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(RESEARCH ARTICLE)

Median lethal dose and sub-chronic toxicity profile of *Azadirachta indica* A. Juss. leaf hexane and ethyl acetate fractionated extracts on albino rats

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Abstract

Azadirachta indica A. Juss. (Neem) is a multipurpose medicinal plant, traditionally used in the treatment of various human ailments. This plant is tagged as having high toxicity profile, and the toxicity might be related to the polarity nature of the solvent used for extraction. Hence, this research was aimed at screening the toxicity profile of *Azadirachta indica* leaf fractionated extracts using Albino rats. *A. indica* leaf was collected, authenticated and extracted using 95 % methanol then fractionated with hexane and ethyl acetate. Median lethal dose (LD_{50}) of each fraction was determined using single oral dose of 5,000 mg/kg body weight (b. wt.) to five (5) rats. For the sub-chronic toxicity screening, the fractions were administered to groups of rats at different concentrations. Group 1 served as control, groups 2-5 received 900, 1800, 2700 and 3600 mg/kg b. wt. respectively. After 28 days, biochemical indices of hepatic and renal functions as well as haematological and histopathological parameters were analyzed. LD_{50} of each of the fractions was greater-than 5000 mg/kg b. wt. All the extract fractions at the administered doses, significantly ($P<0.05$) altered the serum levels of some biochemical indices of the hepatic and renal functions, as well as the levels of some haematological parameters. For the histopathology, hepatic congestion, periportal inflammation, distortion, infiltration and haemorrhage were observed at 1800-3600 mg/kg b. wt. Hence, these results indicated that using hexane or ethyl acetate as solvent of extraction, *A. indica* leaf extracts might not be considered safe at the administered sub-chronic doses.

Keywords*:* *Azadiracta indica*; Media lethal dose; LD_{50}; Sub-chronic toxicity; Fractionated extracts

1. Introduction

For centuries, medicinal plants are the basis for the treatment of various diseases [1]. Nearly 80 % of people living in developing countries still depend on plant-based traditional medicine for their primary health care and almost 75 % of the herbal drugs used worldwide are derived from medicinal plants [2]. However, the quality control of herbal medicine remains a challenge owing to the fact that there is a high variability in the active constituents involved [3]. Hence, World Health Organization (WHO) has approved fingerprint technique or standardized extract for quality assurance of herbal medicines [4].

Many plants synthesize compounds that are useful for the maintenance of health in humans and animals, like aromatic substances, most of which are phenols or their oxygen-substituted derivatives such as tannins [5]. Many of these compounds extracted from natural products could be useful lead compounds in the production of drugs [6]. However, many plants have also been reported to be toxic to both human and animals [7, 8]. It should therefore, be emphasized that for any traditional use of medicinal plant, its safety should be ascertained. Plants in folk medicine should therefore, be evaluated for safety or toxicity and necessary recommendations made on their use. The data of the acute and sub-

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chronic toxicity studies on medicinal plants or preparations derived from them should be obtained in order to increase the confidence in their safety to humans, particularly for use in the development of pharmaceutical agents [9].

Azadirachta indica (Neem tree) belongs to the *Meliaceae* (mahogany) family. It is known as '*Dogon yaro or Darbejiya'* in Hausa language. The tree can grow up to 30m tall with spreading branches covering some 10 m across [10]. The neem tree has long been recognized for its unique properties both against insects and in improving human health [11]. It is grown in most tropical and sub-tropical areas of the world for shade, reforestation and for the production of raw material for natural insecticides and medicines [12]. Every part of the tree has been used as traditional medicine for household remedy against various human ailments, from antiquity [13].

Different parts of Neem tree such as seeds, leaves, flowers and the bark are widely used for different purposes [14]. Different phytochemicals such as quercetin and azadirachtin and liminoids such as nimbin, nimbinin, and nimbidin have been purified from the different parts of the plant. Moreover, the leaves also contain mixture of compounds such as nimbanene, 6-desacetylnimbinene, nimbandiol, nimbolide, ascorbic acid, n-hexacosanol, nimbiol, various amino acids and several other types of ingredients [15].

However, the question arises whether the safety of this plant extends to extracts or pure compounds that may be used in a more concentrated form for treating or preventing different diseases. Toxicological studies suggested that the differences in the solvents and methods used to prepare extracts could affect the toxicity level [16]. Hence, this research was centered at evaluating the effect of hexane and ethyl acetate (as solvents for extraction) on the toxicity profile of *Azadirachta indica* leaf extracts.

2. Materials and methods

2.1. Study area

The research work was conducted within Aliero town, Nigeria. It was performed in Biochemistry Research Laboratory, Department of Biochemistry, Faculty of Life Sciences, Kebbi State University of Science and Technology, Aliero, Nigeria.

2.2. Experimental animals

Adult Wistar albino rats of both sexes aged 3 – 4 months and weighing between 150 – 200 g were used for the experiments. They were purchased from National Veterinary Research Institute, Vom, Nigeria and kept under standard laboratory conditions (22–24 °C; 12:12 h dark/light cycle). The animals were allowed free access to both food (commercial rodents pellets) and water *ad libitum* [17], they were allowed to acclimatize for 2 weeks. Weight of each rat was taken before the commencement of the experiment. All animal experiments were conducted in accordance with the guidelines for the use and care of experimental animals [18].

2.3. Collection and authentication of the plant material

Azadirachta indica leaf was collected within Aliero town, Kebbi State, Nigeria. It was authenticated at the herbarium of the Department of Plant Science and Biotechnology, Kebbi State University of Science and Technology, Aliero, Nigeria and voucher specimen; VN:083 was deposited there.

2.4. Preparation of crude *A. indica* leaf methanol extract

The extract was prepared according to the method of Dupont *et al*., [19]. The collected leaf was cleaned with water and air-dried under shade, pulverized using pestle and mortar. One kilogram (1 kg) of the powdered leaf was measured and soaked in 2.5 l of 95% methanol. The mixture was then kept at room temperature for 24 h and filtered twice; initially with a muslin cloth and later with a Whatman filter paper No.1. The filtrate was evaporated to dryness at 45 °C using rotary evaporator. The residue was further fractionated.

2.5. Solvent-fractionation of crude *A. indica* leaf methanol extract

The crude methanol extract of the *A. indica* leaf was fractionated by liquid-liquid extraction using n-hexane and ethyl acetate in increasing order of polarity. Two hundred grams (200 g) of the dried methanol extract were reconstituted in 400 ml of distilled water in a 1 liter separating funnel. This was then partitioned sequentially with equal volume of n-hexane and ethyl acetate to yield the n-hexane and ethyl acetate fractions. The fractions were concentrated to dryness and the residues were kept in a refrigerator in an air-tight container for further use. Before use, each fraction was reconstituted in 1 % aqueous solution of Tween-80 (polysorbate) and was expressed in terms of dry weight (mg/ml).

2.6. Toxicity screening of the hexane and ethyl acetate fractions

The toxicity studies on the *Azadirachta indica* leaf hexane and Ethyl acetate fractions were carried out on albino rats using the methods described by the Organization for Economic Co-operation and Development, for the acute and sub-chronic toxicity studies respectively [20, 21].

2.6.1. Determination of median lethal dose (LD_{50})

Five (5) rats were used for each fraction (n-hexane and ethyl acetate). A single high dose of 5,000 mg/kg b. wt. of fraction was administered to each rat orally. Each fraction was reconstituted in 1 % aqueous solution of tween-80. The treatment followed an overnight fasting period, and body weights of the rats were determined immediately after the fasting period before administering the extract. The doses were calculated in reference to the body weight, as the volume of the extract solution administered to each rat was 10 ml/kg b. wt. Food was provided to the rats approximately an hour after treatment. Each rat was observed in detail for mortality and any behavioral changes or sign of toxic effect within the first 8 hours, 24 hours and 48 hours after the treatment period, and then daily for a period of 14 days. Mortality of two (2) rats or none indicated that the LD_{50} is greater than 5,000 mg/kg b. wt. If three (3) rats and above died, the LD_{50} is less than 5,000 mg/kg b. wt. [20].

2.6.2. Sub-chronic toxicity screening

For each fraction, rats were distributed into five (5) groups of six (6) rats each (3 males and 3 females). Daily oral administration of different concentrations of the extract was carried out for 28 days. Weights of the rats were taken immediately before the commencement of extract administration, then weekly for four (4) weeks.

- Group 1: received 1% aqueous solution of tween-80 orally and served as normal control for the period of the study.
- Group 2 to 5: received graded doses of the extract (900, 1800, 2700 and 3600 mg/kg body weight respectively). The doses were calculated in reference to the body weight, as the volume of the extract solutions administered to the rats was 10 ml/kg b. wt.

All the groups received same volume of preparations. The weights of the rats were taken weekly and detailed observation for the signs of toxicity was done twice daily for the period of 28 days. The rats were fasted overnight on the 28^{th} and on the 29^{th} day, there after the rats were anesthetized with chloroform and sacrificed, then blood samples were collected into tubes with and without EDTA for haematological and biochemical analyses respectively. The coagulated blood samples for biochemical analyses were centrifuged at 3,000 rpm for 10 min. to obtain the sera. The biochemical and haematological analyses were carried out. The liver was excised and washed with ice cold saline and used for histopathological analysis.

Serum biochemical analyses

The following parameters were analyzed in the sera collected: Aspartate aminotransferase (AST) [22], Alanine aminotransferase (ALT) [22], Alkaline phosphatase (ALP) [23], γ-glutamyl transferase (GGT) [24], Lactate dehydrogenase (LH) [23], Total and Direct Bilirubin [25], Albumin [26], Total protein [27], Urea [28], Uric acid [29], Electrolytes and Creatinine [30].

Haematological analyses

Haematological analyses were performed using an automatic haematological analyzer (Coulter STKS, Beckman Coulter, California, USA) by determining the amount of the following blood parameters [31]: Packed Cell Volume (PCV), Haemoglobin (Hb), Red Blood Cell (RBC), Mean Cell Volume (MCV), Mean Corpuscular Haemoglobin (MCH), Mean Corpuscular Haemoglobin Concentration (MCHC), White Blood Cell (WBC), lymphocytes, neutrophils, monocytes, basophils and platelets.

Histopathological analysis

The collected liver was kept for at least 24 h in buffered formalin, then each one was dehydrated with alcohol and embedded in paraffin wax, then cut into 4-5 μm thick sections. Each was then stained with Haematoxylin-Eosin dye for photo-microscopic observation. The microscopic features of the liver from each rat were compared with that of the control group [32].

2.7. Data analysis

The data generated from the study are presented as mean ± SEM and subjected to one way analysis of variance (ANOVA) and statistical difference between the means were separated using New Duncan's Multiple Range Test at $P<0.05$ with the aid of a statistical package (IBM SPSS Statistics 20).

3. Results

3.1. Median lethal dose (LD_{50}) of the hexane and ethyl acetate fractions

Zero and one (1) death were recorded in rats during the 14 days of monitoring after acute oral administration with 5,000 mg/kg b. wt. hexane and ethyl acetate fractions of the *A. indica* leaf respectively (Table 1). Both the hexane and ethyl acetate fractions at the administered dose of 5000 mg/kg b. wt. produced no mortality after 48 hours of observation. The death of one (1) rat administered with ethyl acetate fraction was observed after eight (8) days. None of the extracts produced any behavioral changes or hazardous signs in the rats. Hence, the median lethal dose (LD_{50}) of each of the fraction was estimated to be greater than 5,000 mg/kg body weight.

Table 1 LD_{50} (48 h) of hexane and ethyl acetate fractions of *A. indica* leaf

Extract fraction	Number of rats used	Dose of extract (mg/kg b. wt.)	Number of death	Number of survival
n-Hexane	5	5000	0	5
Ethyl acetate	5	5000	1	4

The LD_{50} of each fraction was determined to be >5,000 mg/kg body weight.

3.2. Sub-chronic toxicity profile of hexane fraction

Table 2 presents the weights of the rats recorded each week for the 4 weeks period of administration of the hexane fraction. At the administered doses of the plant's fraction, the rats gained weight after each week.

Table 2 Body weights of rats administered with hexane fraction of *A. indica* leaf for 28 days

Week	Animals body weight (g)				
	Control	900 mg/kg b. wt.	1800 mg/kg b. wt.	2700 mg/kg b. wt.	3600 mg/kg b. wt.
0	48.80 ± 5.18	66.85 ± 3.34	72.90 ± 4.10	87.15 ± 2.10	124.90 ± 3.80
1	54.40 ± 1.39	69.50 ± 2.11	73.30 ± 1.20	94.15 ± 3.30	127.60 ± 2.40
2	59.00 ± 6.90	72.00 ± 4.30	77.00 ± 2.90	93.00 ± 3.11	132.50 ± 4.20
3	68.00 ± 1.20	79.50 ± 2.10	85.50 ± 3.10	98.50 ± 7.70	138.50 ± 4.15
4	76.50 ± 2.40	84.60 ± 5.10	87.80 ± 2.20	102.65 ± 7.06	140.55 ± 6.07

Results are presented as Mean ± SEM (n = 6).

During the sub-chronic oral toxicity screening, no abnormal behavioral or physical changes were observed. The biochemical parameters related to hepatic function such as; Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Alkaline Phosphatase (ALP), Gamma-glutamyl transferase (GGT), Lactate Dehydrogenase (LDH), Albumin (ALB), Total Protein (TP), Total Bilirubin (TB) and Direct Bilirubin (DB) were analyzed after 28 days (Table 3). The hexane fraction of the *A. indica* leaf significantly ($P<0.05$) increased the serum levels of AST, ALT, ALP, TB and TP in a dose-dependent manner compared to the control (Table 3). In contrast, the levels of LDH, DB and ALB were not significantly ($P>0.05$) affected. The serum level of GGT recorded a significant dose-dependent decrease when compared to the normal control.

Table 3 Liver function indices of rats administered with hexane fraction of *A. indica* leaf for 28 days

Test parameters	Administered hexane extract (mg/kg b. wt.)				
	Control	**900**	**1800**	**2700**	**3600**
AST (U/l)	87.00 ± 3.95^{a}	268.50 ± 19.09^{b}	276.00 ± 46.89^{b}	284.50 ± 21.71^{b}	332.00 ± 51.56^{c}
ALT (U/l)	25.00 ± 2.08^{a}	212.50 ± 14.85^{bc}	172.50 ± 33.23^{b}	247.50 ± 0.71^{c}	249.00 ± 26.87^{c}
ALP (U/l)	599.00 ± 34.22^{a}	608.03 ± 128.69^{a}	779.50 ± 44.87^{b}	815.00 ± 89.10^{b}	873.00 ± 73.17^{bc}
GGT (U/l)	7.00 ± 0.27^{b}	4.00 ± 1.41^{a}	4.00 ± 2.83^{a}	3.50 ± 0.71^{a}	4.50 ± 3.54^{a}
LDH (U/l)	6930.00 ± 342.03^{a}	7045.00 ± 714.18^{a}	6675.00 ± 500.18^{a}	6715.00 ± 308.15^{a}	6585.00 ± 528.46^{a}
TB (mg/dl)	1.10 ± 0.06^{a}	1.28 ± 0.68^{a}	1.74 ± 0.28^{ab}	1.97 ± 0.47^{b}	2.75 ± 1.44^{bc}
DB (mg/dl)	0.38 ± 0.01^{a}	0.35 ± 0.16^{a}	0.32 ± 0.11^{a}	0.35 ± 0.07^{a}	0.39 ± 0.01^{a}
ALB (g/l)	40.52 ± 3.49^{a}	33.50 ± 3.54^{a}	38.06 ± 2.83^{a}	37.50 ± 3.54^{a}	35.30 ± 6.36^{a}
TP (g/l)	23.23 ± 4.83^{a}	31.50 ± 4.36^{a}	67.64 ± 9.90^{b}	64.51 ± 6.36^{b}	66.76 ± 1.41^{b}

Results are presented as Mean ± SEM (n = 6). Values carrying different superscripts from the normal control for each parameter are significantly (P<0.05) different using ANOVA and Duncan multiple range test. AST = Aspartate Transaminase, ALT = Alanine Transaminase, ALP = Alkaline phosphatase, GGT = γ-Glutamyl Transferase, LDH = Lactate Dehydrogenase, TB = Total Bilirubin, DB = Direct Bilirubin, ALB = Albumin, TP = Total Protein.

Table 4 Renal function indices of rats administered with hexane fraction of *A. indica* leaf for 28 days

Test parameters	Administered hexane extract (mg/kg b. wt.)				
	Control	900	1800	2700	3600
Urea (mmol/l)	8.80 ± 0.48^{c}	6.85 ± 0.64^{b}	5.90 ± 0.14^{ab}	7.15 ± 0.21^{b}	4.90 ± 0.85^{a}
Uric acid (mg/dl)	4.40 ± 0.39^{b}	3.50 ± 0.71^{ab}	3.30 ± 1.27^{ab}	3.15 ± 0.21^{a}	2.60 ± 0.42^{a}
Creatinine (mmol/l)	72.06 ± 3.91^{a}	77.28 ± 18.39^{a}	75.20 ± 9.90^{a}	73.30 ± 31.11^{a}	72.52 ± 33.23^{a}
Na^{+} (mEq/l)	140.00 ± 5.50^{a}	139.50 ± 2.12^{a}	138.50 ± 2.12^{a}	140.50 ± 0.71^{a}	146.50 ± 2.12^{ab}
K^{+} (mEq/l)	7.50 ± 1.42^{a}	8.60 ± 0.00^{ab}	7.80 ± 3.25^{a}	8.65 ± 1.06^{ab}	11.55 ± 0.07^{b}
Cl^{-} (mEq/l)	104.00 ± 3.97^{ab}	102.50 ± 3.54^{a}	104.50 ± 3.54^{ab}	101.00 ± 0.00^{a}	109.00 ± 1.41^{b}
HCO_3^{-} (mEq/l)	30.12 ± 2.61^{a}	29.51 ± 3.54^{a}	27.53 ± 0.71^{a}	30.11 ± 0.86^{a}	27.51 ± 0.71^{a}

Results are presented as Mean ± SEM (n = 6). Values carrying different superscripts from the normal control for each parameter are significantly (P<0.05) different using ANOVA and Duncan multiple range test.

Table 5 Haematological indices of rats administered with hexane fraction of *A. indica* leaf for 28 days

Test parameters	Administered hexane extract (mg/kg b. wt.)				
	Control	**900**	**1800**	**2700**	**3600**
WBC (x10^3/µl)	2.70 ± 0.04^a	7.00 ± 0.42^b	9.05 ± 0.03^b	11.45 ± 3.32^c	12.45 ± 2.05^c
LYM (%)	28.10 ± 2.44^a	72.25 ± 2.09^b	79.85 ± 6.86^{bc}	70.95 ± 5.87^b	79.85 ± 3.71^{bc}
MON (%)	3.70 ± 0.39^a	3.81 ± 0.28^a	2.93 ± 0.14^a	7.26 ± 1.82^b	7.11 ± 0.97^b
NEU (%)	9.65 ± 1.29^a	12.41 ± 2.77^{ab}	13.65 ± 1.44^{ab}	16.57 ± 1.98^{bc}	18.85 ± 2.27^c
EOS (%)	1.03 ± 0.08^b	0.95 ± 0.07^b	0.41 ± 0.04^a	0.54 ± 0.21^a	0.46 ± 0.07^a
BAS (%)	4.62 ± 1.58^a	4.63 ± 0.54^a	3.93 ± 1.13^a	4.71 ± 0.49^a	4.12 ± 0.64^a
RBC (x10^6/µl)	4.82 ± 0.51^a	5.91 ± 0.53^a	4.78 ± 1.61^a	6.62 ± 0.05^a	5.91 ± 0.32^a
HGB (g/dl)	11.82 ± 2.10^a	11.39 ± 0.14^a	10.97 ± 2.83^a	12.31 ± 0.57^a	11.32 ± 0.21^a
HCT (%)	27.37 ± 4.39^a	31.69 ± 0.49^{ab}	26.32 ± 1.51^a	34.40 ± 0.71^b	32.10 ± 0.14^{ab}
MCV (µm^3)	56.62 ± 5.12^a	53.85 ± 3.96^a	55.96 ± 5.23^a	52.04 ± 0.71^a	54.42 ± 3.18^a
MCH (pg)	20.32 ± 2.90^a	19.94 ± 1.48^a	19.16 ± 0.49^a	18.69 ± 0.71^a	19.21 ± 1.34^a
MCHC (g/dl)	35.91 ± 3.71^a	35.74 ± 0.14^a	34.38 ± 2.26^a	35.73 ± 0.92^a	35.33 ± 0.49^a
PLT (x10^3/µl)	64.08 ± 6.29^a	435.05 ± 20.30^b	450.56 ± 24.19^{bc}	483 ± 97.58^c	507.53 ± 15.26^c
MPV (µm^3)	8.56 ± 1.04^c	7.22 ± 0.14^b	7.13 ± 0.14^{ab}	7.10 ± 0.13^{ab}	6.90 ± 0.00^a
PCT (%)	0.05 ± 0.01^a	0.31 ± 0.05^b	0.32 ± 0.08^b	0.35 ± 0.01^{bc}	0.35 ± 0.08^{bc}

Results are presented as Mean ± SEM (n = 6). Values carrying different superscripts from the normal control for each parameter are significantly (P<0.05) different using ANOVA and Duncan multiple range test. LYM = Lymphocytes, MON = Monocytes, NEU = Neutrophils, EOS = Eosinophils, BAS = Basophils, RBC = Red Blood Cells, HGB = Haemoglobin, HCT = Haematocrit, MCV = Mean Cell Volume, MCH = Mean Corpuscular Haemoglobin, MCHC = Mean Corpuscular Haemoglobin Concentration, PLT = Platelets, MPV = Mean Platelets Volume, PCT = Plateletcrit.

The treatment with the hexane fraction of *A. indica* leaf for 28 days significantly (P<0.05) altered the serum levels of some biochemical parameters of renal function (Table 4). Urea and uric acid levels were significantly (P<0.05) reduced in a dose-dependent manner compared to the normal control. The levels of creatinine and HCO_3^- were not significantly (P>0.05) altered when compared to the normal control (Table 4). In contrast, the serum levels of Na^+, K^+ and Cl^- were significantly (P<0.05) increased only at the highest dose (3,600 mg/kg b. wt.) of the hexane fraction.

For the haematological parameters as presented in Table 5, the hexane fraction of the *A. indica* leaf at all doses administered did not significantly (P>0.05) change the levels of BAS, RBC, HGB, MCV, MCH and MCHC compared to the normal control. There were significant dose-dependent increases (P<0.05) in the levels of WBC, NEU, PLT and PCT compared to the normal control (Table 5). The levels of LYM and HCT compared to the control group were significantly increased in all the treatment groups but not in a dose-dependent manner. That of MON, only increased significantly (P<0.05) at the highest administered dose of the hexane fraction (3,600 mg/kg b. wt.). In contrast, the MPV recorded a significant dose-dependent decrease in the treatment groups compared to the normal control (Table 5). But, when compared to the control group, the significant decrease in the level of EOS in the treatment groups administered with the hexane fraction of the *A. indica* leaf was not dose-dependent.

3.3. Sub-chronic toxicity profile of ethyl acetate fraction

The animals' weights recorded per week for the 4 weeks administration of the ethyl acetate fraction is presented in Table 6. It was observed that, the control group showed a significant increase in the body weight and body weight gain compared to the treatment groups. However, at the administered doses of the ethyl acetate fraction, the rats gained weight after each week. Hence, the fraction at the administered doses did not prevent the animals from gaining weight (Table 6).

Table 6 Body weights of rats administered with ethyl acetate fraction of *A. indica* leaf for 28 days

Week	Animals body weight (g)				
	Control	900 mg/kg b. wt.	1800 mg/kg b. wt.	2700 mg/kg b. wt.	3600 mg/kg b. wt.
0	78.86 ± 3.48	76.84 ± 3.64	95.92 ± 0.94	117.17 ± 3.21	149.97 ± 3.85
1	84.44 ± 2.39	90.53 ± 2.71	103.33 ± 4.27	123.19 ± 4.21	152.62 ± 2.42
2	97.15 ± 3.91	108.22 ± 8.39	105.14 ± 5.90	126.20 ± 3.10	157.51 ± 3.23
3	103.09 ± 5.50	110.53 ± 2.12	108.51 ± 2.10	129.59 ± 5.70	161.56 ± 2.10
4	119.52 ± 1.42	118.66 ± 0.50	112.83 ± 3.25	134.66 ± 1.05	163.52 ± 3.07

Results are presented as Mean ± SEM (n = 6).

Daily oral administration of the ethyl acetate fraction of the *A. indica* leaf for 28 days did not show any observable sign of toxicity in the rats, including the highest administered dose of 3,600 mg/kg body weight. No death or abnormal clinical signs were observed in all the groups for the whole experimental period. Physical observation of the treated rats throughout the study period indicated that none of them showed any behavioral changes, diarrhea, tremors, sleep or coma. The food and water consumptions of the treated rats were also not affected.

The biochemical parameters of hepatic function such as; AST, ALT, ALP, GGT, LDH, ALB, TP, TB and DB were analyzed after the 28 days treatment (Table 7). The ethyl acetate fraction of the *A. indica* leaf significantly increased (P<0.05) the serum levels of AST, ALT, ALP, LDH, TB, DB, ALB and TP in a dose-dependent manner compared to the control (Table 7). In contrast, the level of GGT was not significantly (P>0.05) affected in the serum of the animals when compared to the normal control group.

Treatment with ethyl acetate fraction of the *A. indica* leaf for 28 days significantly (P<0.05) affected the serum levels of some biochemical parameters of renal function (Table 8). Urea and uric acid levels were significantly reduced in a dose-dependent manner compared to the normal control. In contrast, the serum levels of creatinine and Na^+ were increased significantly, also in a dose-dependent manner compared to the control group. On the other side, the serum levels of K^+, Cl^- and HCO_3^- were not significantly (P>0.05) altered by the administered doses of the *A. indica* leaf ethyl acetate fraction when compared to the normal control group.

Table 7 Liver function indices of rats administered with ethyl acetate fraction of *A. indica* leaf for 28 days

Test parameters	Administered ethyl acetate extract (mg/kg b. wt.)				
	Control	**900**	**1800**	**2700**	**3600**
AST (U/l)	167.35 ± 5.90^{a}	265.18 ± 70.71^{b}	271.56 ± 38.89^{b}	276.09 ± 38.18^{b}	398.03 ± 5.66^{c}
ALT (U/l)	40.14 ± 3.91^{a}	186.07 ± 11.23^{b}	237.23 ± 24.04^{bc}	257.20 ± 16.26^{bc}	285.52 ± 13.44^{c}
ALP (U/l)	491.06 ± 29.01^{a}	600.28 ± 65.05^{ab}	609.13 ± 40.22^{ab}	662.05 ± 62.23^{b}	670.53 ± 18.09^{b}
GGT (U/l)	5.01 ± 0.37^{a}	4.79 ± 1.01^{a}	4.63 ± 1.41^{a}	4.40 ± 0.00^{a}	4.71 ± 1.41^{a}
LDH (U/l)	5880.02 ± 107.36^{a}	7325.13 ± 186.90^{ab}	7415.73 ± 180.90^{ab}	8020.31 ± 735.40^{b}	8985.83 ± 275.77^{bc}
TB (mg/dl)	0.65 ± 0.02^{a}	1.16 ± 0.90^{ab}	1.94 ± 1.33^{b}	1.77 ± 1.18^{b}	1.82 ± 0.52^{b}
DB (mg/dl)	0.26 ± 0.00^{a}	0.41 ± 0.15^{b}	0.43 ± 0.25^{b}	0.45 ± 0.07^{b}	0.50 ± 0.13^{bc}
ALB (g/l)	34.52 ± 2.06^{a}	35.50 ± 0.71^{a}	37.51 ± 0.71^{ab}	37.10 ± 3.83^{ab}	37.82 ± 7.07^{ab}
TP (g/l)	52.92 ± 7.09^{a}	60.37 ± 3.20^{b}	61.43 ± 4.24^{b}	63.50 ± 3.54^{b}	69.41 ± 9.90^{bc}

Results are presented as Mean ± SEM (n = 6). Values carrying different superscripts from the normal control for each parameter are significantly (P<0.05) different using ANOVA and Duncan multiple range test. AST = Aspartate Transaminase, ALT = Alanine Transaminase, ALP = Alkaline phosphatase, GGT = γ-Glutamyl Transferase, LDH = Lactate Dehydrogenase, TB = Total Bilirubin, DB = Direct Bilirubin, ALB = Albumin, TP = Total Protein.

Table 8 Renal function indices of rats administered with ethyl acetate fraction of *A. indica* leaf for 28 days

Test parameters	Administered ethyl acetate extract (mg/kg b. wt.)				
	Control	**900**	**1800**	**2700**	**3600**
Urea (mmol/l)	8.00 ± 0.55^{d}	6.25 ± 0.07^{bc}	5.93 ± 0.42^{b}	5.47 ± 0.27^{ab}	5.38 ± 0.47^{a}
Uric acid (mg/dl)	4.10 ± 0.09^{c}	2.87 ± 0.07^{b}	2.68 ± 0.14^{b}	2.08 ± 0.78^{a}	2.13 ± 2.55^{a}
Creatinine (mmol/l)	69.11 ± 3.87^{a}	71.54 ± 8.18^{a}	88.23 ± 2.28^{b}	93.47 ± 11.31^{bc}	87.59 ± 12.73^{b}
Na^{+} (mEq/l)	140.54 ± 5.91^{a}	141.58 ± 2.12^{ab}	144.71 ± 0.00^{abc}	145.52 ± 2.12^{bc}	146.63 ± 2.12^{c}
K^{+} (mEq/l)	8.01 ± 0.69^{a}	7.95 ± 0.07^{a}	8.08 ± 0.99^{a}	7.98 ± 0.14^{a}	8.20 ± 0.85^{ab}
Cl^{-} (mEq/l)	103.61 ± 11.30^{a}	106.50 ± 3.54^{a}	105.63 ± 3.54^{a}	106.84 ± 6.71^{a}	107.47 ± 8.70^{a}
HCO_3^{-} (mEq/l)	29.84 ± 2.04^{a}	29.62 ± 1.41^{a}	29.54 ± 2.12^{a}	28.71 ± 1.41^{a}	30.31 ± 4.60^{a}

Results are presented as Mean ± SEM (n = 6). Values carrying different superscripts from the normal control for each parameter are significantly (P<0.05) different using ANOVA and Duncan multiple range test.

Table 9 Haematological indices of rats administered with ethyl acetate fraction of *A. indica* leaf for 28 days

Test parameters	Administered ethyl acetate extract (mg/kg b. wt.)				
	Control	**900**	**1800**	**2700**	**3600**
WBC (x10^3/µl)	16.50 ± 1.39^b	11.37 ± 2.92^{ab}	9.24 ± 1.13^a	9.32 ± 0.97^a	9.11 ± 1.70^a
LYM (%)	80.92 ± 3.01^a	76.17 ± 1.04^a	76.95 ± 3.68^a	80.81 ± 6.92^a	78.83 ± 7.07^a
MON (%)	8.66 ± 0.33^c	5.20 ± 0.08^{ab}	5.65 ± 0.78^b	5.74 ± 1.22^b	4.10 ± 0.14^a
NEU (%)	5.07 ± 0.25^a	16.22 ± 1.15^{bc}	16.56 ± 2.97^{bc}	16.78 ± 2.93^{bc}	22.83 ± 4.87^c
EOS (%)	0.50 ± 0.02^a	0.46 ± 0.11^a	0.50 ± 0.14^a	0.49 ± 0.07^a	0.45 ± 0.03^a
BAS (%)	5.03 ± 1.32^a	4.80 ± 0.16^a	4.45 ± 0.57^a	4.80 ± 0.33^a	5.08 ± 0.99^a
RBC (x10^6/µl)	6.41 ± 0.94^a	6.43 ± 0.97^a	6.27 ± 0.19^a	5.97 ± 0.84^a	5.61 ± 0.04^a
HGB (g/dl)	10.43 ± 2.10^a	10.46 ± 2.33^a	10.45 ± 0.07^a	10.32 ± 1.96^a	10.41 ± 0.42^a
HCT (%)	35.13 ± 3.44^a	35.62 ± 2.16^a	34.60 ± 0.42^a	34.94 ± 2.01^a	36.04 ± 0.85^a
MCV (µm^3)	55.50 ± 6.05^a	54.89 ± 4.29^a	55.03 ± 0.92^a	53.96 ± 2.59^a	54.45 ± 1.63^a
MCH (pg)	19.20 ± 2.19^a	19.32 ± 1.02^a	18.75 ± 0.49^a	18.93 ± 1.42^a	19.51 ± 0.78^a
MCHC (g/dl)	34.74 ± 3.09^a	35.33 ± 3.10^a	35.05 ± 2.21^a	35.40 ± 3.06^a	34.85 ± 0.35^a
PLT (x10^3/µl)	253.93 ± 28.06^a	237.31 ± 63.92^a	248.50 ± 10.35^a	265.00 ± 39.00^a	247.42 ± 22.63^a
MPV (µm^3)	7.07 ± 0.32^a	7.12 ± 0.62^a	7.05 ± 0.87^a	7.11 ± 0.53^a	7.05 ± 0.07^a
PCT (%)	0.24 ± 0.08^a	0.24 ± 0.03^a	0.25 ± 0.07^a	0.21 ± 0.03^a	0.25 ± 0.02^a

Results are presented as Mean ± SEM (n = 6). Values carrying different superscripts from the normal control for each parameter are significantly (P<0.05) different using ANOVA and Duncan multiple range test. LYM = Lymphocytes, MON = Monocytes, NEU = Neutrophils, EOS = Eosinophils, BAS = Basophils, RBC = Red Blood Cells, HGB = Haemoglobin, HCT = Haematocrit, MCV = Mean Cell Volume, MCH = Mean Corpuscular Haemoglobin, MCHC = Mean Corpuscular Haemoglobin Concentration, PLT = Platelets, MPV = Mean Platelets Volume, PCT = Plateletcrit.

The effects of the *A. indica* leaf Ethyl acetate fraction on the haematological parameters are presented in Table 9. All the doses of the extract administered did not significantly (P>0.05) affect the levels of LYM, EOS, BAS, RBC, HGB, HCT, MCV, MCH, MCHC, PLT, MPV and PCT when compared to the normal control. There were significant dose-dependent decreases (P<0.05) in the levels of WBC and MON compared to the normal control (Table 9). In contrast however, the level of NEU recorded a significant dose-dependent increase in the treatment groups when compared to the normal control group.

3.4. Hepatic histopathological profile of hexane fraction

The histopathological signs and the photomicrographs of the liver of rats administered *A. indica* leaf hexane fraction are presented in Table 10 and Figures 1A-E respectively. Hepatic congestion, periportal inflammation and distortion were observed in rats administered 1800, 2700 (Figures 1C and 1D) and 3600 mg/kg b. wt. of the fraction. The number of rats presented with these signs increased as the dose of the extract increased (Table 10). Hepatic infiltration was only observed in rats administered with 2700 and 3600 mg/kg b. wt. of the fraction. Haemorrhage was observed in the liver of the rats administered with the highest dose of 3600 mg.kg b. wt. (Figure 1E).

Table 10 Histopathological signs detected in the liver of rats administered with hexane fraction of *A. indica* leaf for 28 days

Histopathological sign	Administered hexane extract (mg/kg b. wt.)				
	Control	900	1800	2700	3600
Congestion	0/6	0/6	1/6	2/6	4/6
Periportal inflammation	0/6	0/6	1/6	2/6	5/6
Distortion	0/6	0/6	1/6	2/6	3/6
Infiltration	0/6	0/6	0/6	1/6	2/6
Haemorrhage	0/6	0/6	0/6	0/6	3/6
Necrosis	0/6	0/6	0/6	0/6	0/6
Cancerous tumor	0/6	0/6	0/6	0/6	0/6

Results are presented as number of rats with a histopathological sign per total number of rats in a group (n = 6).

3.5. Hepatic histopathological profile of Ethyl acetate fraction

Table 11 and Figures 2A-E present the histopathological signs and the photomicrographs of the liver of rats administered with *A. indica* leaf ethyl acetate fraction respectively. Compared to the control group, hepatic congestion, periportal inflammation and distortion were observed in all the rats administered with the ethyl acetate fraction. The number of rats presented with these signs increase as the dose of the extract increased. Hepatic infiltration was only observed in rats administered with the higher doses of the fraction. Haemorrhage was observed in the liver of rats administered with 2700 and 3600 mg.kg b. wt. dose of the ethyl acetate fraction (Figures 2D and 2E). At all the administered doses, no sign of necrosis or cancerous tumor was observed.

Table 11 Histopathological signs detected in the liver of rats administered with ethyl acetate fraction of *A. indica* leaf for 28 days.

Histopathological sign	Administered ethyl acetate extract (mg/kg b. wt.)				
	Control	900	1800	2700	3600
Congestion	0/6	1/6	2/6	3/6	4/6
Periportal inflammation	0/6	1/6	1/6	1/6	3/6
Distortion	0/6	1/6	2/6	2/6	4/6
Infiltration	0/6	0/6	1/6	1/6	2/6
Haemorrhage	0/6	0/6	0/6	2/6	3/6
Necrosis	0/6	0/6	0/6	0/6	0/6
Cancerous tumor	0/6	0/6	0/6	0/6	0/6

Results are presented as number of rats with a histopathological sign per total number of rats in a group (n = 6).

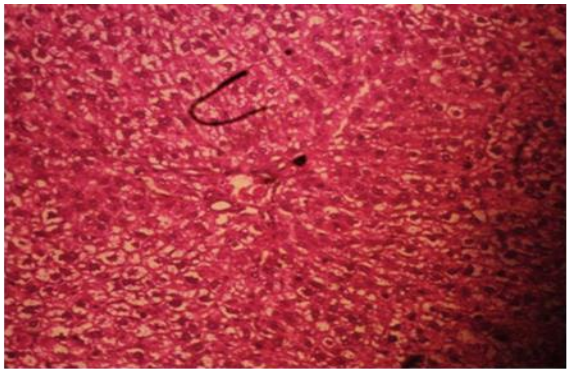

Figure 1A (Control): Normal hepatocytes with central vein.

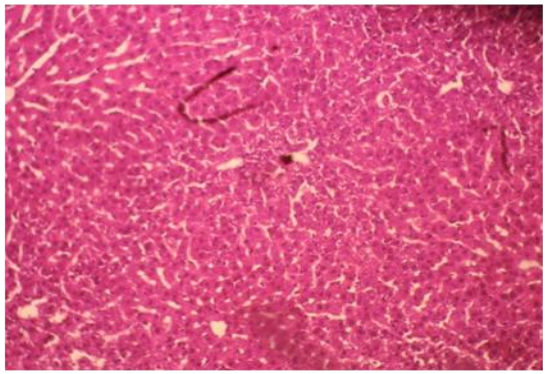

Figure 1B (900 mg/kg): Normal plates of hepatocytes

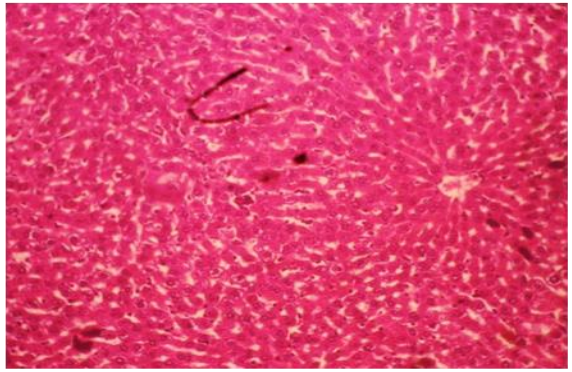

Figure 1C (1800 mg/kg): Normal hepatocytes with interstitial congestion.

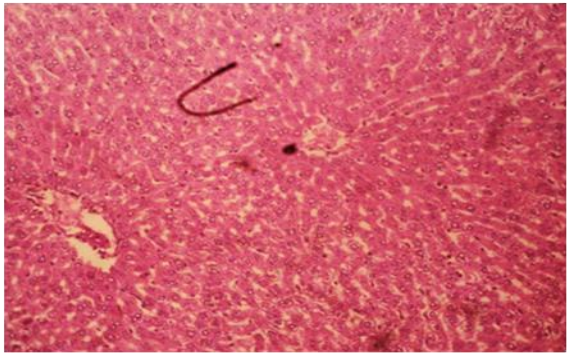

Figure 1D (2700 mg/kg): Irregular hepatocytes with distortion.

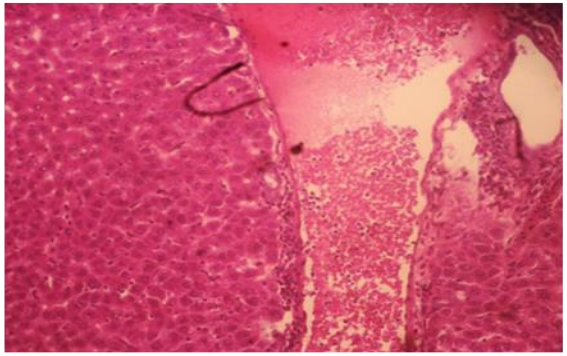

Figure 1E (3600 mg/kg): Abnormal hepatocytes, thick and exposed to blood.

Figures 1 A-E Liver photomicrographs of rats administered sub-chronic doses of *Azadirachta indica* leaf hexane fraction (H&E stain, X100 magnification).

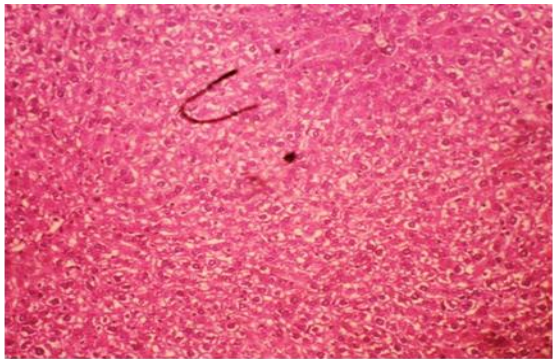

Figure 2A (Control): Normal hepatocytes.

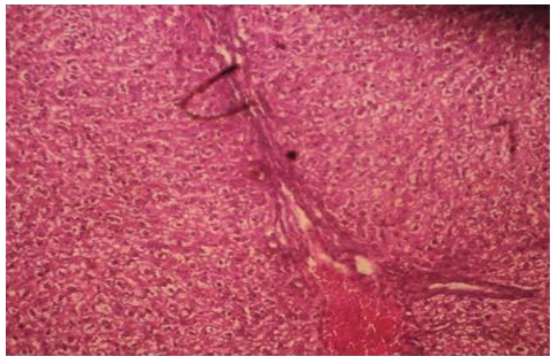

Figure 2B (900 mg/kg b. wt.): Normal hepatocytes with interstitial congestion.

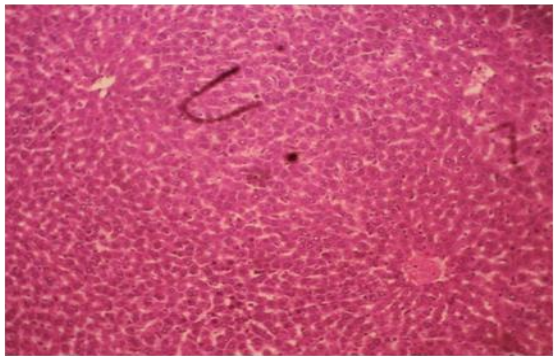

Figure 2C (1800 mg/kg b. wt.): Normal hepatocytes with congestion.

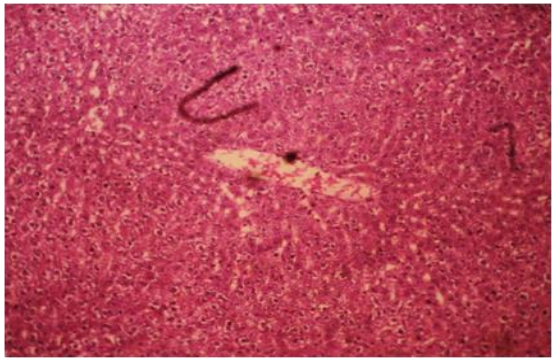

Figure 2D (2700 mg/kg b. wt.): Irregular hepatocytes exposed to blood

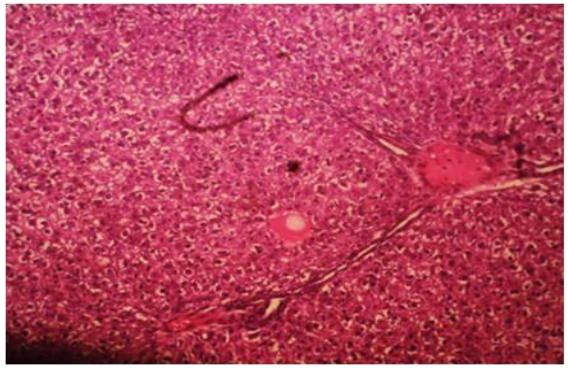

Figure 2E (3600 mg/kg b. wt.): Irregular hepatocytes with central vein. The hepatocytes are exposed to blood on both sides.

Figures 2 A-E Liver photomicrographs of rats administered sub-chronic doses of *Azadirachta indica* leaf ethyl acetate fraction (H&E stain, X100 magnification).

4. Discussion

Although toxicological studies have revealed the potent toxic effects of *Azadirachta indica* extracts [10, 13, 16, 33, 34], this research has indicated that, the hexane and the ethyl acetate fractions of the *A. indica* leaf in acute oral dose of 5,000 mg/kg b. wt. did not produced any death in rats within the first 48 h, suggesting a median lethal dose (LD_{50}) above 5,000 mg/kg b. wt. According to Kennedy *et al*., [35], substances that present LD_{50} higher than 5,000 mg/kg by oral route may be considered practically non-toxic, suggesting that the fractions used in this study are experimentally safe.

Assessment of body weight is a useful index for sense of taste and appetite of the animals during extracts administration period [36, 37]. It was observed in this research that, both the hexane and the ethyl acetate fractions did not significantly affect the weight gain of the rats in all the treatment groups. This was not surprising as the feeding and water intake were not affected in the treatment groups. Hence, water and feed are essential nutrients for growth and development [38]. Any factor influencing water and feed intake will also affect growth and development.

Enzymes such as transferases, phosphatases and dehydrogenases are often found in appreciable quantities in the serum but are not of the extracellular fluid origin. This occurs as a result of tissue damage or disrupted cell membranes that lead to the leakage of such enzymes from the tissue and become elevated in the serum [39, 40]. Therefore, serum enzyme measurement provides valuable information on the effect and nature of pathological damage to the tissues [38].

Compared to the normal control, the significant elevation in the serum levels of AST, ALT, ALP, (at varying doses of the hexane and ethyl acetate fractions) and GGT or LDH (at varying doses of the hexane or the ethyl acetate respectively) in this research could suggest damage in the hepatic cells due to toxic hepatitis or hepatic infiltration [41]. Furthermore, the significant increase in the serum conjugated and total bilirubin for both the hexane and the ethyl acetate fractions could indicate mild haemolysis and obstruction in the normal excretion of bile [42]. Evaluation of serum proteins such as albumin is a good criterion for assessing the secretory ability or functional capacity of the liver [43]. The significant effect of the hexane and the ethyl acetate fractions on the albumin and total protein in the serum of the animals at all the doses investigated could imply that the synthetic and secretory functions of the liver with respect to these proteins were affected.

Renal function indices are usually required to assess the normal functioning of different parts of nephron [44]. Similarly, the serum concentrations of electrolytes, urea, uric acid and creatinine could give an insight into the effect of plant extracts on the tubular and/or glomerular part of the kidney [38, 45]. The significant decrease in the levels of urea and uric acid in the fractions-treated animals in this study signified toxic effect on the glomerular filtration as compared to the normal control. The elevation in the levels of K^+, Na^+ and Cl^- at 3600 mg/kg b. wt. of the fractions suggests interference in the normal homeostasis of these ions.

Assessment of haematological parameters can also be used to determine the extent of deleterious effect of plant extracts on the blood constituents of an animal. Such assessment is relevant for risk evaluation as changes in the haematological system have higher predictive value for human toxicity, when the data are translated from animal studies [46]. The reductions in the levels of WBC and MON out of all the haematological parameters investigated for the ethyl acetate fraction in the present study could imply selective systemic toxicity effect by the extract. Therefore, the reduction in the WBC count at all the doses for the ethyl acetate fraction-treated animals investigated could possibly imply that, its rate of entrance from the bone marrow did not commensurate with the rate of its removal from the circulation or may also be due to underproduction of haematopoietic regulatory elements by the stroma cells and macrophages in the bone marrow at those doses [47]. In the present study, significant increase in the concentrations of WBC, LYM, MON and NEU in the hexane-treated animals may be due to response to foreign substances as a defense mechanism or due to enhancement of their production and reduction in their removal from the circulation in an attempt to defend the system [48]. The present study also revealed that, platelet count was significantly increased in the rats treated with the hexane fraction at all the doses administered. Platelets have a key role in maintaining vascular integrity, as they aggregate at and adhere to exposed collagen to form a physical barrier at the site of vessel injury, accelerate the activation of coagulation proteins and release stored granules that promote vasoconstriction [49]. HGB, RBC and HCT are associated with total population of red blood cells while MCV, MCH, MCHC relates to individual red blood cells. Lack of effect on these parameters indicates that both the individual and total population of the red blood cells were not affected.

Microscopically, the liver of the rats administered with the higher doses of the hexane fraction and at all the doses of the ethyl acetate fraction revealed very clear pathological changes, such as congested vessels channels, periportal inflammation, distortion, infiltration and haemorrhage. Signs of liver damage usually manifest as a result of architectural disarray of the hepatic parenchyma, vascular congestion, hepatocytes necrosis, apoptosis or inflammatory cell infiltration in either acute or chronic conditions [50]. Some of these features were observed in the rats administered

with the higher doses of the fractions compared to the normal control. Generally, cells are reported to die as a result of necrosis or apoptosis when they are challenged with toxins, noxious agents or injuries [51]. These effects were not observed in the present study, hence, the extract fractions were not agents of necrosis or apoptosis.

5. Conclusion

This research has shown that, the hexane and the ethyl acetate fractionated extracts of *Azadirachta indica* leaf in acute oral dose of 5,000 mg/kg b. wt. did not produced any sign of toxicity or death in rats signifying that, the median lethal dose (LD_{50}) of each of the fractions is above 5,000 mg/kg b. wt. On the other side, the sub-chronic oral doses of the fractionated extracts administered, made some alterations in the biochemical indices of the hepatic and renal functions as well as haematological and histopathological parameters. Hence, these results indicated that using hexane or ethyl acetate as solvent of extraction, *A. indica* leaf extracts may still not be considered safe at the administered sub-chronic doses.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

No existing conflict of interest.

Statement of ethical approval

All animal experiments were conducted in accordance with the guidelines for the use and care of experimental animals, National Veterinary Research Institute, Vom, Nigeria.

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