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Histomorphological and Biochemical Changes Induced in Male Wistar Rats by Chronic Oral Doses of *Piper guineense* Schumach. & Thonn.

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ABSTRACT

Histopathological tissue (liver, kidney and testes) and biochemical changes were studied following chronic oral administration of graded doses of ethanol extract of *Piper guineense* Schumach. & Thonn on male wistar rats. Twenty healthy male rats weighing between 190 – 200 g were randomly divided into four groups (A – D) of five rats per group. Group A (control) were orally dosed once daily with 1 ml of the vehicle (distilled water), while the other three groups were administered with 50, 100 and 150 mg/kg body weight of the extract in 1 ml of the vehicle for 42 days treatment period. At the completion of the extract dosing period, the animals were anaesthetized, blood samples collected for biochemical assay (lipid parameters, liver enzymes and liver function test). Tissues were also excised (liver, kidney and testes) for histopathological examination. The liver of the treated groups showed mild to severe vesicular steatosis (fatty change) when compared to the control group which had well preserved lobular architecture, normal hepatocyte with no indication of inflammation. The kidney of the treated groups revealed varying degree of distortion in microanatomy of the cortex when compared to the control with normal histological features. The testes of the treated groups showed severe distortion of seminiferous tubules, no proper coordination between boundaries and delayed maturation of germ cells when compared to the control group with orderly maturation of germ cells. The values for biochemical assay are mean of five replicates ± standard deviation, the values were analyzed using one way analysis of variance (ANOVA) followed by Posthoc Turkey. The lipid parameters of total cholesterol and low density lipoprotein were significantly increased $p < 0.05$, high density lipoprotein was significantly decreased $p < 0.05$, while there was no significant change in triglyceride $p > 0.05$. All liver enzymes were significantly increased $p < 0.05$. Albumin, globulin, total and conjugated bilirubin were significantly increased $p < 0.05$ while the total protein was not significantly changed $p > 0.05$. Prolonged use of herbal formulation of *p. guineense* may herald hepatotoxicity, nephrotoxicity with impaired sexual/reproductive function in male wistar rats.

Keywords: Biochemical, hepatotoxicity, histomorphology, nephrotoxicity, *Piper guineense.*

INTRODUCTION

There is an upsurge in the use of herbal medicine in the treatment and management of various disease conditions all over the world (Adusei-Mensah and Inkum, 2015; Sen and Chakraborty, 2015). Considering cases of adverse drug reactions (Adesina, 2007), that are associated with synthetic drugs and the disease resistant nature of many antibiotics (Lutz and Lee, 2011) coupled with the expensive nature of these drugs, herbal medicine is thus a panacea to these myriads of problems.

The plant *P. guineense* commonly called climbing pepper belongs to the family Piperaceae, they are found in the high forest where it clings onto trees. It is a slender climber up to 12 m high with prominent nodes and clasping roots, the leaves are elliptic in shape about 15 cm long and 7 cm broad, the flowers are small, borne on common stalk as clusters opposite the leaves. The fruits are red, but turn black when dry (Iwu, 1998). The seeds are stomachic and carminative and are indicated for griping stomach ache (Irvin, 1961). The seeds have also been shown to possess antimicrobial (Okigbo and Igwe, 2007), anticonvulsant, antihypertensive, sedative, tranquilizing and insecticidal properties (Iwu, 1998). The leaves are also used for respiratory infections while the seeds are used as spices and as aphrodisiacs (Kpomah et al. 2012). Many of the herbal medicines are not properly administered and dispensed in terms of dosage and duration of usage. The toxicological and aphrodisiac assessment of the diherbal mixture of Z. leprieurii and P. guineense commonly used as sex invigorator in the South Geo-ecological zone of Nigeria has been previously studied (Kpomah et al. 2012a, b).

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This present study is aimed at investigating the individual effects of chronic oral doses of ethanol extract of the seeds of *P. guineense* on histomorphological tissue and some biochemical parameters of male wistar rats.

MATERIALS AND METHODS

Plant source

The seeds of *P. guineense* were bought from a local herb market in Warri, Delta State, Nigeria. The species was identified and confirmed at the Herbarium of the Department of Plant Science and Biotechnology, University of Port Harcourt, Rivers State. Voucher specimen was prepared and deposited in the herbarium of the same department with voucher No: UPH/V/1277.

Preparation of plant extract

The seeds of *P. guineense* were thoroughly washed with distilled water to remove debris and contaminants, they were then dried in an oven at 40℃ until a constant weight was reached, and then pulverized using an electric blender (Blender, 462 Nakai Japan). 100 g of the powdered *P. guineense* was extracted in 300 mL of absolute ethanol for 24 hours at room temperature with constant shaking using a flask shaker (Denly A - 500). The extract was filtered with Whatman No 1 filter paper and the resulting filtrate evaporated to dryness using a Rotatory evaporator at 40℃ to give 3.64 g, the resultant concentrate was then reconstituted in distilled water to give the required doses used in the study.

Experimental animals

A total of twenty healthy male rats weighing between 190 – 200 g were obtained from the animal house unit of the Department of Biochemistry, University of Port Harcourt, Rivers State. The animals were kept in a clean metabolic cage and housed in a well-ventilated room at temperature 28 - 30℃ under natural light and dark cycle with free access to grower's mash and water for a period of one week to acclimatize prior to the commencement of the experiment. All protocols were performed in accordance with the Institutional Animal Ethical Committee (IAEC) as per the directions of the Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA).

Experimental design

The twenty male rats were randomly divided into four groups (A - D), consisting of five rats each. Group 'A' (control) were orally administered once daily with 1mL of distilled water (vehicle), group(s) B, C, and D were orally administered with 50, 100 and 150 mg/kg body weight of the extract in 1ml of the vehicle for 42 days.

Method of collection and handling of serum, liver, kidney and testes

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At the end of the treatment period of 42 days, the animals were anaesthetized and blood samples collected by cardiac puncture into sample bottles, the blood samples were allowed to clot for 10 minutes at room temperature and subsequently centrifuged to obtain serum for biochemical analysis. The liver, kidney and testes were excised and fixed in 10% buffered formalin in preparation for histopathological examination.

Histopathological examination of the liver, kidney and testes

Histomorphological examination of the liver, kidney and testes for inflammation, degeneration and dearrangement was done using the method described by Krause, (2001).

Biochemical assay

The following liver enzymes were studied to investigate the toxicity of the plant extract on the experimental animals used for the study, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined by the colorimetric method of Reitman and Frankel (1957) using commercial assay kit from Randox Laboratories Ltd, Co. Antrim, United Kingdom. Alkaline phosphatase (ALP) was estimated by the colorimetric method of Rec (1972), using assay kits from Randox Laboratories Ltd. Serum protein and serum albumin were estimated by Biuret method and Bromocresol Green (BCG) binding method respectively using a commercial assay kit from Randox Laboratories Ltd. Serum globulin level was calculated as the difference between total protein and albumin, albumin globulin (A/G) ratio was obtained from the division of the values of albumin and globulin. Total and conjugated bilirubin was determined using commercial kits from Randox Laboratories Ltd, using colorimetric method described by Jendrassik and Grof (1938). The levels of cholesterol and triacylglycerol were estimated by colorimetric method as described by Ochei and Kolhatkar (2008). High density lipoprotein (HDL) was determined spectrophotometrically using commercial assay kit from Biosystems S.A. Costa Brava 30, Barcelona (Spain) by adopting the methods of Grove (1979) and (Tietz, 1990). Low density lipoprotein cholesterol (LDL) was also determined spectrophotometrically using commercial assay kit from Biosystem and adopting the method described by Burstein et al. (1980).

Statistical Analysis

The results are expressed as the mean of five replicates ± standard deviation, means were analyzed using one way analysis of variance (ANOVA) followed by Posthoc Turkey. $p < 0.05$ was regarded as significant. The Statistical Package for Social Sciences (SPSS) version 16 was used for data analysis

RESULTS

Effects of *P. guineense* on Histomorphology of the Liver

The effects of the graded dose of the extract in comparison with the control group are shown in plate 1A, 2A, 3A and 4A respectively

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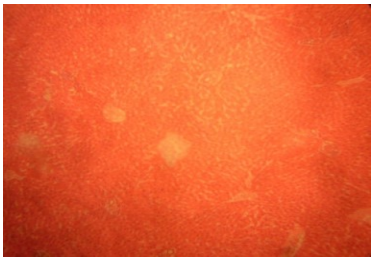

Plate 1A: Photomicrograph of liver administered with distilled water (control). Normal liver with well-preserved lobular architecture, normal hepatocytes, normal central vein, capsules with no indication of adhesion or inflammation. (H & E x 40).

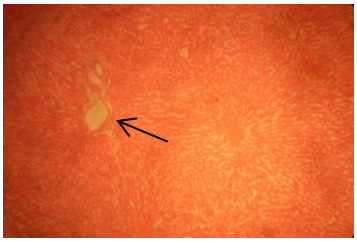

Plate 2A: Photomicrograph of liver administered with 50 mg/kg body weight of *P. guineense*. Arrow showing liver with microvesicular steatosis (fatty change). (H & E x 40).

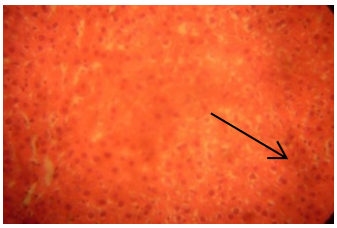

Plate 3A: Photomicrograph of liver administered with 100mg/kg body weight of *P. guineense*. Arrow showing liver with macrovesicular steatosis (fatty change). (H & E x 40).

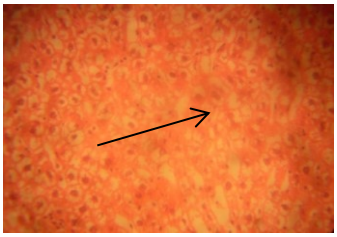

Plate 4A: Photomicrograph of liver administered with 150mg/kg body weight of *P. guineense*. Arrow showing liver with macrovesicular steatosis (fatty change). (H & E x 40)

Effects of *P. guineense* on histopathology of kidney

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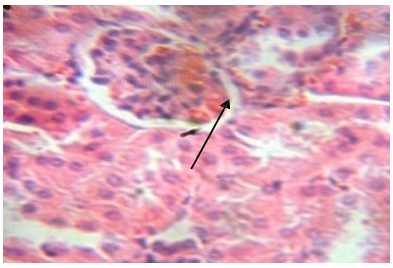

Plate 1B: Photomicrograph of kidney administered with distilled water (control), kidney section shows normal histological features indicating detailed cortical parenchyma and renal corpuscles (H & E × 40)

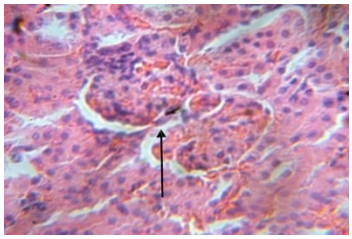

Plate 2B: Photomicrograph of kidney administered with 50 mg/kg body weight of *P. guineense*, kidney histology revealed mild degree of distortion in microanatomy of the renal cortex (H & E × 40)

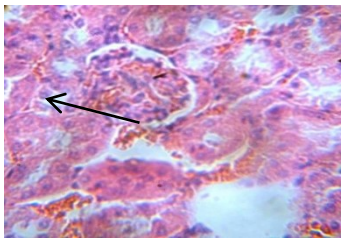

Plate 3B: Photomicrograph of kidney administered with 100 mg/kg body weight of *P. guineense*, kidney histology revealed moderate degree of distortion in microanatomy of the renal cortex (H & E × 40)

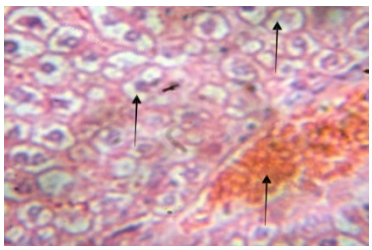

Plate 4B: Photomicrograph of kidney administered with 150 mg/kg body weight of *P. guineense*, kidney histology revealed severe degree of distortion in microanatomy of the renal cortex (H & E × 40)

Effects of *P. guineense* on histopathology of testes

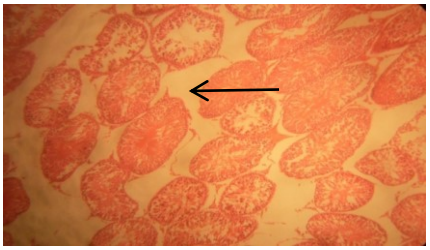

Plate 1C: Photomicrograph of testes administered with distilled water (control). Arrow showing testes with seminiferous tubules containing orderly maturation of germ cells (Normal spermatogenesis), (H & E × 40).

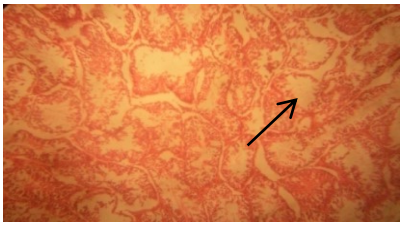

Plate 2C: Photomicrograph of testes administered with 50 mg/kg body weight *P. guineense*. Arrow showingtestes with mild distortion of seminiferous tubules and no proper coordination between boundaries (H & E X 40).

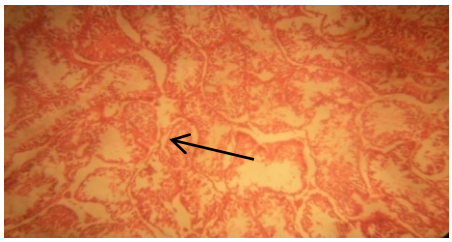

Plate 3C: Photomicrograph of testes administered with 100 mg/kg body weight of *P. guineense* arrow showing testes with severe distortion of seminiferous tubules and delayed maturation of germ cells. (H & E X 40).

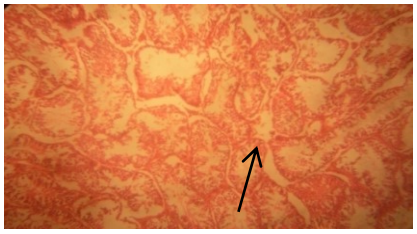

Plate 4C: Photomicrograph of testes administered with 150 mg/kg body weight of *P. guineense* arrow showing testes with severe distortion of seminiferous tubules, no proper coordination between boundaries and delayed maturation of germ cells. (H & E X 40)

Effects of *P. guineense* on some Biochemical Parameters of the Rats
Effects of *P. guineense* on Lipid Parameters

TABLE 1.0: Effect of graded doses of P. guineense on lipid parameters

EXPERIMENTAL GROUP	TC (mg/dL)	TG (mg/dL)	LDL (mg/dL)	HDL (mg/dL)
CONTROL 1ml distilled H_2O	161.00 ± 3.39[a]	122.60 ± 3.97[a]	111.00 ± 2.24[a]	59.20 ± 2.77[a]
50mg/kg body weight	185.20 ± 4.76[b]	121.20 ± 2.39[a]	119.60 ± 2.70[b]	50.80 ± 2.77[b]
100mg/kg body weight	202.60 ± 4.56[c]	119.00 ± 2.35[a]	133.80 ± 3.42[c]	49.60 ± 2.41[c]
150mg/kg body weight	244.60 ± 3.85[d]	116.40 ± 3.05[a]	163.00 ± 3.16[d]	46.40 ± 3.05[d]

Values are mean of five replicates ± standard deviation, values in the same column with superscript letter(s) b, c and d are significantly different from the control group with superscript letter "a" ($p < 0.05$) (One way ANOVA followed by Posthoc Turkey). TC: total cholesterol; TG: Total triglycerides; LDL: Low density lipoprotein; HDL: High density lipoprotein.

Effects of *P. guineense* Liver Enzymes

Table 2.0: Effects of *P. guineense* on liver enzyme activity of rats

EXPERIMENTAL GROUP	AST (U/L)	ALT (U/L)	ALP (U/L)
CONTROL 1 ml distilled H_2O	20.20 ± 1.92 [a]	21.00 ± 2.00 [a]	30.40 ± 2.70 [a]
50 mg/kg body weight	24.40 ± 2.24 [a]	28.00 ± 2.92 [b]	40.40 ± 3.21 [b]
100 mg/kg body weight	29.60 ± 2.97 [b]	31.00 ± 2.74 [c]	50.20 ± 3.49 [c]
150 mg/kg body weight	34.00 ± 2.55 [c]	36.80 ± 2.39 [d]	59.60 ± 3.97 [d]

Values are mean of five replicates ± standard deviation, values in the same column with superscript letter(s) b, c, and d are significantly different from the control group with superscript letter "a" ($p < 0.05$), (One way ANOVA followed by Posthoc Turkey). AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase.

Effects of *P guineense* on liver function

Table 3.0: Effects of oral doses of ethanol extract of *P. guineense* on liver function of rats

EXPERIMENTAL GROUP	TP (g/dL)	ALB (g/dL)	GLO (g/dL)	A/G	TB (nmol/L)	CB (nmol/L)
CONTROL 1ml distilled H_2O	6.60± 0.23[a]	4.12± 0.33[a]	2.54± 0.43[a]	1.69 ± 0.50[a]	6.60 ± 1.14[a]	1.10 ± 0.16[a]
50mg/kg body weight	6.64 ± 0.19[a]	3.44 ± 0.29[b]	3.20 ± 0.19[b]	1.08 ± 0.15[b]	13.00 ± 1.00[b]	1.36 ± 0.11[a]
100mg/kg body weight	6.64 ± 0.21[a]	3.10 ± 0.23[c]	3.54 ± 0.36[c]	0.89 ± 0.16[c]	14.00 ± 1.58[c]	1.68 ± 0.16[b]
150mg/kg body weight	6.72 ± 0.22[a]	2.78 ± 0.31[d]	3.94 ± 0.26[d]	0.71 ± 0.12[d]	15.20 ± 1.64[d]	2.04 ± 0.10[c]

Values are mean of five replicates ± standard deviation, values in the same column with superscript letter(s) b, c and d are significantly different ($p < 0.05$) from the control with superscript letter "a". (One way ANOVA followed by Posthoc Turkey). TP: total protein; ALB: albumin; GLO: globulin; A/G: albumin globulin ratio; TB: total bilirubin; CB: conjugated bilirubin.

DISCUSSION

Alterations in the concentration of major lipids like cholesterol, high density lipoprotein, low density lipoprotein and triglyceride could give useful information on lipid metabolism as well as predisposition of the heart to atherosclerosis and its associated coronary heart disease (Assmann et al. 1984; Yakubu et al. 2009). A number of chemical trials evaluating the risk factors associated with coronary heart disease (CHD) including the role of lipoproteins and lipid metabolism have established that lowering cholesterol reduces death and myocardial infarction in patients with CHD (Yakubu and Afolayan, 2009). Low density lipoproteins are the major artheriogenic lipoprotein and usually account for most of the CHD associated with elevated plasma total cholesterol (CARE, 1996). High density lipoprotein cholesterol protects against CHD, the risk of CHD from arteriosclerosis is inversely proportional to serum levels of HDL (Gordon and Rifkind, 1989; Levine et al. 1995). High triglyceride is associated with high CHD, stroke, diabetes, obesity etc. and it is an indication of cirrhosis, hyperlipoproteinemia, hyperthyroidism, low protein and high carbohydrate diet, poorly controlled diabetes mellitus and pancreatitis (Kruger, 1998). Low levels of triglyceride indicate malnutrition, malabsorption hyperthyroidism, low fats diet and terminal disease (Kruger, 1998). The increase in total cholesterol after 42 days of treatment by the extract at various doses may be due to increase in concentration of cholesterol substrate like Acetyl CoA which may arise from β - oxidation of fatty acids. Long term usage of 100 and 150 mg/kg body weight of *P. guineense* may not be beneficial, as it may predispose the animals to atherosclerosis and hypertension. Findings from this study showed that all doses of the extract had no significant effect ($p > 0.05$) on triglyceride levels but had significant increase ($p < 0.05$) on low density lipoprotein cholesterol (LDLc) levels, this may however not be too healthy for the animals. In the same vein all doses of the extract showed significant decrease ($p < 0.05$) on HDLc, the decrease in HDLc correlates with increased CHD (Enas, 1999; Mayes, 2000).

The liver is the largest, most active and most complex organ in the body, it is basically involved in the regulation, storage and secretion of important nutrients (carbohydrates, proteins, and lipids) and chemicals, as well as the biotransformation of xenobiotics (Garba et al. 2009; Farah et al. 2012; Bhushan et al. 2013). Hepatic insult associated with some toxic phytochemicals found in medicinal plants and the inability to eliminate these metabolic products by the liver often results in marked distortion of the normal function of the liver (Panagiotaks, 2003). Plasma enzyme assay is a vital tool in the toxicological assessment of herbal plants, increase in these enzymes is directly proportional to the degree of tissue damage (Geidam et al. 2004; Ige et al. 2011), due to loss of functional integrity of tissue cell membrane which therefore results in their leakage into the blood stream (Chatterjea and Rana, 2005). AST abound in high concentration in hepatic, renal, cardiac, skeletal muscle cells and erythrocytes thus damage to any of these tissues may increase the plasma AST levels (Lyoussi et al. 2004; Crook, 2006; Lahon and Das, 2011). ALT in the serum is often associated with hepatocellular damage (Geidam et al. 2004; Saka et al. 2011). ALP is a sensitive detector in biliary circhosis, hepatitis and in diseases characterized by inflammation, regeneration, intrahepatic and extrahepatic bile obstruction, osteoblast of bone (Ige et al. 2011; Roland, 2014). After 42 days of treatment, all doses investigated of the extract had significant effect ($p < 0.05$) on AST, ALT and ALP levels and one can suggest that the extracts after 42 days had marked adverse effect on the liver and kidney of the treated rats.

Albumin plays a central physiological role by maintaining osmotic pressure, transport of both endogenous and exogenous substances and serving

as protein reserves. (Panthong et al. 2003). The liver's ability to synthesize albumin and globulin is reduced if the synthetic function of the liver is tampered with (Saidu et al. 2007), and it is an indication of hepatitis and liver cirrhosis (liver damage). After 42 days of extracts dosing serum albumin, globulin and Albumin/globulin ratio were all significantly affected ($p < 0.05$), which is an indication of the liver's impairment to carry out its normal metabolic functions as a result of plant chemicals found in the herbal extract. Bilirubin is formed by the breakdown of haemoglobin in the liver, spleen and bone marrow (Whitby et al. 1989) An increase in tissue or serum bilirubin concentration results in jaundice and it occurs in toxic or infectious disease of the liver e.g. hepatitis or bile obstruction (Vasudevan and Sreekumar, 2005). Bilirubin measurement is also a useful index of determining the excretory function of the liver and assessment of haemolytic aneamia. After the 42 days treatment, both total and conjugated bilirubin were significantly affected ($p < 0.05$) and it is an indication of adverse haemoglobin metabolism and liver function of the treated rats (Edem and Usoh, 2009).

The results obtained for liver enzymes (AST, ALP and ALT) and liver function (ALB, A/G, TB and CB) was however corroborated by results of the histology of the liver and the kidney. The histology of the liver showed micro vesicular steatosis at 50mg/kg body weight of the extract and macro vesicular steatosis (fatty change) for the 100 and 150mg/kg body weight of the extract after 42 days. It is worthy of note that it is sometimes necessary to evaluate renal function alongside the liver function in the assessment of toxicity of phytochemicals present in medicinal plants (Lahon and Das, 2011), thus histomorphological examination of the kidney of the controlled group revealed kidney section with normal histological features indicating detailed cortical parenchyma and renal corpuscles **(Plate 1B)**, while the treated groups with 50, 100 and 150mg/kg body weight **(Plate 2B, 3B and 4B** respectively**)** revealed varying degree of distortion in microanatomy of the renal cortex as this is largely due to the nephrotoxic effect of the extract which may eventually tamper with the normal physiological function of the kidney. Histomorphological examination of the testes of the controlled group **(Plate 1C)** showed cells with seminiferous tubules containing orderly maturation of germ cells and normal spermatogenesis, however the various doses 50, 100, and 150 mg/kg after 42 days of treatment showed **(Plate 2C, 3C and 4C**) testes with mild distortion, slight delay in maturation of germ cells, to severe distortion and delay in maturation of germs cells, this may however, have a negative effect on gametogenesis and sexual function on the extract treated groups.

Conclusion

Chronic oral exposure (abuse) should be greatly discouraged, as results obtained from this study indicated that chronic oral ingestion of ethanol extracts of *P. guineense* induced adverse change in the microstructural integrity of the liver, kidney and testes as well as marked significant change in the biochemical parameters of plasma enzyme, lipid parameters and liver function parameters, thus prolonged usage of the extracts from the seeds of *P guineense* at the various dose studied can interfere with normal hepatic, renal, reproductive function and lipid metabolism with implications on cardiovascular health.

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