African Journal of Pharmaceutical Research and Development
Available online at https://ajopred.com
Vol. 16 No.3; pp. 25-31 (2024)

Original Research Article

TOXICITY AND ANTIOXIDANT ASSESSMENT OF ETHANOL LEAF EXTRACT OF *HIBISCUS SURATTENSIS* IN WISTAR RATS: HEPATOTOXICITY EVALUATION

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ABSTRACT

Hibiscus surattensis is a medicinal plant with a vast beneficial use in the management and cure of a variety of diseases, ranging from urethritis to inflammation and vertigo among others. Notwithstanding the population's increasing use of it, particularly in rural regions, there is still lack of adequate investigation into its toxicity profile. The aim of this study was to investigate the ethanolic extract of Hibiscus surattensis leaf's potential for hepatotoxicity in Wistar rats. The acute toxicity test was carried out in two phases (phases 1 and 2). Phase 1, used nine mice which were randomized into three groups (n=3), receiving graded doses of the extract as follows: 10, 100, and 1000 mg/kg, ip respectively. Phase 2 used three mice which were randomized into three groups of one mouse each and administered the extract in graded doses of 2000, 3500, and 5000 mg/kg, ip, respectively. They were monitored for behavioral abnormalities, toxicity signs, and mortality for 24 hours following treatment. A subacute toxicity experiment was then carried out, in which Wistar rats were given oral doses of H. surattensis extract (141, 282, and 424 mg/kg) every day for eight (8) days. Behavioural changes, haematological and liver function parameters, in-vivo oxidative stress markers and histological changes were then evaluated. The LD50 of H. surattensis leaf extract was estimated to be 1414 mg/kg. Results revealed significant rise ($p<0.01$-0.001) in the levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), conjugated bilirubin (C.B) and total bilirubin (T.B), as well as in white blood cell (WBC) and platelet at high doses of the extract-treated rats compared to control. A significant reduction was recorded in catalase among other antioxidant parameters while the histopathological evaluations showed mild alterations. While caution is urged during long-term administration, administration of H. surattensis ethanolic extract may be safe at the dosages studied in this investigation. .

ARTICLE INFO

Received 30 April, 2024
Accepted 05 December, 2024
Published 20 December, 2024

KEYWORDS

Hibiscus surattensis, Hepatotoxicity, Haematological parameters, Antioxidants

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https://doi.org/10.59493/ajopred/2024.3.4 *ISSN: 0794-800X (print); 1596-2431 (online)*

INTRODUCTION

The use of phytonutrients, also known as nutraceuticals and herbal medicines is still growing quickly worldwide, with many individuals turning to these products in different national healthcare facilities to treat a range of medical conditions [1]. Contrary to common belief, Alastair and Wood reported that the use of herbal medicines can pose significant health hazards. This is understandably so, on the ground that herbal medications are frequently large and bulky, with unquantified doses and most importantly toxicity is not well understood [2]. Although many scientific research publications have documented the activities of several African plants, on the contrary, the toxicity of these plant compounds has not been investigated by many [3]. Nonetheless, Alastair and Wood suggested that assessment of efficacy and safety are crucial factors to take into consideration in the studies on herbal remedies' quality assurance [2].

Hibiscus surattensis (*H. surattensis*) is a medicinal plant that has been exploited over the years and belongs to Malvaceae family. It is an annual plant that can grow up to 6 meters in length on prostrate or ascending stems. The entire plant, even the weak stems and leaf stalks, is coated in tiny, soft prickles and hairs that point downward. *H. surattensis* is commonly used as a potherb in many parts of Africa and Asia. Various medicinal properties of this plant have been documented, such as treatment of cuts, chickenpox, vertigo and penile irritation. Other medicinal properties of *H. surattensis* are venereal sores, urethritis, gonorrhoea, inflammations and boils [4]. In West Africa, leaves of *H. surattensis* have been used for treatment of malaria while crushed leaves are used for wound healing, abscess and gonorrhoea. From the previous report, the whole plant was used for stomachache whereas the flowers were consumed for treatment of hypertension in Nigeria. Significant analgesic and anti-diarrheal properties are reported in *H. surattensis* crude leaf extracts. [5]. Considering its many medicinal values and wide usage, it is imperative that the toxicity assessment of *H. surattensis* be carried out to ascertain its safety profile for human consumption.

MATERIALS AND METHODS

Identification and Collection Of Plant Materials

Fresh leaves of *Hibiscus surattensis* were obtained from Anwa forest in Abak, Abak Local Government Area of Akwa Ibom State, Nigeria. Prof. Margaret Bassey, a taxonomist in the Botany and Ecological Studies Department of the University of Uyo in Uyo, Nigeria, identified and authenticated the plant. A voucher specimen (UUPH45F) was deposited in the University of Uyo's Faculty of Pharmacy Herbarium.

Preparation of Extract.

After being thoroughly washed with distilled water to remove any debris, fresh Hibiscus surattensis leaves were allowed to air dry for ten days at a room temperature of 30 ± 2°C in the laboratory. The dried leaves were ground to a homogeneous powder (pulverized using a Kenwood blender). A 1kg quantity of the powder was macerated in 95% ethanol for 72h. Filtration of the extract was done with Whatman No. 1 filter paper and a Buchner funnel. The liquid filtrate measured was however evaporated till dryness in a water bath set at 40°C. The crude ethanol extract (100 g) was preserved in the refrigerator at -4°C before use.

Chemicals

Absolute ethanol was purchased from Sigma-Aldrich, (via Bristol Scientific company in Lagos). The rest of the biochemical reagents and other chemicals were of commercial analytical grade.

Experimental Animals

Twelve mice with an average weight of 20 g and twenty male albino rats (130-150g) with no signs of ill health were procured from the Animal House of the Faculty of Pharmacy, Madonna University, Nigeria where the research was carried out and then acclimatized for 7 days. The animals were kept in ventilated cages with a regular 12-hour light/dark cycle, and the temperature was maintained at 24±2°C. Standard laboratory feed and water were provided to the animals *ad libitum.* The experimental procedure was carried out in compliance with the Ethics Committee's recommendations regarding the use and care of experimental animals. Madonna University Animal Ethics Committee's Approval was obtained with approval No: MAU/DRC/HD/E/2021/PHARM/021.

Experimental Design

Acute toxicity Test.

The extract's median lethal dose (LD_{50}) was evaluated using Lorke's method [6]. The experimental design had two phases (phases 1 and 2). In phase 1, a total of nine (9) mice were randomized into three (3) groups, each consisting of three mice. The extract was administered to the mice intraperitoneally in graded doses of 10, 100 and 1000 mg/kg respectively. The mice were monitored for 24 hours for behavioural changes and mortality. In phase 2, a total of three (3) mice were randomized into three (3) groups, each consisting of one mouse. The extract was equally administered to the mice intraperitoneally in graded doses 2000, 3500, and 5000 mg/kg, respectively. After treatment, mice were monitored for 24 h for signs of toxicity, behavioural abnormalities (such as restlessness, dullness, and agitation), and mortality.

Sub-acute Toxicity Test

A total of twenty (20) male albino rats were randomly assigned to four (4) groups, each group consisting of five (5) rats. The experimental treatment was as follows:

Group 1 (Control group): Rats received 10 mL/kg body weight of distilled water orally for eight (8) days,

Group 2 (Low dose group): Rats received 141.4mg/kg body weight of the ethanol leaf extract orally for eight (8) days.

Group 3 (Middle dose group): Rats received 282.8mg/kg body weight of the leaf extract orally for eight (8) days.

Group 4 (High dose test group): Rats received 424.2mg/kg body weight of the leaf extract orally for eight (8) days.

Twenty-four hours after administration, all the animals were further weighed before being sacrificed under light chloroform vapour.
The low, middle and high doses were calculated from 10 %, 20 % and 30 % of the estimated LD_{50} respectively.

Collection of Blood Samples and Organs

In all the models, blood was collected both in sterile centrifuge tubes and sterile Ethylene Diamine Tetraacetic acid (EDTA) bottles through cardiac puncture and used instantly. The blood in the sterile centrifuge tubes was immediately centrifuged for fifteen minutes at 2500 rpm for the serum to separate at room temperature and prevent hemolysis. The separated serum was then used for the assay of liver biochemical parameters. The blood collected in the EDTA bottles was used for the analysis of haematological parameters.
The animals' livers were surgically removed and weighed, and a portion was preserved in 10% formaldehyde for histological examinations.

Haematological Analysis.

21-gauge (21G) needles which were mounted on 5mL syringes were used to collect blood samples via cardiac puncture from each light chloroform-anesthetized/sacrificed rat into ethylene diamine tetra-acetic acid (EDTA) coated sample bottle. Using an automated haematology analyzer from Lively Stones Laboratory, Port Harcourt, the blood samples were examined for all haematological indices, including the red blood cells (RBCs), packed cell volume (PCV), haemoglobin (HB), white blood cell (WBC) and its differentials, such as – neutrophils, eosinophils, basophils, lymphocytes and monocytes.

Assay of Liver Function Parameters

The serum was used to estimate biochemical parameters such as Albumin, alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), total protein, total and direct bilirubin and total cholesterol. Using conventional protocols, serum alkaline phosphatase activity was measured at 405 nm [7], while serum aspartate aminotransferase and alanine aminotransferase were both estimated at 340 nm [8]. Fortress Diagnostic Kits® (Fortress Diagnostic, UK) and automated analysers were used for the estimation of all other biochemical parameters, following manufacturer's guidelines.

Assay of Oxidative Stress Markers

Rat's liver homogenates used in this investigation were used to conduct an antioxidant enzyme test. The animals' livers were surgically removed, weighed, and washed in ice-cold 0.9% NaCl before being homogenized with a motor-driven Teflon pestle in a ratio of 1 g of moist tissue to 9 ml of 1.25% KCl. Superoxide dismutase (SOD) activity assay were performed on the supernatants after the homogenates were centrifuged for 10 minutes at 4 °C at 7000 rpm following the procedure described by Marklund and Marklund (1974) [9], catalase (CAT), as well as glutathione peroxidase (GPX) activities, were measured using Fortress Diagnostic Kits®, while malondialdehyde (MDA) content was estimated by Colorimetric TBARS Microplate Assay Kit and reduced glutathione (GSH) activity as described by Ellman (1959) [10].

Histopathological Assessment.

After the collection of blood samples, the livers were immediately excised, wiped with clean tissue paper and weighed. It was later fixed in buffered formalin (10%) for histological procedures. For histopathological examination, the fixed liver was subjected to histopathological procedures [11]. They were then sectioned, stained with haematoxylin and eosin (H&E), mounted with coverslips and viewed using a light microscope set at ×100 magnification.

Statistical Analysis

The obtained data was analyzed statistically with the help of SPSS version 20.0 and results were expressed as arithmetic means ± standard error of the mean (SEM). The results obtained from each drug concentration were compared with measurements from the control experiment using One-way analysis of variance (ANOVA), this was followed by a Bonferroni multiple-comparison test to identify the source of any statistically significant difference and a P value of < 0.05 taken as indicating a statistically significant difference.

RESULTS

Acute Toxicity Effects of Ethanol Leaf Extract of *Hibiscus surattensis*

Following the intraperitoneal administration of ethanol leaf extract of *H. surattensis* to the mice, there was no recorded mortality in phase 1, while total (100%) mortality was observed in all the doses (2000, 3500, and 5000 mg/kg) of phase 2 (Table 1). Using Lorke's method, 1414 mg/kg was determined to be the LD_{50}.
Twenty-four hours post-administration, observations were made to determine groups with the highest dose with no mortality (Do) and the lowest dose with mortality (D100). Thereafter, LD_{50} was calculated in Equation 1.

√(Do x D100) = √(1000 x 2000) mg/kg = 1414mg/kg……..Equation 1

Effects of *Hibiscus surattensis* Extract on Liver Function Parameters

The effects of *H.Surattensis* extract on liver function parameters are shown in Table 2. The result showed an insignificant change in the levels of ALT, AST, TB and CB, in both the low (141 mg/kg) and middle (282 mg/kg) dose-treated groups, while a significant reduction was observed (in both low and middle doses) in the levels of ALP ($p<0.01$), T.P ($p<0.05$) and ALB ($p<0.05$). Conversely, in the high dose (424 mg/kg) treated group, a high significant rise ($p<0.01$-0.001) in the levels of AST, ALP, ALT, TB and CB was noticed when compared with the normal control group. However, the changes in the individual parameters occurred dose-dependently.

Effects *of Hibiscus surattensis* Extracts on Haematological Parameters

The effects of *H.Surattensis* extract on the haematological parameters of rats are presented in Table 3. Administration of low doses (141 mg/kg) of *H.Surattensis* extract to the animals did not cause any significant change in the levels of the haematological parameters when compared with the normal control group. Moreso, treatment of the animals with middle (282 mg/kg) and high (424 mg/kg) doses of the extract resulted in a dose-dependent significant increase in the levels of the WBC ($p<0.001$) and platelets ($p<0.05$-0.01), whereas a dose-dependent significant reduction was observed in the levels of RBC, HB and PCV, at $p<0.01$-0.001, when compared with the normal control group.

Effects *of Hibiscus surattensis* Extracts on Antioxidant Parameters

The effects of *H.Surattensis* extract on the antioxidant parameters of experimental animals are presented in Table 4. Administration of *H.Surattensis* extract to the animals was observed to cause a slight, non-significant and dose-dependent reduction in the enzymatic antioxidant levels (such as GSH and SOD) in the three treatment doses (141-424mg/kg), except in CAT where a dose-dependent, significant ($p<0.05$-0.001) decrease was observed across the three different treatment doses (141-424mg/kg) of the extract, in contrast to the normal control. However, treatment of the animals with the *H.Surattensis* extract did not depict any significant change in the MDA levels, in comparison with the normal control.

Histopathological Studies of Liver Sections

The histopathological photomicrograph of liver sections in both normal and extract-treated groups of rats are shown in Figures 1-4. The result of the histopathological analysis of liver sections taken from the normal control group depicted clear integrity of the hepatocyte membrane. Rats from the normal control group that were not treated with extract showed normal hepatocytes (Hp) with portal vein (Pv), hepatic artery (Ha), well-oriented sinusoids (sn) and the Bile duct (Bd) within the portal area. Infiltration of inflammatory cells as well as necrosis was not observed in this group (Figure 1). Histopathological results confirmed the induction of hepatocyte degeneration by *Hibiscus surattensis*. Liver sections imaging from group 2, low dose group (Figure 2) showed mildly affected liver structures evident by mild disorientation of sinusoids with the presence of red blood cells (RBC) and wide areas of hepatic hyperplasia (hp) when compared to the normal control group. Group 3 liver sections showed mild alteration of the histo-architecture particularly the disorientations of sinusoids (Sn) with the presence of red blood cells (RBC) and the bile duct (Bd) within the portal area (Figure 3). Group 4 depicted moderately altered histoarchitecture, the portal area and hepatic lobules with numerous hepatocytes, portal vein (Pv), presence of proliferating kupffer cells (k), areas with degenerating hepatocytes (D), bile duct (Bd) with hyperplastic ductal epithelium (hp) and displaced ductular epithelial cells (Dc) within the portal connective tissue. (Figure 4).

DISCUSSION

The leaf extract of *H. surattensis* had an LD_{50} of 1414 mg/kg following an acute intraperitoneal toxicity study whereas in an acute oral toxicity investigation conducted by Anoopa *et al* [5] the LD_{50} of H. surattensis leaf extract was found to be 2000 mg/kg. The possible reason for the decreased intraperitoneal LD_{50} value compared to the oral LD_{50} could be due to the different administration routes with intraperitoneally administered extract having increased bioavailability and because of variation in the phytochemical composition of the plant due to environmental factors. Serum enzyme assessments offer excellent insights into the nature and extent of pathological damage to any given tissue, making them an important tool in clinical diagnosis. Therefore, it is important to interpret liver tests within the clinical context. The significant elevation ($p<0.01$-0.001) in the levels of ALT, ALP, AST, TB and C.B in the high dose (424mg/kg) of the extract following liver function test suggests that the *H. surattensis* extract has genuine safety profile at low and the middle doses while the high dose may be hepatotoxic. The observed increase in these enzyme levels across all extract-treated groups may indicate that the bioactive components of the extract are responsible for inducing the enzyme activity [12]. A previous study by Anoopa *et al* [5] reported that *H. surattensis* has been extensively used in conventional medicine as a hepatoprotective agent, where the plant was evaluated for its hepatoprotective activity in the methanolic extract of aerial parts up to a maximum dose of 400mg/kg of the extract. The current result corroborates the above previous study by Anoopa *et al* [5] and lays credence to its safety profile.

Evaluation of the haematological parameters is necessary as parameters such as red blood cells (RBCs) and haemoglobin

Table 1: Acute Toxicity Effects of Ethanol Leaf Extract of *Hibiscus surattensis*

Group	Dose (mg/kg)	N	D/T	Signs of toxicity observed
Phase 1				
A	10	3	0/3	No signs of toxicity were noticed.
B	100	3	0/3	No signs of toxicity were noticed.
C	1000	3	0/3	No signs of toxicity were noticed.
Phase 2				
D	2000	1	1/1	Slight sluggishness was noticed in 2 mice during the first 2 hours.
E	3500	1	1/1	Slight sluggishness was noticed in 2 mice during the first hour.
F	5000	1	1/1	Severe sluggishness was noticed in the 3 mice in the first hour.

Key: *D/T: number of mice that died/total number of mice. **N= number of mice per group

Table 2: Effects *of Hibiscus surattensis* Extracts on Liver Function Parameters

Treatment	AST (U/L)	ALT (U/L)	ALP (U/L)	T.P (g/dL)	ALB (g/dL)	T.B (µmol/L)	C.B (µmol/L)
ControlGroup 10 mL/kg D_W	47.60±7.40	29.80±2.06	40.8±2.78	73.6±1.69	44.6±1.44	9.62±1.38	6.26±1.35
Low dose *H.S* extract (141mg/Kg)	55.40±3.97	28.80±1.43	27.2±1.71**	64.0±1.41*	36.6±1.21*	11.10±0.83	7.22±0.73
Middle dose *H.S* extract (282mg/Kg)	61.60±10.49	35.60±2.38	27.4±2.29**	64.4±3.83*	36.4±2.32*	11.72±1.59	7.64±1.38
High dose *H.S* extract (424mg/Kg)	103.20±5.66***	63.60±1.36***	56.4±1.36***	65.4±1.44	38.4±1.21	19.60±0.99***	13.54±0.69**

Key: Data are expressed as mean ± SEM. Significant at * p<0.05, **p<0.01, ***p<0.001when compared to normal control. D_W=Distilled water, *H.S*= *H. surattensis*

Table 3: Effects *of Hibiscus surattensis* Extracts on Haematological Parameters

Treatment	WBC (x 10^9/L)	LYM (x10^9/L)	NEUT (x10^9/L)	MON (x10^9/L)	EOS (x10^9/L)	RBC (x10^{12}/L)	PLT (x 10^9/L)	HB (g/dL)	PCV (L/L)
Control Group 10 mL/kg D_W	4.74±0.23	52.0±2.81	36.0±3.73	8.6±0.60	3.4±0.51	6.42±0.16	197.8±8.51	14.62±0.36	44.6±0.68
Low dose H.S extract (141mg/Kg)	5.52±0.27	56.0±2.21	34.6±2.14	6.6±1.21	2.8±0.58	6.04±0.23	197.4±10.60	13.82±0.50	41.6±1.50
Middle dose H.S extract (282mg/Kg)	10.10±1.01***	61.6±4.06	28.0±4.57	7.0±0.84	3.4±0.24	4.24±0.16**	246.0±11.15*	9.54±0.51***	28.6±1.54***
High dose H.S extract (424mg/Kg)	13.42±0.72***	58.4±3.54	30.0± 3.86	7.2±0.37	4.4±0.51	4.08±0.23**	266.2±7.43**	9.20±0.76***	28.5±2.44***

Key: Data are expressed as mean ± SEM. Significant at * p<0.05, **p<0.01, ***p<0.001when compared to normal control. D_W=Distilled water, *H.S*= *H. surattensis*

Table 4: Effects *of Hibiscus surattensis* Extracts on Antioxidant Parameters Levels

Treatment	GSH (µg/mg)	CAT (U/mg)	SOD (U/mg)	MDA (nmol/mg)
Control Group 10mL/kg D_W	1.73±0.06	6.26±0.49	0.23± 0.03	0.644±0.03
Low dose H.S extract (141mg/Kg)	1.93±0.05	4.42±0.57*	0.22±0.02	0.556±0.02
Middle dose H.S extract (282mg/Kg)	1.59±0.08	3.54±0.23**	0.20±0.02	0.686±0.04
High dose H.S extract (424mg/Kg)	1.56± 0.09	2.56± 0.19***	0.16±0.04	0.652± 0.05

Key: Data are expressed as mean ± SEM. Significant at * p<0.05, **p<0.01, ***p<0.001when compared to normal control. D_W=Distilled water, *H.S= H. surattensis.*

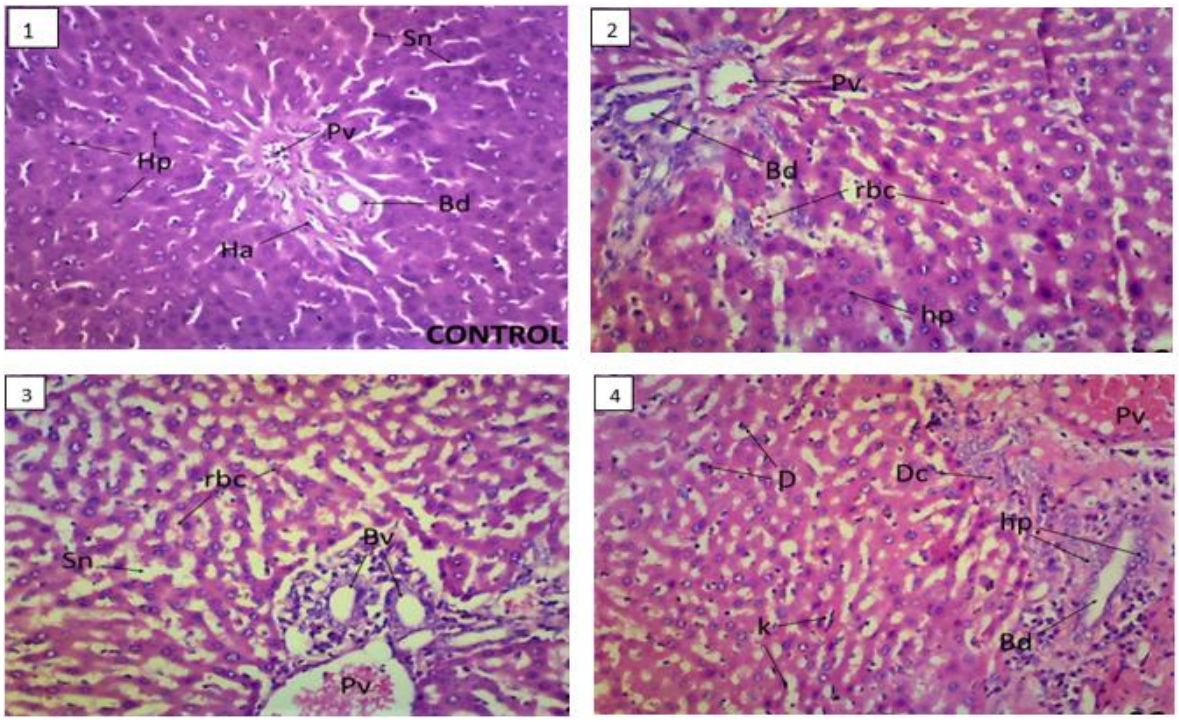

Figure 1-4: Histological sections of livers of experimental rats in normal control group, administered with normal saline 10 mL/kg (1), group 2 treated with 141mg/kg of *H. surattensis* extract (2), group 3 treated with 282 mg/kg of *H. surattensis* extract (3), group 4 treated with 424 mg/kg of *H. surattensis* extract (4) at magnification (x400), stained with H&E technique: Hepatocytes (Hp), Portal vein (Pv), Hepatic artery (Ha), Sinusoids (sn), Bile duct (Bd), red blood cells (rbc), hepatic hyperplasia (hp), bile duct (Bv), proliferating kupffer cells (k), degenerating hepatocytes (D), displaced ductular epithelial cells (Dc).

are major indices used to evaluate circulatory erythrocytes, diagnose anaemia, and also function as helpful indicators of the bone marrow's ability to produce RBCs in mammals as documented by Ozkan et al [13]. The observed significant decrease in the levels of the RBC, haemoglobin (Hb) and PCV in the middle and high dose groups is suggestive of the fact that the extract may cause anaemia when administered in these stated doses. Conversely, platelets play a role in the regular coagulation of blood when they are present in sufficient quantity, size, and function. Therefore, the extract's enhanced thrombopoietic impact may be the cause of the rise in platelet levels in all treated rat groups relative to the control group [14]. The increase in the WBC and lymphocyte count across increasing doses of the leaf extract therefore suggests leucopoietic and possible immunomodulatory effects of the extract as described by Bashir *et al* [15], which boosts the immune components of the animal.

According to Karaca and Güder [16], oxidative stress results from the disruption of the balance between antioxidants and free radicals. The antioxidant defence system enzymes such as; superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT), as well as nonenzymatic antioxidants like Malondialdehyde (MDA), neutralize free radicals [17]. MDA has been severally used as an indicator of oxidative stress where a decrease in the first-line antioxidants causes an increase in the free radicals and result in lipid peroxidation, leading to abnormal metabolism. The increase in MDA levels usually accompanied by decreases in the first-line antioxidants such as SOD, GSH, GPx and CAT usually confirms oxidative stress.

In this study, it was observed that the antioxidant parameters of the treatment groups did not cause any significant change in comparison with the control groups, except for catalase. Although the levels of catalase were significantly lower across the dose-challenged groups, the results from the present findings would not be sufficient to ascertain that the extract caused appreciable oxidative stress as other oxidative stress parameters were not affected.

The histopathological changes observed in the extract-treated groups were mild and did not portray any significant or appreciable alteration in the histoarchitecture of the liver sections as seen in the photomicrographs (Figures 2-4). Consequently, the alterations in the biochemical markers did not significantly manifest at the tissue level, suggesting the safety of the extract especially in the low and middle doses, within the treatment period.

CONCLUSION

Hibiscus surattensis may be safe for human consumption at the doses tested in this study and within the treatment period. Long-term administration, however, requires caution. It is recommended that more research be done on the mutagenic, teratogenic, and carcinogenic effects of Hibiscus surattensis as well as its dose standardization.

ACKNOWLEDGMENT

The authors thank Prof. Margaret Bassey, of the Botany and Ecological Studies Department of the University of Uyo who identified and authenticated the plant, Mr Chimaeze Ogadinma of the Pharmacology and Toxicology animal house, Madonna University for his assistance in animal care.

AUTHORS' CONTRIBUTION

All authors contributed meaningfully to the success of this work. Israel K. Umana conceived the idea, co-planned the work and as well prepared the article with Tobechukwu A. Tochukwu. Israel K. Umana revised the article. Each author provided a substantial financial commitment and re-vetted the final article.

CONFLICT OF INTEREST

The authors do not have any conflicts of interest concerning this article.

FUNDING

This research received no funding from external sources.

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