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Protective Role of *Moringa oleifera* (Sajina) Seed on Arsenic-Induced Hepatocellular Degeneration in Female Albino Rats

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Abstract In an attempt to develop new herbal therapy, an aqueous extract of the seed of *Moringa oleifera* was used to screen the effect on arsenic-induced hepatic toxicity in female rat of Wistar strain. Subchronic exposure to sodium arsenite (0.4 ppm/100 g body weight/day via drinking water for a period of 24 days) significantly increased activities of hepatic and lipid function markers such as alanine transaminase, aspartate transaminase, cholesterol, triglycerides, LDL along with a decrease in total protein and HDL. A notable distortion of hepatocellular histoarchitecture was prominent with a concomitant increase in DNA fragmentation following arsenic exposure. A marked elevation of lipid peroxidation in hepatic tissue was also evident from the hepatic accumulation of malondialdehyde and conjugated dienes along with suppressed activities in the antioxidant enzymes such as superoxide dismutase and catalase. However, co-administration of aqueous seed extract of *M. oleifera* (500 mg/100 g body weight/day for a period of 24 days) was found to significantly prevent the arsenic-induced alteration of hepatic function markers and lipid profile. Moreover, the degeneration of histoarchitecture of liver found in arsenic-treated rats was protected along with partial but definite prevention against DNA fragmentation induction. Similarly, generation of reactive oxygen species and free radicals were found to be significantly less along with restored activities of antioxidant enzymes in *M. oleifera* co-administered group with comparison to arsenic alone treatment group. The present investigation offers strong evidence for the hepato-protective and antioxidative efficiencies of *M. oleifera* seed extract against oxidative stress induced by arsenic.

Keywords *Moringa oleifera* · Arsenic · Liver function · Oxidative stress · Histoarchitecture · DNA fragmentation

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Introduction

Arsenic, the naturally occurring metalloid, exerts its carcinogenic and genotoxic effect on living organism in different parts of the world [1]. Geological distribution of arsenic leads to serious environmental calamity worldwide, but repeated uses of arsenic as herbicides; insecticides, rodenticides, food preservatives, and fossil fuel are drastically contaminating drinking water [2, 3].

Arsenic intoxication is associated with severe health hazards including dermatosis, hyperkeratosis, gangrene, and skin cancer [4, 5] despite of its few beneficial roles in the treatment of certain tropical diseases [6]. Severe diabetic disorders have been found in arsenic-intoxicated humans [7, 8]. Male and female reproductive disorders are also common in arsenic intoxication [9, 10]. Our previous studies on rat model revealed that arsenic is responsible for suppression of ovarian steroidogenesis [11] as well as elevation of adrenocortical steroidogenesis [12] when the level of arsenic is within the range found in the drinking water of West Bengal [13]. Though arsenic is very much toxic, reports reveal that arsenic trioxide is used to treat acute promyelocytic leukemia [14].

Metabolic processing of arsenical compounds is related to the production of free radicals and reactive oxygen species [15, 16], which ultimately results in DNA single-strand breakage [17]. The emerging role of reactive oxygen species in the pathogenesis of arsenic-induced hepatic disorder is established [18, 19].

In recent years, worldwide attention is focused on the potentiality of dietary antioxidants in reducing free radical-induced cellular impairment during different types of stress [15]. Vitamin E, vitamin C, selenium, folate, cobalamin (vitamin B12), and arjunolic acid are suggested to play important role in the detoxification of arsenic in different metabolic and reproductive organs [12, 20–23]. Recently, we have shown that human chorionic gonadotropin and reduced glutathione (GSH) are effective in the restoration of arsenic-mediated ovarian and uterine steroidogenic disorders via the modulation of hypophyseal-gonadal and hypophyseal-adrenal axis [24, 25].

The treatment of arsenic-intoxicated people is a great challenge nowadays. Although few drugs like British Anti Lewsite and dimercaptosuccinic acid are available in the market to combat against arsenic-associated health hazards in human, these metal chelators introduce several moderate to severe side effects such as nausea, itching, abdominal pain, hypertension, and changes in body temperature [26, 27]. Few groups of worker reported the efficacy of some plants extracts (*Phyllanthus fraternus, Terminalia arjuna*, and *Moringa oleifera*) against arsenic-induced alteration of lipid peroxidation and protein contents [28]. Among those, *M. oleifera* has an impressive range of medicinal uses with high nutritional value [29]. Anti-inflammatory efficacy of *M. oleifera* is already established [30]. *M. oleifera* is beneficial for the prevention of hyperlipidemia and hepatocytic disorders caused by iron deficiency [31], and its seed extract has been shown to protect liver from necrotic injury and fibrosis in rat model [32, 33]. Report reveals that *M. oleifera* seed powder is useful in the remediation of arsenic-induced oxidative stress in mouse [34]. Both in vivo and in vitro experimental models show that DNA can be protected from oxidative damage by *M. oleifera* [35, 36]. However, until now, very scanty information is available regarding the hepato-protective activity of *M. oleifera* seed. Thus, the goal of the present study is to determine whether in vivo arsenic-mediated metabolic toxicity of liver and hepatocellular degeneration are ameliorated by the supplementation of *M. oleifera* seed extract and, if so, to delineate whether protection of hepatic tissue is possible through protection at DNA level.

Methods

Preparation of Aqueous Extract of *M. oleifera* Seed

The seeds of *M. oleifera* were collected locally and dried in an incubator for 2 days at 40°C, crushed, and powdered in an electrical grinder. Extraction was performed by taking 50 g powder in 500 ml of distilled water for 18 h in a Soxhlet apparatus, and a deep brown aqueous extract was obtained. The extract was dried at reduced pressure and finally lyophilized.

Animal Selection and Treatment

Female Wistar rats, weighing 150±10 g were selected in this experiment and bred in the Central Animal House, Vidyasagar University, India. The animals were housed in polycarbonate cages in a room with 12-h light–dark cycle, temperature of 32±2°C and humidity of 50–70%. Animals were fed with a standard pellet diet (Hindustan Lever Ltd, Mumbai, India) and water ad libitum. Studies were carried out in accordance with the National Institutes of Health, USA guidelines. Rats were allowed for acclimatization for 10 days and caged in a multi-chambered cage where single rat was placed in a single chamber throughout the experimental schedule.

Rats were randomly separated into three groups having six in each group. Each animal in group II and group III was fed with 0.5 ml drinking water containing sodium arsenite at 6 a.m. at a concentration of 0.4 ppm/100 g body weight/day for 24 days. Remaining group of control was supplied with the same amount of drinking water without arsenic and was continued in the same duration. On the other hand, each rat in group III was supplemented with lyophilized extract of *M. oleifera* seed by gavage at a concentration of 500 mg dissolved in 0.1 ml distilled water/100 g body weight/day at the same time for 24 days. All the animals of group I and group II were supplemented with 0.1 ml distilled water/100 g body weight/day for 24 days by the same procedure. Feeding habits of all the animals were observed carefully throughout the experimental schedule. On 25th day of experiment, final body weights of all the experimental rats were noted. Rats were anesthetized by using light ether, and their blood was collected from the dorsal aorta of by a heparinized syringe (21-gauge needle), and plasma samples were separated. Organs required for biochemical and histological examinations were dissected out.

Biochemical Assay of Transaminases, Phosphatase, and Total Protein

Plasma of 0.1 ml was taken to assay alanine transaminase (ALT) and aspartate transaminase (AST) following the method of Bergmeyer [37] by using L-alanine and α-ketoglutarate as substrate for ALT, whereas L-aspartate and α-ketoglutarate were taken as substrate for the assay of AST. Enzyme activities were measured at 340 nm.

To measure the activity of alkaline phosphatase (ALP), 0.1 ml of plasma was incubated at 37°C in presence of a mixture of Tris–HCl (pH 8.0) and *p*-nitrophenyl phosphate. The activity was measured spectrophotometrically at 405 nm using visible spectrophotometer [38].

Total protein was measured following Biuret method using standard kit from Ranbaxy Diagnostic India Limited, Mumbai, India.

Estimation of Lipid Profile

The lipid components such as total cholesterol [39], HDL-C [40], and triglyceride [41] were estimated in plasma by using standard kits supplied by Ranbaxy Diagnostic Limited, Mumbai, India. VLDL-C and LDL-C were calculated from the value of triglyceride, TC and HDL-C by Friedwald and Fredickson's formula [42].

Estimation of Malondialdehyde and Conjugated Dienes Levels

Hepatic tissue was homogenized (10% *w*/*v*) in ice-cold phosphate buffer (0.1 mol/L, pH 7.4), and the homogenate was centrifuged at 15,000×*g* in 4°C for 3 min. The supernatant was used for the estimation of malondialdehyde (MDA) and conjugated dienes (CD). MDA was determined from the reaction of thiobarbituric acid (Merck, Germany) with MDA [43]. The amount of MDA formed was measured by taking the absorbance at 530 nm ($\varepsilon = 1.56 \times 10^5$ mol^{-1} cm^{-1}).

Conjugated dienes were determined by a standard method [44]. The lipids were extracted with chloroform–methanol (2:1), followed by centrifugation at 1,000×*g* for 5 min. The lipid residue was dissolved in 1.5 ml of cyclohexane, and the absorbance at 233 nm measured the amount of hydroperoxide formed.

Assay of Superoxide Dismutase and Catalase Activities in Liver

Hepatic tissue was homogenized in chilled 100 mmol/l Tris–HCl buffer containing 0.16 mol/l KCl (pH 7.4) to give a tissue concentration of 10% (*w*/*v*) and centrifuged at 10,000×*g* for 20 min at 4°C. The superoxide dismutase (SOD) activity in the supernatant was measured according to a standard protocol [45]. The reaction mixture was prepared by mixing 0.8 ml of TDB (Merck), 40 ml of 7.5 mmol/l NADPH (Sigma), 25 ml of EDTA–$MnCl_2$, and 0.1 ml of the supernatant in Tris–HCl buffer (pH 7.4) containing 0.16 mol/l KCl. The activity of SOD in this mixture was monitored using a UV spectrophotometer (Hitachi) from the rate of oxidation of NADPH.

Catalase (CAT) was assayed colorimetrically [46]. Dichromate in acetic acid was converted to perchromic acid and then to chromic acetate when heated in the presence of H_2O_2. The chromic acetate formed was measured at 620 nm. The catalase preparation was allowed to split H_2O_2 for different periods of time. The reaction was stopped at different time intervals by the addition of a dichromate–acetic acid mixture, and the remaining H_2O_2 was determined spectrophotometrically as chromic acetate. One unit of activity was expressed as a mole of H_2O_2 consumed per minute per milligram protein.

DNA Fragmentation

Liver cell pellet was treated with 500 μl of lysis buffer (50 mM Tris pH 8.0, 20 mM EDTA, 10 mM NaCl, 1% SDS, 0.5 mg/ml proteinase K) for 20 min on ice (4°C) and centrifuged in cold at 12,000×*g* for 30 min. The supernatant was extracted with 1:1 mixture of phenol: chloroform (gentle agitation for 5 min followed by centrifugation) and precipitated in two equivalence of cold ethanol and one tenth equivalence of sodium acetate. After spin down and decantation, the precipitate was resuspended in 30 μl of deionized water–RNase solution (0.4 ml water+5 μl of RNase) and 5 μl of loading buffer for 30 min at 37°C. The 1.2% agarose gel with ethidium bromide was run at 5 V for 5 min before increasing to 100 V and documented in gel documentation system [47, 48].

Statistical Analysis

Results were expressed in terms of mean and standard error of the different groups. The differences between the mean values were evaluated by ANOVA followed by multiple comparison two-tail *t* test. For all instances, $P<0.05$ was considered significant. One-way ANOVA was carried out by SPSS 10.0 software (SPSS Inc., USA).

Results

General Observations

Food and water consumption was unaltered in all groups of animal throughout the experimental schedule. At the end of the experiment, general somatic growth of arsenic-treated or *M. oleifera* co-administered groups of rat did not differ significantly from control (Table 1). After 24 days of arsenic treatment, as shown in Table 1, there was a significant increase in the hepato-somatic index compared to the normal control group ($P<0.01$). However, hepato-somatic indices were unaltered in *M. oleifera* co-administered group.

Liver Function Markers

In order to confirm the hepatocellular degeneration of hepatic tissue, activities of ALT, AST, and ALP were estimated in the arsenic-induced model group. These enzymatic activities were significantly increased though ALP activity was unaltered when compared to the normal control group ($P<0.001$; Fig. 1). Whether co-treatment with *M. oleifera* seed extracts leads to any protective role in the restoration of normal range of the above enzymes was an apparent question. A noticeable lower level of these altered enzymatic activities was observed in arsenic-fed rats with *M. oleifera* administration (Fig. 1).

Lipid Profile

The administration of arsenic in female rats caused a significant increase ($P<0.001$) of total cholesterol in plasma (Fig. 2). Elevated plasma triglyceride, LDL-C ($P<0.001$), along with low level of HDL-C, were observed due to sodium arsenite treatment. *M. oleifera* co-administration to the arsenic-treated rats, however, reduced the arsenic-induced effects significantly (Fig. 2).

Table 1 Effect of *M. oleifera* Seed Extract on General Somatic Growth along with Hepato-Somatic Indices in Arsenic-Treated Rats (Mean±SE, $N=6$) As Compared with Control, $P<0.01$ (ANOVA Followed by Multiple Comparison Two-Tailed *t* Test)

Condition	Body weight (g)		Organosomatic indices (g%)
	Initial	Final	Liver
Control	146±5[a]	170±7[a]	3.06±0.31[a]
Sodium arsenite	150±4[a]	167±6[a]	3.55±0.15[b]
Sodium arsenite + *M. oleifera*	148±7[a]	169±5[a]	3.71±0.42[b]

Same superscript letters did not differ from each other significantly

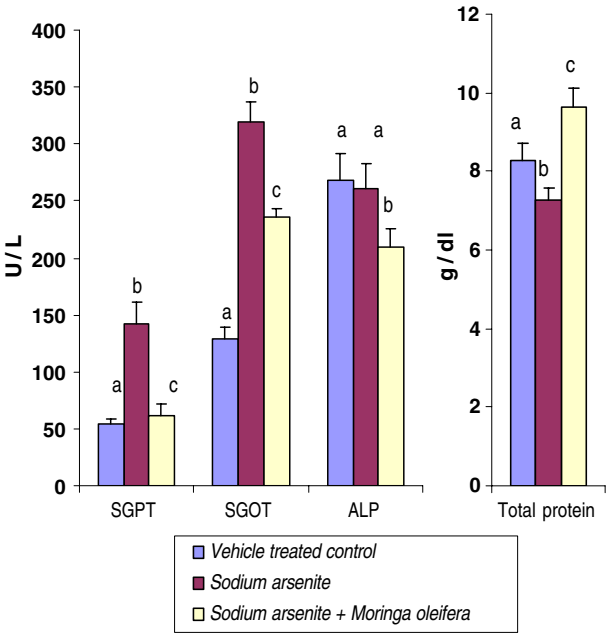

Fig. 1 Effect of *M. oleifera* on arsenic-induced hepatotoxicity (mean ± SE, $N=6$) as compared with control, $P<0.001$ (ANOVA followed by multiple comparison two-tailed t test). In *vertical column*, *same superscript letters* did not differ from each other significantly

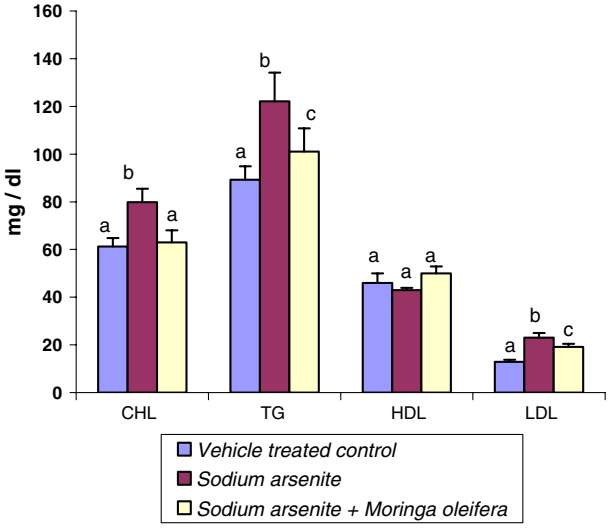

Fig. 2 Effect of *M. oleifera* on lipid profile of arsenic-treated rats (mean ± SE, $N=6$) as compared with control, $P<0.001$ (ANOVA followed by multiple comparison two-tailed t test). *Same superscript letters* did not differ from each other significantly

Status of the Oxidative Stress Markers in Liver

The MDA and CD content in liver tissue homogenates significantly increased in the sodium arsenite-treated group (Fig. 3). However, administration with *M. oleifera* extract combined with arsenic prevented MDA and CD elevation in this metabolic organ when compared to the arsenite-intoxicated group ($P<0.001$; Fig. 3).

There was a dramatic decrease in hepatic SOD and CAT activities in all arsenic-induced groups when compared to the normal control group ($P<0.001$; Fig. 3). Interestingly, higher values of SOD and CAT activities close to control level were observed in the *M. oleifera* co-administered group when compared to the arsenic only treated group ($P<0.001$; Fig. 3).

Liver Histology and DNA Fragmentation

Arsenic ingestion exerted cellular disarrangement of hepatocytes when compared to control. The *M. oleifera* co-administration to rats with arsenic has shown partial but significant protection in histoarchitecture (Fig. 4).

Sodium arsenite caused a significant amount of "ladder" of DNA fragments in liver in comparison to control rats, whereas DNA was partially but significantly protected from fragmentation in rats of *M. oleifera* co-administered group (Fig. 5).

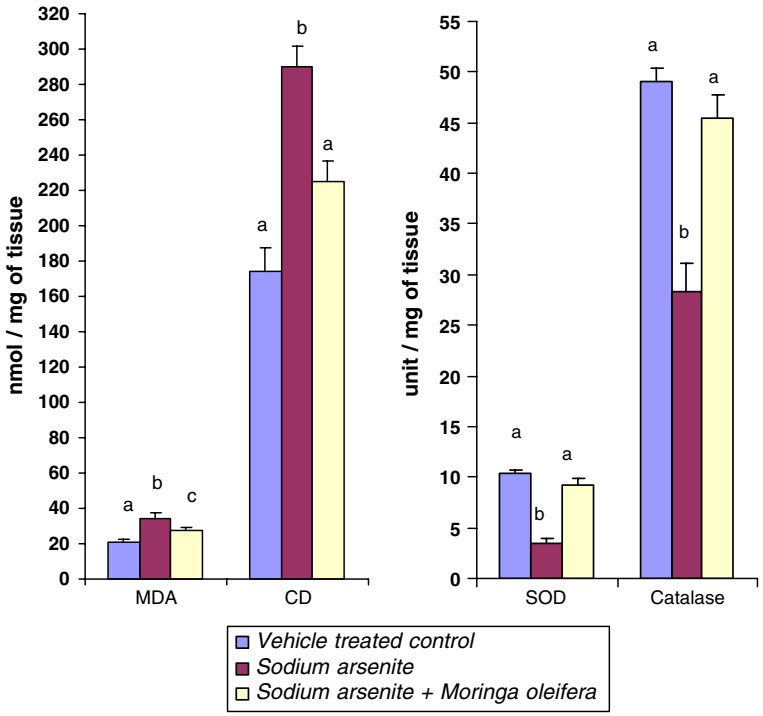

Fig. 3 Effect of *M. oleifera* arsenic-induced hepatic oxidative stress rats (mean ± SE, $N=6$) as compared with control, $P<0.001$ (ANOVA followed by multiple comparison two-tailed *t* test). *Same superscript letters* did not differ from each other significantly

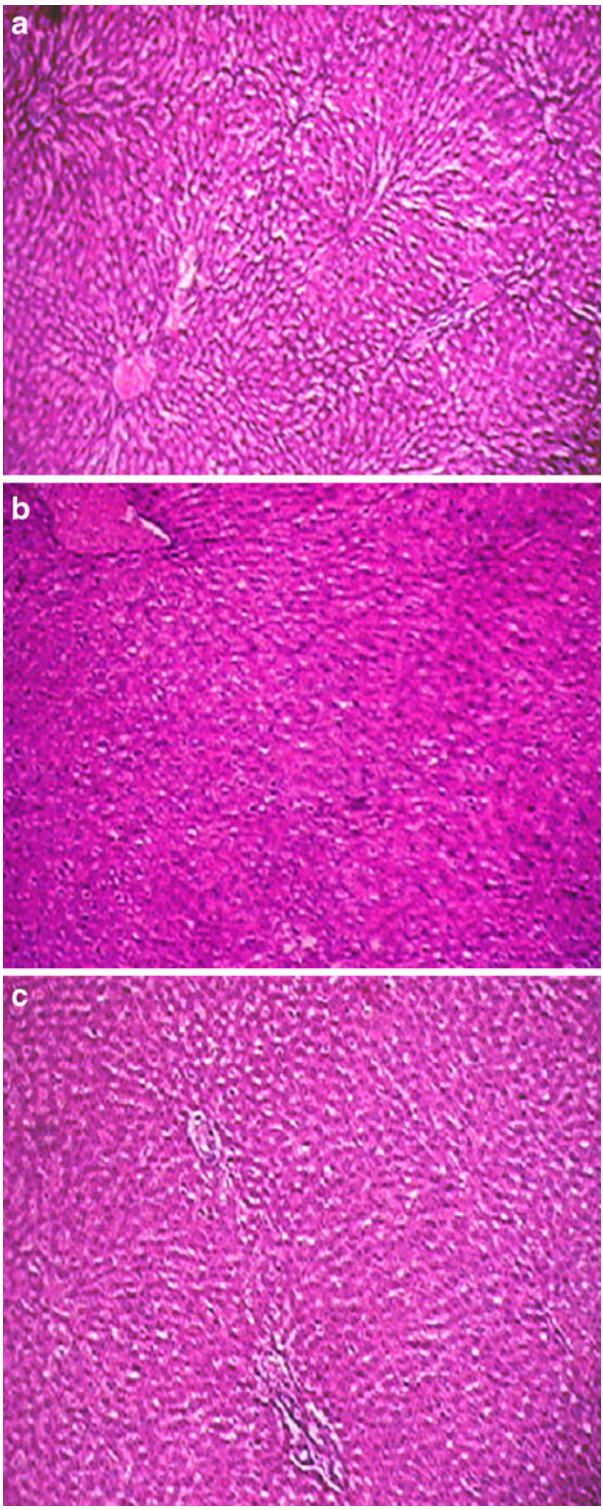

Fig. 4 Hepatic histoarchitecture (magnification ×100) of female rat treated with arsenic. Control rat (**a**) or treated with sodium arsenite (**b**) or sodium arsenite + *M. oleifera* (**c**)

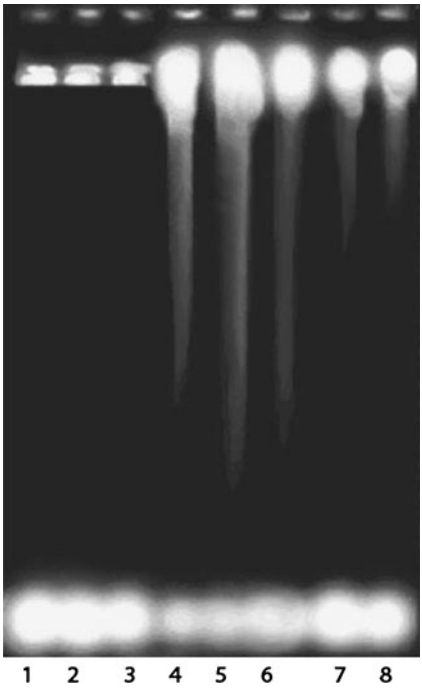

Fig. 5 DNA fragmentation in liver of female rat treated with arsenic. Control rat (*lanes 1, 2, 3*) or treated with sodium arsenite (*lanes 4, 5, 6*) or sodium arsenite + *M. oleifera* (*lanes 7, 8*)

Discussion

In this present investigation, arsenic treatment induced hepatic injury after 24 days with noticeable alterations in its liver function as compared to control rat. An increased mortality from liver cirrhosis has been reported in smelter workers following exposure to inorganic arsenic [49]. Recently, Liu et al. observed that ingestion of arsenic-contaminated drinking water caused distorted histoachitecture in liver biopsy samples [50]. All these findings are supportive in accordance with the present study.

It is evident that the damages in liver tissue occur due to necrosis, apoptosis, and that of their histological manifestations are observable in oxidative stress-induced impaired hepatic functions [22, 51]. The results of the present study reveal a significant decrease in total protein levels in liver after 24 days in rat groups treated with sodium arsenite. This could be related to the inhibition of protein synthesis by accumulation of free amino acids in liver, whereas arsenic exposure alters numerous sulfhydryl-containing proteins [52], thus probably causing a slight but significant reduction in liver protein levels in the present study.

The elevated levels of serum transaminases in sodium arsenite-treated rat may be related to the extensive alterations in the liver histology and indicate liver damage [53]. Similar increases in serum transaminases have been reported in workers exposed to high arsenic concentrations [54]. Our results explored the significant changes of lipid profile in arsenic-treated rats, and this correlates with hyperlipidemic condition where oxidative stress may be one of the major contributors, and this hyperlipidemic condition may also play a crucial role to develop hepatic disorders [55, 56]. Metabolic processing of arsenical compounds is related to the production of free radicals and reactive oxygen species [15, 16] which ultimately results in DNA strand breakage [17]. Genotoxic effects of arsenic are evident in

human lymphocytes [57]. This in turn, could be related to the altered gene expression regulation resulting in pre-malignant skin lesions [58, 59]. Interestingly, these DNA damage in lesioned skin, mammalian V79 cells could be reversed by supplementation of phytochemicals and selenium [60, 61]. This suggests that DNA damage may be the result of free radical-mediated oxidative stress. Arsenic exposure also exhibits oxidative stress through significant reduction of GSH content in liver, cultured lung epithelial cells, and discrete brain areas [62–64], and this finding is corroborated with the result of our present investigation where sodium arsenite leads to the formation of free radical via the inhibition of catalase and SOD. Recent study explored that arsenic-mediated liver injury is associated with increased oxidative stress in liver mitochondria via alteration of mitochondrial permeability transition [65].

M. oleifera co-treatment in arsenic-treated rats was capable of maintaining the hepatic functional markers and lipid profile similar to control level along with normalization of liver histoarchitecture. This protection of the above parameters may be due to the suppression of oxidative stress in arsenic-ingested female rats as evident from this study, where co-administration of *M. oleifera* seed extract with arsenic significantly restored the activities of SOD and catalase with no significant increase in MDA and CD levels in comparison to vehicle-treated animal which is supported by other investigators [28, 33]. Moreover, reduced free radical generation may be the outcome of reduced uptake of arsenic in soft tissues by increased excretion of arsenic after *M. oleifera* co-treatment [33]. Different parts of this plant have been shown to contain a very high level of protein, vitamin, beta-carotene, amino acids, and various phenolics [29, 66].

Though the zinc and calcium content of this plant body is noteworthy, the selenium (Se) content of *M. oleifera* is moderate, and this may be partially helpful for the possible antioxidant activity of this plant [67–69].

In the present investigation, DNA fragmentation assay strongly suggests that arsenic increased DNA damage in liver tissues of female rat which has been significantly prevented in rat co-administered with *M. oleifera* seed extract. The concomitant protection of the antioxidant components in this group compared with the impaired antioxidant status of arsenic-exposed group further indicate that DNA damage was resulted partially, if not completely, by the free radical-mediated attack on DNA strand. Reports reveal that the fruits of this plant contain thiocarbamate- and isothiocyanate-related phenolic compounds. Isothiocyanate group of compounds deserve anti-inflammatory activities, and interaction of this compound with arsenic has been demonstrated. Isothiocyanate has been shown to potentiate arsenic cytotoxicity in leukemic cells and results in apoptosis of cancerous cells [70–72].

So it can be suggested that the antioxidant efficacy of *M. oleifera* seed extract is not only evident against arsenic-induced mutilation of metabolic parameters but it also plays a role as strong geno-protectant agents against free radical-mediated damages. Administration of *M. oleifera* seed extract could be also expected to show its effectiveness on the course of exposure of arsenic to affected people, though wide-ranging study is required for isolation and characterization of active molecules from this plant product.

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