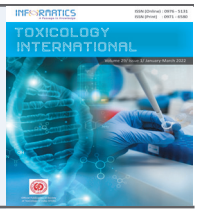
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Toxicological Assessment of Methanolic Fruit Extract of *Zanthoxylum acanthopodium* DC. in Swiss Albino Mice: Acute and Sub-acute Toxicity Study

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Abstract

Zanthoxylum acanthopodium DC. is widely used in traditional plant-based medical practices in India, with different parts of this plant used to treat a variety of ailments. Though this has been a time-tested practice, the side effects and other implications that the plant may have should not be overlooked. This study evaluated the toxicity of the methanolic fruit extract of *Z. acanthopodium* in Swiss albino mice by conducting acute and sub-acute toxicity tests using hematological, biochemical, ultrastructural analysis, sperm abnormality, and genotoxicity as parameters. For the acute toxicity test, mice were treated with a single dose of 5000 mg extract/kg bw of mice. While for sub-acute toxicity test, doses of 200, 500, and 1000 mg of the extract/kg bw were administered to the mice. Furthermore, a satellite group of the respective control group and the highest dose group were employed to observe the post-treatment effect that the plant may have. The acute toxicity test resulted in a significant reduction of the mean body weight gain in treated mice, but, no signs of morbidity and mortality were recorded. Sub-acute toxicity test revealed a negative effect of the plant extract on the blood and biochemical parameters. Light and electron microscopic examination of the liver and kidney showed the damaging effects of the extract on the tissues and the cellular structures. In addition, the extract induced sperm abnormalities with a significant reduction of sperm count and sperm viability, and caused an increase in the percentage of abnormally shaped sperms. The extract treatment also resulted in an increased frequency of chromosomal aberrations as well as cell death.

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1. Introduction

The use of plants for their medicinal properties is a well-established and time-tested concept. In fact, they are a reliable source of modern medicine and serve as templates and precursors for drug development. It is estimated that around 25% of prescribed conventional drugs are of plant origin[1]. Hence, the effectiveness of plants has created a global trust among users (80%) with many of them often neglecting the toxicity aspect of plants thinking that naturally available products carry no phytochemical that could cause any health hazard[2,3]. What is more concerning is that many users self-medicate without any supervision or advice from an expert, leaving their body exposed to countless phyto-compounds that may cause serious health complications[4–7]. Therefore, it is of the utmost importance to conduct toxicity studies on medicinal plants to determine the potential adverse effects that they may pose on the health of the users.

Zanthoxylum acanthopodium DC. (Family: Rutaceae) commonly known as lemon pepper is widely distributed among South-Asian countries. The plant is of great economic importance possessing stimulant, carminative, sudorific[8], anti-aging, anti-acne[9], anti-inflammatory, anti-diabetic[10], antibacterial[11], anthelmintic[10,12], and antioxidant[13] properties. The plant is found to be rich in secondary metabolites such as alkaloids, flavonoids, terpenoids, steroids, tannins, saponins, glycosides, and glycoside anthroquinones[14]. The presence of these phyto-compounds may be responsible for the plethora of medicinal uses of the plant such as treatments for bronchitis, dyspepsia, stomach ache, hair diseases, and rheumatism[12,15]. *Z. acanthopodium* has also been proven to be a potent anticancer agent against different cancer cell lines (4T1 cells and T47D cells)[16,17].

In Northeast India, the fruits of the plant are used for the treatment of chronic fever, headache, toothache, abdominal troubles, appendicitis, cholera, and dysentery. The topical application of the plant cures different skin diseases. A mixture consisting of leaves of *Z. acanthopodium*, *Sarcandra glabra*, *Polygonum alatum* and rhizome of *Pteridium aquilinum* are used for healing leprosy and paralysis[8,18]. An acute toxicity study of the fruits of *Z. acanthopodium* conducted by Bhattacharya *et al.*,[19] at 2000 mg/kg bw showed no toxic effect. However, an acute toxicity study at higher doses was not considered and toxicity on prolonged uses of the plant is unknown. Therefore, this study aimed to assess the toxicological effects of methanolic fruit extract of *Z. acanthopodium* (MFZA), if any, on mice through hematological, biochemical, ultrastructural, sperm abnormality, and genotoxicity analysis.

2. Materials and Methods

2.1 Plant Collection and Extract Preparation

The plant was collected from Meghalaya on October 2018 and was identified by the scientist of the Botanical Survey of India, Shillong. A copy of the herbarium was submitted to the Department of Botany, NEHU, Shillong for future reference (Accession No. NEHU-12378).

The fruits collected were cleaned, dried, and pulverized. 500 mL of methanol was infused with a 100 g sample of the powder, regularly stirred for a week and filtered using Whatman's No. 1 filter paper. Maceration was repeated twice and the solvent was evaporated to dryness under reduced pressure, giving a yield of 8.2%. The extract was collected in a glass vial and stored at 4°C until used.

2.2 Experimental Animals

Swiss albino mice (*Mus musculus*) were procured from Pasteur Research Institute, Shillong, Meghalaya. Healthy mice aged between 8–12 weeks old and weighing 30±5 grams were used for the study. The mice were housed in well-aerated polypropylene cages and the animal room was maintained in the standard condition (lighting cycles: 12 h light/12 h dark; temperature: 22±3°C). The animals were allowed to acclimatize for 5 days before conducting the experiments. Mice were fed with standard laboratory rodents' diet and water *ad libitum*. All experimental procedures were approved by the Institutional Animal Ethics Committee, North-Eastern Hill University (NEHU).

2.3 Acute Toxicity

An acute toxicity study was conducted following Organization for Economic Co-operation and Development (OECD) 423 guidelines[20]. Female mice were divided into two groups, i.e., a control group that received 1% Tween 20 solution (vehicle) and a treated group that received a single dose of 5000 mg MFZA/kg

bw + vehicle orally. Mice were left in a fasted state (3–4 hours) before administration of the extract and after dosing (1–2 hours). They were observed carefully for any behavioral changes, toxic effects, and mortality for a period of 14 days. At the end of the study period, the animals were weighed, sacrificed by cervical dislocation, their organs excised and weighed.

2.4 Sub-acute Toxicity

The sub-acute toxicity study was conducted following OECD 407 guidelines[21]. Six groups of six animals each (3 males and 3 females) were randomly selected and divided into the following groups: Group I: Control received 1% Tween 20 solution, Group II-IV: received MFZA (200 mg, 500 mg, and 1000 mg) suspended in 1% Tween 20 solution, Group V: Control (Satellite) and Group VI: 1000 mg MFZA/kg bw (Satellite). Satellite groups were treated for 28 days and left untreated for another 14 days. Satellite groups were included in the study to determine the reversibility or recovery of the animals from the toxic effects of the plant extract. The animals were given the extract orally once per day for 28 days at a concentration of 1 mL/100 g. During the study period, the animals were weighed weekly and observed daily to confirm morbidity/mortality. After the termination of treatment, the animals were fasted overnight, weighed, and sacrificed via cervical dislocation. Organs were excised, weighed, and calculated for relative organ weight.

$$\text{Relative organ weight (ROW)} = \frac{\text{Absolute organ weight (g)}}{\text{Body weight on the day of sacrifice (g)}} \times 100$$

2.5 Hematological and Biochemical Analysis

The retro-orbital sampling method was employed for blood collection from mice. For hematology, blood was collected in heparinized tubes, mixed thoroughly, and analyzed. While for a biochemical test, blood was collected in non-heparinized tubes and allowed to clot for 45 minutes, followed by centrifuging at 3000 rpm for 10 minutes to obtain serum for analyzing liver and kidney function tests.

2.6 Histopathology Studies

Liver and kidney were fixed in 10% formaldehyde overnight and were washed in phosphate buffer saline. The tissues were dehydrated in gradients of alcohol solutions (70%, 80%, 90%, 95%, and 100%) and cleared in xylene. The samples were embedded in paraffin and were blocked out. Sections cut at 7 microns were processed for Hematoxylin & Eosin staining and viewed in a Leica DM 1000 microscope (Leica, Germany).

2.7 Stereoscan Studies

Liver and kidney excised were cut into small pieces of around 2-3 mm and fixed in 4% glutaraldehyde at 4°C. Samples were washed and post-fixed in 1% OsO_4 buffered with 0.1 M sodium cacodylate. The samples were dehydrated in ascending grades of acetone and dried in tetramethylsilane[22,23]. Dried samples were gold coated in a JFC 110 (JEOL) ion sputtering chamber and were viewed in a JEOL JSM 6360 scanning electron microscope.

2.8 Ultrastructural Studies

The liver and kidney were cut into small pieces of 1 mm^3 and fixed in a modified Karnovsky's fixative for 4 hours at 4°C. Samples were washed and post-fixed in 1% OsO_4 buffered with 0.1 M sodium cacodylate for 4 hours at 4°C. Samples were dehydrated in the ascending grades of acetone and embedded in araldite. Ultrathin sections were stained using uranyl acetate followed by lead citrate and viewed in a JEM 2100 (JEOL) transmission electron microscope operated at 200 kV.

2.9 Sperm Abnormality Test

For this study, thirty male mice (five in each group) were grouped as it was done for the sub-acute toxicity study. At the end of the treatment period (28 days), animals were sacrificed and their testes, as well as epididymis, were weighed. The epididymis was dissected in 1 mL of pre-warmed phosphate buffer saline making numerous cuts to allow the sperm to swim out and disperse freely. The solution was left undisturbed for 15 minutes at 37°C. The suspension was centrifuged for 5 minutes at 500 g and spermatozoa obtained were analyzed for sperm count, sperm viability (using eosin-nigrosin staining method) and sperm morphology (using Papanicolaou's staining method)[24].

2.10 Chromosomal Aberration Test

For this study, twenty-four mice were divided into six groups, and the grouping of the animals was done

similar to the sub-acute toxicity study. At the end of the treatment period (28 days), the animals were injected with 4 mL of 0.05% colchicine intraperitoneally 2 hours before dissection. The femurs were dissected and cut open at both ends. 3 ml of 0.56% of pre-warmed KCl solution (hypotonic solution) was used to flush out the bone content using a syringe in 15 mL centrifuge tubes. It was mixed properly and the solution was incubated for 20 minutes at 37°C. The solution was centrifuged at 800 rpm for 4 min and the supernatant was discarded. Pellets obtained were fixed in 2 mL of freshly prepared chilled Carnoy's fixative (3 methanol : 1 glacial acetic acid) thoroughly mixed, allowed to stabilize for an hour at room temperature, and centrifuged for 4 min at 800 rpm. The pellets obtained were re-suspended in the fixative and this step was repeated 4 times. Debris-free pellets were suspended in 1 mL of Carnoy's fixative and after which, they were ready for chromosomal slide preparation. The cell suspension was dropped on a cleaned and wet slide from the height of 30 cm for the cells to burst. This was followed by gentle heating for proper spreading and staining with 5% Giemsa solution for 20 min. Slides were dipped in distilled water once or twice for the removal of excess stain and mounted using DPX[25]. 100 well-spread metaphase plates were observed in a 1000 DM Leica microscope at 100X using immersion oil.

2.11 TUNEL Assay

The liver was excised and fixed in 4% formaldehyde followed by cryoprotection of the tissue in a gradient of sucrose solution of 10%, 20%, and 30% until the tissue sank to the bottom. It was embedded in the OCT media and sectioned in a Leica CM 1850 cryomicrotomy (Leica Microsystems, Wetz-lar, Germany) at -20°C. The section was cut at 4 µm thickness and processed for DNA fragments detection using the commercial APO-BrdU TUNEL assay kit. In brief, a section was washed in a buffer and labeled with a DNA labeling solution (10 µL of reaction buffer, 0.75 µL of TdT enzymes, 8.0 µL of BrdUTP, 31.25 µL of dH_2O) for an hour at 37°C. It was rinsed with rinsing buffer followed by the addition of Alexa Fluor 488 dye-labeled anti-BrdU antibody (5 µL of antibody in 95 µL of rinse buffer) and incubated for 30 min. The section was stained with 0.5 mL of propidium iodide and observed under an EVOS FL fluorescence microscope (Life technologies, USA).

2.12 Statistical Analysis

Data were analyzed using SPSS software and expressed as mean ± Standard Error Mean. For the acute toxicity study, Student's t-test was used to analyze the data. While for others, statistical significance was determined by a one-way Analysis Of Variance (ANOVA) and Tukey's test for post hoc. $p \leq 0.05$ was considered significant.

3. Results

3.1 Acute Toxicity

Treatment with the plant extract at a single dose of 5000 mg/kg bw did not result in any toxic clinical signs or mortality to the animals during the 14-day study period. However, the plant extract-treated mice experienced a significant mean weight decline when compared to the control mice (Table 1). Relative organs weight showed no significant difference between the two groups (Table 2).

Table 1. Acute toxicity study: Body weight of mice exposed to the methanolic fruit extract of *Zanthoxylum acanthopodium*

Body weight	Initial (g)	Final (g)	Mean weight change (g)
Control	27.10 ± 0.11	27.87 ± 0.07	0.77 ± 0.07
Treated	27.37 ± 0.13	27.23 ± 0.03	-0.13 ± 0.12*

Data are expressed as mean ± SEM (n=3).

Asterisk (*) signifies values that are significantly different from control ($p<0.05$)

Table 2. Acute toxicity study: Relative organ weight (ROW) of mice exposed to the methanolic fruit extract of *Zanthoxylum acanthopodium*

ROW	Liver (%)	Kidney (%)	Spleen (%)	Stomach (%)	Heart (%)	Brain (%)
Control	4.02 ± 0.04	1.01 ± 0.01	0.49 ± 0.02	0.89 ± 0.02	0.54 ± 0.02	1.65 ± 0.04
Treated	3.96 ± 0.04	1.02 ± 0.02	0.49 ± 0.01	0.88 ± 0.01	0.50 ± 0.03	1.73 ± 0.01

Data are expressed as mean ± SEM (n=3).

Asterisk (*) signifies values that are significantly different from control (p<0.05)

3.2 Sub-acute Toxicity

Repeated oral intake of the extract for 28-days caused a significant decline in the weight gain of the animals receiving 200 and 500 mg of MFZA/kg bw respectively. However, the 1000 mg/kg bw treated mice and satellite group did not show any significant reduction in weight gain when compared to their respective control groups (Table 3). Moreover, the extract did not alter the relative weight of different organs except for the liver. The relative weight of the liver of the highest dose treated group increased significantly in comparison to that of the control. Also, the relative weight of the liver of satellite group animals was observed to increase insignificantly (Table 4).

Table 3. Sub-acute toxicity study: Body weight of mice exposed to the methanolic fruit extract of *Zanthoxylum acanthopodium*

	Z. acanthopodium				Satellite	
Body weight (g)	**Control**	**200 mg**	**500 mg**	**1000 mg**	**Control**	**1000 mg**
Initial	28.30 ± 0.87	28.02 ± 0.72	27.68 ± 0.53	28.40 ± 0.95	28.45 ± 0.72	28.45 ± 0.68
Final	32.57 ± 0.83	30.53 ± 0.84	29.95 ± 0.50	31.32 ± 0.81	32.02 ± 0.97	30.68 ± 0.32
Mean weight change	4.27 ± 0.29	2.52 ± 0.55*	2.27 ± 0.27*	2.92 ± 0.25	3.57 ± 0.36	2.23 ± 0.51

Data are expressed as mean ± SEM (n=6).

Asterisk (*) signifies values that are significantly different from control (p<0.05)

Table 4. Sub-acute toxicity study: Relative organ weight (ROW) of mice exposed to the methanolic fruit extract of *Zanthoxylum acanthopodium*

	Z. acanthopodium				Satellite	
ROW (%)	**Control**	**200 mg**	**500 mg**	**1000 mg**	**Control**	**1000 mg**
Liver	3.92 ± 0.07	4.06 ± 0.08	4.11 ± 0.07	4.49 ± 0.13*	3.90 ± 0.08	4.13 ± 0.19
Kidney	1.18 ± 0.07	1.12 ± 0.13	1.31 ± 0.15	1.22 ± 0.05	1.17 ± 0.07	1.28 ± 0.05
Spleen	0.40 ± 0.02	0.45 ± 0.03	0.42 ± 0.05	0.43 ± 0.04	0.49 ± 0.04	0.43 ± 0.03
Stomach	0.77 ± 0.02	0.79 ± 0.02	0.76 ± 0.13	0.84 ± 0.03	0.86 ± 0.03	0.88 ± 0.05
Heart	0.46 ± 0.01	0.50 ± 0.01	0.49 ± 0.01	0.51 ± 0.03	0.52 ± 0.02	0.50 ± 0.02
Brain	1.64 ± 0.06	1.68 ± 0.02	1.72 ± 0.08	1.57 ± 0.05	1.67 ± 0.06	1.62 ± 0.02

Data are expressed as mean ± SEM (n=6).

Asterisk (*) signifies values that are significantly different from control (p<0.05)

Results of the hematological analysis are presented in Table 5. It was observed that the plant extract induced a significant change in the Red Blood Corpuscles (RBCs) and RBC indices in animals treated with 500 mg and 1000 mg. The changes observed, were an increase in RBCs count and the percentage of Packed Cell Volume (PCV). Hemoglobin (Hb) levels were elevated significantly, but Mean Corpuscular Hemoglobin (MCH) and Mean Corpuscular Hemoglobin Concentration (MCHC) declined to a significant level. Animals exposed to 1000 mg of MFZA/kg bw also recorded a reduction in the levels of Mean Corpuscular Volume (MCV) in comparison to that of the control group. Satellite groups showed somewhat similar results, however, changes in RBCs count and MCH levels were the only parameters that were significant when compared to the control satellite. White Blood Cells (WBCs) total count was elevated significantly when animals were exposed to 500 mg and 1000 mg of MFZA/kg bw as well as satellite group. Differential WBC count indicated a significant elevation in the percentage of neutrophils accompanied by a significant reduction in lymphocyte count in the 1000 mg/kg treatment group. Thrombocytes or platelets count was highly altered showing an increase in their levels in the 500 mg/kg bw, 1000 mg/kg bw, and satellite group of treated animals in comparison to the control groups.

Results of the biochemical analysis are presented in Table 6. Mice exposed to 500 and 1000 mg of MFZA/kg bw experienced a significant increase in the ALT and AST levels compared to the control group. The satellite-

Table 5. Hematological values in mice exposed to methanolic fruit extract of *Zanthoxylum acanthopodium*

	Z. acanthopodium				Satellite	
Parameters	**Control**	**200 mg**	**500 mg**	**1000 mg**	**Control**	**1000 mg**
Hb (g%)	14.32 ± 0.12	13.93 ± 0.22	16.08 ± 0.19*	16.57 ± 0.24*	14.32 ± 0.41	15.30 ± 0.14
RBC (10^5/mm^3)	08.21 ± 0.10	08.44 ± 0.12	10.30 ± 0.05*	10.38 ± 0.18*	08.45 ± 0.27	09.62 ± 0.24*
PCV (%)	42.33 ± 1.14	42.25 ± 0.46	54.62 ± 1.28*	52.25 ± 0.50*	43.12 ± 1.87	46.88 ± 0.54
MCV (fl)	52.25 ± 0.68	51.18 ± 0.44	51.15 ± 0.87	48.62 ± 0.43*	52.07 ± 0.82	48.80 ± 0.24
MCH (pg)	17.52 ± 0.19	17.23 ± 0.33	16.20 ± 0.39*	16.13 ± 0.13*	17.10 ± 0.04	15.68 ± 0.22*
MCHC (%)	33.27 ± 0.34	32.23 ± 0.51	30.55 ± 0.64*	31.02 ± 0.54*	33.48 ± 0.63	33.23 ± 0.65
WBC (10^3/mm^3)	06.21 ± 0.19	06.21 ± 0.18	07.50 ± 0.18*	07.76 ± 0.10*	06.05 ± 0.06	06.88 ± 0.22*
Neutrophils (%)	06.00 ± 0.36	05.83 ± 0.31	05.33 ± 0.42	08.67 ± 0.21*	06.67 ± 0.33	08.50 ± 0.22*
Lymphocytes (%)	84.83 ± 0.83	86.17 ± 1.38	86.67 ± 1.08	79.50 ± 0.88*	84.67 ± 1.38	80.83 ± 0.94
Eosinophils (%)	04.17 ± 0.17	04.67 ± 0.61	04.83 ± 0.54	04.50 ± 0.34	04.50 ± 0.22	04.33 ± 0.21
Monocytes (%)	02.83 ± 0.17	02.83 ± 0.48	03.17 ± 0.40	03.50 ± 0.34	02.17 ± 0.17	02.33 ± 0.21
Plt (10^5/mm^3)	09.04 ± 0.07	09.54 ± 0.21	10.95 ± 0.42*	12.41 ± 0.44*	09.25 ± 0.25	11.27 ± 0.29*

Data are expressed as mean ± SEM (n=6).

Asterisk (*) signifies values that are significantly different from control (p<0.05)

Table 6. Biochemical values in mice exposed to methanolic fruit extract of *Zanthoxylum acanthopodium*

	Z. acanthopodium				Satellite	
Parameters	**Control**	**200 mg**	**500 mg**	**1000 mg**	**Control**	**1000 mg**
ALT (U/L)	17.91 ± 0.82	18.77 ± 0.83	21.26 ± 0.53*	22.29 ± 0.32*	18.15 ± 0.25	20.65 ± 0.45*
AST (U/L)	148.42 ± 2.93	148.11 ± 1.36	158.32 ± 0.99*	172.06 ± 1.42*	149.54 ± 0.94	155.20 ± 0.85
ALP (U/L)	78.81 ± 1.97	77.18 ± 1.00	83.58 ± 0.93	90.74 ± 1.66*	79.64 ± 1.07	83.95 ± 1.16
Creatinine (mg/dl)	0.32 ± 0.01	0.33 ± 0.01	0.36 ± 0.01*	0.44 ± 0.01*	0.33 ± 0.01	0.37 ± 0.01*
Urea (mg/dl)	37.94 ± 1.33	36.64 ± 0.97	41.23 ± 0.85	44.93 ± 0.96*	38.13 ± 0.79	41.23 ± 1.33

Data are expressed as mean ± SEM (n=6).

Asterisk (*) signifies values that are significantly different from control ($p<0.05$)

treated group showed an increase only in the ALT levels compared to that of the satellite-control group. Alkaline Phosphatase (ALP) levels were also significantly elevated in the animals exposed to the highest dose of the extract. The renal markers analysis revealed a significant increase in creatinine levels in the mice exposed to 500 and 1000 mg extract/kg bw and the satellite group. Urea levels were found to be elevated in groups treated with 500 mg and 1000 mg of the extract and the satellite group, but only the highest dose treatment group showed significant changes.

Histopathological examination of the liver of mice treated with the plant extract revealed an enlargement in the size of the nucleus and severe infiltration by the leukocytic cells in the hepatic lobule (Figure 1B), which was not observed in the control (Figure 1A). On scanning the surface topography, it was noted that the extract drastically contorted the arrangement of the hepatic plates and the size of the sinusoidal pores (Figure 1D), unlike the control's liver where the hepatocytes are well arranged in the hepatic plates and separated from each other by the sinusoids (Figure 1C). In addition, TEM examination revealed the degenerative impact of the plant on the ultrastructures of the hepatocytes as evident by the rupture of the nuclear membrane, mitochondria, and fragmentation of the endoplasmic reticulum. Heavy deposition of dark granules in the cytoplasm of the treated hepatocytes and collagen fibres occupying the intercellular spaces was observed (Figures 1F and 1G) when compared to control (Figure 1E).

Light microscopy of the kidney exposed to the extract showed an enlargement in the size of the glomerulus which is also called glomerular hypertrophy (Figure 2B). This change was not visible in the control group where the glomerulus is of the normal size and encapsulated in the Bowman's capsule (Figure 2A). Similarly, SEM observations of the control kidney revealed a normal shape and size of the glomerulus with epithelial cell body called podocytes, and from each podocyte cells, primary as well as secondary foot processes originate (Figure 2C). However, treatment with a high dose of the extract resulted in the development of fibrous tissue on the cell body of the glomerulus (Figure 2D). Ultrastructural observations revealed a thickening of the glomerular basement membrane and effacement of the fenestrated endothelium on kidneys of the plant extract-treated group. It also resulted in cirrhosis of the podocyte (Figure 2F) which was not observed in the case of the control group (Figure 2E). The plant extract targeted the nucleus of the epithelial cell of proximal convoluted tubule by degenerating the nuclear membrane and affecting the distribution of the chromatin material (Figure 2H) as compared to control group (Figure 2G).

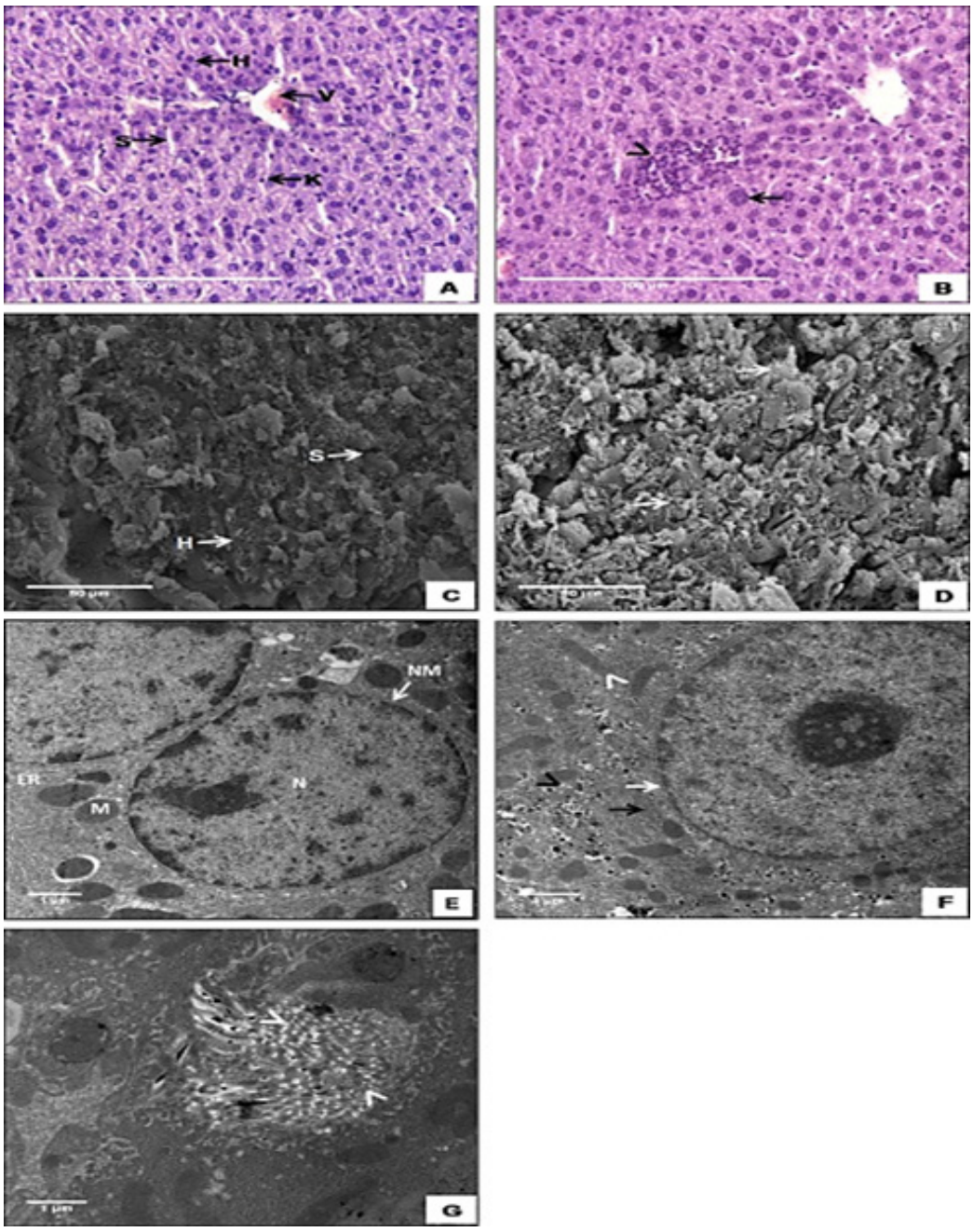


Figure 1. Light microscopy **(A and B)**, scanning electron microscopy **(C and D)** and transmission electron microscopy **(E–G)** images of the liver showing control **(A, C and E)** and methanolic fruit extract of *Zanthoxylum acanthopodium* exposed mice **(B, D, F and G)**. **A.** Liver showing hepatocyte (H), sinusoids (S), Kupffer cells (KC), and hepatic vein (V). **B.** Liver showing nuclear enlargement (arrow) and leukocytic infiltration (arrowhead). **C.** Liver showing hepatic plates (H) and sinusoids (S). **D.** Liver showing deterioration and deformation of the tissue (arrow). **E.** Hepatocyte showing nucleus (N), nuclear membrane (NM), mitochondria (M), and endoplasmic reticulum (ER). **F.** Hepatocyte showing deteriorated nuclear membrane (white arrow), damaged mitochondria (white arrowhead), broken ER (black arrow), and dark granular bodies (black arrowhead). **G.** Intercellular deposition of fibres (arrowhead).

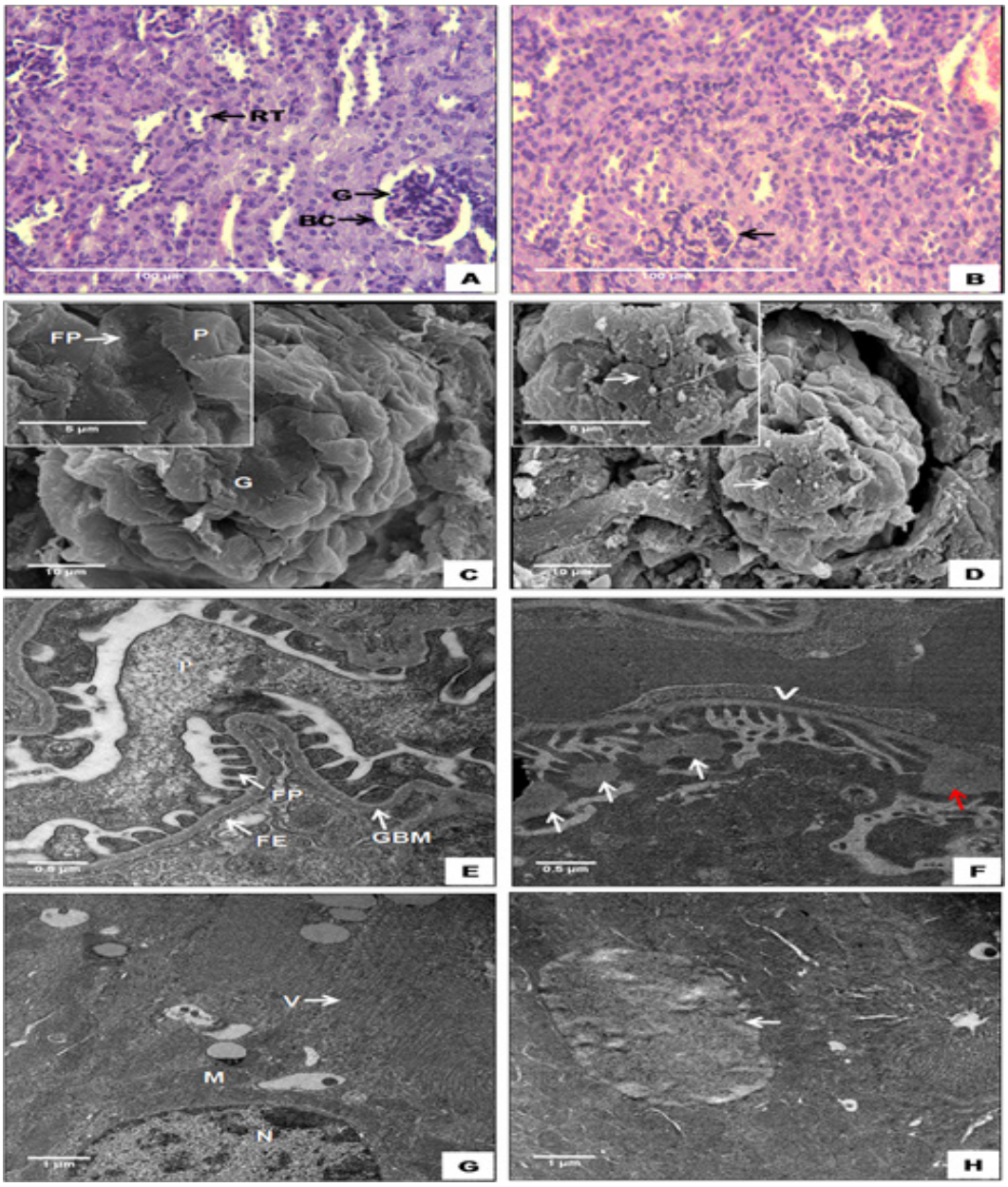


Figure 2. Light microscopy **(A and B)**, scanning electron microscopy **(C and D)** and transmission electron microscopy **(E–H)** of a kidney showing control **(A, C, E and G)** and methanolic fruit extract of *Zanthoxylum acanthopodium* **(B, D, F and H)** exposed mice. **A.** Kidney showing glomerulus (G), Bowman's capsule (BC) and renal tubules (RT). **B.** Kidney showing glomerular hypertrophy (arrow). **C.** Control showing glomerulus (G), podocyte (P) and foot processes (FP). **D.** Kidney showing glomerulus with abnormal fibrous tissue (arrow) on P. **E.** Control kidney showing podocyte (P), foot processes (FP), glomerular basal membrane (GBM) and fenestrated endothelium (FE). **F.** Kidney showing scarred P (white arrow), distended GBM (red arrow) and effaced FE (arrowhead). **G.** Kidney showing epithelial cell of proximal convoluted tubule with nucleus (N), mitochondria (M) and villi (V). **H.** Kidney showing damaged N and abnormal chromatin distribution (arrow).

3.3 Testes, Epididymis and Sperm Abnormality Study

The testicular weight of animals exposed to the extract was slightly higher than that of the control animals, though the changes were not significant. However, the testicular weight of the treated satellite group increased significantly compared to that of the control satellite group. A significant decrease in the epididymal weight only in animals treated with 1000 mg of the extract was recorded (Table 7).

Sperm count and sperm viability results are presented in Table 7. For determination of viability, unstained sperm heads are scored as live or viable, while stained sperm's head is scored as dead or non-viable (Figure 3). A significant decline in the number and viability of spermatozoa in animals receiving 500 mg and 1000 mg of the extracts, and satellite group was recorded.

Table 7. Effects of methanolic fruit extract of *Zanthoxylum acanthopodium* on testicular weight, epididymal weight, sperm count and sperm viability in mice

	Z. acanthopodium				Satellite	
Parameters	**Control**	**200 mg**	**500 mg**	**1000 mg**	**Control**	**1000 mg**
Testicular weight (g)	0.66 ± 0.03	0.78 ± 0.01	0.79 ± 0.05	0.75 ± 0.01	0.68 ± 0.04	0.89 ± 0.02*
Epididymis weight (g)	0.27 ± 0.01	0.26 ± 0.02	0.26 ± 0.02	0.19 ± 0.01*	0.28 ± 0.01	0.28 ± 0.25
Sperm count (x 10^6)	32.10 ± 0.60	32.40 ± 0.58	29.50 ± 0.42*	28.40 ± 0.37*	32.30 ± 0.66	29.00 ± 0.65*
Sperm viability (%)	86.60 ± 0.68	86.00 ± 0.95	80.00 ± 0.71*	76.00 ± 0.45*	86.80 ± 0.97	79.00 ± 0.71*

Data are expressed as mean ± SEM (n=5).

Asterisk (*) signifies values that are significantly different from control (p<0.05)

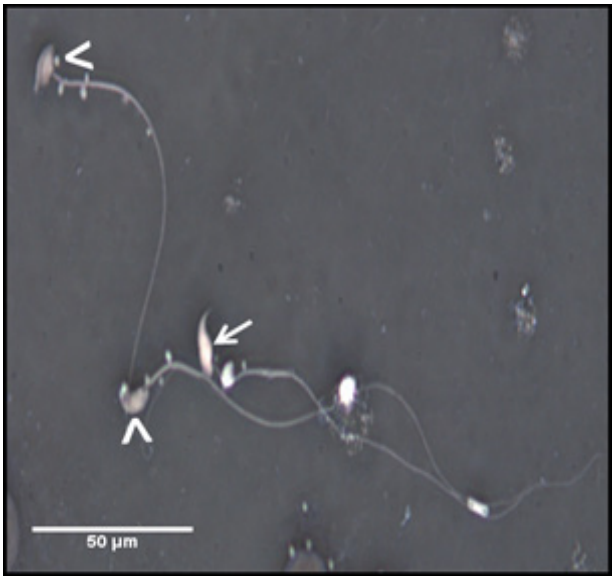


Figure 3. Photomicrograph of Eosin-nigrosin stained spermatozoa showing live sperm (arrow) and dead sperms (arrowhead).

The data on altered sperm morphology are presented in Table 8. It was observed that the number of spermatozoa with abnormal morphology increases in a dose-dependent manner (i.e., 500 mg and 1000 mg MFZA exposed mice) except in the lowest treated group. The types of abnormal sperms recorded were headless sperm, tailless sperm, bent mid-piece sperm, coiled tail sperm, bent head sperm, amorphous head sperm, short sperm, hookless sperm, swollen hook sperm, swollen neck sperm, and coiled sperm (Figure 4). Among them, headless sperm, coiled tail sperm, and amorphous head sperm were the most prevalent in the animals which received 1000 mg MFZA. The treated satellite group showed similar elevation in the numbers of abnormal spermatozoa.

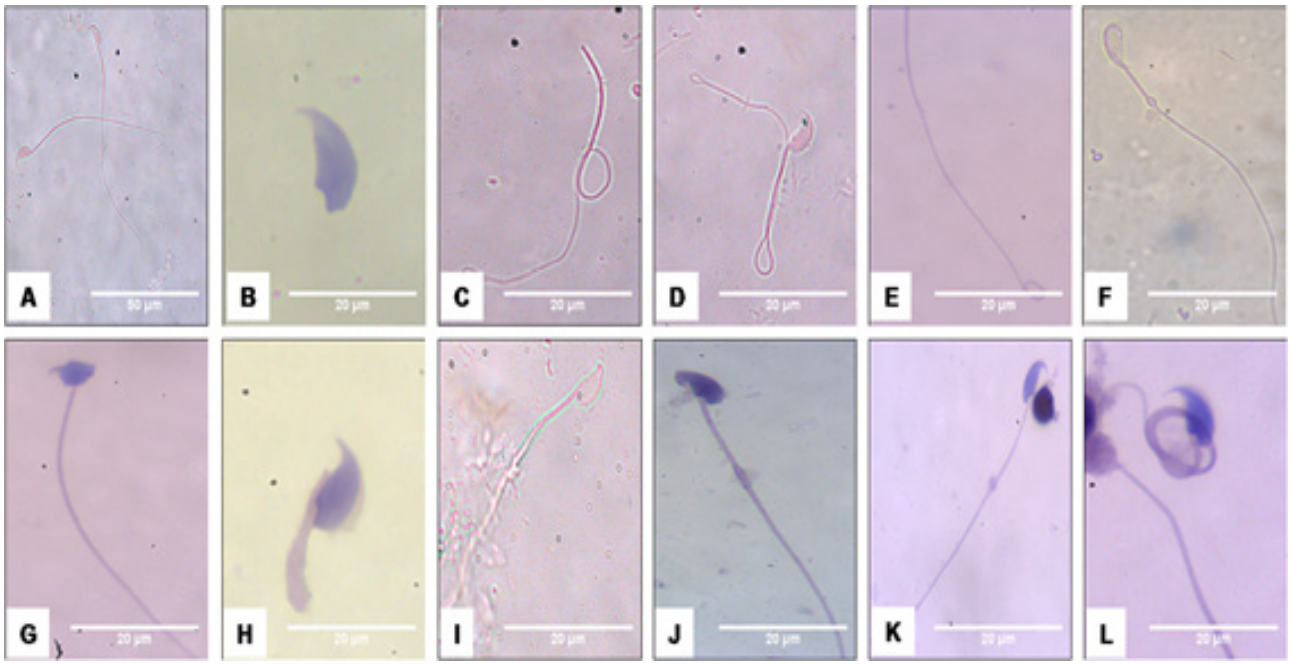


Figure 4. Sperm morphological abnormalities of control and methanolic fruit extract of *Zanthoxylum acanthopodium* treated mice. Papanicolaou stained spermatozoa. **A.** Normal sperm. **B.** Tailless sperm. **C.** Headless sperm. **D.** Bent midpiece. **E.** Coiled tail sperm. **F.** Bent head sperm. **G.** Amorphous head sperm. **H.** Short sperm. **I.** Hookless sperm. **J.** Swollen hook sperm. **K.** Swollen neck sperm. **L.** Coiled sperm.

Table 8. Effects of methanolic extract of *Zanthoxylum acanthopodium* on sperm morphological abnormalities in mice

	Z. acanthopodium				Satellite	
Parameters	**Control**	**200 mg**	**500 mg**	**1000 mg**	**Control**	**1000 mg**
Abnormal sperm	25.20 ± 2.13	25.40 ± 0.51	33.85 ± 0.58*	47.40 ± 0.68*	26.20 ± 1.28	33.60 ± 1.21*
Head less	5.60 ± 1.25	5.80 ± 0.66	6.00 ± 0.32	11.80 ± 1.62*	5.60 ± 0.68	7.80 ± 1.07
Tail less	5.40 ± 1.29	3.20 ± 0.37	4.00 ± 1.52	4.60 ± 0.98	4.80 ± 0.37	6.80 ± 0.37
Bent mid piece	2.00 ± 0.63	2.20 ± 0.73	4.80 ± 0.97	4.00 ± 1.09	4.40 ± 0.60	4.20 ± 0.73
Coiled tail	1.40 ± 0.51	1.40 ± 0.40	2.40 ± 0.51	5.40 ± 1.87*	1.80 ± 0.37	3.60 ± 0.68
Bent head	0.60 ± 0.40	1.00 ± 0.45	1.00 ± 0.55	2.40 ± 0.60	0.80 ± 0.37	2.40 ± 0.51
Amorphous head	3.00 ± 0.71	3.60 ± 0.51	2.80 ± 0.66	7.60 ± 2.06*	1.60 ± 0.40	1.60 ± 0.24
Short sperm	1.60 ± 0.93	2.40 ± 0.68	2.20 ± 0.66	0.80 ± 0.20	1.80 ± 0.37	2.00 ± 0.84

Hook less	0.40 ± 0.24	1.40 ± 0.40	2.00 ± 0.63	1.00 ± 0.45	0.40 ± 0.25	0.60 ± 0.60
Swollen hook	0.00 ± 0.00	0.80 ± 0.37	1.20 ± 0.58	1.00 ± 0.45	0.40 ± 0.25	0.20 ± 0.20
Swollen neck	3.60 ± 1.36	2.80 ± 0.58	6.60 ± 1.63	7.00 ± 1.97	3.80 ± 1.24	2.60 ± 0.98
Coiled sperm	1.00 ± 0.45	0.40 ± 0.25	0.80 ± 0.37	1.80 ± 0.80	0.80 ± 0.49	1.80 ± 0.66

Data are expressed as mean ± SEM (n=5).

Asterisk (*) signifies values that are significantly different from control ($p<0.05$)

3.4 Chromosomal Aberrations (CAs)

The frequency and types of aberrations which resulted from administering the extract to the animals are presented in Table 9 and Figure 5. It was observed that an increased occurrence of CAs was predominant in the animals receiving 500 mg/kg and 1000 mg extract/kg bw of mice as well as the satellite group. CAs recorded in the study are chromatid crossing over, sister chromatid separation, centric fusion, polyploidy, isochromatid break, and chromatid break. Aberrations like chromatid crossing over, sister chromatid separation, and centric fusion were observed in all the groups and these changes

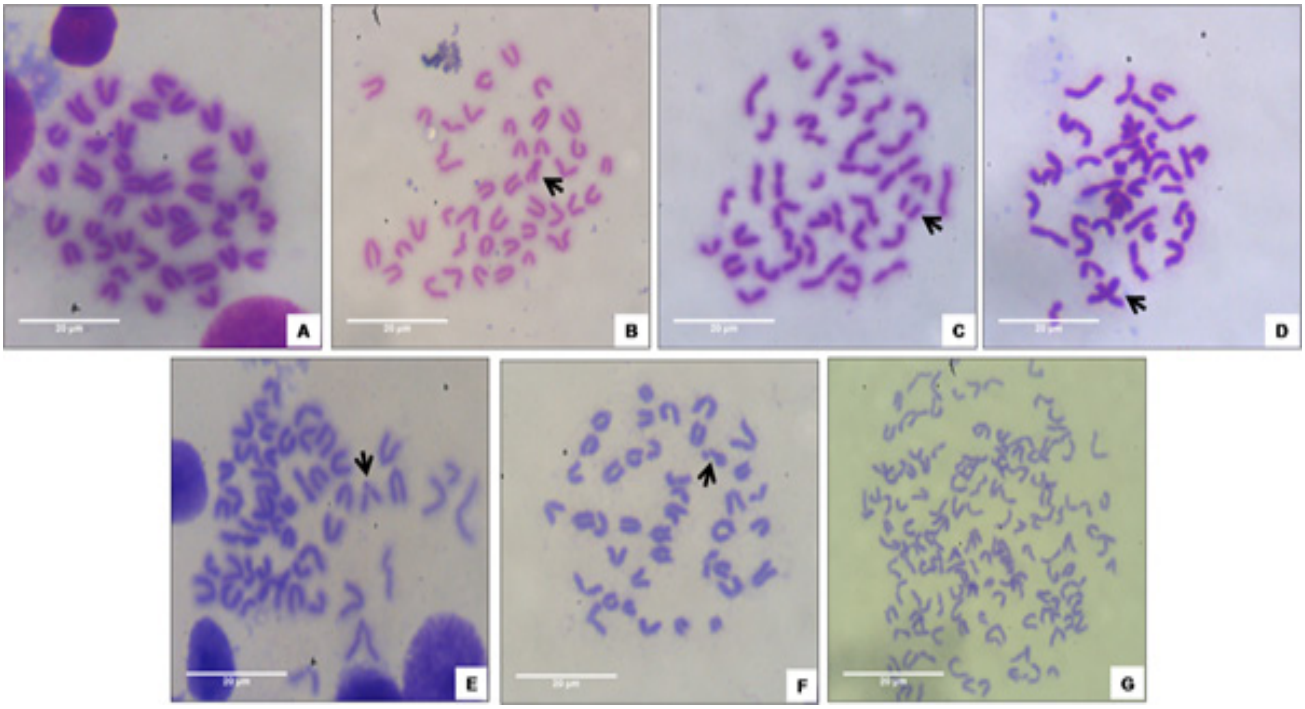


Figure 5. Chromosomal aberrations in bone marrow cells of control and methanolic fruit extract of *Zanthoxylum acanthopodium* treated mice. **A.** Normal chromosomes. **B.** Chromatid crossing over. **C.** Sister chromatid separation. **D.** Centric fusion. **E.** Isochromatid break. **F.** Chromatid break. **G.** Polyploidy.

Table 9. Effects of methanolic fruit extract of *Zanthoxylum acanthopodium* on chromosomal aberrations in mice

	Z. acanthopodium				Satellite	
Parameters	**Control**	**200 mg**	**500 mg**	**1000 mg**	**Control**	**1000 mg**
Aberrations	4.50 ± 0.50	4.25 ± 0.25	8.25 ± 0.48*	17.00 ± 0.71*	4.75 ± 0.48	10.25 ± 0.63*
Chromatid crossing over	1.00 ± 0.00	1.00 ± 0.00	1.25 ± 0.25	2.00 ± 0.41	1.75 ± 0.25	2.00 ± 0.41
Sister chromatid separation	1.50 ± 0.29	1.75 ± 0.25	2.00 ± 0.41	1.50 ± 0.29	1.00 ± 0.41	1.25 ± 0.75
Centric fusion	2.00 ± 0.71	1.50 ± 0.29	1.75 ± 0.25	3.75 ± 0.85	1.75 ± 0.85	1.50 ± 0.64
Polyploidy	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.50 ± 0.50	0.00 ± 0.00	0.00 ± 0.00
Isochromatid breaks	0.00 ± 0.00	0.00 ± 0.00	3.25 ± 0.75*	8.25 ± 0.25*	0.00 ± 0.00	5.00 ± 1.08*
Chromatid break	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.00 ± 0.58	0.00 ± 0.00	0.50 ± 0.29

Data are expressed as mean ± SEM (n=4).

Asterisk (*) signifies values that are significantly different from control ($p<0.05$)

were not significant to that of the control group. It was also observed that aberration like polyploidy was only observed in the highest dose treated group, though the numbers are insignificant. Chromatid break was recorded in the highest treated group as well as satellite group but in both cases, they are not significant. However, isochromatid break was highly significant in 500 mg, 1000 mg/kg bw treated groups, and the satellite group. The frequency of isochromatid breaks was highest in the 1000 mg treated animals which was almost eight times that of the control group.

3.5 DNA Fragmentation

Qualitative analysis of apoptosis using TUNEL assay revealed that the number of apoptotic cells in the group

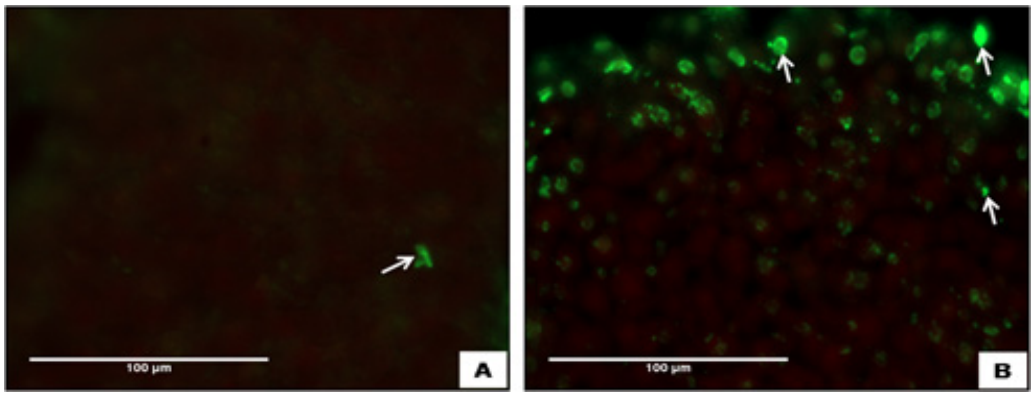


Figure 6. TUNEL stained photomicrograph of control and methanolic fruit extract of *Zanthoxylum acanthopodium* treated mice. **A.** Control liver section showing lack of apoptotic cells. **B.** Treated liver section showing numbers of brightly fluoresced green colored apoptotic cells.

treated with the highest dose of the plant extract has increased significantly when compared to a control group (Figures 6A and 6B).

4. Discussion

Acute toxicity results showed the LD_{50} of the plant extract to be above 5000 mg/kg bw and according to the OECD guidelines[20], the plant extract can be considered safe and testing at higher doses (>5000 mg/kg bw) is not suggested. In our study, the mice selected were in the growing stage and hence, the increase in the mean weight gain of the control group may be due to the normal growth of the animals. Therefore, a decline in the body weight of the treated group indicates that the plant suppresses or inhibits the normal growth and development of the test animals. However, it may also be noted that there were no significant changes recorded in the weight of the organs of the treated animals in comparison to the control group which suggests that a single dose treatment with the plant extract is not enough to alter the weight of the organs. Therefore, the reduction in the body weight may be due to compound(s) that interfere with the metabolism of lipids. An example of compounds known to have this property are saponins which have been reported to be present in *Z. acanthopodium*[14,26].

The sub-acute toxicity study indicates the effect of the plant on the body weight where the plant at a low dose prevents the weight gain of the animals. This kind of result was reported by Jambocus *et al.*,[27] where the scientists tested the plant *Morinda citrifolia* on rats fed with a normal as well as a high-fat diet and found out that the extract prevented the weight gain of the animals in both cases. They suggested that the plant has anti-obesity properties and served as a potent anti-diabetic remedy.

The hematological assessment showed that higher doses of the extract enhance the number of RBCs in circulation which may be an indication of positive erythropoiesis or polycythemia inducing properties of the plant[28]. PCV is the percentage of RBCs in the total blood volume and is usually three times of the RBCs count. Therefore, an increase in the percentage of PCV correlates with the increase in RBCs count. MCV represents the size of the RBC, where high MCV represents a large cell and low MCV represents a small cell. Our investigation suggests the possibility that the plant possesses compounds that cause the RBC to shrink in size[29]. An increase in the Hb concentration is correlated with the production of a huge number of the RBCs in the circulation. On the other hand, MCH count which denotes the amount of hemoglobin per cell reduces significantly. Each RBC carries millions of Hb in them[30]. Therefore, it is obvious that with the reduction in the size of the cell, the Hb carrying capacity decreases as well as the concentration of Hb per cell. Increased RBCs count and reduced MCH persisted in the satellite group signifying that the extract had a prolonged effect on these blood parameters. Our investigation also revealed an increase in leukocyte count which indicates either a case of leukocytosis or that this increase itself is a sign of an immunity boost. Moreover, an increase in neutrophils count has been associated with bacterial infection, damaged tissue, and chronic inflammation. However, a decline in lymphocyte numbers may impair the body's defense against antigens and thereby leaving the body susceptible to diseases[31,32]. An increase in platelet count signifies that the extract might trigger thrombopoietin, a hormone that stimulates the production of thrombocytes. Abnormal production of platelets may result in the formation of a blood clot in the vessels and tannins are secondary metabolites that can inflict these responses[29]. The satellite group showed a somewhat similar result, i.e., an increase in WBC, neutrophils, and platelet count indicating that the negative effects of the plant persisted even after termination of the treatment.

The liver is the vital organ for the metabolism and detoxification of substances or xenobiotics[33]. Hence, damage to the cellular structures of the hepatocytes following the administration of the plant extract may have highly compromised the normal functions of liver tissue. Similar to our findings, Soren *et al.*[34] reported the hepato-toxicity inducing potential of the plant *Cyperus compressus* on the ultrastructures of the parenchymal cell of Swiss albino mice. Additionally, the extract appears to cause an abnormal growth of the nucleus resulting in nuclear enlargement. Nuclear enlargement takes place when a cell undergoes normal DNA replication but fails to enter the mitosis stage by arresting in the G2/M phase[35]. Therefore, it may be possible that the extract can interfere with the cell cycle stages. Our investigation also observed the presence of infiltrative leukocytic cells on the liver tissue, which usually is a response to tissue injury but if it is excessive and prolonged, it might lead

to severe inflammation. This process may result in a heavy deposition of collagen fibres in the tissue causing acute liver diseases[36,37]. Therefore, these results explain the increase in the relative organ weight of the treated liver tissue. It also explains the elevation of ALT and AST which are the liver enzymes that leak into the serum when the hepatocytes are damaged, and ALP when the bile canaliculi are obstructed[38–40]. It may be noted here that after the termination of the extract treatment, the animals (satellite group) showed an increase only in the ALT enzyme level which may be an indication that the liver is recovering from the effects of the extract.

Kidney is another vital organ that performs the function of eliminating the waste particles which develop after metabolic processes[41]. From our investigation, it was evident that the extract alters the glomerular filtration barrier, and developed fibrils on the cell body with an enlargement of the glomerulus. Many plants and their active derivatives have been reported to exert severe effects on the architecture of the glomerulus causing acute renal injury[42–44]. The toxic effect on the ultrastructures of the tubule was restricted only to the nucleus where the chromatin was highly disturbed on exposure to the extract. Nevertheless, alteration of the chromatin material will result in the apoptosis of the epithelial cell and hence, impairs the tubule's function[45,46]. This result correlates with the renal function test markers, i.e., serum creatinine and serum urea, which rise in their levels when the injured kidney fails to perform its normal function[47]. Also, this effect lasted even in the satellite group. It may be noted that several cases of polycythemia associated with erythropoietin and renal injury have been discussed[48,49], and this may explain the high levels of RBCs counts in MFZA treated animals.

For sperm abnormality, we investigated the epididymal spermatozoa since the immature spermatozoa undergo maturation in the epididymis and are stored in the epididymis itself until ejaculation[50]. It is evident from the results that the extract at high concentration reduces the sperm count and sperm viability, while the percentage of abnormal sperms was highly elevated, and these effects persist even after the termination of the treatment as evident from the findings in the satellite group. Reduction in the numbers of spermatozoa may be correlated with the decline in the weight of the epididymis and thus, reducing the storage capacity. Yakubu[51] observed a similar result and suggested that the extract of *Chromolaena odorata* may have interfered with the production of testosterone to the seminiferous tubules and therefore, alters the process of spermatogenesis. Morphological changes observed in the head of the spermatozoa may be due to either an induction of point mutation or an abnormal packing of the genetic material[52]. While an effect to the mid-piece and the tail may be an indication that the extract impairs the energy production and motility of sperms[53]. Effect on sperm viability may be attributed to tannins, which have been reported to reduce cell viability at higher concentrations[54]. Besides tannins, several other phyto-compounds like alkaloids, flavonoids, steroids, terpenoids, and saponins are known to interfere with some steps of spermatogenesis[55–57].

The genotoxic effect was evaluated by *in vivo* chromosome assay from the bone marrow cells of the animals. From our investigation, the high frequency of chromatid and isochromatid break occurrence at doses above 200 mg indicates that at higher concentrations the extract targets the DNA causing double-stranded lesions in both the arms of the sister chromatids[58] and hence, act as a potent genotoxicant. Similar to our study, numbers of medicinal plants and their compounds have been scientifically proven to be a potent genotoxicant when delivered at larger concentrations[59,60]. The effect on the chromosomes was also seen in the satellite group indicating that the plant has a persisting effect on the genetic material. However, when compared to the highest dose treated group, the chromosomal aberrations were slightly lowered suggesting the recovery of the animals from the genotoxic effect.

Genotoxicant property was further supported by the results of the TUNEL assay which suggest that the phyto-compounds target the genetic material resulting in the fragmentation of DNA, and therefore, it can be implied that the extract has the potential to induce apoptosis. Similar to our result, Roy *et al.*[62] showed that the extract of *Carex baccans* and *Potentilla fulgens*, when administered to rats for 14 days at a concentration as low as 400 mg/kg bw, induced apoptosis to liver cells.

5. Conclusion

This study revealed that the methanolic fruit extract of *Zanthoxylum acanthopodium* causes high levels of

toxicity at a concentration of 500 mg/kg bw and above. These effects were reflected in the blood parameters and physiology of vital organs such as the liver and kidney. The extract affected the spermatozoa by reducing the sperm count, sperm viability on the one hand while increasing the frequency of abnormally shaped sperm on the other. It increases the occurrence of chromosomal aberrations and induces cell death. Moreover, the extract has a long-lasting adverse effect on the test animals which was evident from the data of the satellite group. Therefore, from the present investigation, it is strongly suggested that repeated intake of the plant at high concentrations should be discouraged.

6. Acknowledgement

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7. Conflict of Interest

The authors declared that there was no conflict of interest

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