

ANTIMICROBIAL AND TOXICOLOGICAL STUDIES OF *RICINODENDRON HEUDELOTII* (BAILL.)

OMOLARA FAITH YAKUBU¹, ABIODUN HUMPHREY ADEBAYO^{1*}, TOLULOPE OLUWASOMO FAMAKINWA^{1,2},
OLUWATOBI SAMUEL ADEGBITE³, TEMITOPE ARIKE ISHOLA¹, LORETTA ONOSHOWNEN IMONIKHE¹,
OLADIPUPO ALABA ADEYEMI¹, OLUFISAYO ADEBOLA AWOTOYE¹ AND EMEKA EZE JOSHUA IWEALA¹

¹Department of Biochemistry, College of Science and Technology, Covenant University, PMB 1023, Canaan land, Ota, Ogun, Nigeria. ²Department of Biological Sciences, Baze University, Abuja, FCT, Nigeria. ³Department of Chemical Science, Osun State University, Osun State, Nigeria. Email: abiodun.adebayo@covenantuniversity.edu.ng

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ABSTRACT

Objective: This work assessed the antimicrobial activity and toxicological consequence of the ethanolic extract of *Ricinodendron heudelotii* (Baill.).

Methods: The antimicrobial potential of the leaf extract was investigated against 9 different microorganisms using the agar well diffusion method while the minimum inhibitory concentration (MIC) was ascertained. Toxicity evaluations were carried out on five groups of seven albino rats each for 28 days. Rats in Group A received normal saline, while those in Groups B, C, D, and E received 250, 500, 1000, and 2000 mg/kg body weight (b.w.) of the extract. After treatment, they were anesthetized, blood samples collected for biochemical assays; organs isolated, weighed, and processed for histopathological studies.

Results: The phytochemical screening showed the presence of glycosides, tannins, polyphenols, terpenoids, and alkaloids while saponin was absent. Antimicrobial effect was observed against 7 of the 9 microorganisms tested with inhibition zone and MIC ranging from 18 to 36 mm and 31.25 to 62.5 mg/mL, respectively. Liver function tests were significantly ($p < 0.05$) increased, whereas no significant change in total protein and creatinine levels was observed. Histology showed a sinusoidal congestion and vacuolar degeneration of hepatocytes in groups administered higher doses, and a dose-dependent reduction was observed in some hematological parameters.

Conclusion: The results obtained suggest that the *R. heudelotii* may not induce significant toxic effect below 3600 mg/kg b.w. and could serve as an effective antibiotic.

Keywords: *Ricinodendron heudelotii*, Euphorbiaceae, Antimicrobial, Toxicology, Histology.

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INTRODUCTION

Herbal plants, known to contain countless biologically active compounds [1], have been utilized for the management and cure of various ailments throughout human history. Above 50% of all contemporary drugs are from natural products, which play a significant role in drug evolution programs [2]. Nature has been the origin of healing agents over the years. Medicinal plants, for many centuries and today, have been used for curing different types of diseases in virtually all cultures [3]. *Ricinodendron heudelotii* (Baill.) is a member of the Euphorbiaceae family. It is a rapid-growing secondary forest tree native to West and Central Africa [4]. It is traditionally called "Njansang" in Cameroun and "Okwe" in South East Nigeria [5]. *R. heudelotii* has been used in folk tradition for treating cough, yellow fever, anemia, malaria, stomach pain, and intestinal disease and used as a poison neutralizer [6,7]. A blend of the bark of *R. heudelotii* has been reported to stimulate sexual desires and increase passing out of urine in some parts of Cameroon [6]. Bark extracts are also used to prevent abortion [8,9]. Its leaves are used to treat looseness of the bowels while the fruits are used as seasoning [7]. It is cultivated by farmers for topsoil fertility advancement, light wood work, shades, and pasturage [10]. The seed is often used as a thickener in soup making [5]. The seeds have been reported to contain hydrogen cyanide, tannin, alkaloid, phenol, saponin, and flavonoid [5]. The effectiveness of existing antibiotics has been challenged by the advent of drug-resistant pathogens, thus making antibiotic resistance to be a global concern [11]. Thus, in view of the fact that adequate scientific information regarding the use of the plant is lacking, this makes pre-clinical toxicological and therapeutic studies important. This study

is therefore focused on exploring the biochemical, antimicrobial, histological, and hematological effects of *R. heudelotii* in rats.

METHODS

Plant material

The leaves were collected on October 2015 in Covenant University Ota, Ogun, Nigeria, and certified by Dr. J.O. Popoola of the Department of Biological Sciences, Covenant University, Ota, Nigeria. Sample of the leaf was deposited herbarium section of the Forest Research Institute of Nigeria with voucher no FHI 110573. Leaves were dried at room temperature (25°C) and grinded using an electric blender into coarse powder. These powdered samples were sealed in plastic bowls until needed for the study.

Microorganisms

The selected bacterial strains for the current study were *Klebsiella pneumoniae*, *Pseudomonas aureginosa*, *Shigella* sp., *Escherichia coli* (Gram-negatives), *Staphylococcus aureus*, *Bacillus* sp., *Micrococcus* sp., *Streptococcus faecalis*, and *Salmonella* sp. (Gram-positive) while the fungal strain was *Candida albicans*. They were all obtained from culture collection center, Department of Biological Sciences, Covenant University, Ota. All organisms were sustained on nutrient broth (NB) at 37°C and fungus on potato dextrose agar (PDA) at a temperature of 28°C.

Experimental animals and housing

In this study, 35 male adult albino Wistar rats having weight between 170 and 200 g were purchased from Lagos University Teaching Hospital,

Lagos, Nigeria, and kept at a maintained temperature and fed with rat chow (Graceline Feeds Ota, Ogun State) along with water. The rats were permitted to adapt to the environment for 2 weeks before the inception of the experiment. This study was approved by the Biological Sciences Research Ethics Committee, Covenant University. All experimental rats were managed in adherence to the rules of the National Institute of Health for the use and care of laboratory animals [12].

Extraction and phytochemical screening of the plant

The powdered leaf samples (580 g) were extracted by maceration with 3 L of 95% ethanol (Sigma-Aldrich) and kept at room temperature for 3 days. The solution was stirred daily for thorough mixing and sieved thereafter to obtain the filtrate which was further condensed at reduced temperature of 40°C and pressure through a rotary evaporator [13]. The yield of the plant extract obtained was 13.8%.

The ethanolic extract was exposed to preliminary phytochemical tests to ascertain the specific phytoconstituents contained in the leaf such as tannins, alkaloids, glycosides, steroids, flavonoids, terpenoids, saponins, anthocyanin, and phenols. Such tests were determined by the typical color change following guidelines as described by Harborn [14] and Sofowora [15].

Antimicrobial studies of plant extract

The antimicrobial effect of ethanolic extract of *R. heudelotii* was carried out using agar diffusion method described by Benkeblia [16] with slight modifications. Bacterial cell suspensions of about 18–24 h were adjusted to a 0.5 McFarland standard. Mueller-Hinton agar plates were seeded with 100 µL of each of the bacterial cell suspensions, and wells were bored using 0.8 mm diameter cork borer. The extract was dissolved in dimethyl sulfoxide and aseptically introduced into the bored wells, allowed to spread out, and incubated at 37°C for 18–24 h for bacteria, and thereafter, the diameter of the inhibition zones were measured. The same method was employed for fungal isolate which was initially cultured on PDA and incubated at room temperature for 48 h. For standard, standard antibiotic discs of 6 mm diameter (Hi-Media) for gentamicin were used.

Minimum inhibitory concentration (MIC) was determined by preparing different concentrations of the extracts using sterile distilled water. Standardized bacterial suspension (0.1 mL) was introduced into test tubes containing nutrient broth, and different concentrations of the extract were introduced to them after which they were aerobically incubated at 37°C for 18–24 h. The highest dilution where there was no bacterial (or fungal) growth was recorded as the MIC.

Acute toxicity

Lethal dose (LD₅₀) was determined using the method as described by Lorke [17]. This involves two stages. At the initial stage, rats were grouped into 3, each containing 3 rats and received the ethanolic extract of *R. heudelotii* at an oral dose of 10, 100, and 1000 mg/kg body weight (b.w.), respectively. For the second stage, different doses of 2900, 3600, and 5000 mg/kg b.w. were administered to another set of 3 groups of three rats. The rats were monitored over 72 h and 2 weeks period for morbidity or mortality; changes in behavior were recorded.

Experimental design

A total of 35 rats were purchased and equally distributed across five experimental groups. Group I rats was administered 1ml of distilled water while Groups II-IV were given orally 250, 500, 1000, and 2000 mg/kg b.w. of the extract, respectively, for 28 days. At the end of the 28-day treatment, food was withdrawn from the animals overnight, and the following morning, they were anesthetized in diethyl ether. They were afterward dissected from the abdomen; blood samples were obtained through the cardiac puncture into heparinized tubes. Plasma was obtained by centrifuging at 3000 rpm for 15 min [18] and kept at -20°C inside Eppendorf tubes until needed for biochemical assays. The organs (liver and kidney) obtained were used for histological examination.

Biochemical assay

The commercial test kits for liver function test were purchased from Randox Laboratory, United Kingdom. Standard procedures were used to evaluate the protein concentration [19], aspartate aminotransferase (AST) [20], alkaline phosphatase (ALP) [21], alanine aminotransferase (ALT) [22], cholesterol [23], total albumin [24], total bilirubin [25], urea [26], and creatinine [27].

Hematological assays

Hematological parameters were estimated with the aid of an automated hematology system analyzer, mean cell volume, white blood cell count, hemoglobin, red blood cell count, hematocrit (HCT), platelet count (PLT), mean cell hemoglobin (MCH), mean corpuscular hemoglobin concentration, percentage lymphocyte, and percentage granulocyte [28].

Histopathological studies

The method according to Aliyu et al. [29] was adopted. The organ tissues were fixed in normal saline for 72 h and cut into thin slices 2.1 mm thick. The tissues were dehumidified using liquor. They were thereafter treated with paraffin wax and cast into blocks; tissue sections were then slit into 5µm using microtome and allowed to dry on a slide. The slides were afterward soiled with hematoxylin-eosin stain, analyzed using a light microscope, and photomicrographs recorded [29,30].

Statistical analysis

Data were analyzed through one-way analysis of variance (ANOVA) and Tukey's test using the statistical package for the social sciences (SPSS), version 21.0 (SPSS Inc., Chigaco, IL, USA). Probability of *p<0.05 was considered to be statistically significant. All data were represented as mean±standard error mean for 7 animals graphically using Graph pad prism, version 5.0.

RESULTS

Phytochemical analysis

The qualitative phytochemical analysis revealed the existence of tannins, flavonoids, alkaloids, cardiac glycosides, saponins, steroids, and terpenoids in the leaf extract (Table 1).

Acute toxicity

Administration of varying concentrations of ethanolic extract of *R. heudelotii* at doses 10, 100, 1000, and 2900 mg/kg did not cause any changes in behavior. However, there were behavioral changes in the group administered 3600 and 5000 mg/kg b.w. of the extract; decreased locomotive activity, food intake, weight loss, and weakness were observed in these groups. No mortality of rats was experienced all through the period of administration.

Effects of *R. heudelotii* extract on biochemical parameters

The ethanolic extract showed a significant increase (p<0.05) in the activity of ALT and AST (Fig. 1). Similarly, the levels of urea were significantly (p<0.05) elevated (Fig. 2) in Groups II, III, and IV. The activity of ALP significantly increased (p<0.05) in the group treated with 2000 mg/kg b.w. of leaf extract. Total protein levels were significantly decreased (Fig. 1), while creatinine levels were unaltered (Fig. 2) across all the treated groups.

Table 1: Qualitative phytochemical screening of *R. heudelotii*

Phytochemicals	Status
Phenols	Absent
Flavonoids	Present
Terpenoids	Present
Cardiac glycosides	Present
Alkaloids	Present
Tannins	Present
Steroids	Present
Saponins	Present
Anthocyanin	Absent

Histopathological studies

Compared with the control groups, no remarkable alterations were noticed in the morphology of the organ tissues of rat administered 250 mg/kg b.w. However, noticeable cellular alterations such as severe portal and central venous congestion were observed in the liver organ of rats administered 500 mg/kg b.w. of the extract including severe periportal cellular infiltration and vacuolar degeneration of hepatocytes of those given 1000 and 2000 mg/kg b.w. (Fig. 3). The kidney of rats treated with 1000 mg/kg b.w. of extract showed a mild tubular degeneration (long arrow Fig. 4) with interstitial hemorrhage (short arrows Fig. 4) at the renal cortex, while the 2000 mg/kg b.w. group showed mild-to-moderate interstitial hemorrhage (Fig. 4).

DISCUSSION

Toxicological evaluations are essential in determining the safety limit of plant extracts and herbs in animals. These are usually conducted to determine the safety of the plant extract in humans. In this study, no death occurred all through the treatment period. There were neither occurrences of diarrhea nor changes in locomotor activity observed. From the preliminary qualitative phytochemical test carried out on the ethanolic leaf extract, tannins, steroids, terpenoids, flavonoids, saponin, cardiac glycosides, and alkaloids were discovered present (Table 1). These secondary metabolites contribute significantly to biological activities of medicinal plants as some of them have shown excellent antimicrobial, antioxidant, and antimalarial properties. Tannins have

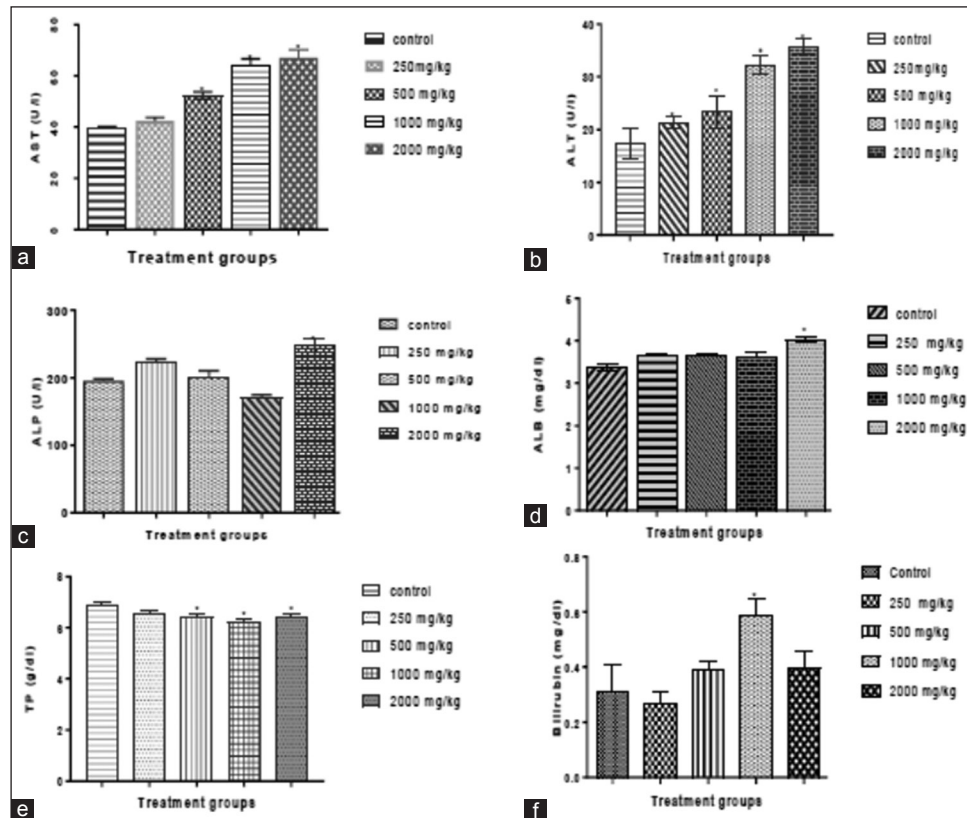


Fig. 1: Effect of *Ricinodendron heudelotii* extract on liver function parameters in albino Wistar rats. (a) AST: Aspartate aminotransferase; (b) ALT: Alanine amino transferase; (c) ALP: Alkaline phosphatase; (d) ALB: Albumin; (e) TP: Total protein; (f) BIL: Bilirubin. Values are presented as mean±standard error of mean of 7 replicates; *significant at $p < 0.05$ as compared with control

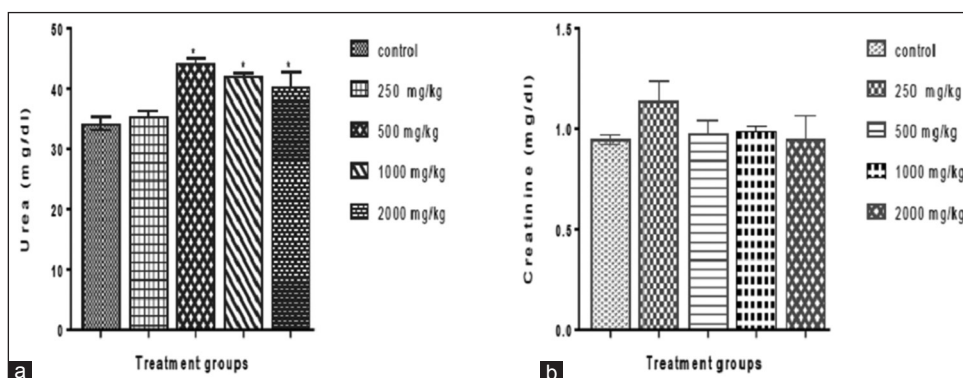


Fig. 2: Effect of *Ricinodendron heudelotii* extract on. (a) Urea and (b) creatinine levels. Values are presented as mean±standard error of mean of 7 replicates; * $p < 0.05$ as compared with control

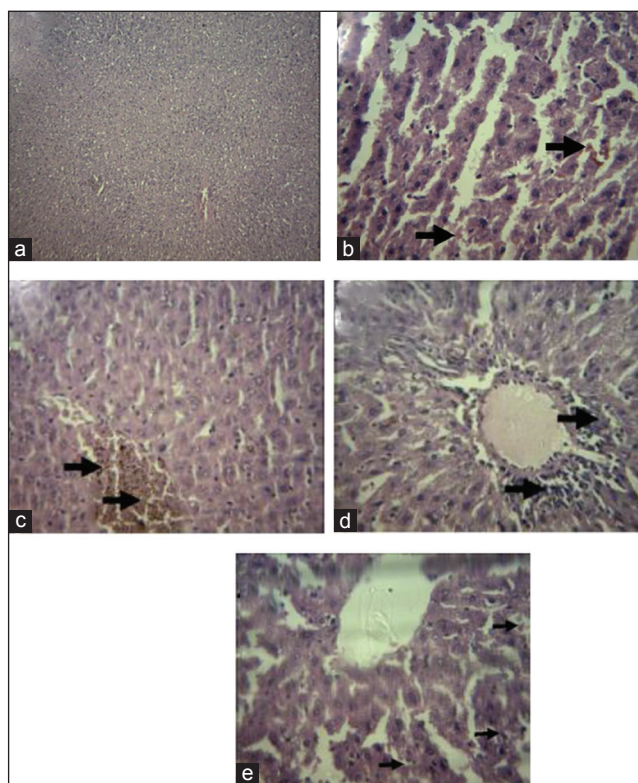


Fig. 3: Photomicrograph of liver tissues (H and E, ×400) of rats (a) with normal features in the control group, (b) treated with 250 mg/kg, (c) treated with 500 mg/kg, (d) treated with 1000 mg/kg, (e) treated with 2000 mg/kg body weight of the extract of *Ricinodendron heudelotii*

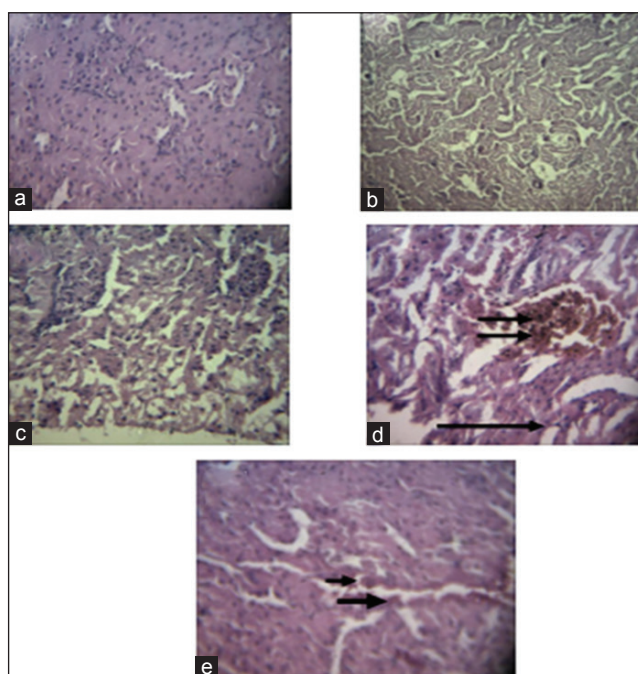


Fig. 4: Photomicrograph of kidney tissues (H and E, ×400) of rats, (a) with normal features in the control group, (b) treated with 250 mg/kg, (c) treated with 500 mg/kg, (d) treated with 1000 mg/kg, (e) treated with 2000 mg/kg body weight of the extract of *Ricinodendron heudelotii*

amazing stringent properties. They are known to hasten the healing of wounds [31], treat bacterial infections, and dysentery [32]. Flavonoids have also been reported to be efficient reactive oxygen species scavengers as their anticancer property ascertained on their chelating and antioxidant attributes [33].

The antimicrobial potential of the leaf extract may be hard to correlate to a specific compound as a result of their complexity [34]; however, tannins have been reported by Hatano *et al.* [35] to strongly inhibit microorganisms, especially effect on *S. aureus*. This is in line with this present study where the leaf extract showed a zone of inhibition of

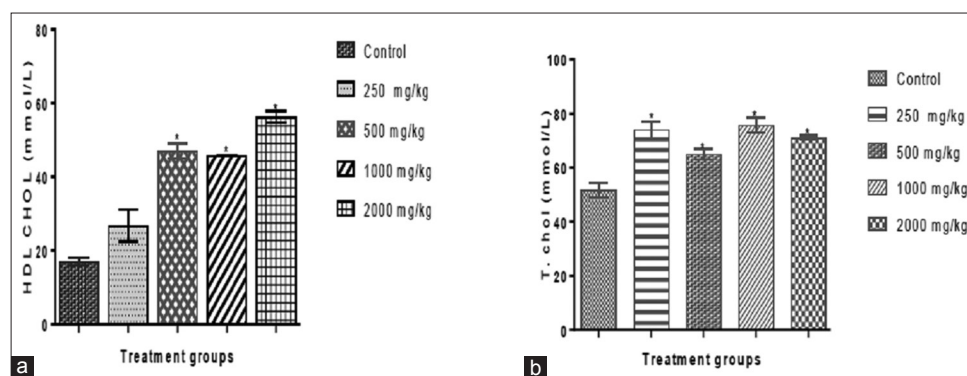


Fig. 5: Effect of *Ricinodendron heudelotii* extract on. (a) High density lipoprotein and (b) total cholesterol. Values are represented as mean±standard error of mean of 7 replicates; *p<0.05 as compared with control

36mm against the organism (Table 4). The class of tannin that possesses antibiotic property is the ellagitannins which was also reported by Hatano *et al.* [35]. Our findings revealed that the ethanolic leaf extract of *R. heudelotii* showed activity against most of the microorganism tested (Table 2). Three of the organisms, *Klebsiella pneumoniae*, *Candida albicans*, and *Salmonella sp* were, however, resistant to the extract. This is in accordance with Oyono *et al.* [36] where they observed resistance of these strains to the bark extract of *R. heudelotii*. The diameter of zone of inhibition varied from 15 to 30 mm and 18 to 36 mm for gentamycin and the extracts, respectively (Table 2). The disparity observed can be linked to either the variation of active ingredients available in it or the mode of action on Gram-negative and Gram-positive bacteria [36]. The MIC for *E. coli* and *Streptococcus faecalis* was 31.25 mg while that of *Bacillus* species, *S. aureus*, *Shigella* species, and *Micrococcus* species was 62.5 mg (Table 3), this implies that small doses can be effective against microorganisms.

Mean LD₅₀ value is often used as the basis for assessing acute toxicity [19]. Our study has shown that ethanolic leaf extract *R. heudelotii* could produce signs of toxicity at very high concentrations (3600 and 5000 mg/kg b.w.) but not death. This shows that the extract is relatively safe or may be slightly toxic just like any xenobiotics since it falls within the range of 1000-5000 mg/kg b.w. [37]. No significant difference (p<0.05) in the weight of the animals was observed. However, the organ weights in the 2000 mg/kg b.w. group showed a significant difference (Table 4), this may be as a result of the enhanced working ability of the organs [38].

Hematological parameters are useful markers used to ascertain the adverse effect of plant extracts or even drugs on blood constituents [39]. In this study, treatment with the plant extract led to a significant decrease (p<0.05) in PLTs in rats in the 2000 mg/kg b.w. group (Table 5). According to McLellan *et al.* [40], reduction in PLT in experimental rats indicates detrimental action on the blood's oxygen transporting ability as well as thrombopoietin. The observed reduction in the PLTs in this study indicates that RH extract may cause disorder in the blood oxygen transporting ability. Reductions in red blood cell (RBC) and HCT were also observed (Table 5). Reduction in RBC was statistically significant in the group treated with 2000 mg/kg b.w. of *R. heudelotii* extract while the reduction in HCT was dose dependent but not significant. This could be as a result of osmoregulatory system disturbance of the blood cells or impairment of the cell membrane. The observed reduction in hematological indices could indicate erythrocyte destruction [41]. Therefore, the reduction observed in RBC count and HCT may be linked to delayed hemopoiesis, shrinkage, and destruction of RBC. Likewise, the oxygen-transporting ability of the blood and the oxygen supplied to the tissues may be disrupted following administration of the extract.

Biochemical analysis is useful for predicting the toxicological effect of the leaf extract in animals and the safety of plant products for human

Table 2: MIC values of extract of *R. heudelotii* leaf against test organisms

Plant/microorganism	<i>R. heudelotii</i> MIC (µg/mL)
<i>K. pneumoniae</i>	-
<i>Shigella sp</i>	62.5
<i>E. coli</i>	31.25
<i>S. aureus</i>	62.5
<i>Bacillus sp.</i>	62.5
<i>Micrococcus sp.</i>	62.5
<i>S. faecalis</i>	31.25
<i>Salmonella sp.</i>	-
<i>C. albican</i>	-

K. pneumoniae: *Klebsiella pneumoniae*, *Shigella sp.*: *Shigella* species, *E. coli*: *Escherichia coli*, *S. aureus*: *Staphylococcus aureus*, *Bacillus sp.*: *Bacillus* species, *Micrococcus sp.*: *Micrococcus* species, *S. faecalis*: *Streptococcus faecalis*, *Salmonella sp.*: *Salmonella* species, *C. albicans*: *Candida albicans*, *R. heudelotii*: *Ricinodendron heudelotii*, MIC: Minimum inhibitory concentration

Table 3: Effects of *R. heudelotii* extract on organ weight after 28 days treatment

Groups	Liver (g)	Kidney (g)
Control	5.25±0.26	0.97±0.02
250 mg/kg	1.03±0.04	5.63±0.19
1000 mg/kg	5.97±0.39	1.15±0.06
500 mg/kg	5.74±0.22	1.10±0.05
2000 mg/kg	6.8±0.30*	1.20±0.37*

Values represent mean±SEM of seven replicates. *p<0.05 compared to control. *R. heudelotii*: *Ricinodendron heudelotii*, SEM: Standard error mean

Table 4: Zone of inhibition of *R. heudelotii* against bacteria and fungus isolate

Organism	Zone of inhibition (mm)	
	<i>R. heudelotii</i>	Gentamycin
<i>K. pneumo</i>	-	27
<i>Shigella sp</i>	28	25
<i>E. coli</i>	24	15
<i>S.aureus</i>	36	20
<i>Bacillus sp</i>	25	20
<i>Micrococcus sp</i>	23	
<i>S. faecalis</i>	18	25
<i>Salmonella sp</i>	-	26
<i>C. albican</i>	-	15

use [42]. ALP, AST, and ALT are distinct markers of hepatic injury [43]. ALT is an enzyme found in highest amount in the liver [31]. Thus, an increase of the enzyme in the blood (Fig. 1) indicates hepatic injury. This is in alignment with work done by Oyono *et al.* [34] when acute

Table 5: Effects of *R. heudelotii* extract on hematological markers after 28-day treatment

Parameters	Group A	Group B	Group C	Group D	Group E
WBC ($\times 10^{12}/L$)	7.13 \pm 0.26	8.75 \pm 0.08	9.60 \pm 0.90	9.20 \pm 0.73	8.23 \pm 1.07
RBC ($\times 10^{12}/L$)	9.22 \pm 0.34	8.75 \pm 0.08	9.41 \pm 0.10	8.94 \pm 0.17	3.03 \pm 0.30*
HGB (g/L)	17.55 \pm 0.80	17.08 \pm 0.28	17.63 \pm 0.21	16.88 \pm 0.43	16.00 \pm 0.57
HCT (L/L)	54.20 \pm 1.78	53.10 \pm 0.97	54.88 \pm 0.25	52.06 \pm 1.06	50.73 \pm 0.92
PLT (L)	715.50 \pm 19.10	590.00 \pm 14.00*	655.00 \pm 18.61	734.67 \pm 18.22	509.33 \pm 29.79*
MCV (fL)	58.83 \pm 0.42	60.73 \pm 0.96	58.60 \pm 0.63	58.48 \pm 0.47	59.50 \pm 0.44
MCH (pg)	18.98 \pm 0.23	19.48 \pm 0.34	18.62 \pm 0.22	19.04 \pm 0.15	19.34 \pm 0.12
MCHC (g/L)	32.28 \pm 0.58	32.13 \pm 0.26	31.88 \pm 0.32	32.60 \pm 0.17	32.56 \pm 0.09
LYM (%)	42.85 \pm 2.75	34.63 \pm 0.88	31.03 \pm 1.34*	34.83 \pm 1.90	33.23 \pm 1.04*
GRAN (%)	35.90 \pm 3.36	41.73 \pm 0.79	60.77 \pm 0.71*	36.83 \pm 1.77	43.61 \pm 2.36

Values represent mean \pm SEM of seven replicates. *p<0.05 compared with control. WBC: White blood cell count, HGB: Hemoglobin, RBC: Red blood cell count, HCT: Hematocrit, PLT: Platelet count, MCH: Mean cell hemoglobin, MCV: Mean cell volume, LYM%: Percentage lymphocyte, MCHC: Mean corpuscular hemoglobin concentration, GRAN%: Percentage granulocyte, *R. heudelotii*: *Ricinodendron heudelotii*

toxicity was conducted on methanolic bark extract of *R. heudelotii*. AST which is also a significant biomarker for liver disease [44] which showed a significant increase (Fig. 1). Since the liver is the major organ of detoxification and mostly exposed to different levels of ingested xenobiotics which can adversely affect hepatic cells [45]. This result suggests that the extract is likely to exert damage to liver cells at higher concentrations. The liver also helps to synthesize proteins that maintain the electrolyte balance in plasma. In line with that, *R. heudelotii* leaf extract reduced the total protein content in the groups administered 500, 100, and 2000 mg/kg b.w. of the extract. Albumin is also an important marker for liver diseases [46]. Our study showed a significant elevation of albumin in the 2000 mg/kg b.w. group.

ALP, a biomarker of liver disease and obstructive jaundice, was found to be significantly increased (p<0.05) (Fig. 1) in the 2000 mg/kg b.w. group; this suggests that the observed liver injury might be linked to biliary obstruction of the liver. This is also in line with research done by Oyono *et al.* [36]. This is further substantiated by a significant and steady rise in the total bilirubin (Fig. 1) concentration in the plasma caused by an obstruction in the bile duct causing an accumulation of bilirubin in the liver. Plasma urea concentration significantly increased (p<0.05) at high extract doses, and this may be due to nephrotoxicity. An elevated plasma urea concentration has been associated with diseases related to nephrotoxicity since the kidney is known to dispose of waste products of metabolism [47]. During renal breakdown, there is a rise in nitrogenous substances such as urea and uric acid. However, plasma creatinine concentration was found to be insignificant.

Toxicological studies also show an increase in cholesterol concentration. High blood cholesterol concentration is one of the important possibilities during cardiovascular disease [13]. Thus, the rise in plasma total cholesterol concentration (Fig. 5) effected by the extract is harmful and may increase the risk of cardiovascular disease. This is, however, combated by a very rapid increase in the HDL-cholesterol concentration (Fig. 5) to mop up cholesterol from the blood vessels. This also shows that, at the right dose, *R. heudelotii* can be used in the treatment of coronary heart disease and cardiovascular diseases [48]. The toxicity exhibited by the ethanolic extract of *R. heudelotii* was reaffirmed through the histological sectioning which revealed sinusoidal congestion and severe vacuolar degeneration of hepatocytes in all treated groups. Vacuolar degeneration detected in the present study may be due to mitochondrial intermembrane space expansion and extension of the outer mitochondrial membrane [49].

CONCLUSION

The ethanol leaf extract of *R. heudelotii* may not induce significant toxic effects when administered in rats below 3600 mg/kg b.w. and thus may be safe for use as a potential candidate for the enhancement of new antimicrobial formulations as it demonstrated high antimicrobial activities. However, one should apply caution on the dosage to be administered as higher concentration could induce liver cell injury.

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