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Toxicological Evaluation of Cod Liver Oil-Enriched *Vernonia amygdalina* Leaf-Based Diet in Wistar Rats

Soji-Omoniwa, O.[1*], Oyindamola, E.O.[1], Ogunbanwo, S.O.[1], Obasawo, S.O.[1], Akinleye, E.O.[1], Ogunbayeje, M.P.[1], Taiwo, B.O.[1], Odoh, M.U.[1], Odejayi, H.O.[1], Oluwadamilola ,S.O.[1], Sanusi, R.O.[1], Oba, A.M.[1], Salawu, A.O.[1] Salihu, L.U.[1a], , OdewoleM.A.[1], Obe, D.A.[1], Salaudeen, I.O.[1].

[1]Department of Biochemistry, Faculty of Life Sciences, P.M.B. 1515, University of Ilorin, Ilorin, Nigeria.

Corresponding author's E-mail: sojiomoniwa.o@unilorin.edu.ng

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ABSTRACT

Cod Liver Oil-Enriched *V.amygdalina* Leaf-Based Diet in our previous study elicited wound-healing effect in Type 2 diabetic rats. As part of the safety evaluation of this nutraceutical formulation, it is important to evaluate it toxicity profile. Therefore the purpose of this study was to determine the toxicological effect of cod liver oil-enriched *V. amygdalina* leaf-based diet (CLVA) in Wistar rats. Acute oral toxicity (limit test) and sub-acute toxicity (repeated dose 28-days oral toxicity) tests were carried out using Organization of Economic Cooperation and Development guidelines. For acute toxicity experiment, 2000 mg/kg body weight (b.wt.) single dose administration was given to 5 overnight-fasted female rats. This was done sequentially in an interval of 48 hr. While for the sub-acute study, 30 male and 30 female rats were assigned randomly to 6 groups constituting 4 main and 2 reversal groups, each containing 10 rats (5 male and 5 females). CLVA at the following doses; 0, 250, 500 and 1000 mg/kg b.wt. were administered daily to Wistar rats for 28 days. Rats in the Main groups (G1, G2, G3 & G4) were sacrificed at day 29 while the reversal groups (G1R & G4R) were monitored for another 14 days without CLVA administration. Data were analysed using the 20th version of Statistical Package for Social Sciences. Results showed that the lethal dose of CLVA in Wistar rat was above 2000 mg/kg b.wt. Water, feed intake, liver function indices (alanine aminotransferase, aspartate aminotransferase, albumin, total protein and total bilirubin), kidney function indices (alkaline phosphatase, blood urea nitrogen, urea, and electrolytes), lipid profile (total cholesterol, triglycerides), glucose concentration and haematological parameters were not altered significantly ($p > 0.05$) in CLVA-fed groups. Histopathological examination of kidneys and livers did not show any treatment-related changes as well. In conclusion, administration of CLVA at the studied doses is safe for consumption and can therefore be recommended for use by patients suffering from diabetes with delayed wound healing.

Keywords: Nutraceutical, Vegetable, Biochemical, Feed, Histopathology

INTRODUCTION

Vernonia amygdalina, also known as bitter leaf is a vegetable often cultivated domestically in Africa (Ndaeyo, 2007). In Nigeria It is often prepared and consumed as soup (Onabanjo and Oguntona, 2003). *V. amygdalina* is a wooded shrub of about 2 to 10 m height that regenerates rapidly after planted. The leaves are petiolated in shape and has a bitter taste. Several studies have reported its medicinal uses both locally (Amira and Okubadejo, 2007), some of which have been validated scientifically. For instance, reports on its anti-diabetic (Ijeh and Ejike, 2011; Ugbogu *et al.*, 2021), anthelmintic, antioxidant (Omojokun *et al.*, 2019), anticancer (Yeap *et al.*, 2010), antimicrobial (Ugbogu *et al.*, 2021), hepatoprotective (Ugbogu *et al.*,

2021), analgesic (Njan *et al.*, 2008), anti-inflammatory and wound-healing (Soji-Omoniwa *et al.*, 2022) have been documented. It is also used as a bittering agent and a hop substitute used for controlling microbial contamination in beer brewing without reducing the quality of malt (Alara *et al.*, 2017). The leaves and roots decoctions are used in ethnomedicine to treat hiccups, fevers, kidney problems and stomach disorder (Alara et al., 2017).

Cod liver oil (CLO) on the other hand, is derived from the liver of cod fish. It is abundant in omega 3 fatty acids, namely, docosahexanoic acid (DHA) and eicosapentanoic acid (EPA). It is also contains vitamins A and D used in both pharmaceutical and food supplements (Stark and Mahar, 2002; Gunston, 2004). CLO components, EPA and DHA, play very crucial roles in cardiovascular disease prevention, nervous system protection and immune function (Moghadasian, 2008). According to Skeie *et al.* (2009) and Stene *et al.* (2000) respectively, CLO exhibited anti-cancer and anti-diabetic activities. Meanwhile, its anti-ulcer, hepatoprotective and neuroprotective benefits have also been established (Khare *et al.,* 2008; Salama *et al.*, 2013; Mohammed *et al.*, 2015). The therapeutic properties elicited by *V. amygdalina* and cod liver oil have been associated with the presence of bioactive compounds present in the duo. For instance, vernodalinol, vernolepin, vernomygdin, hydroxyvernolide, vernolide and vernodalol isolated from *V. amygdalina* were reported to inhibit breast cancer cell growth, exhibited antitumoral and antimicrobial properties (Alara *et al.*, 2017).

Cod liver oil-enriched *V. amygdalina* leaf-based diet (CLVA) was previously formulated and fed to wound-inflicted diabetic rats. Reports of that study showed that its consumption by the experimental rats promoted wound healing by lowering blood glucose concentration and down regulating the pro-inflammatory cytokines studied (Soji-Omoniwa *et al.*, 2022). As part of the safety evaluation of this nutraceutical formulation, it is important to evaluate it toxicity profile. The safety evaluation of herbal dietary supplements is important because natural toxins can be formed in plants as defense mechanisms of such plants and these toxins may pose a health risk to humans (WHO, 2018). Di Lorenzo *et al.* (2015) reported allergic reactions associated with soybean consumption, hypolalaemia and hypertension with consumption of *Glycyrrhiza glabra*, acute hepatitis with green tea and coagulation difficulties with *Ginkgo biloba* consumption. Similarly, the Greek philosopher Socrates, was given a liquid preparation of hemlock plant (*Conium maculatum*), after being sentenced to death for corruption of young men and impiety. The hemlock plant contains a group of toxic piperidine alkaloids, of which the representative members include coniine and γ-coniceine (Lee *et al.*, 2008; Reynolds, 2005). The aim of this study therefore, was to evaluate the acute and subchronic toxicity of CLVA in Wistar rats using the Organization for Economic Cooperation and Development (OECD) guidelines (OECD, 2006; 2008).

MATERIALS AND METHODS

Plant material and Cod liver oil

CLVA comprised 30 % pulverized *V. amygdalina* leaves and 3.5 % cod liver oil incorporated into locally sourced indigenous ingredients (corn starch, cellulose, sucrose, soya bean, vitamin-mineral mix, DL-methionine and L-lysine). *V.amygdalina* leaves were harvested from local farmland (8°29'23.6"N 4°36'44.5" E) in Ilorin, Kwara State of Nigeria, following the guidelines for sustainable harvesting of medicinal plants (WHO, 1993). Cod liver oil was a product of Ecomed Pharma Limited Sango Ota, Ogun State, Nigeria and was procured from a pharmacy store in Ilorin, Kwara State, Nigeria.

Vernonia amygdalina leaves were identified and authenticated at the Plant Biology Department (herbarium unit), University of Ilorin, Ilorin, Nigeria. A voucher specimen number UILH/001/1324/2022 was issued.

Plant preparation and feed processing

The leaves of *V. amygdalina* were subjected to a washing process using tap water and subsequently left to air dry at room temperature (28°C) until a constant weight was achieved. They were thereafter pulverized to smoothness using a blender. Three hundred grams of the ground leaves was mixed into corn starch, maize husk, sucrose, soybean, cod liver oil/corn oil, mineral and vitamin mix (Table 1). Distilled water (40 ml) was added slowly to the homogenous ingredients until the mixture became a paste. The paste was formed into a pellet using wire mesh and dried at 40 °C in a laboratory oven.

Chemicals and Reagents

Assay kits for the biochemical parameters were products of Fortress Diagnostics Limited, Unit 2C Antrim Technology Park, Antrim, BT41 1QS (United Kingdom). The reagents used included Cholesterol reagents, Phosphotungstic Acid, Magnesium Chloride, Lipoprotein Lipase, Glycerol-3-P-Oxidase, Glycerol kinase, p-Chlorophenole, Peroxidase 4-Aminoantipyrine, ATP, Mg^{2+}, Na-cholate and Formalin. All reagent used during the experiment were of analytical grades and were prepared with suitable salts and distilled water in volumetric flasks. The reagents used were not expired, kept at 2-8 °C and protected from light.

Table 1: Formulation of cod liver oil-enriched *V.amygdalina* leaf-based diet

Ingredients (g/kg)	Control	*CLVA
Corn Starch	150	105
Cellulose	50	35
Sucrose	500	350
Soybean	200	140
Corn Oil	50	-
Mineral and Vitamin Mix	45	31.5
D-Methionine	3	2.1
L-Lysine	2	1.4
Cod Liver Oil	-	35
V.amygdalina	-	300

*CLVA is 70 % of control feed for all ingredients except corn oil replaced by cod liver oil and *V.amygdalina* leaves which makes up 30 % of the remaining ingredients

CLVA - Cod liver oil-enriched *V.amygdalina* leaf-based diet

Experimental Animals

Seven weeks old Wistar rats (30 males and 35 females) were purchased from animal rearing section, located in Biochemistry Department, University of Ilorin, Nigeria. They were allowed to acclimatize for 7 d to the housing conditions (temperature: 22 ± 3°C; relative humidity: 45%; 12 h light and dark cycle) before starting the experiments. Throughout the testing period, water and rodent feed were freely available to all of the rats.

Ethical clearance

Ethical clearance for animal care and handling for this study was obtained from University of Ilorin Ethical Committee in accordance with the recommendations for handling animals for research (approval number UERC/LSC 1020/2020).

Experimental design

Acute oral toxicity experiment

This test was done following the OECD Test 425 guidelines. An amount of CLVA equivalent to 2000 mg/kg b.wt. was percolated in distilled water and given by oral gavage as a single dose to 5 female Wistar rats fasted overnight. Each administration per animal was done sequentially in 48 hr interval. After administering the feed, the rats were monitored for mortality and any clinical indicators of toxicity (i.e. decreased motor activity, circling, hunched posture, hyperactivity, jumping/hopping, kicking of rear legs, ruffled fur, standing on front legs, stained eyes and trembling) every hour for the first 4 hrs, followed by daily checks for the next two weeks. Their body weight, feed and water intake were also recorded daily. Rats were then sacrificed by day 15 of the study. They were anaesthetized with dichloromethane and their jugular vein cut using a scalpel. Kidney and liver were isolated for histopathological assessment.

Twenty-eight-day repeated dose oral toxicity study

In this study, OECD Guidelines 407 was used. The male and female rats were divided into six groups through a random selection process, which included four Main groups and two reversal groups. The sample size for each group was ten rats (5 males and 5 females). Group 1 (control), labeled G1, received feed without VA and CLO (1 ml). Groups 2 to 4 labeled G2, G3 and G4, received 250, 500 and 1000 mg/kg b.wt. of CLVA, respectively. The two reversal groups (G1R and G4R) received feed without VA and CLO and 1000 mg/kg b.wt. of CLVA for 28 days. After 28 days, CLVA administration was stopped in the reversal groups and fed regular rodent feed for the next 14 days.

Each dosage formulation was made fresh every day and given at the same volume of 1 ml. The body weight of each rat was recorded every week during the study period. On the 29th day of the experiment, rats belonging to the Main groups G1, G2, G3, and G4 were anesthetized using dichloromethane and then euthanized. Blood samples were obtained from the rats by incising the jugular vein using a scalpel for haematology and biochemical analysis. The kidney and liver were isolated for histopathological assessment. Animals in the reversal groups, on the other hand, were sacrificed by day 43 and treated as earlier described. The reversal groups were included in determining if any effect caused by the administered feed would be reversed following the withdrawal of the feed from the rats.

Table 2: Animal grouping for evaluating twenty-eight-day repeated dose oral toxicity of Cod liver oil-enriched *V.amygdalina* leaf-based diet in rats.

Groups	Number of Rats		Treatment and dose	Definition
	Male	Female		
G1	5	5	1 ml feed without CLO and VA	Main group (control)
G2	5	5	250 mg/kg b.wt. CLVA	Main group
G3	5	5	500 mg/kg b.wt. CLVA	Main group
G4	5	5	1000 mg/kg b.wt. CLVA	Main group
G1R	5	5	1 ml feed without CLO and VA	Reversal group (control)
G4R	5	5	1000 mg/kg b.wt. CLVA	Reversal group

G1- Group 1; G2 – Group 2; G3 – Group 3; G4 - Group 4; G1R – Group 1 reversal; G4R – Group 4 reversal; CLO – Cod liver oil; VA – *V. amygdalina* ; CLVA – Cod liver oil-enriched *V.amygdalina* leaf-based diet

Blood Preparation for Analysis

Rats were anaesthetized with dichloromethane and sacrificed 24 hours after the last day of treatment by incising the jugular vein using a scapel. Blood samples for haematological analysis were collected into EDTA bottles and inverted gently about 8 times before proceeding with the analysis. For biochemical analysis, blood samples were collected into plain sample bottles and allowed to stand for 30 minutes at room temperature for clot formation. Serum was then collected from the sample bottles using Pasteur pipette.

Haematological Assay

Haematological assay in the rats was carried out to determine the effect of CLVA on erythrocyte parameters (red blood cells, hemoglobin concentration, mean corpuscular volume, packed cell volume, mean corpuscular hemoglobin concentration and mean corpuscular hemoglobin) and leukocyte parameters (neutrophils, white blood cells, lymphocytes, eosinophils, basophils, platelets, reticulocytes and monocytes) using a haematology analyzer (Sysmex KX-21N).

Biochemical Assay

Biochemical parameters for the determination of liver functions (i.e. alanine aminotransferase, aspartate aminotransferase, albumin, total protein and total bilirubin), kidney functions (i.e. alkaline phosphatase, blood urea nitrogen, urea, and electrolytes), lipid profile (total cholesterol, triglycerides) and glucose were assessed by carrying out the manufacturer's recommended procedures for each parameter, as described in the assay kits.

Histopathological Assessment

The livers and kidneys were separated for histopathological analysis and then kept in 10% buffered formalin for 48 hours to fix them. Thin slices (about 5 mm) of the fixed tissues were cut, put in tissue cassettes and processed using paraffin wax tissues processing method with the aid of LEICA PT 1020 automatic tissue processor (Avwioro, 2014). The tissue sections were treated with ascending concentrations of alcohol and then stained with hematoxylin and eosin (Drury and Wallington, 1980). Tissue sections (previously stained) were then examined using ×10 objective lens of light microscope for focusing and viewed with ×40 objective lens for higher magnification. Photomicrographs were taken at a magnification of ×100 and high power of ×400.

Analysis of data

Data were expressed as mean ± standard error of mean (SEM) of 5 determinations. Raw data were analyzed using the 20th version of the statistical package for social sciences (SPSS). To identify significant differences among the means, one-way analysis of variance (ANOVA) was employed, and post hoc multiple comparisons were performed using Duncan's multiple range test. The level of significance set at $p < 0.05$ (confidence level = 95 %).

RESULTS

Acute oral toxicity study of CLVA

CLVA at 2000 mg/kg body weight did not cause clinical toxicity or fatalities in female Wistar rats, as determined by the acute toxicity study. Additionally, there were no noticeable changes in their body weight, food, and water consumption during the 14-day monitoring period. The oral LD_{50} for CLVA in female Wistar rats was greater than 2000 mg/kg b.wt. based on the findings. Results of the histopathological assessment of the liver section showed irregular shapes of hexagonal plates of hepatocyte admixed with the central vein. The sinusoid at the margin of the hepatic lobules and course between plates of hepatocytes converge upon the terminal venule. The plates of hepatocytes were one cell thick, and each plate was exposed to blood on both sides. The hepatocytes were round to polygonal with round nuclei, dense chromatin,

and inconspicuous nucleoli. A few of the hepatocytes showed prominent nucleoli. These histological features were consistent with normal histology (Figure 1 a, b, c, and d).

The kidney tissue section showed numerous glomeruli, which demonstrated no abnormalities. The tubules were lined by simple cuboidal epithelium. There were renal arterioles with normal interstitial spaces. The tubules laid back to back with little intervening stroma. The histological features were consistent with normal histology under light microscopy (Figure 1).

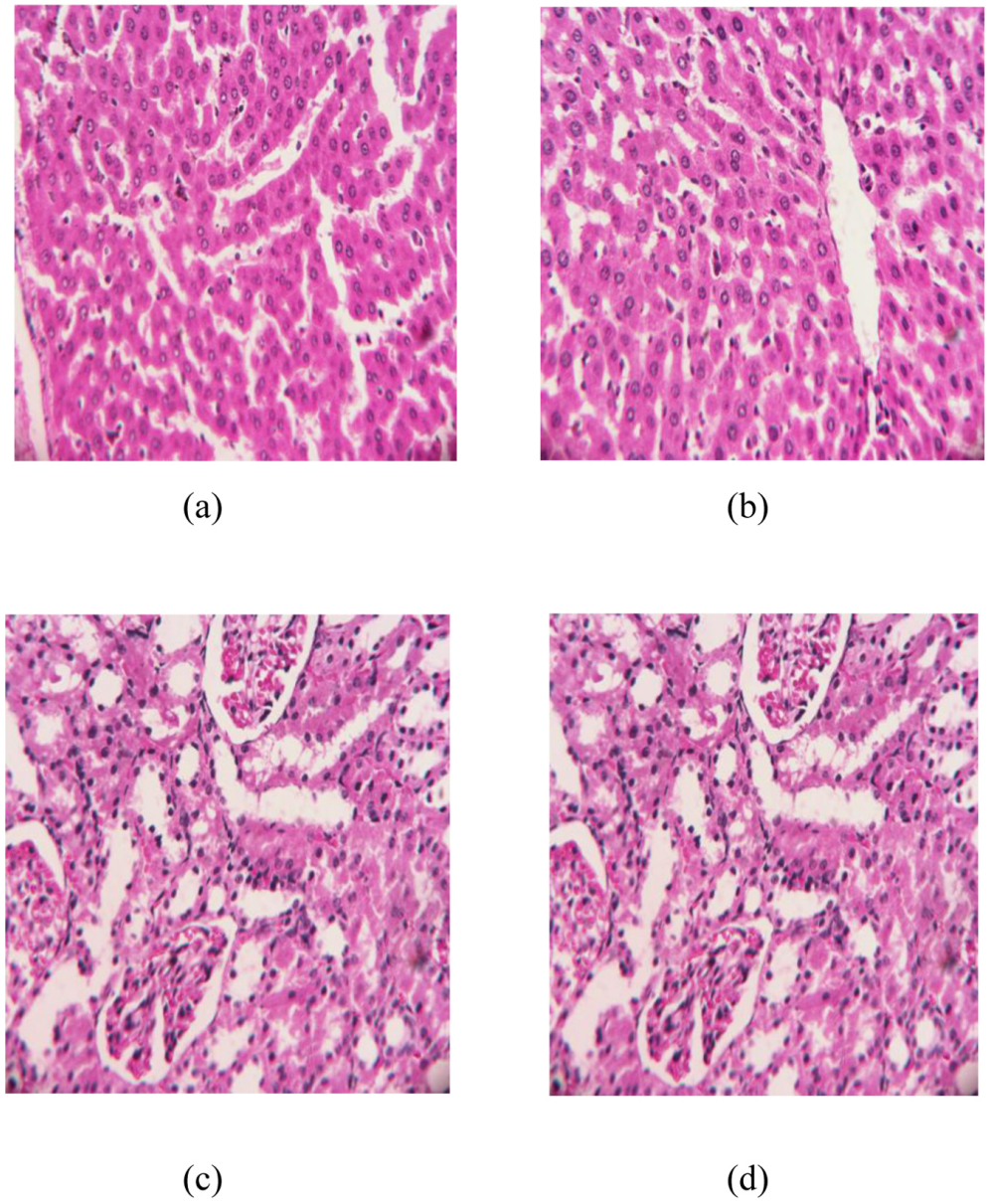

Figure 1: Photomicrographs of the livers and kidneys of female rats administered single-dose 2000 mg/kg b.wt. of CLVA Stain: Hematoxylin & Eosin ; Mag: × 100

(a) and (b) – represent livers; (c) and (d) represent kidneys; CLVA – Cod liver oil-enriched *V.amygdalina* leaf-based diet; b.wt. – body weight

Twenty-eight-day repeated dose oral toxicity study

Body weight of Wistar rats administered CLVA for 28 days

Table 3 shows the results of body weight analysis of female rats administered with CLVA and control. A significant decrease ($p<0.05$) was observed in the body weights of CLVA-administered groups in a dose dependent manner throughout the four weeks of the experiment. This decrease was sustained in the reversal groups 2 weeks after stopping the administration of CLVA compared to the control.

Similarly, a significant decrease ($p<0.05$) in the body weight of the male rats was recorded in this study (Table 4). However, the significant decrease was evident from week 2 to 4 and sustained in the 2 weeks reversal period.

Table 3: Body weight of female Wistar rats administered cod liver oil-enriched *V.amygdalina* leaf-based diet for 28 days

	Body weight (g)					
	Main groups (mg/kg/day)				Reversal groups (mg/kg/day)	
Weeks	G1	G2	G3	G4	G1R	G4R
0	152.08 ± 2.24^{a}	150.60 ± 0.37^{a}	150.67 ± 0.37a	150.79 ± 0.10a	151.95 ± 0.32^{A}	151.63 ± 0.41^{A}
1	158.05 ± 1.47^{a}	154.61 ± 4.30^{a}	153.45 ± 1.04^{b}	152.04 ± 1.71^{b}	164.56 ± 0.74^{A}	153.01 ± 0.40^{B}
2	163.16 ± 1.71^{a}	159.27 ± 1.19^{b}	153.65 ± 0.78^{c}	152.40 ± 1.48^{c}	168.81 ± 1.83^{A}	154.44 ± 2.99^{B}
3	178.32 ± 1.77^{a}	163.42 ± 1.24^{b}	154.09 ± 0.59^{c}	153.59 ± 1.54^{c}	172.12 ± 1.34^{A}	155.98 ± 1.86^{B}
4	184.05 ± 0.42^{a}	165.04 ± 1.16^{b}	155.06 ± 0.67^{c}	154.62 ± 1.38^{c}	178.73 ± 0.88^{A}	158.68 ± 0.89^{B}
5	-	-	-	-	184.17 ± 0.61^{A}	162.12 ± 0.27^{B}
6	-	-	-	-	174.74 ± 0.44^{A}	166.07 ± 0.68^{B}

Values are expressed as mean of 5 determinations ± S.E.M. Values for both Main and Reversal groups with different superscripts across a row are statistically different ($p < 0.05$)

Table 4: Body weight of male Wistar rats administered cod liver oil-enriched *V.amygdalina* leaf-based diet for 28 days

	Body weight (g)					
	Main groups (mg/kg/day)				Reversal groups (mg/kg/day)	
Weeks	G1	G2	G3	G4	G1R	G4R
0	148.58 ± 6.93^{a}	143.38 ± 6.93^{a}	146.03 ± 9.33^{a}	155.57 ± 6.75^{a}	149.08 ± 5.24^{A}	150.57 ± 3.77^{A}
1	157.58 ± 8.60^{a}	151.70 ± 6.10^{a}	158.75 ± 9.82^{a}	153.43 ± 6.87^{a}	152.90 ± 4.08^{A}	150.48 ± 5.21^{A}
2	166.73 ± 6.45^{a}	155.73 ± 6.71^{ab}	150.88 ± 4.36^{b}	150.90 ± 4.21^{b}	164.52 ± 6.15^{A}	148.45 ± 2.71^{B}
3	174.17 ± 6.06^{a}	168.05 ± 3.51^{a}	154.33 ± 3.67^{b}	152.33 ± 4.76^{b}	168.50 ± 5.70^{A}	145.47 ± 2.81^{B}
4	182.45 ± 6.72^{a}	172.04 ± 6.40^{a}	158.24 ± 2.66^{b}	152.05 ± 2.25^{b}	172.02 ± 6.82^{A}	147.34 ± 3.23^{B}
5	-	-	-	-	180.67 ± 5.23^{A}	152.02 ± 2.22^{B}
6	-	-	-	-	195.02 ± 2.30^{A}	161.05 ± 4.46^{B}

Values are expressed as mean of 5 determinations ± S.E.M. Values for both Main and Reversal groups with different superscripts across a row are statistically different ($p < 0.05$)

Table 5: Feed intake of female Wistar rats administered cod liver oil-enriched *V.amygdalina* leaf-based diet for 28 days

	Feed intake (g)					
	Main groups (mg/kg/day)				Reversal groups (mg/kg/day)	
Weeks	G1	G2	G3	G4	G1R	G4R
1	140.67 ± 1.32^{a}	139.00 ± 3.00^{a}	142.80 ± 0.00^{a}	142.77 ± 0.20^{a}	140.87 ± 3.12^{A}	141.17 ± 0.82^{A}
2	142.00 ± 0.20^{a}	140.00 ± 2.00^{a}	141.00 ± 0.01^{a}	142.87 ± 2.12^{a}	141.00 ± 1.00^{A}	140.00 ± 0.79^{A}
3	140.23 ± 2.02^{a}	141.85 ± 2.90^{a}	140.27 ± 1.29^{a}	140.00 ± 0.61^{a}	140.47 ± 1.60^{A}	141.80 ± 1.27^{A}
4	142.52 ± 2.08^{a}	142.32 ± 2.12^{a}	140.95 ± 1.18^{a}	141.62 ± 1.90^{a}	142.05 ± 1.77^{A}	142.70 ± 0.79^{A}
5	-	-	-	-	142.30 ± 1.70^{A}	140.32 ± 3.29^{A}
6	-	-	-	-	140.87 ± 1.12^{A}	141.55 ± 1.00^{A}

Values are expressed as mean of 5 determinations ± S.E.M. Values for both Main and Reversal groups with the same superscripts across a row are not statistically different ($p > 0.05$)

Table 6: Feed intake of male Wistar rats administered cod liver oil-enriched *V.amygdalina* leaf-based diet for 28 days

	Feed intake (g)					
	Main groups (mg/kg/day)				Reversal groups (mg/kg/day)	
Weeks	G1	G2	G3	G4	G1R	G4R
1	147.17 ±2.82^{a}	143.47 ± 4.22^{a}	145.37 ± 1.62^{a}	146.67 ± 3.32^{a}	147.72 ± 2.27^{A}	147.00 ± 0.00^{A}
2	147.12 ±2.87^{a}	146.82 ± 2.26^{a}	148.40 ± 1.02^{a}	147.36 ± 1.59^{a}	148.05 ± 0.95^{A}	148.45 ± 1.19^{A}
3	143.05 ±4.06^{a}	141.70 ± 1.63^{a}	142.12 ± 2.87^{a}	141.22 ± 1.72^{a}	146.03 ± 1.01^{A}	146.06 ± 1.13^{A}
4	146.52 ±2.08^{a}	146.32 ± 2.12^{a}	146.70 ± 1.12^{a}	146.96 ± 1.55^{a}	144.07 ± 2.47^{A}	144.65 ± 2.10^{A}
5	-	-	-	-	148.30 ± 1.70^{A}	147.32 ± 2.29^{A}
6	-	-	-	-	148.87 ± 1.12	147.77 ± 2.17

Values are expressed as mean of 5 determinations ± S.E.M. Values for both Main and Reversal groups with the same superscripts across a row are not statistically different ($p > 0.05$)

Table 7: Water intake of female Wistar rats administered cod liver oil-enriched *V.amygdalina* leaf-based diet for 28 days

	Water intake (ml)					
	Main groups (mg/kg/day)				Reversal groups (mg/kg/day)	
Weeks	G1	G2	G3	G4	G1R	G4R
1	210.50 ± 1.44^{a}	210.33 ± 2.89^{a}	210.83 ± 4.00^{a}	215.83 ± 2.50^{a}	218.75 ± 2.39^{A}	216.00 ± 2.86^{A}
2	212.00 ± 3.85^{a}	215.33 ± 2.25^{a}	215.83 ± 2.21^{a}	217.08 ± 3.15^{a}	217.83 ± 3.65^{A}	219.17 ± 3.23^{A}
3	215.00 ± 2.04^{a}	217.10 ± 3.16^{a}	213.00 ± 3.54^{a}	214.00 ± 3.54^{a}	216.00 ± 2.16^{A}	215.00 ± 2.04^{A}
4	214.38 ± 2.14^{a}	212.19 ± 1.07^{a}	212.49 ± 1.07^{a}	214.38 ± 2.14^{a}	215.56 ± 1.05^{A}	219.56 ± 3.14^{A}
5	-	-	-	-	216.67 ± 2.36^{A}	217.00 ± 2.04^{A}
6	-	-	-	-	213.75 ± 2.39^{A}	214.00 ± 0.01^{A}

Values are expressed as mean of 5 determinations ± S.E.M. Values for both Main and Reversal groups with the same superscripts across a row are not statistically different ($p > 0.05$)

Table 8: Water intake of male Wistar rats administered cod liver oil-enriched *V.amygdalina* leaf-based diet for 28 days

	Water intake (ml)					
	Main groups (mg/kg/day)				Reversal groups (mg/kg/day)	
Weeks	G1	G2	G1	G2	G1	G2
1	182.50 ± 4.33^{a}	183.75 ± 1.25^{a}	183.75 ± 3.15^{a}	182.7 ± 3.15^{a}	180.00 ± 2.04^{A}	182.50 ± 1.44^{A}
2	184.08 ± 1.75^{a}	181.00 ± 1.35^{a}	184.17 ± 2.10^{a}	182.00±3.54^{a}	185.75 ± 4.27^{A}	184.17 ± 3.63^{A}
3	185.00 ± 1.77^{a}	182.50 ± 3.02^{a}	185.00 ± 2.04^{a}	186.67±2.36^{a}	185.00 ± 2.04^{A}	185.00 ± 1.77^{A}
4	183.42 ± 4.40^{a}	180.92 ± 3.15^{a}	182.50 ± 2.02^{a}	181.2 ± 2.72^{a}	183.21 ± 3.39^{A}	184.33 ± 3.12^{A}
5	-	-	-	-	186.67 ± 1.18^{A}	185.83 ± 3.23^{A}
6	-	-	-	-	186.00 ± 2.04^{A}	186.17 ± 2.21^{A}

Values are expressed as mean of 5 determinations ± S.E.M. Values for both Main and Reversal groups with the same superscripts across a row are not statistically different ($p > 0.05$)

Feed and water intake of Wistar rats administered CLVA for 28 days

Tables 5 to 8 showed the results of feed and water intake analysis of both male and female rats administered with CLVA. Compared to the control groups, there was no significant difference ($p>0.05$) in the feed and water intake of the experimental rats for both male and female group, as well as those in the reversal group.

Table 9: Haematological parameters of female Wistar rats administered cod liver oil-enriched *V.amygdalina* leaf-based diet for 28 days

	Main groups (mg/kg/day)				Reversal groups (mg/kg/day)	
Parameters	G1	G2	G3	G4	G1R	G4R
WBC × 10^3/µL	11.37 ± 0.46^a	11.13 ± 0.62^a	11.73 ± 2.36^a	11.67 ± 1.37^a	11.85 ± 1.50^A	11.50 ± 0.40^A
RBC× 10^6/µL	6.76 ± 0.08^a	6.78± 0.20^a	6.84 ± 0.26^a	6.91 ± 0.38^a	6.52 ± 0.17^A	6.59 ± 0.00^A
HGB/dL	10.03± 0.18^a	11.33± 0.17^a	11.53± 0.46^a	11.37 ± 0.50^a	11.50 ± 0.22^A	11.60 ± 0.18^A
PVC %	35.00 ± 0.60^a	35.53± 0.96^a	33.00 ± 1.37^a	35.07 ± 2.02^a	37.50 ± 0.67^A	37.15 ± 0.02^A
MCV fL	51.77 ± 0.33^a	50.80 ±0.07^a	50.70 ± 0.52^a	51.60 ± 0.54^a	57.60 ± 0.45^A	56.30 ± 0.04^A
MCH pg	14.83± 0.15^a	14.93± 0.16^a	14.33± 0.28^a	14.63± 0.13^a	14.60 ± 0.04^A	14.25 ± 0.11^A
MCHC/dL	28.67 ± 0.16^a	29.47 ± 0.31^a	28.87 ± 0.26^a	28.43± 0.51^a	25.30 ± 0.13^A	25.90 ± 0.24^A
PLT × 10^3/µL	884.33±6.40^a	881.67±8.80^a	888.00±2.93^a	886.33±4.97^a	889.50±6.95^A	881.00± 4.02^A
LYM %	65.73± 3.57^a	70.43± 5.45^a	70.57 ± 4.02^a	69.57 ± 7.53^a	73.80 ± 0.58^A	72.90 ± 0.13^A
LYMNO× 10^3/µL	8.20 ± 0.67^a	7.67 ± 0.21^a	8.00 ± 1.33^a	8.17 ± 1.21^a	8.75 ± 1.19^A	8.10 ± 0.04^A
RDWSD fL	29.70 ± 0.57^a	28.50 ± 0.20^a	29.50 ± 0.26^a	29.07 ± 0.63^a	34.25 ± 0.65^A	34.25 ± 0.07^A
RDWCV %	16.10 ± 0.63^a	15.67 ± 0.42^a	16.87 ± 0.66^a	17.43± 0.52^a	17.00 ± 0.58^A	17.70 ± 0.04^A
PDW fL	8.83± 0.17^a	8.83± 1.88^a	9.63± 0.48^a	9.13± 0.48^a	10.50 ± 0.27^A	10.50 ± 0.04^A
MPV fL	7.00 ± 0.06^a	7.53± 1.44^a	7.50 ± 0.10^a	7.03± 0.22^a	7.65 ± 0.02^A	7.85 ± 0.03^A
PLCR %	8.17 ± 0.38^a	8.37 ± 1.52^a	8.43± 0.46^a	8.30 ± 1.10^a	12.95 ± 0.02^A	12.05 ± 0.02^A

Values are expressed as mean of 5 determinations ± S.E.M. Values for both Main and Reversal groups with the same superscripts across a row are not statistically different ($p > 0.05$). WBC: White blood cell, RBC: Red blood cell, HBG: haemoglobin, PCV: Packed Cell Volume, MCV: Mean Corpuscular Volume, MCH: Mean Cell Haemoglobin, MCHC: Mean Corpuscular Haemoglobin Concentration, PLT: Platelet, LYM: Lymphocytes, LYMNO: Lymphocyte Number, RDWSD: Red Cell Distribution Width, PDW: Platelet distribution width, MPV: Mean Platelet Volume, PLCR: Platelet-large cell ratio.

Table 10: Haematological parameters of male Wistar rats administered cod liver oil-enriched *V.amygdalina* leaf-based diet for 28 days

Parameters	Main groups (mg/kg/day)				Reversal groups (mg/kg/day)	
	G1	G2	G3	G4	G1R	G4R
WBC × 10^3/µL	14.27 ± 0.95^a	14.1 ± 1.20^a	13.83 ± 0.54^a	14.63 ± 2.91^a	15.60 ± 1.48^A	15.53 ± 0.16^A
RBC× 10^6/µL	8.22 ± 0.23^a	7.23± 0.26^a	7.28 ± 0.22^a	7.13 ± 0.21^a	7.21 ± 0.49A	7.37 ± 0.06^A
HGB/dL	11.37 ± 0.39^a	10.73± 0.58^a	10.57 ±0.33^a	10.33± 0.33^a	10.10 ± 0.58^A	10.63± 0.12^A
PVC %	40.40 ± 1.23^a	42.43± 1.68^a	40.13± 1.52^a	40.67 ± 0.92^a	40.53± 1.34^A	40.17 ± 0.50^A
MCV fL	49.17 ± 0.24^a	48.53± 0.82^a	48.93± 0.56^a	49.03± 0.57^a	57.07 ± 2.55^A	56.03± 0.31^A
MCH pg	13.83± 0.14^a	13.43± 0.43^a	14.53± 0.06^a	14.47 ± 0.23^a	14.07 ± 0.17^A	14.00 ± 0.07^A
MCHC/dL	28.10 ± 0.16^a	28.20 ± 0.44^a	28.50 ± 0.32^a	28.97 ± 0.19^a	24.83± 0.74^A	24.93± 0.33^A
PLT × 10^3/µL	887.00±9.88^a	885.33±9.24^a	878.33±6.97^a	883.67±4.17^a	870.00±3.74^A	867.00± 4.12^A
LYM %	72.03± 1.32^a	72.47 ± 3.95^a	72.73± 4.02^a	70.23± 0.77^a	80.80 ± 4.36^A	84.47 ± 0.65^A
LYMNO× 10^3/µL	10.23± 0.55^a	10.47 ± 1.54^a	10.90 ± 0.22^a	11.87 ± 2.28^a	12.30 ± 1.04^A	12.60 ± 0.13^A
RDWSD fL	28.77 ± 0.12^a	29.57 ± 6.19^a	29.90 ± 0.45^a	29.50 ± 0.70^a	40.67 ± 6.34^A	45.47 ± 0.18^A
RDWCV %	17.70 ± 0.22^a	17.27 ± 1.45^a	16.90 ± 0.30^a	17.03± 0.44^a	19.97 ± 2.42^A	20.87 ± 0.37^A
PDW fL	8.17 ± 0.17^a	8.07 ± 1.95^a	8.43± 0.19^a	8.70 ± 0.04^a	10.23± 0.13^A	9.97 ± 0.09^A
MPV fL	6.50 ± 0.09^a	6.67 ± 1.48^a	6.70 ± 0.10^a	6.93± 0.08^a	7.60 ± 0.04^A	7.60 ± 0.07^A
PLCR %	6.97 ± 0.36^a	5.03± 1.77a	6.63± 0.87^a	6.90 ± 0.63^a	11.40 ± 0.41^A	10.77 ± 0.09^A

Values are expressed as mean of 5 determinations ± S.E.M. Values for both Main and Reversal groups with the same superscripts across a row are not statistically different ($p > 0.05$). WBC: White blood cell, RBC: Red blood cell, HBG: haemoglobin, PCV: Packed Cell Volume, MCV: Mean Corpuscular Volume, MCH: Mean Cell Haemoglobin, MCHC: Mean Corpuscular Haemoglobin Concentration, PLT: Platelet, LYM: Lymphocytes, LYMNO: Lymphocyte Number, RDWSD: Red Cell Distribution Width, PDW: Platelet distribution width, MPV: Mean Platelet Volume, PLCR: Platelet-large cell ratio.

Haematological parameters of Wistar rats administered with CLVA for 28 days

Tables 9 and 10 showed the haematological parameter of both male and female rats administered with CLVA. There was no significant difference ($p > 0.05$) in the haematological parameters of CLVA-administered groups compared to control for both the male and female groups at the end of the experiment. Similar results were also recorded for the reversal group.

Biochemical parameters of Wistar rats administered CLVA for 28 days

Tables 11 and 12 showed the results of selected biochemical parameters of male and female Wistar rats administered orally with CLVA for 28 days. There was no significant alteration ($p>0.05$) in the investigated parameters of the CLVA-administered groups compared to control in the female rats. However, there was a significant increase ($p<0.05$) in the concentration of creatinine for the groups administered with 1000 mg/kg b.wt. of CLVA (G4) compared to control (G1) in the male groups. Similarly, calcium and chloride concentrations were significantly increased ($p<0.05$) in all the CLVA-administered groups (G2, G3 and G4) compared to G1 in the male group.

Table 11: Selected biochemical parameters in female Wistar rats administered cod liver oil-enriched *V.amygdalina* leaf-based diet for 28 days

Parameters	Main groups (mg/kg/day)				Reversal groups (mg/kg/day)	
	G1	G2	G3	G4	G1R	G4R
Glu (mg/dl)	44.88 ± 3.66^{a}	47.40 ± 2.19^{a}	49.42 ± 1.06^{a}	41.37 ± 3.36^{a}	52.75 ± 3.51^{A}	54.71 ± 0.15^{A}
BUN (mg/dl)	22.07 ± 0.30^{a}	22.70 ± 0.95^{a}	22.97 ± 0.20^{a}	21.07 ± 1.68^{a}	20.91 ± 0.88^{A}	21.21 ± 0.05^{A}
Urea (mg/dl)	47.26 ± 0.64^{a}	48.75 ± 2.03^{a}	50.33 ± 3.42^{a}	46.10 ± 3.61^{a}	44.78 ± 1.89^{A}	44.36 ± 0.18^{A}
Cr (µmol/l)	112.75 ± 3.00^{a}	98.00 ± 8.07^{a}	91.42 ± 7.04^{a}	91.08 ± 6.85^{a}	92.75 ± 8.50^{A}	97.46 ± 5.45^{A}
T. Chol (mg/dl)	89.69 ± 5.46^{a}	90.24 ± 2.78^{a}	91.98 ± 1.24^{a}	87.30 ± 2.50^{a}	90.53 ± 1.53^{A}	91.63 ± 1.73^{A}
Trig (mg/dl)	67.60 ± 3.10a	68.30 ± 3.04^{a}	65.12 ± 0.76^{a}	66.47 ± 0.76^{a}	68.71 ± 1.48^{A}	68.13 ± 0.48^{A}
HDL (mg/dl)	6.96 ± 0.48^{a}	6.62 ± 0.33^{a}	6.74 ± 0.44^{a}	6.51 ± 1.02^{a}	8.53 ± 0.77^{A}	9.03 ± 0.20^{A}
LDL (mg/dl)	68.86 ± 4.55^{a}	67.53 ± 2.25^{a}	70.70 ± 1.19^{a}	67.03 ± 1.44^{a}	68.02 ± 0.69^{A}	68.05 ± 0.64^{A}
T. Bil (µmol/l)	56.17 ± 1.03^{a}	59.99 ± 2.39^{a}	56.51 ±1.77^{a}	57.21 ± 1.60^{a}	41.81 ± 1.66^{A}	40.96 ± 1.39^{A}
AST (U/L)	40.77 ± 2.62^{a}	45.09 ± 3.98^{a}	42.00 ± 0.90^{a}	38.44 ± 2.64^{a}	34.28 ± 0.24^{A}	34.47 ± 0.42^{A}
ALT (U/L)	92.62 ± 2.15^{a}	96.47 ± 3.10^{a}	90.46 ± 1.46^{a}	96.15 ± 2.97^{a}	90.39 ± 0.95^{A}	90.28 ± 0.03^{A}
ALP (U/L)	46.45 ± 1.21^{a}	47.63 ± 2.35^{a}	46.92 ± 2.12^{a}	45.56 ± 1.05^{a}	42.82 ± 0.43^{A}	42.54 ± 0.41^{A}
TP (g/dl)	5.12 ± 0.42^{a}	5.20 ± 0.40^{a}	4.94 ± 0.04^{a}	4.70 ± 0.11^{a}	4.56 ± 0.02^{A}	4.54 ± 0.04^{A}
Alb (g/dl)	1.56 ± 0.11^{a}	1.42 ± 0.14^{a}	1.53 ± 0.15^{a}	1.46 ± 0.06^{a}	1.54 ± 0.18^{A}	1.58 ± 0.05^{A}
Ca (mg/dl)	9.28 ± 0.04^{a}	9.24 ± 0.49^{a}	9.25 ± 0.03^{a}	9.28 ± 0.09^{a}	9.65 ± 0.08^{A}	9.67 ± 0.05^{A}
Phos (mmol/l)	2.51 ± 0.07^{a}	2.44 ± 0.10^{a}	2.50 ± 0.09^{a}	2.48 ± 0.05^{a}	1.65 ± 0.03^{A}	1.55 ± 0.07^{A}
Na (mmol/l)	163.35 ±2.23^{a}	165.32 ± 2.02^{a}	165.95 ±2.18^{a}	162.63 ± 1.72^{a}	154.3 ± 1.20^{A}	154.53 ± 0.13^{A}
K (mmol/l)	7.19 ± 0.33^{a}	7.18 ± 0.53^{a}	7.39 ± 0.34^{a}	7.50 ± 0.27^{a}	6.63 ± 0.42^{A}	6.62 ± 0.32^{A}
Cl (mmol/l)	8.28 ± 0.04a	8.31 ± 0.49^{a}	8.30 ± 0.03^{a}	8.32 ± 0.09^{a}	9.65 ± 0.08^{A}	9.67 ± 0.05^{A}

Values are expressed as mean of 5 determinations ± S.E.M. Values for both Main and Reversal groups with the same superscripts across a row are not statistically different ($p > 0.05$). Glu: Glucose, BUN: Blood urea nitrogen, Cr: Creatinine, T. Chol: Total Cholesterol, Trig: Triglyceride, HDL: High Density Lipoprotein, LDL: Low density lipoprotein, T. Bil: Total bilirubin, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, ALP: Alkaline phosphatase, TP: Total protein, Alb: Albumin, Ca: Calcium, Phos: Phosphate, Na: Sodium, K: Potassium, Cl: Chloride.

Table 12: Selected biochemical parameters of male Wistar rats administered cod liver oil-enriched *V.amygdalina* leaf-based diet for 28 days

Parameters	Main groups (mg/kg/day)				Reversal groups (mg/kg/day)	
	G1	G2	G3	G4	G1R	G4R
Glu (mg/dl)	41.88 ±3.59^{a}	45.77 ± 1.46^{a}	44.57 ± 3.56^{a}	47.30 ± 2.11^{a}	50.49 ± 2.08^{A}	52.61 ± 0.21^{A}
BUN (mg/dl)	20.40 ± 0.24^{a}	21.69 ± 0.56^{a}	20.95 ±1.36^{a}	20.87 ± 0.87^{a}	22.85 ± 1.71^{A}	21.82 ± 0.01^{A}
Urea (mg/dl)	41.54 ± 0.51^{a}	42.87 ± 2.99^{a}	43.95 ± 1.86^{a}	44.69 ± 1.86^{a}	42.26 ± 0.81^{A}	40.29 ± 0.01^{A}
Cr (µmol/l)	88.50 ± 0.10^{a}	88.40 ± 0.10^{a}	88.5 ± 0.01^{a}	88.20 ±1.39^{a}	88.58 ± 0.05^{A}	88.55 ± 0.08^{A}
T. Chol (mg/dl)	90.10 ± 3.58^{a}	93.61 ± 2.09^{a}	100.13 ±8.24^{a}	98.17 ± 3.08^{a}	98.83 ± 1.43^{A}	100.41 ± 0.80^{A}
Trig (mg/dl)	70.92 ± 2.57^{a}	67.05 ± 0.80^{a}	67.03 ± 3.33^{a}	65.67 ± 0.76^{a}	59.05 ± 1.06^{A}	61.42 ± 0.46^{A}
HDL (mg/dl)	7.54 ± 0.79^{a}	7.20 ± 0.51^{a}	7.87 ± 0.66^{a}	7.04 ± 0.51^{a}	7.85 ± 0.56^{A}	8.34 ± 0.44^{A}
LDL (mg/dl)	69.09 ± 2.41^{a}	71.63 ± 1.57^{a}	76.32 ± 7.15^{a}	73.86 ± 2.97^{a}	78.15 ± 0.87^{A}	79.43 ± 0.77^{A}
T. Bil (µmol/l)	46.17 ± 1.03^{a}	47.99 ± 0.39^{a}	46.51 ±1.77^{a}	46.81 ± 1.60^{a}	41.81 ± 1.66^{A}	39.96 ± 1.39^{A}
AST (U/L)	45.16 ± 0.38^{a}	41.90 ± 1.56^{a}	46.23 ± 6.02^{a}	47.50 ± 1.44^{a}	35.59 ± 3.17^{A}	32.95 ± 0.24^{A}
ALT (U/L)	114.74 ± 1.55^{a}	111.40 ± 1.61^{a}	113.12 ±6.65^{a}	111.61 ± 3.71^{a}	95.04 ± 5.92^{A}	98.84 ± 4.04^{A}
ALP (U/L)	40.97 ± 0.80^{a}	42.50 ± 0.66^{a}	43.75 ± 0.83^{a}	40.34 ± 0.47^{a}	44.09 ± 1.64^{A}	44.77 ± 0.56^{A}
TP (g/dl)	5.67 ± 0.09^{a}	5.71 ± 0.06^{a}	6.06 ± 0.60^{a}	5.53 ± 0.01^{a}	4.12 ± 0.02^{A}	4.65 ± 0.17^{A}
Alb (g/dl)	1.48 ± 0.12^{a}	1.60 ± 0.12^{a}	1.67 ± 0.21^{a}	1.52 ± 0.08^{a}	1.64 ± 0.10^{A}	1.67 ± 0.01^{A}
Ca (mg/dl)	9.34 ± 0.19^{a}	9.89 ± 0.03^{b}	10.03 ±0.19^{b}	9.84 ± 0.06^{b}	9.68 ± 0.07^{A}	9.39 ± 0.01^{A}
Phos (mmol/l)	2.02 ± 0.24^{a}	2.30 ± 0.10^{a}	2.34 ± 0.10^{a}	1.92 ± 0.19^{a}	1.69 ± 0.09^{A}	1.97 ± 0.04^{A}
Na (mmol/l)	156.51 ± 3.21^{a}	156.72 ± 1.20^{a}	155.31 ±2.71^{a}	157.14 ± 1.21^{a}	152.00±1.37^{A}	147.23 ± 0.62^{A}
K (mmol/l)	6.79 ± 0.58^{a}	7.32 ±0.25^{a}	7.47 ± 0.51^{a}	7.56 ± 0.07^{a}	5.40 ± 0.14^{A}	5.06 ± 0.01^{A}
Cl (mmol/l)	71.65 ± 3.68^{a}	80.60 ± 0.37^{b}	79.01 ±0.34^{c}	80.74 ± 0.62^{b}	76.92 ± 0.70^{A}	76.43 ± 0.01^{A}

Values are expressed as mean of 5 determinations ± S.E.M. Values for both Main and Reversal groups with different superscripts across a row are statistically different ($p < 0.05$). Glu: Glucose, BUN: Blood urea nitrogen, Cr: Creatinine, T. Chol: Total Cholesterol, Trig: Triglyceride, HDL: High Density Lipoprotein, LDL: Low density lipoprotein, T. Bil: Total bilirubin, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, ALP: Alkaline phosphatase, TP: Total protein, Alb: Albumin, Ca: Calcium, Phos: Phosphate, Na: Sodium, K: Potassium, Cl: Chloride.

Histopathology of kidney and liver of male and female Wistar rats administered orally with CLVA for 28 days

Figure 2 showed the photomicrograph of kidney and liver of control and the CLVA-administered groups for female rats. Sections showed renal tissue with preserved architecture composed of normal glomeruli, tubules and unremarkable interstitium (a, b, c, d and e). Similarly, the hepatic tissue section showed mostly well-preserved architecture composed of normal hepatocytes, normal portal tracts and central veins for the female rats (f, g, h, i, j).

Figure 3 showed the photomicrograph of the kidney and liver of control and the CLVA-administered groups of the male rats. Section showed renal tissue with preserved architecture composed of normal glomeruli, tubules and unremarkable interstitium (A, B, C, D, E). Hepatic tissue section showed well-preserved architecture, composed of normal hepatocytes.

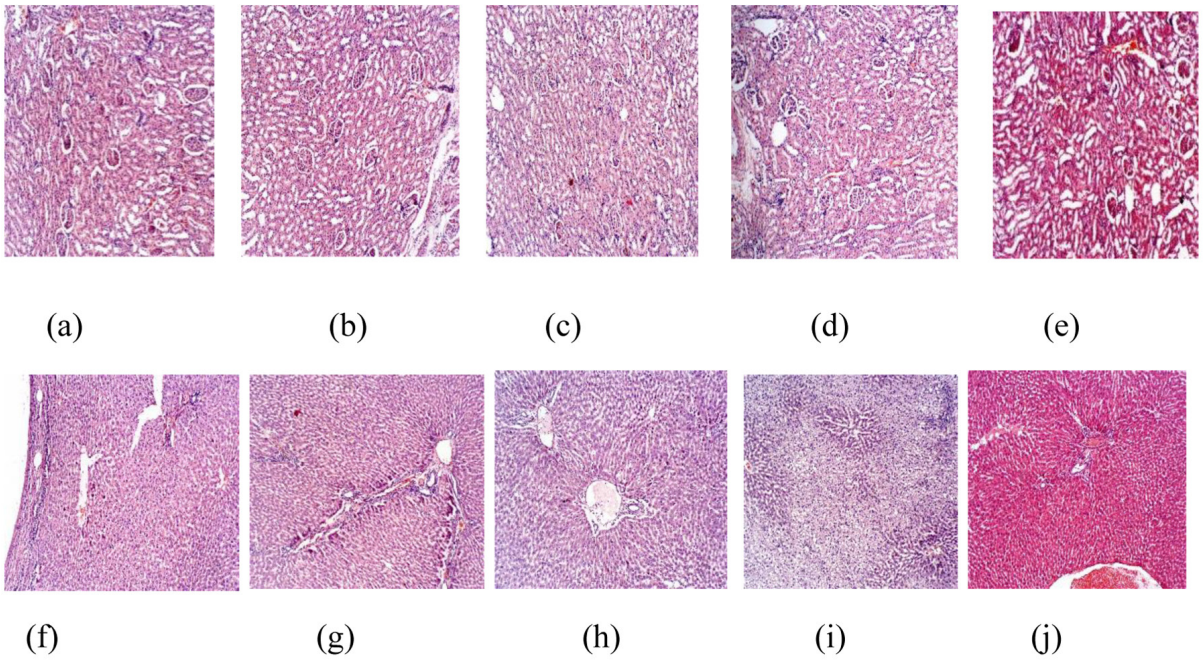

Figure 2: Photomicrographs of the kidney and liver of control and CLVA-fed female rats in the 28-day toxicity study. Stain: Hematoxylin & Eosin-stained : ×100

(a): kidney of control rat (feed without CLVA); (b): kidney of rat (250 mg/kg b.wt. CLVA); (c): kidney of rat (500 mg/kg b.wt. CLVA); (d): kidney of rat (1000 mg/kg b.wt. CLVA); (e): kidney of rat (1000 mg/kg b.wt. CLVA) in reversal group; (f): liver of control rats (feed without CLVA); (g): liver of rat (250 mg/kg b.wt. CLVA); (h): liver of rat (500 mg/kg b.wt. CLVA); (i): liver of rat (1000 mg/kg b.wt. CLVA); (j): liver of rat (1000 mg/kg b.wt. CLVA) in reversal group

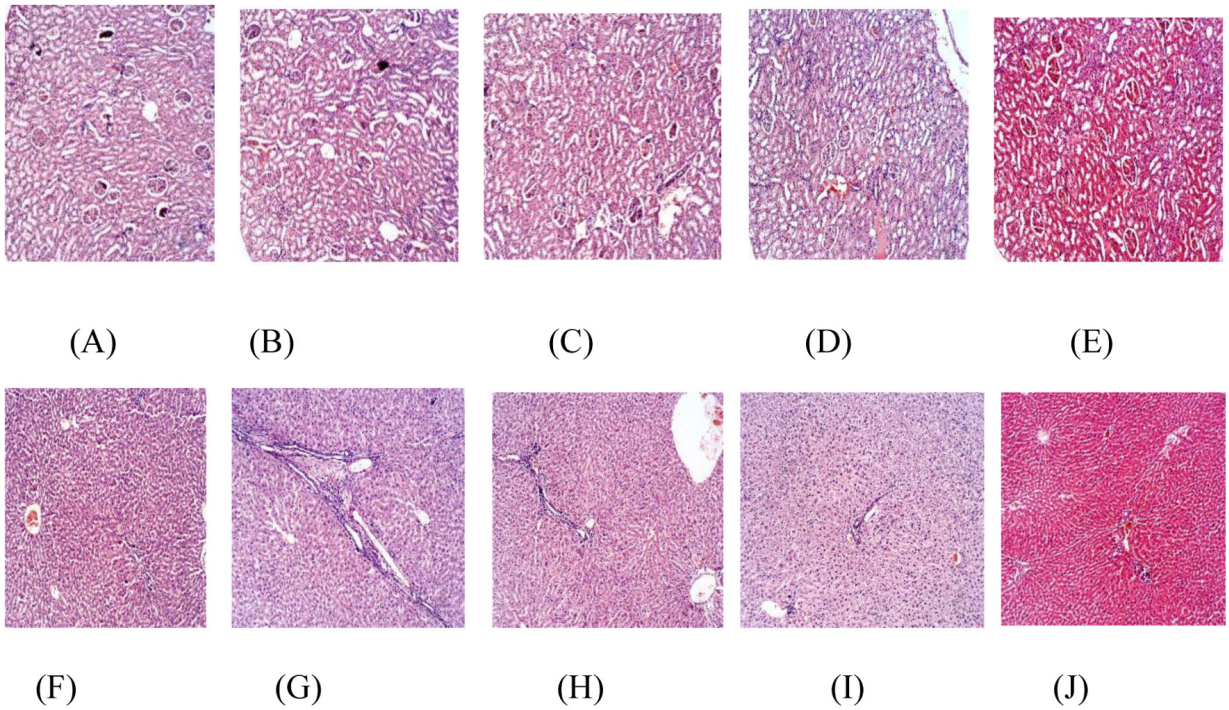

Figure 3: Photomicrographs of the kidney and liver of control and CLVA-fed male rats in the 28-days toxicity study. Stain: Hematoxylin & Eosin ; Mag: ×100

(A): kidney of control rat (feed without CLVA); (B): kidney of rat (250 mg/kg b.wt. CLVA); (C): kidney of rat (500 mg/kg b.wt. CLVA); (D): kidney of rat (1000 mg/kg b.wt. CLVA); (E): kidney of rat (1000 mg/kg b.wt. CLVA) in reversal group; (F): liver of control rats (feed without CLVA); (G): liver of rat (250 mg/kg b.wt. CLVA); (H): liver of rat (500 mg/kg b.wt. CLVA); (I): liver of rat (1000 mg/kg b.wt. CLVA); (J): liver of rat (1000 mg/kg b.wt. CLVA) in reversal group

DISCUSSION

In this study, the formulated feed used for testing contained both cod liver oil and *V.amygdalina* leaves. These ingredients are known to be rich sources of nutrients and have been scientifically proven to provide various health benefits when consumed in sufficient quantities. (Skeie *et al.*, 2009; Ijeh and Ejike, 2011; Mohammed *et al.*, 2015; Soji-Omoniwa *et al.*, 2022; Ugbogu *et al.*, 2021).

Despite the fact that cod liver oil and *V.amygdalina* leaves are food items, it is important to conduct toxicological evaluations of the formulated feed before certifying it as safe for consumption. This is because natural toxins can be formed in food as defense mechanisms of plants, and these toxins may pose a health risk to humans (WHO, 2018). Therefore, it is necessary to evaluate the safety of the formulated feed before recommending it for consumption by patients suffering from hyperglycemia and associated wound-healing complications.

In this study, the experimental rats were given a single dose of CLVA at a high concentration of 2000 mg/kg b.wt. The results showed that this dose did not cause acute toxicity in the rats, as demonstrated in Figure 1. Additionally, the LD_{50} was determined to be greater than 2000 mg/kg b.wt. This finding is consistent with the results reported by previous researchers such as Njan *et al.* (2008), Owen *et al.* (2011), Egharevba *et al.* (2014), Mansurah *et al.* (2013), and Rohman (2017). These researchers also reported no clinical signs of toxicity and insignificant toxicity limits after the consumption of *V.amygdalina* and cod liver oil. Reports of phytochemical analysis of *V.amygdalina* leaves revealed that it is rich in proteins, fats, fibers, amino acids, minerals, vitamins, and carbohydrates (Alara *et al.,* 2017). Alara *et al.* (2017) reported the isolation of several bioactive compounds from the extracts of V.amygdalina. On the other hand, cod liver oil is known to be rich in omega-3 fatty acids, specifically eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). It is also a potential source of vitamins A and D, either in the form of pharmaceutical or food supplements, and the levels of these constituents in both VA and CLO are within the dietary reference intake and tolerable upper limits standards set by IOM (1998) and OASH (2021). This could be a possible explanation for the lack of toxicity observed in the acute phase of the study. Similarly, methods used to process VA and CLO such as washing, pulverizing and oven drying might have reduced the presence of natural or other sources of toxins significantly. Processing methods of food plants have been reported to reduce or remove risk of toxicity significantly in previous studies (Ugwu and Oranye, 2006; Montagnac *et al.*, 2008).

In the sub-chronic toxicity study, the impact of various doses (250, 500, and 1000 mg/kg body weight) of CLVA was examined over a period of 28 days, as well as during a 14-day post-treatment period. Results suggested CLVA has the potential to reduce body weight of both male and female rats (Tables 2 and 3) in a dose dependent manner upon prolonged administration. This observation was sustained during the reversal period in this experiment, suggesting that constituents of VA and CLO might possess the ability to induce lipolysis or modulate appetite in the experimental rats. However, based on the result of the feed intake analysis which showed no significant difference compared to the control group suggests that the observed weight reduction in the experimental rats might be as a result of active metabolism and utilization of stored fat. Typically, fat is stored in fat cells as triacylglycerol. Lipolysis involves the breakdown of triacylglycerol molecules into glycerol and three fatty acids, which is facilitated by hormone-sensitive lipase (HSL). Subsequently, the fatty acids are discharged into the bloodstream and conveyed to the areas where energy is required (Manore *et al.*, 2011). When the muscles are active, they require more FFAs which are delivered to them through increased blood flow. As the fat cells release these FFAs, they reduce in size, resulting in a more slender appearance when the body loses fat (Turcotte, 2000). Previously, studies with extracts from the VA plant extracts (Ekpo *et al.*, 2007; Ezekwe and Obidia, 2001) and leaves incorporated into diet (Ugwu *et al.*, 2009, 2011; Adaramoye *et al.*, 2008) reported the lipid modulating effect in normal adult rats. Terpenes from *V.amygdalina* was reported to exert hypolipidaemic and anti-obesity effects by inhibiting pancreatic lipase (Bustanji *et al.*, 2011). Coumarin, another secondary metabolite was reported to play a role in obesity management by inhibiting early stage adipogenic differentiation (Shin *et al.,* 2010), lignans caused significant loss in weight (Park and Velasquez, 2012) and xanthones was also reported to elicit anti-obesity effect (Liu *et al.*, 2015). It has been reported that taking CLO, which is a good source of DHA, is associated with lower activity of stearoyl-CoA desaturase (SCD) in mice. This enzyme is crucial in the onset of obesity. Therefore, it was suggested that DHA in CLO might hinder SCD activity and thus, prevent the occurrence of obesity and hypertriglyceridemia (Fujita *et al.*, 2015). Even though administration of CLVA resulted in significant weight loss in the experimental rats, feed and water intake of both male and female rats were not altered significantly, suggesting that the observed weight loss might be as a result of lipolysis-inducing constituents present in CLVA. This implies that the appetite and thirst regulation was not altered by consumption of CLVA at the doses investigated.

Similarly, results of the haematological parameters evaluated suggested there was no sub chronic toxicological

effect on the blood parameters investigated. Typically, a decrease in number of red cells in the blood is often associated with development of anemia (Junqueira *et al*., 2006) which could be associated to the stimulation of lipid peroxidative system by toxins. This in turn results in production of lipid peroxides which hemolyses the RBCs. However in this study, there was no alteration in the RBCs of the CLVA-fed groups. As observed for RBCs, Hemoglobin, Packed Cell Volume (PCV), Mean corpuscular volume (MCV) and leucocytes parameters were not altered in the CLVA-fed groups. Hemoglobin, the primary intracellular protein of the RBCs, binds oxygen in the pulmonary artery for transport to tissues and binds tissue carbon dioxide for transport back to lungs for exhalation (Wintrobe and Greer, 2009). It is synthesized in the bone marrow. Hemoglobin is a conjugated protein containing heme as prosthetic group and globin as the protein part apoprotein (Chhabra, 2013). Increased levels of free hemoglobin in blood (hemoglobinemia) might be suggestive of massive hemolysis of the red blood cells which was not the case in this study. Packed Cell Volume (PCV) represents the percentage of red blood cell volume of whole blood volume in animals and is clinically used to signal known or suspected anemia as well (Wintrobe and Greer, 2009). MCV on the other hand is a measure of the average volume or size of a red blood cell. MCV is elevated or decreased in accordance with average red cell size. Low MCV is consistent with iron deficiency, microcytic anemia and thalassaemia syndromes (Aslikinia et al., 2006). Mean corpuscular hemoglobin (MCH) is a calculation of the average amount of hemoglobin inside a single red blood cell (Chhabra, 2013). White blood cells count and its indices play a vital role in immune function. They are formed from pluripotent hematopoietic stem cells found in the red bone marrow (myeloid).

Results from evaluation of biochemical parameters also suggested a no-treatment associated effect on the selected biochemical parameters studied except for the significant increase in creatinine and calcium concentrations of rats administered with the highest dose of 1000 mg/kg body weight CLVA in the male rats only (Tables 10 and 11). This suggests that at high dose, consumed for prolong period, the excretory function of kidney might be impaired but this impairment is reversed once the treatment is stopped as depicted by the result for the reversal period. Creatinine, a breakdown product of creatine and phosphocreatine are involved in energy metabolism of skeletal muscle. It is freely filtered by the glomerulus and to a lesser degree (10%–30%) secreted by the proximal tubule. Any changes in its level in the blood are related to excretion and therefore reflect kidney function. Although, higher than normal creatinine concentration and blood urea nitrogen (BUN) can be indicative of dehydration, there was no alteration in the water intake level of the experimental rats to support this (Mayer and Donnelly, 2013). The kidney is an important organ that perform a critical function of ensuring balance between the internal milieu and external environment. Kidney failure would disrupt a number of homeostatic mechanisms which also control calcium metabolism (Hill Gallant and Spiegel, 2017).

As depicted by the photomicrograph of the kidneys and livers, consumption of CLVA did not cause any structural alteration to these tissues. Histopathology plays a critical role in safety assessment of nonhuman models used in drug development through identification and interpretation of microscopic tissue changes. The absence of treatment-related alterations in the kidneys and livers of the CLVA-fed groups suggests that constituents of CLVA are not injurious at the investigated doses to the tissues studied. This result supports the findings of function capacity of both the liver and the kidney which also showed no significant alterations discussed earlier. This findings support the reports of previous researchers that consumption of *V. amygdalina* and CLO individually did not elicit toxic effect (Lips, 2003; Njan *et al.* 2008; Imaga and Bamigbetan 2013; Egharevba, *et al.*, 2014; Akowuah *et al*., 2015; Alara *et al.*, 2017)

CONCLUSION

CLVA at 250, 500, 1000 and 2000 mg/kg b. wt. did not elicit acute and sub chronic toxicity on the functions of the liver, kidney and blood in both male and female rats but caused a reduction in body weight. Therefore, CLVA might be safe for consumption and can be recommended for use by patients suffering from diabetes with impaired wound healing.

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