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Acute and Subchronic Toxicity Studies of *Combretum collinum* Methanol Root Extract in Albino Rats

S. W. Hassan[1], A. N. Ukwuani-Kwaja[1], U. D. Nuhu[1*] and R. D. Jabaka[2]

[1]Department of Biochemistry, Faculty of Life Science, Kebbi State University of Science and Technology, P.M.B. 1144, Aliero, Kebbi State, Nigeria.
[2]Department of Microbiology, Faculty of Life Science, Kebbi State University of Science and Technology, P.M.B. 1144, Aliero, Kebbi State, Nigeria.

Authors' contributions

This work was carried out in collaboration among all authors. Authors SWH, UDN and ANUK designed the study, wrote the protocol, manage the analysis of the study and wrote the first draft of the manuscript. Authors UDN and RDJ manage the literature reviews and performed the statistical analysis. All authors read and approved the final manuscript.

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ABSTRACT

Combretum collinum root extract has been recognized long ago as traditional medicinal plant in curing several diseases among the indigenous people of Alela-land (Zuru), Kebbi State, Nigeria. This research work was carried out to evaluate the toxicological effects of *Combretum collinum* methanol root extract (CCME) in albino rats. Acute toxicity was performed by a fixed single oral administration at a dose of 10, 100, 1000 mg/Kg and 1600, 2900, 5000 mg/Kg. Subchronic toxicity studies of CCME was conducted at doses of 32, 63, 126 and 253 mg/Kg for 28 days. The result showed that acute administration of CCME resulted at mortality and general behavioral changes at 1000 to 5000 mg/Kg. Therefore, the estimated lethal dose (LD_{50}) of CCME was 316.23 mg/Kg. Subchronic oral administration of CCME revealed a significant (P<0.01) decrease in body weight in rats receiving 63 to 253 mg/Kg throughout the study period compared with the control group. The results also showed a significant (P<0.01) increase in serum ALT, AST, creatinine, potassium and

**Corresponding author: E-mail: dabainuhu@gmail.com;*

bicarbonate in rats administered with 126 and 253 mg/Kg of the extract. Haematological analysis of the same extract revealed a significant (P<0.01) increase in WBC, HCT, MCV, MCH, MCHC, PLT, LYM and NEUT in rats receiving 126 and 253 mg/Kg only. Histopathological examination of liver revealed severe periportal inflammation, hypertrophy, areas of hydropic changes, cancerous tumor, areas of infiltration and necrosis of the hepatic cells while the kidney showed a mild mesengial hyperplasia, compressed blood vessels, glomerular degeneration, tubular degeneration and tubular widened lumen in rats treated with 63 to 253 mg/Kg. Therefore, caution should be applied as *C. collinum* root extract has a low mean lethal dose and would be toxic at higher concentrations.

Keywords: Combretum collinum; oral administration; acute lethal dose (LD_{50}); severe periportal inflammation (SPI); hypertrophy (HY); Infiltration (INF) *and mild mesangial hyperplasia (MMH).*

1. INTRODUCTION

Natural product remedies are popular and gaining a lot of acceptance among people in the prevention and cure of some diseases. The general belief that the products of natural origin are considered safe and free from severe side effects which the synthetic drugs provoke, drives people towards using natural medicinal plants [1]. However, many of the natural formulations available in the market do not have sufficient scientific data on their safety and toxicological profile and the consumption of such medicinal plants as conventional medications or and as curatives may cause adverse toxicological effects to human health [2]. This assumes particular importance since the natural products are more often used under self-medication without authentic medical supervision. Thus one of such plant is *Combretum collinum* (*Combretaceae*) which its botanical description was reported by Komakech.and Omujal [3] Hence, proper scientific knowledge about the toxicity and safe administration level of the natural medicines is crucial. In other words proper scientific evaluation of medicinal plants with pertinent emphasis on toxicological paradigms is imperative while assessing their efficacy.

In Alela-land (Zuru), Kebbi State, Nigeria *Combretum collinum* root extract has been recognized or noticed long ago as traditional medicinal plant in curing several diseases including sleeping sickness among the indigenous people. *C. collinum* is a small to medium-sized, semi-deciduous tree growing up to 18 m in height, with a rounded crown. The bark is light grey, creamy-brown, reddish-brown or brown-black, transversely cracked. The leaves are opposite and alternate, simple, narrowly elliptic to broadly ovate, dark green above, paler green to silvery below. The flowers cream to yellow, up to 5 mm in diameter while the 4-winged fruit is rusty red when young, becoming dark brown when mature. Young shoots are densely covered with short, soft hairs [3].

Komakech [3] reported that the preparation of the herbal remedy from *C. collinum* can be in the form of infusions, or as a decoction. It can also be ground into powder and mixed with soup, localbrew, porridge or milk and then taken. In some cases, the juice/sap from the plant parts is extracted by chewing or pounding, depending on a given disease state or condition. The leaf is the most frequently used part of the plant by the traditional healers to treat and manage many disease conditions. For instance, the leaf-powder decoction or suspension in water is used for a bath, administered orally, or the powder is put into fire and the smoke inhaled depending on the targeted disease condition. The leaf decoction is used as a purgative to relieve one from constipation and for treatment of malaria. A fresh or dried leaf infusion is widely administered orally as a cholagogue, diuretic and is also taken as a blood tonic.

The decoction is also used to treat gastro-intestinal problems, including diarrhea, dysentery, stomachache and ascariasis conditions. In addition, a combination of leaf and twig decoction is drunk and fresh roots are chewed to treat coughs, bronchitis, tuberculosis, jaundice as well as snakebites. The leaf sap is applied to treat wounds, leprosy and is also used as ear drops to treat earache. The crushed leaves are applied as poultice and also added to bathing water to treat fatigue and rheumatism. The *C. collinum* stem-bark powder is eaten in porridge to treat rectal prolapse and haemorrhoids. The hot water bark extract is also used for the treatment of diarrhea, anal bleeding, malaria, headache and general body pain. The

bark also yields an edible/chewing gum which is used to cure toothache or to plug a carious tooth [3].

Although wild plants are important sources of nutrients and phytocompounds that play a role in protection against conditions such as cardiovascular disease and cancer, they also contain other compounds that may lead to hepatic or tubular necrosis [4]. To our knowledge, information on the toxicity of the root extract of *C. collinum* in Alela-land (Zuru), Kebbi State, Nigeria is scanty. Therefore, this paper reports the evaluation of the toxic effect of root extract of *C. collinum* by acute and sub chronic oral administration in rats.

2. MATERIALS AND METHODS

2.1 Reagents and Chemicals

All the chemicals and reagents used were of analytical grade (Appendix I, Table 1).

2.2 Sample Collection, Identification and Preparations of Plant Extract

2.2.1 Collection and identification of plant sample

Fresh plant sample of Bush willow was collected from Udaba hills, Dabai village, along Koko road, Zuru, Kebbi State, Nigeria; in June 2017. The whole plant sample (including the leaves, flowers, stem and roots) was taken to Herbarium Section for botanical authentication at Botany unit, Department of Biological Sciences, Kebbi State University of Science and Technology, Aliero, and a voucher specimen number was obtained as 321A.

2.2.2 Preparation of plant sample

The collected *C. collinum* root sample was washed with clean water and scrapped to remove the root bark which was air dried under shade for two weeks. The dried root bark was pulverized into small pieces using a mortar and pestle. The weight was measured on an electronic balance and the whole sample collected was well packaged and stored in a clean specimen container for safe keeping before use.

2.2.3 Preparation of crude plant extract

A reflux extraction method was used to obtain crude methanol extract of *C. collinum* dried root bark [5]. Five hundred grammes (500 g) of the prepared pulverized pieces of *C. collinum* root bark plant sample was weighed and packed into a round bottom flask (1000 ml) and 800ml of pure methanol was poured into the sample flask and a reflux apparatus was set-up with the flask containing the mixture fixed on a heating mantle through which heat (45^{o}C) was applied to the flask for 4hours as the plant sample was extracted continuously. Therefore, the brownish extract obtained was filtered through a clean muslin tissue (2 mm thickness) and the filtrate was further concentrated to dryness on a water bath by steam heating at 45^{o}C. This procedure was repeated for another 500 g of *C. collinum*. The percentage yield of the residue was calculated as:

$$\text{Percentage (\%) yield} = \frac{\text{Amount of extract obtained}}{\text{Amount of initial sample}} \times 100$$

2.3 Qualitative Phytochemical Screening of *C. collinum* Methanol Extract

A preliminary qualitative phytochemical screening of five grammes (5g) of the dried *C. collinum* root bark methanol crude extract was carried-out by dissolving in 40ml of distilled water to qualitatively detect the presence of Alkaloids, Resins, Saponins, Tannins, Phenol, Flavonoids, Terpenoids, Anthraquinones, Steroids and Phlobatannins using standard analytical methods [9,7,6,8] and [10].

2.4 Toxicity Study

2.4.1 Experimental animals

Thirty nine adult *Rattus norvegicus Wistar* (weighing 170-200g and of age 3-4 months old) to be used for the test, were purchased from the Animal House Facility, Nigerian Institute of Trypanosomiasis Research, Kaduna. All the animals were kept in well ventilated wooden cages under a standard laboratory condition, at the animal house of Kebbi State University of Science and Technology, Aliero, Kebbi State, Nigeria. The rats obtained were exposed to natural 12 hours day light and dark cycles and were allowed free access to growers feed (mash) (commercial rodents pellets, vital feeds, Nigeria) and water *ad libitum* throughout the study period. They were allowed for a 2-weeks period of acclimatization to the laboratory environment at temperature of 30-35^{0}C before the commencement of research. Weight of each rat was recorded before the commencement of the experiment. This research work was conducted,

in compliance with animal care guidelines on animal use protocol review of Canadian Council and international accepted principles for laboratory animal use [11,12].

2.4.2 Acute toxicity assay of *C. collinum* methanol crude extract

The acute oral toxicity test of methanol root extract of *C. collinum* was evaluated in albino rats as reported by the method described by [13].

Nine (9) *Rattus norvegicus Wistar* were divided into three (I, II and III test groups) of three rats per group, following an overnight fast, the rats were weighed and the dose was calculated in reference to their body weight. The crude extract was suspended in a vehicle (normal saline) and 1ml of 10, 100 and 1000 mg/kg extract body weight were orally administered for each rat in every group respectively. The rats were keenly observed for about 30 mins for any signs of toxicity or mortality, and further observations were made for the first 3 to 4 hours, and every 8 hours for 24 hours, subsequently for 72hours and thereafter daily for 14 days after administration of the extract. The signs of toxicity such as physical activity and general appearance, paw-licking, salivation, stretching, gastrointestinal signs: Dropping (Feces), rubbing of nose on the floor and walls of cage, sedation, coma, convulsion and death was critically observed. The number of deaths in each group within 24hours was recorded and then the lowest dose, which killed one rat (minimum toxic dose) and the highest dose, which had not killed any rat (maximum tolerated dose) was observed, and the geometric mean of these two doses make up the LD_{50}. The presence of mortality in this stage does not necessitate proceeding to the second stage. Therefore, the LD_{50} was computed using the formula below:

The mathematical expression of Geometric Mean (LD_{50}) is given as:

$$\text{Geometric Mean } (LD_{50}) = \sqrt{x \times y}$$

Where *x* and *y* represents the lowest lethal dose (minimum toxic dose) and the highest non-lethal dose (maximum tolerated dose) respectively.

2.4.3 Sub-chronic toxicity studies of *C. collinum* methanol extract

Thirty (30) *Rattus norvegicus Wistar* were randomly grouped in to five (5) (I, II, III, IV and V) of six (6) rats each (3 males and 3 females). Group I: was administered with 0.5mL of normal saline once daily for 28 days and served as the control. Groups II-V: were orally administered with graded doses of *C. collinum* crude extract (10, 20, 40 and 80% based on the calculated LD_{50}) once daily for 28 days. The doses were calculated in reference to the body weight, as the volume of the extract solutions administered to the rats was 1 ml/kg.

The weight of the rats was measured before administration of the extract and subsequently every week. Detailed observation for the signs of toxicity was carried out twice daily for the period of 28 days. The rats were fasted overnight on the 28th and 29th day. On the 29th day of the research, following an overnight fast of 8hours, all animals in various groups were anesthetized under chloroform and blood samples were collected by cardiac puncture into heparinised and non-heparinised bottles for haematological and biochemical analysis respectively. Blood samples collected into clean non-heparinised bottles were allowed to clot and centrifuged at 3,000 rpm for 10 min to obtain the sera according to groups; and serum was separated from the clot into clean bottles for the biochemical analysis. The liver, kidneys and heart were excised from dissected rats, which was immediately cleaned of blood by using physiological saline and weighed. The liver and kidneys were fixed in 10% formalin for histopathological examination.

2.5 Haematological Analysis

Haematological analysis was performed by determining the amount of the following blood parameters: The hematocrit Packed Cell Volume (PCV) or Hemoglobin (HGB), Red Blood Cell (RBC), Mean Cell Volume (MCV) Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC), White Blood Cell (WBC) Lymphocytes, Neutrophiles and Platelets (PLT) was examined [14] and [15].

2.6 Serum Biochemical Analysis

The following parameters were analyzed in the sera: Alanine Aminotransferase (ALT) [16], Alkaline Phophatase (ALP) [17], Aspartate Aminotransferase (AST) [16], Total Protein [18], Albumin [19], Total and Direct Bilirubin [20], Electrolyte and Creatinine [22], Uric acid [21] and Urea [23].

2.7 Histopathological Assay

Histopathological investigation was conducted according to methods described in the literature [24,25] and [26]. The organs were collected and kept for 24 hours in the buffered formalin, each one was dehydrated with alcohol, then embedded in paraffin wax, and sliced into 4-5µm thick sections, and was stained with haematoxylin-eosin dye for photomicroscopic observation [27]. The microscopic features of the organs from each rat were compared with that of the control groups.

2.8 Data Analysis

The data collected was subjected to statistical analysis as the average body weight, haematology and serum biochemistry were expressed as Mean ± SEM using Instat Graphpad software version 21 and mean value was further subjected to statistical comparison using SPSS version 20 where the data was analyzed by using one way analysis of variance (ANOVA) and Dunnette, Tukey-Kramer Multiple Comparisons Test (i.e the significant differences between groups was determined using one way ANOVA with the aid of SPSS soft ware and $P< 0.05$ was considered as significant).

3. RESULTS

3.1 Percentage Yield

Methanol extraction of 1000 g (1 Kg) *C. collimun* root yielded 19.03%, and the extract was soluble in water, brown in colour with a sticky texture and has a pleasant smell.

3.2 Preliminary Phytochemical Consti tuent of *C. collinum* Methanol Extract

The preliminary qualitative phytochemical screening of *C. collinum* revealed the presence of some secondary metabolites (Table 1) while some were not detected.

3.3 Acute Oral Toxicity Assay Result of CCME

The first stage of acute toxicity assay of CCME produces no remarkable signs of toxicity at 10 and 100 mg/Kg. While at 1000 mg/Kg of CCME signs of toxicity such as restlessness, dropping of feces and rubbing of nose and mouth on the cage walls was observed for the first 30mins and 3-4hours and consequently one of the rats died after 28 hours of CCME administration at 1000 mg/Kg and a significant ($P<0.05$) gross decrease in body weight was observed for 14 days suggesting that the extract could be toxic at 1000 mg/Kg (Fig. 1). Therefore, the mortality observed in this first stage did not necessitate proceeding to second stage, as the median lethal dose (LD_{50}) was calculated as 316.23 mg/Kg [14].

Table 1. Qualitative phytochemical composition of *C. collinum* methanol extract

Phytochemicals	CCME
Alkaloids	+
Saponins	+
Tannins	+
Phenols	+
Flavonoids	+
Terpenoids	+
Anthraquinones	+
Steroids	-
Phlobatanins	+
Resins	-

CCME: C. collinum methanol extract, (+) Present and (-) Not detecte

3.4 Sub-chronic Oral Toxicity Assay Result of CCME

There was neither sign of toxicity nor death of the experimental rats during the 28 days period of observation after every daily oral administration of the CCME.

3.4.1 Body weight change in rats administered with CCME

Toxicity assessment was carried out by observing body weight loss which revealed a significant ($P<0.05$) decrease in body weight which was noticed in rats administered with 126 and 253 mg/kg from the 2nd to 4th week when compared with the control group while no significant ($P>0.05$) decrease in body weight was observed in rats administered with 32 and 63 mg/Kg from the 1st week onwards (Table 2).

Likewise the weight of some organs (liver, kidney and heart) in the experimental rats was observed, in which a significant ($P<0.01$) increase in liver, kidney and heart weight was noticed at 63, 126 and 253 mg/Kg CCME treated groups whereas 32 mg/Kg CCME revealed no significant ($P> 0.01$) increase in any of the organs weight when compared with the control group (Table 3).

3.4.2 Haematological analysis result

The haematological result in this study revealed that, CCME has a mild significant (P<0.01) increase on WBC, MCV, MCH, MCHC, PLT and LYM in rats administered with 126 and 253 mg/Kg body weight, with an exceptional high significant (P<0.01) increase on WBC and PLT for rats administered with 253 and 126mg/Kg CCME respectively, while HCT showed significant decrease at 126 and 253 mg/kg as NEUT and MXD showed significant decrease at 253 mg/kg when compared with control group. Therefore, at 32 and 63 mg/Kg CCME no significant (P>0.01) increase was observed on all the haematological indices compared with the control group (Table 4).

3.4.3 Serum biochemical analysis result

Some hepatic functional indices (biomarkers) were examined at different doses administered to experimental rats after 28 days of daily CCME in take. The liver function test conducted revealed a very significant (P<0.01) increase in ALT and AST at 126 and 253 mg/Kg CCME treated group with a very significant (P< 0.01) decrease in AST and ALP observed at 63 and 253 mg/Kg CCME treated group respectively compared with control group, whereas a mild significant (P< 0.01) decrease in Total protein at 32 and 63 mg/kg, Total bilirubin at 126 and 253 mg/kg and Direct bilirubin was observed at 63 and 253 mg/Kg CCME treated groups while no significant difference (P> 0.01) was noticed for ALB at all doses when compared with the control (Table 5).

In the same vein some renal function indices (biomarkers) were examined at different doses showing a significant (P< 0.01) increase in creatinine, and bicarbonate HCO_3^- at 126 and potassium (K) at 253 mg/Kg CCME treated group, while a significant (P<0.01) decrease in sodium (Na) was observed at 32 mg/Kg CCME treated group, whereas the same extract administered at the same dose revealed no significant difference or effect on BUN, Urea, Uric acid, and chloride when compared with the control group, (Table 6).

3.4.4 Histopathological assay result

The histopathological examination of the liver sections in the control untreated rats group showed a normal histological photomicrographic picture. The central vein lies at the centre of the lobule surrounded by the hepatocytes with strong lyeosinophilic granulated cytoplasm, distinct nuclei, and strands of hepatocytes and the hepatic sinusoids are all exhibited as shown in (Plate. 1a and b). Photomicrograph of the liver in rats administered with 10% LD_{50} (32mg/Kg) CCME revealed a mild periportal inflammation (MPI) and congestion (CON) of the hepatic cells (Plate. 2a and b), also the photomicrograph of the liver in rats administered with 20% LD_{50} (63mg/Kg) CCME showed a hydropic changes (H.C), mild periportal inflammation (MPI) and congestion (CON) of the hepatic cells (Plate. 3a and b) when compared with the control group. However, the photomicrograph of the liver in rats administered with 40% LD_{50} (126mg/Kg) CCME presented a severe periportal inflammation (SPI) and hypertrophy (HY) (Plate. 4) while that of the liver in rats administered with 80% LD_{50} (253mg/Kg) CCME revealed areas of hydropic changes (HC), cancerous tumor (CT) and areas of infiltration (INF) and necrosis of the hepatic cells (Plate. 5a and b) when compared with the control group.

The histopathological examination of the kidney sections in the control group has presented a normal renal photomicrographic picture with normal renal cells (RCs), renal tubule (RT), Bowman's capsule and glomeruli (G) (Plate. 6a and b). Likewise the kidney in rats administered with 32mg/Kg (10% LD_{50}) CCME showed no changes from the normal renal cells, renal tubule (RT) and glomeruli (G) (Plate. 7) as compared with the control group. However, photo micrograph of kidney in rats administered with 63mg/Kg (20% LD_{50}) CCME revealed a mild mesengial hyperplasia (MMH) at the renal tubule and the glomeruli region (Plate. 8), while the kidney in rats administered with 126mg/Kg (40% LD_{50}) CCME presented a compressed blood vessels (CBV) and mild mesengial hyperplasia (MMH) at the renal tubule and the glomeruli (Plate. 9) and the kidney in rats administered with 253mg/Kg (80% LD_{50}) CCME showed a mild mesengial hyperplasia (MMH), glomerular degeneration (GD), tubular degeneration (TD) and tubular widened lumen (TWL) (Plate. 10) when compared with the control group respectively. Therefore, this confirms the effect of kidney biochemical changes in the parameters observed in this study.

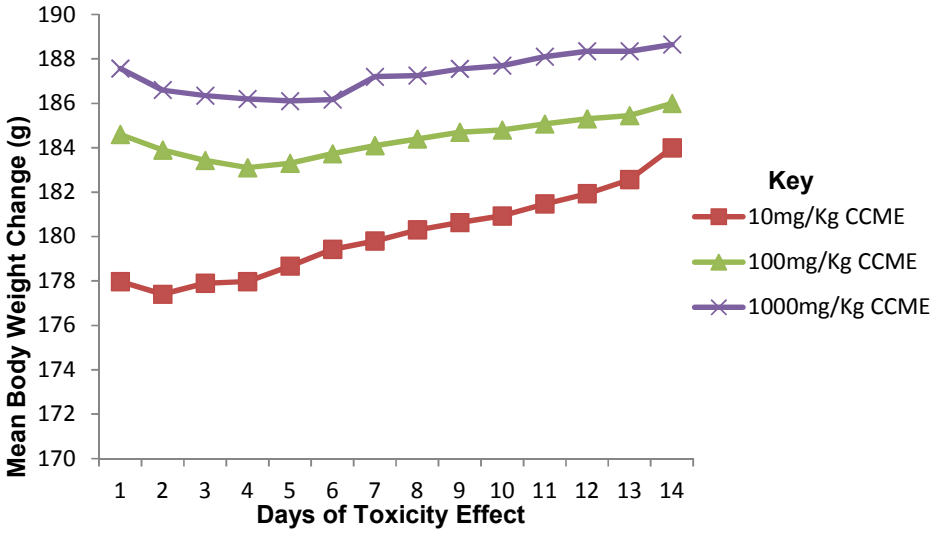
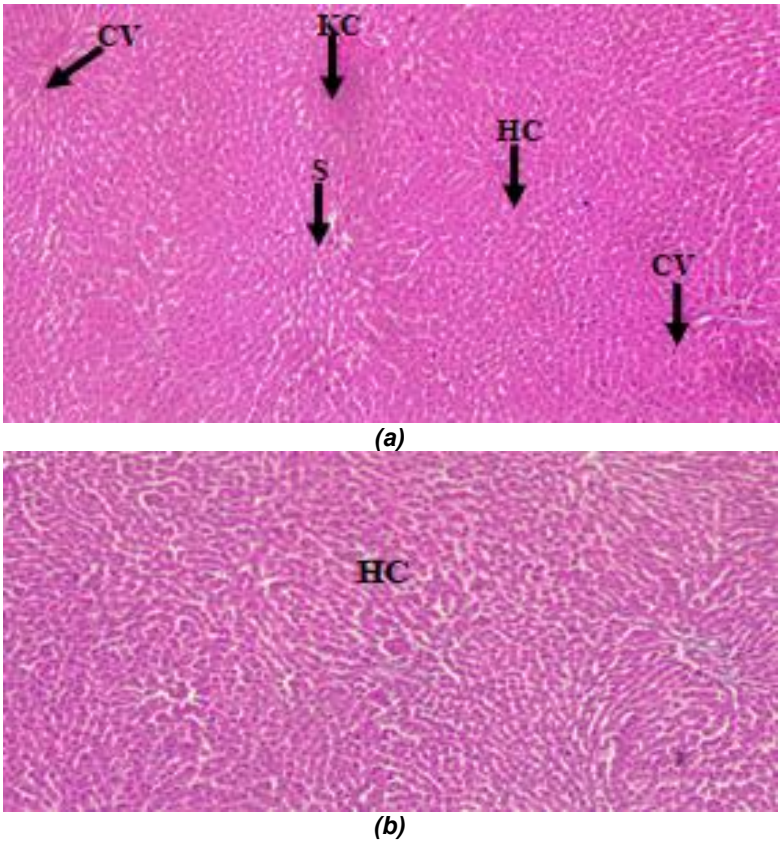


Fig. 1. Mean body weight change of rats during acute toxicity assay of CCME



(a)

(b)

Plate 1. Photomicrograph (Histological pattern) of control group liver administered with 0.5 mls/Kg 0.9% Normal saline (NaCl): a) showing normal hepatocytes architecture with normal hepatic cells (HC), central vein (CV), sinusoids (S) and kupffer cells (KC). b) showing normal hepatocytes architecture (×100).

Table 2. Body weight change of rats (g) as affected by doses of *Combretum collinum* methanol extract after 28 days of administration

DOSE (mg/Kg b. wt.)	WEEK 0	WEEK 1	WEEK 2	WEEK 3	WEEK 4
Control	174.02±0.62	174.75±0.45	175.65±0.62	176.32±0.64	177.45±0.47
CCME (32)	178.05±0.46	178.27±0.37	177.60±0.45	177.72±0.41	178.57±0.47
CCME (63)	182.77±0.61	182.82±0.94	182.42±0.76	182.28±0.92	182.13±0.87
CCME (126)	184.92±0.59	184.10±0.59	183.72±0.57	183.08±0.60*	183.20±0.62*
CCME (253)	186.58±0.53	185.53±0.56	183.95±0.71*	183.28±0.76*	182.53±0.51*

*Values are expressed in Mean ± standard error or mean (SEM) of six rats per group, * = significantly different **(P<0.05)** when compared with control group, using one way analysis of variance (ANOVA)*

Table 3. Weight of some organs (g) as affected by different doses of *Combretum collinum* methanol extract after 28 days of administration

Organ weight (g)	Control	CCME (32mg/Kg)	CCME (63mg/Kg)	CCME (126mg/Kg)	CCME (253mg/Kg)
Liver	4.08±0.23	4.92±0.42	6.27±0.32*	6.73±0.17*	6.67±0.14*
Kidney	1.27±0.09	1.62±0.07	1.98±0.09*	2.03±0.08*	2.00±0.08*
Heart	0.53±0.04	0.65±0.04	0.87±0.06*	0.97±0.07*	0.93±0.05*

*Values are expressed in Mean ± standard error of mean (SEM) of six rats per group; * = significantly different **(P<0.01)** when compared with control group, using one way analysis of variance (ANOVA)*

Table 4. Effect of *Combretum collinum* methanol extract on haematological indicies after 28 days of administration in rats

Haem. Indices	Control (0.5 mls/Kg)	32 mg/Kg CCME	63 mg/Kg CCME	126 mg/Kg CCME	253 mg/Kg CCME
WBC (µL)	$(10.48\pm1.51)10^3$	$(10.33\pm1.43)10^3$	$(11.467\pm1.67)10^3$	$(14.68\pm1.96)10^{3*}$	$(20.52\pm2.09)10^{3*}$
RBC (µL)	$(9.34\pm 0.06)10^6$	$(9.27\pm 0.05)10^6$	$(8.68\pm0.04)10^6$	$(8.59\pm 0.16)10^6$	$(8.62\pm0.36)10^6$
HGB (g/dl)	16.48±0.17	15.80±0.42	16.68±0.07	16.72±0.60	16.70±0.88
HCT (%)	61.87±0.71	59.43±2.74	59.13±2.32	57.83±1.33*	56.50±0.92*
MCV (fl)	64.93±0.66	62.28±0.73	64.72±0.91	66.15±1.65*	67.27±1.74*
MCH (pg)	18.50±0.11	19.05±0.20	19.02± 0.15	19.18±0.26	19.67±0.36*
MCHC (g/dL)	28.53±0.17	29.63±0.77	29.82±0.37	30.28±0.26*	30.38±0.36*
PLT (µL)	$(463.67\pm14.52)10^3$	$(596.00\pm48.78)10^3$	$(506.00\pm35.32)10^3$	$(824.00\pm33.78)10^{3*}$	$(693.00\pm83.89)10^{3*}$
LYM (%)	74.87±1.95	73.52±4.10	72.77±3.59	77.32±1.26*	77.42±1.69*
MXD (%)	6.33±0.97	5.42±0.29	5.68± 0.31	6.47±0.74	5.02± 0.16
NEUT (%)	19.20±1.24	18.73±0.46	20.02±1.02	24.67±2.64*	17.83±2.04*

*Values are expressed in Mean ± standard error of mean (SEM), * = significantly different **(P<0.01)** when compared with control group, using one way analysis of variance (ANOVA)*

Table 5. Effect of *Combretum collinum* methanol extract on liver function indices after 28 days of administration in rats

LFI	0.5 mL/Kg (Control)	CCME (32 mg/Kg)	CCME (63 mg/Kg)	CCME (126 mg/Kg)	CCME (253 mg/Kg)
ALT (U/l)	72.33±2.08	60.17±5.28	65.17±2.49	83.16±71.68*	85.000±1.83*
ALP (U/l)	121.00±5.55	125.00±7.48	121.00±5.56	127.17±12.97	118.50±7.68*
AST (U/l)	214.20 ±7.97	214.21±7.97	172.36 ±17.30*	254.41±8.94*	259.69±13.73*
T. P (G/l)	81.83±2.86	75.67±1.41*	74.33±1.12*	80.50 ±3.29	84.83±2.17
ALB (G/l)	39.17±1.19	37.17±0.83	34.17±1.08	37.83±2.41	39.50±1.06
T.Bil (mg/dL)	0.48±0.07	0.54±0.05	0.37±0.04	0.29±0.03*	0.26±0.03*
D.Bil (mg/dL)	0.20 ±0.03	0.18±0.02	0.13±0.01*	0.16±0.01	0.14±0.01*

*Values are expressed in Mean ± standard error or mean (SEM) of six rats per group, * = significantly difference (P<0.01) when compared with control group, using One Way Analysis of Variance (ANOVA). ALT: alanine transacetylase, ALP: alanine phosphatase, AST: aspartate transacetylase, T.P: total protein, ALB: albumin, T. Bil: total bilirubin and D. Bil: direct bilirubin.*

Table 6. Effect of *Combretum collinum* methanol extract on kidney function indices after 28 days of administration in rats

KFI	0.5 mL/Kg (Control)	CCME (32 mg/Kg)	CCME (63 mg/Kg)	CCME (126 mg/Kg)	CCME (253 mg/Kg)
BUN (mmol/L)	5.85 ±0.37	5.03±0.25	6.93±0.58	5.97±0.92	5.100±0.16
Creatinine (mmol/L)	157.33±2.40	158.30±32.20	153.33±13.98	166.67±0.71*	174.67±2.54*
Urea	8.383±0.65	7.72±0.83	7.22±0.57	8.68±0.25	9.92±0.27
U. acid (mg/dl)	3.45±0.43	3.63±0.39	3.83±0.36	4.38±0.51	5.23±0.22
Na (mmol/L)	166.65±1.16	157.62±1.54*	159.32±4.55	169.20±4.88	169.57±1.74
K (mmol/L)	9.98±0.24	9.06±0.39	8.42±0.58	9.15±0.44	11.91±0.45*
Cl (mmol/L)	105.87±9.84	107.27±0.70	103.83±0.42	105.33±3.05	106.73±0.96
HCO_3^- (mmol/L)	23.27±10.42	30.22±5.17	21.20 ±5.10	61.02 ±2.73*	71.05 ±2.71*

*Values are expressed in Mean ± standard error or mean (SEM) of six rats per group, ***= significantly different (P< 0.01) when compared with control group, using One Way Analysis of Variance (ANOVA). BUN: Blood Urea Nitrogen, U. acid: Uric acid, Na: Sodium, K: Potassium, Cl: Chlorine and CO_2: Carbon dioxide.*

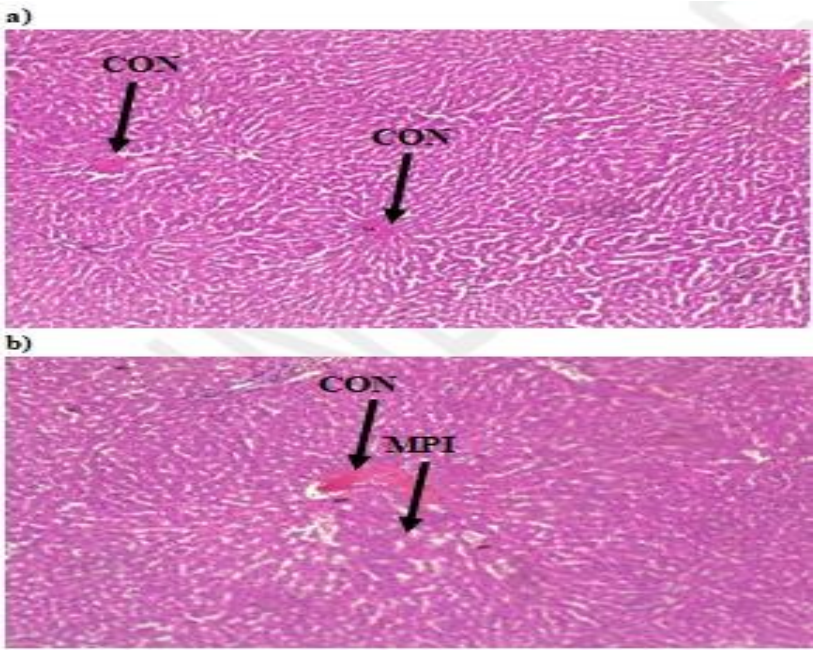


Plate 2. Photomicrograph (Histology pattern) of the liver in rats administered with 10% LD50 (32 mg/Kg) CCME: a) Showing congestion (CON). b) Showing a mild periportal inflammation (MPI) and congestion (CON) (×100)

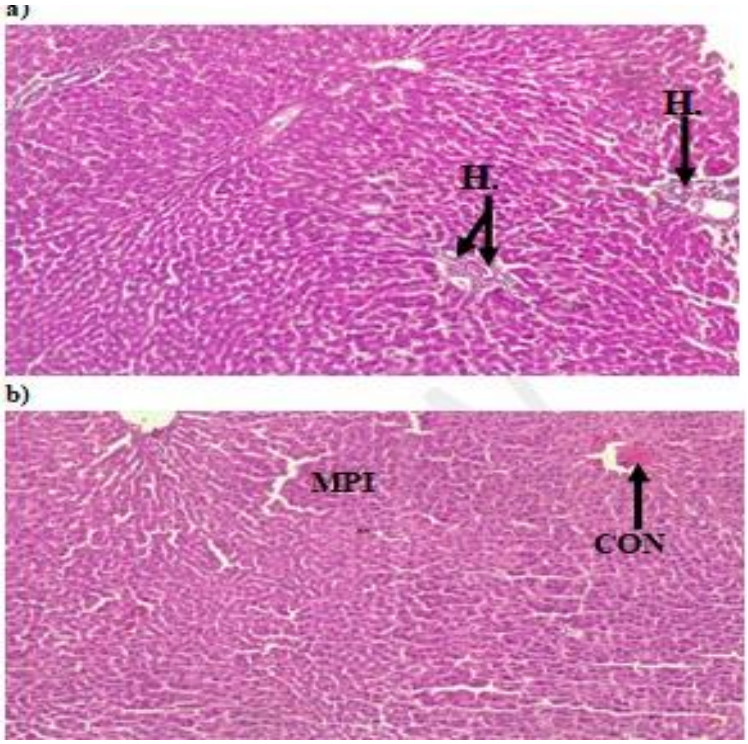


Plate 3. Photomicrograph (Histological architecture) of the liver in rats administered with 20% LD50 (63 mg/Kg) CCME: a) Showing a hydropic changes (H.C). b) Showing a congestion (CON) and mild periportal inflammation (MPI) (×100)

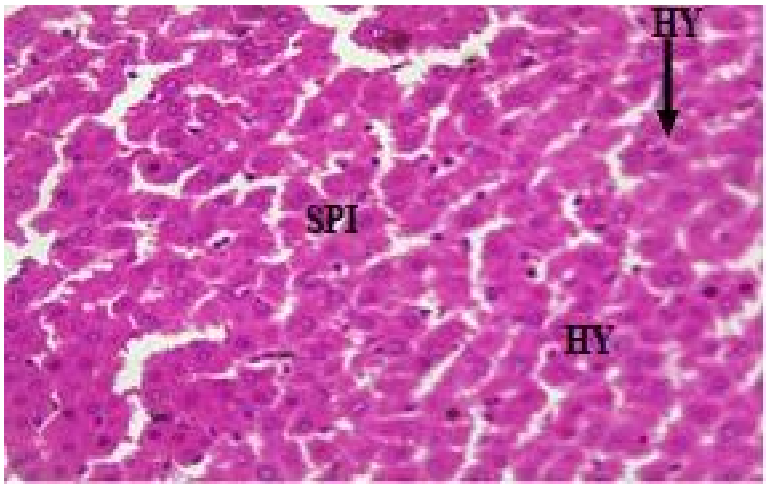


Plate 4. Photomicrograph (histological pattern) of the liver in rats administered with 40% LD50 (126 mg/Kg) CCME: Showing a severe periportal inflammation (SPI) and hypertrophy (HY) (×100)

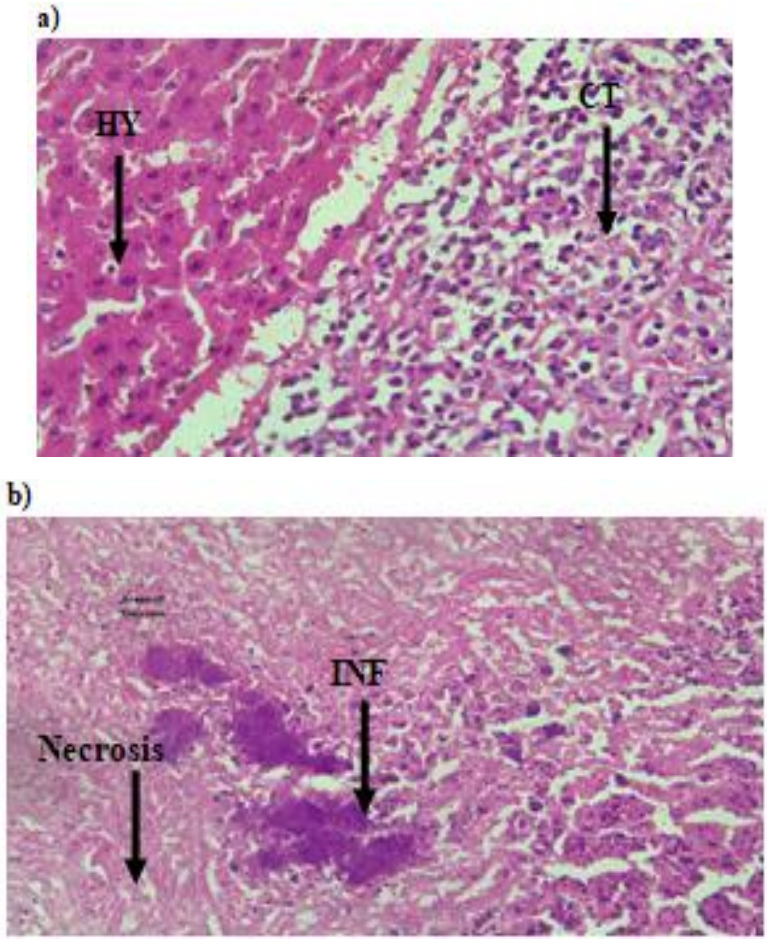


Plate 5. Photomicrograph (Histological pattern) of the liver in rats administered with 80% LD50 (253 mg/Kg) CCME: a) Showing areas of hydropic changes (HY) and Cancerous Tumor (CT). b) Showing areas of Infiltration (INF) and Necrosis (×100)

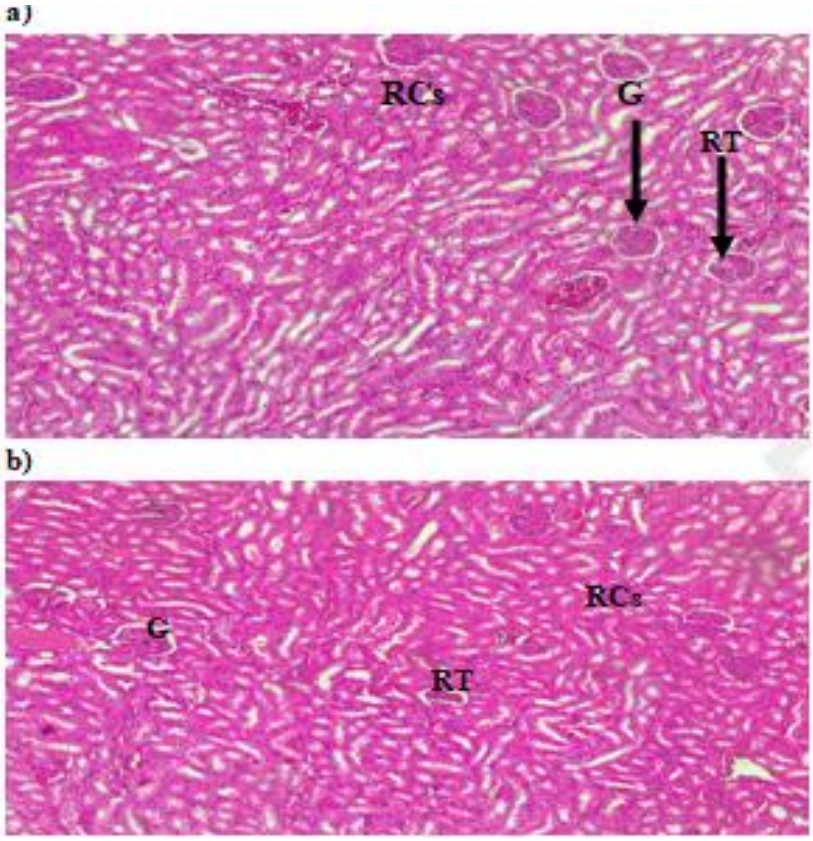


Plate 6. Photomicrograph (Histological architecture) of control group kidney in rats administered with 0.5mls/Kg 0.9% Normal saline (NaCl): a) Showing normal renal cells (RC), renal tubule (RT) and glomeruli (G). b) Showing normal renal cells (RCs), renal tubule (RT) and glomeruli (G) (×100)

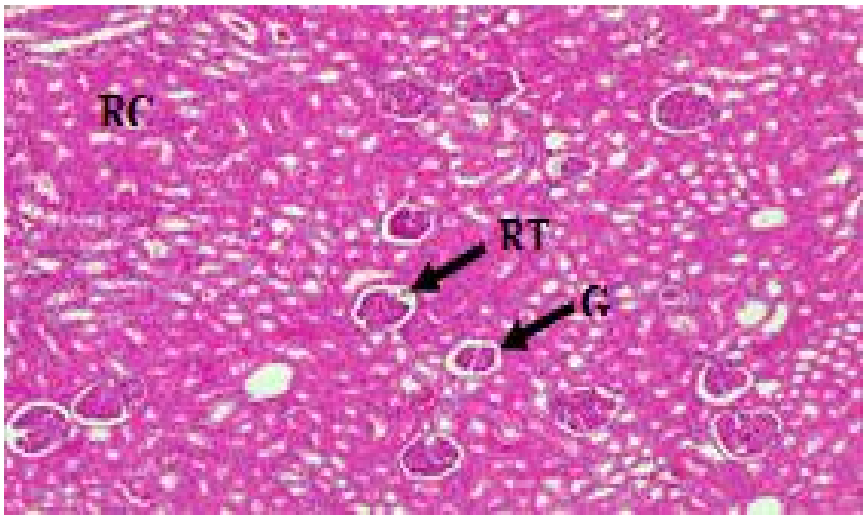


Plate 7: Photomicrograph (Histological pattern) of kidney in rats administered with 32mg/Kg (10% LD50) CCME: Showing no significant change from the normal renal cells, renal tubule (RT) and glomeruli (G) as compared with the control group (×100)

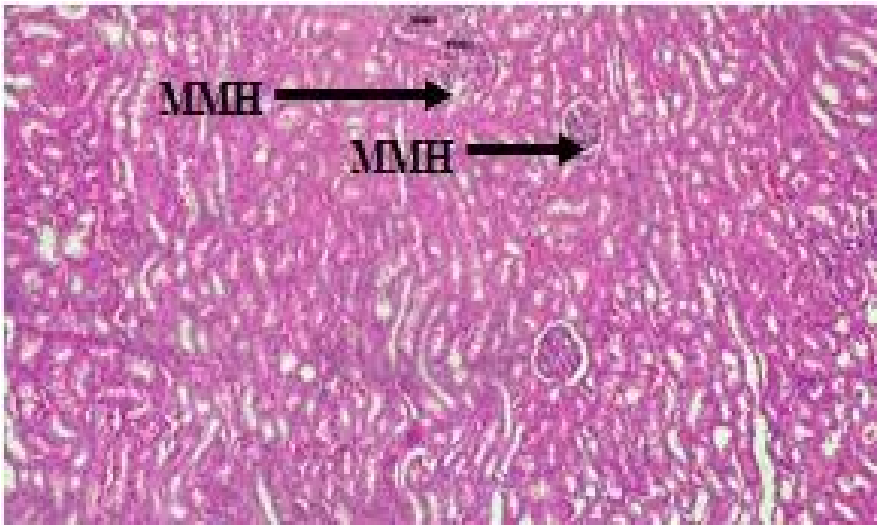


Plate 8. Photomicrograph (histological pattern) of kidney in rats administered with 63mg/Kg (20% LD50) CCME: Showing a mild mesangial hyperplasia (MMH) at the renal tubule and the glomeruli region (×100)

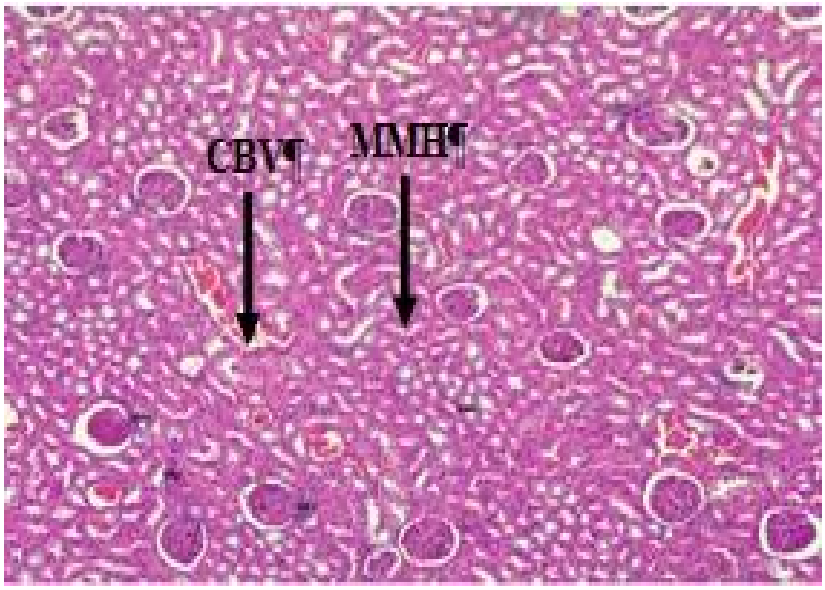


Plate 9. Photomicrograph (Histological pattern) of kidney in rats administered with 126mg/Kg (40% LD50) CCME: Showing a compressed blood vessels (CBV) and Mild Mesangial Hyperplasia (MMH) at the renal tubule and the glomeruli (×100)

4. DISCUSSION

Recent research on the consumption of medicinal plants as conventional medications or and as curatives revealed that, excessive intake may cause adverse toxicological effects to human health [2]. Tannins for example have biological properties that may favour the prevention and management of many ailments and may decrease protein quality by reducing palatability and digestibility [28,29]. However, excess tannins may be toxic because tannins as metal ion chelators can decrease the bioavailability of iron which often leads to anaemia [30]. Saponins consumption often times causes deleterious effects such as haemolysis and permeability of the intestines [31]. Though, saponins have been reported to help in the

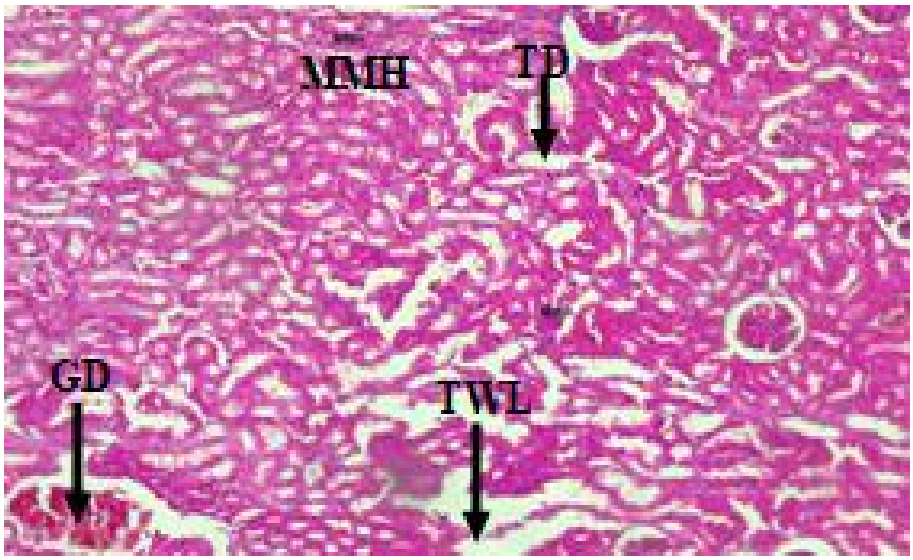


Plate 10. Photomicrograph (Histological pattern) of kidney in rats administered with 253mg/Kg (80% LD50) CCME: Showing a mild mesangial hyperplasia (MMH), glomerular degeneration (GD), tubular degeneration (TD) and tubular widened lumen (TWL) (×100)

control of blood cholesterol levels, bone health, building of the immune system and cancer [32].

In the present study CCME showed no remarkable signs of toxicity and mortality at dose level of 10 and 100 mg/Kg in rats through-out the period of acute toxicity assessment. However, at a dose level of 1000 to 5000 mg/Kg, signs of toxicity such as, shivering, bulging of the eyes, clustering together and dullness, restlessness, dropping of feces and rubbing of nose and mouth on the cage walls, convulsions or coma were observed and mortality was recorded from the third to twelfth day. The median lethal dose (LD_{50}) of CCME was estimated to be 316.23mg/Kg. The LD_{50} (Oral) of CCME indicates that the plant is toxic in accordance with toxicity classification/scale of toxic substances [33]. It has been argued that even if LD_{50} values could be measured exactly and reproducibly, the knowledge of its precise numerical value would barely be of practical importance, because an extrapolation from the experimental animals to man is hardly possible [14]. However, it still serves a great purpose as a first pointer to the safety or toxic potential of a substance whose toxicity profile is not yet known [34].Therefore the findings in this study suggest that at higher concentrations, CCME has lethal effect on the tested animals.

According to Diallo et al. [35], reductions in body and internal organs weights have been considered as a sensitive index of toxicity after exposure to toxic substance. In the 28 days repeated dose oral toxicity study, administration of CCME showed a significant (P<0.05) decrease in body weight of rats administered with 126 and 253 mg/kg from 2nd to 4th week onwards when compared with the control. Likewise organs weight (liver, kidney and heart) in the experimental rats showed a significant (P<0.01) increase in liver, kidney and heart weight at 63 to 253 mg/Kg CCME treated rats compared to the control, hence an indication of the extract toxicity [35].

The assessment of the heamatological parameters are important indices of the physiological and pathological status for both animals and humans as it can point directly to the systemic effects caused by the administered extract [36,1]. In herbal toxicity studies, increase in WBC, LYM and NEUT may indicate the impact of plant extracts in inducing the immune response of treated animals [37]. On the other hand, significant (P<0.01) decrease in the WBC, LYM, and NEUT of the blood indicates a decline in the production of leukocytes called leukopenia, means that the body is less able to fight off infections. The hematological analysis of this study demonstrated that WBC, LYM, and NEUT count showed a significant (P<0.01) increase at doses of 126 and 253mg/Kg compared to the control. This result may indicate that the CCME does possess chemical substances capable of

inducing leukocytosis, which is an abnormally high number of WBC, LYM, and NEUT in the blood circulation or in suppression of normal production of WBC, LYM, and NEUT [38,39].

According to Peters et al. [40], Packed Cell Volume (PCV) or haematocrit (HCT), hemoglobin (HGC), Mean Corpuscular Haemoglobin (MCH) and Mean Corpuscular Haemoglobin Concentration (MCHC) are major indices for evaluating circulatory erythrocytes, and are significant in the diagnosis of anemia, and serve as a useful index for the bone marrow capacity to produce red blood cells as in mammals [41,42], [43]. The effect of CCME on HCT, MCV, MCH, and MCHC showed a significant (P<0.01) increase at 126 and 253 mg/Kg in treated rats compared to the control. These observations suggest that CCME caused a remarkable toxic effect on the levels of analyzed red blood cell (RBC) indices at both doses and may result to primary or secondary polycythemia. Therefore, this implies that the affected rats were anaemic and at risk of been infected with other blood diseases.

In platelet count, thrombocytopenia is a condition of abnormally low number of platelets in the circulation, may result from decreased production or increased destruction of platelets [37]. Some drugs provoke platelet antibodies and platelet destruction, resulting in thrombocytopenia [38]. On the other hand, thrombocytosis is an abnormal increase in the number of circulating platelets [44]. However, in the present study there was significant (P<0.01) increase in the platelets count compared to the control group at 126 and 253 mg/Kg CCME treated rats. This result suggests that CCME at both doses is capable of inducing thrombocytopenia or thrombocytosis.

The primary organs prone to toxic effects of medicines or extracts are the liver and kidney, which play a key role in assessing changes in biochemical parameters. The safety study was accomplished by the implementation of general pre-clinical toxicity experiments to uncover potential poisonous effects of the extract majorly in liver and kidney of animals [45]. A mild inflammation and tissue damage to these organs, is an indication that the permeability of the cell membrane will increase and release cytoplasmic enzymes such as LD, ALP, and AST, while necrosis will release mitochondrial ALT as well as AST leaking into the blood and increase in levels [46,47]. The biochemical indices monitored in the serum such as the electrolytes and other secretory substances of the liver and kidney can be used as 'makers' for assessing the functional capacities of the organs [48]. These parameters of organ function will impair the normal function of the organs if altered.

The ALT and AST are liver specific enzyme markers of necrotic injury and cholestasis [49]. The findings of this present study revealed significant (P<0.01) increase in serum level of ALT and AST at the dose level of 126 to 253 mg/Kg, which is an indication of hepatocellular pattern of liver injury or damage or inflamed liver. Although in clinical practice, it is not uncommon to see elevations of AST, ALT or both in common non-hepatic conditions such as myocardial infarction and rhabdomyolysis. The significant (P<0.01) increase could be due to damage to the hepatic cell or heart attack [50] and may have been induced by some phytocompounds of CCME. ALP is a marker enzyme for the plasma membrane and endoplasmic reticulum [51]. The significant (P<0.01) increase or decrease in the serum ALP could be due to renal or intestinal damage, biliary track damage, fasting, pernicious anemia, hypothyroidism and inflammation [52]. In this present study, the observed significant (P<0.01) decrease in ALP at dose level of 253 mg/Kg could be attributed to enzyme deactivation by the phytochemical constituents of CCME.

Albumin is synthesized by the liver and as such, it represents a major synthetic protein and is a marker of the ability of the liver to synthesize proteins [53]. The decrease in the serum total protein and albumin indicates that the synthetic function of the liver has been affected though malnutrition can cause decrease in albumin (hypo albuminemia) without associated liver disease [54]. The findings of this study showed a significant (P<0.01) decrease in total protein at dose level of 32 and 63mg/Kg compared to the control, suggesting that CCME is capable of inducing a synthetic malfunctioning effect of the liver.

Bilirubin is a major break down product of haemoglobin [52]. The water solubility of bilirubin allows it to be excreted in the bile; the bile is then used to digest food. As the liver becomes irritated, the total bilirubin may rise. As presented in this study, there was a significant (P<0.01) decrease in total bilirubin in the serum of rats administered with 63 to 253 mg/Kg CCME which suggests a risk factor of diseases accompanied

by non-hemolytic anemia as well as a phenomenon observed in seasonal depression [55].

Renal function indices are usually required to assess the normal functioning of different parts of the nephrons [56]. Serum urea, uric acid, creatinine and electrolytes are markers of damage to renal function and could give an insight into the effect of extract on the tubular and or glomerular part of the kidney [57,58]. The significant (P<0.01) increase in creatinine, potassium (K) and bicarbonate (HCO_3^-) in rats treated with 126 and 253 mg/Kg are also signs of impaired renal function [58].

Histopathological examinations provide information to strengthen the findings on biochemical and heamatological parameters [59]. In this present study a gross histological examination of liver sections of rats treated with 126 to 253mg/Kg CCME showed various treatment-related toxicological changes such as severe periportal inflammation (SPI), hypertrophy (HY), areas of hydropic changes (HY), cancerous tumor (CT), and areas of inflammatory infiltrate (INF) inside sinusoidal capillaries and necrosis of the hepatic cells when compared with the control. Also the histopathological examination of the kidney sections of rats treated with 126 and 253 mg/Kg CCME showed some treatment-related toxicological changes such as compressed blood vessels (CBV), mild mesangial hyperplasia (MMH), glomerular degeneration (GD), tubular degeneration (TD) and tubular widened lumen (TWL) when compared with the control. The observed hepato-renal toxicity at 126 to 253mg/Kg dose is also supported by marked elevations of ALT, AST, ALP, total protein, creatinine, and potassium in biochemical analysis conducted, which are indicators of liver and kidney functions [60,61]. Therefore, these two findings suggest that administration of the CCME at 126 and 253 mg/Kg dose for 28 days induces significant damage to the liver and kidneys of treated rats. In general, the sub-chronic study indicates that CCME administration did induce detrimental changes and morphological alterations in liver and kidney of experimental rats.

5. CONCLUSION

The present findings clearly demonstrated that *C. collinum* methanol root extract may be capable of inducing hepatorenal index changes at high doses; caution should be applied as *C. collinum* root extract has a low mean lethal dose and would be toxic at higher concentrations.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Appendix 1. List of chemicals and reagents used

Table 1. List of chemicals and reagents used

S/NO.	Chemicals/ Reagent	Grade	% purity	Manufacturer	Conc. Used
I	Magnesium Chips	-	-	-	
II	Normal saline (NaCl)	-	99.0	Kermel	0.9%
III	Methylated Spirit	-			
IV	Methanol (CH_3OH)	BDH	99.5	BDH, Chemical limited	99.5%
V	Distilled water (H_2O)	AR	98.0	SD fine chemical limited	98.0%
VI	Ammonium hydroxide (NH_4OH)	AR	96.0	Kiran-light laboratory	10%
VII	0.5M Ferric solution	AR	95.0	Kiran-light laboratory	0.5M
VIII	Conc. H_2S0_4	BDH	98.0	SD fine chemical limited	Concentrated
IX	Chloroform	GPR	96.0	Alderich chemical limited	96.0%
X	Hexane	BDH	99.0	BDH chemical, England	99.0%
XI	Sodium Chloride (NaOH)	BDH	99.0	Kiran-light laboratory	10%
XII	Hydrochloric acid (HCl)	BDH	99.0	BDH chemical, England	1% and 10%
XIII	Glacial acetic acid	BDH	95.0	BDH chemical, England	95.0%
XIV	n-Butanol	BDH	99.0	BDH chemical, England	99.0%
XV	Ethyl acetate	BHD	99.0	BDH chemical, England	99.0%
XVI	Ammonia (NH_3)	AR	96.0	Kiran-light laboratory	10%
XVII	Ferric Chloride	BDH		BDH chemical, England	0.1% and 10%
XVIII	Mayer's Reagent (1.36g $HgCl_2$ and 5g KI)			Biochemistry Lab KSUSTA,	

GPR- General Purpose Reagent, AR - Analytical Reagent, BDH - British Drug House

Peer-review history:
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