



(RESEARCH ARTICLE)



Evaluation of toxicological profile of methanol leaf extract of *Waltheria indica* (Sterculiaceae)

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GSC Biological and Pharmaceutical Sciences, 2021, 17(02), 034–043

Publication history: Received on 06 September 2021; revised on 25 October 2021; accepted on 27 October 2021

Article DOI: <https://doi.org/10.30574/gscbps.2021.17.2.0267>

Abstract

Waltheria indica has been claimed to be used in managing several diseases in traditional medicine, although substantial scientific data are not available as regards its safety despite its pronounced efficacy in management of some ailments. Therefore, methanol leaf extract of *W. indica* was evaluated for its effects on some toxicological parameters using experimental animals. However, acute and sub-acute toxicity were carried out using experimental animals as described by standard methods. Absence of death reported after 24 hours of single oral administration of *W. indica* indicated that the crude drug is safe orally and the sample also had no effect on both hematological and biochemical parameters, with mild toxicity observed with some vital enzymes and organs upon continuous oral administration of *W. indica* for 21 days. However, it can be concluded that prolong usage of the plant for disease management should be discouraged, which implies that usage of the plant for short time usage in disease management.

Keywords: Acute toxicity; Biochemical parameters; Sub-acute toxicity; *W. indica*

1. Introduction

Medicinal plants are usually regarded as a rich source of chemical entities which can be used in drug development and synthesis. Besides these plants play a critical role in the development of human cultures around the world [1]. Medicinal plants have been discovered for decades and employed in indigenous medicine practices ever since prehistoric times. Hundreds of biochemical compounds are biosynthesized by plants for functions including defense against insects, herbivorous mammals, and microbial attack. However, as a single plant has widely diverse phytochemicals, the effect of using a whole plant as medicine is not certain due to associated toxicity, apart from its inherent biological or pharmacological attributes. Further, the constant development of modern scientific research bioassays and analytic procedures and techniques are expected to further enhance not only purity and efficacy but also safety of these medicinal plants and their corresponding natural drugs and led compounds [2].

However, A large number of plants can cause adverse effects when ingested by animals or people. Plant toxicity is due to a wide diversity of chemical toxins that include alkaloids, glycosides, proteins and amino acids. There are several notable toxic plants for which a specific chemical responsible for toxicity has not been determined. There are many examples of species differences in terms of their sensitivity to intoxication from plants. Pets, such as dogs and cats, and people, especially children, are frequently exposed to the same toxic plants due to their shared environments [3]. On the other hand, livestock are exposed to toxic plants that are rarely involved in human intoxications due to the unique

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environments in which they are kept. Fortunately, adverse effects often do not occur or are generally mild following most toxic plant ingestions and no therapeutic intervention is necessary [3]. However, some plants are extremely toxic and ingestion of small amounts can cause rapid death. The diagnosis of plant intoxication can be challenging, especially in veterinary medicine where a history of exposure to a toxic plant is often lacking. Analytical tests are available to detect some plant toxins, although their diagnostic utility is often limited by test availability and timeliness of results. With a few notable exceptions, antidotes for plant toxins are not available. However, general supportive and symptomatic care often is sufficient to successfully treat a symptomatic patient [3].

Waltheria indica (WI) is a common plant used in traditional medicine in Nigeria, usually found in wild and used by locals in preparation of many local medicinal herbs for disease management. The plant is distributed widely in tropical regions of the world and known for its bitter taste and astringent property. Hence, it is used to clear damp heat, poison and to cool blood [4]. However, WI in northern Nigeria is locally called as 'kafafi' in Fulani, 'hankufah' or 'hankubah' in Hausa, 'efu-abe' in Nupe, 'korikodi' in Yoruba [5, 6]. It is utilized by native populations in different regions of the globe to treat several pathological conditions due to various classes of secondary metabolites present in the plant. WI have also been reported to be used in management of the following diseases, cataract [7], diabetics [8], asthma [4], anemia [9], male sexual dysfunction [10] and cancer [11].

2. Material and methods

2.1. Laboratory animals

Swiss albino mice of both sexes (20 - 25 g) were chosen for acute toxicity studies and Wistar albino rats of weight ranging between 150 - 200 g of both sexes were likewise selected for sub-acute toxicity procedure. The laboratory animals were sourced from Department of Pharmacology animal house and were likewise given free access to food. They were maintained under standard laboratory conditions which included 12-hour light-dark cycle and temperature of 28-30°C. Moreover, the experimental animals are allowed for two weeks acclimatization period for adaptation prior to the entire toxicity studies. The experimental protocol was initially approved by the IAEC (Institutional Animal Ethical Committee) prior to carrying out of the entire experimental activities and care of the laboratory animals were carried out using the CPCSEA guidelines.

Acute and sub-acute toxicity studies were carried out on the extract as explained below:

2.2. Acute Toxicological Study

Lethal doses (LD₅₀) of the crude extracts were examined using 48 mice (consisting of both male and female weighing 20 - 25 g) for each extract. They were fed with standard rat pelleted diet (Bendel feeds, Edo state, Nigeria) and water *ad-libitum* and maintained under hygienic standard laboratory condition in animal house of Department of Pharmacology, Faculty of Pharmacy, and University of Benin, Nigeria. The experiment was initiated only after the approval of the Animal Ethics Committee of Faculty of Pharmacy, University of Benin. Single and daily oral doses of 50, 100, 300, 500, 1000 and 5000 mg/kg of the extracts were administered to six groups of animals (each consisting of seven animals) while 5% Tween-80 were administered to the control group. The mice were observed continuously for 1hr for any gross behavioural changes and deaths and intermittently for the next 6 hours and then again at 48 hrs after dosing. Survival animals were kept under observation for 7 days.

2.3. Sub-Acute toxicity

Rats were grouped into four (4), namely: A, B, C and D. However, the animals in group A which served as the control were orally administered with 1 mL of distilled water while those in groups B, C and D received orally extract of *W. indica* at doses of 250, 500 and 1000 mg/kg body weight respectively. The administration was done once daily. The animals were handled according to the guidelines of [12]. The animals were sacrificed 21 days of administration of the crude extract. The blood of the animals was used for both biochemical and hematology analysis while histopathology studies of the liver and kidney were carried out.

2.3.1. Preparation of serum and plasma

The rats were anaesthetized in a jar containing cotton wool soaked in chloroform. Blood samples were collected (via cardiac puncture) into sample bottles and allowed to clot at room temperature for 20 minutes and thereafter centrifuged at 1398 x rpm for 15 minutes. Clear colorless serum was collected with the aid of Pasteur pipette. Moreover, plasma obtained after centrifugation of heparinized blood was used for hematological analysis.

2.3.2. Biochemical analysis

Biochemical analyses were performed on serum obtained after centrifugation of whole blood (without anticoagulant) at 3000 rpm for 5 min. The following biochemical analyses were carried out.

Kidney function test

A) Assay for Urea

This test exploits the hydrolysis of urea to ammonia in the presence of urease enzyme. 100 μ L of reagent 1 was added to 10 μ L serum samples in test tubes. 100 μ L of reagent 1 was also added to 10 μ L distilled water to serve as blank. Solutions in the test tubes being thoroughly mixed for incubation at 10 minutes of 37°C. 2.5 mL of reagents 2 and 3 drops were added in each test tube. Solutions in test tubes were mixed and further incubated for 10 minutes at 37 °C. Absorbances of samples and standard were read against the blank using the wavelength of 546 nm. Urea values were calculated as thus.

Urea concentration (mg/dL) = Absorbance of sample/ Absorbance of standard x 80.

b) Assay for Creatinine

This test exploits the reaction of creatinine present in alkaline mixture using picric acid to produce colored complex. The quantity of complexity formed is directly proportional to creatinine concentration. 0.2 mL sample macro and semi micro was added to the test tube. 0.2 mL standard macro and 0.1 mL semi micro were added to the standard solution test tube. 2 mL of standard macro, 1 mL of semi micro, 2 mL of sample macro and 1 mL of semi micro were added to the working reagent test tube. The solutions in the test tubes were mixed. The absorbances of the samples and standard were measured at 492 nm. Creatinine concentration being calculated as thus:

Creatinine concentration units (mg/dl) = Absorbance of sample/Absorbance of standard x 2.

c) Assay for Electrolytes (Sodium, chloride, bicarbonate and potassium)

Bicarbonate

The assay for bicarbonate exploits the reaction between bicarbonate ions with dilute hydrochloric acid to yield carbon dioxide. The excess acid is titrated with dilute sodium hydroxide using phenol red as indicator. 0.01 N HCl was added to 200 μ L of serum sample. The solution was mixed and 1 drop of phenol red indicator added. The solutions were titrated using 0.01 N sodium hydroxide to attain brick red colouration which serves as endpoint [13].

Bicarbonate was calculated as thus: Bicarbonate (μ mol/l) = 50 - Titre

Titre = Endpoint x 100.

Chloride

The assay for chloride exploits the formation of chloride precipitate in a sample using mercuric nitrate. When chloride is titrated with standard solution of mercuric ion, undissociated but stable mercuric chloride is formed. The excess mercuric chloride nitrate reacts with diphenylcarbazone to produce a violet coloration. 2 mL of deionized water was added to 200 μ l serum sample. This solution was mixed thoroughly and 3 drops diphenylcarbazone indicator and 1 drop of nitric acid was added. The mixture was titrated via mercuric nitrate to give a violet endpoint. The same procedure was repeated for chloride standard solution [14]

Chloride calculation such as:

Chloride (μ mol/l) = Titre of sample/Title of standard x 100.

Sodium and potassium

The assay method for sodium and potassium entails the injection of solutions containing these elements in flame leaving solid salt, which dissociates to neutral ground state. The atoms become excited in the flame, thus moving to a higher energy state. The excited atoms then fall back to ground state emitting light of characteristic wavelength (590 nm

sodium and 770 nm potassium). The light surpasses through viable filter onto photosensitive element while, the quantity of current flows was relatively the amount of potassium and sodium found in serum sample [15]

d) Determination of Serum Total Bilirubin and Unconjugated Bilirubin

Total bilirubin can be anticipated via acid diazo technique as illustrate by [16], by assay kits (Randox Laboratories Ltd).

Principle

Sulphanilic acid can be diazotized using nitrous acid synthesized from reaction involving hydrochloric acid (HCL) and sodium nitrite (NaNO₂). Direct conjugated bilirubin interacts with diazotised sulphanilic acid to produce azobilirubin. Pink acid (azobilirubin) can be changed to blue acid (azobilirubin) using alkaline tartrate. Total bilirubin usually can be resolute in caffeine presence, which liberates albumin hurdle bilirubin via response to diazotized sulphanilate. Unconjugated bilirubin can be through their differences.

Procedure

Total bilirubin; at precisely, 0.05 mL reagent 2, composed of sodium nitrate (NaNO₂) being placed into test tubes enclosed with 0.2 mL of the reagent 1, composed of hydrochloric acid and sulphanilic acid of 1.0 mL reagent 3, has sodium benzoate and caffeine was added with 0.2 mL of serum, thoroughly mixed and kept in a place for 10 minutes room temperature. Reagent 4 (1 mL), have tartarate, also sodium hydroxide (NaOH) was added, properly mixed and kept for 10 minutes room temperature. Absorbance of samples against blank (ATB) was measured using wavelength of 560 nm in spectrophotometer. Calculations

$$\text{Total bilirubin (mg/dl)} = 10.8 \times \text{ATB}$$

$$\text{Unconjugated bilirubin units (mg/dl)} = 10.8 \times \text{Absorbance of total bilirubin (ATB)} - 14.4 \times \text{Absorbance of direct bilirubin (ADB)}$$

Liver function test

A volume of blood from each rat was put into plain bottles and allowed to clot at room temperature for 4 hours before centrifuging using Hettich centrifuge at 4000 rpm for 10 minutes. The sera obtained were used to assay for liver function test endpoints using standard diagnostic kits on automated clinical system in line with manufacturer instructions. Assays were carried out as follows:

Determination of Serum Alanine Aminotransferase (ALT)

The serum alanine aminotransferase was determined as described by Reitman and frankel (1957) using end-point technique.

Assay principle



Alanine aminotransferase is measured by monitoring the concentration of pyruvate hydrazone formed with 2, 4-dinitrophenylhydrazine at 540 nm and 37°C.

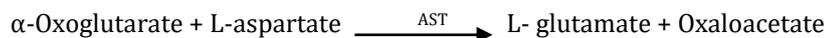
Procedure

Exactly, 0.5µL of reagent 1 which is made up of phosphate buffer, L-alanine and α-oxoglutarate was added in a clean test tube containing 0.1 mL of serum, mixed and 43 incubated for exactly 30 minutes at 37°C. 0.5 mL of reagent 2 which is made up of 2, 4-dinitrophenylhydrazine was added, mixed and allowed to stand for exactly 20 minutes at 20-25°C. Then 0.5 mL of sodium hydroxide solution was added, mixed and absorbance was read against the blank (also prepared) at 540 nm after 5 minutes. The ALT concentration (U/L) was determined from the standard calibration table provided in the manual of Randox Lab. Ltd, UK Reagent Kit.

Determination of Serum Aspartate Aminotransferase (AST)

The serum aspartate aminotransferase was determined as described by [17] using end-point technique.

Assay Principle



Aspartate aminotransferase is measured by monitoring the concentration of oxaloacetate hydrazone formed with of 2, 4-dinitrophenylhydrazine using 540 nm at 37°C.

Procedure

Exactly, 0.5µL of reagent 1, made up of phosphate buffer, L-aspartate and α-oxoglutarate were added to a test tube containing 0.1 mL of serum, mixed and incubated for exactly 30 minutes at 37°C. 0.5 mL of reagent 2, made up of 2,4-dinitrophenylhydrazine was added, mixed and allowed to stand for exactly 20 minutes at 20-25°C. Then 0.5 mL of sodium hydroxide solution was added, mixed and absorbance was read against the blank (also prepared) at 540nm after 5 minutes. The AST concentration (U/L) was determined from the standard calibration table provided in the manual of Randox Lab. Ltd, UK Reagent Kit.

Determination of Serum Alkaline Phosphatase (ALP)

The procedure described by [18] as modified by [19] using Randox kits was used for the assay.

The substrate p-nitrophenyl phosphate is hydrolyzed by alkaline phosphatase from the sample in the presence of magnesium ions, to form nitrophenol which is yellow and can be read at 405 nm. The intensity of color produced is proportional to the activity of alkaline phosphatase.

Principle



Alkaline phosphatase is measured by monitoring the concentration of phosphate hydrazone formed with 2, 4-dinitrophenylhydrazine at 405 nm and 37°C.

Procedure

In a cuvette, 10 µL of sample was mixed with 500 µL of the reagent. The initial absorbance was read at 405 nm, and subsequently over 3 minutes. The mean absorbance per minute was used in the calculation.

2.4. Hematological parameters

The heparinized blood was used for the analysis of hematological parameters such as hemoglobin, red blood cell count, white blood cell count, platelet count was measured using fully automated hematology analyzer (PE 6000)

2.5. Histological study

The organs (Liver, Kidney and heart) were carefully removed and weighed individually and fixed in 10% (vol/ vol) formaldehyde, cleaned up in xylene and embedded in a paraffin wax (melting point at 56 percent). Tissue sections were prepared according to the method of [20] and stained with eosin/ hematoxylin. Photomicrographs were taken at ×400 using a digital camera.

2.6. Statistical analysis

The statistical significance was evaluated by analysis of variance (ANOVA) followed by Dunnett's test. P values ≤ 0.05 were considered to be statistically significant. The experiments were carried out in six replicates and results are presented as mean ± standard error of the mean (Mean ± SEM).

3. Results and discussion

Toxicological tests are carried out to ascertain the safety of plant material. Different analyses were carried out on *W. indica* to analyze the toxicological profile of the methanol extract of *W. indica* and results are obtained are shown below:

3.1. Acute toxicity of *W. indica*

The behavior parameters observed were wobbling, hyperactivity and increased respiration.

The LD₅₀ > 5000 mg/kg when both extracts were orally administered, since no animal died at 5 g/kg (highest dose administered). Therefore, both plants were noted to be safe orally.

3.2. Sub-acute toxicity study of *W. indica*

Based on the biochemical analyses, no significance was observed in any of the biochemical parameters in the kidney function test when different doses of methanol extracts of *W. indica* were administered to the animals. This indicates that the methanol extracts of *W. indica* has no toxic biochemical effect on the kidney s shown in Table 1 below.

However, the results showing the effect of *W. indica* on some vital enzymes and hematological parameters after administration for 21 days are shown in Table 2 and 3 respectively.

Table 1 Effect of *Walteria indica* on biochemical parameters of rats after 21 days of administration

Biochemical parameters	Doses			
	Control (Distilled water)	100 mg/kg	200 mg/kg	400 mg/kg
Na (mmol/l)	125.00 ± 2.38	118.00 ± 4.19	136.30 ± 4.89	139.10 ± 4.71
K (mmol/l)	13.20 ± 2.17	14.50 ± 4.52	15.60 ± 5.90	16.70 ± 3.75
Cl (mmol/l)	81.41 ± 3.55	95.50 ± 4.89	99.80 ± 4.26	89.50 ± 3.85
Bicarbonate (mmol/l)	28.20 ± 2.10	28.60 ± 2.99	27.30 ± 4.11	31.70 ± 3.28
Urea (mg/dl)	22.40 ± 1.66	24.90 ± 2.80	37.60 ± 4.24	29.89 ± 5.27
Creatinine (mg/dl)	0.20 ± 0.11	0.20 ± 0.11	0.20 ± 0.19	0.10 ± 0.02
Total Bilirubin (mg/dl)	0.10 ± 0.04	0.30 ± 0.05	0.20 ± 0.08	0.20 ± 0.11
Direct Bilirubin (mg/dl)	0.15 ± 0.09	0.10 ± 0.08	0.10 ± 0.04	0.20 ± 0.02
Indirect Bilirubin (mg/dl)	0.10 ± 0.06	0.20 ± 0.13	0.10 ± 0.02	0.10 ± 0.02

The values above are mean of six replicates. n=6. Mean± SEM. Dose with no superscript (*) indicate no significant difference at P<0.05 when compared to mean of control for each biochemical enzymes using one-way ANOVA (Kruskal – Wallis test).

However, no significant difference in the alkaline phosphate, AST/SGOT and ALT/SGPT at 250 mg/kg and 500 mg/kg respectively when compared with the amount of the enzymes obtained from animals administered with the vehicle (control; 10 mL/kg). An increase in the dose to 1000 mg/kg shows a significant difference in the alkaline phosphate but no significant difference in the AST/SGOT and ALT/SGPT when compared with the amount of the enzymes obtained from animals administered with the vehicle (control; 10 mL/kg). Increase in the alkaline phosphate indicates that a high dose the methanol extract of *W. indica* maybe hepatotoxic.

The methanol extract of *W. indica* shows no significant effect on the blood cells which indicates that the methanol extract of *W. indica* is not toxic from hematological point of view.

The values above are mean of six replicates. n=6. Mean± SEM. Doses with no superscript * indicate no significant difference at P<0.05 when compared to mean of control (for each hematological parameter using one-way ANOVA (Kruskal – Wallis test).

Table 2 Effect of *W. indica* on biochemical enzymes in sub-acute toxicity using rats

Liver enzymes	Doses			
	Control (10 mL/kg)	250 mg/kg	500 mg/kg	1000 mg/kg
Alkaline Phosphate	142.90 ± 10.31	141.00 ± 23.51	211.20 ± 11.41	289.97±12.11*
AST/SGOT	78.60 ± 7.19	72.67± 6.29	69.61± 13.38	67.68± 11.76

Difference at $P < 0.05$ when compared to mean of control for each liver enzyme using one way ANOVA (Kruskal – Wallis test).

Table 3 Effect of *W. indica* on hematological parameter in sub-acute toxicity study using rats

Haematological Parameter	Control	<i>W. indica</i>		
		100 mg/kg	200 mg/kg	400 mg/kg
WBC	142.41 ± 8.22	148.41 ± 8.72	147.20 ± 4.28	197.50 ± 2.32
LY	77.98 ± 6.29	78.65 ± 4.22	78.52 ± 4.29	55.55 ± 3.16
MO	25.37 ± 6.61	24.89 ± 6.77	23.89 ± 3.29	28.53 ± 6.39
GR	122.34 ± 8.99	129.78 ± 5.88	135.31 ± 18.18	141.23 ± 13.29
LY%	12.77 ± 3.67	13.91 ± 5.86	13.50 ± 4.89	13.90 ± 5.42
MO%	92.82 ± 8.70	96.27 ± 7.25	94.64 ± 9.22	88.50 ± 4.28
GR%	27.11 ± 2.30	31.45 ± 5.10	25.90 ± 6.23	27.34 ± 7.13
RBC	37.23 ± 4.18	29.25 ± 1.82	39.51 ± 1.22	29.23 ± 3.11
Hgb	0.25 ± 0.05	0.22 ± 0.10	0.28 ± 0.12	0.31 ± 0.12
HCT	0.20 ± 0.01	0.20 ± 0.14	0.20 ± 0.12	0.20 ± 0.11
MCV	0.03 ± 0.01	0.10 ± 0.01	0.10 ± 0.01	0.20 ± 0.11
MCH	0.10 ± 0.03	0.10 ± 0.02	0.10 ± 0.01	0.10 ± 0.01

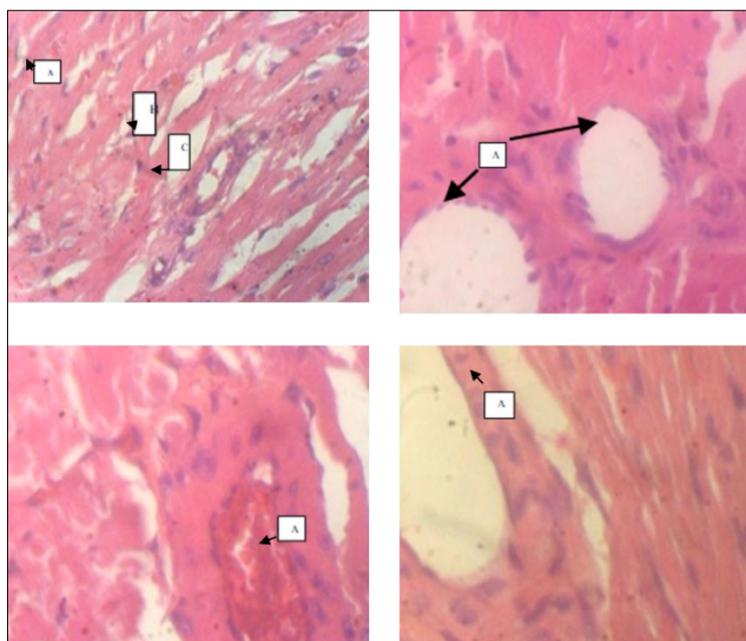


Figure 1 (A) Control Rat heart composed of; A, bundles of myocardial fibres, B, coronary vessel and C, interstitial space, (B) 250 mg/kg crude extract of *W. indica* showed A, moderate vascular dilation, (C) 500mg/kg crude extract of *W. indica* showed A, moderate vascular congestion, and (D) 1000mg/kg crude extract of *W. indica* showed A, moderate vascular dilatation (H&E) at day 21

Histological examination of the methanol extract of *W. indica* shows some histopathological changes on the various organs (heart, spleen, liver and kidney) examined. Some of these changes includes: the dilation of bundles of myocardial fibres in the heart at 100 mg/kg and 250 mg/kg and congestion of the vessels at 500 mg/kg and mild follicular atrophy in the spleen at 250 mg/kg, a mild vascular congestion and moderate Kupffer cell activation in the liver and a mild interstitial congestion in the kidney. These histopathological changes maybe as a result of the secondary metabolites present in the plant.

However, the administration of graded doses of *W. indica* over a period of 21 days caused dilation of bundles of myocardial fibres at both 250 and 100 mg/kg while 500 mg/kg induced congestion of the vessels when compared with the normal control as shown in Figure 1.

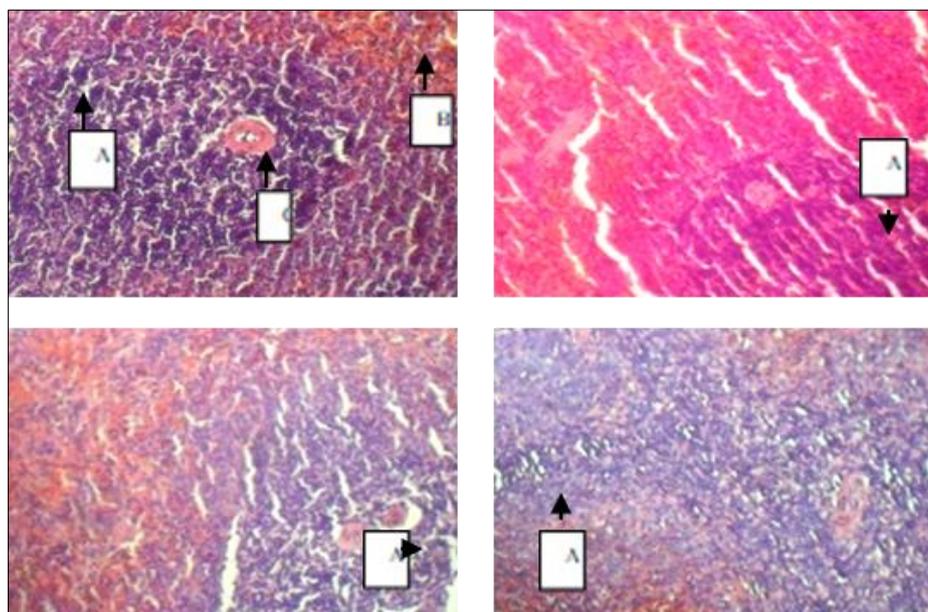


Figure 2 (A) Control Rat spleen composed of A, white pulp, B, red pulp and C, arteriole, (B) 250 mg/kg crude extract of *W. indica* showed A, mild follicular atrophy, (C) 500mg/kg crude extract of *W. indica* showed A, normal follicular architecture and (D) 1000mg/kg crude extract of *W. indica* showed A normal follicular architecture (H&E x 100) at day 21

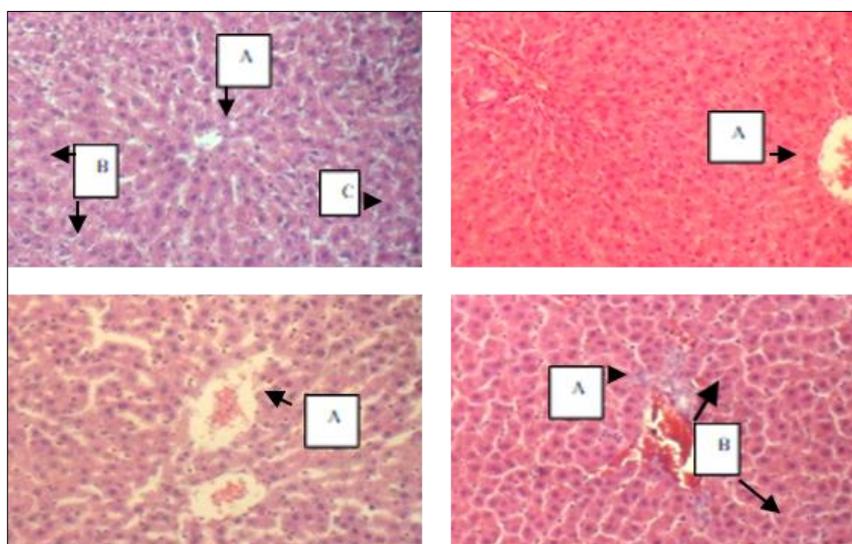


Figure 3 (A) Control Rat liver composed of A, central vein, B, hepatocytes and C, sinusoids, (B) 250 mg/kg crude extract of *W. indica* showed: A, mild vascular congestion, (C) 500 mg/kg crude extract of *W. indica* showed: A, mild vascular congestion and (D) 1000 mg/kg crude extract of *W. indica* showed A, mild vascular congestion and B, moderate Kupffer cell activation (H&E x 100) of hepatocytes.

In addition, administration of 250 mg/kg of plant extract resulted into a mild follicular atrophy induced when administered for 21 days. Moreover, normal follicular architecture was observed at 500 and 100 mg/kg when compared with normal control at day 21 as shown in Figure 2.

The administration of graded doses of crude extract of *W. indica* induced mild vascular congestion and moderate Kupffer cell activation to the liver when compared with Control as shown in Figure 3

The administration of graded doses of crude extract of *W. indica* induced mild interstitial congestion in the kidney when compared with Control (distilled water) as shown in Figure 4 below

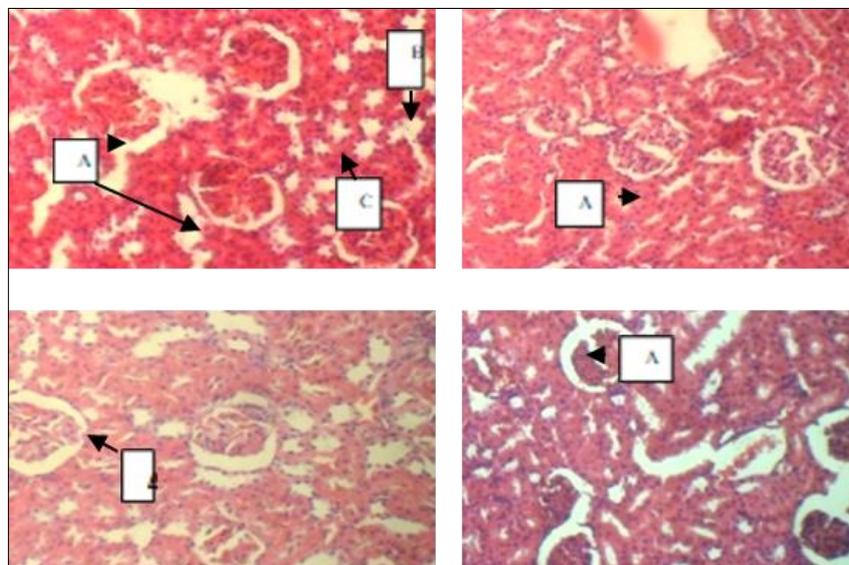


Figure 4 (A) Control Rat kidney composed of A, glomerulus, B, tubules and C, interstitial space, (B) 250 mg/kg crude extract of *W. indica* showed A, mild interstitial congestion, (C) 500 mg/kg crude extract of *W. indica* showed A, mild interstitial congestion and (D) 1000 mg/kg crude extract of *W. indica* showed A, mild interstitial congestion (H&E x 100) in kidney

4. Conclusion

The study has shown that *W. indica* leaf possess no toxic effect on blood parameters and kidney but mild toxicity was observed on some key serum enzymes as well as organs upon prolong days of administration. However, it can be concluded that prolong usage of the plant for disease management should be discouraged.

Compliance with ethical standards

Acknowledgments

The Authors are grateful to the entire staff of Department of Pharmacognosy and Pharmaceutical chemistry, College of Pharmacy, Igbinedion University, Okada, Nigeria for their assistance during this research.

Disclosure of conflict of interest

No conflict of interest.

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