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Acute and Sub-Acute Toxicological Evaluation of Aqueous Leaf Extract of *Nauclea latifolia* (Rubiaceae) in Albino Rats

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Authors' contributions

This work was carried out in collaboration between all authors. Author RIO conceptualized, designed and supervised the study which was the final year project topic for author NU but assisted in the laboratory by authors BEO and DOU. Histology was done by author EEU. The study was written up by author RIO but the final version was approved by all authors.

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ABSTRACT

Objective: Parts of *Nauclea latifolia* Smith (Rubiaceae) have been used extensively in ethnomedicine. Despite increasing popularity, there is paucity of information regarding its safety. We therefore evaluated the toxicological profile of the aqueous leaf extract.
Methods: Acute doses were administered and the animals were observed for signs and symptoms for 14 days. In the sub-acute evaluation, the rats were given oral doses of 0.5, 1 and 2.5 g/kg/day for 28 consecutive days after which hematological and biochemical analyses were done. Kidneys, livers, spleens, lungs and hearts of the rats were assessed histologically.

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Results: There were no signs of toxicity up to an acute maximum dose of 10 g/kg. In the sub-acute evaluation, weight initially gained was lost by the 28th day but organ-to-body weight ratios were not significantly affected. Platelet count decreased significantly (P<0.001) but packed cell volume increased significantly (P<0.01) in the extract-treated groups. Alkaline phosphatase level increased significantly (P<0.01) in the group that received 2.5 g/kg/day. Plasma sodium decreased significantly (P<0.001) in all the extract-treated groups. The levels of other hematological parameters and enzymes (aspartate aminotransferase, alanine aminotransferase) were not significantly altered. The levels of total protein, albumin, bilirubin, urea, creatinine and potassium also remained comparable with the control group. Histology showed acute tubular necrosis at the dose of 2.5 g/kg/day and acute lung inflammation and bronchopneumonia at all dose levels.
Conclusions: While low doses of the aqueous extract appear safe, the daily use of high doses above 0.5 g/kg may be injurious to health.

Keywords: Nauclea latifolia; acute; sub-acute; aqueous extract; toxicological profile.

1. INTRODUCTION

Plants have remained a useful source of new medicines. It is estimated that in some African and Asian countries as many as 80 percent of the population depend on herbal medicine for their primary healthcare needs [1]. For example in Nigeria, about 80 percent of the population use herbal medicine almost exclusively while about 95 per cent use it concurrently with Western medicine [2]. In the Western world, there is a growing interest in herbal medicine as a component of alternative and complementary medicines [3]. The increasing popularity of herbal medicine is due to factors such as: their easy availability, lower price, and close association with the belief and culture of the users [4,5]. A major criticism often associated with the use of plant based medicines is the absence of information on their safety profile since many of them have turned out to be toxic [6-8]. Evaluation of herbal medicine safety becomes even more pertinent when a particular herbal product gains popularity.

Nauclea latifolia Smith (syn: *Sarcophelus latifolius*) of the family Rubiaceae, is a straggling shrub or small spreading tree that is easily identified by its broad compound leaves. It is found in rain forests and savannah of West and Central Africa [9]. The infusions and decoctions of various parts of *N. latifolia* are used traditionally as cure against several disease states in different countries including: Cameroon [10], Congo [11], Mali [12], Nigeria and elsewhere [13]. The powdered dry leaves are often sold by herbal medicine hawkers in many cities in Nigeria. Various scientific studies have been conducted on parts of *N. latifolia* some of which include: antimalarial [14,15] antiviral [16], antidiabetic [17,18], antiamoebic and spasmolytic [11], and antidiarrhoeal [19]. Others include antifungal activity [20], antinociceptive and antipyretic [10,15], anticonvulsant [21]; treatment for neuropathic pain [10]; antihelminthic [22] and anti-hypertensive [23]. The leaves of *N. latifolia* also possessed strong antioxidant potential [24]. The root extract has been developed for the treatment of uncomplicated malaria by the Nigeria-based National Institute for Pharmaceutical Research and Development [25]. These scientific reports have further increased the popularity of the plant as a herbal medicine.

Polyphenols have been identified as the major constituents of the leaf extract of *N. latifolia* while different alkaloids including strictosamine have been found in its leaves and stem bark [11,26]. Despite the acclaimed and documented uses, there appears to be no information on the safety associated with repeated and prolong use of the leaf of the plant for chronic diseases such as diabetes [17], neuropathic pain [10] and hypertension [23]. Considering the increasing popularity of *N. latifolia*, the present study was designed to evaluate the safety of this potential herbal medicine. The results of acute and sub-acute toxicity studies on the aqueous leaf extract of *N. lautifolia* in rats are presented in current communication.

2. MATERIALS AND METHODS

2.1 Plant Material and Preparation of Extract

Fresh leaves of *Nauclea latifolia* were collected from Ekosodin, Edo State, Nigeria in October, 2014. The plant was identified and authenticated by Mr. Sunny Nweka of the Department of Pharmacognosy, Faculty of Pharmacy, University

of Benin, Benin City, Nigeria. A herbarium sample with reference number UBHr0281 has been deposited in the Department of Plant Biology, University of Benin, for record.

The leaves were washed with tap water and air-dried for one week before grinding to coarse powder using an impact mill. The powdered leaves (300 g) were soaked in a glass jar of 2 L distilled water for 48 h with regular stirring. This was followed by filtration and concentration, first over a hot water bath followed by keeping it in an oven at 40℃. The extract was packed in an amber-colored sample bottle and stored in a refrigerator. The aqueous extract was reconstituted in distilled water and administered according to the experimental protocol.

2.2 Animals

Experiments were performed using adult albino rats of either sex weighing 150-200 g. The animals were bred locally in the Department of Anatomy, University of Benin, Benin City and were acclimatized for two weeks in the animal house of the Department of Pharmacology and Toxicology, University of Benin, Benin City. They were housed in standard plastic cages and allowed free access to rat pellets (Bendel Feeds and Flour Mill Ltd, Ewu, Nigeria) and free access to water. The animals were exposed to 12 h light/dark cycle with room temperature at 28.0±1.0℃ and were handled according to standard protocols for the use of laboratory animals [27]. The study was overseen by institutional ethical committee. The extract was administered by using orogastric tubes (CUFNC 16-3).

2.3 Acute Toxicological Assessment

The oral median lethal dose (LD_{50}) was evaluated by using modified Lorke method [28]. Rats were randomly assigned to three groups (n = 3 per group; both sexes represented) in the first phase. The groups were administered the extract at doses of 0.01, 0.1 and 1 g/kg body weight respectively. In the second phase using different rats, doses of 5, 7.5 and 10 g/kg of the extract were administered to one rat each. Control rats were given 2 ml/kg of distilled water. After the administration of the extract, animals were observed for death and symptoms of toxicity within three days in the first instance and then for 30 min each day for another eleven days. Gross toxicological symptoms monitored include writhing, piloerection, ptosis, diarrhea, constipation, depression, convulsion and hypermotility [13].

2.4 Sub-acute Toxicological Assessment

Rats of either sex were assigned to four groups of 8 rats each (males = females). One group was used as control and administered 2 ml/kg of distilled water for 28 days. The remaining three groups were each administered doses of 0.5, 1.0 and 2.5 g/kg/day respectively for 28 days. The dose of the extract was chosen based on the method of Bautista et al. [29,30]. On the 28th day, the animals were anesthetized with chloroform vapor in a chamber and blood samples were withdrawn from the abdominal aortas for biochemical and hematological assays. General visceral condition of each animal was observed and compared with that of control group animals. The hearts, kidneys, lungs, and spleens were taken out, and kept on absorbent papers for 5 min before they were weighed. The isolated organs were fixed with 10% v/v formaldehyde in saline solution for histological analyses [13].

2.5 Hematological Evaluations

This was done according to a previously reported method [5,13,29,31,32]. In brief, blood samples preserved in sodium edetate bottles were drawn into capillary tubes and placed in an automated blood analyzer (Symsmex, UK). Evaluated parameters include white blood cell count (WBC) and differentials (lymphocytes, monocytes and granulocytes), platelet count (PLT), hemoglobin (Hb) concentration and packed cell volume (PCV).

2.6 Biochemical Assays

Blood samples in lithium heparin bottles were centrifuged at 3000 rpm and plasma was separated by using Pasteur pipettes into labeled bottles. The plasma samples were stored in a deep freezer at –20℃ until analyzed using Randox® test kits. Alkaline phosphatase was analyzed using the method described by Raymond-Habecher and Lott [33]. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were assayed by the method described by Schumann et al. [34]. Bilirubin was quantified by Jendrassik-Group method [35]. Total protein was assayed by the Biuret method [36], and albumin was quantified by the method described by Doumas et al. [37].

Creatinine and urea were assayed using commercial kits and in accordance with previously reported principles [38,39]. Sodium and potassium were measured by standard Flame Atomic Absorption Photometry procedure [40].

2.7 Histological Assessment

The tissue specimens were dehydrated in serial grades of alcohol, processed with the Leica® automatic tissue processor over 24 h in xylene and then embedded in paraffin wax before sectioning with a microtome. Sections (5 µm thick) of the tissues were made and stained with hematoxylin and eosin [13]. All sections were viewed under a Leica® light microscope (Model DM500) at x 100 magnification by a histopathologist who did not know the experimental grouping and protocols.

2.8 Statistical Analysis

Data are presented as mean ± SEM (standard error of mean) and "*n*" represents the number of rats per experimental group. Data were analyzed using one-way ANOVA with Tukey post hoc test (GraphPad Prism 6 software, UK) and $P< 0.05$ indicates statistically significant difference between compared data.

3. RESULTS

The results of the present study are presented in Tables 1-5, and Figs. 1 and 2.

Table 1. Result of acute toxicological test on aqueous leaf extract of *N. latifilia* in rats

Dose (g/kg)	Number of death	Mortality (%)	Symptoms
0.01	0/3	0	None
0.1	0/3	0	None
1	0/3	0	None
5	0/1	0	None
7.5	0/1	0	None
10	0/1	0	None

Animals were first observed for 72 h and then 30 min each day for the next 11 days

3.1 Acute Toxicity

The oral LD_{50} of the leaf extract of *N. latifolia* was indeterminable as there was no death in any of the doses used even after 14 days. There were also no obvious signs and symptoms of toxicity at all the dose levels (Table 1).

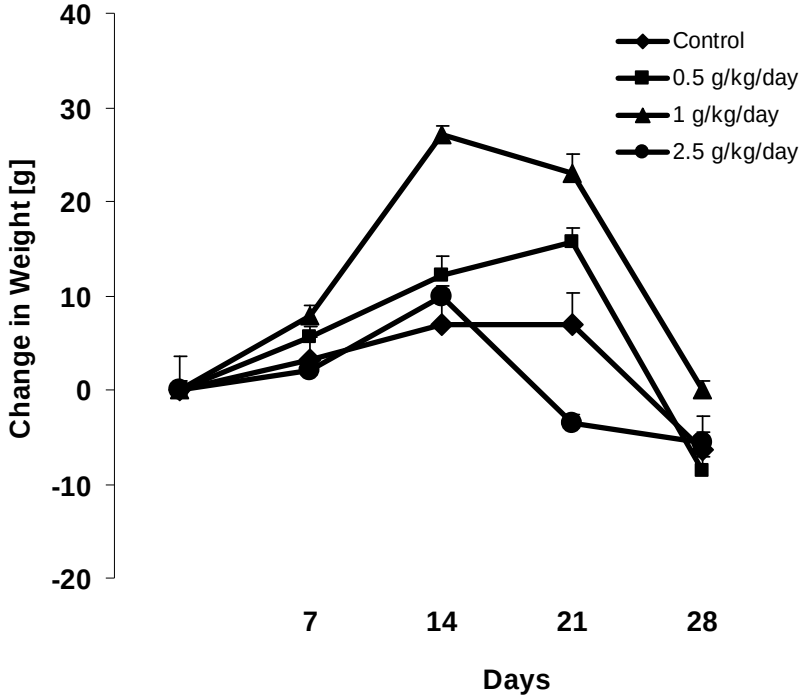

Fig. 1. Changes in whole body weight of rats treated with doses of aqueous leaf extract of *N. latifolia* for 28 consecutive days. Initial weight gains were lost by the 28th day. n = 8 per group

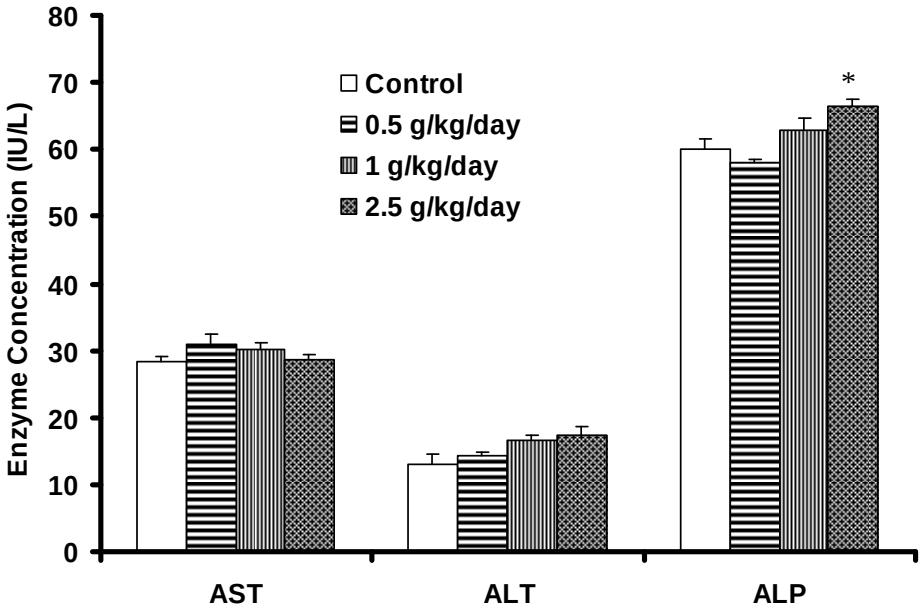

Fig. 2. Levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) of rats treated with doses of aqueous leaf extract of *N. latifolia* for 28 consecutive days

**P< 0.01 compared with control. n = 8 per group*

Table 2. Organ-to-body weight ratios following daily oral treatment of rats with doses of aqueous leaf extract of *N. latifolia* for 28 days

	Ratios ($x10^{-3}$)				
	LV:BW	**H:BW**	**K:BW**	**L:BW**	**S:BW**
Control	15.9±5.5	4.0±0.6	6.2±0.4	35.0±0.9	3.8±0.4
0.5 g/kg	10.5±0.9	4.2±0.3	7.1±0.3	32.8±0.7	6.1±1.1
1 g/kg	9.0±0.5	4.5±0.2	7.4±0.4	34.4±1.0	4.1±0.6
2.5 g/kg	11.4±1.8	4.2±0.4	6.6±0.3	32.5±1.3	4.3±0.3

Organ-to-body weight ratios are not significantly different from controls. LV:BW, liver-to-body weight; H:BW, heart-to-body weight; K:BW, kidney-to-body weight; and S:BW, spleen-to-body weight. n = 8 per group

Table 3. Hematological indices following daily oral doses of *N. latifolia* aqueous leaf extract for 28 days

	WBC ($x\ 10^3$/µl)	**LY (%)**	**MO (%)**	**GR (%)**	**PLT ($x\ 10^{-3}$/µl)**	**PCV (%)**	**Hb (g/dl)**
Control	9.6±1.1	79.9±1.9	8.7±3.9	11.5±4.5	663.7±6.0	37.3±1. 8	16.4±0.4
0.5 g/kg	11.5±0.7	83.6±1.7	8.3±2.1	8.1±5.9	389.7±8.6 *	50.9±1.5*	17.1±1.0
1 g/kg	10.0±0.8	78.5±2.1	10.4±2.1	9.0±6.3	427.0±6.7*	48.1±1.1*	15.9±0.6
2.5 g/kg	11.8±1.7	73.0±2.3	12.7±2.3	11.8±7.6	503.6±1 4.0*	44.9±0.7†	14.9±0.5

**P<0.001 versus Control; †P<0.01 versus Control. n = 8 per group. WBC, white blood cell count; LY, lymphocytes; MO, monocytes; GR, granulocytes; PLT, platelets: PCV, packed cell volume (hematocrit); and Hb, hemoglobin*

3.2 Sub-acute Toxicity

At the end of 28 days, the organ-to-body weight ratios were found not to show any statistically significant differences between the control and treatment groups (Table 2). The mean weight change data (Fig. 1) showed that the control, 0.5, and 1 g/kg/day treatment groups gained weight steadily until day 21. Later, a decline in weight started. It is worth stating that in the group of animals treated with 2.5 mg/kg/day the weight decline started on day-14.

Table 4. Some biochemical parameters and electrolytes after daily oral administration of doses of aqueous leaf extract of *N. latifolia* for 28 days

	TP (mg/dl)	ALB (mg/dl)	TB (mg/dl)	DB (mg/dl)	Cr (mg/dl)	Urea (mg/dl)	Na^+ (mMol/l)	K^+ (mMol/l)
Control	6.3±0.7	3.9±0.4	2.1±0.2	0.8±0.4	9.2±0.8	61.1±2.2	193.9±5.4	3.6±0.9
0.5 g/kg	7.1±0.4	2.5±0.4	1.0±4.2	0.5±0.3	13.5±1.5	77.8±0.8	122.0±9.3*	1.7±0.5
1 g/kg	7.1±0.2	5.9±0.7	0.5±0.4	0.6±0.1	12.9±0.8	53.1±1.8	117.6±4.1*	1.8±0.6
2.5 g/kg	6.5±0.3	3.1±0.3	1.6±0.8	0.7±0.4	11.3±5.3	58.0±1.6	106.1±7.0*	1.6±1.0

**P<0.001 versus Control. n = 8 per group. TP, Total Protein; ALB, Albumin, TB, Total Bilirubin; DB, Direct Bilirubin; Cr, Creatinine.*

Table 5. Histopatological effects of daily oral treatment with aqueous leaf extract of *N. latifolia* for 28 days

	Spleen	Kidney	Liver	Heart	Lungs
Control	Normal	Normal	Normal	Normal	Normal
0.5 g/kg	Normal	Normal	Normal	Normal	Acute inflammation and, bronchopneumonia
1 g/kg	Normal	Normal	Normal	Normal	Acute inflammation and bronchopneumonia
2.5 g/kg	Normal	Acute tubular necrosis	Normal	Normal	Acute inflammation and bronchopneumonia

Slide for each organ were prepared from two different animals in a group and viewed with an Leica® microscope DM500 at x 100 magnification with eosin (E) and hematoxylin (H) as stains

Hematological parameters are shown in Table 2. All doses significantly (P<0.001) reduced the platelet counts in the treatment groups as compared with the control group. Packed cell volume (PCV, hematocrit) on the other hand was significantly increased by all dose levels as compared with the control group. The increase in PCV, was in the reverse order of the doses used: being highest with 0.5 g/kg/day group (P<0.001) and lowest with the 2.5 g/kg/day group (P<0.01).
The serum levels of some enzyme biomarkers of toxicity are shown in Figure 2. As compared with the control group; there was no significant increase in the level of aspartate aminotransferase (AST) and alanine aminotransferase (ALT). However, a statistically significant (P<0.01) increase was observed in the level of alkaline phosphatase (ALP) at the treatment with dose: 2.5 g/kg/day.

Total proteins (TP), albumin (ALB), and direct bilirubin (DB) were all comparable across the groups (Table 4). In the same table, the values of other biochemical parameters such as creatinine (Cr), urea, and K^+ were all comparable across the groups. Nevertheless, there was a significant (P<0.001) reduction in the serum level of Na^+ in all the extract-treated groups.

Histopathological results presented in Table 5, demonstrated that there were signs of acute inflammation, and bronchopneumonia in all animals in the treatment groups as compared with the control. In addition, the dose of 2.5 g/kg/day resulted in acute tubular necrosis in the kidneys of rats. The livers, hearts and spleen of all animals did not reveal any histological changes and were comparable to the control group.

4. DISCUSSION

The determination of median lethal dose (LD_{50}) has remained a useful tool in safety assessment of substances, despite some criticisms in the scientific literature [29]. In the present study, LD_{50} of the aqueous leaf extract of *N. latifolia* was indeterminable since the maximum dose of 10 g/kg did not cause death or any other gross toxicological symptoms in the rats of treatment groups. The general visceral condition of the animals, both in the treatment groups as well as control groups were normal and comparable. Our results are substantiated by earlier studies where a single oral dose of the aqueous leaf extract (higher than 400 mg/kg) could not induce any adverse effects [17,22,23]. In addition, an acute dose of extract up 5 g/kg was found to be safe when used to determine the index of safety [41,42].

All these findings support the extract under investigation to be a candidate for the category of non-toxic substances according to the Hodge

and Sterner scale for toxicity [43] and classified under the category of substances with low toxicity according to OECD [44].

According to another method of determining the toxic potential of a crude drug extract, much higher dose (5 g/kg) could give information on the safety of an extract in a possible event of overdose or homicide [32]. In the acute experiments, the extract under present study could not be classified under poisonous plants. Nonetheless, the absence of gross toxicological symptoms following an acute dose does not preclude the need for more detailed assessment of possibly delayed adverse effects, and general safety when a substance is used for chronic diseases. Based on the fact that *N. latifolia* is used for such chronic disease states, hence sub-acute toxicity assessment as well as chronic toxicity studies were considered imperative [31,30].

Organ weight indices have often provided useful toxicological information [45]. Although organ to-body weight ratios did not change within 28 days, interestingly, in the present study, there was loss of the initially gained weight in all the groups due possibly to daily handling of the animals. The significant decrease in platelet counts seen alongside the significant increase in the hematocrit is suggestive of alteration in hematopoiesis [46]. While increase in hematocrit through possible erythropoiesis may be helpful, thrombocytopenia may affect hemostasis [47]. Values of ALT and AST are commonly used for the qualitative assessment of underlying cellular injury [48,49]. The elevations often suggest non-specific injuries to internal organs such as the liver, kidney and lungs [48,50,51,49,52]. In this study, there were no significant alterations in the levels of the enzymes AST and ALT; however, ALP level was significantly elevated at the dose of 2.5 g/kg/day. This indicates possible injury to internal organs at that dose [50,52]. Destruction of the glomeruli causes significant decrease in the glomerular filtration rate and an increase in blood urea and creatinine resulting in chronic renal failure [49]. This implies that even if there were kidney-related adverse effects in this study, they did not alter the level of these biomarkers. Significant reduction in sodium levels which was consistent with extract-treated groups could have been due to enhanced excretion, an effect associated with diuretic action [49]. It is not clear if this is related to the reported hypotensive effect of the extract [23].

A consistent histological finding across all extract-treated groups is the presence of acute inflammation and bronchopneumonia. Although this is poorly related to the biochemical findings of the current investigations, it is nonetheless important. Aside from causing pulmonary lesion, the highest dose of 2.5 g/kg/day caused acute tubular necrosis.

5. CONCLUSION

This study has shown that the administration of doses of the aqueous leaf extract of *N. latifolia* in excess of 0.5 g/kg/day for as long as 28 days has the potential for renal and pulmonary injuries in rats and these high doses may not be safe in humans. Based on the findings of the present study, the plant seems to induce some hazardous effects. Hence detailed toxicity studies including the effects of chronic treatment are recommended to conclude the toxicity profile of this herbal medicine.

CONSENT

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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