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Acute and sub-acute toxicity profile of methanol leaf extract of Geophila obvallata on renal and hepatic indices in Wistar rats

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1. Introduction

Globally, there is an increase in the rate of herbal formulations consumption [1] because of the belief that they are organic, harmless and effective in the treatment of diseases [2]. Developing countries in Africa mostly use herbal formulations as alternative treatment for various illnesses due to perceived disparities in conventional medicine [3, 4]. According to Fabricant and Farnsworth [5], herbal formulations are safer and less damaging to biological systems with fewer side effects when compared with synthetic drugs. This is as a result of the presence of bioactive constituents translating into low animal and human toxicity. The World Health Organization recommends that complementary medicine should be adopted by member states in developing proactive policies that will strengthen the use of medicinal plants in keeping populations healthy [6].

One of the many medicinal plants used in Nigerian folkloric medicine is *Geophila obvallata* commonly known as*"avbovbotor"* and "*ekoro*" by the Edo and Yoruba tribes of Nigeria, respectively [7]. Its taxonomic classification includes; kingdom plantae, order Gentianales, family Rubiaceae and genus *Geophila* [8, 9]. It is an edible rainforest plant that grows extensively in the tropical rain forest floors especially the *Gelegele* forests, Okomu oil palm reserves and Rubber Research Institute Iyanomo, located in Edo State, Nigeria [10]. This herb has been used by the rural natives of Edo state as a decoction in the treatment of abdominal troubles, headache, hypertension, tooth ache, jaundice, diabetes, stroke and cardiovascular diseases [7]. Aqueous and methanol leaf extracts of the plant were reported to possess antioxidant qualities as a result of its bioactive components [11]. However, there has been no scientific evaluation of the toxicological implications on biological systems on short or long-term basis. This study therefore aims to assess the acute and sub-acute toxicity effects of GOE on some renal and hepatic indices in Wistar rats.

2. Materials and Methods

2.1. Chemicals

The chemicals were purchased via a local vendor from Randox Ltd (USA) with a high quality.

2.2. Collection of plants and preparation of the extract

The fresh leaves of *G. obvallata* (GO) were collected by following leads supplied by a local healer at Ugbowo Quarters, Benin City, Nigeria. They were confirmed by Dr. Akinigboso (taxonomist), at the life science Department, Uniben, Benin City and voucher number UBHa 0312 was assigned to it. GO was then deposited at the Plant Biology and Biotechnology herbarium, Uniben for future references.

A method modified by [12] was adopted. Fresh leaves were washed and air-dried for seven days. Air-dried leaves were blended by a grinding machine (hammer type) (Meecan, CM/L-2264458, UK) until a smooth texture was obtained, and was later weighed and packaged. About 87.52 g of the blended leaves were extracted in the Soxhlet extractor using methanol (70%) (1:10 w/v) [13] followed by homogenization and continuous agitation for two days. Whatman's paper (No. 1) was used to filter the homogenate and the filtrate was concentrated to aridness at 40°C [14] within 24 hrs to obtain about 46.20 g of methanol extract, and then, dried over anhydrous $CuSO_4$ in a dessicator. The dried residues were stored in airtight containers at 4°C before laboratory experiments.

2.3. Experimental animals

Rats used in this experiment were obtained from the animal house, Uniben. They were caged in a hygienic, conducive habitat with proper lighting. The rats weighed between 130 and 200 g. The rats were fed orally with rat pelleted feed (Agro feeds, Nigeria), they had access to dirt-free drinking water and they were housed in steel cages, in compliance with US revised guidelines [33]. Ethical principles regulating the use of living animals for research were strictly adhered to as adopted by Ward and Elsea [15]. The research procedures for animal handling were endorsed by the ethical committee of the institution (NHREC/01/01/2007).

2.4. Acute toxicity study

A slight modification of Lorke's method [16] was employed in this study. Mixed genders of 20 Wistar rats were chosen and organized into four sets of five rats per set. The control rats were given tap water (10 ml/kg/ body weight) while the other three sets were orally administered with a single dose of GOE at 1600, 2900 and 5000 mg/kg body weight. Observation for signs of toxicity was carried out 1, 2 and 4 hr after treatment and periodically during the first 24 hr, then, daily for two weeks following treatment. Changes in the skin, eyes and mucus membrane, body weight and behavioural patterns were noted during the test period [17]. Animals were sacrificed for analysis at the end of the experiment.

2.5. Sub-acute toxicity

This investigation was completed in 28 days according to the OECD guidelines 407 [18]. Experimental animals were divided into four sets of five rats per set of mixed sexes, both sexes were placed in separate cages to prevent mating. Set 1 served as control (i.e. the rats were fed without extract), while the other sets were daily fed by oral administration of GOE at different doses (100, 500, 1000 mg/kg) for 28 days.

On day 28, the rats were anaesthetized using ether after fasting for the night while blood samples were taken for biochemical and haematological analysis using both EDTA and non-EDTA vials while the kidney and liver were harvested for histology investigation.

2.5. Relative organ and body weights study

The changes in body weights were recorded on a weekly basis, while the organs (the liver, kidneys, brain and heart) were weighed using standard weighing balance to calculate relative organ weight for the different sets on the sacrifice day.

Relative organ weight (%) = [Absolute weight of organ (g) /weight of rat on sacrifice day (g)] x100

2.6. Haematological analysis

The indices analysed in the blood samples included haematocrit (HCT), corpuscular volume (CV), erythrocyte count, lymphocytes (LYM), neutrophils (NEU), monocytes (MONO), thrombocyte count, basophils (BASO), leucocyte count (WBC) were performed by means of an automated analyzer (BiopacBS-1100i, Shanghai, China).

2.7. Serum biochemistry and lipid profile assay

Dry tubes were used to collect blood samples which were spun at 5°C for 10 min at 3000 rpm to get the serum isolates used in the measurement of biochemical indices. The serum isolates were examined using an automated biochemistry analyzer (Operon touch, BC-3003432).

2.8. In vivo antioxidant study of GOE

2.8.1. Malondialdehyde (MDA) determination

Lipid peroxidation level was evaluated using spectrophotometry [19] by measuring, malondialdehyde (MDA) which interacts with thiobarbituric acid (TBA) to produce a coloured complex at 532nm in an acidic medium.

2.8.2. Determination of super oxide dismutase (SOD)

The SOD assay was carried out according to Xin et al., [20]. Adrenaline solution was formed by dissolving (5mg) adrenaline in 10 ml of distilled water. Then, 0.10 ml of serum was agitated in potassium buffer at pH 7.8. Buffer was mixed with 0.3ml of adrenaline solution which was then added to 0.2ml of the extract inside a cuvette, agitated and read at 450nm.

2.8.3. Estimation of catalase

The method of Aebi [21] was employed in estimating catalase activity. This is based on the ultraviolet absorption and decomposition of hydrogen peroxide (H_2O_2) by catalase over time. Absorbance is easily measured at 240nm.

2.8.4. Determination of reduced glutathione (GSH)

The technique of Xifan et al., [22] was employed in GSH determination. It is based on the principle that GSH interacts with alloxan and O_2 in alkaline medium at a wavelength (320nm).

2.9. Histopathology

On the 28th day, the liver and kidneys excised from the sets administered with the extracts and the control groups were collected and weighed and quickly set in 10% buffered formalin at pH 7.4 and developed for histology studies. Following fixation, tissues were cleansed in graded series of alcohol, washed in xylene, inserted into paraffin, segmented by a microtome (5-µm thin) and tainted with dye in glass slides. Segments were viewed by a standard microscope (at X 100 and X 400) magnification [23].

2.10. Analysis of data

ANOVA (One- way) was used to analyze data and data are presented as mean ± SEM. ANOVA was followed by Dunnett's multiple comparison test. A $p<0.05$ was considered statistically significant. Statistical analysis of data was done by using Minitab 16.

3. Results

3.1. Acute toxicity analysis

No signs of lethality or morbidity were detected in the rats given different doses up to 5000 mg/kg of GOE for two weeks. Therefore, the median lethal dose (LD_{50}) of GOE was higher than 5000 mg/kg.

3.2. Sub-acute oral toxicity study

Administration of GOE for 28 days continuously did not induce morphological changes or general behavioural changes in treated rats compared to the control group. No deaths were observed during the period.

3.2.1. Body weights

The body weight alterations of rats given graded doses of GOE are indicated in Figure 1. Daily administration of GOE at different doses (100, 500 and 1000 mg/kg) did not result in significant changes in the body weight of GOE-fed rats when compared with the control.

3.2.2. Relative organ weights

The weights of GOE-treated rats' organs were non-significantly different from the control set (Figure 2).

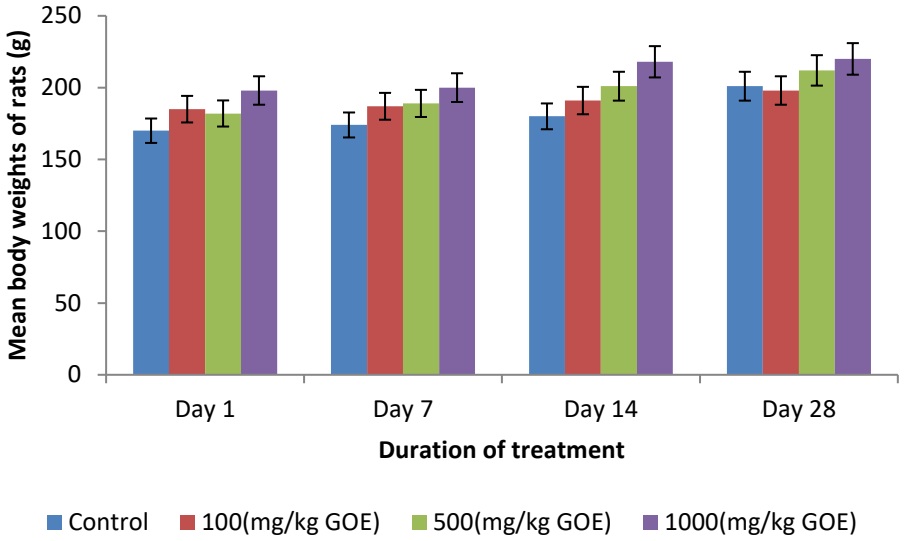

Figure 1. Effect of methanol extract of *Geophila obvallata* (100, 500 and 1000 mg/kg) on mean body weights of rats in sub-acute toxicity study. Values are mean±SEM of five rats. Compared to the control group (one-way ANOVA followed by Dunnet's *post-hoc* test).

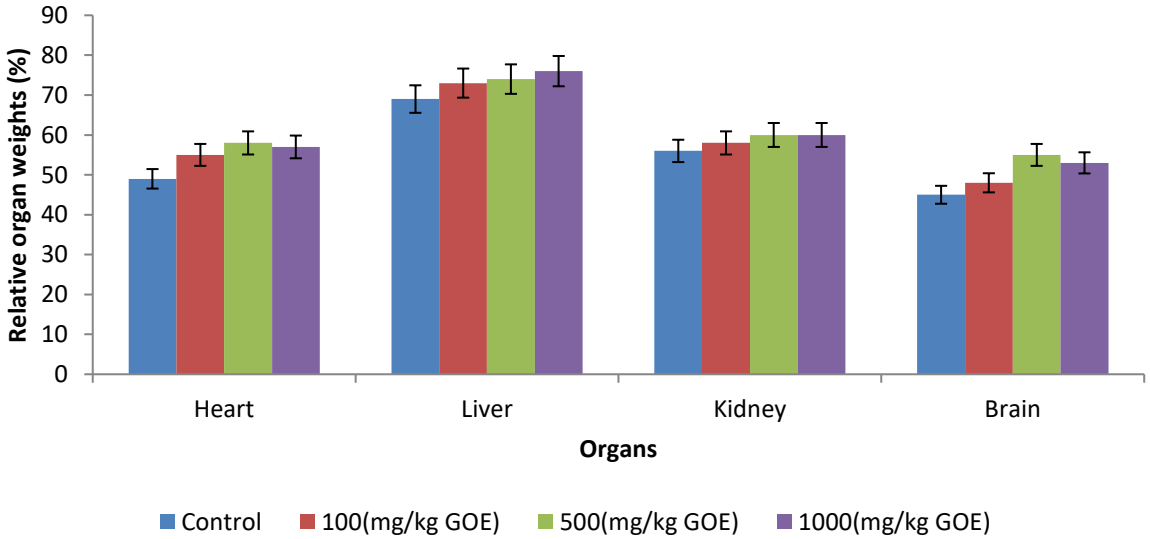

Figure 2. Effect of sub-acute administration of *Geophila obvallata* extract on the relative weight of organs. Values are mean±SEM of five rats. Compared to the control group (one-way ANOVA followed by Dunnet's *post-hoc* test).

3.2.3. Biochemical Analysis

3.2.3.1. MDA (mg/dl of wet tissue) activity

MDA (mg/dl of wet tissue) levels in both control and GOE-fed rats are indicated in Table 7. The results revealed a significant increase ($p<0.05$) in MDA levels in the liver, kidneys and brain after sub-acute treatment with 500 and 1000 mg/kg bw doses of GOE for 28 days.

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3.2.3.2. Catalase activity

The catalase activity of both control and GOE-fed rats are indicated in Table 1. The results indicated no significant difference in catalase activity after sub-acute treatment with different doses of GOE for 28 days, when compared to control set.

3.2.3.3. Superoxide dismutase activity

Superoxide dismutase activities of GOE-treated and control rats are shown in Table 2. The results indicated a significant increase ($p<0.05$) in superoxide dismutase activity in the liver after sub-acute treatment with 500 and 1000 mg/kg bw of GOE for 28 days.

Table 1. Effect of sub-acute administration of *Geophila obvallata* extract on the catalase activity (unit/mg of wet tissue)

Organs	Control	*G. obvallata* (mg/kg BW)		
		100	500	1000
Heart	53.01±2.10	48.66±6.37	52.42±1.42	52.45±2.50
Liver	52.82±0.81	52.30±2.73	53.70±1.50	53.35±0.90
Kidneys	50.96±1.72	51.76±0.50	52.17±0.92	53.03±2.06
Brain	54.83±1.51	54.00±3.00	54.48±0.32	54.57±2.63

Values are mean±SEM of five rats. Compared to the control group (one-way ANOVA followed by Dunnet's *post hoc* test).

Table 2. Effect of sub-acute administration of *Geophila obvallata* extract on the superoxide dismutase activity (unit/mg of wet tissue)

Organs	Control	*G. obvallata* (mg/kg BW)		
		100	500	1000
Heart	7.56±0.12	8.82±0.21	8.45±0.10	8.40±0.51
Liver	6.24±1.41[a]	7.08±0.34	11.56±0.83*	15.44±1.70*
Kidneys	8.36±0.57	9.43±0.55	9.52±0.84	9.58±0.13
Brain	6.56±0.37	7.28±0.12	8.54±0.20	8.74±0.50

Values are mean ± SEM of five rats. * Significantly different from the control sets ($P<0.05$).

3.2.3.4. GSH activity (mmol/GSH of wet tissue)

The reduced glutathione activities of GOE-fed rats and control rats are shown in Table 3. The results indicated no significant difference ($p>0.05$) in superoxide dismutase activity in the liver after sub-acute treatment with different doses of GOE for 28 days, when compared to control set.

3.2.4. Effects of GOE on haematological profiles of Wistar rats

The effects of sub-acute administration of GOE on haematological parameters are illustrated in Table 4. Daily administration of GOE for 28 days did not cause any significant difference in most of the haematological parameters when compared with the control group. However, there was a significant decrease ($p<0.05$) in hematocrit and haemoglobin (HB) concentration at 1000 mg/kg.

3.2.5. Effects of GOE on liver indices

The effect of sub-acute administration of GOE on liver indices is presented in Table 5. A significant increase ($p<0.05$) in ALP activity at 1000 mg/kg was observed while other liver markers showed normal levels.

3.2.6. Effects of GOE on kidney function in rats

Sub-acute administration of GOE on kidney function (Table 6) caused no significant difference ($p>0.05$) in kidney parameters (bicarbonates ion, creatinine, uric acid, sodium ion, potassium ion, urea and chloride ion levels) between the extract-fed and control rats for all doses.

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3.2.7. Effects of GOE on lipid profiles in rats

Effects of sub-acute administration of GOE on the lipid profile of GOE-fed rats are illustrated in Table 8. GOE resulted in a significant decrease ($p<0.05$) in TC and TG concentrations at 100 mg/kg in rats exposed to graded doses of extract in comparison to the control set. Alternately, GOE at 100 and 500 mg/kg resulted in elevated HDL levels of GOE-fed rats when compared to the controls.

Table 3. Effect of sub-acute administration of *Geophila obvallata* extract on the activity of GSH (mmol//GSH of wet tissue) of treated rats.

Organs	Control	*G. obvallata* (mg/kg BW)		
		100	500	1000
Heart	19.97±0.11	21.67±0.16	21.46±0.57	21.68±0.43
Liver	20.72±1.32	22.56±0.22	22.43±0.31	21.88±1.02
Kidneys	19.63±0.55	20.76±0.32	20.61±0.19	21.34±1.20
Brain	21.39±0.25	22.56±0.14	22.52±1.03	22.56±0.91

Values are mean ± SEM of five rats. Compared to the control group (one-way ANOVA followed by Dunnet's *post hoc* test).

Table 4. Effect of sub-acute administration of *Geophila obvallata* extract on haematological profiles

Parameters		*G. obvallata* (mg/kg B.W)		
	Control	100	500	1000
WBC (10^3/mm^3)	7.11±0.23	6.62±0.12	6.78±1.23	5.59±0.88
RBC (10^6/mm^3)	7.01±0.83	5.05±0.49	6.49±0.50	6.63±0.23
Hematocrit (%)	41.45±0.16	40.02±1.40	39.94±0.15	24.68±0.14*
Hemoglobin (%)	15.98±0.50	14.15±0.43	13.80±1.6	9.73±0.48*
MCV (µm^3/red cell)	59.50±0.45	60.75±0.21	60.25±0.42	59.25±1.50
MCH (pg/red cell)	19.46±1.52	20.95±1.80	19.98±1.00	19.30±0.50
MCHC (g/dL)	32.68±0.90	34.53±2.80	32.90±0.70	32.08±1.10
Platelets (10^3 cells/mm^3	391.80±160.1	388.80±169.30	382.00±83.10	397.20±122.60
Lymphocytes (%)	58.52±1.40	50.08±1.23	52.10±0.50	53.47±0.25
Neutrophils (%)	34.80±0.11	35.70±1.20	36.90±0.55	37.20±0.32
Monocytes (%)	3.30±0.32	3.47±2.00	2.33±1.02	3.34±0.20
Basophils (%)	0.04±1.11	0.05±0.48	0.06±0.42	0.03±1.50
Eosinophils (%)	1.07±0.05	1.15±0.23	1.09±0.98	1.71±0.01

Values are mean ± SEM of five rats. * Significantly different from the control sets ($P<0.05$).

Table 5. Effect of sub-acute administration of *Geophila obvallata* extract on liver indices

Parameters	*G. obvallata* (mg/kg B.W)			
	Control	100	500	1000
AST (IU/L)	19.06±1.77	20.92±1.78	20.88±0.50	21.66±1.54
ALT (IU/L)	14.68±1.45	16.50±1.23	16.25±0.76	15.48±0.06
ALP (IU/L)	65.17±1.63	61.46±1.09	64.63±0.02	89.12±0.07*
Total bilirubin (mg/dL)	0.70±0.54	0.98±0.10	1.01±0.61	1.13±0.59
Total protein (g/dL)	7.57±1.70	7.41±2.23	7.61±0.87	8.69±0.96
Albumin (mg/dL)	3.49±0.50	3.63±0.5	3.74±0.99	3.81±0.81

Values are mean ± SEM of five rats. * Significantly different from the control sets ($P<0.05$).

Table 6. Effect of sub-acute administration of *Geophila obvallata* extract on kidney indices

Parameters	*G. obvallata* (mg/kg B.W)			
	Control	100	500	1000
Urea (mg/dL)	44.81±2.50	41.81±9.09	40.84±0.63	45.16±3.30
Creatinine (mg/dL)	0.69±0.27	0.62±0.09	0.65±0.39	0.75±0.11
Uric acid (mg/dL)	11.25±0.42	12.14±0.25	14.09±0.37	13.27±0.12
Na^+ (mEq/L)	140.44±2.93	143.41±6.88	143.57±8.53	144.62±5.34
K^+ (mEq/L)	2.44±0.67	2.48±0.45	2.38±0.97	2.43±0.39
Cl^- (mEq/L)	77.42±2.50	76.77±1.20	71.94±1.38	71.24±0.25

HCo_3^-(mEq/L)	39.11±2.29	43.13±0.54	37.15±0.90	42.25±0.61

Values are mean±SEM of five rats. Compared to the control group (one-way ANOVA followed by Dunnet's *post hoc* test).

Table 7. Effect of sub-acute administration of *Geophila obvallata* extract on the activity of MDA (mg/dl of wet tissue

***G. obvallata* (mg/kg B.W)**	**Organs**			
	Heart	**Liver**	**Kidney**	**Brain**
Control	1.33 ± 0.16	1.64 ±0.28	1.63±0.20	0.80±0.14
100	1.37 ± 0.51	1.65±1.03	1.65±0.06	0.87±0.01
500	1.39 ± 0.03	1.82±0.09*	1.69±0.12	0.89±0.03
1000	1.41 ± 0.14	1.98±0.10*	2.20±0.01*	0.91±0.19

Values are mean ± SEM of five rats. * Significantly different from the control sets (P<0.05).

Table 8. Effect of sub-acute administration of *Geophila obvallata* extract on the activity on lipid profiles in rats

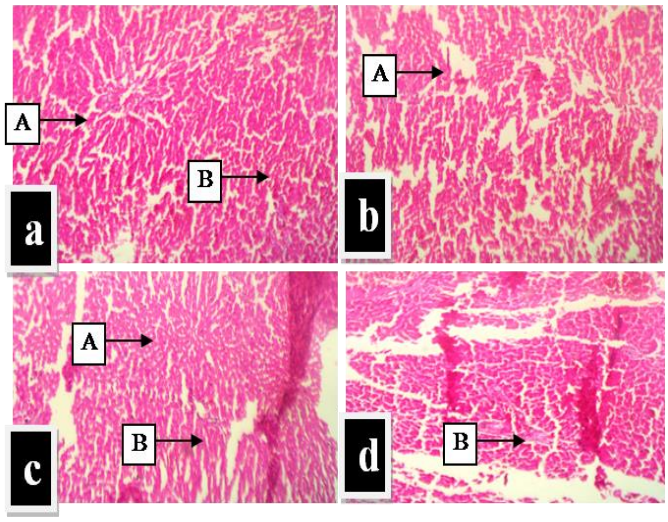
***G. obvallata* (mg/kg B.W)**	**Lipid Profile**				
	TC	**TG**	**HDL**	**LDL**	**VLDL**
Control	0.40 ± 0.12	0.43 ±0.20	0.63±0.04	0.35±0.22	0.29±0.14
100	0.42 ± 0.01	0.45±0.03	0.78±0.07	0.37±0.18	0.20±0.01
500	0.61 ± 0.02*	0.60±0.02*	0.62±0.11	0.39±0.30	0.31±0.03
1000	1.20 ± 0.10*	1.98±0.10*	0.32±0.08*	0.51±0.19*	0.25 ±0.19

Values are mean ± SEM of five rats. * Significantly different from the control sets (P<0.05).

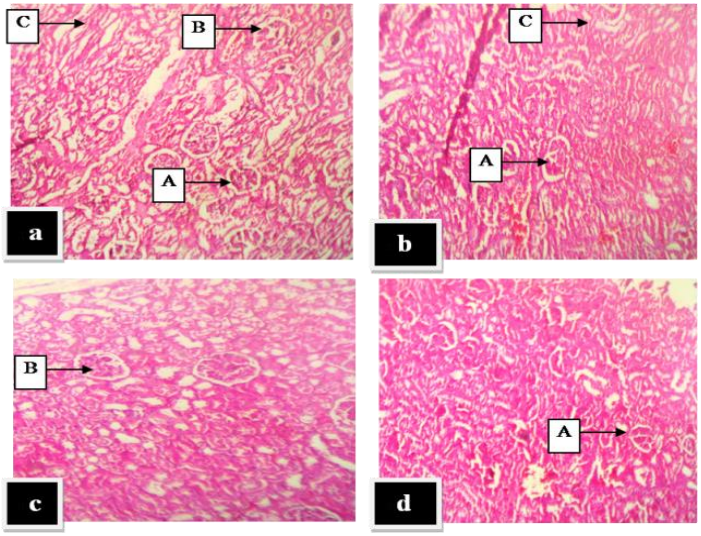
3.2.8. Histopathology analysis

Histology results of assessment of GOE effects on the liver after 28 days of administration is shown in Figure 5a-d. The microscopic examination revealed no significant pathological alterations in the liver for all experimental groups. It revealed unambiguous, observable rows of normal liver cells resulting from (**A**) central veins (**B**) hepatic sinusoids after 28 days of extract administration.

The histopathological effect of GOE on the kidney after 28 days of administration is revealed in Figure 6a-d. The microscopic examination revealed no significant pathological alterations in the kidney for all experimental groups. It also revealed very clear and visible glomeruli as indicated by (**A**) renal tubules (**B**) renal corpuscles (**C**) medullary ray after 28 days of extract administration.



Figures 3 (a-d). Effect of *Geophila obvallata* on histology of the liver of rats after 28 days of extract administration (Haematoxylin and eosin staining; X 100). Key: a= Normal control showing normal hepatic histology; b= Administered with 100 mg/kg body weight of GOE, showing normal hepatic structure; c= Administered with 500 mg/kg body weight of GOE, showing normal hepatocytes; d = Administered with 1000 mg/kg body weight of GOE, showing more or less normal hepatic structure (A) central veins (B) hepatic sinusoids.



Figures 4 (a-d). Effect of *G. obvallata* on histology of the kidney of rats after 30 days of extract administration (Haematoxylin and eosin staining; X 100*)* Key: a= Normal control showing intact renal tubules, corpuscles and medullary rays; b= Administered with 100 mg/kg body weight of GOE, showing normal renal architecture and medullary rays; c= Administered with 500 mg/kg body weight of GOE, showing normal renal corpuscles; d= Administered with 1000 mg/kg body weight of GOE, showing more or less normal renal architecture (A) Renal tubules (B) Renal corpuscles (C) Medullary rays.

4. Discussion

Information regarding the toxic effects of *Geophila obvallata* methanol extract in health care does not exist in previous research archives. To guarantee the quality of GOE for human consumption, a methodical toxicity assessment was needed to estimate the dangers of toxicity and to provide a basis for safe dose selection and scientific data in humans.

The acute toxicity study revealed that there were no signs of morbidity or death after two weeks of treatment. The rats were able to tolerate higher doses of GOE. Therefore, the LD_{50} of GOE is above 5000mg/kg body weight when taken orally. Consequently, GOE should be considered a class 5 drug based on the OECD 423 guidelines [17] adopted worldwide synchronized categorization system (GSH) for chemical materials and concoctions.

In summary, it can be said that oral treatment with GOE caused no striking negative effects on the body weights and relative organ weights of the fed set in the sub-acute assessment. This is in agreement with Olorunnisola et al., [24] who reported that 28 day oral feeding of Wistar rats with graded doses of *Tulbaghia violacea* rhizomes had zero negative consequences to the organs of the extract-fed sets.

The bone marrow is a major location for novel blood cell manufacture and a vulnerable tissue targeted by toxic compounds in the hematopoietic system [25]. In this study, there was a slight decrease in hematocrit and haemoglobin (HB) concentrations at 1000 mg/kg when compared with the control groups. However, these alterations were considered minor and toxicologically insignificant. This implies that GOE has no lethal implication on the hematopoietic system.

The liver biomarkers are specific tools in examining liver toxicity during drug biotransformation [26]. The assessment of liver and kidney functions in this research revealed that GOE consumption at graded doses, had no effects on the ALT and AST liver indices, although, ALP levels increased significantly ($p<0.05$) at 1000mg/kg as an indication of biliary duct obstruction or cholestatic disease at higher doses [27].

According to Roberts et al. [34], the notable potential of the liver to rejuvenate its cells makes it exceptional in overcoming various forms of necrosis and perturbations. This is in accordance with Tarkang et al's., [28] investigations using *Carica papaya* extracts in rats. An

increase in ALP was observed implying that hepato-biliary damage can be an effect of GOE consumption at higher doses thereby causing destruction of the liver cells. Illnesses like cholestasis of the liver and biliary cirrhosis are associated with elevated liver indices [29].

Kidney disease can be detected by measurements of kidney indices like creatinine, uric acid, urea, bicarbonates, potassium,sodium, and chlorides and their normal levels reflect a reduced likelihood of renal problems [30]. In the present study, no significant ($P > 0.05$) alterations in plasma creatinine, uric acid, urea, bicarbonates, potassium, sodium, and chlorides levels in *Geophila obvallata* extract fed rats when compared to the control was observed. This indicates that the functional integrity of the kidney was not compromised after treatment with graded doses of the extract.

GOE effects on lipid peroxidation were evaluated by measuring malondialdehyde (MDA) levels, GSH levels, and SOD and catalase enzyme activities. Reduction in catalase, GSH, SOD activities and increases in MDA levels connotes an elevation in oxidative stress in biological entities thereby interfering with the system's antioxidant defence mechanisms [31]. However, in this study, GOE administration at 500 and 1000 mg/kg bw significantly increased ($p<0.05$) the MDA and SOD levels, especially in the liver of fed sets in comparison to the control. Also, GSH and catalase levels remained within the normal ranges. This suggests that *G. obvallata* methanol extract possesses beneficial properties due to its content of phytochemicals, in boosting the body's defence [11].

GOE oral administration significantly increased ($p<0.05$) total cholesterol (TC), serum triglyceride (TG) and LDL levels while HDL levels significantly decreased at elevated doses of 500 and 1000 mg/kg bw. This is in agreement with Moller's [32] research which recommended that GOE administration may prove effective in the management of cardiovascular ailments, diabetes as well as deregulated blood pressure. This information is particularly relevant to the trado-medical practitioners who prepare the leaves of this plant in a decoction for its anti-hypertensive and cardiovascular potentials.

Histological observations of kidney and liver sections from the experimental animals demonstrated no significant pathological conditions in the test group as the liver and kidney tissues of the test groups were consistent with the normal histology of the control.

5. Conclusions

Oral doses of *G. obvallata* leaf extracts can be considered non-toxic especially at 100 mg/kg, as the extract did not elicit lethality in the acute and sub-acute toxicity studies in rats. The findings of this study give credence to the application of *G. obvallata* in folkloric traditional medicine. However, further pre-clinical assessments should be carried out to validate its effectiveness and long-term toxicological safety.

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Conflicts of interest

The authors have declared that there is no conflict of interest.

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