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ORIGINAL ARTICLE

Sub-acute toxicity study on hydromethanolic leaves extract of *Combretum hypopilinum* (Combretaceae) Diels in Wistar rats

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Abstract

The plant *Combretum hypopilinum* Diels (Combretaceae) has been utilized in Nigeria and other African nations to treat many diseases including liver, inflammatory, gastrointestinal, respiratory, infectious diseases, epilepsy and many more. Pharmacological investigations have shown that the plant possesses anti-infective, antidiarrhoeal, hepatoprotective, anti-inflammatory, anticancer, sedative, antioxidant, and antiepileptic potentials. However, information on its toxicity profile is unavailable despite the plant's therapeutic potential. As such**,** this work aimed to determine the acute and sub-acute oral toxic effects of the hydromethanolic leaves extract of *C. hypopilinum*. The preliminary phytochemical evaluation was carried out based on standard procedures. The acute toxicity evaluation was conducted by oral administration of the extract at the dose of 5000 mg/kg based on the guideline of the Organization of Economic Co-operation and Development (OECD) 423. To investigate the sub-acute toxicity effects, the extract was administered orally to the animals daily for 28-consecutive days at the doses of 250, 500, and 1000 mg/kg. Mortality, body weight and relative organ weight were observed. The hepatic, renal, haematological, and lipid profile parameters were investigated. The liver, kidney, heart, lung, small intestine, and stomach were checked for any histopathological alterations. The results of the phytochemical investigation showed cardiac glycosides, tannins, steroids, flavonoids, alkaloids, saponins, and triterpenes. Based on the acute toxicity investigation outcome, no death and signs of toxic effects were observed. The result showed that the oral median lethal dose (LD_{50}) of the extract was more than the 5000 mg/kg. The extract remarkably reduced the weekly body weight of the animals at 500 mg/kg in the first and second weeks. It also significantly decreased the relative kidney weight, alkaline phosphatase, glucose, potassium, and low-density lipoprotein. There was a remarkable elevation in the percentage of eosinophils, basophils, monocytes, and granulocyte. There were histopathological abnormalities on the kidney, lung, stomach, and small intestine. The extract is relatively safe on acute exposure but moderately toxic at higher doses on sub-acute administration, particularly to the kidney.

Keywords Acute toxicity · Biochemical parameters · *Combretum hypopilinum* · Haematological parameters · Histopathological changes · Sub-acute toxicity

Abbreviations

AC	Alveoli congestion
ALP	Alkaline phosphatase
ALT	Alanine transaminase
ANOVA	Analysis of variance
AN	Alveoli necrosis
AST	Aspartate transaminase
CNS	Central nervous system
CVS	Cardiovascular diseases
EDTA	Ethylenediaminetetraacetic acid
FDA	Food and drug administration
GAE/g	Gallic acid equivalent per gram of the extract
GC–MS	Gas chromatography-mass spectrometry
HCT	Haematocrit
HDL	High-density lipoprotein
HMECH	Hydro methanolic leaf extract of *Combretum hypopilinum*
HPLC	High-performance liquid chromatography
IA	Intestinal atrophy
KH	Kupffer cell hyperplasia

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LD_{50}	Median lethal dose
LDL	Low-density lipoprotein
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
MTN	Moderate tubular necrosis
NA	Normal alveoli
NF	Normal features
NG	Normal glomerulus
NH	Normal features
NM	Normal myocardium
NV	Normal villi
NT	Normal tubules
OECD	Organization of economic cooperation and development
QEQ/g	Quercetin equivalent per gram of the extract
RBC	Red blood cells
ROW	Relative organ weight
Rpm	Revolution per minutes
SHN	Slight hepatic necrosis
SN	Slight mucosa necrosis
VA	Villi atrophy
WBC	White blood cells
WHO	World Health Organization

Introduction

Herbal products have been employed to source new bioactive compounds to treat many diseases [1]. Even though there has been an emergence of modern and advanced scientific methods of drug discovery, medicinal plants are the leading sources of drugs [2]. Approximately 80% of the world population consider and utilize herbal preparations for their basic health [3]. Several medicinal plants were scientifically investigated to evaluate their therapeutic potentials, thus providing insight into drug discovery and development [4, 5]. There has been a perception that plant-derived substances are devoid of harmful effects [6]. The rising need for alternative medicine, global attention in phytomedicine and herbal treatments, and the high cost of conventional drugs lead to a rapid increase in the use of herbal products from ethnomedicinal experience [7]. Although some of these medicinal plants used traditionally by many indigenous people are relatively safe, many of them are highly toxic when taken for a short or long period [8]. There has also been limited scientific information on many medicinal plants' clinical, pharmacological, and toxicological profiles [9], including the plant *Combretum hypopilinum* [10]. As such, it is important to provide scientific evidence regarding the short and long term toxic effects of herbal products and their extracts to raise confidence in their use for human safety in an attempt to discover novel therapeutic compounds [11, 12]. The scientific investigation of a medicinal plant's toxicity profile is vital in providing safety information and potential therapeutic purposes [13].

The plant *C. hypopilinum* (synonym: *Combretum collinum*) is a shrub that is part of the family Combretaceae [14, 15]. The plant is average in size with many stems, deciduous and annually sheds its leaves. It has many branches that are 12–17 m tall [15]. The local names of the plant are *Jar taramniya* or *jar ganye* (Hausa), *buski daneehi* (Fulani), *katankara* (Kanuri), and *aro* (Yoruba) [16]. The plant *C. hypopilinum* in its natural habitat is shown in Fig. 1.

The fresh leaves of *C. hypopilinum* are utilized against cholestasis, diarrhoea, dysentery, stomach pain [17], cough, bronchitis, tuberculosis, jaundice, and snakebite [18]. The leaves are used as a diuretic and purgative [17]. The fresh leaves of *C. hypopilinum* are applied topically to alleviate fatigue and rheumatism [19]. Additionally, the leaves of *C. hypopilinum* are used to enhance blood production [19]. The leaves have also been used against diarrhoea [10, 14, 17, 20], inflammatory conditions, ulcer, earache, ascariasis and bacterial infections [21]. The leaves are used as decoction against malaria and dysentery [14]. Amino acids such as arginine, cysteine, leucine, proline, isoleucine, threonine, lysine, hydroxyproline, serine, histidine, tyrosine, phenylalanine, valine, aspartic acid, methionine, glycine, glutamic acid, and alanine were isolated from the gum fluid of *C. hypopilinum* [22]. Some bioactive compounds, including combretastatin A and B, stilbenoids, and phenanthrenes, were identified and isolated from the plant [23]. Furthermore, an analytical study on the gum exudates of *C. hypopilinum* revealed arabinose, xylose, galactose, rhamnose, mannose and glucuronic acid [24]. Glucoside, xyloside derivatives, mollic acid, myricetin, and 3β-O-arabinoside were isolated from the plant's leaf using the high-performance liquid chromatography (HPLC) technique [21, 23]. Additionally, Marquardt et al. [21] identified various acids (lignoceric, mallic, arachidic, palmitic, myristic,

Fig. 1 The plant *Combretum hypopilinum* in its natural habitat

stearic, behenic and oleic acids). Other constituents identified include pentacosane, hexacosane, heptacosane, octacosane, nonacosane, triacontane, nonadecane, squalene, campesterol, and stigmasterol from the n-hexane leaves of the plant [21]. In 2018, Idoh et al. [15] quantified the total phenolic (583.56 ± 5.95 mg GAE/g) and flavonoids contents (76.11 ± 5.97 QEQ/g) in the root of *C. hypopilinum*.

The work of Marquardt and colleagues [21] showed the inhibitory effects of ethanol leaves extract of the plant on *Pseudomonas aeruginosa, Staphylococcus epidermis, staphylococcus aureus, Klebsiella pneumoniae* as well as *Escherichia coli*. Besides, the leaves of *C. hypopilinum* possesses antifungal [15], antitumor [19], larvicidal [25], antioxidant, anti-inflammatory, hepatoprotective [15], antidiarrhoeal [10, 16] sedative and anticonvulsant activities [26]. In 1977, Bamgbose and Dramane reported the hypotensive and central nervous system actions of the plant. Recently, Ahmad et al. [16] have shown that *C. hypopilinum* leaves possess antidiarrhoeal activity via interaction with intestinal opioid and adrenergic receptors.

Despite the increase and promising use of *C. hypopilinum* in traditional medicine and its therapeutic potential for managing many diseases, there is a lack of safety information on the plant's short and long-term administration. Therefore, this experiment was done to determine the safety profile of *C. hypopilinum* following the acute and sub-acute oral administration to stimulate further research to discover novel bioactive compounds for drug development.

Materials and methods

Collection and authentication of the plant

The leaves of *C. hypopilinum* were sourced from the Giwa Area, Kaduna State of Nigeria. Then the identification of the plant material was done at the Botany Department, Faculty of Life Sciences, Ahmadu Bello University (ABU), Zaria, Nigeria by a taxonomist Mallam Namadi Sanusi. A voucher number of 012063 was obtained.

Animals

Adult Wistar rats (120–160 g) of both gender aged 10–12 weeks were sourced from the experimental animal facility located in the Pharmacology and Therapeutics Department of the Faculty of Pharmaceutical Sciences, Ahmadu Bello University (ABU), Zaria, Nigeria. The rats used for the experiment were kept in well-ventilated cages and provided with a rodent feed (Vital feed, Jos, Nigeria) with access to water ad libitum. The rats were maintained under an optimum laboratory environment (temperature 22 ± 3 ^{0}C, relative humidity of 30–70% with 12-h light and 12-h dark cycle). They were kept in polypropylene cages for 2 weeks to acclimatize to the laboratory condition before starting the experimental procedures. The experimental methods were granted permission by Ahmadu Bello University Ethical Committee on Animal Use and Care Research Policy (ABUCAUC) with an approval number of ABUCAUC/2020/40 and conducted as per the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. After completing the experimental procedures, all the animals were anaesthetized with chloroform and quickly euthanized by cervical dislocation. They were immediately buried deeply according to the institutional guideline for proper disposal of laboratory animal remains.

Extraction

The leaves of the plant *C. hypopilinum* were kept under shade environment to dry. The leaves were subsequently size-reduced with pestle and mortar. Then 1000 g of the powdered material were extracted using 70% $^{v}/_{v}$ aqueous methanol (2.5 L) with the aid of soxhlet apparatus for 72-h. We then concentrated the extract by removing the solvent on a water bath set at 45 ^{0}C. Then the crude extract was weighed and packed in a well-closed container and labelled as "hydromethanolic leaf extract of *C. hypopilinum*" (HMECH). Then the extractive value of the extract was determined as follows:

$$\text{Percentage yield}\,(\%) = \frac{\text{Weight of the crude extract}\,(g)}{\text{Weight of the powdered plant material}\,(g)} \times 100$$

Preliminary phytochemical investigation

The phytochemical investigation of the HMECH was done based on the standard method [27] described below:

Test for flavonoids

Shinoda test: 100 mg of the extract was dissolved in 2 ml of 50% methanol and warmed on a steam bath. Then few pieces of metallic magnesium chips and four drops of concentrated hydrochloric acid were added. The appearance of a reddish colour showed the presence of flavonoids.

Sodium hydroxide test: Few drops of 10% sodium hydroxide were added to an aqueous solution of the HMECH. Yellow colouration was formed, which indicated the presence of flavonoids.

Test for cardiac glycosides

Keller Killiani's test: 1 ml of glacial acetic acid containing one drop of $FeCl_3$ was used to dissolve 20 mg of the extract.

The mixture was transferred into a dry test tube, and then 1 ml of concentrated sulphuric acid was added along the side of the test tube to form a layer at the bottom. Formation of purple ring colour at the interface was observed which indicated the presence of cardiac glycosides.

Test for saponins

Frothing test: 1 ml solution of the HMECH was shaken with 3 ml of distilled water vigorously for 30 s and allowed to stand for 30 min in a vertical position. Formation of honey comb froth which persisted for more than 30 min was observed, indicating the presence of saponins.

Test for tannins

Ferric chloride test: To 1 ml of the HMECH solution, 3 drops of ferric chloride solution were added. A greenish-black precipitate was formed which indicated the presence of condensed tannins.

Lead sub-acetate test: The HMECH (0.5 g) was dissolved in 2 ml of water, and then 3 drops of lead sub-acetate solution were added and observed for the formation of a black-green coloured precipitate which indicated the presence of tannins.

Test for steroids and triterpenes

Lieberman-Burchard's test: To 1 ml of the chloroform solution of the extract, an equal volume of acetic acid anhydride was added and mixed gently. Then 1 ml of concentrated sulphuric acid was added down the side of the test tube to form a lower layer and observed immediately for one hour. A brownish-red colour was observed immediately at interphase, which indicated triterpenes and blue-green at the upper layer indicated the presence of steroids.

Test for alkaloids

The MECH (0.5 g) was stirred with 5 ml of 1% aqueous HCl and then filtered. The filtrate was tested carefully with some alkaloidal reagents as follows:

Dragendoff's test: To a few ml of the filtrate, two drops of Dragendoff's reagent were added by the side of the test tube. The orange-red precipitate was observed which indicated the presence of alkaloids.

Wagner's test: To a few ml of the filtrate, two drops of Wagner's reagent were added by the side of the test tube. Reddish-brown precipitate was observed which confirmed the presence of alkaloids.

Test for anthraquinones

Bontrager's test: To 500 mg of the extract in a dry test tube, 5 ml of chloroform was added, and then the test tube was stoppered, shaken for at least 5 min and filtered. The filtrate was shaken with an equal volume of 10% ammonia solution. The upper aqueous layer was observed for bright pink colour for the presence or absence of free anthraquinones.

Preparation of the extract for the experiment

For the daily preparation, 2.5 g of the HMECH was accurately weighed and transferred into a cleaned and dried porcelain mortar containing 10 ml of distilled water and appropriately mixed in one direction until a homogenous mixture was formed (concentration: 250 mg/ml). The solution of the extract was administered to the rats by oral gavage based on the body weight.

Acute toxicity evaluation

The determination of the acute toxic effect of HMECH was done based on the guideline of the Organization of Economic Co-operation and Development (OECD) 423 [28]. We used non-pregnant and nulliparous female rats to estimate the oral median lethal dose (LD_{50}) of the extract. The rats were deprived of food overnight before the administration of the HMECH. Three rats each received 5000 mg/kg of HMECH via the oral route and were deprived of foods for two hours with the provision of water ad libitum. We subsequently observed the rats for signs of toxic effects including tremor, convulsion, salivation, lacrimation, diarrhoea, lethargy, sleep, respiratory, behavioural pattern, the onset of toxicity if any, and length of the recovery period as well as the time of death every 30 min for the first four hours. The observation of the animals continued every day for 14 consecutive days.

Sub-acute toxicity

The 28-days repeated oral toxic effects of HMECH was conducted following OECD 407 guidelines for testing chemicals [29]. Twenty-four (24) rats were grouped into four groups ($n = 6$). The group I as the control received distilled water (1 ml/kg) orally. Simultaneously, groups II, III, and IV received 250, 500, and 1000 mg/kg of HMECH, respectively, one time in a day for 28-consecutive days. The weekly body weights of the animals were checked and monitored for any signs and symptoms of toxic effects and death. Following completion of the experimental procedure, the rats were deprived of food overnight with adequate water and anaesthetized with chloroform on the 29th day. The blood sample was collected from each rat

through cardiac puncture in ethylenediaminetetraacetic acid (EDTA)-containing tubes and plain tubes to investigate haematological and biochemical indices, respectively. The animals were euthanized quickly by a physical method of euthanasia (cervical dislocation). They were confirmed dead following the euthanasia and immediately buried deeply according to the institutional guideline for proper disposal of laboratory animal remains.

Biochemical analysis

Blood sample for the analysis of biochemical parameters was kept at room temperature for 1-h and allowed for proper clotting. The centrifugation of the blood samples was done at 3,000 revolutions per minute (rpm) for 10 min. The serum obtained after the centrifugation was used to investigate any alterations in the biochemical biomarkers including alkaline phosphatase (ALP), alanine transaminase (ALT), aspartate transaminase (AST), bilirubin (total and direct), protein, albumin, urea, glucose, creatinine, and electrolytes (potassium, sodium, chlorine, and bicarbonate). Besides, the lipid parameters namely, triglycerides, high-density lipoproteins (HDL), cholesterol, and low-density lipoprotein (LDL) were evaluated.

Hematological analysis

The haematological analysis was done to determine any changes in the levels of haemoglobin (HGB), red blood cells (RBC), mean corpuscular haemoglobin (MCH), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), platelet, granulocytes, monocytes, lymphocytes, neutrophils, basophils, eosinophils and white blood cell (WBC).

Evaluation of relative organ weight (ROW)

Following the completion of the experimental procedures, each rat was weighed. After the blood collection, the livers, kidneys, lungs, hearts, stomachs, and small intestines of each rat were removed and weighed. The following formula was used to calculate the ROW:

$$\mathrm{ROW} = \frac{\text{Absolute organ weight}\,(g)}{\text{Final body weight of the animal}\,(g)} \times 100$$

Histopathological analysis

The liver, kidneys, lung, heart, stomach, and small intestine removed from each animal were processed for embedment in paraffin wax after fixation in 10% formalin. The sections from the livers, kidneys, hearts, lungs, stomachs, and small intestines were cut 4–5 µm with rotary microtone. The staining was done with hematoxylin and eosin. The photomicrographs of the sections were analyzed for any histopathological abnormalities.

Statistical analysis

We performed the analysis of the data with IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp. The data were represented as mean ± standard error of the mean (SEM) in figures and tables. We used one-way variance analysis (ANOVA) to analyse ROW and biochemical, haematological, and lipid profile parameters followed by Dunnett's post hoc test. In contrast, repeated measure ANOVA was applied to analyse weekly body weight. The $p \leq 0.05$ values were taken as significant.

Results

Extractive value

The extraction of the 1000 g leaves of *C. hypopilinum* produced 126.24 g of the extract. Therefore, the percentage yield was 12.62% $^{W}/_{W}$.

Phytochemical constituents

Phytochemical investigation of HMECH indicated the presence of flavonoids, triterpenes tannins, cardiac glycosides, saponins, alkaloids and steroids.

Acute toxicity

Following the oral administration of HMECH to the rats at the dose of 5000 mg/kg, there were no death and clear signs of toxic effects. Therefore, the oral LD_{50} of the HMECH was higher than the administered dose (5000 mg/kg).

Weekly body weight of rats following 28-days repeated administration of HMECH

The sub-acute oral toxicity evaluation demonstrated that the HMECH (250, 500, and 1000 mg/kg) produced no deaths in rats. Besides, no alteration in the weekly body weight of the rats was observed in the groups that received the lowest (250 mg/kg) and the highest dose (1000 mg/kg) of the extract. However, the body weight of the rats that received 500 mg/kg of the HMECH reduced ($p < 0.05$) in the first and second weeks relative to the control. Table 1 indicates the results of the sub-acute oral administration of HMECH on the weekly body weight.

Table 1 Weekly body weight of rats following 28-days repeated administration of HMECH

Treatments (mg/kg, *p.o*)	Mean body weights (g)				
	Week 0	Week 1	Week 2	Week 3	Week 4
DW (1 ml/kg)	145.83 ± 6.54	163.00 ± 6.79	175.17 ± 5.22	182.17 ± 5.01	190.67 ± 5.67
HMECH 250	145.00 ± 6.28	141.17 ± 5.47	154.67 ± 8.88	161.00 ± 9.10	167.50 ± 9.17
HMECH 500	146.00 ± 5.54	134.83 ± 6.47*	144.50 ± 6.82*	153.67 ± 7.50	162.17 ± 7.66
HMECH 1,000	145.00 ± 6.40	151.50 ± 4.28	166.00 ± 6.90	180.00 ± 9.07	192.17 ± 9.46

The Data are represented in the table as Mean ± SEM

DW distilled Water, *HMECH* hydromethanolic leaf extract of *Combretum hypopilinum*, n = 6

*$p < 0.05$ in relation to control group (Repeated measure ANOVA then followed by Dunnet's post hoc test)

Relative organ weights of rats following 28-days repeated administration of HMECH

The repeated administration of HMECH at all doses showed no alteration in the ROW of the liver, heart, lung, stomach, and small intestine. However, the MECH significantly reduced the relative kidney weight at the higher doses of 500 and 1000 mg/kg relative to the distilled water group. The effects of the MECH on the ROW of the animals are presented in Fig. 2.

Hepatic parameters of rats following 28-days repeated administration of HMECH

The HMECH did not show any remarkable difference in ALT, AST, albumin, total protein, total and direct bilirubin. However, it reduced ($p < 0.01$ and $p < 0.001$) ALP level at the doses of 500 and 1,000 mg/kg, respectively, and a remarkable ($p < 0.01$) decreased glucose at the dose of 1,000 mg/kg relative to the control. The effects of the extract on the hepatic parameters following 28-days of repeated oral administration are presented in Table 2.

Renal parameters of rats following 28-days repeated administration of HMECH

The administration of HMECH did not produce a significant difference in urea, creatinine, sodium, chlorine, and bicarbonate. However, the extract revealed a remarkable ($p < 0.01$ and $p < 0.001$) decline in the potassium level in comparison to the group that received distilled water. The effects of the HMECH on renal biomarkers are presented in Table 3.

Haematological parameters of rats following 28-days repeated administration of HMECH

The HMECH did not alter the WBC, lymphocytes, HGB, RBC, HCT, MCV, MCH, and MCHC in all the groups. However, the HMECH at the dose of 1,000 mg/kg remarkably ($p < 0.05$) elevated the percentage of eosinophils, basophils, monocytes and granulocytes. The extract also increased ($p < 0.01$) the level of platelets at 500 mg/kg. The effects of the HMECH on the haematological parameters are presented in Table 4.

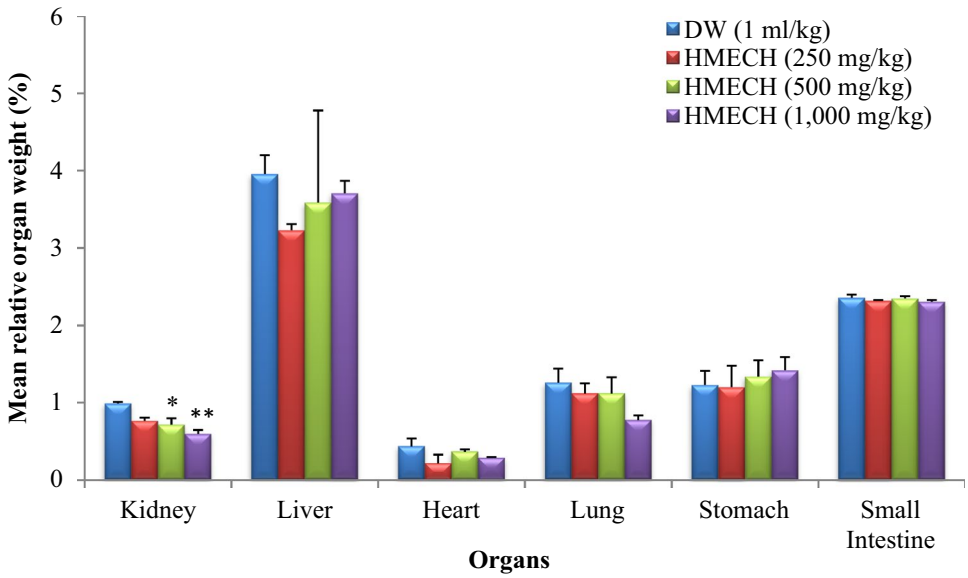

Fig. 2 Relative organ weight of rats following 28-days repeated administration of HMECH. The data are documented as Mean ± SEM; *$p < 0.05$ and **$p < 0.01$ compared to control group (One-way ANOVA then followed by Dunnet's post hoc test), *DW* distilled water, *HMECH* hydromethanolic leaf extract of *Combretum hypopilinum*, n = 6

Table 2 Hepatic parameters of rats following 28-days repeated administration of HMECH

Treatments (mg/kg)	ALT (I.U/L)	AST (I.U/L)	ALP (I.U/L)	TP (mg/dL)	ALB (mg/dL)	TB (mg/dL)	DB (mg/dL)	GLU (mg/dL)
DW (1 ml/kg)	61.17 ± 0.98	106.33 ± 5.16	49.82 ± 2.94	11.73 ± 1.14	2.98 ± 0.60	11.48 ± 0.46	4.88 ± 0.34	90.67 ± 5.58
HMECH 250	54.00 ± 2.38	105.00 ± 4.68	50.55 ± 2.47	12.30 ± 0.51	3.12 ± 0.87	11.38 ± 0.66	5.68 ± 0.19	78.33 ± 4.71
HMECH 500	62.00 ± 3.29	89.67 ± 11.02	31.67 ± 4.01*	14.47 ± 0.78	2.90 ± 0.93	10.58 ± 0.61	12.25 ± 6.98	72.00 ± 6.33
HMECH 1,000	54.50 ± 3.23	87.00 ± 15.39	26.22 ± 3.03**	12.28 ± 0.70	3.03 ± 0.61	11.62 ± 0.55	10.28 ± 6.54	51.83 ± 9.04*

The data for the effects of the MECH on hepatic parameters are represented as Mean ± SEM

DW distilled water, *HMECH* hydromethanolic leaf extract of *C. hypopilinum*, *AST* aspartate transaminase, *ALT* alanine transaminase, *ALP* alkaline phosphatase, *TP* total protein, *ALB* albumin, *TB* total bilirubin, *DB* direct bilirubin, *GLU* glucose, *I.U* International Unit, n = 6

*$p < 0.01$ and **$p < 0.001$ compared to control group (One way-ANOVA then followed by Dunnet's post hoc test)

Table 3 Renal parameters of rats following 28-days repeated administration of HMECH

Treatments (mg/kg)	Urea (mmol/L)	Creatinine (mmol/L)	Sodium (mmol/L)	Potassium (mmol/L)	Chlorine (mmol/L)	Bicarbonate (mmol/L)
DW (1 ml/kg)	37.02 ± 1.74	0.78 ± 0.05	137.32 ± 1.91	27.93 ± 1.32	26.17 ± 1.96	89.75 ± 3.21
HMECH (250)	33.27 ± 4.02	0.80 ± 0.05	148.69 ± 6.04	22.92 ± 0.67*	24.17 ± 1.82	82.50 ± 2.06
HMECH (500)	33.95 ± 1.16	0.78 ± 0.05	157.53 ± 2.59	20.00 ± 0.57**	22.17 ± 1.45	76.67 ± 2.38
HMECH (1,000)	36.07 ± 1.92	0.85 ± 0.06	160.57 ± 1.20	19.85 ± 1.01**	23.33 ± 1.05	87.00 ± 2.84

The data for the effects of the MECH on renal parameters are represented as Mean ± SEM

DW distilled water, *HMECH* hydromethanolic leaf extract of *C. hypopilinum*, n = 6

*$p < 0.01$and **$p < 0.001$ compared to control group (One-way ANOVA then subsequently by Dunnet's post hoc test)

Table 4 Haematological markers of rats following 28-days repeated administration of HMECH

Haematological parameters	Treatments (mg/kg)			
	DW (1 ml/kg)	HMECH (250)	HMECH (500)	HMECH (1,000)
WBC($\times 10^9$/L)	5.70 ± 0.58	5.82 ± 1.07	6.10 ± 0.74	6.88 ± 0.75
LYM (%)	31.88 ± 2.17	33.68 ± 1.94	36.92 ± 2.51	32.90 ± 1.86
MID (%)	5.22 ± 0.59	7.23 ± 1.66	9.13 ± 1.24	10.92 ± 0.78*
GRAN (%)	44.03 ± 1.86	47.22 ± 3.16	53.15 ± 2.36	56.13 ± 2.04**
RBC ($\times 10^{12}$/L)	4.80 ± 0.06	5.08 ± 0.31	4.65 ± 0.15	4.96 ± 0.12
HGB (g/dL)	15.14 ± 0.81	14.73 ± 0.45	13.77 ± 0.32	14.58 ± 0.47
HCT (%)	45.33 ± 2.40	42.83 ± 1.99	42.00 ± 1.24	46.17 ± 2.12
PLT ($\times 10^9$/L)	222.67 ± 24.17	336.80 ± 36.20	400.67 ± 25.72**	311.67 ± 25.92
MCV (fl)	85.11 ± 1.75	87.48 ± 1.55	86.88 ± 1.48	88.08 ± 1.75
MCH (pg)	32.48 ± 1.66	29.05 ± 0.91	30.27 ± 0.48	31.12 ± 0.52
MCHC (g/dl)	34.96 ± 0.64	33.62 ± 0.47	33.83 ± 0.35	34.22 ± 0.27

The data are for the effects of MECH on blood parameters are represented as Mean ± SEM

DW distilled water, *HMECH* hydromethanolic leaf extract of *C. hypopilinum*, *WBC* white blood cell count, *LYM* lymphocytes, MID % percentage of eosinophils, basophils and monocytes, *GRA* granulocytes, *RBC* red blood cell count, *HGB* hemoglobin, *HCT* hematocrit, *PLT* platelet, *MCV* mean corpuscular volume, *MCH* mean corpuscular haemoglobin, *MCHC* mean corpuscular haemoglobin concentration, n = 6

*$p < 0.05$ and **$p < 0.01$ compared to control group (One-way ANOVA then subsequently by Dunnet's post hoc test)

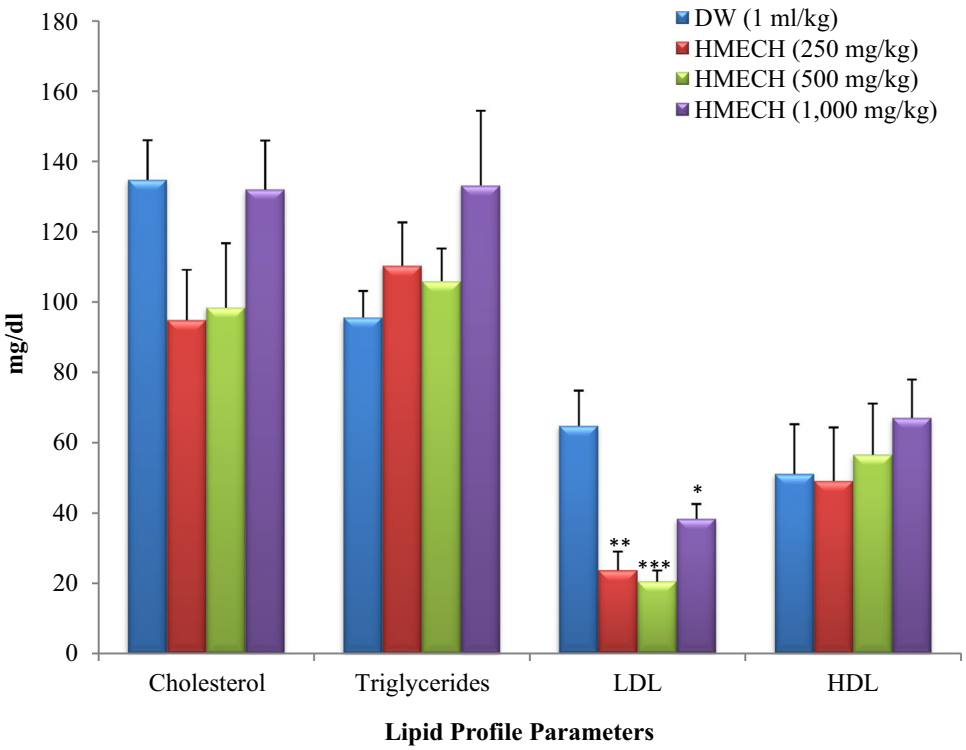

Fig. 3 Lipid profile of rats following 28-days repeated administration of HMECH. The data for the effect of MECH on lipid parameters are represented as Mean ± SEM; $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ compared to control group (One way-ANOVA then subsequently by Dunnet's post hoc test), *DW* distilled water, *HMECH* hydromethanolic leaf extract of *C. hypopilinum*, *LDL* low-density lipoprotein, *HDL* high-density lipoprotein, n = 6

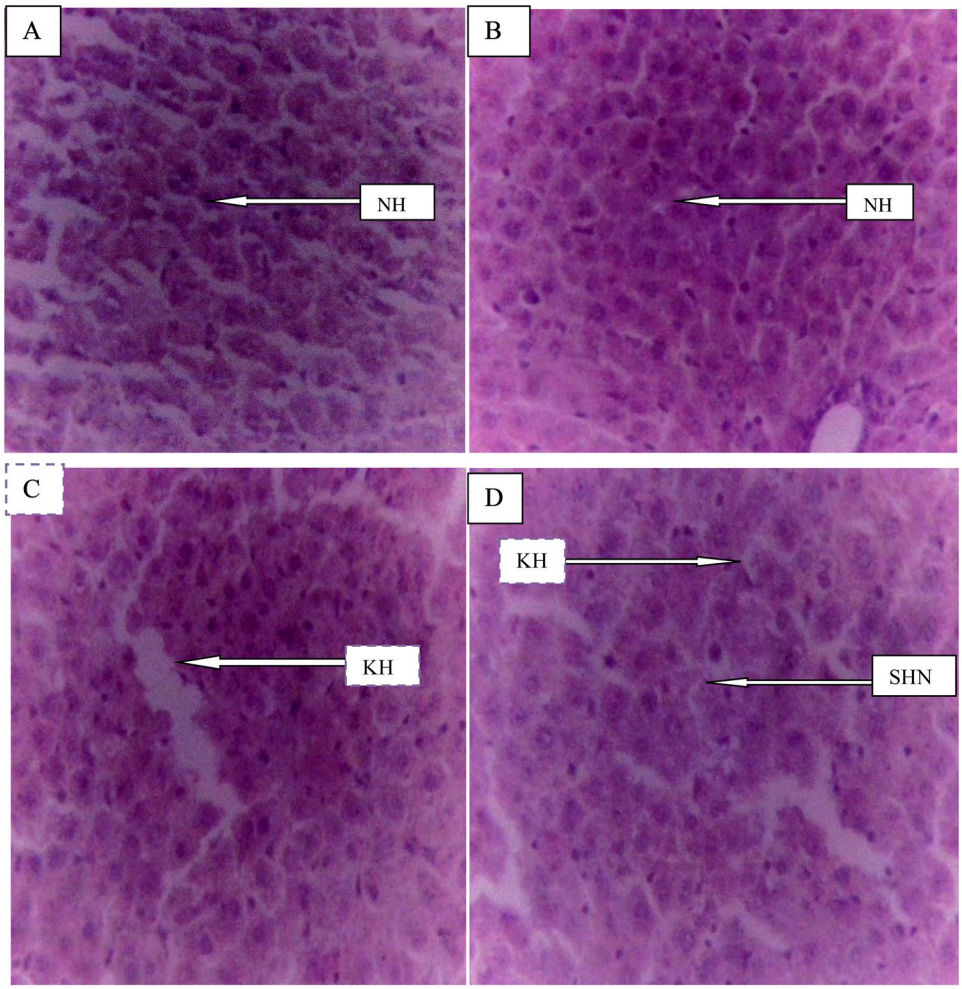

Fig. 4 Photomicrographs of liver sections of rats following 28-days daily oral administrations of HMECH (Haematoxylin and eosin-stained at × 250). **a** Control (Distilled water, 1 ml/kg), **b** HMECH (250 mg/kg), **c** HMECH (500 mg/kg), **d** HMECH (1,000 mg/kg), *NH* normal hepatocytes, *SHN* slight hepatic necrosis, *KH* Kupffer cell hyperplasia, *HMECH* hydromethanolic leaf extract of *Combretum hypopilinum*

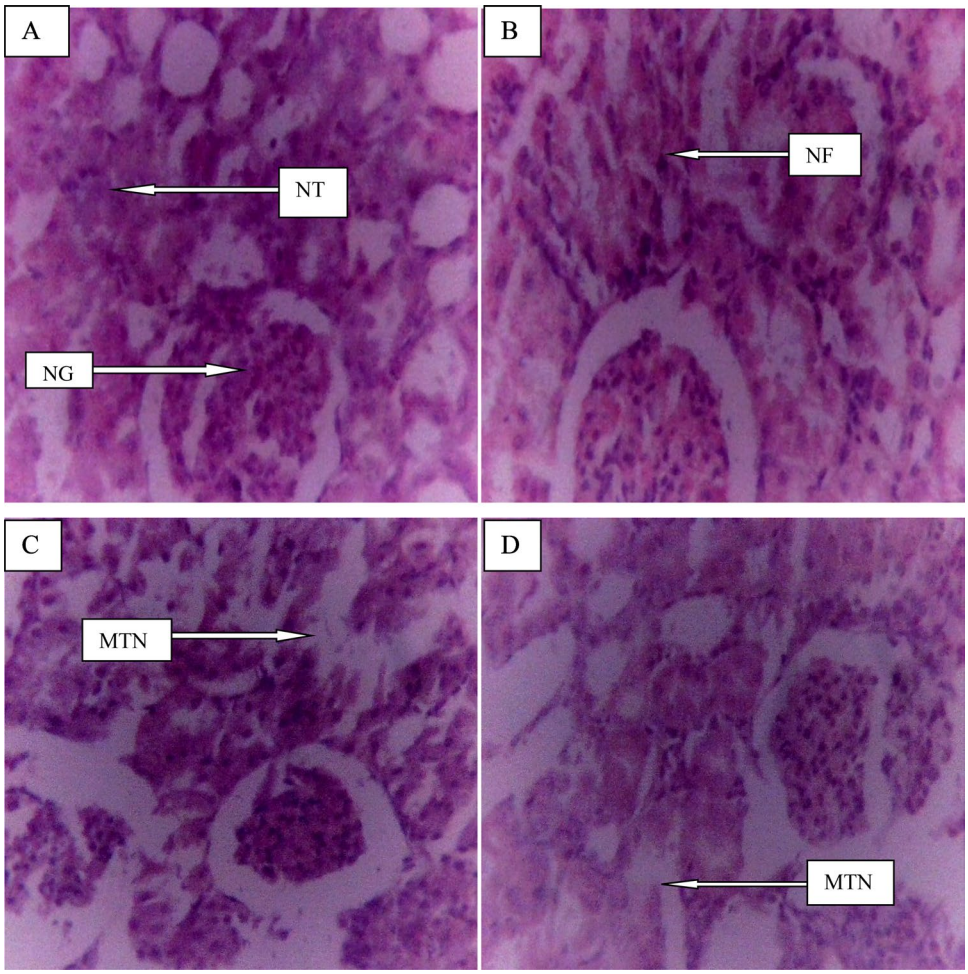

Fig. 5 Photomicrographs of kidney sections of rats following 28-days daily oral administration of HMECH (Haematoxylin and eosin-stained at × 250). **a** Control (Distilled water, 1 ml/kg), **b** HMECH (250 mg/kg), **c** HMECH (500 mg/kg), **d** HMECH (1,000 mg/kg), *NG* normal glomerulus, *NT* normal tubules, *NF* normal features, *MTN* moderate tubular necrosis, *HMECH* hydromethanolic leaf extract of *Combretum hypopilinum*

Lipid profile of rats following 28-days repeated administration of HMECH

The HMECH did not change the cholesterol, triglycerides, and HDL levels in all the groups. However, it remarkably decreased the LDL level compared to the control group. The outcomes of the effects of the HMECH on lipid profile are presented in Fig. 3.

Histology of liver, kidney, lung, heart, stomach, and small intestine of rats following 28-days repeated administration of HMECH

The extract did not show any histopathological abnormalities on the liver of the rats at the lowest dose. However, the animals that were administered the 500 mg/kg of the HMECH revealed kupffer cell hyperplasia. The HMECH at the highest dose (1000 mg/kg) revealed slight hepatic necrosis and kupffer cell hyperplasia (Fig. 4). In contrast, no histopathological abnormalities were observed on the kidney structures of the rats that received the 250 mg/kg of the HMECH. The animals that received the higher doses (500 and 1000 mg/kg) of the test substance showed moderate tubular necrosis (Fig. 5). The extract did not reveal any histopathological abnormalities in the rats' heart muscles at all doses (Fig. 6).

The groups that received the 250 and 500 mg/kg of HMECH revealed typical lung features. However, the HMECH at the highest dose revealed moderate alveoli congestion and slight alveoli necrosis (Fig. 7). Similarly, the group treated with 250 mg/kg showed normal gastric mucosa, while the groups that received 500 and 1,000 mg/kg of the HMECH showed slight necrosis of the gastric mucosa (Fig. 8). Furthermore, the extract at 500 mg/kg indicated clear intestinal villi, while the groups that received the 250 and 1,000 mg/kg of the test extract revealed slight small intestinal atrophy and moderate villi atrophy, respectively (Fig. 9). The extract did not produce any histopathological alterations on the heart, liver, lung, kidney, stomach, and small intestine in the control group (Figs. 4, 5, 6, 7, 8, 9).

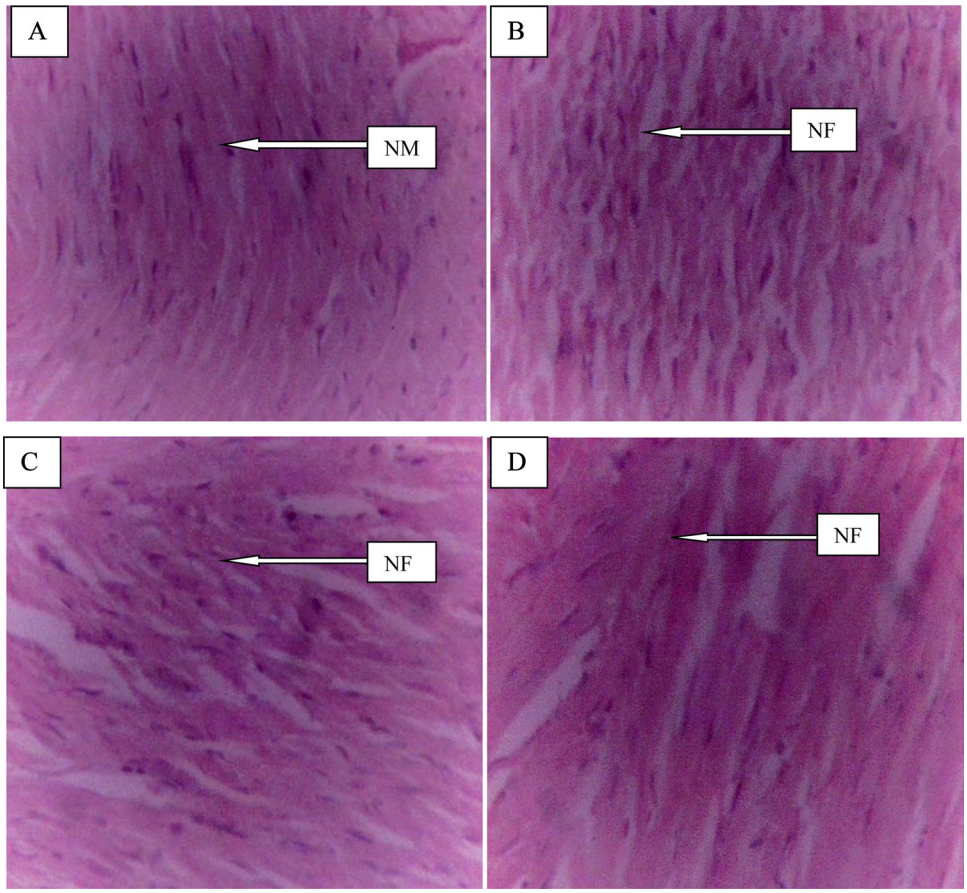

Fig. 6 Photomicrographs of heart sections of rats following 28-days daily oral administration of HMECH (Haematoxylin and eosin-stained at × 250). **a** Control (Distilled water, 1 ml/kg), **b** HMECH (250 mg/kg), **c** HMECH (500 mg/kg), **d** HMECH (1,000 mg/kg), *NM* normal myocardium, *NF* normal feature, *HMECH* hydromethanolic leaf extract of *Combretum hypopilinum*

Discussion

Generally, medicinal plants used in traditional medicine possess phytochemical compounds that may have therapeutic effects [30, 31]. However, scientific studies have identified some of these medicinal plants as toxic [30]. The evaluation of animals' general behaviour, body weight, biochemical and haematological indices, as well as histopathological investigations could determine the toxicity effects of medicinal plants [32].

The phytochemical determination of the HMECH indicated flavonoids, steroids, tannins, triterpenes, glycosides, alkaloids and saponins, which corroborates to the study conducted by Ahmad et al. [10, 33]. Many of these secondary metabolites in medicinal plants elicit various biological effects [31]. Besides, they are not free from toxicity effects [34].

Evaluation of acute toxicity effects is usually employed to investigate the possible toxicity of bioactive compounds after single and short-term administration [35]. It is the first step used in evaluating the biological activity of unknown chemical substances, particularly the evaluation of LD_{50} of the chemical substances [31]. The result of this research has shown that the LD_{50} of the HMECH could be greater than 5000 mg/kg and non-toxic following acute oral exposure. This outcome agrees with the work of Li and his team members [36] on hesperidin obtained from orange peel. Similarly, the oral and intravenous LD_{50} of the leaf and seed of *C. hypopilinum* were higher than 5000 mg/kg [10, 33] and 2300 mg/kg [37], respectively.

Any change in animals' body weight and internal organs after exposure to potentially harmful substances indicates toxicity from such chemicals [38]. Additionally, changes in animals' body weight may occur due to fat accumulation and physiological response of the animals to the extract instead of the toxic effects of the bioactive substances, which causes a reduction in appetite and low energy consumption [30]. Therefore, the body weight reduction at the dose of 500 mg/kg in the first and second week could be due to physiological adjustment exhibited by the animals in response to the extract as the animals regained their average body weight in the subsequent weeks (3rd and 4th weeks). Besides, the reduction in the kidney's ROW indicated a possible renal adverse effect by the extract's toxicity due to abnormal atrophy [39]. Indeed, herbal medicines used in traditional medicine form a vital cause of nephrotoxicity [40, 41].

The liver's metabolic function is determined by serum hepatic biomarkers such as ALT, AST, and ALP [42].

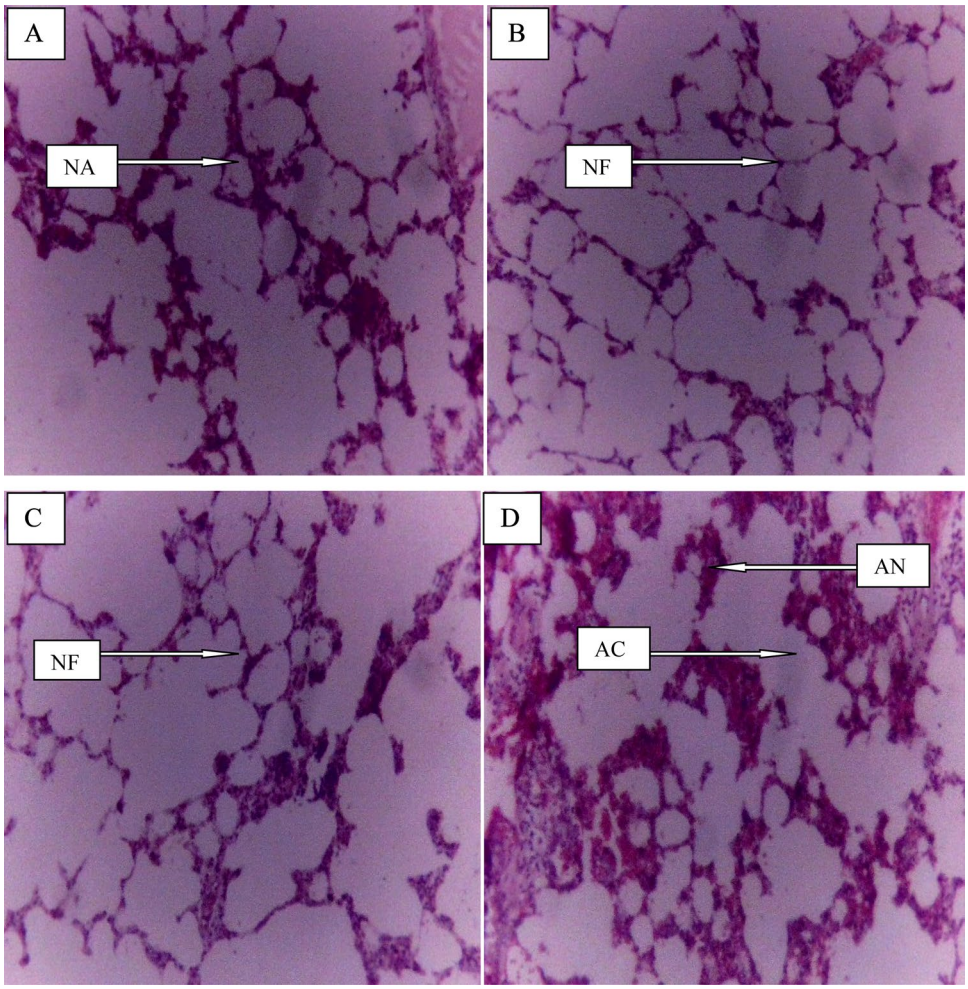

Fig. 7 Photomicrographs of lung sections of rats following 28-days daily oral administration of HMECH (Haematoxylin and eosin-stained at × 250). **a** Control (Distilled water, 1 ml/kg), **b** HMECH (250 mg/kg), **c** HMECH (500 mg/kg), **d** HMECH (1,000 mg/kg), *NA* normal alveoli, *NF* normal feature, *AC* moderate alveoli congestion, *AN* slight alveoli necrosis, *HMECH* hydromethanolic leaf extract of *Combretum hypopilinum*

The ALT is an enzyme present in the hepatic cytoplasm which significantly increases during hepatocellular toxicity. Also, AST is an enzyme found in different body tissues such as the pancreas, liver, skeletal muscles, kidneys, heart and RBC released after cellular injury or alteration in cell membrane transportation ability [36]. The cells lining the hepatic biliary duct contain ALP which is vital in checking biliary duct disease [42]. The non-significant alterations in ALT and AST suggested that the extract may not be hepatotoxic. However, the ALP level reduction in this study by the HMECH could be attributed to malnutrition, mineral (zinc and magnesium) and vitamin (vitamin C and B12) deficiency and may interfere with the metabolic activities [43]. Besides, the hypoglycemic effects of the HMECH could be explained by a previous study that shows that polyphenols and flavonoid-containing substances elicit hypoglycemic effects by inhibiting α-glucosidase and α-amylase enzymes, increasing glucose utilization by peripheral tissues, and stimulating the release of insulin from pancreatic β-cell [42]. Creatinine and urea are metabolic end-products excreted from the body by glomerular filtration, and their elevation in plasma serves as an index for nephrotoxicity [44, 45]. The significant decrease in potassium level in this study could be due to renal tubular damage that impairs the absorption of electrolytes [46], consistent with the reduced relative kidney weight and renal tubular necrosis observed.

The blood-forming system is a target to many harmful substances and is an essential indicator of animals' healthy and diseased state [42]. It is well-known that the reduction in the levels of blood indices such as RBC, MCH, MCV, MCHC, as well as HGB is an indicator of anaemia [47]. The results of this work have suggested the lack of interference of the extract with RBC production and, therefore, may not induce anaemia. Our result corresponds with a study reported by Jatsa and team members [48] on *Clerodendrum umbellatum* leaves. WBC contributes to the body's cellular defensive mechanisms against infectious agents, tissue damage, and inflammatory mechanisms [49] and its reduction is related to bone marrow suppression, which may cause leucopenia [50, 51]. Based on this study, the HMECH

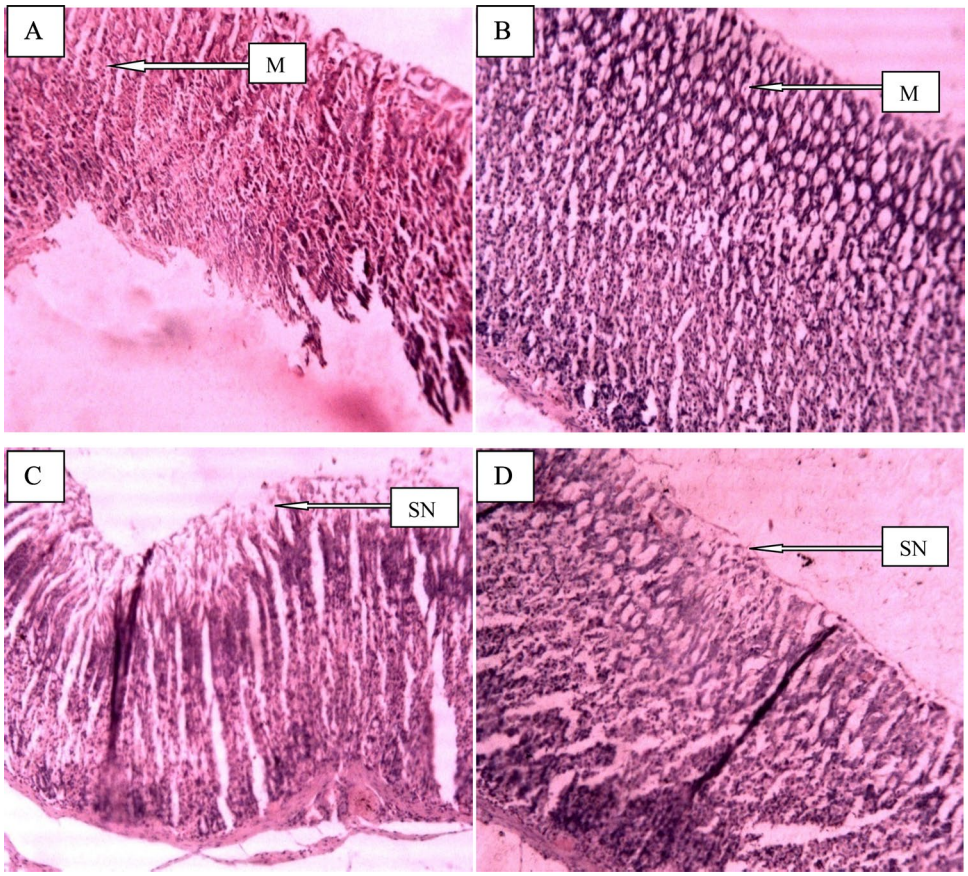

Fig. 8 Photomicrographs of stomach sections of rats following 28-days daily oral administration of HMECH (Haematoxylin and eosin-stained at × 250). **a** Control (Distilled water, 1 ml/kg), **b** HMECH (250 mg/kg), **c** HMECH (500 mg/kg), **d** HMECH (1,000 mg/kg), *M* normal gastric mucosa, *SN* slight mucosa necrosis, *HMECH* hydromethanolic leaf extract of *Combretum hypopilinum*

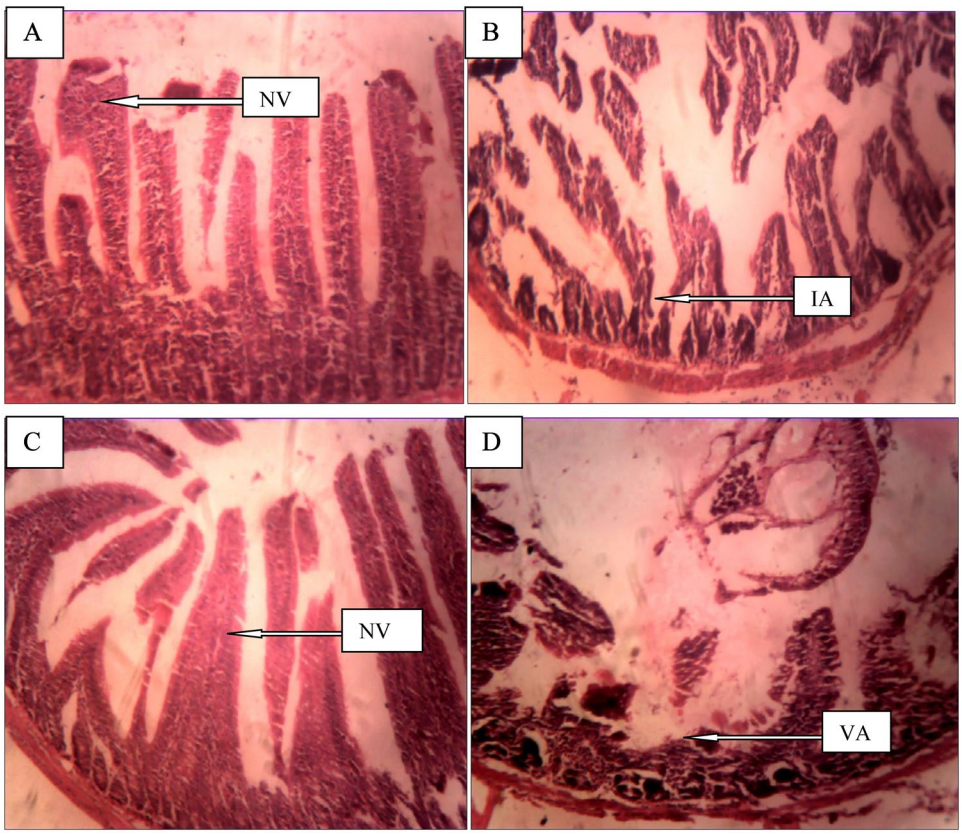

Fig. 9 Photomicrographs of small intestine sections of rats following 28-days daily oral administration of HMECH (Haematoxylin and eosin-stained at × 250). **a** Control (Distilled water, 1 ml/kg), **b** HMECH (250 mg/kg), **c** HMECH (500 mg/kg), **d** HMECH (1000 mg/kg), *NV* normal villi, *IA* slight small intestinal atrophy, *VA* moderate villi atrophy, *HMECH* hydromethanolic leaf extract of *Combretum hypopilinum*

might not cause leucopenia and bone marrow and immune system suppression. Our study corroborates with the work of Bhushan et al. [52] that reported the lack of effects on WBC following sub-acute administration of *Jasminum mesnyi*, *Clerodendrum inerme* and *Callistemon citrinus.* Besides, the elevation in granulocytes and percentage of eosinophils, basophils, and monocytes at the highest dose indicates a possible involvement of the HMECH in innate immune response, particularly phagocytosis [30], whereas the elevation in the platelet suggests that it could stimulate thrombopoietin production to prevent haemorrhages [53]. The finding in this experiment correlates with that of Ehile et al. [49], and Ugwah-Oguejiofor et al. [54] on *Macaranga barteri* (Euphorbiaceae) and aqueous leaves extract of *Carralluma dalzielii* (Apocynaceae), respectively.

The high blood level of cholesterol, triglycerides and LDL contributes to cardiovascular (CVS) diseases [48, 55]. In contrast, an elevation of HDL level in the blood prevents atherosclerosis and other CVS diseases [48]. Therefore, the HMECH in the current study may not adversely affect lipid metabolism and could have a beneficial effect against CVS risk, including atherosclerosis. Also, Jatsa et al. [48] reported the *C. umbellatum* leaves' safety on lipid profile.

The safety investigation of the bioactive substance also involves macroscopic and microscopic evaluation of histopathological changes of the vital organs of the treated animals [56]. Kupffer cells situated in the liver sinusoids have an essential role in host defence-mechanisms by preventing the pathogens derived from the portal and systemic circulation from getting into the liver by phagocytosis and restraining hepatocellular and liver damage [51, 57]. The liver's kupffer cells hyperplasia in this work further suggested the lack of hepatotoxic effects of the HMECH. Despite the observed slight hepatic necrosis, the extract may not be considered toxic because there was no malfunction associated with the hepatic biomarkers and synthetic liver ability. Also, the liver forms new cells, clears necrotic cells, and restores the hepatic structure and functions [55].

The moderate tubular necrosis caused by the HMECH at 500 and 1,000 mg/kg may result from the delivery of toxic substances to the kidney from systemic circulation and may subsequently cause malfunction of the renal tubular system [46], which concurs with the reduction in the ROW of the kidney related to possible nephrotoxic effects. The alveoli congestion and mild necrosis showed that the HMECH could interfere with oxygen diffusion and other gaseous substances across the alveoli epithelium and into the pulmonary circulation. The stomach secretes mucus, which inhibits gastric erosion induced by gastric juices and gastric hormones. In this study, the mild gastric mucosal necrosis at the higher doses indicates a possible gastro-toxicity. Besides, the intestinal and villi atrophy indicate an intestinal malfunction and structural alterations, reducing the height of intestinal villi, crypt depth, and surface area that could interfere with normal absorption of nutrients [56]. However, the lack of morphological alterations on the heart muscles at all doses suggested that the extract is non-toxic to the heart.

In conclusion, the findings of this work showed that the HMECH could be relatively safe on acute exposure and at the lowest dose on sub-acute administration. However, it is moderately toxic at the higher doses on sub-acute administration, particularly to the kidney. The plant is a potential therapeutic agent that deserves further in-depth toxicity evaluations. Therefore, we recommend more research to further determine the sub-chronic toxicity, chronic toxicity, mutagenic, teratogenic, and genotoxic effects of the plant. Furthermore, the traditional herbalists should be sensitized on the potential toxicity associated with the long-term consumption of *C. hypopilinum,* particularly the nephrotoxic effects despite its promising therapeutic properties as part of the process of the safe use of the medicinal plant.

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Authors contributions MHA: Conceptualisation, Investigation, Data curation, Writing—Original Draft and editing. AUZ: Supervision, Project administration, Review, validation. SBA: Supervision, Project administration, Review, validation. OYA: Resource, Writing. MM: Formal analysis and Review. SM: Writing and Review. AAB: Writing and Review. SM: Resources and Review. SMJ: Resources, Writing—Review. ASW: Formal analysis. AIJ: Critically reviewed the whole manuscript. All the authors read and approved the final manuscript.

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Data availability statement The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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