NJB, Volume 36 (2), 201-212, Dec., 2023

Pharmacological and Histopathological Assessment of *Phyllobotyron spathulatum* Müll.Arg.) in rats

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Received 15th November, 2023; accepted 20th December, 2023

ABSTRACT

Presently, information on toxicity and anti-inflammatory studies of *Phyllobotyron spathulatum* appear scarce. This study adopted different methods to determine the anti-inflammatory potentials, toxicity, enzyme activity and histopathological examination of *P. spathulatum* using rats. The leaf samples of *P. spathulatum* were collected. Active ingredients were extracted by percolation using ethanol. The dried crude extracts were stored in the refrigerator at 4ºC under aseptic conditions for subsequent use. Acute toxicity testing of the leaf of the plant showed that the extract was well tolerated at a dose of 2000 mg/kg as the animals showed no sign of toxicity or death after 72 hours. A sub-chronic study using the relative weight as well as liver and kidney enzyme profile showed that the extract of the leaf of *P. spathulatum* at various concentrations did not significantly alter the relative weights. *In vivo* anti-inflammatory studies using animal model tests showed that the plant extract at 200 mg/kg concentration inhibited carrageenan-induced paw oedema by up to 81% when compared to 0% in water and the 92% in standardised drug after 3 days. Activity in the analysed liver enzymes as well as the kidney function markers were not affected at the extract concentration used. The safety of the plant material was further confirmed by the histopathological examinations which did not show any changes in the kidney and liver tissues examined. Results obtained from this study showed that the leaf of this plant is safe and can be employed in the management of inflammatory-related diseases.

Key words: *Phyllobotyron spathulathum;* anti-inflammation; toxicity; histopathology
https://dx.doi.org/10.4314/njbot.v36i2.8

INTRODUCTION

Identification and documentation have been a major challenge in the use of plants and their products for disease control. Overall, knowledge is a very important aspect in the identification, preparation, safety and efficacy of such plants especially those used as medicinal plants. Modi *et al*. (2010) stated that pharmacognosy is a direct and reliable tool, by which adequate information of the crude drug from plants can be analysed. Although medicinal plants are considered generally safe at certain concentrations or volumes, they are known to contain natural but potentially toxic, mutagenic and/or carcinogenic substances that are harmful to living tissues (van-Vuuren, 2008; Gosh, 2015; Anyanwu and Okoye, 2017; Mitsunari *et al.*, 2021).

Ideally, it is recommended that pharmacological studies should always be accompanied by toxicological screening. A safe extract is one with oral LD_{50} above 1000 mg/kg in rats and is considered as being of low toxicity or relatively safe while a substance with intraperitoneal LD_{50} in rats ranging between 50 and 500 mg/kg is considered toxic (Saganuwan, 2012; Ngulde *et al*., 2013). One of the common disorders or symptoms associated with some illness is inflammation. This is commonly treated with herbal medicine. Friedman and Hughes (2002) noted that the cardinal signs of inflammation are pain, oedema, loss of function, redness and heat.

The moist tropical Nigerian environment facilitates the growth and development of a wide variety of plant species that have been used in Nigerian traditional medicine even before the introduction of conventional, refined drugs (Lifongo *et al*., 2014). Hence, the Nigerian flora is not only rich in diversity but it is also mostly endemic to some plant species. It is estimated that there are about 250,000 to 500,000 species of plants on Earth of which a relatively minute fraction (1 to 10%) is used as foods by both man and other animal species (Heinrich and Gibbons, 2001). It has been reported that possibly, a higher fraction of these estimated species is used for medicinal purposes (Moerman, 1996) with more species still unknown and underutilised (FAO, 1998; Demele and Abebe, 2004).

In Cross River National Park, Akamkpa, there is a unique plant, *Phyllobotryon spathulatum*, with the unique feature of bearing flowers in the leaf. An inquiry from the rangers and villagers indicates that the plant has no local name or known use within the locality. Literature reviewed showed lack of information on the taxonomy, phytochemical yield and medicinal benefits of *P. Spathulatum*. Considering the role of plants in ethno-medicine, the plant was collected for identification and further investigated to unravel its potential in medicine and to mitigate the effects of lack of drugs in developing countries of Africa where it grows. At present, the toxicity and anti-inflammatory studies of the Salicaceae appear scarce. Akbar (2020) reported that ethanol flower extract of *Salix caprea* L. (Salicaceae), like any other plant, exhibited significant anti-oxidant and anti-inflammatory effects. No report is available on the toxicity and anti-inflammatory effects of the present investigated plant. This study was aimed at studying the anti-inflammatory effects and other pharmacological activities of *Phyllobotryon spathulatum* leaf extract on albino rats and mice.

MATERIALS AND METHODS

Collection of Plant Samples

The leaf samples of *Phyllobotyron spathulatum* were collected from the Okwangwo division of Cross River National Park measuring about 4000 km^2. The park forms a continuous linear corridor with the Korup National Park in the neighbouring Cameroun. It lies Southwest of the Obudu Plateau and immediately to the east of the Afi River Forest Reserve, separated from this reserve by the Mbe Mountains Community Forest (BirdLife International, 2010).

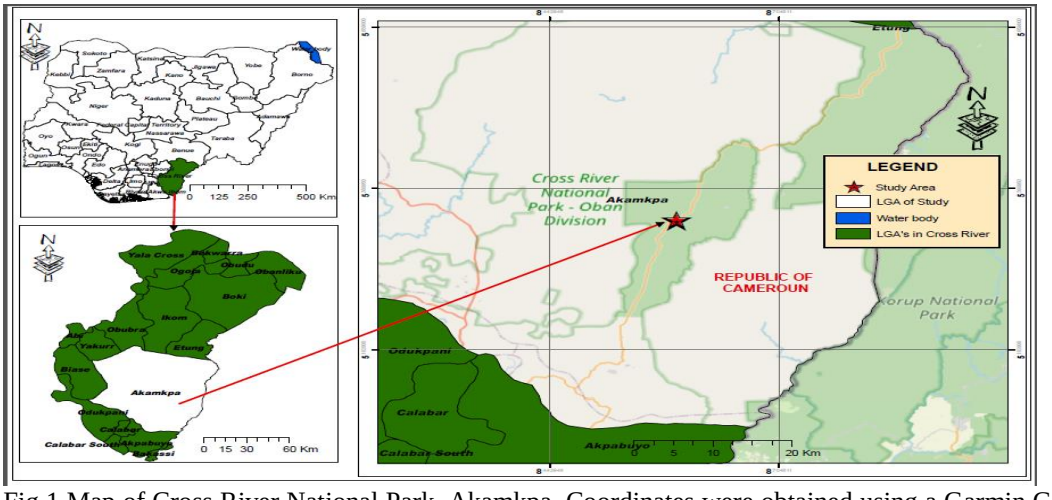


Fig 1 Map of Cross River National Park, Akamkpa. Coordinates were obtained using a Garmin GPS 72H device and the map was generated using ArcGIS software

Extraction of Plant Material

The leaves of the plants were collected, rinsed with water and air-dried at room temperature for 14 days. The dried leaves were pulverised using a milling machine to obtain fine powder. The active ingredients were extracted by percolation using ethanol. Fifty grams (50 g) of each leaf powder was added to 200 ml of ethanol. The mixture was covered and then allowed to stand for 42 hours for extraction. The mixture was then separated by passing through a clean muslin cloth, after which the filtrate was evaporated to dryness in an oven at 35^{o}C. The dried crude extracts were stored in the refrigerator at 4^{o}C under aseptic conditions for subsequent use.

Collection and Preparation of Experimental Animals/ Ethical Clearance

Fifteen (15) Wistar albino rats of both sexes weighing 90–105 g and twenty-five (25) mice of both sexes weighing 70-85 g obtained from the laboratory Animal Unit of the Faculty of Veterinary Medicine, University of Nigeria, Nsukka, Enugu State, were used for the study. They were maintained in accordance with the recommendations of the Guide for the care and use of laboratory animals. The rats were acclimatised for two weeks prior to the study at the animal house of the College of Veterinary Medicine, Michael Okpara University of Agriculture Umudike, Abia State, Nigeria.

The animals were kept at the Animal Laboratory House, Department of Physiology and Pharmacology, College of Veterinary Medicine, MOUAU, and were housed in separate stainless steel cages, given water and feed (Vital Feed® grower's mash) *ad libitum* daily. All the experimental animals were kept under ambient atmospheric and 12-hour light/dark conditions. All procedures were carried out in strict compliance with the institutional ethical instructions for the work, as well as adequate consultation with the Experimental Ethics Committee (EEC) guidelines to laboratory animal care and use contained in NCCLS (1993) and Louhimies (2002).

Acute Toxicity Testing

The crude extract of the leaves of *P. spathulatum* was tested for acute toxicity effect in Wistar rats. The up-and-down method described by Lorke (1983) was adopted for this study. A total of fifteen (15) rats were used for the study. The 15 rats were divided into three (3) groups (A, B and C) of five (5) rats each. An initial stock solution of 100 mg/ml (w/v) concentration in distilled water was prepared. Group A received distilled water at 5 ml/kg, to serve as the control thereafter, while doses of 500 mg/kg and 2000 mg/kg body weight of the crude extract were administered by oral gavage to groups B and C, respectively. The rats were allowed free access to feed and water *ad libitum* and observed for both acute and delayed signs of toxicity and possibly death over a 72-hour period. The median lethal dose (LD_{50}) of the crude extract was then calculated using the formula of Khan *et al.(* 2013):

$$LD_{50} = \sqrt{(\text{least dose with mortality} \times \text{Highest dose without mortality})}.$$

Sub-chronic Toxicity Study

It was carried out according to the method of Rispin *et al.* (2002) and modified by Saganuwan (2015). A total of 25 mice were used. The mice were randomly divided into four (4) groups of five (5) mice each labelled A - D. On a trial basis, three different non-toxic doses (50, 100 and 200 mg/kg body weight) of the plant crude extract were selected. Mice in group A received 0.2 ml of distilled water and served as the negative control while mice in groups C-D were administered orally with the extracts at 50 mg/kg 100 mg/kg and 200 mg/kg body weight, respectively, for a period of 28 days to evaluate the sub-chronic effect of the extract. At the end of the extract administration, the mice were sacrificed and blood was collected for serum biochemical evaluation. The liver and kidney organs were harvested from each mouse for histopathological study. Assay of Aspartate Aminotransferase (AST) activity, Alanine Aminotransferase (ALT) activity and Alkaline Phosphate (ALP) activity were determined by the method of King (1965).

Anti-inflammatory Test

Carrageenan–induced paw oedema

This was done using the volume displacement method of Owoyele *et al*. (2004). The rats were fasted overnight and had free access to water before the day of the experiment but were denied access to feed and water during the experiment. Thirty albino rats were weighed and randomly divided into five groups (A–E) of 6 rats each. Group A was given 10 ml/kg of distilled water (negative control), group B was treated with 20 mg/kg of acetylsalicylic acid (aspirin) (positive control) and groups C, D and E were treated with 100, 200 and 400 mg/kg of the plant extract, respectively. One hour after treatment, paw oedema was induced by injecting 0.1 ml of 0.6% solution of carrageenan into the sub-plantar surface of the hind right paw. Their left paw volumes were measured using the volume displacement method, as control. Thereafter, the right paw volume was determined at 1, 2, 3, 24 hours and 3 days post-treatment. This increase in paw volume was calculated as follows:

The increase in paw volume = Right paw volume - Left paw volume

Histopathological Examination

The liver and kidney tissues were manually processed for histopathological studies after fixing in 10% neutral buffered formalin (NBF) for 48 hr. After dehydration with ethanol and cleared in chloroform overnight, the tissues were infiltrated and embedded with paraffin. Sections of the samples were cut using a rotary microtome (Shandon ThermoFisher, England). The sections were then mounted on glass slides, de-paraffinised in xylene, stained, counter-stained in ethanol and cleared in xylene. The sections were covered with cover slips in dibutylpththalaten-polystyrene-xylene (DPX) moutant for light microscopy. Photomicrographs of the sections were obtained using Motic Images plus 2.0 digital camera.

Statistical Analysis

Analysis of the data obtained was done using Genstat version software. Comparison of data sets and mean separation were done using LSD in one way ANOVA.

RESULTS

Acute toxicity of *P. spathulatum* leaves

After 72 hours of administration of the crude extract and the distilled water, neither signs of toxicity nor death were recorded both in the distilled water group and the crude extract even at the highest dose of 2000 mg/kg. The result of the acute toxicity results showed that the crude extract had a high safety margin ≥ 2000 mg/kg body weight.

Sub-chronic toxicity of *P. spathulatum* leaves

Effect of ethanol leaf extract of *P. spathulatum* on viscerosomatic index (relative organ weight)

The effect of the leaf extract on the relative organ weight of the tested animals and the control is shown in Table 1. The study showed that the plant extract did not affect the weight of the liver, kidney spleen, heart and lung. There was no significant increase or decrease in the weight of the organs between the control group and the extract group even at increasing level of extract administration at 95% confidence level.

Table 1: Effect of *P. spathulatum* on viscerosomatic index (relative organ weight)

Group (Treatment) (mg/kg)	Relative Liver wt(%)	Relative paired kidney wt(%)	Relative spleen wt(%)	Relative Heart wt(%)	Relative lungs wt(%)
A (water)	4.7±0.7	1.6±0.2	0.4±0.1	0.4±0.1	0.4±0.2
B(50)	4.8±0.0	1.7±0.3	0.4±0.0	0.4±0.0	0.6±0.0
C(100)	4.8±1.3	1.6±0.1	0.6±0.0	0.8±0.3	0.7±0.1
D(200)	5.0±0.2	1.5±0.1	0.7±0.1	0.9±0.0	0.7±0.0
LSD	NS	NS	NS	NS	NS

Values are expressed as means of triplicates plus standard deviation

Effect of the leaf extract on enzyme activity

Table 2 shows the effects of different concentrations of the leaf extract on some liver and kidney enzymes. Liver enzymes Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT) and Alkaline Phosphate (ALP) were not significantly altered as the level of extract used increased at 95% confidence level.

Table 2: Effect of the leaf extract on enzyme activity

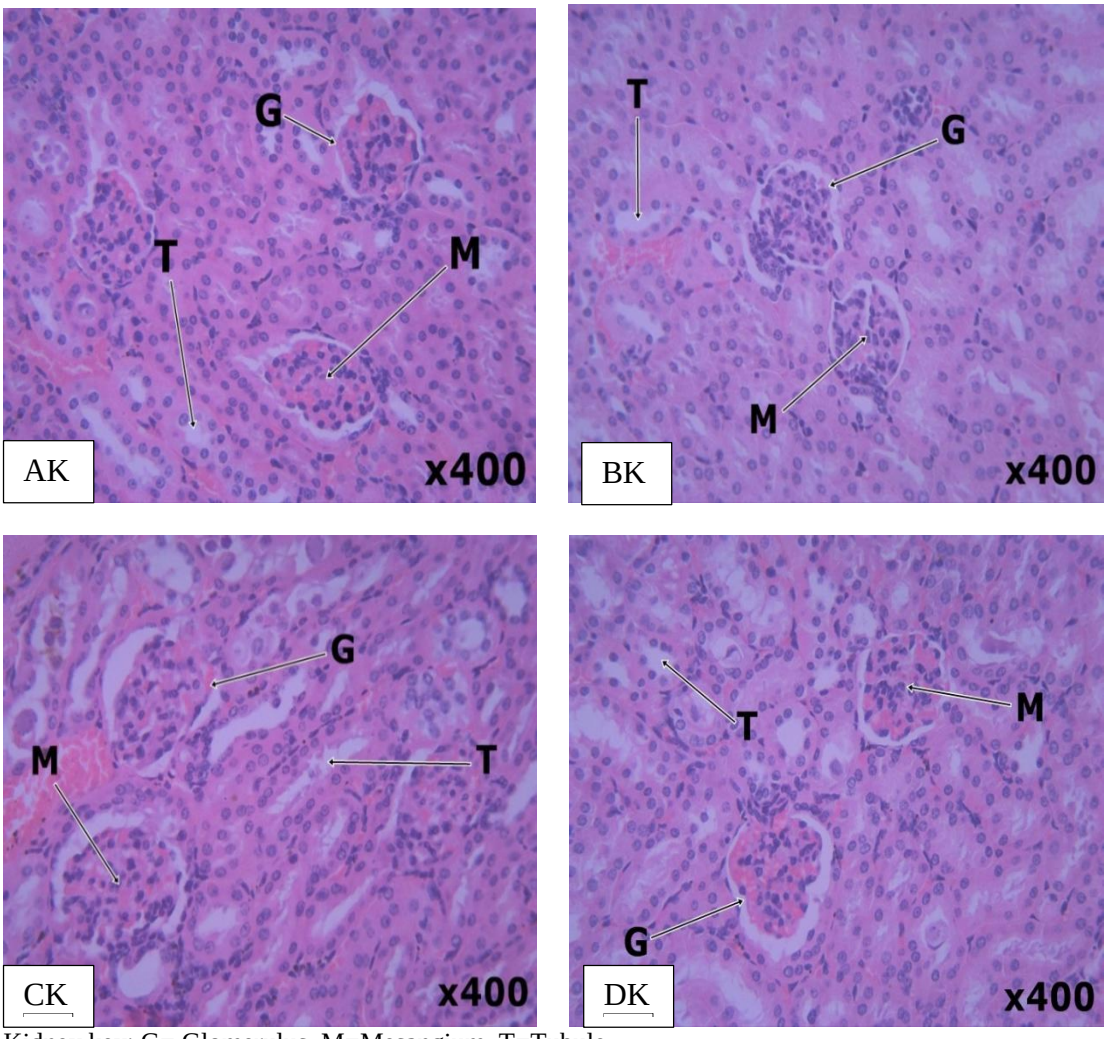
Group (mg/kg)	AST (u/l)	ALT (u/l)	Urea (mg/dl)	Creatinine	ALP (u/l)
A(water)	72.57±2.3	38.22±1.2	18.09±3.3	0.85±0.0	97.27±7.8
B(50)	72.22±1.1	37.57±0.3	13.84±5.3	0.79±0.0	91.06±7.9
C(100)	71.58±1.3	37.80±1.4	19.29±7.6	0.83±0.0	95.35±6.9
D(200)	69.40±1.5	37.78±3.2	18.00±5.0	0.70±0.1	82.57±2.7
LSD	NS	NS	NS	NS	NS

Values are expressed as means plus standard deviation

Table 3: Effects of the leaf extracts of P. spathulatum on kidney of the test animals

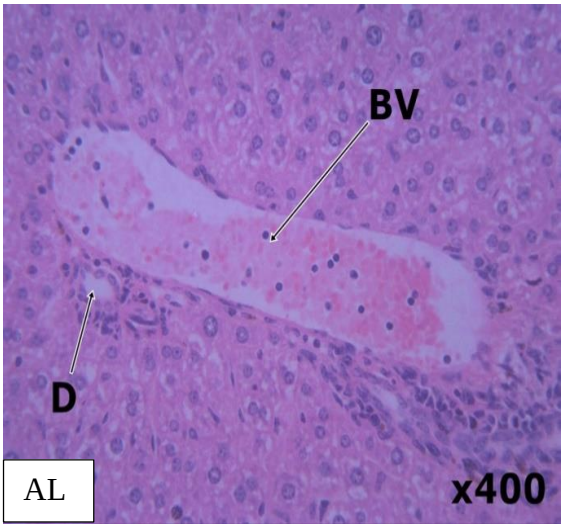
AK	BK	CK	DK
Photomicrographs showed evenly distributed glomeruli (G), of similar size, with normal mesangial (M) cellularity. There are numerous open glomerular capillaries and normal endothelium. The tubules (T) are of normal density and tubular epithelium is viable.	Compared to AK, there is no pathology	Compared to AK, there is no pathology	Compared to AK, there is no pathology

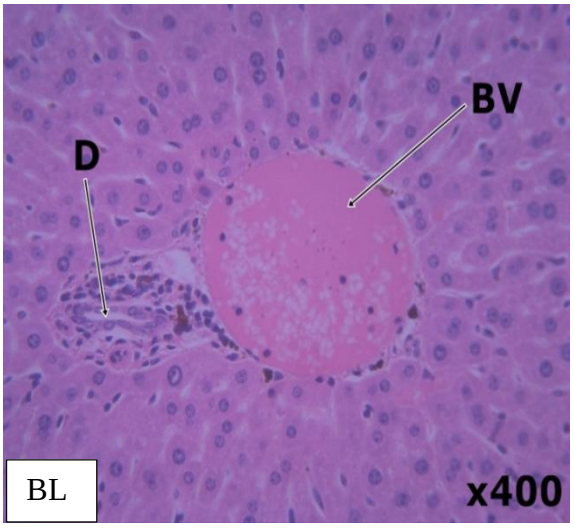
AK = kidney of water-treated rats, BK= kidney of 50mg/kg extract-treated rats. CK= kidney of 100 mg/kg extract-treated rats. DK= kidney of 200 mg/kg extract-treated rats



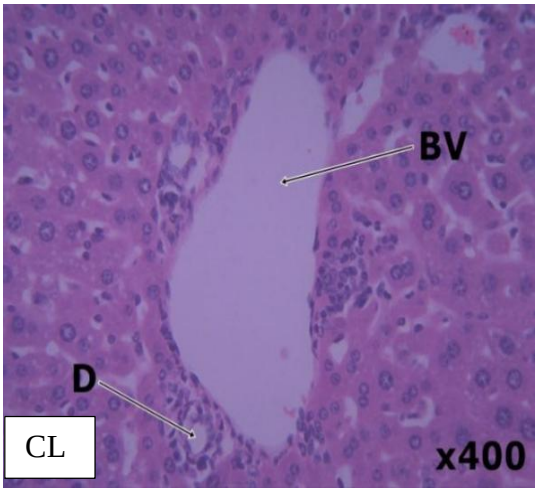
Kidney key: G= Glomerulus. M=Mesangium. T=Tubule

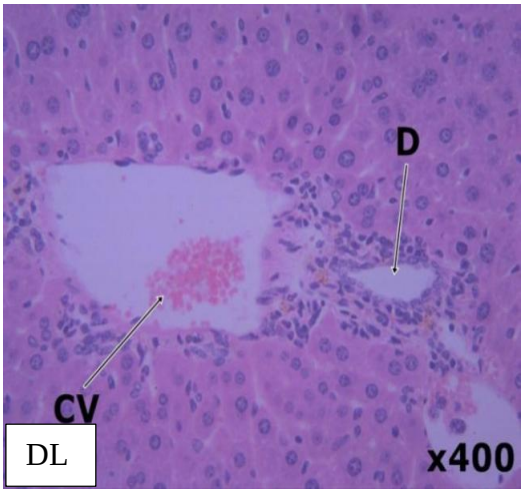
As contained in plates AL-DL, the effects of the leaf extracts of *P. spathulatum* on the liver tissues of the test animals are summarised in Table 3.





AL	**BL**	**CL**	**DL**
Photomicrographs show well-preserved liver architecture. The portal triads are evenly spaced around a central vein and there is neither portal inflammation nor fibrosis. There is no steatosis.	Compared to AL, there is no pathology	Compared to AL, there is no pathology	Compared to AL, there is no pathology





LIVER keys: IC= Inflammatory cells. BV= Blood vessel. D= Hepatic ductile. CV- Central vein

Key: AL = Liver of water-treated rats, BL= Liver of 50 mg/kg extract-treated rats. CL= Liver of 100mg/kg extract-treated rats. DL= Liver of 200 mg/kg extract-treated rats

Anti-inflammatory effect of the leaves of *P. Spathulatum*

Table 4: Anti-inflammatory effect of the leaves of *P. spathulatum*

Extract/ Dose (mg/kg)	Mean decrease in Paw volume (g)					% Inhibition				
	1 Hr	3 Hr	6 hr	24 hr	3 Days	1 Hr	3 Hr	6 Hr	24 Hr	3 Days
Water	0.40	0.68	0.87	0.88	0.88	-	-	-	-	-
Aspirin	0.19	0.55	0.51	0.32	0.07	52.5	23.6	41.3	64.0	92.0
100	0.27	0.55	0.65	0.43	0.22	32.5	23.6	25.2	51.1	75.2
200	0.27	0.58	0.56	0.36	0.16	32.5	17.2	35.6	59.0	81.8
400	0.36	0.57	0.61	0.39	0.23	10.0	19.3	29.8	55.6	73.8

Figures are expressed as mean values of three replicates

DISCUSSION

Plants are considered generally safe; at certain concentrations or volume, they are known to contain natural but potentially toxic substances that are harmful to living tissues (Anyanwu and Okoye, 2017). After administration, neither signs of toxicity nor death were recorded both in the control group and the group administered with the crude extracts (200-2000 mg/kg). The result of the acute toxicity showed that the crude extract was safe at $\geq$ 2000 mg/kg body weight. Clark and Clark (1977) and Ejeh *et al.* (2017) reported that substances with an LD_{50} > 1000 mg/kg are of low toxicity or are relatively safe. Similarly, Saganuwan (2021) reported that an LD_{50} of 50-500 mg/kg is considered toxic. A similar result to this study was reported by Onoja *et al*. (2017), who observed that the extract of *Justicia secunda* was well tolerated in mice as no signs of toxicity or death were noticed at 2 g/kg during the period under review. Tiwari *et al.* (2013) attributed the toxicity, or otherwise, of an extract to the concentration or dose used in a system.

Adeneye *et al.* (2006) noted that the aqueous extract of *Musanga cecropioides* at a higher dose of 3000 mg/kg, was relatively safe toxicologically when administered orally. The safety of a plant extract may be connected to the degradability or digestibility of their by-product in living tissues causing no traceable harm to the system involved. Leaf extracts of *P. spathulatum* could be described as toxically safe for use in ethno- medicine and for other pharmaceutical purposes at the present concentration.

In furtherance to the confirmatory toxicity tests of *P. spathulatum*, the sub-chronic toxicity was determined through the evaluation of enzyme activity, viscero-somatic index and histo-pathological examination. The study showed that the plant extract did not affect the investigated relative organ weight of the test animals. The relative weights of the liver, paired kidney, spleen, heart and relative lung were not significantly altered despite increases in treatment levels. Adeneye *et al*. (2006), working on the extract of *Musanga* showed that the extract did not affect the organ weights, other serum electrolytes, liver enzymes and other haematological indices in test rats. Narhari *et al*. (2015) observed no significant variation in the body and organ weights between the control and the treated group after 28 days of treatment with extract of plant origin. Abiodun *et al.* (2010) reported a significant effect and increase in the biochemical and viscerosomatic index in the plant studied. This may be attributed to the concentration of the extract used.

Liver enzymes, namely Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT) and Alkaline phosphate (ALP) were not significantly altered as the level of extract concentration increased. Kidney enzymes (urea and creatinine) were also not affected by the increase in dose of test drug. Narhari *et al*. (2015) observed a similar unchanged effect on Urea, Adenosine Monophosphate (AMP) deaminase, Creatinine and ALP content by plant extract. The haematological and serum biochemical parameters of the extract *Commelina benghalensis* treated-rats showed no significant change as compared to the control as reported by Tiwari *et al*. (2013).

Onoja *et al*. (2017) used histopathological examination to show the infiltration of mononuclear cells such as plasma cells, lymphocytes, macrophages and occasional eosinophils in liver tissues of cat infested by *Taenia taeniaeformis*. In this study, photomicrographs of the kidney tissues showed that no pathology was induced in the rats treated with different concentrations of the extracts of *P. spathulatum*. Similarly, no pathology was observed in the photomicrographs of the tissues of the liver treated with the plant extract when compared with the control. Photomicrographs showed well-preserved liver architecture; portal triads were evenly spaced around a central vein and there was neither portal inflammation nor fibrosis. There was also no steatosis observed in the test groups. Diallo *et al*. (2010) reported that an absence of significant alterations in the levels of kidney and liver enzymes is a good indicator of liver and kidney functions. This implies that the administration of plant extract did not alter hepatocytes and kidneys of rats. Blood chemical enzymes are the index of kidney and liver function, suggesting that the extract did not induce toxicity to the kidneys and liver. This report is in line with the findings of Narhari *et al.* (2015) in which histopathological evidence showed neither gross abnormalities nor histopathological changes in the mice treated with *Terminalia citrina* leaf extracts.

Documented works abound on the use of plant-based products to cure various ailments (Edeoga *et al.,* 2005; Rodriguez, 2021; Yang *et al.,* 2021; Onoja *et al.,* 2023). Carrageenan-induced rat paw oedema has been widely used for the discovery and evaluation of anti-inflammatory drugs (Mujumdar and Misar, 2004; Udegbunam *et al.,* 2015; Onoja *et al*., 2017). The anti-inflammatory effects of the leaves of *P. spathulatum* showed a mean decrease in paw volume of the rats treated with different concentrations of the leaf extract of the plant as well as the aspirin-treated group when compared with the control group. After one hour of inducing inflammation in the rats using carrageenan, the mean paw decreased from 0.40 g in the distilled water-treated rats to 0.27 g and 0.36 g in 100 mg/kg, 200 mg/kg and 400 mg/kg leaf extract-treated rats, respectively. A dose-dependent decrease was observed in subsequent time-intervals. A similar dose-dependent reduction in anti-inflammation in rats using extracts of plant origin was reported by Ezeja *et al*. (2015). Aspirin, being a refined drug with specificity, was the most efficacious in reducing the paw volume of the inflammatory rat limbs from 0.88 g in water-treated rats to 0.07 g at the three-day period of reading. This report agrees with that of Tiwari *et al*. (2013) who observed close but lesser anti-inflammation of *Commelina benghalensis* to the synthetic drug. Amongst the different levels of plant extract used, 200 mg/kg reduced the inflammation by 81% is against the 92% observed in the synthetic drug, aspirin. This result is in agreement with the study of Tiwari *et al*. (2013), who reported that a dose of 400 mg/kg exhibited significant anti-inflammatory reduction (up to 66% inhibition) compared to the control group of 72% inhibition in mice. Ezeja *et al*. (2015) showed a dose-dependent increase in percentage oedema inhibition from 0% in the negative control group to 41% in the 6th hour at 600 mg/kg. Onoja *et al*. (2017) observed 60% inhibition in paw oedema volume using *Justicia secunda* extract which was also competitive with the reference drugs (aspirin and pentazocine). The anti-inflammatory effect of a plant extract may be due to the inhibition of production and/or migration of inflammatory mediators such as histamine and lysosomes (Rodriguez, 2021).

CONCLUSION

The results from the anti-inflammatory and toxicity studies established the pharmacological evidence for the possible uses of *P. spathulatum* in inflammatory diseases. Oral administration of 2000 mg/kg of the extract in rats did not indicate any toxicity or death showing that the extract is safe. The viscerosomatic index shows that the relative organ weight of the animals was not significantly altered by the extract neither did the extract change the enzyme activity of the kidney and liver tissues. Histological examination revealed no changes in the architecture of the organs investigated, confirming the safety of the extract of leaves of *P. spathulatum*. It could be inferred from the foregoing that the extract of *P. spathulatum* is safe, non-lethal, has potentials in inhibiting tissue inflammation and could be employed for pharmacological purposes.

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