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28 Days Sub Acute Toxicity Studies of the Methanol Stem Bark Extract of *Combretum hypopilinum Diels* (Combretacea) in Rats

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Abstract

The aim of this study was to evaluate the safety profile of *Combretum hypopilinum* stem bark extracted with Methanol (70%v/v). Preliminary Phytochemical screening of the crude methanol stem bark extract was carried out, and revealed the presence of secondary metabolites such as steroids, flavonoids and alkaloids. *Initial oral acute toxicity test was carried out using the Limit Dose Test to ascertain the safety of the extract in rats. Sub-acute toxicity testing was conducted by 28 days oral administration of* 400 mg/kg, 800 mg/kg and 1600 mg/kg body weight to three groups of ten rats. The fourth group was administered distilled water 10 ml/kg. No major changes were observed in body weight of the animals following 28 days of daily oral administration. Biochemical parameters such as Total Protein, Total Bilirubin, Creatinine, Aspartate Transaminase (AST) and Alanine Transaminase (ALT), were found to be within normal ranges. The levels of marker enzymes in the vital organs did not show any significant changes between control and treated groups. Histopathological examination of the major vital organs (liver, brain, and kidney) revealed no significant pathological changes in the treated groups of rats. The results of the present work suggested that the methanol stem bark extract of *Combretum hypopilinum* is relatively safe for use at the tested doses.

Keywords: Toxicity, Methanol stem bark extract, *Combretum hypopilinum, sub*-acute, *aspartate* transaminase.

Introduction

The traditional use of medicinal plants in the treatment of diseases is as old as the existence of man. This practice was the major means of disease management practiced by more than 80% of people in Africa (Ekor, 2014; Merriam 2013). Recent reports confirmed that traditional medicine use in Africa is lower than the previously quoted figure (Oyinlola *et al*., 2016). There exist more than 250,000 species of plants and many of these have been reported to have therapeutic values. Such plants are therefore used for treatment of different ailments that afflict the society (Saleh *et al.,* 2015). Plants have been known to have both biological and therapeutic effect. An aspect of the biological effects could be its toxic effect. There is a need therefore to evaluate the toxicity of any plant or part used as a therapeutic agent as this will be followed by a recommendation from the researcher on the appropriate safe dose of the regimen. *Combretum hypopilinum* (Combretaceae) is a small to medium-sized, semi-deciduous tree of about 6-13 m in height, and has a rounded heavy crown. The shoots of the plant are covered with short, soft hairs and the bark is light grey, reddish-brown or brown-black, fissured, transversely cracked, with smooth scales of various sizes. The leaves are opposite to alternate, simple, narrowly elliptic to broadly ovate. *Some documented uses of the plant include;* use traditionally, in the treatment of epilepsy. In Africa, the dried leaves and root bark of the plant are used as purgatives and diuretics (Eloff, 1999). The plant leaves infusion is also used to treat gastrointestinal disorders, such as stomach ache, dysentery, and diarrhoea (Kokou

et al., 2018). Fresh roots of the plant are employed in treatment of lungs problems and these include: tuberculosis, cough, and bronchitis, and also snake bites and jaundice. Infertility in men and women, and gonorrhoea are treated with maceration or decoction of root bark. Roots and leaves infusions are used as a blood tonic (Kokou *et al.,* 2018).

Regardless of the widespread research on the stem bark extract of *Combretum hypopilinum*, a scarcity of information on the toxicological profile of this plant still exists. This work is therefore aimed at evaluating the acute and sub chronic toxicity effects of the extract on rats.

Materials and Methods

A sample of the plant of *Combretum hypopilinum* was collected from Zuru Local government area, Kebbi State, Northern-Nigeria. The plant was authenticated by Dr. Mshelia H.E. and assigned the voucher number PCG/UDUS/Mor/0001. Subsequently the stem bark of the plant was collected, washed with distilled water, shade-dried, pulverized, labelled and stored in air-tight container prior to extraction.

The powdered plant material (500g) was continuously extracted with 2000mL of 70% methanol by maceration for three (3) days; extract obtained was filtered and concentrated in a rotary evaporator at 45°C. A brown product called methanol stem bark extract of *Combretum hypopilinum* was obtained. The extract was dried in an oven at a temperature of 40-45°C to remove any remaining moisture and stored at room temperature

Six to eight weeks old, Wistar rats weighing (160-200g) were obtained from the Animal House facility of the Department of Pharmacology and Therapeutics, ABU, Zaria. Animals were allowed to acclimatize prior to the study. During acclimatization, the animals were housed in groups of five in separate cages, with free access to animal feeds (from Excel Feeds Kaduna, Nigeria) and water *ad libitum.* The approval for the use of animals was obtained from the Department of Pharmacology and Toxicology, UDUS Animal Ethics Committee and assigned the number (PTAC/Nh (MO)/008-18**).**

Acute toxicity study

According to the OECD guideline 423 for acute oral toxicity studies, ten animals of equal numbers of male and female rats were used and each received a single oral-dose of 2000 mg/kg. The rats were fasted overnight and administered with a single oral dose of 2000 mg/kg of the extract one at a time (OECD, 2002). Each rat was observed for signs of toxicity and mortality every 30 minutes for the first four hours and then two hourly for forty-eight hours. Each animal was further observed daily for fourteen days. Since all the animals survived, the limit dose was terminated and the LD_{50} was assumed to be greater than 2000 mg/kg.

Sub-Acute toxicity study

The sub chronic toxicity of the methanol extract of *Combretum hypopilinum* was carried out by method described by OECD Test Guidelines 408. A total of forty rats of both sexes were employed. *Combretum hypopilinum* methanol stem bark extract was weighed and solubilised in water. The rats were randomly divided into 4 groups of 10 rats each. Group 1, control group, received orally normal saline 0.5mL on daily bases for 28 days. The extract was administered orally at doses of 400, 800 and 1600 mg/kg-body on daily bases for 28 days to group 2, group 3, and group 4 respectively.

The behaviour of the animals was monitored daily for any abnormality, and their weights were recorded once per week. At the end of the experiment (28 days), all the rats were anaesthetized under diethyl ether inhalation and their blood samples were collected via cardiac puncture into non-heparinized and EDTA containing tubes for biochemical and haematology analysis respectively (Diallo *et al.,* 2010). All rats were sacrificed after the blood collection; the internal organs and some tissues were weighed to determine relative organs weights and observed for gross lesions. All tissues were preserved in 10% neutral buffered formalin or Bouine solution, for histopathological examination.

Haematological analysis was performed at the Haematology Laboratory of Usmanu Danfodiyo University Teaching Hospital (UDUTH) Sokoto.

Biochemical analyses were performed at the Chemical Pathology Laboratory of Usmanu Danfodiyo University Teaching Hospital (UDUTH) Sokoto. A histological analysis was also performed at the Department of Veterinary Anatomy Laboratory Usmanu Danfodiyo University Sokoto and Department of Pathology (UDUTH) Sokoto. Histology was conducted on three organs: liver, kidney and brain.

Statistical analysis

Results were expressed as mean ± standard error of mean (S.E.M.). Statistical significance was determined by one-way analysis of variance (ANOVA) and post hoc Dunnett's test. *P* value less than 0.05 were considered significant.

Results

Extraction of *Combretum hypopililnum* yielded 85.5g of extract which is equivalent to 17.10%. The methanol stem bark revealed the presence of Steroids, Glycosides, Flavonoids, Tannins, Alkaloids, Saponins, Carbohydrates, Reducing Sugars, and Proteins.

Acute toxicity studies

The median lethal dose (LD_{50}) in rats was found to be >2000mg/kg body weight. Both female and male rats fed with the extract at a dose of 2,000 mg/kg did not show any signs of toxicity in the entire period of observation.

Effect of the methanol extract of *Combretum hypopilinum on* Body Weight changes following 28 Days Sub chronic Oral Treatment in Wister Rats

There was a general increase in body weight of the treated animals. However, the increases were not significant (Table 1).

Table 1: Results of Body Weight (g) changes of Rats following 28 days oral administration of methanol stem bark extract of *Combretum hypopilinum*

Treatment (mg/kg)	Week 0	Week 1	Week 2	Week 3	Week 4	Total Weight Gain on day 28
N/S	100.71±7.74	117.29±7.65	123.57±7.40	129.71±6.41	133.57±5.50	32.86
400CHME	89.57±5.21	93.71±5.52	97.00±6.04	105.14±7.79	109.43±7.72	19.86
800CHME	115.57±4.25	110.29±7.33	115.00±8.70	123.29±7.61	128.14±6.91	12.57
1600CHME	102.29±6.25	123.43±4.86	123.29±5.29	125.57±6.27	128.14±6.22	25.85

CHMSE= *Combrettium hypopilinum* crude methanol stem bark extract; N/S= Normal saline (n=10) *=$p<0.05$; compared to control**.** One way ANOVA followed by Dunnett's Post *hoc.*

Effects of the Methanol Stem Bark Extract of Combretum hypopilinum on Functional Integrity of Kidney in Rats following 28 Days Oral Administration

The result showed that, there was insignificant ($p<0.05$) changes in the values of sodium ion, chloride ion, potassium ion, bicarbonate, creatinine, and urea in all treated groups when compared with control (Table 2).

Table 2: Effects of the Methanol Stem Bark Extract of *Combretum hypopilinum* on Functional Integrity of Kidney in Rats following 28 Days Oral Administration

Parameters (mmol/L)	N/S 10 mL/kg	CHMSE 400mg/kg	CHMSE 800mg/kg	CHMSE 1600mg/kg
Sodium	133.33±2.43	125.50±2.86	131.83±4.16	129.83±6.20
Potassium	4.18±0.20	3.63±0.07	4.05±0.17	4.45±0.15
Bicarbonate	21.33±0.42	21.83±0.54	21.33±0.67	20.00±0.37
Urea	9.02±0.35	7.78±0.50	9.58±0.64	10.92±1.05
Creatinine	0.77±0.12	0.55±0.85	1.00±0.12	1.05±0.14
Chloride	103.33±7.14	94.17±1.89	92.17±2.10	93.00±2.38

Effects of the Methanol Stem Bark Extract of *Combretum hypopilinum* on Liver Function Indices in Rats following 28 Days Oral Administration

The result indicated statistically significant ($p<0.005$) changes in the value of Total Protein in groups treated with highest dose of the extract (Table 3). There were no statistically significant changes in the values of other parameters.

Table 3: Effects of the Methanol Stem Bark Extract of Combretum hypopilinum on Liver Function Indices in Rats following 28 Days Oral Administration

Parameters (mmol/L)	N/S 10 mL/kg	CHMSE 400mg/kg	CHMSE 800mg/kg	CHMSE 1600mg/kg
T. Protein	6.40±0.17	5.93±0.17	6.05±0.19	5.68±0.13*
Albumin	3.08±0.05	3.05±0.11	3.15±0.85	2.98±0.15
T.BIL (mg/dL)	1.04±0.06	1.10±0.07	0.98±0.05	1.08±0.05
ALP (U/L)	365.50±38.59	365.33±40.24	453.00±17.93	431.50±13.63
AST (U/L)	232.00±28.81	223.50±25.73	301.00±10.99	278.50±13.07
ALT (U/L)	81.50±6.22	90.00±9.69	96.17±3.49	91.83±4.32

*CHMSE= Combrettium hypopilinium crude methanol stem bark extract, N/S= Normal saline (n=10) *=p<0.05; compared to control. One way ANOVA followed by Dunnets' post hoc*

Effect of 28 Days Oral Administration of Methanol Stem Bark Extract of Combretum hypopilinum on Haematological Parameters in Rats

The result showed that, there was no significant (p<0.05) changes in the values of all the parameters used in all treated groups (Table 4).

Table 4: Effect of Oral Administration of the Methanol Stem Bark Extract of Combretum hypopilinum on Haematologic Parameters Following 28 days oral Administration in rats

Parameters	N/S 10 mL/kg	CHMSE (400mg/kg)	CHMSE (800mg/kg)	CHMSE (1600mg/kg)
WBC ($x10^6$ /ul)	13.10±2.67	11.23±1.70	10.95±2.33	14.53±2.62
LYMPHOCYTES	84.05±1.85	83.53±0.89	80.52±0.90	78.93±3.21
RBC ($x10^6$ /ul)	6.62±0.25	6.79±0.15	6.81±0.25	7.32±0.16
HGB g/dl	12.52±0.45	14.40±0.88	13.35±0.45	13.45±0.15
HCT %	35.62±0.97	33.55±0.09	37.27±1.48	38.18±0.36
MCV (fl)	32.82±1.26	56.18±1.56	55.20±0.36	51.97±1.21
PLT (x103/ul)	366.17±74.49	400.83±29.75	382.67±17.66	459.67±28.21

Data are presented as mean ± SEM of 6 rats/treatment. RBC= Number of red blood cells, HGB= Haemoglobin, HCT= Volume of haematocrit, MCV= Mean corpuscular volume, PLT=Platelets, CHMSE= Combrettium hypopilinium crude methanol stem bark extract. Statistical tool: ANOVA (one way). Dunnett's multiple comparison tests.

Effects of the Methanol Stem Bark Extract of Combretum hypopilinum on Histology of the Brain following 28 Days Oral Administration in Rats.

Histopathological observation of the cerebellum (as shown in plate 1) revealed decrease in number, size and shape of Purkinje cells of the molecular germinal layer at highest dose of the extract (1600 mg/kg). At moderate dose (800mg/kg) the cells decrease in size and shape but not in number. At low dose (400mg/kg) none of the cells is affected in terms of size and shape.

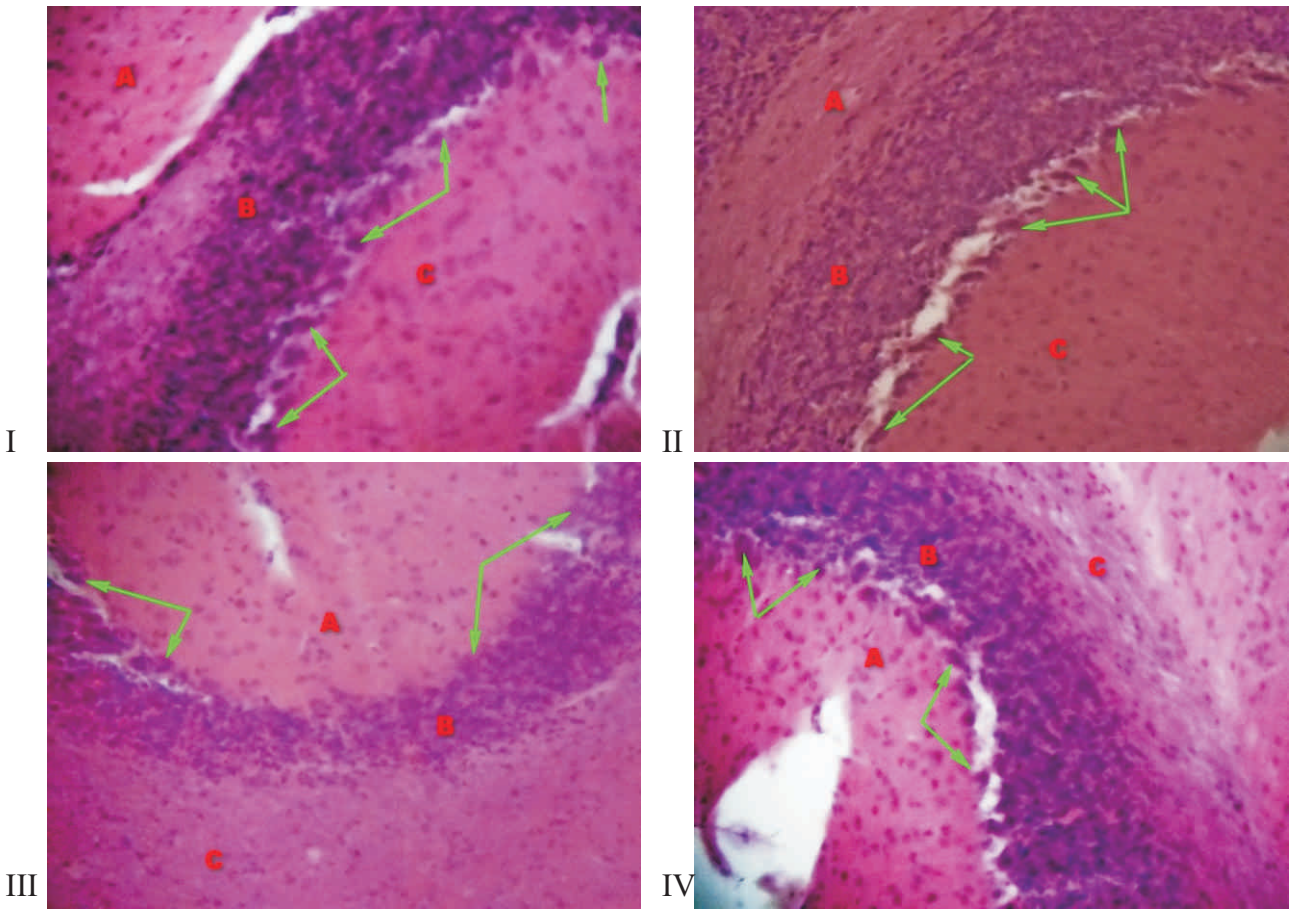

Plate 1 Photomicrograph of a section of the rat brain treated with CHMSE following 28 days oral administration (H &E x 200. I). 400mg/kg of CHMSE showing external germinal layer (**A**), molecular layer Purkinje layer (**B**), with normally arranged Purkinje cells (green arrows) and internal granular layer(**C**). II). 800mg/kg of CHMSE showing external germinal layer (**A**), molecular layer Purkinje layer (**B**), with normally arranged Purkinje cells (green arrows) and internal granular layer(**C**). III). 1600mg/kg of CHMSE showing external germinal layer (**A**), molecular layer Purkinje layer (**B**), with less no of Purkinje cells (green arrows) and internal granular layer (**C**). IV).10ml/kg N/S showing external germinal layer (A), molecular layer Purkinje layer (B), with normally arranged Purkinje cells (green arrows) and internal granular layer(C).

Effects of Methanol Stem Bark Extract of Combretum hypopilinum on Histology of the Kidney following 28 Days Oral Administration in Rats.

Histopathological differentiation of the kidney (as shown in plate 2) revealed moderate degenerative changes on glomerulus at highest dose (1600mg/kg) with no effect on Juxter Glomerulus Complex, with slight changes in the glomerulus at a moderate dose (800mg/kg). No visible changes observe in low dose (400mg/kg).

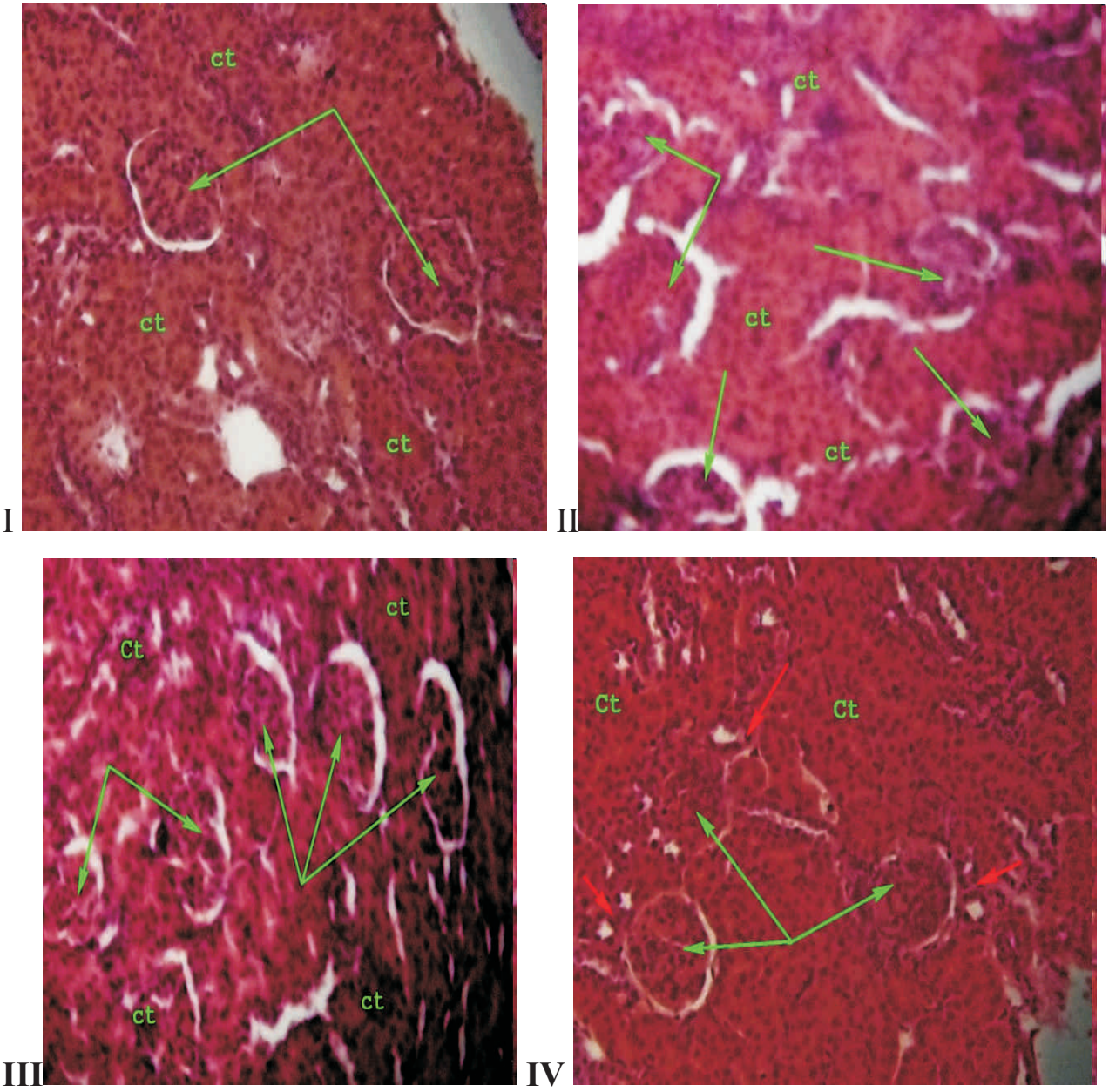

Plate 2: Photomicrograph of a section of the rat kidney treated with the CHMSE *following* 28 days oral administration (H &E x 200). I). 400mg/kg of CHMSE showing normal glomerulus (Green arrow) with normal collecting ducts (Ct). II). 800mg/kg of CHMSE showing normal glomerulus (Green arrow) with normal collecting ducts (Ct).111). 1600mg/kg of CHMSE showing normal and slight degenerated glomerulus (Green arrow) with normal collecting ducts (Ct). IV).10ml/kg N/S showing normal glomerulus (Green arrow) with normal collecting ducts (Ct).

Effect of Methanol Stem Bark Extract of *Combretum hypopilinum* on Histology of the Liver following 28 days Oral Administration in Rats

Result of the histopathology of the liver (as shown in plate 3) revealed that at highest dose of the extract (1600 mg/kg) there was moderate cellular infiltration of inflammatory cells at sinusoid level with aggregate zone of necrotic tissues (focal necrosis). Furthermore, no degenerative or infiltration of cells was observed in the central vein at moderate dose (800mg/kg) only cellular infiltration of the inflammatory cells was observed with clear central vein. No degenerative or inflammatory change was observed at low doses (400mg/kg) of the extract.

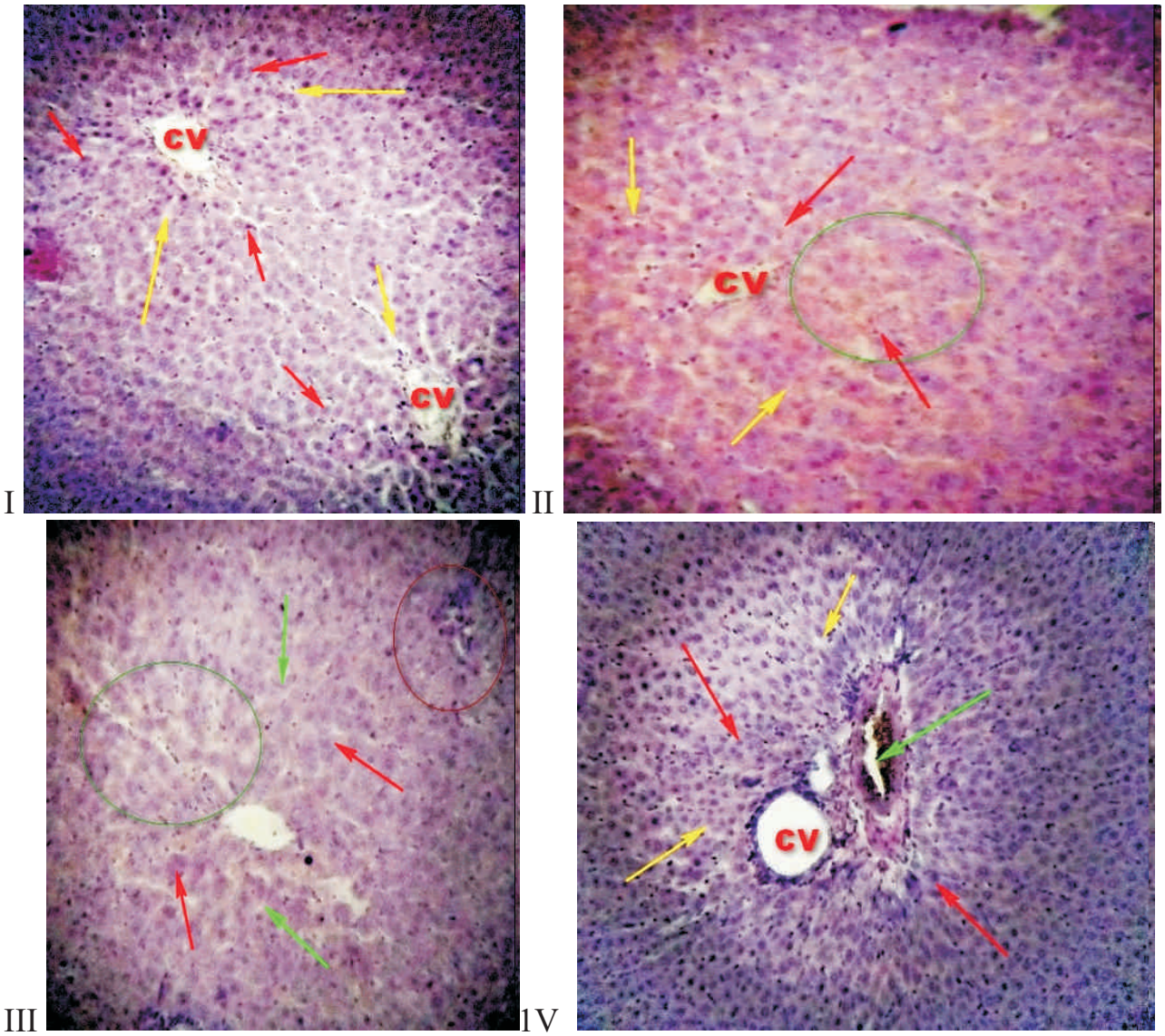

Plate 3: Photomicrograph of a section of the rat liver treated with the CHMSE *following* 28 days oral administration (H &E x 200). I). 400mg/kg of CHMSE showing normal orientation of hepatocyte (Red arrow) normal sinusoid (Red arrow) with severely cellular infiltrated central vein (CV). II). 800mg/kg of CHMSE showing moderate disorientation of hepatocyte (Red arrow), congested sinusoid (Red arrow) with severe cellular infiltration in the sinusoid (Green circle) and central vein (CV)**.** III). 1600mg/kg of CHMSE showing moderate disorientation of hepatocyte (Red arrow), Circumscribed necrotic cell (Red circle), congested sinusoid (Green arrow) with moderate cellular infiltration within the sinusoid (Green circle) and central vein (CV)**.** IV) 10ml/kg N/S showing normally distributed hepatocyte (Red arrow), normal orientation of sinusoid (Yellow arrow) with normal clear central vein (CV) and Blood vessels (Green arrow).

Discussion

Sub-acute toxicity studies in animals are essential in predicting potential toxic effects of a substance from which the response may be correlated with human. It also gives an idea of the effect of the test substance on certain organs or system. Decrease in body weight is considered an important parameter of toxicity after exposure to toxic substances (Uma *et al.,* 2013). Oral administration of 400, 800, and 1600 mg/kg of CHMSE for 28 days presented no decrease in body weight but rather an increase which was consistent across the test groups. Body weight changes serve as a sensitive indication of the general health status of animal (Salawu *et al.,* 2009). The result of the present study is similar to the findings of Uma *et al.* (2013).

Kidney is the major excretory organ of the body, therefore, when a kidney is damaged, it becomes inefficient in excreting both urea and creatinine and this consequently results in accumulation of these substances in the blood. Hence, the high level of blood urea and creatinine implies kidney damage (Ajayi *et al.,* 2014). Thus, level of urea, creatinine, and electrolyte in blood can be used as parameters to determine damage to the kidneys (Ajayi *et al.,* 2014). The normal values of the biochemical parameters such as urea and creatinine and other electrolytes suggests that the methanol stem bark extract of *Combretum hypopilinum* does not produce any kind of disturbance in the renal function. Similar observations have been made by (Uma *et al.,* 2013).

Liver is an important organ involved actively in different metabolic functions in the body (Mohammed *et al.,* 2016). Liver damage caused by chemicals or infectious agents is associated with disturbances of these metabolic functions and may result to progressive liver fibrosis and ultimately cirrhosis and liver failure (Abd-Allah *et al.,* 2015). Hepatic damage is identified by distortion in the normal functions of the liver and is diagnosed by determining the serum concentration of liver enzymes (ALT, AST, and ALP), bilirubin, total protein and albumin (Gowda *et al.,* 2009). These enzymes were reported to be higher than normal level in the blood in occasions of impaired liver function (Gowda *et al.,* 2009). Hence, these enzymes are used as serum markers of liver damage. Serum protein measurement may be helpful in the assessment of liver function because protein metabolism impairment may lead to increase serum protein concentration. The result of this study shows that the methanol stem bark extract of *Combretum hypopilinum* had no significant effect on the values of ALT, ALP, Albumin, Total bilirubin when compared with control which implies that the function of the liver is not affected by the extract.

Findings from the haematological test indicate that parameters such as haemoglobin, RBC, platelets, MCV, haematocrit, lymphocytes in the extract treated animals were within normal limits. Haematological changes such as anaemia are often accompanied with bone marrow toxicity. According to Uma *et al. (*2013) anaemia that results after administration of agent can be a result of lysis of blood cells. However, no such anaemia was observed after chronic treatment with the extract implying that there is no lysis of blood cells. Our finding that the values of blood parameters was within the normal range show that the drug is non-toxic. The white blood cells in all treated groups were within the normal range when compared to the control. Analysis of blood parameters with respect to animal studies has a high relevance and predictive value for humans (Uma *et al.,* 2013).

The histopathological studies of the rat brain revealed little to none deleterious effect, though the 1600 mg/kg treated revealed slightly decreased number of Puckinje cells, this therefore calls for caution when administering large doses of the extract.

Conclusion

In conclusion the result of the present study may be considered as a preliminary investigation necessitating further probe into the chronic administration of the methanol stem bark extract of *C. hypopilinum*.

Conflict of Interest

Authors declare no conflict of interest

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