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Acute and sub-chronic toxicity studies of 5-methylcoumarin-4-β-glucoside isolated from *Vernonia glaberrima* in Wistar rats

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Abstract

5-methylcoumarin-4-β-glucoside is a natural product isolated from Vernonia glaberrima – a plant used in African traditional medicine for the treatment of cancer and skin diseases. This compound possesses significant in-vitro antiproliferative potentials against colon cancer cell lines. However, safety assessment in several laboratory animals is required before commencing the in-vivo anticancer evaluation of the compound. This study is designed to evaluate the safety of 5-methylcoumarin-4-β-glucoside using 28 days sub-chronic toxicity model in Wistar rats. Acute toxicity study was carried out at a single oral dose of 10 mg/kg, 100 mg/kg and 1000 mg/kg body weight (phase 1) for 24 hours, and 1600 mg/kg, 2900 mg/kg, and 5000 mg/kg body weight (phase 2). Sub-chronic toxicity was conducted by oral administration of distilled water, 1%hydroxypropyl cellulose in distilled water as controls, and graded doses of 10 mg/kg, 50 mg/kg, and 100 mg/kg body weight of 5-methylcoumarin-4β-glucoside freshly prepared in 1% hydroxypropyl cellulose as vehicle to five groups of six rats each for 28 days. The result indicates that 5-methylcoumarin-4β-glucoside at a dose of 100 mg/kg body weight is non-toxic as serological indices like liver and kidney functioning test, serum lipid profile, oxidative stress markers and hematological indices remained in the normal range after the 28 days sub-chronic administration of the compound. Moreover, the histopathology findings revealed no alteration in the normal tissue architecture. Overall, the findings in Wistar rats corroborate the initial findings in mice. Conclusively, the compound 5-methylcoumarin-4β-glucoside is safe at the prescribed doses for further preclinical in vivo investigations.

Keywords: 5-methylcoumarin-4-β-glucoside, Safety profile, Toxicity, *Vernonia glaberrima*, Wistar rat

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INTRODUCTION

In recent years, there has been an upsurge in the utilization of plants or plant products for therapeutic purposes and enhancement of wellbeing (Jamshidi-Kia, 2018). This practice has gain widespread acceptance owing to the perception that herbal medicines are natural and thus safer than the orthodox medicine (Lynch and Berry, 2007). However, most of these plants have constituents that could be toxic to vital organs of the body (Hussin, 2001). Herbal medicines consumption reportedly contributes up to 35% of cases of acute kidney injury in the developing world (Luyckx and Naicker, 2008). Thus, the rising level of liver and kidney failures currently obtainable in Northern Nigeria could be associated with the indiscriminate consumption of herbal products. Therefore, it is of paramount importance to investigate the toxicity profile of medicinal plants as well as their phytoconstituents in order to establish their safety.

Vernonia glaberrima (Welw. ex O.Hoffm.) H. Rob is a medicinal plant that is used traditionally in the treatment of cancer and skin related disorders. This plant has been reported to possess several pharmacological properties which include; analgesic, antimalarial, antidiabetic, antimicrobial and anticancer activities (Alhassan *et al*., 2018).

Previous research on this plant reveals that the leaves contain large quantity of 5-methylcoumarin-4β-glucoside (Figure 1) as the major constituent (Alhassan *et al*., 2018). This compound was reported to display significant cytotoxic activity against human malignant melanoma (A375), human caucasian colon adenocarcinoma (HT-29) and human breast adenocarcinoma (Alhassan *et al*., 2018). It was also demonstrated to be relatively safe in mice (Abubakar *et al*., 2022). Thus, it is a potential anti-cancer agent for further *in vivo* investigations. However, based on literature search, there is paucity of information on the toxicity profile of 5-methylcoumarin-4β-glucoside that will guarantee its safety as a potential anti-cancer agent or lead compound. Toxicological screening is not only aimed at demonstrating the safety of a candidate molecule, but it is also aimed at characterizing the possible toxic effects the molecule can produce (Cunny & Hodgson, 2010). Due to variations in absorption, distribution, metabolism and excretion capabilities, different laboratory animals have been shown to react differently to potential toxic compounds (Spurgeon *et al*., 2020; Gokulan *et al*., 2021). A number of laboratory rodent toxicity studies is a vital component of the *in vivo* evaluation process of any substance that is intended to be used for medicinal purpose. Using several rodents during preclinical toxicity testing reveals organ- and dose-specific toxic effects of medicinal products (Parasuraman, 2011). Therefore, the research investigated the acute and sub chronic toxicity effect of 5-methylcoumarin-4β-glucoside in Wistar albino rats to ascertain the safety of the compound and further buttress its preclinical safety for further investigations.

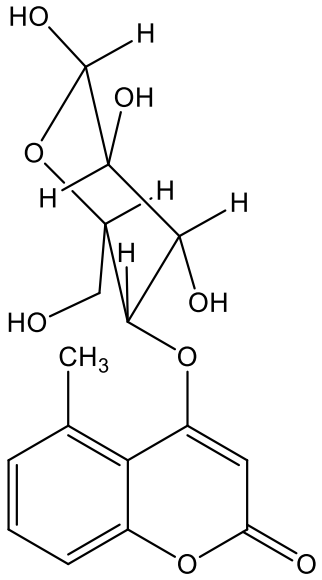

Figure 1: Structure of 5-methylcoumarin-4β-glucoside

MATERIALS AND METHOD

Chemicals and reagents

All solvents (analytical grade) were purchased from Merck (Merck KGaA, Darmstadt, Germany). Biochemical assay kits for liver and kidney function tests were purchased from Randox laboratories LTD, (England). ISE electrodes for sodium, potassium and chloride analysis were obtained from LandWind LTD (China) while diluent and haemoglobin lyse were procured from Absolute Medical Services INC. (New York).

5-methylcoumarin-4-β-glucoside

The 5-methylcoumarin-4-β-glucoside used in this study was isolated from *Vernonia glaberrima* following a previously described method (Alhassan *et al*, 2018). The structure of the compound was confirmed by spectroscopic analysis and found to be the same with data reported in literature (Alhassan *et al*., 2018)

Sample preparation

The stock solution of 5-methylcoumarin-4β-glucoside was prepared using 1% hydroxypropyl methyl cellulose (HPMC) as vehicle and dispersed completely. Different concentration of the compound was then prepared via dilution of the stock solution with appropriate volume of distilled water.

Experimental Animals and their Housing

Wister rats were used during the present investigation as experimental animals. A total of forty-two (42) rats, weighing 160 ± 20 g aged 4–6 weeks, were obtained from animal Care Centre, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria. The animals were housed in rat cages and allowed to acclimatize to their new environment. They were fed with pelletized growers feed (Vital), obtained from Grand Cereal Soil Mills Limited, Jos, Nigeria. The animals were allowed access to clean drinking water *ad libitum* throughout the experimental period. The experiment was carried out in the Animal House of the Faculty of Pharmaceutical Sciences, Usmanu Danfodiyo University, Sokoto.

Ethical Considerations
All the experimental protocols followed the Animal Ethics Committee of Usmanu Danfodiyo University, Sokoto, Nigeria as well as internationally accepted practices for use and care of laboratory animals.

Experimental Design for Acute Toxicity
This study was carried out following the method described by Lorke *et al*., (1983). The animals were subjected to fasting for a period of 24 hours prior to the commencement of study. The tests were conducted in two phases. In the Initial phase, nine animals of either sex were randomly divided into three groups of three rats each. The first, second and third group were given 10 mg/kg, 100 mg/kg and 1000 mg/kg of 5-methylcoumarin-4β-glucoside respectively through oral administration. The animals were observed at different time intervals for 24 hours for mortality and any sign of toxicity such as lacrimation, salivation, tremor convulsion sedation and drowsiness. In the second phase, three rats were used, one per group and dosed with 1600 mg/kg, 2900 mg/kg and 5000 mg/kg of 5-methylcoumarin-4β-glucoside and the same clinical observations were made. The LD_{50} was determined by calculating the geometric mean of the highest dose that the rats survived and lowest dose that killed the rats.

$$LD_{50} = (D_O - D_{100})^{1/2}$$

Where D_O = highest dose that gave no mortality
D_{100} = Lowest dose that produced mortality

Experimental Design for Sub-acute Toxicity Study
The 28-day sub-acute toxicity study was performed according to the Organization for Economic Cooperation and Development (OECD) guideline 407 (OECD 407, 2008). Thirty rats of either sex were randomly divided into five groups of six rats each (3 males and 3 females). The experimental groups were given 5-methylcoumarin-4β-glucoside at graded doses of 10, 50 and 100 mg/Kg by gavage for 28 consecutive days. While the control group was treated with the vehicle (1% carboxymethylcellulose in distilled water). The rats had access to food and water throughout the duration of the experiment (28 days). The body weight and adverse signs of toxicity were observed daily during treatment. At the end of the treatment, all rats were anaesthetized with formalin, and blood samples were immediately collected for hematological and biochemical analyses. The liver and kidney were collected and weighed to calculate the relative organ weights, while part of the tissues was used for histopathological analysis.

Biochemical analysis
The blood serum obtained by centrifuging the non-heparinized blood samples at 1500×g for 15 min, was used to evaluate the following biochemical parameters: aspartate transaminase (AST), alanine transaminase (ALT), total protein, Albumin, total bilirubin, urea, creatinine, total cholesterol, high density lipoprotein (HDL), triglyceride (TG), low density lipoprotein (LDL), very low density lipoprotein (VLDL) spectrophotometrically using S23A spectrophotometer U.S.A. sodium (Na^+), and potassium (K^+), were estimated by ISE method. Superoxide dismutase (SOD), catalase (CAT), and malondialdehyde (MDA), were analysed by ELISA methods.

Haematological analysis
The blood samples collected and kept in sterile EDTA tubes were analysed using ACT5 autumated haematology analyzer (Beckman Coulter, Califonia USA) for the following

haematological parameters: total white blood cell (WBC) count, haemoglobin, total red blood cell (RBC) count, platelets (PLT), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), differential leucocyte count (neutrophils, eosinophil, monocytes, lymphocytes), red Cell distribution width coefficient of Variation (RDW-CV), red Cell distribution width standard deviation (RDW-SD), mean platelet volume (MPV), platelet distribution width (PDW), mean platelet volume (MPV), platelet distribution width (PDW), plateletcrit (PCT) and platelet to large cell ratio (P-LCR).

Histopathological assessment

After euthanasia, the tissues of liver and kidney were fixed in 10% formol-saline (Das *et al*., 2015) were inserted in paraffin after dehydration in graded alcohol, cut into thin sections and stained using hematoxylin-eosin (H and E) histological staining technique. The sections were examined using x40 and x100 objective piece for presence of negative features such as hemorrhage, sinusoidal congestion, inflammation, cirrhosis, sinusoidal congestion, necrosis, and disruption of central vein.

Statistical analysis

Data analysis was done using statistical package for social sciences (SPSS 21) and the results expressed as mean ± SEM. Data analysis was carried out using One-way ANOVA with a post-hoc Tukey's HSD test where a significant difference was realized. Values were considered significant at values of $p \leq 0.05$.

RESULTS

The acute toxicity study did not reveal any sign of toxicity (Table 1). The oral LD_{50} for 5-Methylcoumarin-4-β-glucoside in mice was determined to be above 5,000 mg/kg.

Table 1: Clinical observation during acute toxicity study of 5-methylcoumarin-4β-glucoside in Wistar rats

S/N	Parameter	10 mg/kg	100 mg/kg	100 mg/kg	1600 mg/kg	2900 mg/kg	5000 mg/kg
1	Lacrimation	Negative	Negative	Negative	Negative	Negative	Negative
2	Pilo-erection	Negative	Negative	Negative	Negative	Negative	Negative
3	Tremor	Negative	Negative	Negative	Negative	Negative	Negative
4	Convulsion	Negative	Negative	Negative	Negative	Negative	Negative
5	Increased motor activity	Negative	Negative	Negative	Negative	Negative	Negative
6	Drowsiness	Negative	Negative	Negative	Negative	Negative	Negative
7	Anesthesia	Negative	Negative	Negative	Negative	Negative	Negative
8	Tonic erection	Negative	Negative	Negative	Negative	Negative	Negative
9	Arching and rolling	Negative	Negative	Negative	Negative	Negative	Negative
10	Straub reaction	Negative	Negative	Negative	Negative	Negative	Negative
11	Salivation	Negative	Negative	Negative	Negative	Negative	Negative
12	Exophthalmos	Negative	Negative	Negative	Negative	Negative	Negative
13	Hyperesthesia	Negative	Negative	Negative	Negative	Negative	Negative
14	Depression	Negative	Negative	Negative	Negative	Negative	Negative
15	Ptosis	Negative	Negative	Negative	Negative	Negative	Negative
16	Writing	Negative	Negative	Negative	Negative	Negative	Negative
17	Opisthotomus	Negative	Negative	Negative	Negative	Negative	Negative

18	Blanching hypnosis	Negative	Negative	Negative	Negative	Negative	Negative
19	Cynosis and analgesia	Negative	Negative	Negative	Negative	Negative	Negative
20	Stimulation	Negative	Negative	Negative	Negative	Negative	Negative
21	Average weight	162	159	160	164	161	163
22	Mortality	Negative	Negative	Negative	Negative	Negative	Negative

Key; Negative = not detected

There was no significant difference in weight among the various interventions during the course of the 28 days sub-acute toxicity study (Table 2). The weekly increase in weight was uniform among all the groups of rat.

Table 2: Effect of 5 –methylcoumarin-4β-glucoside on body weight changes following 28 days sub-acute oral treatment in Wistar rats

Treatment group	**N**	**Average weight (g)**			
		Week 0	**Week 1**	**Week 2**	**Week 3**
Distilled water	6	147 ± 3.96	150 ± 5.20	164 ± 4.23	166 ± 4.40
Vehicle (1% HPMC)	6	154 ± 5.70	156 ± 6.63	167 ± 8.15	170 ± 9.65
10 mg/kg	6	152 ± 4.10	155 ± 3.32	163 ± 6.87	168 ± 7.03
50 mg/kg	6	140 ± 6.05	144 ± 5.34	152 ± 5.39	155 ± 6.48
100 mg/kg	6	146 ± 3.77	148 ± 5.45	159 ± 7.03	163 ± 8.95

Data are presented as mean ± standard error of mean, N = number of rats.
The weight of the livers and kidneys of all the rats in the treatment groups were not different from the control group (Table 3).

Table 3: Effect of 5-methylcoumarin-4-β-glucoside on relative organ weight

Treatment group	**N**	**Average organ weight (g)**	
		Liver (g)	**Kidney (g)**
Distilled water	6	0.032 ± 0.003	0.003 ± 0.000
Vehicle (1%HPMC)	6	0.030 ± 0.003	0.003 ± 0.000
10 mg/kg	6	0.028 ± 0.003	0.028 ± 0.001
50 mg/kg	6	0.035 ± 0.002	0.003 ± 0.002
100 mg/kg	6	0.030 ± 0.016	0.003 ± 0.000

Data are presented as mean ± standard error of mean, N = number of rats. HPMC = Hydroxypropyl cellulose.

All the investigated liver enzymes did not differ from one another when compared (Table 4).

Table 4: Effect of 5-methylcoumarin-4β-glucoside on liver function indices following 28 days of sub-chronic oral treatment in wistar rats

Treatment group	N	AST (IU/L)	ALT (IU/L)	ALB (g/L)	TP (g/L)	T. BIL (μmol/L)
Distilled water	6	83.93 ± 7.40	31.01 ± 4.44	29.90 ± 0.45	64.17 ± 5.82	5.73 ± 1.40
Vehicle (1% HPMC)	6	83.62 ± 8.69	41.81 ± 4.27	29.23 ± 0.39	63.90 ± 1.71	4.28 ± 1.54
10 mg/kg	6	94.36 ± 0.19	32.84 ± 2.94	29.10 ± 0.45	68.73 ± 1.25	3.14 ± 0.88
50 mg/kg	6	71.27 ± 6.25	35.18 ± 3.24	29.66 ± 0.70	71.48 ± 3.25	3.91 ± 0.65
100 mg/kg	6	76.85 ± 5.29	28.77 ± 1.80	31.78 ± 0.92	63.76 ± 4.32	4.31 ± 1.25

Data are presented as mean ± standard error of mean, N = number of rats. HPMC = Hydroxypropyl cellulose, AST= Aspartate aminotransferase, ALT= Alanine aminotransferase, ALB = Albumin, TP= Total protein, T. BIL= Total bilirubin.

Sodium, chloride and urea levels showed significant difference at 100 mg/kg dose when compared to control (Table 5). Other serum biochemical parameters related to renal function did not differ between groups

Table 5: Effect of 5-methylcoumarin-4β-glucoside on renal function indices following 28 days of sub-chronic oral treatment in Wistar rats

Parameter	Unit	N	Distilled Water	1%(HPMC)	10 mg/kg	50 mg/kg	100 mg/kg
Urea	mmol/L	6	4.92 ± 0.51	5.26 ± 0.70^{c}	4.06 ± 0.20	3.05 ± 0.41^{c}	3.10 ± 0.49^{c}
Creatinine	mmol/L	6	103.84±5.94	110.40±5.03	116.53±14.59	107.30±9.07	117.87 ± 8.21
Uric acid	mmol/L	6	136.14±23.57	99.17±28.40	150.43±37.91	107.80±24.45	187.80 ± 42.84
Chloride	mmol/L	6	96.33 ± 1.91^{c}	101.69 ± 2.00	102.70 ± 0.49	105.85 ± 1.15^{c}	103.33 ± 1.48^{c}
Sodium	mmol/L	6	135.51 ± 1.73	138.15 ± 1.50^{b}	131.55 ± 0.98^{b}	134.79 ± 0.97	139.48 ± 0.48^{a}
Potassium	mmol/L	6	4.90 ± 0.60	5.01 ± 0.1	5.07 ± 0.56	4.79 ± 0.36	4.62 ± 0.25
Bicarbonate	mmol/L	6	25.79 ± 0.62	24.46 ± 1.06	26.02 ± 0.99	25.44 ± 0.64	25.60 ± 0.44

Data are presented as mean ± standard error of mean, HPMC = Hydroxypropyl cellulose, N = number of rats, a = $p \leq 0.001$, b = $p \leq 0.01$, c = $p \leq 0.05$.

Catalase enzyme levels in the 10 mg/kg and 50 mg/kg groups showed significant difference when compared to their respective controls (Table 6). The other antioxidant enzymes (SOD and MDA) didn't show any difference.

Table 6: Effect of 5-methylcoumarin-4β-glucoside on antioxidant enzymes and lipid peroxidation markers following 28 days of sub-chronic oral treatment in Wistar rats

Treatment group	N	SOD (ng/ml)	CAT ng/L	MDA (mmol/L)
Distilled water	6	64.16 ± 1.20	4.15 ± 0.44^{b}	0.93 ± 0.32
Vehicle (1% HPMC)	6	44.01 ± 1.46	2.63 ± 0.50	0.76 ± 0.09
10 mg/kg	6	44.017 ± 0.99	4.08 ± 0.22^{b}	0.85 ± 0.06
50 mg/kg	6	42.12 ± 2.14	1.87 ± 0.21^{b}	0.76 ± 0.55
100 mg/kg	6	43.66 ± 1.29	3.33 ± 0.55	0.89 ± 0.37

Data are presented as mean ± standard error of mean, HPMC = Hydroxypropyl cellulose, N = number of rats, b = $p \leq 0.01$, SOD = superoxide dismutase, CAT = catalase, MDA = malondialdehyde

There was no difference among all the lipid profile parameters quantified when compared with the control group (Table 7).

Table 7: Effect of 5-methylcoumarin-4β-glucoside on lipid profile parameters following 28 days of sub-chronic oral treatment in Wistar rats

Treatment	N	T. CHOL. (mmol/L)	TG (mmol/L)	VLDL (mmol/L)	HDL (mmol/L)	LDL (mmol/L)	AI
Distilled water	6	2.29 ± 0.32	0.43 ± 0.10	0.19 ± 0.04	1.55 ± 0.22	0.31 ± 0.08	13.49 ± 0.06
Vehicle (1% HPMC)	6	1.90 ± 0.05	0.33 ± 0.05	0.15 ± 0.02	1.27 ± 0.09	0.48 ± 0.08	9.75 ± 2.45
10 mg/kg	6	2.00 ± 0.12	0.36 ± 0.03	0.16 ± 0.01	1.19 ± 0.13	0.64 ± 0.08	13.78 ± 1.87
50 mg/kg	6	1.97 ± 0.10	0.38 ± 0.08	0.17 ± 0.03	1.56 ± 0.17	0.54 ± 0.09	16.21 ± 1.59
100 mg/kg	6	1.78 ± 0.07	0.37 ± 0.08	0.17 ± 0.03	1.13 ± 0.09	0.46 ± 0.09	9.98 ± 1.29

Data are presented as mean ± standard error of mean, HPMC = Hydroxypropyl cellulose, N = number of rats, T. CHOL. = total cholesterol, TG = triglyceride, VLDL = very low density lipoprotein, HDL= high density lipoprotein, LDL = low density lipoprotein, AI = antherogenic index.

There was no difference among all the haematoligical parameters quantified when compared with the control group (Table 8).

Table 8: Effects of 5-methylcoumarin-4β-glucoside on haematological parameters

Parameter	Unit	N	Distilled water	Vehicle (1% HPMC)	10 mg/kg	50 mg/kg	100 mg/kg
WBC	10^9/L	6	13.05 ± 2.17	9.75 ± 2.45	13.78 ± 1.87	16.21 ± 1.59	9.98 ± 1.29
LYM	10^9/L	6	10.43 ± 1.97	7.88 ± 2.28	11.20 ± 1.47	12.65 ± 1.45	8.01 ± 1.08
MID	10^9/L	6	1.68 ± 0.32	1.16 ± 0.23	1.48 ± 0.24	1.83 ± 0.22	1.18 ± 0.18[c]
GRA	10^9/L	6	0.93 ± 0.22	0.70 ± 0.12[c]	1.95 ± 0.43[c]	1.73 ± 0.24	0.78 ± 0.19
LYM%	%	6	80.20 ± 1.48	76.15 ± 4.20	76.16 ± 2.36	77.15 ± 2.51	79.80 ± 2.34
MID%	%	6	12.50 ± 1.03	12.71 ± 0.79	10.78 ± 1.00	11.30 ± 0.88	11.80 ± 0.80
GRA%	%	6	7.30 ± 0.71	9.40 ± 2.65	12.36 ± 3.30	11.55 ± 2.29	8.41 ± 1.75
RBC	10^12/L	6	4.39 ± 0.36	5.38 ± 0.35	4.77 ± 0.33	4.96 ± 0.25	5.34 ± 0.17
HGB	g/dl	6	10.91 ± 0.86	12.31 ± 0.45	9.83 ± 1.80	12.33 ± 0.71	13.10 ± 0.31
MCHC	g/dl	6	41.75 ± 0.84	39.50 ± 1.51	40.47 ± 1.33	40.22 ± 1.20	41.37 ± 0.89
MCH	Pg	6	24.96 ± 1.02	23.25 ± 1.05	24.13 ± 0.55	25.15 ± 0.74	24.56 ± 0.44
MCV	fL	6	59.76 ± 1.74	58.81 ± 1.20	60.01 ± 0.81	61.71 ± 1.34	59.51 ± 0.78
RDW-CV	%	6	13.03 ± 0.32	15.60 ± 2.82	12.93 ± 0.30	12.96 ± 0.11	12.58 ± 0.09
RDW-SD	fL	6	35.73 ± 1.68	34.23 ± 1.07	35.66 ± 1.10	37.20 ± 1.18	34.40 ± 0.73
HCT	%	6	26.53 ± 1.88	31.61 ± 2.07	29.15 ± 2.19	30.66 ± 1.69	31.85 ± 1.32
PLT	10^9/L	6	566.50 ± 81.99	816.66 ± 81.26	744.00 ± 40.39	679.00 ± 54.56	776.33 ± 44.54
MPV	fL	6	7.28 ± 0.13	7.05 ± 0.09	7.35 ± 0.15	7.38 ± 0.16	7.18 ± 0.18
PDW	fL	6	12.00 ± 0.98	12.86 ± 0.14	12.95 ± 0.17	12.85 ± 0.08	12.88 ± 0.19
PCT	%	6	0.40 ± 0.04	0.57 ± 0.04	0.54 ± 0.02	0.49 ± 0.03	0.55 ± 0.03
P-LCR	%	6	11.50 ± 1.14	9.51 ± 0.54	9.51 ± 0.54	11.31 ± 1.08	10.28 ± 0.99

Data are presented as mean ± standard error of mean, HPMC = Hydroxypropyl cellulose, N = number of rats, c = $p \leq 0.05$, WBC=white blood cell, LYM=lymphocyte, GRA=granulocyte, RBC=red blood cell, HGB=haemoglobin, MCHC=mean corpuscular haemoglobin concentration, MCH=mean corpuscular haemoglobin concentration, MCV= mean cell volume, RDW-CV=red cell distribution width coefficient of variation, RDW-SD=red cell distribution width standard deviation, HCT= haematocrit, PLT=platelet, MPV=mean platelet volume, PDW=platelet distribution width, PCT=plateletcrit, P-LCR=platelet to large cell ratio.

The photomicrograph of the liver (Figure 2 A-E) and the kidney (Figure 3 A-E) were unremarkable. The liver photomicrographs shows normally radiating hepatocytes. The central veins were normal and the sinusoidal lining cells have flattened condensed nuclei. The kidney photomicrograph has displays a normal architecture. The glomerulus, parietal layer and visceral layer appeared normal.

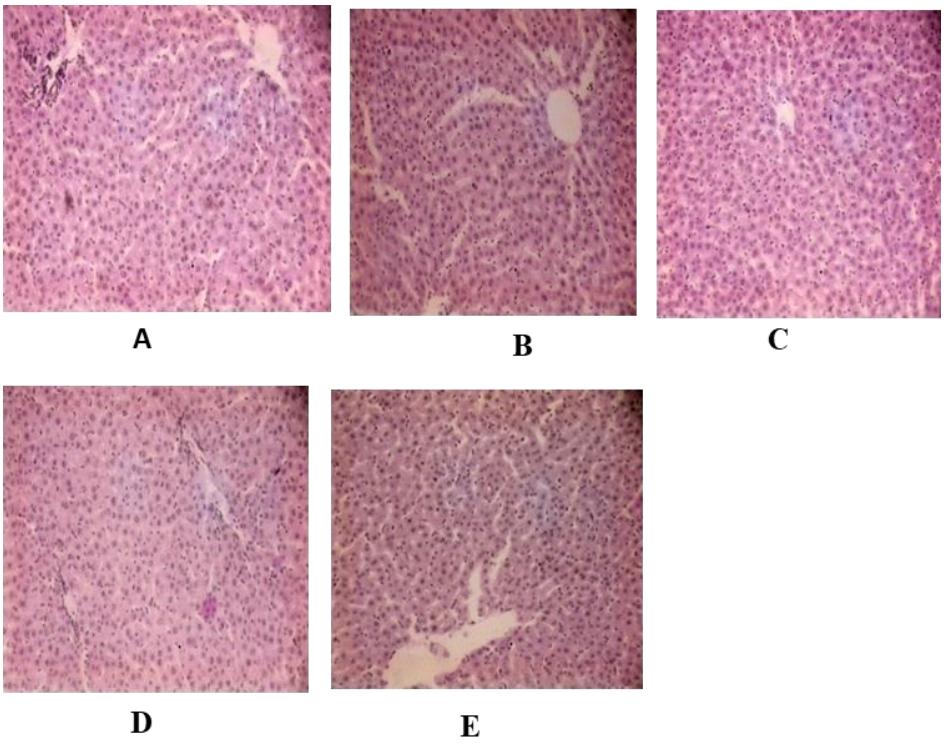

Figure 2. A normal section of the liver: A= Distilled water-a portal triad, B= 1% HPMC-Central vein, C=10 mg/kg-hepatocyte, D=50 mg/kg-central vein, E=100 mg/kg-potal triad.

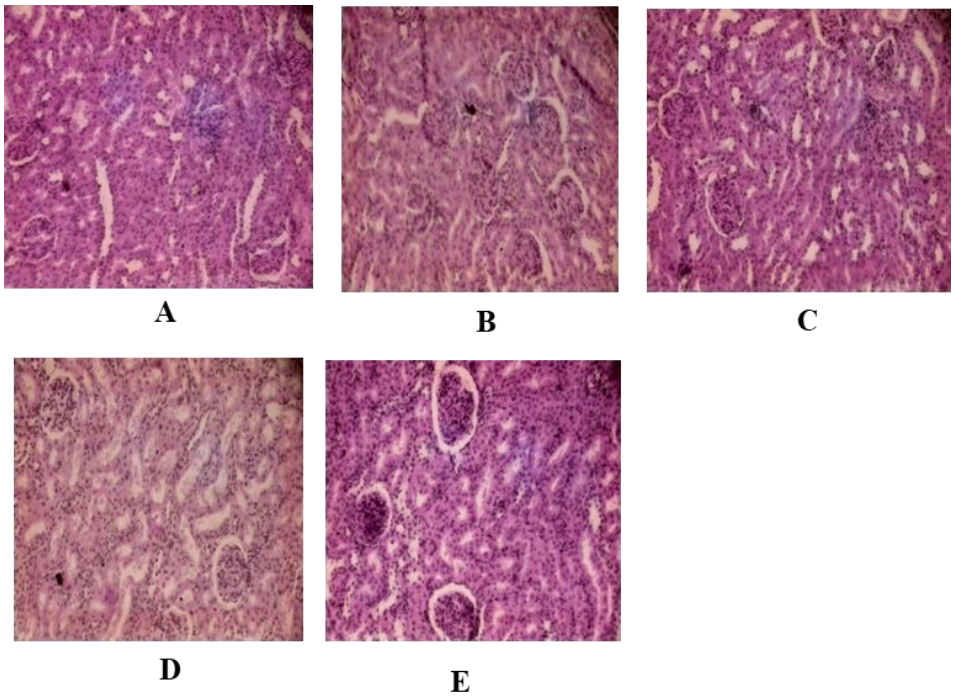

Figure 3. Kidney section.: A = Distilled water - a normal distal convulated tubules, B = 1% HPMC- a normal glomeruli, C=10 mg/kg - a normal distal convulated tubule, D=50 mg/kg - a normal glomeruli, E=100 mg/kg- a mild infiltrated glomeruli.

DISCUSSION

In the acute toxicity study, all experimental animals were carefully observed for development of any toxic signs or symptoms of both autonomic and central nervous system abnormalities at different time intervals within a time frame of 24 hrs. The administration of 5000 mg/kg body weight of 5-methylcoumarin-4β-glucoside in rats produced no mortality or morbidity over a period of 24 hours indicating that the LD50 is above 5000 mg/kg. In addition, there were no apparent signs of abnormalities in clinical parameters throughout the acute toxicity study (Table 1). These findings suggested that the compound has wide safety margin as similarly determined by Abubakar *et al.* (2022).

The results of absolute body and relative organ weights of the experimental animals demonstrate that the oral administration of 5-methylcoumarin-4β-glucoside for the stated period of time did not interfere with the normal growth of the animals. Liver is perhaps the most important organ responsible for the metabolism of food, drugs and other chemicals. Liver failure is often associated with high morbidity and mortality. Drug related hepatotoxicity is a common cause of liver injury with herbal products contributing the highest percentage (Stournaras and Tziomalos, 2015). Assessing the liver function is therefore critical in the evaluation of drug and chemical safety. Results for effects of sub-chronic administration of 5-methylcoumarin-4-β-glucoside on liver function parameters are presented in Table 4. The results show that there were no statistically significant differences in the liver function parameters which include, aspartate aminotransferase (AST), alanine aminotranferase (ALT), albumin, total protein and total bilirubin in all the treatment groups as compared to the control's groups. Absence of any treatment-related significant change in AST and ALT indicates absence of both cell membrane and infiltrative damage with resultant intact hepatocytes integrity (Robson *et al*., 2013). Non-significant differences in the serum levels of albumin and total protein levels indicates normal hepatic function. From these findings it can be construed that oral administration of 5-methylcoumarin-4β-glucoside for the period of 28 days did not interfere with normal hepatic functions within the dose range tested.

The kidneys play a vital role in the excretion of drugs and other xenobiotics. Orthodox drugs and herbal preparations are among the major causes of nephrotoxicity (Luyckx and Naicker, 2008). Hence, assessment of renal function parameters is an important aspect of toxicity evaluation of drugs and related substances. The results of kidney function tests are displayed in Table 5. There were no treatment-related effects in serum creatinine, potassium, uric acid, and bicarbonate concentrations as all differences among the treated and the control groups are statistically non-significant. There was significant decrease in serum urea concentration in the groups administered with 10 mg/kg, 50 mg/kg, and 100 mg/kg in a non-dose dependent manner compared to the control group ($p<0.05$). Sodium concentration with a dose of 10 mg/kg body weight decreased significantly yet within normal values compared with the control. There is significant difference but non-clinically important variation within the treated groups. The significant decrease in serum urea levels observed in the treatment groups may be caused by decreased urea production, increased urinary excretion or a combination of both. Physiological and pathological factors associated with reduced serum urea levels include, low protein diet, starvation, over-hydration and hepatobiliary damage (Lum and Leal-Khouri, 1989). Considering the results of liver function test obtained in this study, the possibility of hepatobiliary damage in the 5-methylcoumarin-βD-glucoside treated animal is improbable. Further study is however needed to corroborate this finding. On the general note,

the results suggest that sub-chronic administration of 5-methylcoumarin-βD-glucoside at the tested doses do not have negative clinical effect on the normal kidney function.

Oxidative stress and lipid accumulation in blood are major risk factors in the development of chronic metabolic disorders such as cardiovascular diseases, diabetes mellitus and cancer (Castelao and Gago-Dominguez, 2008). Thus, oxidative stress markers and lipid profile assays are often employed in assessing the sub-chronic and chronic toxicity effects of drugs and herbal supplements. The results for the sub-chronic administration of 5-methylcoumarin-4β-glucoside on serum levels of antioxidant enzymes and lipid profile are shown in Tables 6 and 7 respectively. For the antioxidant enzymes assay, the result shows that there is no significant difference in the levels of SOD and MDA in all the graded doses compared with the normal and test controls. Similar results were obtained from the liver profile assay. There was no any observed significant difference in total cholesterol, triglyceride (TG), high density lipoprotein (HDL), low density lipoprotein (LDL), very low-density lipoprotein (VLDL) and antherogenic index (AI) compared to the control groups. These findings demonstrated that sub-chronic administration of 5-methylcoumarin-4β-glucoside do not interfere with the body antioxidant system as well as lipid profile parameters within the tested doses. Hence administration of the compound may not expose the system to oxidative stress and lipid associated diseases.

Evaluation of haematological parameters is a vital and sensitive index that is important in the toxicity studies of drugs and herbal products. Available evidence has shown that the consumption of some medicinal plants or their purified products can cause significant alteration in haematological parameters as a consequence of which organ dysfunctions ensue (Arome and Chinedu, 2013). The effects of sub-acute administration of 5-methyl-4-β-glucoside on haematological parameters are presented in Table 8: Almost all haematological parameters, such as total WBC, total RBCs, haemoglobin, WBCs, neutrophils, lymphocytes, monocytes, and platelet count, in treated groups were not significantly different from the control groups. The haematological parameter findings are in harmony with those of Abubakar *et al*. (2022). Granulocyte in all the graded treatment doses 50 mg/kg and 100 mg/kg increased non-significantly with the normal control group. This finding indicates that the sub-chronic treatment with 5-methylcoumarin-4β-glucoside at maximum dose of 100 mg/kg body weight did not cause haematological disorders like anemia, leukocytosis and thrombocytopenia. This findings further buttress the good safety profile of this compound within the tested doses.

Light microscopic examination of sections of liver and the kidney of control and treated groups showed a normal histology (Figure 2 and 3) and absence of any gross pathological lesions except in the dose of 100 mg where mild infiltration of the glomeruli was noted. The absence of pathological lesions on both the liver and kidney tissues indicates the absence of toxic effect on the organs. This finding is in line with those of biochemical assays of liver and kidney function of Abubakar *et al* (2022) and it further confirm the safety of the compound within the tested doses.

CONCLUSION

The results of this study indicated that oral administration of 5-methylcoumarin-4β-glucoside did not reveal any significant toxic effect in both acute and sub-acute studies in Wistar rats. The parameters analyzed predominantly did not exhibit significant difference compared to the controls. Although there was fluctuation in few parameters, they were considered not clinically significant. Therefore, 5-methylcoumarin glycoside could be considered non-toxic

at the tested doses. Nevertheless, further research is needed to determine the effect of the compound at higher doses and on chronic administration. In addition, investigations at molecular level may also be required to assess the effect of the compound on gene expression in order to guarantee it safe use.

CONFLICT OF INTEREST

The authors wish to declare that there is no conflict of interest.

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