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EFFECTS OF ETHANOLIC LEAF EXTRACT OF *PIPER GUINEENSE* ON THE ORGAN HISTOLOGY OF MALE WISTAR RATS (*RATTUSNOVERGICUS*)

Ani, C. P., Okwuonu, E. S., Aham, C.,* Abasilim, O. R. and Ekeh, F. N.
Department of Zoology and Environmental Biology, University of Nigeria, Nsukka.
Corresponding Author's Email: christopheraham@gmail.com

ABSTRACT
This study focused on the adverse effects of ethanolic leaf extract of *Piper guineense* on selected organs (Kidneys, Liver and Spleen) of male wistar rats. 20 male albino rats were divided into four different groups with 5 rats each and were administered concentrations of 6g/kg, 4g/kg, 3g/kg extract and standard laboratory feed as control, all according to the bodyweight of the animals. The organs were harvested after 28days of extract administration and were collected for histological screenings. Gross examination of the organs showed no differences from the normal. However, histological examination showed marked dose-dependent changes in the organs of the experimental groups. Coagulative necrosis of the tubules, hyperaemic renal vessel, mild centrilobular vacuolar hepatopathy, hepatocellular necrosis, massive haemosiderosis in the red pulp and erythroid hyperplasia in the white pulp were some of the effects seen in the kidneys, liver and spleen respectively. Statistical analysis was done using Dunnet's comparison test to ascertain the effects of extract administration on the weight of the animals. Consumption of *Piper guineense* as a leaf extract or as spice may not have any adverse histological effects on the organs if taken at levels below 3g/kg BW, however, consumption above this dose will cause marked histological changes in the organs as observed in the results of this research

Keywords: Kidneys, Liver, Spleen, *Piper guineense*, Wistar rats.

INTRODUCTION
Piper guineense (Uziza or black pepper) is a West African tropical plant that is widely used as a spice. It has also been known to possess medicinal properties (Iwu, 1993). Spices are fragrant vegetable substances that are used in small amounts as seasonings, preservatives, or flavorings to improve the taste of food, beverages, and pharmaceuticals (Marcel *et al.,* 2011). *Piper guineense*, otherwise referred to as Climbing black pepper, bush pepper or Benin pepper;

belongs to the plant family Piperaceae (Rehm and Espig, 1991; Amusam and Okorie, 2002, Asawalam, 2006).Of the five most cultivated species, *Piper nigrum* is the most common followed closely by *P. longum*, *P. cubeba*, and *P. guineense*. Black pepper is the most intense and flavorful of all peppers, and it comes in whole or cracked peppercorns, as well as powdered form. Black peppercorns are prepared by harvesting the pepper berries when they are half ripe and just ready to turn red, they are then dried until they shrivel and turn to a dark color. Green peppercorns on the other hand are picked while still green and unripe, while white peppercorns are picked when very ripe and then soaked in brine to remove their dark outer shell leaving just the white pepper seed (Jirovetz *et al*., 2002; Adebowale and Olayinka, 2007). Apart from their use as spices and condiments, various studies have demonstrated that these plants have a variety of therapeutic uses in the local treatment and management of ailments. Indigenous people regard the plants for both their ethno-medicinal and culinary applications (Ndukwu and Ben-Nwadibia, 1993). *P. guineense* is used as an anticonvulsant (Abila *et al*., 1993).The fruits and leaves are used as spice for preparing soup for postpartum women. Powder from the dried fruits mixed with honey acts as carminative and relieves stomach aches (DeWitt, 2006).Black pepper extract has also been reported to stimulate the digestion of foods by stimulating the secretion of digestive enzymes, pancreatic amylases, trypsin, and chymotrypsin (Platel and Srinivasan, 2000) and is therefore used for the treatment of stomach upset. The leaves of the plant are used to treat respiratory infections, rheumatism, and syphilis. The leaves are also used to cure male and female infertility while the fruits are used as an aphrodisiac (Mbongue *et al.,* 2005). The pepper is valued not just for its culinary applications as a spice and for its capacity to add flavor to dull foods, but also for its ability to keep foods fresh for long periods (The George Mateljan Foundaion, 2006). *Piper guineense* is also used in traditional African medicine to treat ailments such as typhoid fever, malaria, cough, bronchitis, and rheumatism. The plant's methanolic extracts have also been claimed to be effective in the treatment of bilharzia (Nwaehujor *et al.,* 2014; Echo *et al.,* 2012). It has also been opined to help with convulsions, leprosy, and rheumatoid arthritis (Udoh *et al.,* 1999). To ascertain the effects of the ethanolic leaf extract of *Piper guineense*, the kidney, liver and the spleen are of particular interest.

The kidneys are a pair of organs that clear the blood plasma of metabolic wastes, regulate osmolality of fluids and volume, eliminate foreign chemicals, maintain electrolyte balance, and help maintain the acid-base balance of the body. In addition to these, the kidneys have properties of endocrine organs (Gartner and Hiatt, 2007).Each kidney has three major components: the cortex, the medulla, and the collecting system. In the hemi-sectioned kidney, the outer pale region is the cortex, which has a granular appearance. The medulla is a series of pyramidal structures. Cortical parenchyma extends into spaces between adjacent pyramids and is known as the columns of Bertin. A renal lobe consists of a medullary pyramid with both columns of Bertin as well as the sub-capsular cortex.

The rat liver is a large multi-lobulated gland occupying the anterior third of the abdominal cavity. In rats, the liver mass represents approximately 5% of the total body weight. The mean weight is 6-15 g and its transverse diameter measures from 7.5 to 8.0 cm. The superior-inferior diameter measures from 3.8 to 4.2 cm, while the anterior-posterior ranges from 2.2 to 2.5 cm (Martins and Neuhaus, 2007). The surface of

the rat liver is covered by a thin serosa from which reticular connective tissue invaginate to form the supporting pillars for the blood vessel, bile ducts and hepatic cells (Cook, 2008). The hepatocytes synthesize bile and secret it from the cell into the bile canaliculi. From the hepatic cords, the bile flows towards the portal triad at the periphery of the hepatic lobules (Duker, 2007).The origin and course of the major vessels, supplying the rat liver are similar to those of humans (Martins and Neuhaus, 2007).Most of the blood in the liver (70-80%) comes from the portal vein, arising from the stomach, intestines, and spleen; the smaller percentage (20-30%) is supplied by the hepatic artery (Krause, 2005).

The spleen is the second-largest immune organ in the body and is responsible for initiating immune reactions to blood-borne antigens and for filtering the blood of foreign materials and old or damaged red blood cells. These functions are carried out by the white and the red pulps of the spleen. The spleen is dark red to blue-black in color and is located in the cranial left abdomen. The gross appearance and size of the spleen are variable, depending on the species and the degree of distension; nonetheless, the ratio of splenic to bodyweight remains fairly constant regardless of the weight and in rats, the weight is typically around 0.2% to the body weight of the organism (Losco*et al.,* 1992).The red pulp of the liver is a blood filter that removes damaged erythrocytes. It is also the storage site for iron, erythrocytes, and platelets. In rats, it is a site of hematopoiesis, particularly in fetus and neonates. The spleen is the largest secondary lymphoid organ contributing about one-fourth of the body's lymphocytes and initiates immune responses to blood-borne antigens (Patrick, 2017).

Piper guineense may be causing a variety of histopathological problems that have never before been seen in humans. As a result, the aim of this study is to identify the histological changes incurred by the ingestion of ethanolic leaf extract of *Piper guineense* leaves on selected organs of male wistar rats. The objectives are: to determine the effects of *Piper guineense* on the organ histology of male wistar rats, to identify and compare histological effects of ethanolic leaf extract of *Piper guineense* when consumed at different doses on the selected organs (kidney, liver and spleen), and to successfully harvest the kidney, liver and spleen and study the extent of necrosis and vacuolization in male wistar rats.

MATERIALS AND METHODS

Fresh leaves of *Piper guineense* were purchased from Ogige market, Nsukka, Enugu State, Nigeria. The leaves were identified and confirmed at the Department of Plant Science and Biotechnology, Faculty of Biological Sciences, University of Nigeria, Nsukka. The leaves were left to dry at room temperature (25±2°C). The dried leaves were ground to a fine powder and weighed until a constant weight was achieved. 1kg of the leaf powder was soaked in 1500ml (1.5 liters) of absolute ethanol for 72hours (3 days). The soaked leaves were then filtered out with a tiny spore sieve and filter paper. The filtrate was collected, evaporated under the fan and the fine powder collected. This fine powder is the extract and its concentration is absolute by weighing and re-weighing until it maintains a constant weight. The absolute weight of *Piper guineense* obtained after extraction was 207.3g. The extraction procedure is as described by Harborne (1984) in (Saba and Tomori, 2007).Twenty-nine male wistar rats weighing 120g-200g were purchased from a breeder's farm at Ejuona-Obukpa in Nsukka Local Government Area of Enugu State. They were kept in metal cages with iron netting at the Zoological garden, University of Nigeria, Nsukka, and were let to

acclimatize for two weeks while being fed with growers' mash. Approval to use the wistar rats was obtained from the ethical committee of University of Nigeria, Nsukka, adherence to the full protocol for animal experimentation was observed throughout the period of this study.

Acute toxicity/LD50

Nine of the rats were used for the acute toxicity testing using the method described by Lorke, (1983). The rats were placed in groups of three (3), according to their body weights.

Group 1: 120g
Group 2: 138g
Group 3: 153g

The animals were given graded doses of 100mg, 200mg, and 400mg/kg BW respectively. After four hours, the animals were observed for signs of toxicity.

Experimental Design

The rats were divided into 4 groups of 5 animals; with group 4 serving as the control. Groups 1, 2, and 3 were administered with graded levels of the ethanolic leaf extract of *P. guineense* at graded levels of 6g/kg, 4g/kg, and 3g/kg of body weight respectively for 28 days, while group four was fed with grower's mash (Chicken feed) throughout this period. This high dosage was because adverse effects were not observed during the acute toxicity testing period. The rats in both the test and control group were allowed free access to food and water throughout the period of the experiment. Administration of the extract was carried out orogastrically using an insulin syringe and intubator to determine the actual dose that each animal group received. The animals received their doses every day of the week (including Saturdays and Sundays) for the whole period of the experiment. At the end of the experiment, the animals were sacrificed per group 24hrs after the last administration of doses. They were subjected to ether anesthesia (absolute chloroform). An incision was made on the midline of the ventral surface of the rats to properly harvest the organs and incur minimal damage. In each sacrifice, the kidneys, spleen, and liver were dissected out, labeled, and put into specimen bottles containing 10% formal saline for preservation until tissue processing.

Tissue Processing

The kidney, liver and spleen were harvested from each group and preserved in labeled containers infused with 10% formal saline. The organs were allowed to rest in the solution for 24hrs to enhance fixation. Standard histological procedures were followed – dehydration, clearing, impregnation, embedding, sectioning, and staining. Xylene was used to clear the tissues to remove any trace of alcohol and impregnation with paraffin wax was done at 58°C for 2hrs in the Bionic® oven (BST/HA0-1124, Bionics scientific Laboratory, Delhi). Sectioning of the tissues was conducted using a Rotary microtome of 5-6 microns' thickness (MRS 3500, Histo-line laboratories, Italy). The sectioned tissues were mounted on slides using a thin film slide coated with egg albumen. De-waxing was conducted under a hot plate and then cleared in two changes of xylene. Hydration was performed using varying grades of alcohol after which the slides were stained using hematoxylin and eosin stain. The slides were finally mounted and examined for pathological changes under a photo Olympus Scientific China and Boreal light microscope at different magnifications. Results of the variations were obtained by photomicrography using a digital photographic microscope.

Data Analysis

The data was presented as mean and ± standard error for the control and test groups. The differences between the means were analyzed using one and two-way

ANOVA. Values at $p < 0.05$ were accepted as significant.

RESULTS

The results of toxicity testing showed no toxicity of the extract to the test animals. To assess for toxicity beyond the 2.5g/kg - 3g/kg mark as reported by various researchers, increasing doses of 3g, 4.5g, and 5g/kg BW were administered. The rats were observed for signs of writhing, choking, stretching, or dying. No deaths were recorded even at doses as high as 5g/kg BW.

However, during the orogastric administration of the ethanolic leaf extract, we observed animals in groups 1-3 (6g/kg, 4g/kg, and 3g/kg BW respectively), to be dizzy immediately after administration, but regained agility after a short while. On day 3 of the first week of administration, one animal died in group 2, but this was not as a result of the extract. We presumed this to be because of environmental conditions (the period of study was at the peak of the rainy season –July). No other mortalities were recorded in any of the groups throughout the study. In the first two weeks of dose administration (days 1-14), we observed an increase in weight, however the rats were observed to reduce in weight during the last two weeks (days 15- 28) of the experiment (**table 1** shows the % weight difference). At weeks three and four, the animals were observed to consume food more rapidly than the previous weeks. They increased in agility during this period too (weeks 3-4). They were also noticed to be a little more aggressive during this period (weeks 3-4) especially, animals in group 3 (administered with 3g/kg BW). The mean weight per week interval however explains this significant increase in weight at $p<0.05$.

Table 1: The percentage weight difference per week interval of rats administered with ethanolic leaf extract of *Piper guineense*

		Weight Difference (%)			
Sample	**Dose**	**Week 1**	**Week 2**	**Week 3**	**Week 4**
Extract	6g/kg	11.6	8.5	3.4	-1.7
Extract	4g/kg	10.9	9.9	5.5	-0.8
Extract	3g/kg	11.7	10.9	5.9	-4.1
N. control	5ml/kg	14.8	10.1	8.1	5.5

PHOTOMICROGRAPHY

The results of histological examination of the organs showed marked histological change. In the kidney, cortical hyperemia, massive perivascular eosinophilic infiltration, and coagulative necrosis of the internal kidney structure was observed (**plates 3 and 4**). In the liver, necrosis of the liver cells, hepatocellular degeneration, intravascular edema, and sinusoidal hyperemia were observed in all the test groups (**plates 6-8**). Histological examination of the spleen of test animals shows erythroid hyperplasia (abnormal increase in the number of cells), sever and massive hemosiderosis (abnormal deposit of hemosiderin; a symptom of hemochromatosis), in the red pulp (**plates 10-12**).

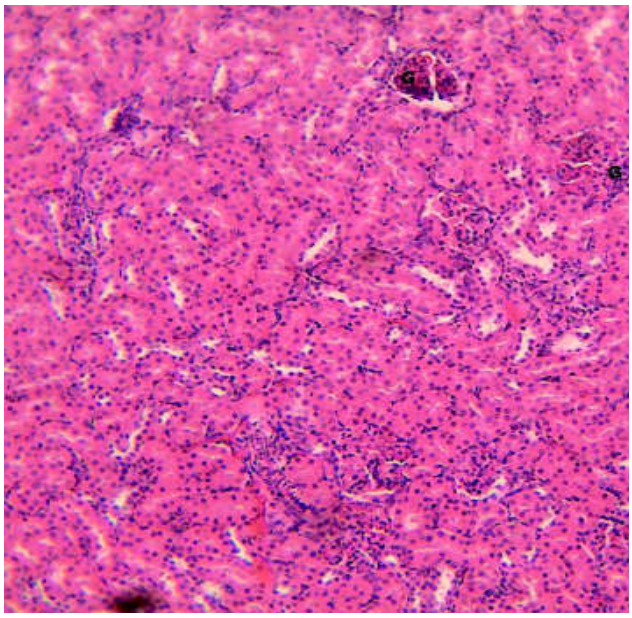


Plate 1: Kidney section for group 4 (control) showing normal glomeruli (G) and renal tubules. H & E ×100.

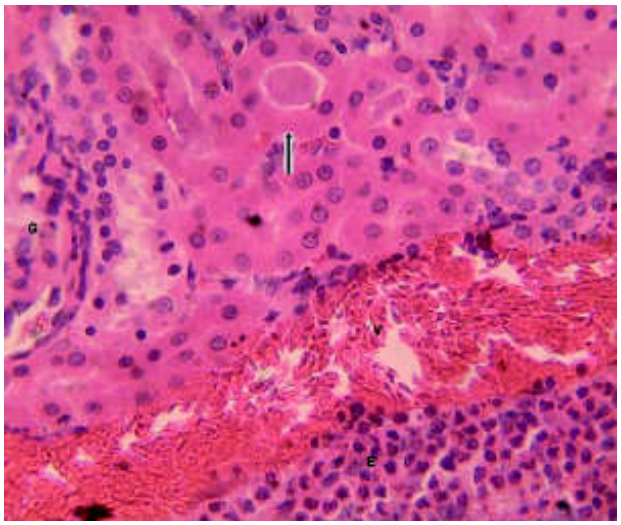


Plate 3: Kidney section for group 2 showing coagulative necrosis of renal tubules(arrows), hyperaemic renal vessel (V), and massive perivascular eosinophilic infiltration (E). Note the glomerulus (G). H & E stain ×400.

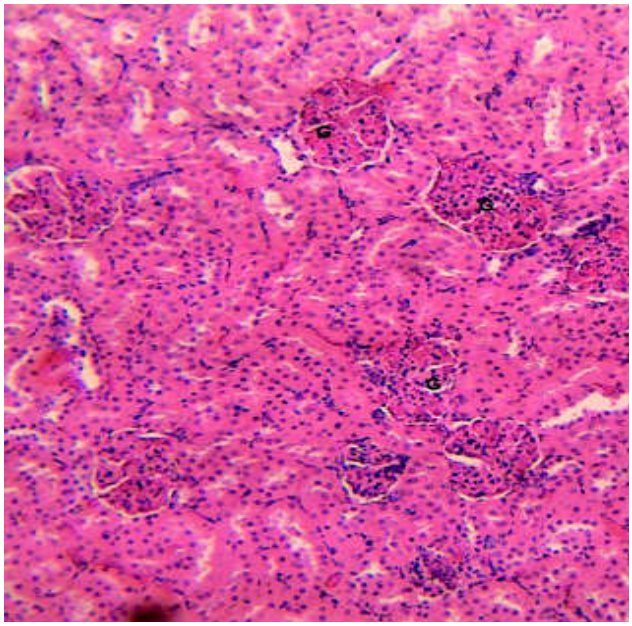


Plate 2: Kidney section for group 3 showing normal glomeruli (G) and renal tubules. H & E stain ×100.

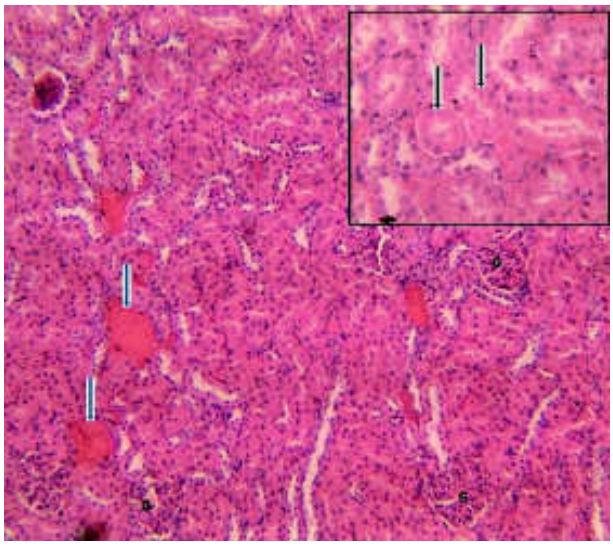


Plate 4: Kidney section for group 1 showing central cortical hyperemia (blue arrows) and renal tubular-necrosis (inset). Note the glomeruli (G). H & E stain ×100.

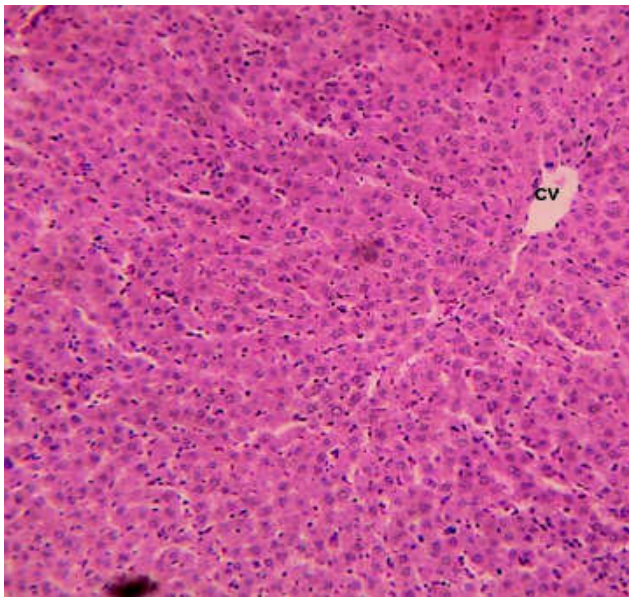


Plate 5: Liver section for group 4 (control) showing normal chords of hepatocytes around the central vein (CV). H & E stain ×100.

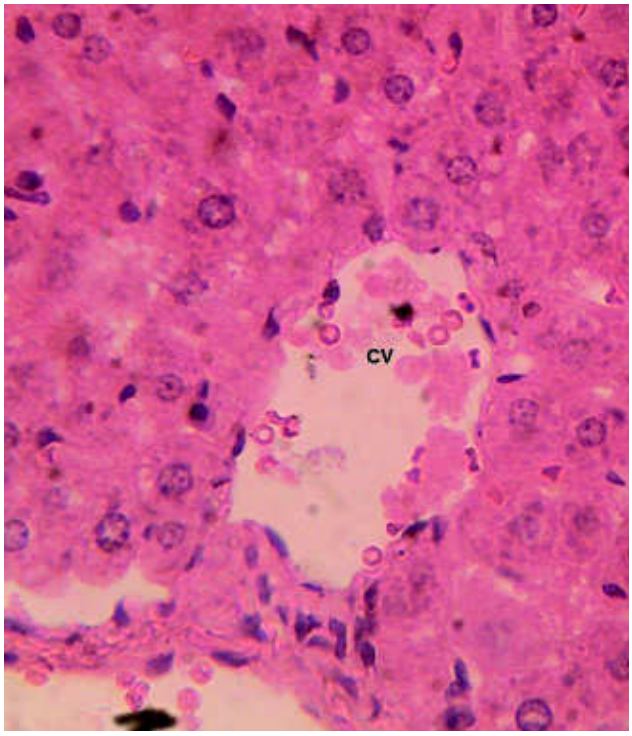


Plate 6: Liver section for group 3 showing mild centrilobular vacuolar hepatopathy and hepatocellular necrosis. Note the central vein (CV). H & E stain ×400.

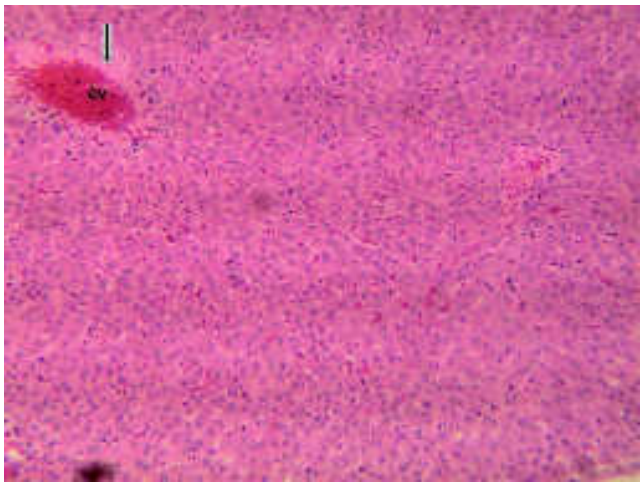


Plate 7: Liver section for group 2 showing severe congestion of the central vein (CV) with intravascular edema (arrows). Note sinusoidal hyperemia and mild random hepatocellular necrosis. H & E stain ×100.

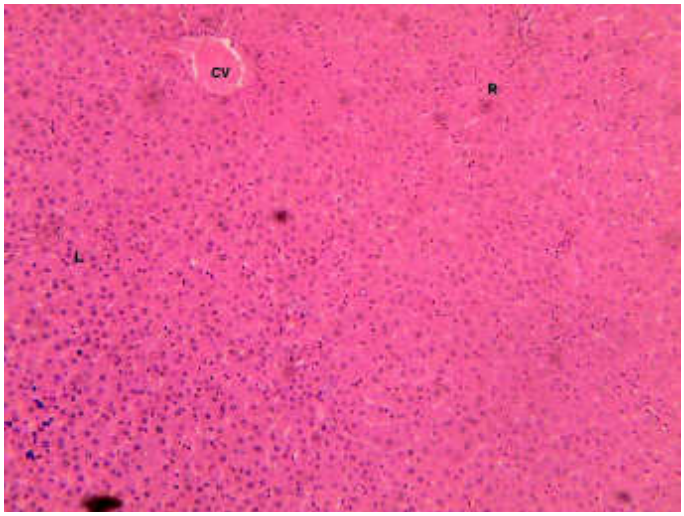


Plate 8: Liver section for group 1 showing a large focal area of hepatocellular degeneration and necrosis (K) and a relatively normal area (L). Note the central vein (CV). H & E stain ×100.

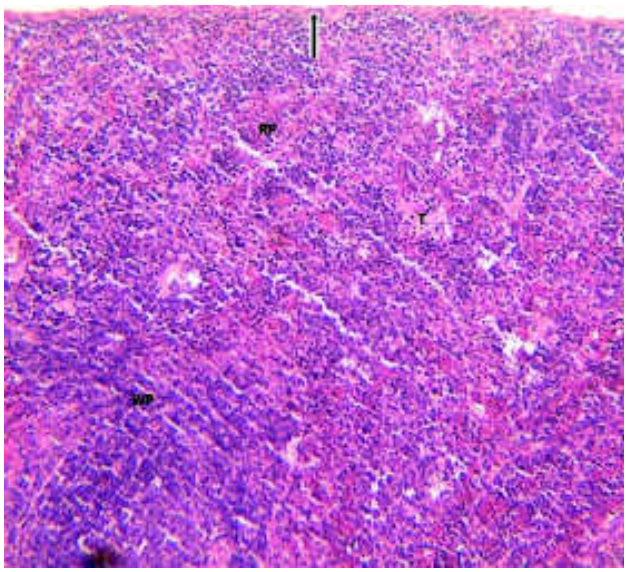


Plate 9: Section of the spleen for group 4 (control) showing the White Pulp (WP) (note that it is moderately hyperplastic), Red pulp (RP), trabecula (T), and splenic capsule (arrow). H & E stain ×100.

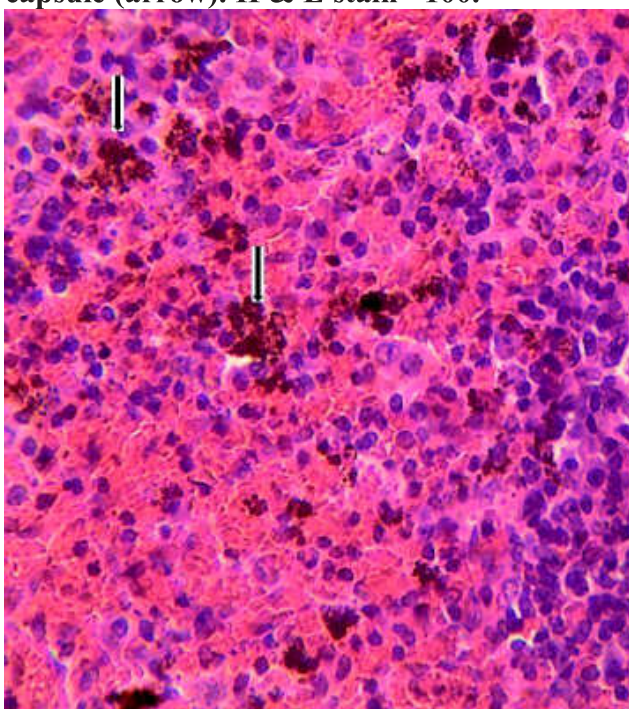


Plate 10: Section of the spleen for group 3 showing massive hemosiderosis in the red pulp (arrows) and erythroid hyperplasia. Note the white pulp (WP). H & E stain ×400.

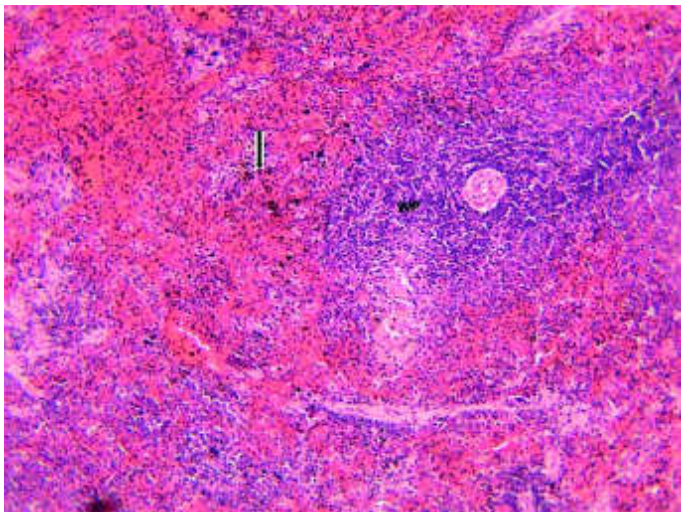


Plate 11: Section of the spleen for group 2 showing erythroid hyperplasia in the red pulp, with haemosiderosis (arrow). Note the white pulp (WP) with the central arteriole. H & E stain × 100.

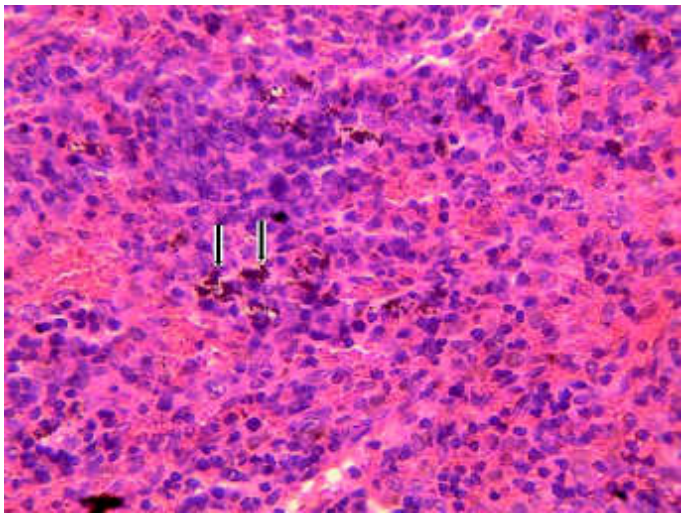


Plate 12: Section of the spleen for group 1 showing erythroid hyperplasia with severe hemosiderosis (arrows) in the red pulp.H& E ×400.

DISCUSSION

The results of the acute toxicity test showed that at doses as high as 5g/kg body weight, the animals showed no signs of toxicity. This suggests that the ethanolic leaf extract of *P. guineense* may not present any adverse effects during general use. The results of the histological examination, showed marked histological changes in the kidney, liver and spleen. Cortical hyperemia, massive perivascular eosinophilic infiltration, and coagulative necrosis of the internal kidney structure, were observed in the kidneys at 4g/kg and 6g/kg BW. Histological changes such as necrosis of the liver cells, hepatocellular degeneration, intravascular edema, and sinusoidal hyperemia were observed at 3g/kg, 4g/kg, and 6g/kg BW. In the spleen, erythroid hyperplasia (abnormal increase in the number of cells), and severe and massive hemosiderosis (abnormal deposit of hemosiderin; often a symptom of thalassemia or hemochromatosis) in the red pulp were observed at doses of 3g/kg, 4g/kg and 6g/kg BW.

The findings of this study differ with the findings of Wassawa *et al.,* (2017), who found good preservation of the sinusoids and no evidence of congestion or necrosis; although this study has gone beyond the study of the effects of *Piper guineense* leaves on just the liver to include the effects of the ethanolic leaf extract on the kidneys, liver and the spleen. This disparity in results could be in part because ethanolic leaf extract was administered. The results of the effects of the leaf extract on the liver however agree with the study by Ebeye *et al.,* (2007), whose study showed necrosis of the liver and other cyto-architectural changes.

The findings obtained from this study also show a significant increase in weight across the test groups, during the first two weeks of dose administration. This agrees with the findings of Wassawa *et al.,* (2017), who reported a significant increase in body weight of the animals in the first two weeks of the experiment. This mean increase in body weight suggests that *Piper guineense* may possess anabolic properties as opined by Mbongue *et al.,* (2005), although continued use of extract may lead to rapid reduction in weight as observed in the weight loss in the fourth week across groups 1-3. The impact of the extract on the weight also suggests that the extract may contain hypo-lipidemic (a lower than normal concentration of lipids in the blood) properties or toxins that impact weight gain.

CONCLUSION

Consumption of *Piper guineense* may have no significant effect on the kidneys, liver, and spleen if consumed at doses as low as 300mg – 500mg/kg BW or 800mg- 2g/kg BW as shown in the results of this research. If consumed intermittently and daily as done in this research, it may have body build-up properties (anabolic properties) as observed in the weight gain of the animals during the first two weeks of administration of doses. Further research however should be done to confirm this.

RECOMMENDATION

Further studies should be carried out on the toxicological effects of the ethanolic leaf extract of *Piper guineense* on wistar rats. The model for the research should also be expanded to include other test models such as guinea pigs and rabbits to better extrapolate the effects on humans.

CONFLICT OF INTEREST

The authors agree that there is no conflict of interest.

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