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Original Research Article

Sub-Acute Toxicological Evaluation of Methanol Leaf Extract of *Nymphaea lotus* Linn (Nymphaeceae) in Wistar Rats

Musa G. Rege[2], Lydia O. Ayanwuyi[1], Abdulkadir U. Zezi[1], Saidi Odoma[3,4]*, Muhammed Bisalla[5].

[1]*Department of Pharmacology and Therapeutics, Ahmadu Bello University, Zaria, Nigeria.*
[2]*Department of Pharmacy Technician Training, Kebbi State School of Health Technology, Jega, Nigeria.*
[3]*Department of Pharmacology, College of Health Sciences, Kogi State University, Anyigba, Nigeria.*
[4]*Department of Pharmacology and Toxicology, Kampala International University, Bushenyi, Uganda.*
[5]*Department of Veterinary Pathology, Ahmadu Bello University, Zaria, Nigeria.*

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ABSTRACT

Nymphaea lotus has been used for centuries as an astringent, aphrodisiac, sedative, analgesic, anti-inflammatory, and for the treatment of infectious diseases. This study is aimed at evaluating the subacute toxicity profile of methanol leaf extract of *Nymphaea lotus* in Wistar rats. Rats were administered the crude methanol leaf extract orally for 28 days at 250, 500, and 1,000 mg/kg. Weekly body weight, food, and water intake were recorded. On the 29th day, the rats were sacrificed, and their hematological and biochemical parameters were assessed, as well as histological examination of the kidney, liver, stomach, and intestine. The extract had no effect on the body weights, relative organ weights, or food and water intakes of the animals. It had no impact on hematological markers at the tested doses (hemoglobin, packed cell volume, red blood cell, white blood cell, mean corpuscular hemoglobin, mean corpuscular volume, and platelets concentration), except for alkaline phosphate, which was significantly ($p \leq 0.01$) greater at the 500 mg/kg. There was no significant effect on liver function parameters evaluated (total bilirubin, direct bilirubin, alanine aminotransferase, aminotransferase, and aspartate). Urea, creatinine and chloride levels were significantly elevated ($p \leq 0.01$ and $p \leq 0.05$) at 250 and 500 mg/kg doses, but not at the 1,000 mg/kg dose. Histopathological evaluation of the liver, kidneys, stomach, and intestine revealed no notable histological abnormalities. Based on the results, the methanol leaf extract of *Nymphaea lotus* appears to be generally safe when taken orally at these doses.

Keywords: biochemical, ethnopharmacology, haematology, histopathology, *Nymphaea lotus*, subacute toxicity..

Introduction

Nymphaea lotus (Linn) is a herbaceous aquatic plant belonging to *Nymphaeaceae*. It has white flowers, and its leaves float or are submerged in water. It is native to West Africa, particularly Nigeria, as well as North Africa, Africa's tropical mountains, the southern and central Europe, Asia, and the Middle East.[1] It is one of the earliest aquatic macrophytes found in watery areas of Nigeria.[2] Water lily is the common name;[3] nevertheless, it is also known locally (in Nigeria) by the names *Badoo* in Hausa, *Gwidbi* in Zuru, *Osibata* in Yoruba, and *Enge* in Ibo.

The entire plant, including the leaves, has long been used for a variety of ailments, including as an astringent, aphrodisiac, sedative, pain reliever, and to cure infectious and inflammatory diseases.[3] Numerous studies done on lab animals demonstrate that the leaf extract has antinociceptive, anti-inflammatory, anxiolytic, and antidepressant properties; the stem extract has anti-inflammatory and analgesic potentials; that the flower extract has aphrodisiac properties; and that the rhizome extract has anti-diarrheal properties.[4-8]

*Corresponding author. E mail: odoma.s@ksu.edu.ng
Tel: +2348027547778

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During a preliminary phytochemical screening of its leaf extract, alkaloids, cardiac glycosides, phenols, tannins, proanthocyanidins, flavonoids, saponins, and steroids were discovered.[4,9]

Users of herbal medicines frequently believe that these drugs are always safe and efficient because they are "natural" in nature. Contradictory evidence can be found, though. There are some unfavorable side effects associated with the use of herbal medicines or natural products (some of which are life threatening).[10] Hence, contrary to popular belief, herbal medicine can also be harmful to the health.[11] Thus, the significance of determining the toxicity of herbal medicines cannot be overstated.

We previously described the acute toxicity profile of methanol leaf extract of *N. lotus* in rats.[4] Additionally, the leaf extract's subacute toxicity profile in rats, at dosages of 50, 100, and 200 mg/kg were also reported.[12] The goal of this study is to further investigate the subacute toxicity profile of methanol leaf extract in rats in a repeated-dose 28-day oral toxicity study using higher doses of 250, 500, and 1,000 mg/kg, as recommended by the Organization for Economic Corporation and Development (OECD) guidelines.[13] This will enhance its pharmacological profile and promote its investigation as a potential future medication.

Materials and Methods

Plant material collection

Nymphaea lotus leaf was obtained from a farm in Zaria, Kaduna State, Nigeria, in December 2019. A plant taxonomist, Mallam Namadi Sanusi, of the Herbarium division, Department of Biological Sciences, Faculty of Sciences, Ahmadu Bello University, Zaria, identified and authenticated the leaf. For future reference, the voucher specimen number ABU/894/005 was collected.

Preparation of the plant extract
The *Nymphaea lotus* leaf was cleaned and air-dried for many days until a consistent weight was achieved. Using a mortar and pestle, the dried leaf was ground into a coarse powder. Approximately 725 g of powdered leaves were extracted for 72 hours using the cold maceration technique with (30-70 v/v) aqueous-methanol with periodic shaking. After that, the extract was filtered and concentrated in a water bath at 50°C.[14] The extract was stored in an airtight container for future use. For each experiment, fresh stock solutions were prepared. Subsequently, it was referred to as "*Nymphaea lotus* Extract" (NLE).

Animals
The experiment employed 24 Wistar rats of either sex, weighing 150-200g, obtained from the Animal House facility of the Department of Pharmacology, Ahmadu Bello University, Zaria. The animals were kept in conventional animal cages, at room temperature; and fed a regular rat's feed (Vital feeds), and had free access to tap water, except where experimental protocol required otherwise.

Twenty-eight (28)-day oral toxicity study (repeated doses)
The OECD 407 (2008) guidelines were followed while treating rats orally for a total of 28 days. Twenty-four rats of both sexes were separated into four groups, each with six rats. Distilled water (1 ml/kg) was given to Group 1, which served as the standard control. NLE was given to rats in groups 2, 3, and 4 at doses of 250, 500, and 1,000 mg/kg body weight, respectively. Throughout the experiment, the rats had unrestricted access to food and water, and they were monitored daily for signs of toxicity and death. Animals' body weights were measured once a week and food and water intake were recorded daily.

Blood and organ samples collection
On the twenty-ninth day of the experiment, rats were humanely sacrificed via cervical dislocation. For blood chemistry and hematological examination, blood samples were taken through jugular vein puncture into heparinized tubes and EDTA tubes, respectively. The blood samples used in chemistry were allowed to coagulate for 30 minutes before being centrifuged for 10 minutes at 7000 RPM, to separate the serum. The rats' liver, kidney, stomach, and small intestine were carefully harvested for histopathological studies. The relative organ weight was calculated using the formula:

$$\text{Relative organ weight} = \frac{\text{Organ weight (g)}}{\text{Bodyweight of animal on the day of sacrifice (g)}} \times 100$$

Histopathology
The liver, kidney, stomach, and small intestine of the rats were removed and fixed in 10% formalin. The tissue was dehydrated by passing it through a series of grades of alcohol ranging from 70 percent to 100 percent for 16 hours, following which it was cleaned with xylene for 4 hours. The tissue was imbedded in molten paraffin wax. A Leica microtome was used to cut 5 micron slices, which were subsequently stained using the hematoxylin and eosin (H and E) staining procedure.[15] A pathologist from Ahmadu Bello University Zaria's Faculty of Veterinary Medicine examined the tissues under a light microscope for pathological abnormalities. At (H and E) 400, photomicrographs of slices of the various tissues were obtained.

Hematological parameters
The hematological parameters of the albino rats were analyzed by aspirating 2mls of the blood samples into the chamber of the fully automated hematology analyzer (Pentra-XL 80, Horiba ABX, USA). The samples were then diluted with isotonic saline solution. The levels of the packed cell volume (PCV), white blood cell (WBC), hemoglobin (HB), red blood cell (RBC), mean cell volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and platelets (PLT) in the blood were subsequently determined.[13,16]

Biochemical analysis
A completely automated chemistry analyzer (Mindray BS-200, China) was used to determine aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), urea, bilirubin, creatinine, sodium, potassium, chloride, and bicarbonate,[13,16] according to the manufacturer's instruction.

Ethical consideration and approval
The studies were carried out in compliance with the requirements specified in the National Institutes of Health's Guide for the Care and Use of Laboratory Animals (Publication No. 80-23, revised 1996). The Ahmadu Bello University Committee on Animal Use and Care (ABUCAUC) approved the studies (Protocol Number: ABUCAUC/2021/125).

Statistical analysis
The mean and standard error of the mean are used to express the results. When applicable, tables and figures were utilized to show the data. One-way analysis of variance (ANOVA) was used to evaluate single point data, while repeated measure ANOVA was used to investigate time-dependent point data; when applicable, Dunnett and Bonferroni's post hoc tests were used, with findings considered significant at $p \leq 0.05$.

Results and Discussions

It was previously reported that the acute oral toxicity (LD_{50}) of *Nymphea lotus* methanol leaf extract is larger than 5,000 mg/kg in mice and rats, indicating that the extract is virtually harmless when taken orally.[4] Following the OECD recommendations,[13] rats were orally administered the leaf extract, in a repeated doses for 28 days, to assess its subacute toxicity profile.
The weight increase, relative organ weight, food intake and water intake of rats were not affected by the oral administration of the extract for 28 days (results not shown). The results show no statistically significant difference in these parameters across the groups throughout the cause of the studies.
In toxicity research, determining the effects of plant extract on the organ and body weights of experimental animals is critical.[17] After exposure to potentially toxic chemicals, a change in relative organ weight or overall body weight may suggest an impairment in the normal functioning of the organs and serve as a toxicity indicator.[18,19] The administration of the *Nymphea lotus* methanol leaf extract daily via the oral route for 28 days did not result in any significant differences in body and relative organ weight of the treated groups when compared to the control group, suggesting that the extract is not harmful to the organs. This finding is consistent with prior research, which found that *Nymphaea lotus* leaf extract had no effect on the weight of rats or their organs.[12]
The impact of *Nymphea lotus* methanol leaf extract treatment for 28 days on hematological markers is shown in Table 1. At the studied doses of NLE, daily ingestion of methanol leaf extract for 28 days had no significant effect on hematological markers. The results show no statistically significant difference in these parameters across the groups. The hematopoietic system is a sensitive indicator for pathological states and a significant target for harmful substances (such as medications and toxins).[20] At the doses of NLE examined, the results revealed no significant changes in hematological parameters. This indicates that NLE is unlikely to be hazardous to the hematopoietic system. This tally with the practically nontoxic effect from the LD_{50} determination, that NLE has a safe toxicity profile.[4] This finding agrees with previous studies.[12]
The impact of NLE treatment for 28 days on liver function tests is shown in Table 2. Most of the indicators, such as alanine aminotransferase, aspartate aminotransferase, alkaline phosphate, direct bilirubin, and total bilirubin, did not significantly change after 28 days of daily administration of the methanol leaf extract. However, at 500mg/kg, NLE caused a significant ($p < 0.01$) serum elevation of ALP. LFT are routinely used tests in clinical practice. They are used to check for liver illness, track the progression of any known disease, and assess the effects of potentially hepatotoxic medicines. Bilirubin, alkaline phosphate (ALP), and serum aminotransferases are the most prevalent LFT parameters. Hepatic intercellular enzymes such as aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are circulating hepatic intercellular enzymes that act as indicators of hepatic

damage. Bilirubin and Alkaline phosphate (ALP) are indicators of cholestasis and biliary function.[21] The assessment of enzyme levels such as ALT and AST is primarily utilized in diagnosing liver damage caused by medications or other hepatotoxins.[22] The enzymes are released into circulation as a result of necrosis or membrane damage, and may thus be evaluated in serum.

High levels of AST may suggest liver impairment, although they are also utilized as a cardiac marker and are not specific for liver injury. Alanine is converted to pyruvate and glutamate by ALT, which is released in the same way. As a result, ALT is a superior criterion for diagnosing liver damage since it is more specific to the liver.[23] Serum ALP levels, on the other hand, are linked to hepatic cell activity. The increased production of hepatic enzymes in response to increased biliary pressure causes a rise in serum ALP.[24]

The concentrations of alanine aminotransferase (ALT), alkaline phosphate (ALP), direct bilirubin (DB), and total bilirubin (TB) did not change significantly after 28 days of NLE administration. However, NLE produced a significant serum elevation of ALP at 500 mg/kg. Since ALP was significantly elevated in this experiment, it suggests increased activity in the liver.

The impact of 28 days of NLE treatment on kidney biochemical markers in rats is shown in Table 3. At 250 mg/kg, the concentration of urea was significantly increased ($p< 0.05$) than in the control group (distilled water group). The creatinine level increased significantly ($p<0.01$) in the rat groups given 250 mg/kg and 500 mg/kg. The blood level of the electrolyte chloride was similarly greater in the 250 mg/kg and 500 mg/kg groups ($p<0.01$ and $p<0.05$, respectively). When compared to the distilled water control group, the observed elevation in kidney biochemical markers all returned to practically normal levels at the dose of 1,000 mg/kg. The observed effect might be due to the dose-response relationship of the extract. In some substance, there is a high-level of adverse effect, when administered at low doses. The adverse effects decrease with increase in dose. As the dose is increased to the point that the deficiency no longer exists, and no adverse response is therefore detected, the organism reaches a state of homeostasis. This is known as the U-shaped dose-response relationship.[25] The serum, potassium, sodium, and bicarbonate levels did not alter significantly.

In the kidney function tests, urea was significantly elevated following 28-days of oral administration of NLE at the dose of 250 mg/kg. In contrast, creatinine levels were significantly elevated at 250 and 500 mg/kg doses but not at 1,000 mg/kg. Chloride levels were also increased at 250 and 500 mg/kg doses, suggesting minor kidney damage since the other parameters were not elevated. The kidney histology did not show any pathology.

The electrolyte variation in sodium, potassium, and chloride reflects transitory stages of hydration. Remarkably, the increase of these electrolytes can lead to dehydration.

Table 1: Effect of NLE on hematological parameters of rats

Hematological Parameters	DW 1 ml/kg	NLE 250 mg/kg	NLE 500 mg/kg	NLE 1000 mg/kg
PCV (%)	36.00 ± 2.07	39.33 ± 0.66	37.40 ± 1.43	43.33 ± 2.40
HB (g/dL)	12.34 ± 0.42	13.26 ± 0.22	12.72 ± 0.39	13.90 ± 0.50
WBC ($10^{9}/L$)	4.78 ± 0.08	4.43 ± 0.11	4.50 ± 0.15	4.46 ± 0.21
RBC ($10^{6}/L$)	5.75 ± 0.10	5.80 ± 0.07	5.60 ± 0.11	5.73 ± 0.14
Platelets ($10^{5}/L$)	6.98 ± 0.03	7.08 ± 0.09	6.94 ± 0.05	6.96 ± 0.08
MCV (cu)	62.69 ± 4.14	67.92 ± 1.85	67.03 ± 3.60	75.74 ± 5.07
MCH (µg)	21.46 ± 0.89	22.91 ± 0.62	22.79 ± 1.02	24.28 ± 1.15
MCHC (%)	34.49 ± 1.08	33.73 ± 0.25	34.05 ± 0.41	32.14 ± 0.63

No statistically significant difference in these parameters across the groups

Table 2: Effect of 28-day oral administration of NLE on liver function test in rats

Liver function Parameters	DW 1 ml/kg	NLE 250 mg/kg	NLE 500 mg/kg	NLE 1000 mg/kg
AST (iu/L)	73.00 ± 13.52	66.16 ± 12.16	55.80 ± 7.87	64.66 ± 15.07
ALT (iu/L)	26.40 ± 2.06	23.50 ± 1.87	26.60 ± 2.01	32.33 ± 2.18
ALP (iu/L)	81.29 ± 7.94	83.45 ± 4.27	121.27 ± 5.28*	105.83 ± 11.26
Direct Bilirubin (mg/dL)	0.29 ± 0.02	0.30 ± 0.06	0.20 ± 0.04	0.21 ± 0.03
Total Bilirubin (mg/dL)	0.55 ± 0.04	0.52 ± 0.08	0.37 ± 0.07	0.29 ± 0.02

* = $p<0.01$ vs DW.

Table 3: Effect of NLE on kidney function test in rats

Kidney function Parameters	DW 1 m/kg	NLE 250 mg/kg	NLE 500 mg/kg	NLE 1000 mg/kg
Urea (mg/dL)	48.96 ± 2.91	63.35 ± 3.51*	52.58 ± 4.43	41.95 ± 1.76
Creatinine (mg/dL)	0.24 ± 0.05	0.68 ± 0.09**	0.68 ± 0.10**	0.30 ± 0.11
Sodium (mg/L)	99.81 ± 4.97	102.87 ± 5.74	112.88 ± 2.90	93.35 ± 16.44
Potassium (mg/L)	8.17 ± 0.96	6.78 ± 0.32	6.08 ± 0.25	6.59 ± 0.32
Chloride (mg/dL)	83.80 ± 1.35	97.50 ± 2.35**	94.60 ± 3.23*	80.33 ± 0.88
Bicarbonate (mg/dL)	20.00 ± 0.83	28.50 ± 3.07	25.40 ± 2.56	20.66 ± 0.88

* $p<0.05$, ** $p<0.01$ versus Control (DW)

Electrolytes play a central role in the inter compartmental water balance and gaseous exchange.[26] Elevation or depletion of serum electrolytes may result from hyper or hypo-functioning of the related tissues or organs, respectively.[27] The kidney function is assessed with clinical electrolytes such as potassium, chloride, sodium, and bicarbonate ions. An increase or decrease in the concentration of any clinical electrolytes indicates kidney dysfunction. In this experiment, the kidney biochemical parameters were normal at the 1,000 mg/kg dose; urea, creatinine, and chloride were elevated at 250 and 500 mg/kg, while sodium, potassium, and bicarbonate were not elevated at any of the doses. This suggests that there was a minor renal dysfunction probably due to the inhibitory effect of NLE on COX-2, which did not result in kidney damage as other parameters were not elevated, and there was no observed pathology on the kidney. Renal-derived prostanoids are essential for renal development and maintenance of its function.[28]

Histopathology gives support for the haematological and biochemical findings in toxicological research.[29] In the histopathological examination of the organs, we observed no significant findings concerning hypotrophy, hypertrophy, and necrosis. This shows that the change in several serum biochemical markers after 28 days of oral NLE treatment was minor and did not result in organ damage. This may further suggest that the NLE is safe at the doses tested at a subacute level for consumption and use as a medicinal plant.

Figures 1-4 show the photomicrographs of the liver, intestine, stomach, and kidney sections of rats treated with distilled water and different doses of the NLE for 28 days. The various section revealed no significant observable pathological changes in all the treatment groups. However, widening the bowman capsule was observed in the group treated with 1,000mg/kg of NLE (Figure 2D).

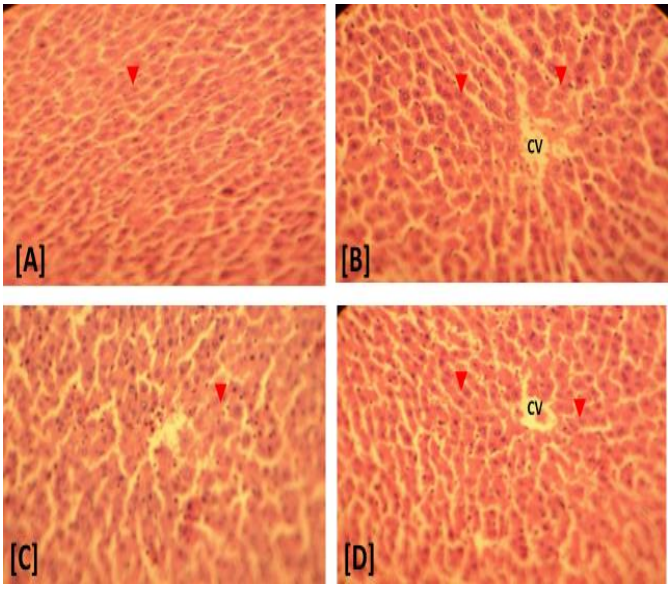

Figure 1: Photomicrographs of sections of the liver of rats treated with distilled water and different doses of *Nymhaea lotus* extract for 28 days (H and E ×400).
Normal histo-architecture after treatment with A (distilled water, 1 ml/kg), B (NLE, 250 mg/kg), C (NLE, 500 mg/kg) and D (NLE, 1,000mg/kg). CV=Central Vein; hepatocyte cord (red arrowhead).

Conclusion

Subacute studies showed that *Nymphea lotus* methanol leaf extract, following daily administration for 28 days, did not significantly affect the body weight and relative organ weight of the animals. Similarly, there was no significant effect of the *Nymphea lotus* methanol leaf extract on the haematological parameters. However, in the biochemical liver test, NLE 500 mg/kg elicited an increase in the level of ALP. Urea was also elevated at the dose of 250 mg/kg, while creatinine and chloride were increased at 250 and 500 mg/kg, but not at 1,000 mg/kg compared to the control group. The histopathological examination of the liver, kidney, and intestine, showed that NLE is practically nontoxic at the tested doses of NLE.

The methanol leaf extract of *Nymphea lotus* was found to be relatively safe for human consumption when taken orally. Nonetheless, long-term administration should be approached with caution.

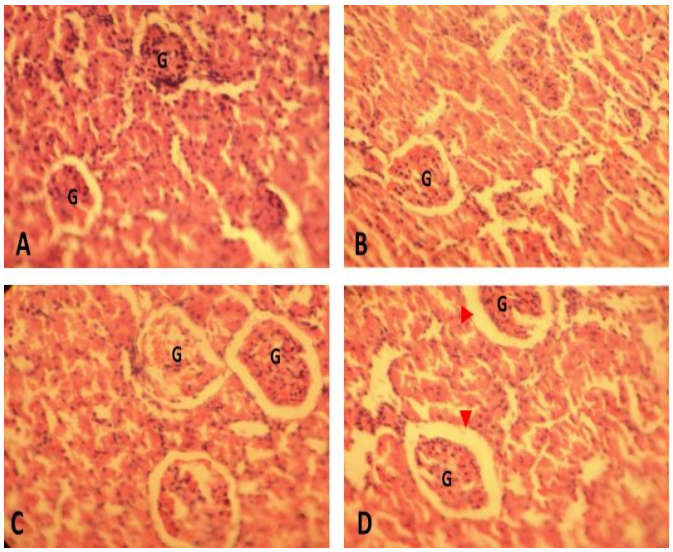

Figure 2: Photomicrographs of sections of the kidney of rats treated with distilled water and different doses of *Nymphaea lotus* extract for 28 days (H and E ×400)
Normal histo-architecture after treatment with A (distilled water, 1 ml/kg), B (NLE, 250 mg/kg), C (NLE, 500 mg/kg) and D (NLE, 1,000mg/kg). G = Glomerulus; widening of the bowman capsule (red arrowhead).

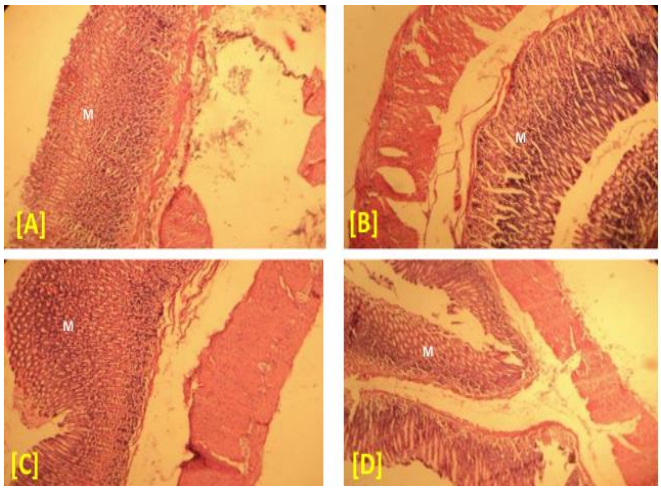

Figure 3: Photomicrographs of sections of the stomach of rats treated with distilled water and different doses of *Nymphaea lotus* extract for 28 days (H and E ×400).
Normal histo-architecture after treatment with A (distilled water, 1 ml/kg), B (NLE, 250 mg/kg), C (NLE, 500 mg/kg) and D (NLE, 1,000mg/kg). M= Mucus Layer.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

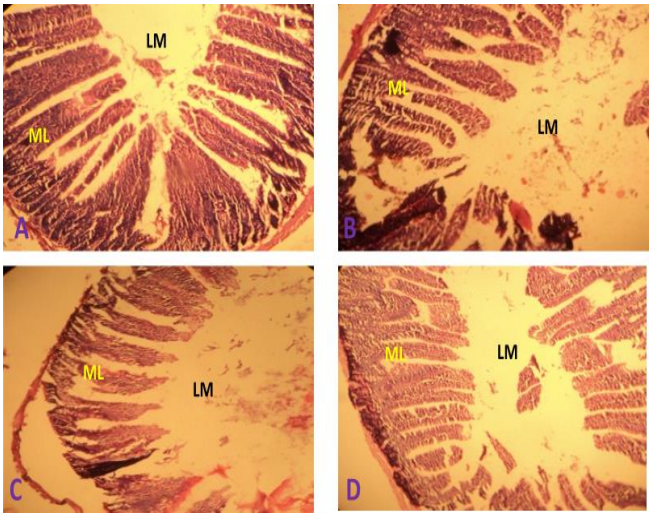

Figure 4: Photomicrographs of sections of the intestines of rats treated with distilled water and different doses of *Nymhaea lotus* extract for 28 days (H and E ×400).
Normal histo-architecture after treatment with A (distilled water, 1 ml/kg), B (NLE, 250 mg/kg), C (NLE, 500 mg/kg) and D (NLE, 1,000mg/kg). ML= Mucus Layer, LM= Lumen.

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