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Purification and dose-dependent toxicity study of abrin in swiss albino male mice

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Abstract

Abrin, a phytotoxin obtained from the seeds of the *Abrus precatorius* plant, is highly toxic with an estimated human fatal dose of 0.1-1 µg/kg. In this study, abrin was purified and characterized through SDS PAGE and mass spectrometry analysis; further study on toxicity was carried out to investigate the alteration in biochemical, and hematological variables through histopathological observations in mice. The intraperitoneal LD_{50} value of purified abrin for mice was found to be 0.91µg/kg of body weight. Mice were exposed to 0.4 and 1.0 LD_{50} abrin doses intraperitoneally and observed on days 1, 3, and 7. Plasma GOT and GPT levels increased significantly at both doses. At 1.0 LD_{50} dose, alkaline phosphatase, bilirubin, urea, uric acid, and creatinine levels increased, whereas albumin, total protein, glucose and cholesterol levels decreased significantly. Abrin intoxication also altered the hemoglobin, WBC, and RBC counts significantly at 1.0 LD_{50} dose. Liver GSH levels decreased while lipid peroxidation increased significantly in a dose-dependent manner. Biochemical changes were supported by the histological investigation, which also showed the degenerative changes in organs. In conclusion, abrin intoxication caused toxic effects and severe damages on studied organs mediated through alteration in biochemical and hematological variables, lipid peroxidation, and degeneration.

Key words: Abrin, lethal dose LD_{50}, oxidative stress, purification, toxicity.

Introduction

Jequirity bean or rosary pea (Abrus precatorius L) has been known for its medicinal use and its toxicity for more than a century (1, 2). Various preparations of *A. precatorius* seeds, roots and leaves have been used as medicinal therapeutics such as an analgesic, aphrodisiac, abortifacient, anticonvulsant, laxative, sedative, insecticide, for eye diseases, fevers, coughs and colds, worms, venereal diseases and other conditions (3). All parts of *A. precatorius* plant are toxic, but the seeds contain the highest concentration of toxin. Abrin, a proteinacious toxin produced by this plant, had been implicated in the toxicity of these seeds. Abrin is a member of the class II ribosome-inactivating proteins (RIPs), a group of proteins that share the property of catalytically damaging ribosomes in an irreversible manner (4). Abrin and ricin are structurally and functionally related protein toxins and, next to botulinum toxin, are among the most poisonous substances known. Abrin is a heterodimeric protein made up of A and B subunits linked by a single disulfide bond (5, 6, 7, 8). Abrin, through its B subunit, binds to cell-surface receptors containing terminal galactose, enters the cell by receptor-mediated endocytosis (9), and is transported to the endoplasmic reticulum (ER) by a retrograde pathway (10). The intersubunit disulphide bond, which is essential for its toxicity (11), is reduced in the ER followed by the translocation of the A-chain to the cytosol by an ER-associated degradation pathway (12). The A subunit has RNA-N-glycosidase activity, which catalyzes the cleavage of the adenine residue at 4324 in the 28S rRNA this subsequently arrests protein synthesis and eventually leads to cell death (13, 14).

Abrin is highly toxic in nature, with an estimated human fatal dose of 0.1-1 µg/kg body weight and death has been reported after accidental and intentional poisoning (15). The median lethal intraperitoneal (i.p.) dose for abrin in female BALB/c mice has been estimated to be 2–20 µg/kg of body weight on the basis of previous reports of abrin poisoning (16). Several poisoning cases due to abrin were reported in India (17,18,19). Clinical signs and symptoms of abrin poisoning commonly include nausea, vomiting, diarrhea, and abdominal pain. Gastrointestinal bleeding may ensue with bloody diarrhea (20, 21) and hematemesis (15). Contact with the eyes may cause conjunctivitis and even blindness. Death occurs due to dehydration and shock (22).

Abrin may be a potential candidate to be used as a bioweapon because of its low cost of isolation and ease of use either by aerosolization as a dry powder or liquid droplets or by addition to food and water as a contaminant. Abrin is many times more potent than ricin. It would be very easy to cause fear and panic among people with this kind of a deadly toxin. Hence, an understanding of the mechanism of action and therapeutic strategies against this toxin is of utmost importance in the scenario of an eventuality of its use.

Few previous studies have shown that the abrin caused alteration in hematological and biochemical variables, but still the information is insufficient to understand its dose and time dependent toxicological effects especially in respect of histopathological changes and oxidative stress (*in vivo* conditions). The present study intended to purify, characterize, and attempt to investigate the minimum dose and time at which abrin alters biochemical, hematological and histopathological variables and induces oxidative stress.

Materials and methods

Chemicals

Abrin seeds were purchased from the local market. Sepharose 6B and chemicals used for electrophoresis were purchased from Sigma (St. Louis, MO, USA). Bio-Gel P-100 was purchased from Bio-Rad Laboratory Pvt. Ltd India. Molecular weight markers were obtained from Fermentus (EU). The biochemical kits were obtained from Merck (India). All other chemicals used were of analytical grade.

Animals

Swiss albino male mice randomly bred in Institute's Animal Facility were used. They weighed between 20 and 25 g. The animals were fed with standard pelleted diets (Ashirwad Brand, Chandigarh, India). They were housed in clean polypropylene cages with sterilized rice husk as bedding material, and the temperature was maintained at 25 ± 2 °C with a 12 h light and 12 hour dark cycle. Food and water were given *ad libitum*. The experiments were carried out according to the guidelines of Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA, India). This study was approved by the Institutional Animal Ethical Committee.

Purification of abrin

Abrin was purified in our laboratory from *Abrus precatorius* seeds as described by Hegde et al. (23). In brief, abrin was extracted andfurther purified by using a lactamyl-sepharose affinity column. The sepharose 6B was activated by introducing an epoxy group, amination of an epoxy activated gel, and subsequent coupling of ligand lactose. Under these conditions, the lectins bound to the column matrix and were eluted with 0.4 M lactose. The lectins were then separated based on their size difference using Bio-Gel P-100 Gel (Bio-Rad). Chromatographic purification was achieved using the protein purification system, Bio-Rad, USA (Biologic HR model). The fractions corresponding to peaks were pooled, dialyzed extensively against water, and lyophilized.

Sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gel electrophoresis

Samples were diluted (1:1) with sample buffer (50 mM Tris pH 6.8, 2% SDS, 20% glycerol, 2% 2-mercaptoethanol and 0.04% bromophenol blue) and were boiled for 5 min. Approximately 10 µg of samples were loaded in each well for the electrophoresis. SDS–PAGE under reduced and non-reduced conditions was performed to assess the purity of abrin according to the standard method described by Laemmli (24). Separation was done using Bio-Rad electrophoretic apparatus.

Molecular weight measurement by mass spectrometry

Mass spectrometric analysis was performed using-Matrix-assisted laser desorption ionization-Time-of-Flight (MALDI-TOF, Bruker Microflex LRF-20, Flex Control Workstation, Bremen, Germany) equipped with delayed extraction and a UV ionization laser (N2, 337 nm) with a 3-ns pulse width. The accelerating voltage was 20 Hz and the grid voltage was set to 18.3 kV. Protein molecular weight determination was carried out in positive ion linear mode. One hundred laser shots were averaged per spectra. The dried droplet method was used for routine MALDI analysis of the purified protein. The protein sample (1µg) was applied to the MALDI plate and was allowed to dry at room temperature. For determination of molecular weight of pure protein, spectra were analyzed.

Determination of median lethal dose (LD_{50})

The Organization for Economic Co-operation and Development (OECD) guidelines TG-425 for acute oral toxicity Up and Down Procedure (UDP) adopted 3 October 2008 was used for the present study. The Up-and-Down procedure is an alternative acute toxicity test that provides a way to determine the toxicity of chemicals with fewer test animals by using sequential dosing steps. This test guideline also enables the test article's characterization and allowsthe test article to be ranked and classified according to the United Nations. The median lethal dose (LD_{50}) of abrin with 95 % confidence limits for intra-peritoneal (i.p.) route was determined by the Up and Down procedure as directed by the OECD-425 guidelines. One mouse was used for each dose. After administration of abrin, animals were observed for toxicity-related symptoms and mortality for 7 days.

Hemagglutination assay

The hemagglutination assay (25) was performed in microtiter plates (Nunc A/S, Denmark). Each well contained 100 µl of 0.05 M Tris–HCl/0.1 M NaCl, pH 7.6 in which 100 µl of lectins (0.2 mg/ml) were serially diluted. Fifty microliter of a 2% suspension of sheep red blood cells in 0.15 M NaCl was added to each well and gently agitated. The plates were incubated at room temperature for 3 h. The hemagglutination titer was expressed as the reciprocal of the dilution factor of the last well showing haemagglutination activity.

Toxicity studies

The animals were divided into three groups of fifteen animals. Group 1 was the control. Groups 2 and 3 were injected with 0.4 and 1.0 LD_{50} dose of abrin respectively, as a single dose through i.p. administration. Group 1 mice were injected with a comparable volume of sterilized normal saline. The body weights of the animals were monitored daily. Studies have been carried out for three different time points 1, 3 and 7 day. At the end of each designated study period, five animals from each group were anesthetized with ether, and blood was collected from the retro orbital plexus in vials containing heparin on days 1, 3 and 7. The animals were then sacrificed by cervical dislocation and vital organs such as the liver, spleen and kidney were collected. The tissues were blotted, freed from blood and adhering tissues, and weighed for organ body weight index (OBWI). Some portion of the liver was quickly processed for estimation of GSH and lipid peroxidation, and the remaining was fixed in buffered formalin for histopathological studies using standard procedures (26).

Hematological Variables

White blood cells (WBC), red blood cells (RBC),

haematocrit (HCT), haemoglobin (Hb), and platelet (PLT) were measured on a Sysmex Hematology Analyzer (model K4500).

Biochemical assessment

Plasma GOT, GPT, ALP, total bilirubin, total protein, albumin, blood glucose, cholesterol, uric acid, urea and creatinine was carried out using diagnostic kits from Merck, India.

Liver Glutathione (GSH) levels

Liver GSH levels were measured as described by Hissin and Hilf (27). Briefly, 0.25 g of tissue sample was homogenized on ice with 3.75 ml of phosphate–EDTA buffer and 1 ml of 25% metaphosphoric acid, which was used as a protein precipitant. The total homogenate was centrifuged at 10,000 x g for 30 min at 4 °C. For the tissue GSH assay, 0.5 ml supernatant and 4.5 ml phosphate buffer (pH 8.0) were mixed. The final assay mixture (2.0 ml) contained 100 µl supernatant, 1.8 ml phosphate–EDTA buffer and 100 µl O-phthaldehyde (OPT; 1000 g/ml in absolute methanol, prepared fresh). After mixing, samples were incubated at room temperature for 15 min. Fluorescence was measured at 350 nm (Ex)/420 nm (Em) with Shimadzu RF- 5000 spectrofluorometer.

Thiobarbituric acid reactive substances (TBARS)

Tissue lipid peroxidation was measured by the method of Ohkawa et al. (28). Tissue homogenate was incubated with 8.1% SDS (w/v) for 10 min. followed by the addition of 20% acetic acid (pH 3.5). The reaction mixture was incubated with 0.6% thiobarbituric acid (w/v) for 1 h in a boiling water bath. The pink color chromogen formed was extracted in a butanol–pyridine solution (15:1) and read at 532 nm. The amount of TBARS was calculated using a molar extinction coefficient of 1.56×10^5/ (M cm).

Histopathological Examination

Liver, kidney and spleen tissues fixed in formalin were dehydrated through a graded ethanol series and embedded in paraffin according to standard procedures. Liver, kidney and spleen were sectioned at 5 µm thicknesses, which was followed by haematoxylin and eosin staining (H&E stain) and analyzed using light microscope (Leica, MEC 53A-V).

Statistical analysis

Results were analyzed by One Way Analysis of Variance (ANOVA) followed by Dunnett's test and the p values < 0.05 were considered statistically significant.

Results

Purification of abrin

Abrin extracted from *Abrus precatorius* seeds was first purified by affinity chromatography on sepharose 6B lactamyl column (Fig. 1A). Under these conditions, the lectins (i.e., agglutinin and abrin) bind to the lactose moieties available on the gel matrix. The matrix bound proteins were eluted with 0.4 M lactose (corresponds to Peak 2). Peak 1 corresponds to unbound protein eluted during washing of the column. Peak 3 corresponds

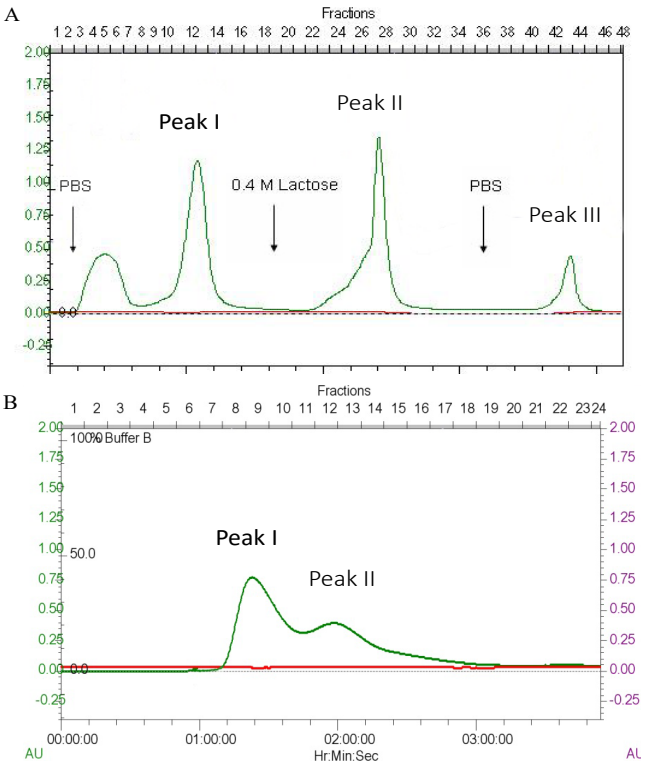

Figure 1. (A) Elution profile of abrin from lactamylsepharose affinity matrix. Peak I corresponds to non-lectin protein; Peak II represents abrin desorbed with 0.4 M lactose in water. **(B)** Gel-filtration chromatography of lactamylsepharose purified abrin (abrin and agglutinin) on Bio-gel P-100 column. The Peak I represents Agglutinin; Peak II corresponds to abrin.

to the proteins that were not characterized. The separation of affinity purified lectins (abrin and agglutinin) on Bio-gel P100 column by gel filtration chromatography is presented in (Fig. 1B). This resolved into 2 peaks, where peak 1 corresponded to agglutinin and peak 2 corresponded to abrin.

Electrophoretic characterization of Abrin

The purity of the lectins was evaluated by SDS polyacrylamide gel electrophoresis. The relative migration rate of abrin under reducing and non-reducing conditions to those of molecular weight markers has been presented in Fig. 2. Under non-reducing conditions, abrin gave a single band in 60-65 kDa regions, whereas under reducing conditions, it gave two bands in the region of 30-35 kDa region which represent A and B subunits of

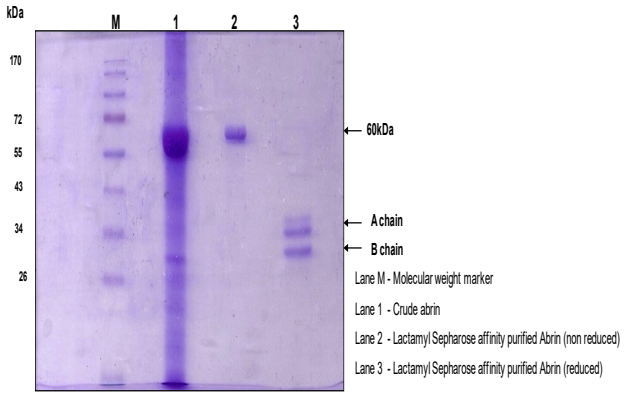

Figure 2. SDS -PAGE of abrin on 12% acrylamide gel. Lanes: (M) Standard markers; 1) Crude abrin; 2) Purified abrin (non-reduced); 3) Purified abrin in the presence of 2-mercaptoethanol (reduced).

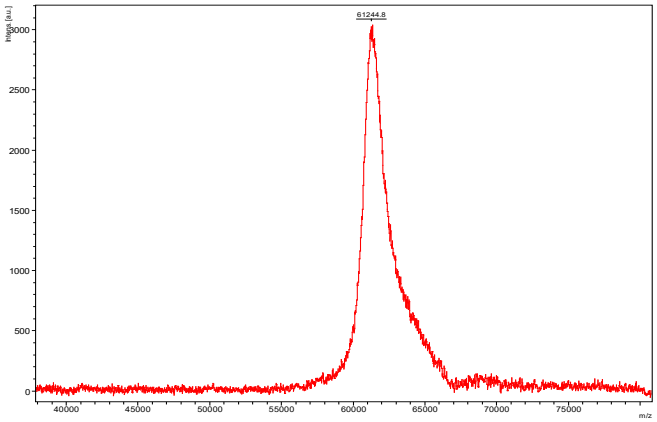

Figure 3. MALDI-TOF Mass spectrum of abrin showing $[M+H]^+$ 61,2448 in the linear mode using sinapinic acid as matrix.

the abrin, respectively.

MALDI-TOF/MS

The MALDI spectra of purified abrin in linear positive ion mode using sinapinic acid as matrix are shown in Fig. 3. Acquired spectrum through MALDI TOF showed a single intense peak corresponding to the molecular weight (m/z 61,2448) of abrin.

Determination of median lethal dose (LD_{50})

The median lethal dose calculated after administration of abrin intraperitoneally was found to be 0.91 µg/kg of body weight. Approximate 95% confidence limit is 0.5 µg/kg to 1.5 µg/kg of body weight.

Hemagglutination

Hemagglutination titers for crude and purified abrin were determined on the sheep red blood cells as shown in Table 1. The hemagglutination titer for crude abrin was 8 whereas for purified abrin it was 2. The concentration of the last point showing hemagglutination was 2.5 µg of sample (25 µg/ml) in the well (Fig. 4). The low hemagglutination ability of purified abrin in comparison to crude abrin further confirms its purity and higher toxicity.

Change in body weight and organ body weight index (OBWI)

The change in body weight and OBWI is shown in Fig. 5 and Table 2 respectively. The body weight of all abrin exposed animals decreased significantly from the 3rd day to the 7th day compared to vehicle treated animals. Also, in the case of OBWI, the significant changes were seen mainly in the liver and spleen of abrin- exposed animals in 1.0 LD_{50} dose compared to the vehicle

Table 1. Hemagglutination activity of crude and purified abrin from *Abrus precatorious* seeds.

Abrin	Agglutination**
Crude abrin	1:8
Purified abrin	1:2

** Agglutination is the reciprocal of the lowest dilution factor of abrin variants that causes red blood cells agglutination.

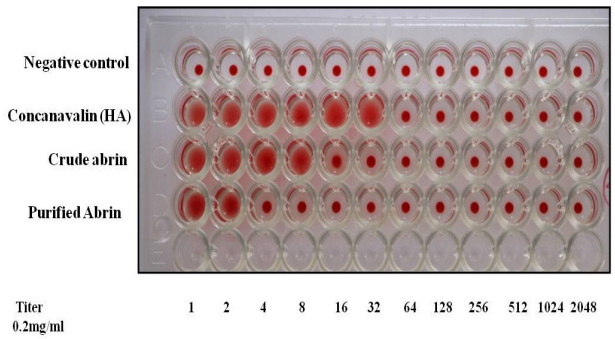

Figure 4. Hemagglutination test for crude and purified abrin in a microtiter plate. Sheep erythrocytes standing with abrin variants for 3h at room temperature. Lectins concentrations (0.2 mg/ml) were in a series of consecutive two-fold dilution.

treated control.

Hematological variables

The results are shown in Table 3. The count of WBCs increased, whereas count of RBCs and hemoglobin decreased significantly, when the animals were exposed to abrin at the dose of 1.0 LD_{50} on the 3rd and the 7th day of exposure. There was no significant change seen in the hematocrit and platelets count at any time or dose of exposure.

Biochemical parameters

The results are shown in Table 4. Most of the biochemical parameters were significantly different in the animals exposed to both doses of abrin at various time points compared to the vehicle-treated control. The levels of liver enzymes like SGOT, SGPT and ALP increased significantly at both doses. However, the levels of total protein and albumin were found to decrease significantly on the 3rd and 7th day in animals exposed to a higher dose of abrin. Total bilirubin increased significantly in the animals exposed to a higher dose of abrin. Urea and uric acid levels were found to increase significantly in animals exposed to both doses of abrin at different time intervals, whereas the level of creati-

Table 2. Effect of abrin on organ body weight index (OBWI) following intraperitoneal route in male mice.

Organs	Groups						
	I (Control)	II (0.4 LD_{50})			III (1.0 LD_{50})		
		1st day	3rd day	7th day	1st day	3rd day	7th day
Liver	5.7 ± 0.1	5.4 ± 0.1	5.3 ± 0.2	5.1 ± 0.1	5.2 ± 0.2	3.8 ±0.2**	4.2 ± 0.1*
Lung	0.7 ± 0.01	0.62 ± 0.03	0.68 ±0.09	0.73 ±0.05	0.6 ± 0.08	0.66 ±0.07	0.67 ± 0.06
Heart	0.6 ± 0.05	0.5 ± 0.01	0.6 ± 0.04	0.58 ±0.07	0.5 ± 0.01	0.53 ±0.08	0.58 ± 0.07
Kidney	1.3 ± 0.03	1.3 ± 0.07	1.3 ± 0.1	1.3 ± 0.09	1.2 ± 0.01	1.1 ± 0.01	1.3 ± 0.05
Spleen	0.56 ±0.04	0.42 ± 0.04	0.5 ± 0.03	0.51 ±0.02	0.49 ±0.03	0.3 ± 0.03**	0.48 ± 0.02

Data was analyzed using analysis of variance (One-way ANOVA) followed by Dunnett's test.

Values are expressed as mean ± SEM; n=5. Values are statistically significant at $^{**}p< 0.01$, $^{*}p< 0.05$ vs. control group respectively.

Table 3. Effect of abrin on hematological parameters following intraperitoneal route in male mice.

	Groups						
	I (Control)	II (0.4 LD_{50})			III (1.0 LD_{50})		
		1st day	3rd day	7th day	1st day	3rd day	7th day
WBC ($X10^3$/µl)	6.6 ± 1.6	7 ± 0.7	7.8 ± 1.2	6.9 ± 1.0	8.1 ± 1.2	13.1±1.3**	11.8 ± 0.8**
RBC ($X10^6$/µl)	9.9 ± 1.5	9.8 ± 1.1	9.7 ± 0.8	10.2 ± 1.5	9.2 ± 1.4	7.30 ± 1.0*	7.36 ± 1.3*
Hemoglobin (gm/dl)	11.7 ± 0.7	11.5 ± 1.0	10.9 ±1.2	10.5 ± 0.8	10.8 ± 0.7	8.7 ± 0.5*	9.0 ± 0.8*
Hematocrit (%)	42.7 ± 2.6	41.2 ± 1.3	40.4 ± 2.5	41.8 ± 2.7	41.3 ± 2.5	40.1 ± 2.8	40.9 ± 1.9
Platelets ($X10^3$/µl)	873 ± 54.1	880 ± 64.1	876 ± 46.3	885 ± 53.4	887 ± 41.2	896 ± 51.1	880 ± 45.6

Data was analyzed using analysis of variance (One-way ANOVA) followed by Dunnett's test.
Values are expressed as mean ± SEM; n=5. Values are statistically significant at $^{**}p< 0.01$, $^{*}p< 0.05$ vs. control group respectively.

Table 4. Effect of abrin on biochemical parameters following intraperitoneal route in male mice.

	Control	0.4 LD_{50}			1.0 LD_{50}		
		1st day	3rd day	7th day	1st day	3rd day	7th day
SGOT(IU/L)	38.6±2	75.9±5**	80.7±3.5**	43.45±2	92.4±2**	110.65±1**	53.8±1*
SGPT(IU/L)	34.9±2	48.6±2*	59.4±2**	40±2	59.8±3**	76.14±4**	49.75±1**
ALP(IU/L)	19.3±3.1	27.2±3.3*	31.5±2.3*	20.4±2.5	28.3±3.1*	42.4±2.9**	29.8±4.1*
Total Protein (g/dL)	4.7±0.6	4.4±1	5.3±0.5	5.2±1	4.8±0.42	2.7±0.46**	3.0±0.49**
Albumin (g/dL)	3.25±0.51	3.1±1	3.2±0.8	3.2±1	2.9±0.22	2.0±0.2**	2.1±0.3**
Total Bilirubin (mg/dL)	0.48±0.1	0.53±0.07	0.58±0.03	0.49±0.03	0.85±0.1*	1.07±0.08**	1.03±0.05**
Urea (mg/dL)	33.1±1	40.2±2.5*	49.1±3.6**	33.4±5.2	62.7±1**	69.5±5**	60.7±2**
Uric acid (mg/dL)	2.2±0.2	3 ±0.2	3.5±0.4*	2.3±0.2	3.5±0.4*	4 ±0.8**	3.8±0.4**
Creatinine (mg/dL)	23.5±0.6	25±0.5	22.1±1.8	24.4±1.3	41.3±0.1.4*	59.3±0.1.5**	38.5±0.2*
Cholesterol (mg/dL)	114.2±2.8	111.5±5.19	116±7.4	121.2±4.4	108 ±5.5	99 ±4.9*	110.2±4.2
Glucose (mg/dL)	183.2±8.5	182.5±8.5	176.2±7.2	179.2±6.9	180.5±9.03	162.5±6.1**	168.6±7.6*

Data was analyzed using analysis of variance (One-way ANOVA) followed by Dunnett's test.
Values are expressed as mean ± SEM; n=5. Values are statistically significant at $^{**}p< 0.01$, $^{*}p< 0.05$ vs. control group respectively.

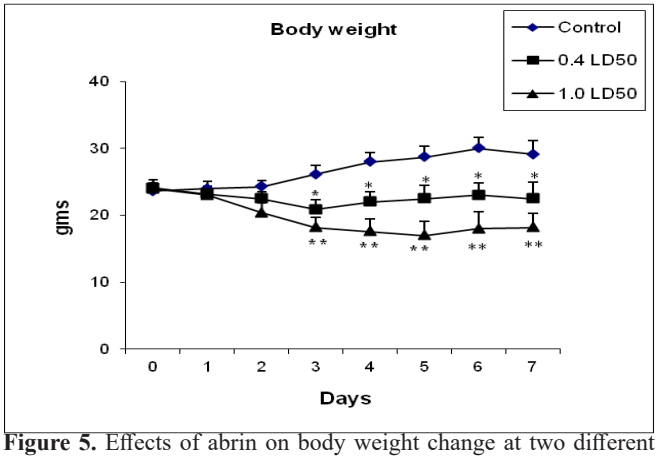

Figure 5. Effects of abrin on body weight change at two different doses following intraperitoneal route in male mice. Values are expressed as mean ± SEM; n=5. Data was analyzed using analysis of variance (One-way ANOVA) followed by Dunnett's test. Values are statistically significant at $^{**}p< 0.01$, $^{*}p < 0.05$ vs. control group, respectively.

nine increased significantly in animals exposed to the 1.0 LD_{50} dose only. On the other hand, the levels of glucose and cholesterol were found to decrease in the animals exposed to a higher dose of abrin at different time intervals.

Effect on hepatic glutathione (GSH) and lipid peroxidation (LPO)

The results are shown in Fig. 6. The level of hepatic GSH were shown to be significantly depleted at both doses and different time points in animals exposed to abrin compared to the vehicle treated control. The level of thiobarbituric acid reactive substances (TBARS, the end product of LPO) increased significantly at all time points in 1.0 $LD_{50,}$whereas at 0.4 LD_{50}, it increased significantly on days 1 and 3, but on day 7, it was restored to normal levels.

Histopathological Examination

A comprehensive account of various histopathological lesions caused by abrin on the 7th day of exposure of the toxin in the dose of 1.0 LD_{50} in various vital organs is shown in Fig. 7. At the dose of 0.4 LD_{50}, these vital organs did not show the major histopathological effects (results not shown).

In the liver at day 1, congestion in hepatic capillaries and moderate basophilic stippling was observed. At day 3, congestion, blood filled sinusoids, granulation of cytoplasm, pyknosis of hepatocytes, and hepatocytes necrosis in the vicinity of the central canal was found (results not shown). At day 7, condensation of chromatin in the nucleus, hypertrophy, increased number of leukocytes in the sinusoids, degeneration of hepatocytes, and vacuolar changes were observed.

In the spleen on day 1, hypocellularity and splenic paranchymal degeneration was observed. At day 3, severe hypocellularity, megakaryocyte formation, atrophy of white pulp, and degeneration was observed (results not shown). On 7thday of post-exposure of toxin, degeneration, hypocellularity, fibrosis, and appearance of large number of megakaryocytes were found.

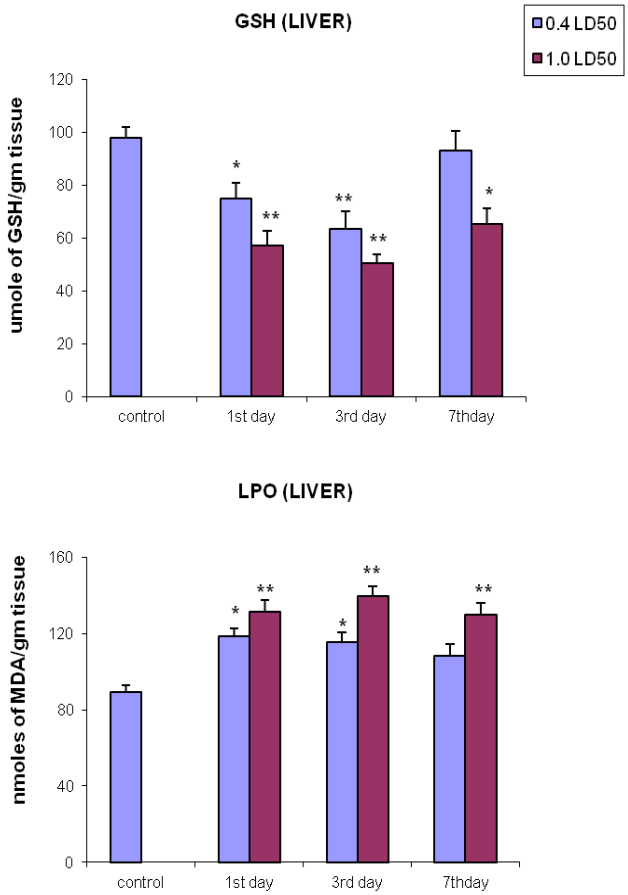

Figure 6. Effect of abrin on hepatic GSH (reduced) and LPO level at two different doses following intraperitoneal route in male mice. Values are expressed as mean ± SEM; n=5. GSH: Reduced glutathione as unit µmoles of GSH/gm of tissue and LPO: Lipid peroxidation as nmoles of MDA/g tissue. Data was analyzed using analysis of variance (One-way ANOVA) followed by Dunnett's test. Values are statistically significant at $^{**}p < 0.01$, $^{*}p < 0.05$ vs. control group, respectively.

In the kidney day 1 post-toxin, moderate congestion, tubular necrosis, and obliteration of chromatin material was observed. At day 3,the severity of these lesions was increased (results not shown). At day 7, congested glomerulus, severe tubular necrosis, liquificative degeneration of renal parenchyma, severe inflammatory cells infiltration, formation of thrombus, and condensation of chromatin material was found.

Discussion

In order to elucidate the potency as well as the mode of action of any toxin in a biological system, study of the alterations in cell integrity is required. This may be achieved by studying various aspects especially through biochemical alterations caused by the toxin. An example is the alterating of the specificity of the enzyme or inhibition of the enzymatic process. An understanding of the mechanism of action of any toxin, therefore, depends on biochemical changes in the biological system.

Abrin is a toxic protein obtained from the seeds of *Abrus precatorius* (jequirity bean), which is similar in structure and properties to ricin, although it is more potent than ricin. The information available is insufficient to understand the mechanism of toxicity of abrin. There is very little literature available about the histopathological changes and oxidative stress (*in vivo* conditions) caused by abrin. In the present study, abrin was purified, characterized, its LD_{50} was determined, and we made an attempt to investigate the minimum dose and time at which abrin causes alterations in biochemical, hematological and histopathological variables, and induced oxidative stress. As per the literature, this may be the first report of abrin's ability to induce oxidative stress and alter histological and biochemical variables below the lethal concentration within 24h in mice.

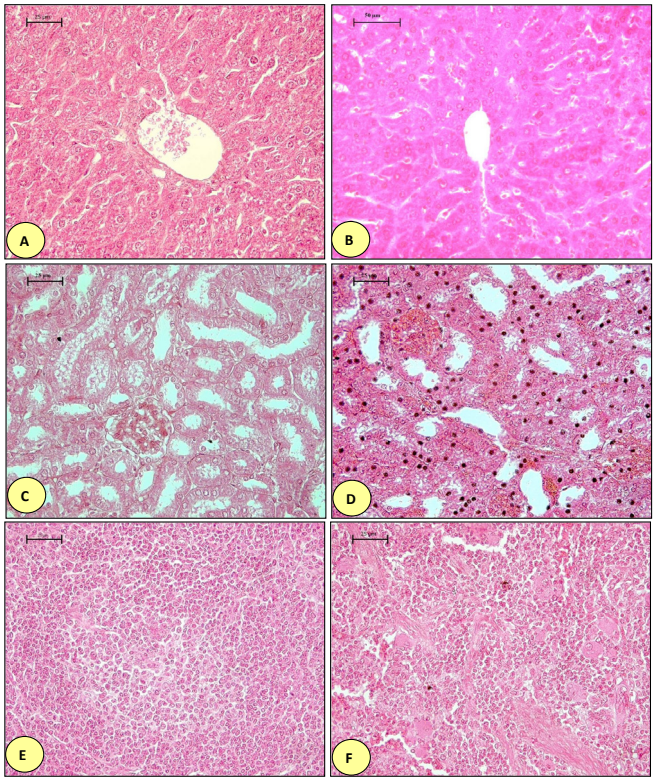

Figure 7. Photomicrographs of control group mice and abrin-treated mice liver, kidney, spleen; H & E X 100. (A) Control mice liver showing normal histoarchitecture with normal hepatic cord patterns, central canal and hepatocytes. (B) Abrin treated mice liver (7th day) showing condensation of chromatin materials, granulation of cytoplasm and degenerative changes in the hepatocytes by the process of necrosis. (C) Control mice kidney showing normal glomerulus, bowman's space and renal parenchyma. (D) Abrin treated mice kidney (7th day) showing severe tubular necrosis, condensation of chromatin materials and degeneration of renal parenchyma. (E) Control mice spleen showing normal splenic parenchyma, germinal centre, red and white pulp. (F) Abrin treated mice spleen (7th day) showing severe hypocellularity, splenic parenchymal degeneration and appearance of large number of megakaryocytes.

Abrin was extracted and purified from the *Abrus precatorius* seeds to apparent homogeneity by lactamyl sepharose affinity matrix followed by gel filtration chromatography on Bio-gel P100 column. The purified abrin so obtained was characterized by SDS-PAGE (under reducing and non-reducing conditions) to check the integrity of the protein and subunit composition. The purified abrin showed a single band in 60-65 kDa region under non-reducing conditions and two bands in 30-34 kDa region under reducing conditions. Estimation of molecular weight of abrin by MALDI-TOF had not yet been done. MALDI-TOF analysis of the purified protein was conducted, and a single, intense peak corresponding to the molecular weight (61.24 kDa) of abrin was found which further confirmed the identity

of the purified protein as abrin. Our results agreed with Olsnes and Pihl (29) who reported the molecular weight of abrin was 65 kDa, and the molecular weights of its a and b subunits were 35 and 30 kDa. Agglutination of red blood cells has been cited as a further mechanism of toxicity, however previous studies shows that the pure abrin has only low hemagglutination ability and that the in vitro agglutination caused by jequirity bean extract is mainly due to *Abrus* agglutinin (15). In other study, abrin-a at 0.8 µg/ml concentration level agglutinated human O-type erythrocytes, whereas abrin-b showed no such activity (30). In the present study, low agglutination was evident with purified abrin, as there were no agglutinins present, which further corroborated our findings. The median lethal dose of abrin through intraperitoneal administration was determined in our experiment (0.91 µg/kg of body weight) to be more than 2 times lower than the previous value reported of 2-20 µg/kg body weight (16). These differences may be due to variations in the source, purity of the abrin, or in the experimental conditions.

The animals exposed to abrin were suffering from abdominal pain, bloody diarrhea, inappetence, and their general well being was adversely affected. Indices of body weight gain and organ body weight ratios are simple and highly sensitive measures of toxicity after exposure to toxic substances (31). A significant decrease in the body weight of animals exposed to abrin at both doses was observed. A significant decrease in liver and intestinal weight following ricin intoxication was reported (32). Here, a significant decrease in liver and spleen organ body weight index was observed at a higher dose of abrin exposure.

The results of the present study showed that abrin intoxication affects various biochemical and hematological variables. We found that even 0.4 LD_{50} dose of abrin induced a significant increase in the levels of serum AST, ALT and alkaline phosphatase with in 24h post exposure, which indicate that abrin led to severe hepatic damage. The increase in the activity of transaminases is mainly due to the leakage of these enzymes from the injured hepatic cells into the blood stream. The levels of urea and uric acid were also found to be significantly increased at both the doses even at 24h post-exposure of abrin. These results suggest impaired kidney function. Our results found to be similar to the previous study in which increased levels of AST, ALT, urea, uric acid, ALP, creatinine, albumin were reported at 1.0 and 2.0 LD_{50} doses (33). In contrast to the finding of Bhasker et al. (34), we observed a significant decrease in the level of albumin and total protein, which were similar to the results obtained by Fodstad et al. (35). WBC count was found to be significantly increased on the 3rd day and 7th day of 1.0 LD_{50} dose exposure. The increase in blood levels of white blood cells and decrease in albumin levels are consistent with the development of a systemic inflammatory response syndrome resulting in vascular permeability changes with exudation of plasma such as albumin into the tissues resulting in hypoalbuminemia. Previous studies with abrin showed vascular leakage syndrome as one of the feature of abrin poisoning (15). In the present study the level of bilirubin was significantly increased, on the other hand hemoglobin count was found to be decreased at higher dose. The increased level of bilirubin may be due to the dysfunctioning of the liver or breakdown of hemoglobin. Further, the exposed group had significantly lowered levels of glucose and cholesterol at 3rd day post exposure of 1.0 LD_{50} dose of abrin, which may be due to the liver damage or starvation and malabsorption due to abrin toxicity. Hematological investigations showed evidence of anaemia, with a fall in hemoglobin concentration and reticulocytosis (35). Here, we also observed significant decrease in hemoglobin and RBC count at higher dose.

Reduced glutathione (GSH) is the main non-enzymatic antioxidant defense within the cell, reducing different peroxides, hydroperoxides and radicals (36). GSH is involved in molecular protective mechanisms that modulate cellular responses to toxic chemicals (37, 38). It is usually assumed that GSH depletion reflects intracellular oxidation. In previous study, GSH depletion occurred at 8 h and 24h of post exposure of abrin in human myeloleukemic cells, U937 (34). In the present study, a remarkable decrease in the concentration of liver GSH was observed even at day 1 of post exposure of both doses of abrin. This reduction might be attributed to the fact that abrin creates an imbalance between the pro-oxidants and the antioxidants inside the body which causes it to be unable to withstand the stress, leading to reduction in the liver GSH. Depletion of GSH commonly precedes or accompanies lipid peroxidation and oxidative stress.

Lipid is considered as an index to monitor the function of membrane integrity, while thiobarbituric reactive substances (TBARS, the end product of LPO) are measured as the index of lipid peroxidation (39). We have earlier reported that ricin intoxication even at 0.4 LD_{50} dose can induce increase in the level of TBARS with in 24h in mice liver (40). Our results are similar to these investigations; there was a marked increase in TBARS level in the liver of abrin exposed mice, even at 0.4 LD_{50} dose of abrin on the 1st day post-exposure.

Abrin toxicity produces ultrastructural changes in various tissues following intraperitoneal route. The literature contains no information, about the histopathological effects in liver, kidney, spleen caused by abrin injected by intraperitoneal route. Abrin caused severe damage in brain (33). After inhalation of abrin, liver, spleen and kidney showed severe passive venous congestion with hepatic sinusoidal ectasia (41). In earlier studies, intravenous administration resulted in the appearance of fat vacuoles in periportal hepatocytes and increased accumulation of neutrophils in hepatic sinusoids at 24 h post-ricin toxin A chain treatment. Neutrophils often were fragmented and engulfed by enlarged Kupffer cells (42). In our previous study, ricin (similar to abrin) caused congestion in hepatic capillaries, and necrosis in hepatocytes, hypocellularity and megakaryocytes formation in the spleen, congestion and degeneration in renal parenchyma in kidney of mice when injected intraperitoneally (43). The results obtained in the present study were similar to these reports. These changes appeared to be necrotic rather than apoptotic in nature and suggest that mechanisms other than direct effect of abrin may be involved.

In conclusion, our study provides detailed information about the toxic effects caused by abrin in mice. Understanding the mechanism of action of abrin, a toxin

produced by a medicinal plant and potentially a potent bioweapon, is vital. It also reveals that abrin purified from our laboratory is highly pure and toxic. It can be further used for the screening of antidotes and the development of sensitive detection systems. To date, many studies have been reported on the mechanism of abrin toxicity, but these studies are performed mainly in *in vitro* conditions. Here, we present detailed toxicological effects of abrin in *in vivo* conditions. We believe this is the first study where visual and clear histopathological effects of abrin in selected vital organs such as kidney, liver, and spleen have been reported.

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