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RESEARCH ARTICLE

Protective effects of *Citrus limon* supplementation against arsenic-induced clinicopathological, ultrastructural, and genotoxic alterations in Swiss Albino mice

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ABSTRACT

Arsenic exposure poses significant toxic effects on various biological systems, and this study investigates the ameliorative potential of *Citrus limon* (lemon) on arsenic-induced toxicity in mice. For the experimental study, 24 Swiss albino mice were randomly divided into four groups of six mice each. Group A served as control group (C). Group B (SA) was exposed to arsenic for 28 days. Group C (CL) was supplemented *C. limon* and Group D (SA+CL) received arsenic treatment for 28 days along with *Citrus limon* supplementation. Hemato-biochemical analysis revealed that arsenic exposure resulted in macrocytic hypochromic anemia, significantly elevated liver enzymes (alanine aminotransferase, aspartate aminotransfera, alkaline phosphatase)), creatinine, cholesterol, and total bilirubin levels, and significantly reduced total protein and albumin levels. Oxidative stress markers indicated increased lipid peroxidation (LPO) and decreased levels of superoxide dismutase (SOD), glutathione reductase (GR), and catalase. Group D (SA+CL)) revealed fewer ultrastructural changes like irregular nuclear membranes, fragmented chromatin, lipid droplet accumulation, and swelling of mitochondria and rough ER and microscopic changes like hydropic degeneration, fatty changes and inflammatory changes in liver as compared to group B (SA). The sperm head abnormalities were significantly reduced and the sperm count was normal in lemon supplemented arsenic toxicity group. The formation of micronuclei in normochromatic and polychromatic RBCs was reduced in arsenic treated lemon supplemented group. In conclusion, *C. limon* administration in mice exhibited a significant protective effect against arsenic-induced alterations in hematological and biochemical parameters, along with a marked reduction in oxidative stress, pathological lesions, and genotoxic damage.

Keywords: Anemia, biochemical enzymes, gross lesions, hematology, histopathology, lemon, micronuclei, oxidative stress, sperm abnormalities

INTRODUCTION

Arsenic is a hazardous metalloid that contaminates air, water, soil, and food sources[1]. It exists in multiple chemical forms, including elemental arsenic (As^0), arsenite (As^{3+}), arsenate (As^{5+}), and arsine gas. Arsenite (As^{3+}) is considerably more toxic than organic forms[2]. Natural processes such as mineral dissolution, groundwater movement, geothermal activity; human activities like mining, industrial operations, and use of arsenic-based pesticides contribute to its environmental dissemination. Groundwater contamination with arsenic has emerged as a critical public health issue, especially in regions of India, and other parts of Southeast Asia. Human and animal exposure occurs primarily through ingestion, inhalation, and dermal absorption, with contaminated drinking water and food particularly rice and seafood being the main sources. Pesticides and insecticides, dipping fluid used to treat ectoparasites, are also significant sources of arsenic exposure for livestock.The WHO safe limit for arsenic in drinking water is 10 µg/L; however, groundwater arsenic levels in India frequently exceed this limit. Severe contamination is reported from the Gangetic plains and states such as West Bengal (50 to 3700 µg/l), Punjab, Haryana, Bihar, Assam, Uttar Pradesh, Jharkhand, Chhattisgarh, Karnataka, Madhya Pradesh, Rajasthan and northeastern states including Manipur and Tripura, with concentrations reaching up to 9,886 µg/L[50]. In West Bengal, arsenic is also elevated in rivers and ponds. Elevated blood arsenic levels, particularly noted in Bihar, have been associated with increased disease susceptibility and a higher prevalence in carcinoma patients, suggesting a link between arsenic exposure and cancer.[51] Acute exposure can cause gastrointestinal distress, neurological symptoms, and, in

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extreme cases, death. Chronic exposure is associated with a range of health complications, including cardiovascular diseases, various forms of cancer, and neurotoxicity[2]. At the cellular level, arsenic induces toxicity through the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), disruption of enzymatic functions, impairment of mitochondrial activity, and interference with key cellular signalling pathways involved in cell development, proliferation and death. These effects can lead to apoptosis and a variety of pathological conditions[3]. It also alters DNA methylation and histone modifications, which can be passed down to subsequent generations via sperm from exposed males[52].

Given the widespread exposure and severe health consequences of arsenic toxicity, the search for effective protective agents is of paramount importance. Natural compounds with antioxidant and detoxifying properties may show promise in this regard. As arsenic affects the intracellular anti-oxidant machinery; therefore, exogenous anti-oxidant supplementation can counteract the anti-oxidant stress caused by it. *Citrus limon* (lemon) is known for its high vitamin C content, flavonoids, limonoids, terpenes, fiber, pectin and other phytochemicals that offer antioxidant, vascular-protective, anti-inflammatory, anti-microbial, anti-allergic and potential anticancer effects[5]. Its phenols and flavones are known to suppress tumoro-genesis and neoplasia. It has demonstrated the ability to combat oxidative stress and enhance detoxification pathways, making it a promising candidate for the alleviation of arsenic-induced health effects. So, the present study was performed to evaluate the ameliorating effec of *Citrus limon* fruit supplementation in experimentally induced sub-acute arsenic toxicity in Swiss albino mice.

MATERIALS AND METHODS

Collection and Identification of Plant Materials

Citrus limon were collected from Local agriculture farm and were authenticated by Department of Pharmacognosy, Faculty of Pharmacy, Bhupal Nobles' University, Udaipur.

Phytochemical Analysis

Qualitative Phytochemical Analysis

The phytochemical analysis was done as per standard tests. The detection of alkaloid was done by Mayer's test; phenol by lead acetate test; flavonoid by sulphuric acid test; cardiac glycosides by Keller-Kiliani test; and terpenoids by Salkowski test[6,7].

Quantitative Phytochemical Analysis

Determination of Total Phenol by Folin-Ciocalteu Reagent Method

Total phenol content was determined by Folin-Ciocalteu reagent method with some modification and employing gallic acid as standard. The absorbance was measured for solution by using UV-spectrophotometer (UV 5704 SS, ECIL, India) at constant wavelength 750 nm. The yield of total phenolic compound was expressed in mg gallic acid per ml of plants.

Determination of Total Flavonoids by Colourimetric Method

The total flavonoids content was measured by using a modified colourimetric method with employing quercetin as standard (mg/L). The total flavonoids contents of crude extracts were estimated by aluminium chloride colourimetric method. The absorbance of samples was measured at 510 nm wavelength using spectrophotometer. Quercetin standard was used for the calibration curve.

Determination of in vitro antioxidant activity of plants by 2, 2-di-phenyl-1-picrylhydrazyl (DPPH) method

The potential of extracts to scavenge DPPH radicals was determined according to the method of Blois (1958)[8]. The absorbance of the samples and control solutions were determined at 517 nm against water. The % DPPH radical scavenging activity was calculated as follows:

% DPPH radical scavenging activity =

$[1– (A_{517}\text{nm sample} / A_{517}\text{nm control})] \times 100$

Experimental animals

A total of twenty-four healthy Swiss albino mice, weighing between 25–30 grams, were procured from the Disease-Free Small Animal House, College of Veterinary Sciences, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar. The animals were housed in the Animal House Facility of the institute under strict hygienic conditions and a controlled environment, in accordance with the guidelines of the Committee for Control and Supervision of Experiments on Animals (CCSEA). All mice had free access to pelletized feed and clean drinking water throughout the study. Prior to the commencement of the experiment, the animals were acclimatized for one week. Necessary approval was obtained from the Institutional Animal Ethics Committee (IAEC/RES/04/01) before the initiation of the experiment.

Experimental Design

Twenty-four mice were randomly divided into four groups, each consisting of six mice. Group A served as the control group. Group B, the toxicity group, received 3 mg/kg body weight (BW) of sodium arsenite (SA) orally, once daily for 28 days. Groups C was treated with 10 ml/kg BW of *Citrus limon* juice orally, once daily for 28 days. Group D received sodium arsenite and *Citrus limon* juice orally, once daily for 28 days.

Hematological analysis

Blood was collected from the retro-orbital sinus of each mouse in all experimental groups at the time of euthanasia on 28th day. Samples were drawn into dry, sterilized vials containing ethylene diamine tetra acetic acid (EDTA) and hematological parameters [Hemoglobin content (Hb),

Total Erythrocyte count (TEC), Packed Cell Volume (PCV), Total Leucocyte count (TLC), Different Leucocyte count (DLC), Total platelets count (PLT), Mean platelet volume (MPV), were analyzed using a Mindray hematology analyzer (Model No. RM-303-03, Serial No. 3903).

Biochemical analysis

To evaluate changes in serum enzyme levels induced by arsenic toxicity in Swiss albino mice, blood samples were collected in clean, sterile tubes without anticoagulant. The samples were centrifuged at 3000 rpm for 15 minutes at 4°C to separate the serum, which was then stored at -20°C until further analysis. Serum enzyme levels were estimated by using liquid stable reagent kits (Aekray Healthcare Pvt. Ltd., Surat, India). The serum analysis was carried out with the help of a biochemistry auto analyzer (CAT No. BGS–246, Biogen). The various parameters such as total protein, albumin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), creatinine, blood urea nitrogen (BUN), cholesterol (CHOL), triglyceride (TRIG) and total bilirubin (TB) were analyzed.

Oxidative stress

The liver tissue homogenate was prepared and assessed for oxidative stress as well as antioxidant enzyme activities by microprocessor UV-VIS double beam spectrophotometer (Chino Scientific Instruments MFG, Ajmer, India).

Preparation of liver tissue homogenates

A 500 mg of individually weighed liver tissue samples were dissected and washed with phosphate buffer saline (PBS). After washing, samples were taken in 5ml ice-cold PBS (pH 7.4). By the help of a teflon homogenizer MSW 346 (IKA), under ice cold condition, 10% tissue homogenate was prepared in PBS, then it was centrifuged (3000 rpm under 4°C) for 10 min to purge cellular debris and supernatant was collected. The resulting supernatant was used for determination of oxidative stress marker.

Lipid peroxidation (LPO)

Lipid peroxidation was evaluated in terms of malondialdehyde (MDA) production by using thiobarbituric acid-reactive substances (TBARS) test[9]. The absorbance was read at 535 nm. Calculation was done using the molar extinction coefficient (EC) of MDA-TBA complex (TBARS) at 535 nm, i.e. $1.56x10^8$/M/cm. The amount of LPO was expressed as nanomole of MDA formed per gm of tissue.

$$\text{LPO (nmole MDA. G}^{-1}) = \frac{\text{OD of test}}{\text{EC}} \times \frac{\text{Total volume of reaction mixture}}{\text{Volume of sample taken}} \times 10^9 \times \text{DF}$$

DF = Dilution factor i.e. 10

Superoxide dismutase

Superoxide dismutase (SOD) was estimated as per the method described earlier[10]. The absorbance was read at 570 nm against distilled water (blank). The result was expressed as SOD units (one unit of SOD was the amount of protein required to inhibit the MTT reduction by 50%).

Glutathione reductase activity

Glutathione reductase (GR) activity from tissue was estimated as per method described earlier[11] with some modification. A high GSH/GSSG ratio is essential for protection against oxidative stress and the oxidation of NADPH to NADP+ is accompanied by a decrease in absorbance at 340 nm. The enzyme activity was expressed as µmole of NADPH oxidized to NADP/mg of protein/min using the molar extinction coefficient of 6200/M/cm at 340 nm

Catalase

Catalase activity was determined by measuring the decomposition of hydrogen peroxide (H_2O_2) at 240 nm according to the method decribed earlier[12]. The decomposition of H_2O_2 was followed directly by the decrease in extinction per unit time at 240 nm. The difference in extinction per unit time is a measure of catalase. Activity of catalase was calculated using the molar extinction coefficient of 43.6 cm^{-1} and expressed a mmoles of H_2O_2 decomposed/min/mg protein.

Pathological studies

Thorough necropsy examination of all mice was carried out and gross lesions were recorded. For histopathological examination, organs (lungs, heart, liver, spleen, kidney, brain, stomach, intestines and testes) were collected in 10% neutral buffered formalin. The formalin fixed tissue were washed and processed for paraffin embedding technique[53] and then stained with routine hematoxylin and eosin staining method[54].

Ultrastructural studies

Liver samples of two mice from arsenic treated groups were processed for transmission electron microscopy following the standard method described earlier[55,56,57] with slight modifications at AIIMS, New Delhi.

Semen Evaluation

Semen was collected after the termination of study from the entire cauda epididymis. Sperm count[13] and sperm head abnormality assays (SHA) were done in mice of all groups.

Micronucleus test

The occurrence of micronuclei in control, arsenic treated and all other treatment groups were assessed by the test as described earlier[14,15].

Statistical analysis

The data for various parameters were subjected to

statistical analysis using analysis of variance (ANOVA) and t-test. Differences between group means were evaluated for statistical significance using Duncan's multiple range test at $P< 0.05$[16].

RESULTS

Phytochemical Analysis

Qualitative Estimation

Phytochemicals help to protect against various diseases due to the presence of alkaloids, flavonoids, terpenoids, cardiac glycosides and phenolic compounds. The phytoconstituents present in *Citrus limon* juice were phenols, terpenoids and flavonoids.

Quantitative Estimation

The total phenol and total flavonoid contents in *Citrus limon* juice were 4.65 mg/ml and 1.67 mg/ml, respectively.

In vitro Antioxidant activity assay

DPPH radical scavenging activity was evaluated using serial two-fold dilutions (corresponding to 0.125–8 ml juice equivalents) of *Citrus limon* juice. The results indicated a dose-dependent antioxidant activity for *Citrus limon* (lemon) juice, with higher concentrations exhibiting greater radical-scavenging activity, increasing from 27.21% to 51.41% .

Hemato-biochemical analysis

The present study revealed decreased total RBC count, hemoglobin levels, elevated Mean corpuscular volume (MCV) and reduced Mean corpuscular hemoglobin (MCH) and Mean corpuscular hemoglobin concentration (MCHC) values in arsenic-exposed mice (Table 1). Mean values of hemoglobin (g/dl) value was significantly (P<0.05) lower in arsenic intoxicated group B (SA) as compared to *C. limon* group C, and arsenic intoxicated group supplemented with Citrus limon groupD (SA+CL). The arsenic intoxicated group B had a significantly (P<0.05) higher total leukocyte count as compared to groups C (CL), and D (SA+CL) (Table 2). The group B had significantly (P<0.05) lower platelet count compared to group D (Table 2).

The results of various biochemical parameters are presented in Table 3. The AST level of arsenic intoxicated group B (SA) mice was significantly (P<0.05) higher as compared to control group A and *Citrus limon* group C (CL). The ALT levels of group B (SA) were significantly (P<0.05) higher as compared to all other groups. Similarly, ALP levels of group B (SA) were significantly (P<0.05) higher as compared to groups A, C and D (arsenic intoxicated group supplemented with *Citrus limon*). Additionally, creatinine levels in group B (arsenic intoxicated) were significantly (P<0.05) higher than other groups. The total protein level in group B (arsenic intoxicated) was significantly (P<0.05) lower compared to groups A (control), C (CL), D (arsenic intoxicated group supplemented with *Citrus limon*). Similarly, albumin level was significantly (P<0.05) lower in arsenic intoxicated group B as compared to control group A. Regarding serum cholesterol values, group B (SA) showed significantly (P<0.05) higher levels than all other groups and group C (CL) showed significantly lower level as compared to control group. Triglycerides

Table 1. Mean (Mean ± SEM) values of erythrocyte parameters of different experimental groups.

Groups	Mean ± SEM Value of Parameters					
	RBC ($10^6/\mu l$)	Hb (g/dl)	HCT (%)	MCV (fL)	MCH (pg)	MCHC (g/dl)
A (Control)	$8.63^{ab} \pm 0.24$	$15.35^{b} \pm 0.55$	$56.38^{a} \pm 1.58$	$65.55^{a} \pm 2.44$	$17.78^{a} \pm 0.34$	$27.30^{b} \pm 1.16$
B (SA)	$7.80^{a} \pm 0.20$	$13.67^{a} \pm 0.35$	$58.38^{a} \pm 1.46$	$74.96^{b} \pm 1.38$	$17.58^{a} \pm 0.57$	$23.47^{a} \pm 0.72$
C (CL)	$8.20^{ab} \pm 0.17$	$15.18^{b} \pm 0.37$	$56.87^{a} \pm 0.74$	$69.48^{ab} \pm 1.80$	$18.54^{a} \pm 0.56$	$26.68^{b} \pm 0.36$
D (SA+CL)	$8.30^{ab} \pm 0.19$	$16.03^{b} \pm 0.29$	$56.95^{a} \pm 1.51$	$68.60^{ab} \pm 0.60$	$19.37^{a} \pm 0.55$	$28.24^{b} \pm 0.83$

All values are represented as Mean ± SEM; n=6 in each group; values bearing different superscript in the same column differ significantly at P<0.05.

Table 2. Mean values of leucocyte and platelet parameters of different experimental groups.

Group	Lymphocyte ($10^3/\mu l$)	Other granulocytes ($10^3/\mu l$)	Neutrophils ($10^3/\mu l$)	Platelets ($10^3/\mu l$)	Mean Platelet Volume (fL)
A(Control)	$6.73^{a} \pm 0.42$	$0.45^{a} \pm 0.12$	$9.27^{ab} \pm 0.63$	$421.17^{b} \pm 8.57$	$8.52^{a} \pm 0.14$
B (SA)	$5.85^{a} \pm 0.74$	$0.78^{a} \pm 0.16$	$10.88^{b} \pm 0.81$	$383.17^{a} \pm 10.79$	$8.22^{a} \pm 0.18$
C (CL)	$6.37^{a} \pm 0.60$	$0.65^{a} \pm 0.29$	$8.20^{a} \pm 0.65$	$403.00^{ab} \pm 10.63$	$8.35^{a} \pm 0.20$
D (SA+CL)	$6.17^{a} \pm 0.54$	$0.72^{a} \pm 0.09$	$8.25^{a} \pm 0.51$	$420.50^{b} \pm 6.51$	$8.28^{a} \pm 0.18$

All values are represented as Mean ± SEM; n=6 in each group; values bearing different superscript in the same column differ significantly at P<0.05.

Table 3. Mean values of biochemical parameters in mice of different experimental groups.

Groups	Mean ± SEM Value of Parameters								
	Aspartate aminotrasferase (U/L)	Alanine aminotransferase (U/L)	Alkaline phosphatase (U/L)	Blood urea nitrogen (mg/dl)	Creatinine (mg/dl)	Total Protein (mg/dl)	Albumin (mg/dl)	Cholesterol (mg/dl)	Triglycerides (mg/dl)	Total bilirubin (mg/dl)
A (Control)	$55.16^{a} \pm 4.58$	$38.40^{a} \pm 4.11$	$56.80^{a} \pm 4.46$	$44.50^{a} \pm 1.86$	$0.41^{a} \pm 0.03$	$5.87^{b} \pm 0.44$	$4.60^{c} \pm 0.41$	$76.06^{a} \pm 3.49$	$259.56^{a} \pm 10.86$	$0.86^{ab} \pm 0.10$
B (SA)	$74.37^{b} \pm 6.57$	$53.25^{b} \pm 5.23$	$85.69^{b} \pm 3.30$	$48.34^{a} \pm 3.49$	$0.58^{b} \pm 0.08$	$4.77^{a} \pm 0.19$	$3.22^{a} \pm 0.13$	$128.41^{c} \pm 9.54$	$342.29^{b} \pm 13.56$	$1.26^{b} \pm 0.18$
C (CL)	$56.27^{a} \pm 3.63$	$34.43^{a} \pm 2.41$	$57.48^{a} \pm 4.88$	$42.44^{a} \pm 2.33$	$0.33^{a} \pm 0.03$	$5.83^{b} \pm 0.19$	$3.95^{abc} \pm 0.11$	$74.17^{b} \pm 5.13$	$275.82^{a} \pm 9.69$	$0.86^{ab} \pm 0.11$
D (SA+CL)	$62.32^{ab} \pm 5.01$	$44.58^{ab} \pm 2.60$	$60.81^{a} \pm 4.85$	$43.36^{a} \pm 4.19$	$0.33^{a} \pm 0.03$	$5.80^{b} \pm 0.35$	$3.57^{ab} \pm 0.25$	$61.99^{a} \pm 3.37$	$280.45^{a} \pm 13.54$	$0.85^{ab} \pm 0.13$

All values are represented as Mean ± SEM; n=6 in each group; values bearing different superscript in the same column differ significantly at P<0.05.

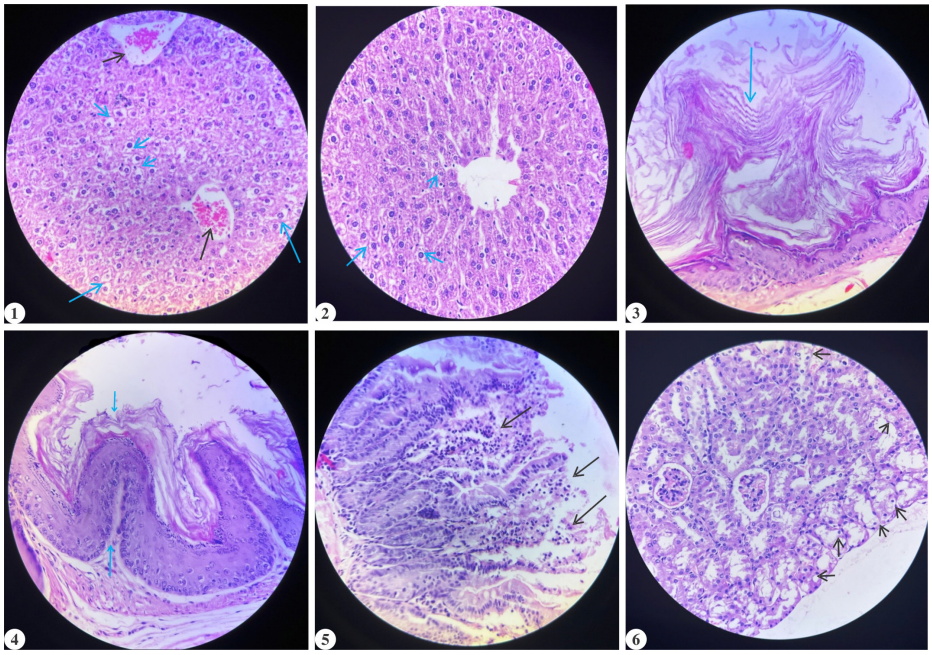

Fig. (1) and (2). Liver of arsenic intoxicated group B (1) showing hydropic degeneration and congested veins, while arsenic intoxicated and *Citrus limon* supplemented group D (2) showing mild degenerative changes (H&E 400X); **Fig. (3) and (4)**. Stomach of arsenic intoxicated group B (3) revealing hyperkeratosis in mucosa, and arsenic intoxicated and *Citrus limon* supplemented group D (4) revealing mild hyperplasia (H&E 400X); **Fig. 5.** Intestine of arsenic intoxicated group B showing villus destruction, and lymphocytic infiltration (H&E 400X); **Fig. 6.** Kidney of arsenic intoxicated group B showing hydropic degeneration in tubules (H&E 400X).

Table 4. Mean oxidative satress related parameters of mice of different experimental groups.

Groups	Mean ± SEM Value of Parameters			
	Superoxide dismutase (U/mg protein)	Lipid peroxidation (nmol/μL)	Glutathione reductase (μg/mg tissue)	Catalase (U/mg protein)
A (Control)	$17.51^{c} \pm 0.59$	$73.09^{a} \pm 0.93$	$3.86^{bc} \pm 0.23$	$40.12^{c} \pm 0.58$
B (SA)	$13.66^{a} \pm 0.41$	$93.3^{b} \pm 0.44$	$1.58^{a} \pm 0.11$	$23.67^{a} \pm 0.68$
C (CL)	$19.33^{d} \pm 0.61$	$71.84^{a} \pm 1.05$	$3.87^{bc} \pm 0.10$	$41.13^{c} \pm 0.73$
D(SA+CL)	$15.94^{b} \pm 0.26$	$73.88^{a} \pm 0.89$	$3.63^{b} \pm 0.09$	$37.28^{b} \pm 0.74$

All values are represented as Mean ± SEM; n=6 in each group; values bearing different superscript in the same column differ significantly at P<0.05.

values in group B (arsenic intoxicated) were significantly (P<0.05) higher than all other groups.

Oxidative stress

The mean values of different antioxidant enzymes are presented in Table 4. LPO (nmol/μL) levels observed was significantly (P<0.05) higher in arsenic intoxicated group B as compared to control group A, *C. limon* group C, and arsenic intoxicated group D supplemented with *Citrus limon*. SOD (U/mg protein) levels were significantly (P<0.05) lower in group B compared to all other groups (A, C, and D);. Additionally, SOD activity in group B was significantly (P<0.05) lower than in groups A, D, but remained higher than in group B. While group D showed significantly higher SOD levels than group A indicating antioxidant effect

Table 5: Mean (Mean ± SEM) values of total sperm count, and number of normal sperms, and abnormal sperms (out of total 200 sperms examined) in mice of different experimental groups.

Groups	Sperm Count ((x10^6 sperm/ml))	Normal	Sperm Head Abnormality Assay				
			Head less tail	Detached head	Pairing phenomena	Amorphous head	Hook less Head
A	36.67^{bc} ± 2.47	183.83^{b} ± 0.60	6.17^{ab} ± 0.31	5.17^{a} ± 0.54	4.82^{a} ± 0.48	0.00^{a} ± 0.00	0.00^{a} ± 0.00
B	26.67^{a} ± 1.67	177.50^{a} ± 0.76	8.50^{b} ± 0.43	6.83^{b} ± 0.60	5.65^{bc} ± 0.56	0.83^{b} ± 0.17	0.67^{b} ± 0.42
C	41.67^{c} ± 4.22	184.50^{b} ± 0.97	5.50^{a} ± 0.43	5.33^{ab} ± 0.40	4.66^{a} ± 0.42	0.00^{a} ± 0.00	0.00^{a} ± 0.00
D	30.83^{ab} ± 3.75	183.33^{b} ± 0.84	4.82^{a} ± 0.48	5.67^{ab} ± 0.56	5.50^{b} ± 0.43	0.50^{a} ± 0.22	0.17^{a} ± 0.17

All values are represented as Mean ± SEM; n=6 in each group; values bearing different superscript in the same column differ significantly at P<0.05.

Fig. 7. Kidney of arsenic intoxicated group B showing marked interstitial inflammatory cell infiltration, mainly in the peritubular and perivascular areas (mainly lymphocytes and some macrophages) (H&E 400X); **Fig. 8.** Arsenic intoxicated *Citrus limon* supplemented group D Kidney section shows marked perivascular mononuclear cell infiltration with congestion and mild tubular epithelial degeneration (H&E 400X); **Fig. 9.** Testes of arsenic intoxicated group B show degeneration of seminiferous tubule characterized by disorganization of germinal epithelium and reduction in mature spermatozoa within the lumen (H&E 400X); **Fig. 10.** Testes of arsenic intoxicated *Citrus limon* supplemented group D show normal testicular structure (H&E 400X); **Fig. 11.** Arsenic intoxicated group B indicates perivascular cuffing, slight increase in small, dark glial nuclei scattered in neuropil (H&E 400X); **Fig. 12.** Arsenic intoxicated *Citrus limon* supplemented group D showing mild clear spaces surrounding neurons and small vessels representing edema (H&E 400X).

of plant extract that led to increased SOD activity and better neutralization of free radicals formed as a result of arsenic toxicity.

Glutathione reductase (µg/mg tissue) activity was significantly lower (P<0.05) in group B compared to groups A, C, and D. Catalase (µg/mg tissue) activity was significantly lower (P<0.05) in group B compared to all other groups. Additionally, catalase activity in groups A, and C, was significantly (P<0.05) higher than in groups B, and D. Similarly, arsenic intoxicated group D supplemented with *C. limon* showed significantly (P<0.05) higher catalase activity compared to arsenic intoxicated group B.

Pathological studies

Control group A and *Citrus limon* group C did not show pathological lesions in any organ. Arsenic exposure (group B) induced mild to moderate gross pathological alterations in liver, lung, heart, kidney, spleen, brain, stomach, intestines and testes. Microscopically, the liver of arsenic intoxicated group B exhibited edema, hepatocellular vacuolation, hydropic degeneration and congested veins, while arsenic intoxicated group D supplemented with *Citrus limon* showed mild changes (Fig. 1and 2). Gastric lesions in group B included mucosal congestion, and hyperkeratoses, these changes were milder in group D (Fig.3 and 4). The intestine of group B revealed mucosal congestion, hemorrhages, focal villus destruction, and lymphocytic infiltration (Fig. 5). These effects were absent in treated groups, which showed normal architecture. Similarly, kidneys of group B showed congestion, tubular degeneration, vacuolization and lymphocytic infiltration (Fig. 6 and 7). Marked perivascular mononuclear cell infiltration with vascular congestion and mild

Table 6. Mean values of polychromatic erythrocytes (PCE), normochromatic erythrocytes (NCE) and micronucleated polychromatic (MNPCE) and micronucleated normochromatic erythrocytes (MNNCE) in mice of different experimental groups

Groups	PCE (%)	NCE (%)	PCE/NCE	MNPCE (%)	MNNCE (%)
A (Control)	$28.57^{b} \pm 1.07$	$71.43^{b} \pm 1.07$	$0.40^{b} \pm 0.02$	$0.16^{a} \pm 0.03$	$0.16^{a} \pm 0.03$
B (SA)	$35.00^{c} \pm 0.34$	$65.00^{a} \pm 0.34$	$0.54^{c} \pm 0.01$	$0.26^{b} \pm 0.03$	$0.29^{b} \pm 0.03$
C (CL)	$26.63^{ab} \pm 1.35$	$73.37^{bc} \pm 1.35$	$0.37^{ab} \pm 0.03$	$0.12^{a} \pm 0.02$	$0.13^{a} \pm 0.02$
D(SA+CL)	$27.73^{ab} \pm 0.90$	$72.27^{bc} \pm 0.90$	$0.38^{ab} \pm 0.02$	$0.15^{a} \pm 0.01$	$0.17^{a} \pm 0.02$

All values are represented as Mean ± SEM; n=6 in each group; values bearing different superscript in the same column differ significantly at P<0.05.

tubular epithelial degeneration and were noted in group D (Fig. 8). Lastly, the testes of group B showed degeneration of seminiferous tubules, characterized by disorganization of germinal epithelium, reduced stratification of spermatogenic cells, marked reduction in mature spermatozoa within the lumen, detached germ cells visible within tubular lumen and slight widening of interstitial spaces (Fig. 9) while group D maintained normal testicular structure (Fig. 10), indicating protective efficacy against arsenic-induced testicular damage. Lung examination revealed congestion, and edema in group B, while groups D had mild changes. Brain of arsenic intoxicated group B, revealed mild cerebral edema and perivascular cuffing, slight increase in small, dark glial nuclei scattered in neuropil (Fig. 11), while group D revealed no marked perivascular cuffing, though clear spaces surrounding neurons and small vessels (Fig. 12) were noticed.

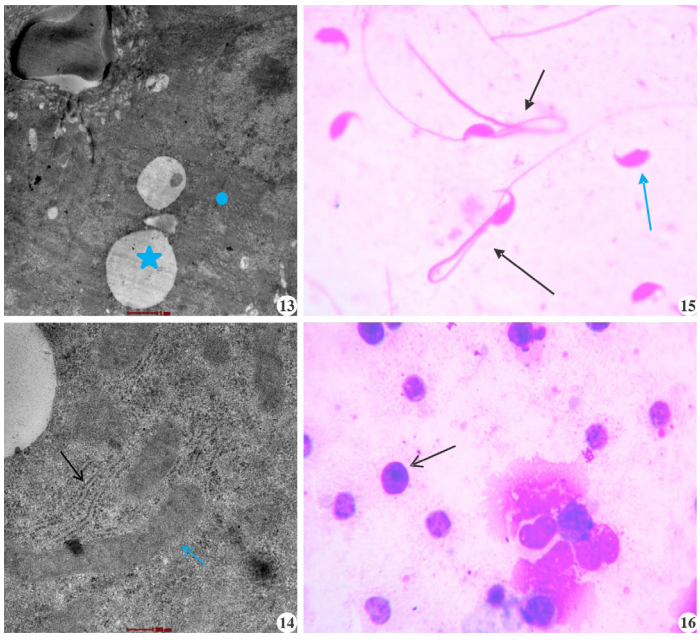

Fig. 13. Arsenic intoxicated group B liver tissue revealed vacuolation in the cytoplasm (*) and unclear mitochondrial cristae and RER (•); **Fig. 14.** Arsenic intoxicated *Citrus limon* supplemented group D the endoplasmic reticulum (ER) showed normal ribosomal attachment, mild mitochondrial swelling and mild vacuolation was observed; **Fig. 15.** Photomicrograph of sperms of group B showing Pairing Phenomena (black arrow) and detached head (blue arrow) (1000X); **Fig. 16.** Bone marrow slide of group B showing polychromatic erythrocytes (PCEs) with moderate basophilia and prominent small, dark purple body consistent with micronuclei (1000X).

Ultrastructural studies

The ultra-structural analysis of arsenic intoxicated group B liver tissue revealed significant cellular alterations, including vacuolation in the cytoplasm indicating hydropic degeneration, an irregular nuclear membrane, mitochondrial cristae and intact RER were not clearly defined (Fig. 13). In group D (arsenic intoxicated group supplemented with *Citrus limon*), the endoplasmic reticulum (ER) showed normal ribosomal attachment, mild mitochondrial swelling and mild vacuolation was observed (Fig. 14).

Semen evaluation

Sperm count- The mean values of sperm count of all mice of different experimental groups are presented in Table 5. The sperm count was significantly lower (P<0.05) in arsenic intoxicated group B compared to all other groups A, C and D (arsenic intoxicated group supplemented with *Citrus limon*).

Sperm head abnormality assay (SHA)

The mean value of frequency of sperm head abnormalities in all groups of mice are shown in Table 5. Head-less tail, detached head, amorphous head, and hook-less head was significantly (P<0.05) increased in arsenic intoxicated group B compared to groups A, and C. Pairing phenomena (Fig. 15) were also significantly (P<0.05) higher in group B compared to control D group .

Micronucleus test (MNT)

The mean value of frequency of PCE, NCE and PCE/NCE ratio and mean value of frequency of MNPCE (Fig. 16), and MNNCE of all groups of mice are shown in Table 6. The PCE (%) was significantly (P< 0.05) higher in group B (arsenic intoxicated) compared to all other groups. The NCE (%) was

significantly (P< 0.05) lower in group B as compared to other groups (A, C and D). The PCE/NCE ratio was significantly (P<0.05) higher in group B as compared to the other groups. The percentages of MNPCE, and MNNCE were significantly (P<0.05) increased in group B compared to groups A, C and D.

DISCUSSION

The phytochemical analysis of *C. limon* fruit revealed the presence of flavonoids, terpenoids, and phenolic compounds, similar to other researchers work[17,18]. The phenolic compounds are recognized for their ability to mitigate oxidative damage. These compounds can directly neutralize free radicals or engage with antioxidant enzymes to scavenge them. Additionally, phenolic substances have been shown to contribute to the stabilization of lipid peroxidation. The flavonoids present in *C. limon* also contribute to good free radical scavenging activity of these plants and play a crucial role in mitigating the toxicity of heavy metals in plants. These bioactive compounds act as potent antioxidants, chelating metal ions and reduce oxidative stress induced by metal toxicity and therefore protect cellular structures from damage[19].

Hematological parameters are critical indicators of an animal's health status, providing insights into the physiological impact of intoxication. In the present study, arsenic exposure in Swiss albino mice led to significant hematological alterations, notably a decrease in RBC count and hemoglobin levels, alongside elevated mean corpuscular volume (MCV) and reduced mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC). These changes suggest arsenic-induced macrocytic hypochromic anemia, likely due to liver dysfunction, disrupted erythropoiesis and oxidative damage to erythrocytes. Ola-Davies and Akinrinde (2016)[20] reported that arsenic inhibits porphyrin and haem synthesis, resulting in decreased hemoglobin and RBC levels.

In contrast, supplemented group showed hematological values comparable to the control group, indicating the protective role of *C. limon* (lemon). Their ameliorative effects are attributed to bioactive compounds such as polyphenols, flavonoids, ascorbic acid, steroidal saponins, and alkaloids.. Lemon juice has been shown to improve RBC count and hemoglobin levels, attributed to its rich antioxidant content[23]. Flavonoids in lemon juice protect hemoglobin from oxidative degradation, and antioxidants mitigate erythrocyte destruction[23].

Arsenic exposure significantly increased total WBC count (P<0.05) in group B compared to other groups, with elevated neutrophil levels, while lymphocytes and other [mid-sized cells (MID) *viz*. Monocyte, eosinophils and basophils)cell counts remained unchanged. These findings suggest an immune response to arsenic toxicity, consistent with previous reports linking arsenic exposure to leukocytosis and immune system activation[24]. The supplemented groups exhibited no significant changes in total and differential leukocyte counts, suggesting immune-protective effects of *C. limon*. Vitamin C, a key constituent of *C. limon*, supports immune function by accumulating in neutrophils and enhancing chemotaxis, phagocytosis, and microbial killing[25].

Biochemical markers of liver and kidney function such as ALT, AST, ALP, creatinine, and urea were significantly elevated in arsenic-exposed mice, indicating hepatocellular damage and renal impairment. These findings were consistent with earlier findings attributing these changes to arsenic-induced oxidative stress, lipid peroxidation, and membrane disruption[26]. Elevated creatinine further confirms nephrotoxicity. Serum total protein and albumin levels were significantly reduced in arsenic-exposed mice, reflecting impaired liver function and possibly increased renal protein loss. These findings corroborated previous studies reporting arsenic-induced hypoproteinemia, likely due to oxidative damage and altered protein metabolism[20]. Additionally, arsenic exposure disrupted lipid metabolism, causing hepatic fat degeneration and elevated serum cholesterol and triglycerides[27]. Arsenic-induced hyperbilirubinemia may result from hemolysis, impaired hepatic uptake, and oxidative modification of bilirubin. In this study, arsenic exposure significantly increased total cholesterol, triglycerides, and total bilirubin levels. Co-treatment with *C. limon* restored serum ALT, AST, ALP, BUN, and creatinine levels to near normal[28].

Arsenic exposure disturbs redox homeostasis by inducing excessive ROS production, resulting in oxidative damage to cells. The arsenic-induced oxidative stress leads to an overproduction of superoxide anion radicals, resulting in a significant reduction in superoxide dismutase (SOD) activity. This reduction causes the down-regulation of SOD2 gene expression, which has been linked to oxidative stress in various tissues. Additionally, reduced SOD and catalase levels indicate a compromised antioxidant defense system, unable to neutralize the excessive ROS generated during arsenic metabolism[29]. Similarly, in the current study the significant decrease (P<0.05) in Superoxide dismutase activity in As-exposed mice group compared to the control group was observed. Arsenic-induced oxidative damage reduces GR activity, impairing GSH regeneration and causing cellular dysfunction[30]. Similarly, in the current study the significant (P<0.05) decrease in glutathione reductase activity in As-exposed mice group compared to the control group was observed. In the present study, a significant decrease (P<0.05) in CAT

activity was observed in arsenic-exposed mice compared to the control group. Arsenic exposure reduces CAT activity by depleting NADPH, which is essential for reactivating CAT from its inactive form during arsenic metabolism. Similar to the present results, Khuntia *et al*. (2023)[31] reported a significant reduction (P<0.05) in CAT activity in arsenic trioxide-treated groups compared to controls[31].

In the present study, *C. limon* reduced the free radical stress by increasing the activity of antioxidant enzymes. Lemon juice exhibits strong antioxidant and hepatoprotective properties due to its rich content of citrate, flavonoids, vitamin C, vitamin E, and limonoids[32]. It restores SOD and CAT activities in pesticide-treated animals, reducing ROS accumulation. Additionally, lemon juice lowers MDA levels and improves glutathione redox status, supporting liver health[33,34].

Arsenic-induced hepatotoxicity primarily results from oxidative stress, leading to cellular injury. Histopathological findings in arsenic-intoxicated animals revealed hepatocyte vacuolation, hydropic degenration, mild haemorrhages, neutrophil infiltration, and vascular congestion. Similar degenerative changes, including vacuolar degeneration, swollen hepatocytes, and bile duct hyperplasia, have also been reported by other researchers[35]. *Citrus limon* significantly mitigated hepatic damage in arsenic intoxicated group D. It preserved hepatocyte integrity, reduced necrosis, and protected against oxidative lipid damage. Renal histology in arsenic-treated mice showed tubular vacuolization, glomerular hyaline degeneration, increased Bowman's space, hemorrhage, and inflammatory infiltration, consistent with previous reports[36]. These changes correlate with biochemical alterations and are attributed to increased glomerular filtration and protein leakage. *C. limon* supplementation reduced renal damage, with near-normal histology observed in group D. The nephroprotective effects are linked to their phenolic content and antioxidant activity. Arsenic intoxicated mice exhibited hyperkeratinization in the stomach mucosa and intestinal villus necrosis with leukocyte infiltration, similar to prior observations. These effects were ameliorated in groups receiving *C. limon*. Vitamin C, abundant in *C. limon*, enhances antioxidant defense and immune function reducing gastrointestinal tissue damage.

Arsenic exposure caused pulmonary congestion, hemorrhage, and inflammation, aligning with previous studies[35]. In arsenic intoxicated and *C. limon* supplemented group D, *C. limon* significantly reduced lung injury. Arsenic neurotoxicity results from ROS-induced oxidative stress, leading to neural degeneration, edema, and inflammation. The brain's vulnerability is intensified by its high oxygen demand and low antioxidant defenses. Histological changes included vacuolar changes and gliosis. Supplementation with *C. limon* reduced these alterations, likely due to their antioxidant properties. Vitamin C has demonstrated neuroprotective effects by preserving brain architecture and reducing oxidative damage[37]. The present study found that *C. limon* supplementation preserved spleen architecture. Histological changes in seminiferous tubules were due to arsenic-induced oxidative stress. These were markedly reduced in antioxidant-supplemented groups, indicating the protective efficacy of *C. limon.*

The ultra-structural analysis of group B liver tissue revealed irregular nuclear membrane and fragmented chromatin, swelling of both mitochondria and rough ER. Additionally, lipid droplet accumulation in the cytoplasm was noted In contrast, group D showed normal ultrastructural features, showing only minor lipid droplet presence, indicating mild cellular injury and apoptotic changes. This mild injury suggests the potential ameliorating effects *Citrus limon* in mitigating arsenic-induced hepatotoxicity.

Arsenic exposure triggers oxidative stress, leading to the generation of reactive oxygen species (ROS) that damage sperm cells. These cells are particularly vulnerable due to their limited cytoplasm and high concentration of unsaturated fatty acids. The combined effects of hormonal imbalance and oxidative stress contribute to sperm abnormalities and DNA damage, ultimately leading to infertility. The histological studies in the present study have revealed that arsenic exposure caused damage to seminiferous tubules. Moreover, arsenic exposure has been reported to induce oxidative stress and damage to spermatogenic cells within the seminiferous tubules and significantly reduce sperm count as reported by other researchers[38,39]. Due to the toxic effects of arsenic, dietary interventions like *Citrus limon* (lemon) has been investigated for its potential protective benefits. The use of this plant showed reduced injury to testes in the present study. Moreover, lemons contain vitamin C, which acts as an antioxidant and supports male reproductive health by maintaining sperm function, testicular integrity, and stimulating testosterone production. Vitamin C enhances sperm concentration, morphology, and motility while protecting sperm from oxidative damage caused by free radicals[40]. It neutralizes ROS, preventing DNA damage and oxidative stress. Additionally, lemon juice contains essential minerals like potassium, calcium, and phosphorus, which play vital roles in sperm motility and testosterone production[41].

Reproductive toxicity is also assessed by evaluating sperm head abnormalities, which is considered one of the most predictive methods for analysing *in-vivo* germ cell genotoxicity. An increase in sperm head abnormalities may result from chromosomal aberrations during the

packaging of genetic material in the sperm head or due to point mutations in testicular DNA. ROS increases genetic material damage in sperm[42]. Ijaz *et al*. (2023)[39] reported increased sperm defects in arsenic-exposed rats, affecting the head, mid-piece, and tail. Similarly, Morakinyo *et al*. (2010)[43] observed a significant rise (P<0.01) in sperm abnormalities in arsenite-treated rats. Furthermore, Kesari *et al*. (2012)[44] observed a significant increase in sperm head abnormalities in male mice due to the genotoxic effects of arsenic exposure.

Presence of micronuclei serves as a key biomarker for chromosomal damage caused by clastogenic or aneugenic mechanisms[45]. Arsenic disrupts normal cellular and mitotic processes, potentially by inhibiting DNA repair mechanisms and interfering with the mitotic spindle apparatus[46]. Its genotoxic effects are largely attributed to the generation of ROS, which trigger oxidative stress and DNA damage[47]. ROS can lead to double-stranded DNA breaks, chromosomal fragmentation, and mitotic spindle disruption, ultimately resulting in the formation of micronuclei (MN)[1]. Sub-chronic arsenic exposure significantly increases micronucleus formation due to its clastogenic potential[48]. A dose-dependent increase in micronucleus formation in polychromatic erythrocytes, further reinforces the link between arsenic exposure and cytogenetic damage[49]. Similarly, in the present study the significant increase (P<0.05) in percentages of micronucleated polychromatic erythrocytes (MNPCE), micronucleated normochromatic erythrocytes (MNNCE) and micronucleated erythrocytes (MNE) was observed in sodium arsenite exposed mice.

CONCLUSION

The present study demonstrates that *Citrus limon* supplementation offers a significant protection against arsenic-induced toxicity in mice. Arsenic exposure caused marked haematological alterations, liver and kidney dysfunction, oxidative stress, tissue damage, and genotoxic effects. Co-administration of *C. limon* effectively ameliorated these adverse changes by improving blood parameters, restoring liver and kidney function markers, enhancing antioxidant enzyme activity, and reducing lipid peroxidation. Additionally, *C. limon* supplementation minimized histopathological and ultrastructural tissue alterations, normalized sperm count, reduced sperm head abnormalities, and decreased micronuclei formation. Overall, *C. limon* exhibits substantial antioxidative and cytoprotective potential, indicating its promise as a protective agent against arsenic-induced systemic toxicity. Long-term exposure models and dose-dependent studies could provide deeper insights into its efficacy and safety profile. Additionally, exploring its protective potential in other animal species and in human populations exposed to arsenic may broaden its applicability. Molecular studies focusing on gene expression, signalling pathways, and epigenetic alterations would further clarify its role in mitigating arsenic-induced oxidative stress, organ damage, and genotoxicity.

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