Journal of Pharmaceutical Research International

20(2): 1-16, 2017; Article no.JPRI.38137
ISSN: 2456-9119
(Past name: British Journal of Pharmaceutical Research, Past ISSN: 2231-2919, NLM ID: 101631759)

In vivo and *in vitro* Toxicity Studies of Crude and Partially Purified Leaf Extracts of *Jatropha curcas* in Wistar Albino Rats

A. Mahe[1], Y. Y. Muhammad[2], A. Ibrahim[2], A. Muhammad[3], I. K. Adam[1], M. K. Atiku[2] and A. A. Imam[2*]

[1]Department of Biochemistry, Faculty of Science, Federal University Dutse, Jigawa State, Nigeria.
[2]Department of Biochemistry, Faculty of Biomedical Sciences, Bayero University, Kano, Nigeria.
[3]Department of Pure and Applied Chemistry, Faculty of Science, Bayero University, Kano, Nigeria.

Authors' contributions

This work was carried out in collaboration among all the authors. Authors AAI, A Mahe and A Muhammad designed the study and performed the experiments, statistical analysis and wrote the first draft of the manuscript. Authors AI, YYM, MKA and IKA supervised data collection and reviewed and made corrections to the manuscript. All authors read and approved the final draft of the manuscript.

Article Information

DOI: 10.9734/JPRI/2017/38137
Editor(s):
(1) Jongwha Chang, University of Texas, College of Pharmacy, USA.
Reviewers:
(1) Afagnigni Alian Desire, University of Yaounde I, Cameroon.
(2) Sangeetha Arullappan, Universiti Tunku Abdul Rahman & Jalan Universiti, Malaysia.
(3) Md. Jakaria, Konkuk University, South Korea.
Complete Peer review History: http://www.sciencedomain.org/review-history/22407

Original Research Article

Received 14th November 2017
Accepted 12th December 2017
Published 22nd December 2017

ABSTRACT

Aims: *Jatropha curcas* (*J. curcas*) is a plant that has numerous medicinal and industrial applications. Evaluation of *in vitro* and *in vivo* acute/sub chronic hepato-renal toxicity of crude methanol *J. curcas* leaf extract on albino rats, *in vitro* toxicity of the fractions and characterization of these fractions were carried out in this study.
Study Design: Twenty albino rats were grouped into four, orally administered; 0, 100, 200, and 250 mg/kg daily of the crude extract for 28 days.
Place and Duration of Study: Department of Biochemistry, Bayero University Kano and Department of Pure and Applied Chemistry, Bayero University Kano between July 2016 and April 2017.

**Corresponding author: E-mail: aaimam.bch@buk.edu.ng;*

Methodology: Partially purified fractions of the extract were obtained by column chromatography. Brine Shrimp Lethality assay of the crude extract and fractions were carried out. FTIR, GC-MS and LC-MS were used to characterize the fractions, one-way anova and probit analysis were used for data analysis.
Results: The oral LD_{50} of *J. curcas* extract was found to be 2,391.65 mg/kg. Alkaline phosphatase (ALP), Direct bilirubin (D. BIL) and Albumin (ALB) showed significant ($p < 0.05$) differences while Alanine amino transferase (ALT), Aspartate amino transferase (AST), Total bilirubin (T. BIL) and Total protein (T.P) showed slight variations in some of the treated groups. Histological features of the liver showed dose dependent mild toxic effect of the extract. Creatinine and potassium levels showed significant ($p < 0.05$) differences while urea, chloride, sodium and bicarbonate levels slightly differ in some of the treated groups when compared to the control. Abnormal kidney histological features were only observed in the highest dose group. The crude leaf extract, fraction 1, and fraction 2 gave LC_{50} values of 109.07 µg/ml, 100 µg/ml, and 883.26 µg/ml respectively. Fraction 1 indicated the presence of ursolic acid, betulinic acid, and oleanolic acid while fraction 2 showed fragments of hydroxyl hopanone.
Conclusion: This study showed various toxicity indices that revealed slight toxic effect of the crude methanol leaf extract.

Keywords: Jatropha curcas; methanol leaf extract; LD_{50}; LC_{50}; liver function test; renal function test.

1. INTRODUCTION

Toxicology is the study of the negative impacts of chemicals on living organisms [1]. Many materials have greatly enhanced our quality of life by improving health and hygiene. Studies on the toxicity of *J. curcas* leaves on various human organs are few compare to the seeds [2]. Plants derived therapeutics are by and large thought to be protective [3], however many have harmful potentials. Toxicity is a serious issue with the increased utilization of home grown cures, hence it is basically important that all herbal medicines should be safe and suitable for consumption. Plants extract ought not to be just strong but rather safe for utilization [4]. The utilization of traditional medicine and plant nutrients continue to widen rapidly across the globe with numerous population presently utilizing these products for treatment of many health issues in various national healthcare system [5]. For quite a while, plants have given a wellspring of developing current prescription and medication mixes, as plant derived pharmaceuticals have made expansive commitments to human wellbeing [6]. *J. curcas* is a species of blooming plant from the spurge family, *Euphorbiaceae* that is local to the American tropics, most likely Mexico and Central America [7], popularly known as Barbados nut, Purging nut and Physic nut. It is known as bini da zugu, bita da zugu or binda zugu among Hausa speaking people of Nigeria [8].

Herbal plants such as *J. curcas* have been utilized for many medicinal purposes, to treat bacterial and fungal infections. All parts of *Jatropha* (seeds, leaves, bark, etc) have been utilized as a part of customary solution and for veterinary usage for quite a while. The plant crude leaves extracts demonstrated active anti plasmodial activity [9], it also has a wide variety of medicinal uses traditionally against sicknesses like malaria, cough, jaundice, neuralgia, paralysis, scabies, sexually transmitted diseases, stomach ache, dermatitis, rheumatism, haemorrhoidal disorder, snake bites, ringworms, tooth ache and sores [6,10]. The plant is used as a laxative, purgative, abortifacient, depurative, anodyne, styptic and vulnerary agent [11]. The anti-oxidant, anti-inflammatory, anti-cancer, anti-diarrheal, coagulative, anti-leukaemic, anti-microbial and antihyperglycaemic properties of the plant parts have been documented [12-14]. The latex of the plant is also used for the production of cyclosporine, an immunosuppressant [15]. However, despite this array of medicinal applications, extensive data on possible toxicological properties of *J. curcas*, especially on major organ system, is lacking. The present study is aimed at evaluating the *in vitro* and *in vivo* acute/sub chronic hepato-renal toxicological properties of the crude, *in vitro* toxicological properties of fractions of the crude methanol leaf extracts of the plant and characterization of these fractions.

2. MATERIALS AND METHODS

2.1 Plant Collection

The fresh plant sample; leaves of *J. curcas,* were obtained in the month of July, 2016 from Lambu

Village, Kumbotso Local Government Area, Kano State with Global Positioning System Coordinates: $11^0 30^l N$ $8^0 30^l E$. The leaves were identified by experts in Department of Plant Biology of Bayero University, Kano, with Herbarium Accession Number: BUKHAN 0060.

2.2 Preparation of Extract

Preparation of extract of the plant leaves was done using the method described by Veeramuthu [16]. The leaves were air dried under shade and ground to powder using grinding mill. Each 100 g of the 1500 g powdered material was dissolved in 1000 ml of methanol for 48 hours. The extract was obtained by sieving, evaporation using a rotary evaporator and drying at ambient temperature. The extract obtained was dark green in colour with gummy texture. It weighs 101 g which gave a percentage yield of 6.7 % for the extract.

2.3 Column Chromatography of Crude Extract

Using silica gel (60-120 mesh), the crude extract of *J. curcas* (58 g) was utilized for column chromatography. The extract was mixed with the silica gel until a solid homogenous mixture was formed. The mixture was then carefully applied onto a silica gel column that was packed with washed silica gel in n-hexane (slurry method). An additional portion of silica gel was added to form a protective layer on top of adsorbent. The column was eluted in order of solvent ascending polarity with n-hexane (3 litres), n-hexane:chloroform (8:2, 3 litres), hexane:chloroform (6:4, 3 litres), hexane:chloroform (5:5, 3litres), hexane:chloroform (4:6, 3litres), hexane:chloroform (2:8, 3litres), chloroform (3 litres), chloroform:ethyl acetate (6:4, 3 litres), hexane:chloroform (4:6, 3 litres), ethyl acetate (3 litres), ethyl acetate:methanol (8:2, 3litres), ethyl acetate:methanol (6:4, 3litres), ethyl acetate:methanol (5:5, 3litres), methanol (3litres).

2.4 Thin Layer Chromatography

Thin layer chromatography (TLC) was performed on 4 × 5cm pre-coated aluminium TLC plates. TLC bands were viewed under sun rays, ultraviolet (UV) lamp (at 254 nm and 356 nm) and iodine crystals.

2.5 Brine Shrimp Lethality Assay

The method described by Meyer [17] and Fatope [18] were used to screen the extract and the partially purified fractions against *Artemia salina* (brine shrimps). A hatching chamber containing sea water (250 ml) was added 25 mg of *Artemia salina* (leach) eggs. For 48 hrs, the hatching chamber was kept under an inflorescent bulb for the eggs to hatch into shrimp larvae. 20 mg of the crude extract and two partially purified fractions of *J. curcas* leaf extract (F1 and F2) were separately dissolved in 2 ml of methanol, from the solution, 500, 50 and 5 µl of each solution was transferred into vials corresponding to 1000, 100 and 10 µg/ml respectively. Each concentration was in triplicate. The vials (9 per test fraction) and control were allowed to evaporate to dryness for about 48 hrs at room temperature. To each sample vial including the control, a drop of DMSO and 3 ml of ocean/sea water were added. To each vial, ten (10) larvae of *Artemia salina* were introduced using a Pasteur pipette and sea water was added up to 5 ml. The LC_{50} values were determined at 95% confidence intervals. The LC_{50} values of the brine shrimps obtained were recorded.

2.6 Acute Toxicity Testing (Determination of LD_{50})

LD_{50} was determined using the method of Lorke [19]. Nine rats were divided into three groups of three rats each in the first phase. They were treated with crude extract of *J. curcas* leaf extract at 10, 100, and 1000 mg/kg orally. The treated rats were monitored for 24 hours for mortality and general behaviour. Next, five rats were used and grouped into five groups of one rat each in the second phase. They were treated with the crude extract at doses of 1400, 1800, 2200, 2600 and 3000 mg/kg orally. They were monitored again for 24 hrs. The geometric mean of the least dose that killed rats and the highest dose that did not kill rats was taken as median lethal dose.

LD_{50} (oral) = √min.conc.full death X max.conc.no death
Lorke [19].

2.7 Experimental Design

2.7.1 Sub chronic toxicity test

Twenty albino rats weighing 110 – 130 g aged between 7 and 9 weeks were randomly divided into four groups, five in each group designated; A-D with similar average weight. Group A was

the control given no extract, groups B-D were the test groups orally treated with the leaf extract of *J. curcas* at: 100, 200, 250 mg/kg for 28 days respectively. The animals were given free access to water and food (Wheat bran, Crown flour mills, Nig. Ltd), during four weeks of treatment.

2.8 Collection and Preparations of Blood Samples

The neck area was cleared and the jugular veins were exposed to avoid contamination with interstitial fluid, the veins after being slightly displaced were sharply cut with a sterile blade and 5 ml of blood was collected into a container with lithium heparin for each. This was then centrifuged at 2000 rpm for 10 min using Uniscope Laboratory Centrifuge. The plasma was thereafter aspirated using Pasteur pipettes into clean, dry, sample bottles and were stored frozen overnight before being used for assaying liver and renal parameters [20].

2.9 Assay of Liver and Kidney Function Indices

All parameters of liver function and renal function were analysed using standard kits (Randox and TECO test kits). Alanine amino transferase and Aspartate amino transferase were determined using the method of Reitman and Frankel [21]. Alkaline phosphatase was assayed according to the method of Rec [22]. Total protein was determined according to Biuret method [23]. Albumin was determined using the method of Doumas [24]. Bilirubin was determined using the method of Jendrassik and Grof [25]. Urea was assayed according to the method of Kaplan [26]. Creatinine was determined using the method of Butler Jaffe's reaction [27]. Sodium was determined using the method of Trinder [28]. Potassium was determined using method of Henry [29]. Chloride was determined using the method of Schonfeld and Lewellen [30]. Bicarbonate was determined using the method of Forrester [31].

2.10 Histological Studies

The animals were dissected, livers and kidneys were removed and kept in 10% formal saline solution. Histological analysis was done using the method of Avwiora [32]. Tissues were dehydrated, cleared, impregnated, embedded, sectioned and stained using haematoxylin and eosin.

2.11 FTIR

Agilent FTIR spectrometer with a Model 223 Gilson autosampler and a positive displacement micropump was used, it was equipped with a deuterated triglycine sulfate (DTGS) detector that uses 100 and 500 mm CaF2 transmission flow cells.

2.12 GC-MS

Agilent 6890N gas chromatography having the 5973 mass selective detector operated in EI mode was utilize to get spectra of fractions. Separations were on the HP – 5 ms column with 30 m X 0.25 mm ID X 0.25 micron film thickness. Helium was the carrier gas with a linear velocity of 35 cm/sec.

2.13 LC-MS

Fractions were analysed with a tandem MS system using a methanol/water gradient at 0.2 ml/min. Each solvent contained 1 vol % formic acid with the gradient starting at 50 % methanol and increasing to 100 % over 50 min with a ten minute hold. AS3000 auto sampler was used for injections.

2.14 Statistical Analysis

All data were expressed as mean ± standard error of mean (SEM). Comparison were done using Probit Analysis, one-way anova (SPSS 20), Significance was reported at $P < 0.05$.

3. RESULTS AND DISCUSSION

3.1 Results

Results of acute toxicity studies of methanol crude extract of *J. curcas* on white albino rats was presented in (Table 1) it showed the first and second phases of acute toxicity test. There was no death recorded in the first phase, while in the second phase death was recorded in animals administered 2600 mg/kg and 3000 mg/kg body weight of the extract.

In the sub chronic toxicity section, four rats were used for the liver and kidney toxicity analysis due to mortality in highest dose group which could be attributed to mild toxicity. The effect of the sub chronic administration of *J. curcas* leaf extract on liver function parameters in rats (Table 2), showed significant higher levels of ALP and

D.BIL. Significant lower level of ALB was also recorded at $p < 0.05$. ALT, AST, T.BIL, and T.P showed slight differences. Observations in this study (Table 2) showed high activity of serum ALT in rats treated with 100 mg/kg, 200 mg/kg and 250 mg/kg of the extract which was proportional to dose, though statistically not significant ($p > 0.05$). There were variations in pattern of AST compared to the control, with a rise in AST activity in group administered with 250 mg/kg though statistically not significant ($p > 0.05$). Slightly higher level of total and direct bilirubin concentration were observed in all the treated groups, but high level in direct bilirubin concentration in highest dose group was found to be statistically significant ($p < 0.05$) when compared to the control. It was also observed that methanol crude *J. curcas* leaf extract administration produced slightly low level of total protein in all the treated groups compared to the control which was seen to depend on dose. There was also low albumin level in all the treated groups, the low levels in groups administered with 100 mg/kg and 200 mg/kg were statistically not significant, but that in group given 250 mg/kg was found to be statistically significant ($p < 0.05$) when compared to the control group.

(Table 3) showed the effect of sub chronic administration of *J. curcas* leaf extract on renal function parameters in rats. It showed there was significant higher levels of potassium and creatinine at $p < 0.05$. High levels in potassium concentration in all the treated groups when compared to the control were observed, groups given 200 mg/kg and 250 mg/kg were found to be statistically significant when each was compared to the control ($p < 0.05$). High levels of creatinine in all the treated groups when compared to the control group were also observed, it was found to be statistically significant ($P < 0.05$) in highest dose group when compared to the control group and to the group given 100 mg/kg. High level of urea was obtained in all the treated groups though statistically not significant. In present study, there was high level of plasma sodium observed in all the treated groups when compared to the control group, but these were found to be statistically not significant ($p > 0.05$) in all the treated groups. The study also showed slightly low levels in serum bicarbonate in groups administered with 100 mg/kg and 250 mg/kg when compared to the control, group given 200 mg/kg showed high level of bicarbonate when compared to the control, which gives variations not related to dosage. Further, this study observed slightly high level of serum chloride in groups administered with 200 mg/kg and 250 mg/kg. There was deviation in level of serum chloride in group given 100 mg/kg which showed slight lower level.

Histopathological features of liver tissues of rats treated with crude methanol leaf of *J. curcas* (Fig. 1) showed mild toxic effect of the extract when compared to the control, these changes were seen to be dose dependent. As shown in fig. 1, group administered with the highest dose of 250 mg/kg of methanol crude concentrate of *J. curcas* leaf showed greater effect.

Histological analyses of the kidney tissues of rats treated with crude methanol leaf of *J. curcas* (Fig. 2) showed a mild toxic effect in only the highest dose group administered with 250 mg/kg of the extract. The kidney of control group showed normal glomeruli likewise the kidneys of groups given 100 mg/kg and 200 mg/kg showed normal appearing glomerular tubules and vesicles with no tubular necrosis, and no interstitial inflammation. But the kidney of group given 250 mg/kg showed mild interstitial haemorrhage and vascular congestion but glomeruli, tubules, vesicles all appeared normal.

Fig. 3. Brine Shrimp Lethality Assay showing survival after 24 hrs against concentration. KEY: C: crude, F1: fraction number 1, F2: fraction number 2. The crude methanol extract of *J. curcas* leaf (C), fraction number one (F1), and fraction number two (F2) showed good brine shrimp larvicidal activity in present study. Fraction 1 having higher activity followed by the crude extract while fraction 2 has the lowest larvicidal activity.

(Table 4) gives the number of shrimp nauplii that survived after treatment with the crude, fraction 1 and fraction 2, their LC_{50} as 109.07 µg/ml, 100 µg/ml and 883.26 µg/ml respectively. It also gives the percentage mortality in Brine Shrimp Lethality assay.

Tables 5a and 5b gives FTIR result for fraction 1 and fraction 2 in comparison with the literature. Table 6a and 6b gives GC-MS phytoconstituents characterization of partially purified fraction 1 and fraction 2 of *J. curcas* leaves extract.

Fig. 4a and b gives the mass fragmentation pattern of compounds in Fractions 1 and 2 using LC-MS results obtained from major peaks.

Table 1. Results of acute toxicity studies of methanol crude extract of *J. curcas* on white albino rats

Doses (mg/kg)	Result
Phase I	
10	0/3
100	0/3
1000	0/3
Phase II	
1400	0/1
1800	0/1
2200	0/1
2600	1/1
3000	1/1

LD_{50} (oral) = 2391.65 mg/Kg.

3.2 Discussion

This study evaluated the acute toxicity of methanol crude extract of *J. curcas* leaf by determining the LD_{50}. The oral LD_{50} obtained (table 2) was consistent with previously established LD_{50} of *J. curcas* leaf extract; the oral LD_{50} of 50% ethanol concentrate of *J. curcas* leaf [33] and that of the aqueous leaf concentrate of *J. curcas* leaf [34]. The lower LD_{50} value in this study compared to that obtained by Shanti et al. (2010) [33] and Dangambo et al. (2015) [34] could be due to variations in degree of polarity of solvents used which might have an effect on the degree of solubility of constituents of *J. curcas* leaf. With this LD_{50} value, the plant extract may be considered as slightly toxic according to Hodges and Sterner scale for toxicity classification [35].

From results of this study, it can be seen that rats administered methanol crude extract of *J. curcas* leaf for 28 days showed dose dependent slight toxic effect in both hepato-renal toxicity indices. Appraisal of liver function capacities utilizing exploratory animals involves measurement of different enzymes and metabolites and in addition assessment of liver tissue design [36]. ALT, AST and ALP activity and serum bilirubin level are to a great extent utilized as most basic biochemical markers to assess liver damage [37]. These enzymes are significant and assume understood part in determination of liver cytolysis and harm to the plasma layer of the liver cells [38]. It has been reported in several studies that whenever liver cells are destroyed and liver enzymes are leaked into the blood, enzyme activity in the plasma is elevated [39]. This could be because of all over production of the enzyme or an adjustment by the liver to the effect of the plant extract [40]. Variation in level of AST observed in this study might have been as a result of AST not only particularly found in the liver and can be affected by other factors such as haemolysis [41].

Total protein and total bilirubin are measured to assay for the synthetic and excretory capacity of the liver respectively [42]. An increased bilirubin level is a sign of impedance of practical limit of the liver, such major liver damage could lead to rise in serum total and conjugated bilirubin level. Additionally, it is observed that levels of serum bilirubin are conversely identified with danger of certain heart ailments [43,44].

The liver is the main producer of many of the plasma proteins. The parenchyma cells are in charge of production of albumin, fibrinogen, and

Table 2. Effect of sub chronic administration of *J. curcas* leaf extract on liver function parameters in rats

GP	ALT (U/L)	AST (U/L)	ALP (IU/L)	T.BIL. (µmol/L)	D.BIL. (µmol/L)	ALB (g/dL)	T.P (g/dL)
I	25.28 ± 5.36	122.27 ± 8.23	48.37 ± 8.14[a]	8.53 ± 2.66	4.39 ±1.93[a]	2.86 ± 0.31 [a]	6.99 ± 0.73
II	26.22 ± 8.97	90.73 ± 21.82	61.58 ± 11.52	8.67 ± 3.05	6.01 ±1.09	2.56 ± 0.08	6.59 ± 0.53
III	29.19 ± 5.82	106.81 ± 19.48	56.40 ±11.43[b]	9.93 ± 3.08	6.72 ± 2.26	2.53 ± 016	6.44 ± 0.15
IV	37.37 ±10.74	138.96 ± 31.95	180.07 ±74.93[ab]	16.35 ± 4.00	12.76 ±3.54[a]	1.75 ± 0.47 [a]	6.30 ± 0.33

Normal, II: Treated with 100mg/kg, III: Treated with 200 mg/kg, IV: Treated with 250 mg/kg mean ± SEM (n= 4 rats/ group). Values with the same superscripts within columns are significantly different at p<0.05
Key: ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, ALP: Alkaline phosphatase, TB: Total bilirubin, DB: Direct bilirubin, ALB: Albumin, and TP: Total protein.

Table 3. Effect of sub chronic administration of *J. curcas* leaf extract on renal function parameters in rats

GP	K^+ mmol/L)	Urea (mmol/L)	Cl^- (mmol/L)	Na^+ (mEq/L)	HCO_3^- (mmol/L)	Creat. (mg/dL)
I	10.06 ± 0.54[ab]	9.04 ± 0.18	189.18 ± 14.21	73.08 ± 13.07	375.60 ± 22.04	1.22 ± 0.38[a]
II	13.97 ± 1.08	9.43 ± 0.34	175.25 ± 3.45	107.73 ± 44.33	281.94 ± 78.17	1.68 ± 0.40[b]
III	14.68 ± 1.26[a]	9.48 ± 0.44	223.11 ± 41.13	112.00 ± 20.46	420.17 ± 10.10	2.45 ± 0.75
IV	15.29 ± 2.02[b]	9.83 ± 0.36	240.42 ± 33.68	143.55 ± 10.08	285.71 ± 92.51	3.32 ± 0.50[ab]

I: Normal, II: Treated with 100 mg/kg, III: Treated with 200 mg/kg, IV: Treated with 250 mg/kg
Values are expressed as mean ± SEM (n= 4 rats/ group). Values with the same superscripts within columns are significantly different at p<0.05. Key: K+: Potassium, Cl-: Chloride, Na+: Sodium, HCO3-: Bicarbonate, and Creat: Creatinine

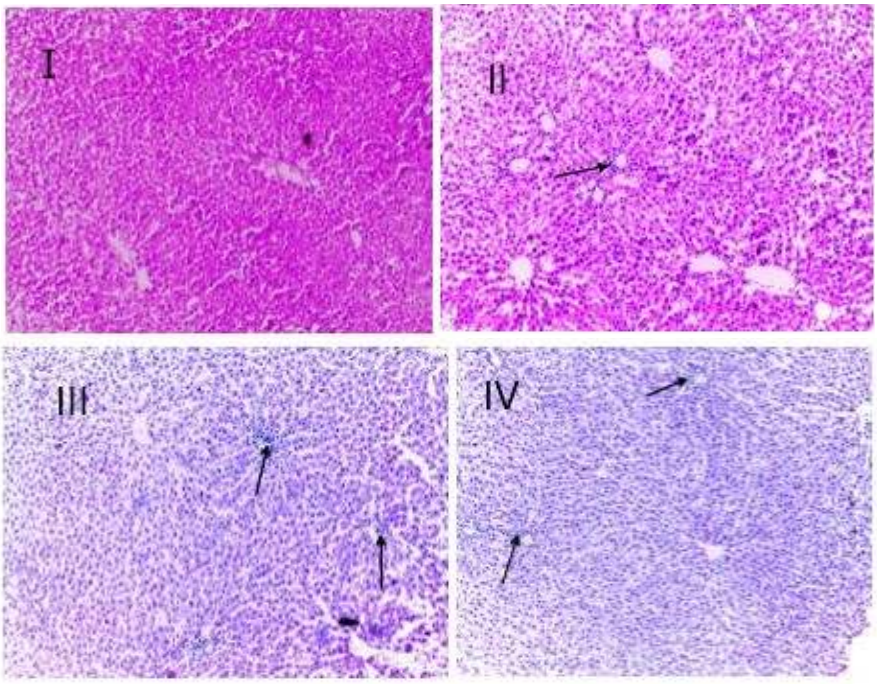

Fig. 1. Histopathological features of liver tissues of rats treated with crude methanol leaf of *J. curcas* showing dose dependent mild toxic effect of the extract
KEY: I: Normal mag x 100, II: Treated with 100 mg/kg, III: Treated with 200 mg/kg, IV: Treated with 250 mg/kg
The arrows indicate inflammation at portal triad by the aggregation of cells

other coagulation factors and most of the alpha and beta globulins. Total protein is the aggregation of albumin (60%) and globulins in the serum, a decrease in the concentration of serum total protein may show hindrance of protein synthesis [45]. The total protein produced by the liver which is mainly albumin is required by the body to battle diseases and perform different capacities, reduction in such levels might indicate liver damage or diseases. Albumin is a useful indicator of hepatic capacity since liver is the major source of albumin [46]. The extract used in this study might have inhibited the production of some proteins, hence resulting in decrease in the serum total protein. Generally, low protein level results when there is major liver injury, but evaluation of serum albumin is mainly preferred to establish the synthetic capacity of the liver [47]. The levels of albumin, globulin and bilirubin in the serum can give the state of the

liver and can also be used to obtain different types of liver injury [40]. This study indicates that sub-chronic administration of *J. curcas* leaf extract showed mild toxic inflammatory effect on liver cells which was dependent on dose. The ordered lipid bilayer of the plasma membrane might have been disrupted which resulted in release of the enzymes to the extracellular fluid, the serum and this was in agreement to the histological studies obtained.

Urea is the significant nitrogen containing metabolic result of protein catabolism [48]. Higher level of urea is an indication that the extract might have affected the clearance capability of the kidney. The major source of creatinine in the body is a high energy compound known as creatine-phosphate when hydrolysed. Creatinine has been accounted for to be a marker of renal capacity with elevated level regularly taken as a sign of

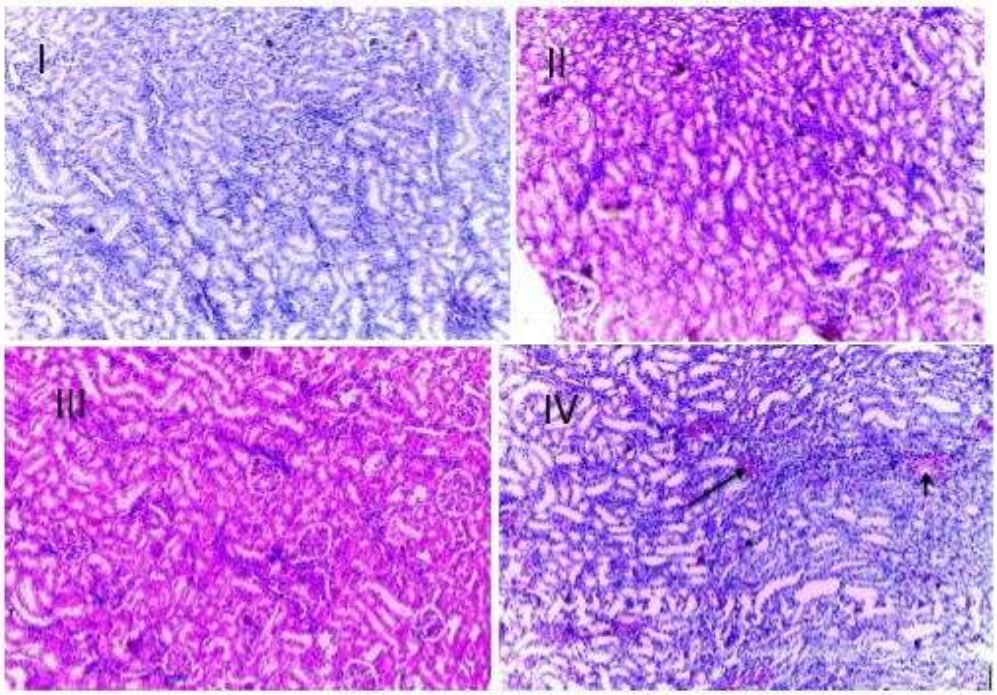

Fig. 2. Histopathological features of Kidney tissues of rats treated with crude methanol leaf of of *J. curcas* showing effect of the extract only in the highest dose group (250 mg /kg)

KEY: I: Normal mag x 100, II: Treated with 100mg/kg, III: Treated with 200 mg/kg, IV: Treated with 250 mg/kg The arrows indicate vascular congestion and interstitial haemorrhage

Table 4. The number of shrimp nauplii that survived after treating with the crude, F1 and F2 and the percentage mortality in Brine Shrimp Lethality Assay

Sample	Concentration (µg/ml)	Initial	Replica	Survival after 24hrs	Total death	% Mortality	LC_{50} (µg/ml)
Crude	1000	10	3	00,00,00	30	100	
	100	10	3	07,05,05	13	43.33	109.07
	10	10	3	10,09,10	01	3.33	
F_1	1000	10	3	00,00,00	30	100	
	100	10	3	06,04,05	15	50	100
	10	10	3	09,10,08	03	10.0	
F_2	1000	10	3	04,04,05	17	56.67	
	100	10	3	08,09,08	05	16.67	883.260
	10	10	3	10,09,10	01	3.33	

KEY: C: Crude, F1: Fraction number 1, F2: Fraction number 2.

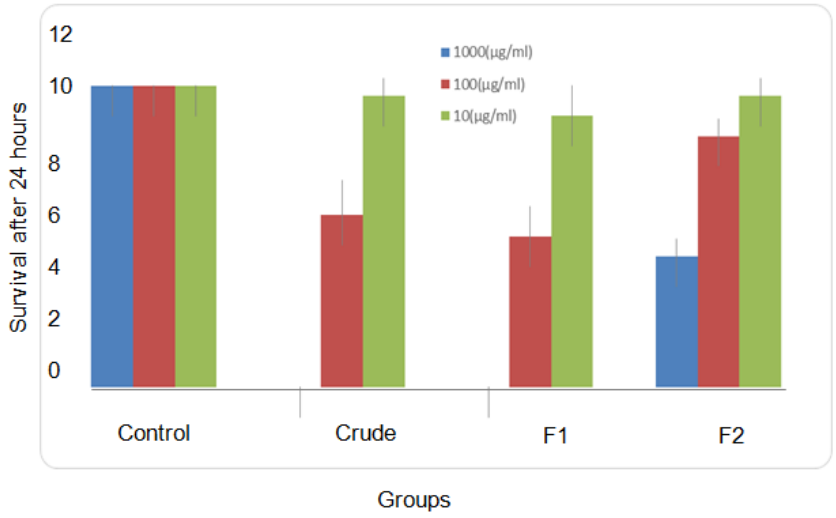

Fig. 3. Brine Shrimp Lethality Assay showing survival after 24 hrs against concentration
KEY: C: Crude, F1: Fraction number 1, F2: Fraction number 2
C is the crude methanol extract, F1 is fraction 1 and F2 is fraction 2. These fractions were obtained from the crude.

Table 5a. FTIR result for the fraction F1 in comparison with the literature, (b) Table 5b: FTI R result for the fraction F2 in comparison with the literature

(a) Literature value (cm-1)	F1 value (cm-1)	Probable functional group
3400-2400	3363	R-C-OH (Carboxylic acid)
3000-2850	2963	C-H (Alkanes)
3000-2850	2922	C-H (Alkanes)
1725-1705	1721	R-C-OH (Carboxylic acid)
1690-1640	1655	C=C (Olefine)

(b) Literature value (cm-1)	F2 value (cm-1)	Probable functional group
3400-3200	3275	O-H (H-bonded)
3000-2850	2925	C-H (Alkanes)
1680-1630	1655	C=C (Alkenes)
1640-1550	1607	C-C (Alkenes)
1450-1375	1376	CH_3(Alkanes)

muscular dystrophy. Creatinine is not mainly found in red blood cells, hence not affected by haemolysis and is also not affected by liver diseases [49].

Potassium ions assume a vital role in the route in which nerve driving forces are spread along the nerve cells and transmitted to receptor cells. The sodium pump keeps up the intracellular K^+ level of 140 mM as against the extracellular K^+ level of 5 mM [50]. Higher net endogenous acid creation in view of biomarkers (urea nitrogen and potassium) was humbly connected with speedier endless kidney illness progression [51].

Hypernatraemia indicates a deficiency of water in connection to the body's sodium stores. It can also result from net water loss or hypertonic sodium gain. Managed hypernatraemia can happen just when thirst or access to water is impeded [52]. Saima et al. [53] reported that excessive loss of water caused by lessened repletion of liquids is indicated in hypernatraemia. A marginally however essential ascent in serum chloride level with no adjustment in anion gap, occurs ahead of chronic renal impairment. Patients with low benchmark serum bicarbonate level bear a somewhat higher danger of a composite renal result [54]. Low serum bicarbonate level is

a free indicator of kidney work decline and mortality in patients with endless kidney ailment [55].

Higher serum chloride concentration may be seen in dehydration, hyperventilation, congestive heart valve, and prostatic or other types of urinary obstruction [56]. Variations seen in; chloride and bicarbonate in present study might be due to differences in weight of rats and doses of extract used. It may also be linked to physiological variation of experimental animals among the groups. As the kidney have significant role in the removal of metabolic by-products and in maintenance of intracellular fluid volume, electrolyte composition and acid – base balance, the functional capacity of the kidney is important to total body homeostasis [57]. Renal function tests are used either to show the presence or absence of injury to the kidney, or to ascertain the normal functioning capacity of different parts of the functioning unit, nephron [58].

In this study, effect on the liver may be due to damage to the hepatocytes by the extract though mildly observed. From the histological evaluation of the kidney of treated rats when compared to the liver, milder effect on the kidney compared to the liver might be attributed to the fact that the liver is the major target organ affected when exposed to toxic substances after being absorbed in the intestines and metabolised to other products [59]. Toxicity of a drug/xenobiotic can be linked to the level of injury on the organ/tissues to a reasonable extent [60]. Histological examination of tissues could be used as supportive proof to enzyme studies towards uncovering any injury to the normal architecture of the tissue cells [61]. The liver and kidney were used for histological analyses in light of the fact that they are part of the essential organs primarily influenced by metabolic reaction due to toxicant [62]. The provocative invasion showed by the kidney might be because of accumulation of immune complexes most likely created by these compounds.

Table 6a. GC-MS phytoconstituents characterization of partially purified fraction 1 of *J. curcas* leaves extract

No	RT	Name of Compound	MF	MW (g/mol)	Area %	m/z
1	17.423	Nonane, 3-methyl-	$C_{10}H_2$	142.286	2.50	57
2	18.230	Carbonic acid, nonyl vinyl ester	$C_{12}H_{22}O_3$	214.305	4.79	43
3	20.410	Dodecane, 2,6,10-trimethyl-	$C_{15}H_{32}$	212.421	10.17	71
4	21.057	Tetradecane	$C_{14}H_{30}$	198.394	6.12	57
5	21.887	Naphthalene, 2,7-dimethyl-	$C_{12}H_{12}$	156.228	6.97	156
6	22.722	Hexadecane	$C_{16}H_{34}$	226.448	2.76	57
7	27.197	Benzene, (1-butyloctyl)-	$C_{18}H_{30}$	246.438	1.98	91
8	29.394	Benzene, (1-pentylheptyl)-	$C_{18}H_{30}$	246.438	2.78	91
9	29.520	Benzene, (1-butyloctyl)-	$C_{18}H_{30}$	246.438	2.49	91
10	29.800	1,5-Decadiyne	$C_{10}H_{14}$	134.222	1.98	91
11	30.778	Cyclopentane, butyl-	C_9H_{18}	126.243	2.05	69
12	31.768	1-Chloroundecane	$C_{11}H_{23}Cl$	190.755	2.26	41
13	33.674	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	228.42	23.48	74
14	35.018	2-Pentadecanol	$C_{15}H_{32}O$	426.729	2.76	43
15	35.654	1-Hexacosanol	$C_{26}H_{54}O$	382.717	3.53	43
16.	37.199	6-Octadecenoic acid, methyl ester, (Z)-	$C_{19}H_{36}O_2$	296.495	9.93	41
17	37.679	Heptadecanoic acid, 16-methyl-,methyl ester	$C_{19}H_{38}O_2$	298.511	6.65	87
18	44.294	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.43	3.25	43
19.	44.711	Oleic Acid	$C_{18}H_{34}O_2$	282.468	3.54	47

RT: Retention Time, MF: Molecular formular, MW: Molecular weight, m/z: mass to charge.

(b) Table 6b. GC-MS phytoconstituents characterization of partially purified fraction F2 of *J. curcas* leaves extract

No	RT	Name of compound	MF	MW (g/mol)	Area %	m/z
1	5.138	13-Octadecenal, (Z)-	$C_{18}H_{34}O_2$	266.469	1.21	41
2	38.537	Dodecanoic acid, 1,2,3-propanetriy l ester	$C_{39}H_{74}O_6$	639.015	13.34	57
3	40.649	Dodecanoic acid, 1,2,3-propanetriy l ester	$C_{39}H_{74}O_6$	639.015	62.33	57
4	42.371	Lauric anhydride	$C_{24}H_{46}O_3$	382.629	23.13	180

***RT**: Retention Time, **MF**: Molecular formular, **MW:** Molecular weight, **m/z:** mass to charge.*

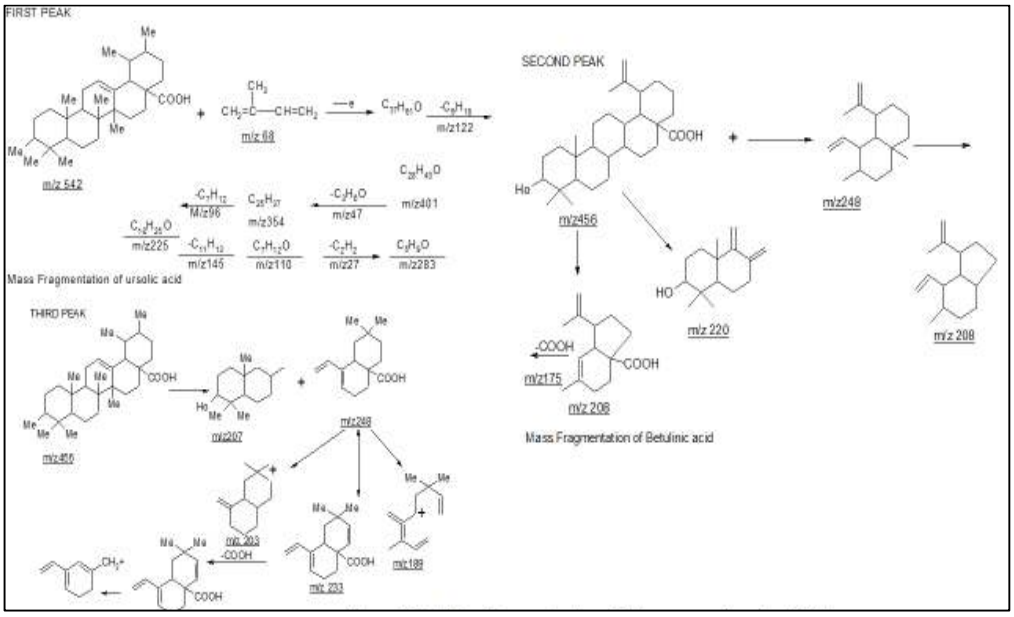

Fig. 4a. Mass fragmentation of F1 compounds using LC-MS

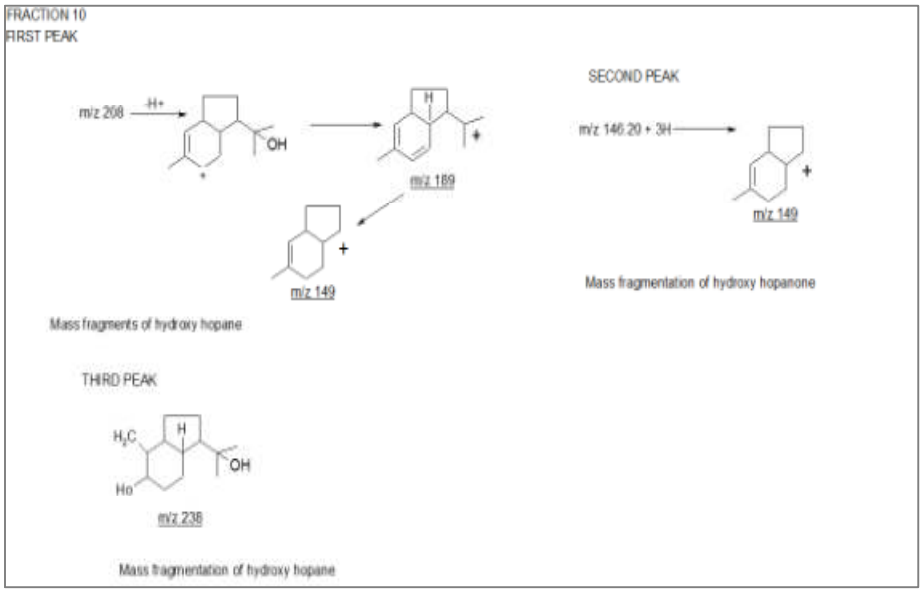

Fig. 4b. Mass fragmentation of F2 compounds using LC-MS

From the result obtained, the level of lethality was proportionate to the strength of extract / fraction used. The LC_{50} obtained for the methanol crude extract of *J. curcas* is consistent with the oral LD_{50} of the same extract that was obtained in this study; both results showed that the crude extract is slightly toxic.

Result of FTIR for fraction 1 indicates an agreement with what was obtained in the fraction's LC-MS results likewise that of Fraction 2. GC-MS results revealed that fraction 1 showed presence of more compounds than Fraction 2. Both benzene and fatty alcohols present in Fraction 1 were reported to have toxic effect on cells. After ingestion or inward breath of benzene, it targets organs including: liver, kidney, heart, brain. Benzene is metabolized majorly in the liver by Cyt P450 mutifunctional oxygenase framework, it also causes haemato-toxicity through its phenolic metabolites. Liver microsomes assume critical role in biotransformation of benzene [63]. Fatty alcohols were also reported to have harmful impact on cells [64]. LC-MS results of this study was in agreement with the molecular ion obtained from the three major peaks in LC-MS, mass fragmentation pattern from LC-MS and the functional groups in the FTIR of the fraction (F1). Also, these three pentacyclic triterpenes, betulinic acid from lupane type, oleanolic acid from oleane type and ursolic acid from ursane type triterpenes were seen in the ethyl acetate partition of dried methanol extract of aerial parts of *Euphorbia microsciadia* (*Euphorbiaceae*) [65], *J. curcas* and this plant belong to the same family.

Triterpenes are a category of natural products discovered particularly in plants. The triterpene acids display critical organic and pharmacological roles, including anti-inflammatory, antimicrobial, antiviral, cytotoxic and cardiovascular effects [66]. Triterpenoids have been accounted for in an assortment of basic European plants and organic products [67]. Uric acid (3β-hydroxyurs-12-en-28-oic) is a pentacyclic triterpene and a phytosterol. Triterpenes are to a great extent obtained from vegetables oils, vegetarian foods, medicinal herbs, cereals, and fruits. They are viewed as normal segments of human diets [68]. The naturally occurring pentacyclic triterpenoid, betulinic acid has been shown appeared to have hostile to tumour action and conquer resistance by actuating apoptosis in an assortment of human malignancy. Its specific cytotoxicity against malignancy was first depicted on human melanoma both *in vitro* and *in vivo*. Curiously, betulinic acid has additionally been effectively connected *in vitro* in adolescence malignancy, namely medulloblastoma, glioblastoma, neuroblastoma, and leukaemia. Aggregated test prove demonstrates that betulinic acid causes particular morphological changes in touchy cells, for example, cell shrinkage, DNA fragmentation, nuclear condensation [69]. From natural purpose of view, the most imperative triterpenoid structures are ursane, oleane, lupane, and dammarane-euphane. Uric acid has been appeared to show different pharmacological activities under in vitro and in vivo conditions. This ubiquitous pentacyclic triterpene has been researched for its anti-inflammatory, hepatoprotective, analgesic, antimicrobial, antimycotic, virostatic, immunomodulatory, diueretic, anti- spasmodic, anti-atherosclerotic, anti-tumor and anti HIV activity [70-72].

4. CONCLUSION

In conclusion, *in vivo* and *in vitro* toxicity of methanol extract of *J. curcas* at acute and sub-chronic level showed mild toxicity of the extract due to numerous phytochemicals in the extract which were characterized in this study. Therefore, use of crude extract of *J. curcas* leaf for medicinal purposes should be with care. However, this study could not isolate the toxic compounds. Further studies is recommended to isolate these compound(s), this will aid to maximize use of the extract for numerous medicinal applications such as anti-plasmodial, anti-oxidant, anti-inflammatory, anti-cancer, anti-diarrheal, coagulative, anti-leukaemic, anti-microbial and antihyperglycaemic properties among others.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. David EA, Curtis DK. Introduction to toxicology. Principles of Toxicology: The McGraw-Hill Companies; 2001.
2. Kulkarni ML, Sreekar H, Keshavamurthy KS, Shenoy N. *Jatropha curcas*-poisoning. Indian Journal of Pediatriatric. 2005;72:75-76.
3. George P. Concerns regarding the safety and toxicity of medicinal plants-An overview. Journal of Applied Pharmaceutical Science. 2011;1:40-44.
4. Eko M. The growing use of herbal medicines: Issues relating to adverse reactions and challenges in monitoring safety. Frontiers in pharmacology. 2014; 11:1-10.
5. WHO (World Health Organization). Guidelines on safety monitoring of herbal medicine in pharmacovigilance system. Geneva, Switzerland. World Health Organisation; 2004.
6. Prasad DMR, Izman A, Khan MR. *Jatropha curcas*: Plant of medicinal benefits. Journal of Medicinal Plants Research. 2012; 6(14):2691-2699. PubMed.
7. Janick J, Robert EP. The encyclopaedia of fruits & nuts. CABI. 2008;371-372.
8. Roger B, Dendo M. Hausa names for plants and trees, 2nd edition. Cambridge CBI 2AL. 2007;9.
9. Imam AA, Salim MA, Bala M, Aisha MI, Yahaya S. Phytochemistry and antiplasmodial properties of aqueous and methanol leaf extract of *Jatropha curcas: Bayero* Journal of Pure and Applied Sciences (BAJOPAS). 2016;1:93-98.
10. Sofowora A. Medicinal plants and traditional medicine in Africa. 2nd Edn, Spectrum Books Ltd., Ibadan, Nigeria. 1993;13:289.
11. Duke JA, Wain KK. Medicinal plants of the world. Computer Index with More than 85,000 entries, Plants Genetics and Germplasm Institute, Agriculture Research Service, Beltsville, Maryland. 1981;3.
12. Mujumdar AM, Misar AV, Salaskar MV, Upadhye AS. Antidiarrhoeal effect of anisolated fraction (JC) of *Jatropha curcas* roots in mice. Journal of Natural Remedies. 2001;1:89-93.
13. Shanti BM, Vijayakumar M, Sanjeev KO, Amita V. Antidiabetic effect of *Jatropha curcas L*. Leaves extract in normal and alloxan-induced diabetic rats. International Journal of Pharmaceutical Sciences. 2010; 2(1):482-487.
14. Oskoueian E, Abdullah N, Saad WZ, Omar AR, Ahmad S. Antioxidant, anti-inflammatory and anticancer activities of methanolic extracts from *Jatropha curcas* Linn. Journal of Medicinal Plants Research. 2011;5:49-57.
15. Parawira W. Biodiesel production from *Jatropha curcas*: A review. Sci. Res. Essays. 2010;5:1796-1808.
16. Veeramuthu D, Muniappan A, Savarimuthu I. Antimicrobial activity of some ethnomedicinal plants used by paliya tribe from Tamil Nadu India; 2006.

    DOI: 10.1186/1472-6882-6-35
17. Meyer BN, Ferrigni NR, Putnam JE, Jacobsen LB, Nichols DE, McLaughlin JL. Brine shrimp: A convenient general bioassay for active plant constituents. Planta Medica. 1982;45:31-34.
18. Fatope MO, Ibrahim H, Takeda Y. Screening of higher plants reported as pesticides using the brine shrimp lethality assay. International Journal of Pharmacognosy. 1993;31(4):250-56.
19. Lorke D. A new approach to practical acute toxicity testing. Archives of Toxicology. 1983;53:275-287.
20. Yakubu MT, Akanji MA, Oladiji AT. Aphrodisiac potentials of aqueous extract of *Fadogia agrestis* (Schweinf. Ex Heirn) stem in male albino rats. Asian Journal of Andology. 2005;7(4):399-404.
21. Reitman S, Frankel AS. A colorimetric method of determination of serum glutamic oxaloacetic and glutamic pyruvic transaminases. American Journal Clinical Pathology. 1957;28:53-63.
22. Rec GSCC. Optimised standard colorimetric methods. Journal of Clinical Chemistry and Clinical Biochemistry. 1972; 10:182.

23. Plummer DT. In: An introduction to practical biochemistry. 2nd ed, McGraw-Hill, London. 1978;144-145.
24. Doumas BT, Watson WA, Biggs HG. Albumin standards and measurement of serum-albumin with bromocresol green. Clinical Chemistry. Acta. 1971; 31:87.
25. Jendrassik L, Grof P. Colorimetric method of determination of bilirubin. Biochemistry Z. 1938;297:81-82.
26. Kaplan A. Urea nitrogen and urinary ammonia. In: Standard Method of Clinical Chemistry, ed. Meites S. Academic Press Inc., New York. 1965;245 – 256.
27. Butler AR. The Jaffe reaction. Identification of the coloured species. Clinica Chimica Acta. 1975;59:227-232.
28. Trinder P. A rapid method for the determination of sodium in serum. Analyst. 1951;76:596-599.
29. Henry RF, Cannon DC, Winkleman. Clinical chemistry principles and techniques: Harper and row, Hagerstown, M.D., 2nd Edition. Hargers town, Maryland, U.S.A. 1974;248-260.
30. Schonfeld RG, Lewellen CJ. A colorimetric method for determination of serum chloride. Clinical Chemistry. 1964;10:533.
31. Forrester RL, Wataji LJ, Silverman DA, Pierre KJ. Enzymatic method for the determination of CO_2 in serum. Clinical Chemistry. 1976;22:243.
32. Avwiora OG. Histochemistry and tissue pathology: Principles and techniques. 2nd Edition. University Press Delta State University, Abraka, Nigeria; 2010.
33. Shanti BM, Vijayakumar M, Sanjeev KO, Amita V. Antidiabetic effect of *Jatropha curcas L*. Leaves extract in normal and alloxan-induced diabetic rats. International Journal of Pharmaceutical Sciences. 2010; 2(1):482-487.
34. Dangambo MA, Kabara HT, Alhassan AJ, Sule MS, Atiku MK, Wudil AM, Mashi JA. Acute toxicity and hepatocurative effect of aqueous leaf extract of *Jatropha curcas* in rats. British Journal of Pharmaceutical Research. 2015;5(1):5.
35. Hodges A, Sterner B. Toxicity classes. In: Canadian center for occupational Health and safety. Copyright @ 1997 – 2010; 2005. Available:http://www.ccohs.ca/oshanswers/chemicals/ld50.htm).04/02/2010
36. Singh K. Evaluation and interpretation of biomarkers of liver diseases. International Journal of Research in Health Sciences. 2013;1(3):213-223.
37. Ajayi GO, Adeniyi TT, Babayemi DO. Hepatoprotective and some haematological effects of *Allium sativum* and vitamin C in lead exposed wistar rats. International Journal of Medicine and Medical Sciences. 2009;1(3):064-067.
38. Shahjahan M, Sabitha KE, Jainu M, Shymala Devi CS. Effects of *Solanum trilobatum* against carbon tetrachloride-induced hepatic damage in albino rats. Indian Journal of Medical Research. 2004; 120:194-198.
39. Chang S. Liver enzymes on Right health Available:http://www.html (cited on)
(Accessed 11th Feb 2013)
40. Yakubu MT, Akanji MA, Salau IO. Protective effect of ascorbic acid on some selected tissues of ranitidine-treated rats. Nigerian Journal of Biochemistry and Molecular Biology. 2001;16(2):177-182.
41. Bolarin DM. Bolarin's aid to chemical pathology. Lantern Books NIG. 2nd Edition. 2013;389-443.
ISBN: 978-978-100-624-1
42. Rosalki SB, Mcintyre N. Biochemical investigations in management of liver disease. Oxford Textbook of clinical Hepatology. 2nd ed. New York; Oxford University Press. 1999;503-521.
43. Novotny L, Vetek L. Inverse relationship between serum bilirubin and atheroschlerosis in men: A meta-analysis of published studies. Experimental Biology and Medicine. 2003;228(5):568 -71.
44. Schwertner, Harvey A, Vitet L. Gilbert Syndrom, UGTIAl*28 allelel, and cardiovascular disease risk: Possible protective effects and therapeutic applications of bilirubin. Atherosclerosis. 2008;198(1):1- 11.
45. Musa TY, Adebayo OJ, Egwim EC, Owoyele VB. Increased liver phosphatase and amino transferases activities following administration of ethanolic extract of *Khaya senegalensis* stem bark to rats. Biochemistry. 2005;17(1):27.
46. Rosalki SB, Mcintyre N. Biochemical investigations in management of liver disease. Oxford Textbook of Clinical Hepatology. 2nd ed. New York; Oxford University Press. 1999;503-521.

47. Corless J, Middleton H. Normal liver function, a basis for understanding hepatic disease. Archives Internal Medicine. 1993; 143:2291-2294.

48. Chawla R. Kidney function tests. In: Practical Clinical Biochemistry Methods and Interpretation. 2nd edition. JAYPEE Brothers Medical Publishers (P) Ltd., New Delhi, India. 1999;90-2.

49. Twee TD. Muscular disease workup. Drugs and diseases in Orthopaedic Surgery. 2016;Article 1259041.

50. Horton AR, Moran LA, Ochs KS, Rawn JD, Scrimgeour KG. Principles of biochemistry. Niel Patterson Publisher/Prentice: Hall Englewood Cliffs: New Jersey; 1993.

51. Scialla JJ, Asplin J, Dobre M, Chang AR, Lash J, Hsu et al. Higher net acid excretion is associated with a low risk of kidney disease progression in patients with diabetes. Kidney International. 2017;91: 204-215.

52. Duggal AK, Yadav P, Agarwal AK, Rewari BB. Clinical approach to altered serum sodium levels. Journal, Indian Academy of Clinical Medicine. 2006;7(2):91-103.

53. Saima N, Yasir K, Sadia N, Hassan B. Hypernatraemia: Correction rate and hemodialysis. Hindawi. 2014;Article ID 736073:1-4.

54. Adeva-Andany MM, Fernandez CF, Bayolo M, Castro EQ, Montero AD. Sodium bicarbonate therapy in patients with metabolic acidosis. The Scientific World Journal. Hindawi. Article. 2014; ID 627673: 13.

55. Schutte E, Heerspink HJI, Lutgers HL, Bakker SJL, Vart P, Wolffenbuttel et al. Serum bicarbonate in diabetic nephropathy. American Journal of Kidney Disease. 2015;66(3):450-458.

56. Tietz NW, Prude EL, Sirgard AO. In: Tietz textbook of clinical chemistry. (Burtis CA and Ashwood ER, eds). W.B. Saunders Company: London. 1994;1354-1374.

57. Orisakwe OE, Hussaini DC, Afonne OJ. Testicular effects of sub-chronic administration of *Hibiscus sabdariffa* calyx aqueous extract in rats. Reproductive Toxicology. 2003;18:295-298.

58. Panda NC. Kidney. In: Textbook of biochemistry and human biology (3rd edition). GP Talwar, LM Srivastava and KD Moudgil (eds); Prentice-Hall of India. Private Ltd., 2004;276-292.

ISBN-13: 978-8120319653

59. Rhiouania H, El-Hilalya J, Israili Z. H, Lyoussia BJ. Acute and sub chronic toxicity of an aqueous extract of the leaves of *Herniaria glabrain* rodents. Journal of Ethnopharmacology. 2008; 118:378-386.

60. Akanji MA, Nafiu MO, Yakubu MT. Enzyme activities and histopathology of selected tissues in rats treated with potassium bromate. African Journal of Biomedical Research. 2008; 11:87–95.

61. Singh I. Textbook of human histology with colour atlas. 4th edn, Jaypee Publisher, India. 2002;249-258.

62. Dybing E, Doe J, Groten J, Kleiner J, Brien J. Hazard characterization of chemicals in food and diet: Dose response, mechanism and extrapolation issues. Food and Chemical Toxicology. 2002;42:237-282.

63. Rana SV, Verma Y. Biochemical toxicity of Benzene. Journal of Environmental Biology. 2005;26(2):157-168.

64. Dyer SD, Sanderson H, Waite SW, Companolle RV, Bradford P, Nielson AM. Assessment of alcohol ethoxylate surfacants and fatty alcohols mixtures in river sediments and prospective risk assessment. Environmental Monitoring and Assessment. 2006;20(1):45-63.

65. Ayatollahi A, Ghanadian M, Afsharypour S, Abdella OM, Mirzai M, Askari G. Pentacyclic triterpenes in *Euphorbia microsciadia* with Their T-cell Proliferation Activity. Iranian Journal of Pharmaceutical Research. 2011;10(2):287-294.

66. Silva ML, David JP, Silva LCRC, Santos R AF, David JM, Lima LS, Pedro SR, Fontana R. Bioactive oleanane, oupane and ursane triterpene acid derivatives. Molecules. 2012;17:12197–12205.

67. Kristo TS, Terdy PP, Simandi B, Szoke, Lemberkovics E, Kery A. Acta Pharmacology Hung. 2001;71:318.

68. Moreau, RA, Whitaker BD, Hicks KB. Phytosterols, phytostols and their conjugates in foods: Structure diversity, quantitative analysis and walth-promoting

uses. Progress in Lipid Research. 2002; 41:457-500.

69. Eichenmuller M, Hemmerlein B, Schweinitz DV, Kappler R. British Journal of Cancer. 2010;103(1):43-51.

70. Harden L, Edwards R. Identification of three triterperiods in almond hills. Journal of Agricultural and Food Chemistry. 2000; 48:3437-3439.

71. Li J, Gou W, Yang Q. Effects of unsolic and obanolic acid on human colon carcinomalell line HCT15. Wold Journal of Gastroenterology. 2002;8(3):493-495.29.

72. Neto CC. Cranberry and its phytochemicals: A review of in vitro anticancer studies. Journal of Nutrition. 2007;137:1865-1935.

Peer-review history:
The peer review history for this paper can be accessed here:
http://sciencedomain.org/review-history/22407