers and possess persuasive reducing capability[41]. Hence, in the present study we investigated the role of the bioactive compounds rich MoSE to prevent the formation of ROS.

The development of cellular (mitochondrial) ROS by HFD altered both the hepatic oxygen gradients and mitochondrial function reasonably *in vivo*. This leads to interruption in fatty acid oxidation, depressed bioenergetics and increased oxidative stress arising from enhanced generation of ROS and RNS (reactive nitrogen species)[42]. Pessayre *et al.*[6], also reported that HFD triggers ROS production by a vicious cycle in hepatocytes. These phenomena supposedly played a pivotal role in generation of pro-oxidative stress on HFD consumption, thereby leading to certain cellular and biochemical changes as found in this study (Table 2).

Aqueous MoSE has shown inhibition of lipid peroxidation, protein oxidation, superoxides, hydroxyl and nitric oxide radicals induced degradation and prevention of oxidative DNA damage[41]. Present results not only substantiated these findings with polyphenol rich ethanolic preparation but also demonstrated by biochemical tests that the MoSE scavenged DPPH, $ABTS^{+}$ or hydroxyl radicals

efficiently, inhibited peroxidation of linoleic acid and possessed potent reducing capability. Further, it showed that MoSE prevents membrane and nuclei damage *in vitro* (Figs 1 and 2).

Stress developed due to HFD or any other reason may lead to exhaustion of antioxidant defense pool by lowering the levels of antioxidant enzymes like CAT, GPx and glutathione reductase[43] and/or by increasing ROS production and diminishing GSH content. We found 68% reduction in GSH content in HFD group compared to control (P <0.05) and higher FRAP and GSH value in MoSE treated animals (Table 2). In addition, higher SOD, catalase and GPx activity was found in MoSE treated group compared to HFD fed group. SOD catalyzes the conversion of superoxides to H_2O_2 which is further converted to H_2O by catalase and GPx. Higher SOD content in MoSE treated group ensures this ctalaytic conversion of superoxides. Lipid peroxidation is the result of interaction between free radicals and membrane lipids. The end products of LPO are thiobarbituric acid reactive substances (TBARS) such as MDA that may cause further ROS production cyclically[6]. LPO products and ROS together may continue their detrimental effects. Low MDA content in MoSE treated group ensures lowering of lipid production and interruption in further ROS generation in this group. Higher content of NO, a significantly reactive oxidizing and inflammatory agent is already reported in HFD fed animals[40]. Similarly, in present study higher TNC content is found in HFD fed group whereas lower TNC content has been observed in MoSE treated group. MoSE antioxidant pool made it possible by scavenging elevated ROS and RNS. Endogenous tissue antioxidant pool remains intact in MoSE treated group since the phytocompounds scavenge the reactive species. Another possibility is that MoSE active components may influence cellular antioxidant mechanisms by (i) inducing enzyme activity through allosteric modulation and/or; (ii) increasing total enzyme molecule by inducing gene expression.

HFD not only develops metabolic stress but also triggers superoxide and hydroxyl anions. Several redox sensitive signaling agents activate molecules and trigger the chronic disorders. HFD alters transcript levels of more than 18 genes, reduces the expression of genes involved in free-radical scavenging and increases the expression of genes involved in stress response and signal transduction[44]. Present study confirms that this HFD induced oxidative stress has been successfully reduced by the MoSE. Apart from polyphenols, MoSE contains several other phytochemicals *viz*., flavonoids, alkaloids, phytosterols along with vitamin C and E. Taken together, this MoSE treatment not only boosts or replenishes the *in vivo* antioxidant store but also provides a protective milieu which helps to combat the oxidative insult mediated by reactive species. The present laboratory has evidenced the preventive role of phytocomponents of leaf of this plant at the molecular depth addressed with signaling pathways[19,22,24,45]. The leaf and seed share majority of the compounds. Recently, the bioactivities of Moringa flower extract have shown leishmania parasitic load reduction in liver and spleen[46]. Our laboratory has already demonstrated the therapeutic potential of Quercetin[2,24,45], the major flavonoid of the leaf and its glycone derivative Rutin[3,45], Beta Sitosterol[2], a phytosterol and Ferulic acid[23]. In most situations, the metabolic stress and the generation of overt oxidative state are major offenders of the physiological systems. The beneficial phytocomponents with their unique chemical structure and biochemical attributes prevent the disorder.

The seed or the pod of *Moringa oleifera* is consumed by a large population throughout India and its neighbour countries. It is used as nutritive health food across many ethnic populations of Afro-Asian countries. It serves as a therapeutic validation against fat diet induced oxidative stress.

Conclusion

The present study has demonstrated that MoSE or its components prevent HFD induced ROS generation and subsequent stress *in vivo*. Moreover, it prevents hydroxyl radical mediated damage of cell nuclei in the *in vitro* condition. This potential of MoSE can be used as alternative yet complementary, cost effective and safe therapeutic agent after thorough validation.

Acknowledgements

Authors acknowledge the University with potential for excellence (UPE) scheme of University of Calcutta, Kolkata from University Grant Commission, Government of India [Ref no. UGC/ST-13/Fellow UPE (SC/T)] and Department of Biotechnology, Government of India [BT/PR/10974/GBD/27/131/ 2008 dated 08.08.08] for funding. Author, SD is faculty of Centre for Research in Nanoscience and Nanotechnology and Centre for Potential for Excellence in Particular Area, UGC fund to University of Calcutta.

References

1 Durkar M, Patil RR & Naik SR, Hypolipidemic and antioxidant activity of ethanolic extract of *Symplocos racemosa* Roxb. in hyperlipidemic rats: An evidence of participation of oxidative stress in hyperlipidemia. *Indian J Exp Biol*, 52 (2014) 36.

2 Sikder K, Das N, Kesh SB & Dey S, Quercetin and β-sitosterol prevent high fat diet induced dyslipidemia and hepatotoxicity in Swiss albino mice. *Indian J Exp Biol*, 52 (2014) 60.

3 Niture NT, Ansari AA & Naik SR, Anti-hyperglycemic activity of Rutin in streptozotocin-induced diabetic rats: An effect mediated through cytokines, antioxidants and lipid biomarkers. *Indian J Exp Biol*, 52 (2014) 720.

4 Suguna P, Geetha A, Aruna R & Siva GV, Effect of thymoquinone on ethanol and high fat diet induced chronic pancreatitis—a dose response study in rats. *Indian J Exp Biol*, 51 (2013) 292.

5 Mehra P, Garg M, Koul A, Bansal DD, Effect of (+)-catechin hydrate on oxidative stress induced by high sucrose and high fat diet in male Wistar rats. *Indian J Exp Biol*, 51 (2013) 823.

6 Pessayre D, Mansouri A & Fromenty B, Nonalcoholic Steatosis and Steatohepatitis.V. Mitochondrial dysfunction in steatohepatitis. *Am J Physiol Gastrointest Liver Physiol*, 282 (2002) G193.

7 Zhang X, Dong F, Ren J, Driscoll MJ & Culver B, High dietary fat induces NADPH oxidase-associated oxidative stress and inflammation in rat cerebral cortex. *Exp Neurol*, 191 (2005) 318.

8 Rao YPC & Lokesh BR, Modulatory effects of α-linolenic acid on generation of reactive oxygen species in elaidic acid enriched peritoneal macrophages in rats. *Indian J Exp Biol*, 52 (2014) 860.

9 Barnard RJ, Prevention of cancer through lifestyle changes. *Evidence-based Compl Alt Med*, 1 (2004) 233.

10 Aggarwal A, Singla SK & Tandon C, Urolithiasis: Phytotherapy as an adjunct therapy. *Indian J Exp Biol*, 52 (2014) 103.

11 Datta S, Sinha M, Das D, Ghosh S & Dhar P, Protective effect of secondary plant metabolites from *Ipomoea aquatica* Forsk. against carbofuran induced damages. *Indian J Exp Biol*, 51 (2013) 1109.

12 Shukla K, Dikshit P, Shukla R, Sharma S & Gambhir JK, Hypolipidemic and antioxidant activity of aqueous extract of fruit of *Withania coagulans* (Stocks) Dunal in cholesterol-fed hyperlipidemic rabbit model. *Indian J Exp Biol*, 52 (2014) 870.

13 Manik S, Gauttam V & Kalia AN, Antidiabetic and antihyperlipidemic effect of allopolyherbal formulation in OGTT and STZ-induced diabetic rat model. *Indian J Exp Biol*, 51 (2013) 702.

14 Agarwal R, Gupta SK, Agarwal P & Srivastava S, Topically applied standardized aqueous extract of *Curcuma longa* L. suppresses endotoxin-induced uveal inflammation in rats. *Indian J Exp Biol*, 51 (2013) 797.

15 Yousefi K, Soraya H, Fathiazad F, Khorrami A, Hamedeyazdan S, Maleki-Dizaji N & Garjani A, Cardioprotective effect of methanolic extract of *Marrubium vulgare* L. on isoproterenol induced acute myocardial infarction in rats. *Indian J Exp Biol*, 51 (2013) 653.

16 Anwar F, Latif S, Ashraf M & Gilani AH, *Moringa oleifera*: a food plant with multiple medicinal uses. *Phytother Res*, 21 (2007) 17.

17 Krishnamurthy PT, Vardarajalu A, Wadhwani A & Patel V, Identification and characterization of a potent anticancer fraction from the leaf extracts of *Moringa oleifera* L. *Indian J Exp Biol*, 53 (2015) 98.

18 Bhatnagar M, Parwani L, Sharma V, Ganguli J & Bhatnagar A, Hemostatic, antibacterial biopolymers from *Acacia arabica* (Lam.) Willd. and *Moringa oleifera* (Lam.) as potential wound dressing materials. *Indian J Exp Biol*, 51 (2013) 804.

19 Das N, Sikder K, Ghosh S, Fromenty B, Dey S, *Moringa oleifera* Lam. leaf extract prevents early liver injury and restores antioxidant status in mice fed with high-fat diet. *Indian J Exp Biol*, 50 (2012) 404.

20 Banji OJF, Banji D & Kavitha R, Immunomodulatory effects of alcoholic and hydroalcoholic extracts of *Moringa olifera* Lam leaves. *Indian J Exp Biol*, 50 (2012) 270.

21 Ranjan R, Swarup D, Patra RC & Chandra V, *Tamarindus indica* and *Moringa oleifera* extract administration ameliorates fluoride toxicity in rabbits. *Indian J Exp Biol*, 47 (2009) 900.

22 Sinha M, Das DK, Datta S, Ghosh S & Dey S, Amelioration of ionizing radiation induced lipid peroxidation in mouse liver by *Moringa oleifera* Lam. leaf extract. *Indian J Exp Biol*, 50 (2012) 209.

23 Kesh SB, Sikder K, Manna K, Das DK, Khan A, Das N & Dey S, Promising role of Ferulic acid, Atorvastatin and their Combination in Ameliorating High Fat Diet-Induced Stress in Mice. *Life Sci*, 92 (2013) 938.

24 Das N**,** Sikder K, Bhattacharjee S, Majumdar-Bhattacharya S, Ghosh S, Majumdar S & Dey S, Quercetin alleviates inflammation after short term treatment in high fat fed mice. *Food Funct*, 4 (2013) 889.

25 Babu V, Gangadevi T & Subramoniam A, Antidiabetic activity of ethanol extract of *Cassia kleinii* leaf in streptozotocin induced diabetic rats and isolation of an active fraction and toxicity evaluation of the extract. *Ind J Pharmacol*, 35 (2003) 290.

26 Taga MS, Miller EE & Pratt DE, Chia seeds as a source of natural lipid antioxidants. *J Am Oil Chem Soc*, 61 (1984) 928.

27 Oktay M, Gulcin I & Kufrevioglu OI, Determination of *in vitro* antioxidant activity of fennel seed extracts. *Lebensmittel-Wissenschaft and Technologie*, 36 (2003) 263.

28 Re R, Pellegrini N, Proteggente A, Pannala A, Yang M & Rice-Evans C, Antioxidant activity applying an improved ABTS radical cation decolorizing assay. *Free radical Biol Med*, 26 (1999) 1231.

29 Oyaizu M, Studies on products of browning reaction: antioxidative activities of products of browning reaction prepared from glucosamine. *Jpn J Nutr*, 44 (1986) 307.

30 Singh RP, Murthy CKN & Jayaprakasha GK, Studies on the antioxidant activity of pomegranate (*Punica granatum*) peel and seed extracts using in-vitro models. Antioxidant activities of grape pomace extracts. *J Agric Food Chem*, 50 (2002) 81.

31 Takao T, Kitatani F, Watanabe N, Yagi A & Sakata K, A simple screening method for antioxidants and isolation of several antioxidant produced by marine bacteria from fish and shellfish. *Biosci Biotechnol Biochem*, 58 (1994) 1780.

32 Anai M, Funaki M, Ogihara T, Kanda A, Onishi Y, Sakoda H, Inukai K, Nawano M, Fukushima Y, Yazaki Y, Kikuchi M, Oka Y & Asano T, Enhanced Insulin Stimulated Activation of Phosphatidyl Inositol 3-Kinase in the liver of High Fat Fed Rats. *Diabetes*, 48 (1999) 158.

33 Lowry OH, Rosebrough NJ, Farr AL & Randall RJ, Protein Measurement with the folin phenol reagent. *J Biol Chem*, 193 (1951) 265.

34 Benzie IF & Strain JJ, Ferric Reducing Ability of Plasma as a measure of antioxidant power, The FRAP assay. *Anal Biochem*, 239 (1996) 70.

35 Tietze F, Enzymatic method for quantitative determination of nanogram amounts of total and oxidized glutathione, *Anal Biochem*, 27 (1969) 502.

36 Marklund S & Marklund G, Involvement of the Superoxide Anion Radical in the Autoxidation of Pyrogallol and a convenient Assay for Superoxide Dismutase. *Eur J Biochem*, 47 (1974) 469.

37 Yumoto I, Ichihashi D, Iwata H, Istokovics A, Ichise N, Matsuyama H, Okuyama H & Kawasaki K, Purification and Characterization of a Catalase from the Facultatively Psychrophilic Bacterium *Vibrio rumoiensis* S-1^T Exhibiting High Catalase Activity. *J Bacteriol*, 182 (2000) 1903.

38 Wandel A, Enzymatic Basis of Detoxification. *Academic Press*, NY, 1 (1980) 333.

39 Niehaus Jr WG & Samuelsson B, Formation of malonaldehyde from phospholipid arachidonate during microsomal lipid peroxidation. *Eur J Biochem*, 6 (1968) 126.

40 Rahman MM, Varghese Z & Moorhead JF, Paradoxical increase in nitric oxide synthase activity in hypercholesterolemic rats with impaired renal function and decreased activity of nitric oxide. *Nephrol Dial Transplant*, 16 (2001) 262.

41 Mantena SK, Vaughn Jr DP, Andringa KK, Eccleston HB, King AL, Abrams GA, Doeller JE, Kraus DW, Darley-Usmar VM & Bailey SM, High fat diet induces dysregulation of hepatic oxygen gradients and mitochondrial function *in vivo*. *Biochem J*, 417 (2009) 183.

42 Singh BN, Singh BR, Singh RL, Prakash D, Dhakarey R, Upadhyay G & Singh HB, Oxidative DNA damage protective activity, antioxidant and anti-quorum sensing potentials of *Moringa oleifera*. *Food ChemToxicol*, 47 (2009) 1109.

43 Hsu C & Yen G, Effect of gallic acid on high fat diet-induced dyslipidaemia, hepatosteatosis and oxidative stress in rats. *Br J Nutr*, 98 (2007) 727.

44 Sreekumar R, Unnikrishnan J, Fu A, Nygren J, Short KR, Schimke J, Barazzoni R & Nair KS, Impact of high-fat diet and antioxidant supplement on mitochondrial functions and gene transcripts in rat muscle. *Am J Physiol Endocrinol Metab*, 282 (2002) E1055.

45 Sikder K, Kesh SB, Das N, Manna K & Dey S, The high antioxidative power of quercetin (aglycone flavonoid) and its glycone (rutin) avert high cholesterol diet induced hepatotoxicity and inflammation in Swiss albino mice. *Food Funct*, 5 (2014) 1294.

46 Singh MK, Paul J, De T & Chakraborti T, Bioactivity guided fractionation of *Moringa oleifera* Lam. flower targeting *Leishmania donovani*. *Indian J Exp Biol*, 53 (2015) 747.