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Acute and sub-chronic toxicity evaluation of the aqueous extract of *Codiaeum variegatum* leaves on *Wistar albino* rodents of both sexes

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ABSTRACT

Background/Aim: The decoction of *Codiaeum variegatum* leaves is used by Cameroonian local population in the treatment of amoebic dysentery. The present study was carried out to investigate the safety of the aqueous extract of *Codiaeum variegatum* leaves on *Wistar albino* mice and rats of both sexes.

Methods: For the acute toxicity study, seven groups of eight mice (four males and four females) each received orally once distilled water (10 ml Kg^{-1}) and the aqueous extract in a single dose of 2, 4, 8, 12, 16, and 24 g Kg^{-1}. These mice were observed for mortality after 48 hours and thereafter, for clinical signs daily for 14 days. In the sub-chronic toxicity evaluation, four groups of 12 rats (six males and six females) each received distilled water (10 ml Kg^{-1}) and the aqueous extract at doses of 50, 100, and 200 mg Kg^{-1} by oral gavage for 28 consecutive days. The body weight was evaluated every 2 days for each animal and after sacrifice, the relative weight of vital organs was determined. Blood serum was used for the analysis of biochemical markers of renal and hepatic toxicity.

Results: The administration of the aqueous extract in both acute and sub-chronic toxicity evaluation did not cause neither significant visible signs of toxicity nor mortality and no significant changes were observed on body weight, relative organ weight and biochemical parameters in treated groups compared to the control groups.

Conclusion: These results demonstrated that the aqueous extract of *Codiaeum variegatum* leaves is non-toxic and may be safely used for its therapeutic application.

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Introduction

Codiaeum variegatum, commonly known as "*garden croton*", belongs to the family of Euphorbiaceae and is an ornamental shrub with diverse beautiful and attractive foliage. This houseplant is native of tropical forests from Indonesia, the Philippines to New Guinea and Australia [1]. *Codiaeum variegatum* is more often used for decorations, and there are various species which are usually hybridized to produce the most decorative potted plants. More than 300 cultivars of *C. variegatum* known as mutants or hybrids are reported around the world; and these cultivars are grouped into nine species based on their leaf morphology [2]. Some cultivars of *C. variegatum* are also used for their medicinal properties in the treatment of various diseases. Freeze-dried leaves decoction of *C. variegatum* is taken as tea by Filipinos and eating crushed leaves cures diarrhea [3]. The root and bark are used against syphilis, constipation, stomach-ache, loss of appetite, and dysuria. In Cameroon, the decoction of *C. variegatum* (var. *mollucanum*) leaves is used by local population in the treatment of amoebic dysentery. In our previous study, it was reported that the

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aqueous extract of *C. variegatum* leaves (AECVL) exhibited significant anti-amoebic activity on polyxenic and axenic culture of *Entamoeba histolytica* [4,5]. Moreover, optimal conditions were defined for leaves' collection to maximize the anti-amoebic activity of the aqueous extract and a sub-fraction (SF9B) was identified with significant higher anti-amoebic activity compared to the unfractionated aqueous extract. Anti-amoebic activity of the most potent SF9B was confirmed with the morphological characteristics of induced death in trophozoites of *E. histolytica* through the destabilization of Gal/GalNAc lectin, an abundant parasite cell surface protein. Differential gene expression analysis using high-throughput RNA sequencing implies that these sub-fraction acts by targeting ceramide, a bioactive lipid involved in the disturbance of biochemical processes within the cell membrane including differentiation, proliferation, cell growth arrest, and apoptosis [4]. Therefore, the rational and safe usage of *C. variegatum* is absolutely important to gain benefit from its therapeutic value. Previously, the *in vitro* toxicity of the AECVL on non-competent or metabolic competent cell lines showed that this extract is neither genotoxic, nor mutagenic at non-toxic or moderately toxic concentrations [6]. This result suggested that the AECVL could be safely used at lower doses for medicinal purpose. To further ensure the safety of this extract, toxicity studies should be done also *in vivo* on animals. The laboratory animals considered closer to humans have been used for the *in vivo* evaluation of toxicity, which represents a key step in the safety evaluation as it may allow extrapolation of results to humans. Thus, it is recognized that a positive effect in an *in vivo* toxicity study on laboratory animals is indicative of a possible adverse effect in humans. Mice and rats are small rodents that are widely used in biomedical research since their genetic, biological and behavior characteristics closely resemble those of humans. The aim of the present study was to evaluate the acute and sub-chronic toxicity of the AECVL on rodents of both sexes.

Material and Methods

Preparation of the aqueous extract

Fresh leaves of *Codiaeum variegatum* (var. *mollucanum*) were collected in the morning in Mbankomo, a sub-division around Yaoundé, Cameroon. A voucher specimen of the plant material identified under the number 33570 HNC has been deposited at the National Herbarium of Cameroon. The leaves collected were thoroughly washed with tap water, rinsed with distilled water, dried at room temperature and grinded. From the powder obtained, 200 g from each batch was mixed with 2 l of distilled water for the preparation of the aqueous extract by decoction for 1 hour. After filtration with the Whatman No.1 filter paper, the filtrate collected was dried by lyophilization. The yield of the extract (24.20%, w/w) was calculated with respect to the initial weight of the dried plant powder.

Phytochemical screening of the aqueous extract

Phytochemical screening was carried out by the method described by Trease and Evans [7]. In brief, phytochemical composition of the AECVL was determined by analyzing the presence of some groups of compounds such as alkaloids, tannins, sterols, polyphenols, coumarins, leucocyanins, sugar, flavonoids, and terpenoids.

Experimental animals

Healthy adult *Wistar albino* mice (20–30 g; 9–11 weeks) and *Wistar albino* rats (170–210 g; 8–10 weeks) of both sexes were used respectively for the acute and the sub-chronic toxicity experiments. They were obtained from the Animal House of the Laboratory of Pharmacology and Toxicology (University of Yaoundé I, Cameroon) and were housed in polypropylene cages under normal laboratory conditions (12 hours light/dark cycle; 23 ± 2°C). Before the experiment, the animals were grouped for an acclimatization period of one week. All the animals were given food and tap water *ad libitum*, and the experiment was conducted according to international guidelines [8,9].

Acute toxicity study

Acute toxicity of the aqueous extract was evaluated on *Wistar albino* mice of both sexes, as per the World Health Organization guidelines [8]. Twenty-eight animals per sex were equally divided into seven groups of four animals each. The AECVL was dissolved in distilled water and administered orally with the aid of a blunt end needle and syringe. The control group received distilled water (10 ml Kg^{-1}) and the test groups received the AECVL at doses of 2, 4, 8, 12, 16, and 24 g Kg^{-1}. Mice were closely observed for the initial 4 hours after administration and clinical signs of toxicity such as aggressiveness, reaction to stimuli, locomotion, social interactions, aspects of feces and convulsions or coma were also observed. After this period, the animals were

supplied with food and water *ad libitum*. Dead animals were counted in each group within 48 hours following the administration of the AECVL, and the median lethal dose (LD_{50}) values were determined in both sexes. The animals were observed daily for the following 14 days and their body weight was registered every 2 days.

Sub-chronic toxicity study

Sub-chronic toxicity of the AECVL was evaluated on *Wistar albino* rats of both sexes, as per the Organization for Economic Co-operation and Development guidelines [9]. Twenty-four animals per sex were equally divided into four groups of six animals each. The control group received distilled water (10 mL Kg^{-1}), while the test groups received the AECVL at doses of 50, 100, and 200 mg Kg^{-1} body weight respectively for 28 consecutive days. These doses were chosen based on the approximation and extrapolation made on the efficient doses applied by traditional healers in the treatment of amoebic dysentery. Animals were observed for clinical signs of toxicity during the treatment period, and the body weights of animals were recorded every 2 days. At the end of the treatment, animals were fasted overnight, but allowed free access to water. These animals were anesthetized with ether and were sacrificed by cervical dislocation for the collection of blood samples in dry test tubes without anticoagulant. Vital organs such as heart, liver, lung, kidney, and spleen as well as genital organs such as prostate, seminal vesicles, testes, epididymis (for males), and ovaries, uterus (for females) were collected and the relative organ weight (weight of organ as proportion to the total body weight of each rat) was calculated and compared with the weight of the corresponding organ in the control group animals.

Preparation of serum samples and liver homogenates

The blood collected in dry test tubes was allowed to stand for complete clotting in ice for 1–2 hours. The clotted blood samples were centrifuged at 3,000 rpm for 15 minutes and serum samples were aspired off and conserved in aliquots at −20°C for the analysis of biochemical markers of renal and hepatic toxicity. The homogenate of the liver was prepared in a buffer solution (Tris 50 mM, KCl 150 mM; pH 7.4) at 20% (i.e., 20 g of the organ in 100 ml solution). In fact, the liver was cut into pieces with scissors and crushed in a mortar covered with ice. The homogenate was then centrifuged at 5,000 rpm for 30 minutes, and the supernatant was collected and conserved in aliquots at −20°C for the quantification of total protein.

Analysis of biochemical parameters

Total protein in blood serum samples was analyzed using the method of Biuret [10], while total protein in liver homogenate was analyzed using the Bradford method [11]. Creatinine was analyzed in blood serum samples using a kinetic method [12], and transaminases (Aspartate aminotransferase: AST; Alanine aminotransferase: ALT) were analyzed according to the method of Reitman and Frankel [13].

Statistical analysis

For each analyzed parameter, the data are expressed as mean ± standard deviation (SD) between different animals within each group. Comparisons between different groups were performed by the one-way analysis of variance. Significant difference between the control and experimental groups was assessed by Dunnett's test using the software *GraphPad Instat 3.0* (GraphPad software Inc., USA). The data were considered as significant when p value is less than 0.05.

Results

Chemical composition of the aqueous extract

The phytochemical screening of the AECVL reveals the presence of some groups of compounds such as polyphenols, tannins, sugars, and coumarins.

Acute toxicity

Oral administration of AECVL at doses up to 24 g Kg^{-1} induced no significant abnormal signs of toxicity. In fact, apart from the decrease in aggressiveness, locomotion and reaction to stimuli observed immediately after administration of the unique dose of AECVL, at doses greater than 16 g Kg^{-1}, all animals presented similar behavior in 4 hours postadministration and no mortality was registered in 48 hours after treatment. Figure 1 describes the body weight percentage variation per sex during the 14 days postadministration of the AECVL, and it is observed that all animals from both sexes gained weight during the study. An exception was noted in rats from groups that received the AECVL at doses greater than 16 g Kg^{-1}. In fact, the weight loss observed up to the 4th day would be due to the loss of appetite caused by the administration of high doses of extract. Overall, no

mortality was noted up to 14 days postadministration and LD_{50} of the AECVL was then estimated as greater than 24 g Kg^{-1} body weight.

Sub-chronic toxicity

Body and organ weights

As summarized in Figure 2, the body weight of the rats increases relatively during the study. When compared with the control groups, AECVL treatments did not cause significant change in the body weight of rats of both sexes. Similarly, the oral ingestion of AECVL over 28 days caused no significant changes in the weight of the studied organs (liver, kidneys, heart, lung, and spleen) regardless of whether the rat took the extract (test groups) or not (control groups) (Table 1). Likewise, the relative weights of genital organs from the treated rats of both sexes were not significantly affected during the study (Table 2).

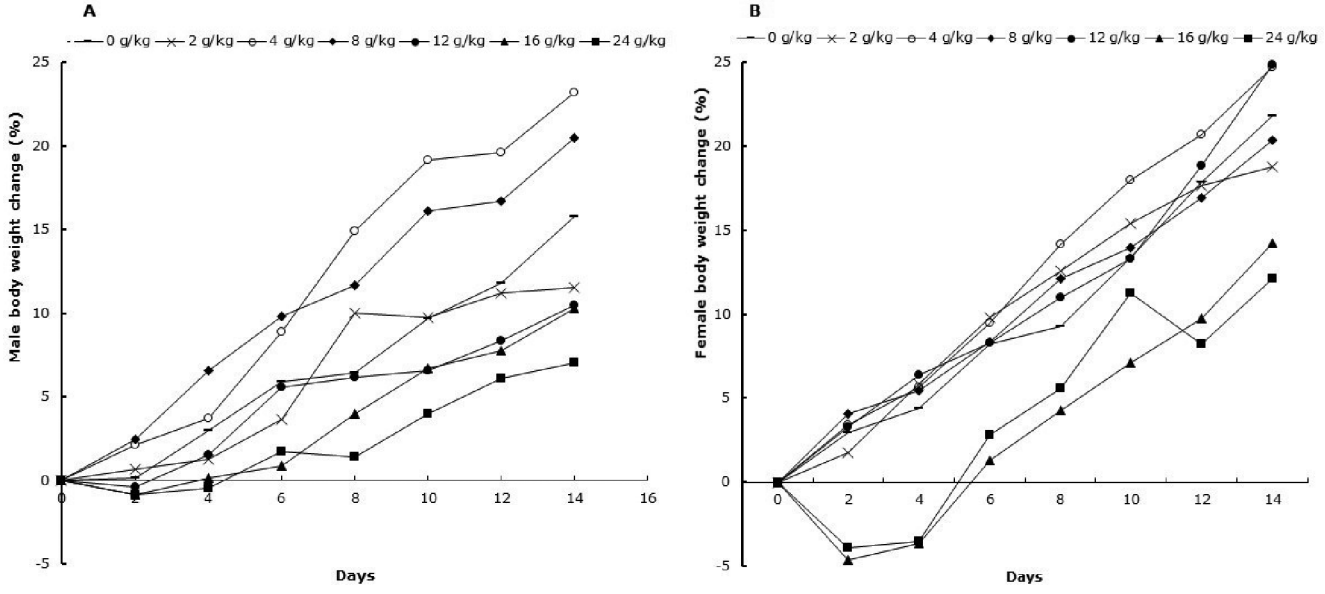

Figure 1. Percentage of body weight change of male (A) and female (B) mice during the 14 days after the administration of different single doses (0, 2, 4, 8, 12, 16, 24 g Kg^{-1}) of the aqueous extract of *Codiaeum variegatum* leaves. Each value represents the average between 4 animals per group.

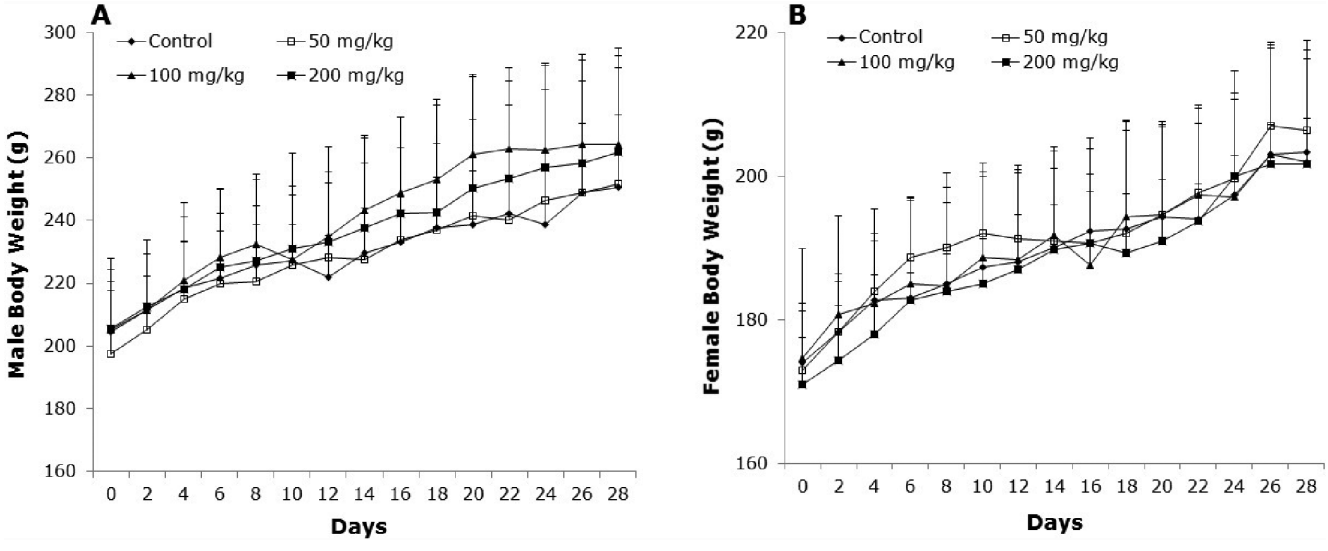

Figure 2. Body weight evolution of male (A) and female (B) rats during 28 consecutive days of oral administration of different repeated doses (0, 50, 100, 200 mg Kg^{-1}) of the aqueous extract of *C. variegatum* leaves. Each value represents the mean ± SD between six animals per group. (Dunnett test: * means significant difference compared to the control group; $p \leq 0.05$).

Biochemical parameters of renal and hepatic toxicity

Quantification of biochemical parameters through the determination of total protein in blood serum samples and liver homogenate, the transaminases (AST, ALT) and creatinine levels, showed no significant difference as compared with the control groups of rats of both sexes (Table 3). A slight increase was observed in the level of transaminases after the treatment of male and female rats with AECVL. But, this increase remains statistically non-significant as compared to control groups. Notwithstanding, the calculation of AST/ALT ratio in all groups is less than 2, which is approximately to the normal value

Table 1. Relative weight of vital organs (g Kg^{-1} body weight) of male and female rats after administration of the aqueous extract of *Codiaeum variegatum* leaves at different doses (0, 50, 100, 200 mg Kg^{-1}) for consecutive 28 days.

Sex	Doses (mg.Kg^{-1})	Organ relative weight (g Kg^{-1} body weight)				
		Liver	Kidneys	Heart	Lungs	Spleen
Male	0	28.70 ± 1.94	6.61 ± 0.52	3.27 ± 0.27	5.89 ± 1.10	4.09 ± 1.35
	50	28.94 ± 0.80	6.54 ± 0.52	3.35 ± 0.35	5.81 ± 1.07	4.19 ± 0.77
	100	29.53 ± 1.21	6.48 ± 0.46	3.45 ± 0.20	5.90 ± 0.60	3.89 ± 0.66
	200	28.85 ± 1.52	6.51 ± 0.43	3.28 ± 0.36	5.95 ± 1.24	3.47 ± 0.73
Female	0	28.68 ± 0.90	6.25 ± 0.48	3.93 ± 0.13	5.87 ± 0.76	4.17 ± 0.91
	50	28.46 ± 1.63	6.34 ± 0.11	3.78 ± 0.22	5.54 ± 0.75	3.79 ± 0.94
	100	28.68 ± 1.31	6.37 ± 0.25	3.37 ± 0.28*	6.15 ± 0.34	4.52 ± 0.99
	200	29.78 ± 1.65	6.52 ± 0.29	3.53 ± 0.15	6.13 ± 1.16	4.90 ± 1.17

Each value represents the mean ± SD between six animals per group. (Dunnett test:* means significant difference compared to the control group, $p < 0.05$).

Table 2. Relative weight of genital organs (gKg^{-1} body weight) of male and female rats after administration of the aqueous extract of *Codiaeum variegatum* leaves at different doses (0, 50, 100, 200 mg Kg^{-1}) for consecutive 28 days.

Sex	Genital organs	Doses (mg Kg^{-1})			
		0	50	100	200
Male	Prostate	1.51 ± 0.50	1.39 ± 0.18	1.72 ± 0.49	1.48 ± 0.60
	Seminal vesicles	3.79 ± 1.29	3.82 ± 1.06	3.63 ± 1.25	4.77 ± 1.30
	Testes	11.23 ± 1.03	12.02 ± 1.79	11.25 ± 1.14	10.78 ± 1.15
	Epididymis	4.27 ± 0.60	4.34 ± 0.91	3.97 ± 0.32	3.79 ± 0.46
Female	Ovaries	0.69 ± 0.20	0.71 ± 0.17	0.86 ± 0.15	0.72 ± 0.12
	Uterus	2.88 ± 1.43	2.99 ± 0.84	2.41 ± 0.31	2.47 ± 0.56

Each value represents the mean ± SD between six animals per group. (Dunnett test:* means significant difference compared to the control group, $p < 0.05$).

Table 3. Biochemical markers of renal (serum creatinine) and hepatic (transaminases, total protein) toxicity of male and female rats after administration of the aqueous extract of *Codiaeum variegatum* leaves at different doses (0, 50, 100, 200 mg Kg^{-1}) for consecutive 28 days.

Sex	Doses (mg Kg^{-1})	Total serum protein (mg mL^{-1})	Total hepatic protein (mg 100 g^{-1})	ALT (UI L^{-1})	AST (UI L^{-1})	Creatinine (mg L^{-1})
Male	0	94.96 ± 4.95	3382 ± 286	56.25 ± 5.77	111.75 ± 7.07	8.35 ± 0.92
	50	91.88 ± 4.10	3393 ± 425	66.35 ± 11.83	120.08 ± 5.64	8.06 ± 0.90
	100	97.44 ± 15.21	3456 ± 221	68.65 ± 12.55	120.92 ± 7.28	8.63 ± 0.47
	200	115.48 ± 12.56	3461 ± 291	70.63 ± 10.29	139.15 ± 11.41	9.15 ± 0.49
Female	0	106.97 ± 10.27	3267 ± 382	42.81 ± 11.31	86.13 ± 9.15	9.23 ± 0.90
	50	99.79 ± 7.41	3276 ± 459	45.52 ± 10.50	83.52 ± 5.72	8.99 ± 1.74
	100	92.82 ± 10.53	3345 ± 170	53.96 ± 14.30	101.23 ± 9.15	10.32 ± 1.24
	200	107.38 ± 11.65	3317 ± 265	52.81 ± 12.79	115.50 ± 12.43	9.31 ± 0.82

Each value represents the mean ± SD between six animals per group. (Dunnett test:* means significant difference compared to the control group, $p < 0.05$). AST: aspartate aminotransferase; ALT: alanine aminotransferase.

of this ratio after drug administration. In fact, after drug administration, changes may be observed in AST and ALT levels in blood, but the AST/ALT ratio should not vary much and should be less than 2, in this case no damage happens to the liver. Therefore, an AST/ALT ratio of 2:1 or greater is suggestive to cell liver injury or other liver diseases.

Discussion

The present study constitutes a part of our ongoing project which intends to valorize the medicinal value of *Codiaeum variegatum* (var. *mollucanum*) in the treatment of intestinal amoebiasis. This infection is a real public health problem in developing countries. The drug of choice (metronidazole) used in the treatment of this disease is less efficient on some strains of the parasite [14] and toxic effects of this drug have been reported [15]. In view of this, medicinal plants have been recognised by the World Health Organization as potential alternatives in the treatment of diseases due to their composition with various primary and secondary metabolites. Therefore, this current investigation, in addition to our previous studies, is realized in order to support the safety profile of the AECVL. In this study, no significant adverse effect was observed in the acute toxicity study after administration of a single dose of the AECVL up to 24 g Kg^{-1} body weight. All animals treated with the AECVL survived beyond the 14 days observation period. The LD_{50} of AECVL was above 24 g Kg^{-1} body weight (b.w.). According to Delongeas et al., 1983 and Kennedy, Ferenz, and Burgess, 1986 [16,17], substances that present LD_{50} higher than 15 g Kg^{-1} by oral route is classified at the toxicity index class 6 and can be considered as relatively inoffensive. Therefore, it can be suggested that AECVL is non-toxic after acute oral administration.

In the sub-chronic toxicity, a relative increase was observed in the body weight, as well as in organ weights of male and female rats after administration of the AECVL for 28 days consecutively (Fig. 2). However, this increase was non-significantly different as compared to the corresponding body and organ weights for the control groups. Also, no significant changes were observed in the levels of the biochemical parameters (transaminases, creatinine, total serum, and liver proteins), speculating that there was no extract-induced toxic effects at the administered dose levels on the major organs involved in various vital functions, specifically, liver and kidney [18]. In fact, transaminases (AST and ALT) are important enzymes in assessing the liver function. ALT is specific to the liver while AST is associated to the liver and heart. All these two enzymes are mainly found in the cytoplasm of animal cells [19,20]. An increase in the level of these enzymes in the serum simply indicates permeability or cell rupture resulting in their secretion into the bloodstream [21]. The slight increase observed in the levels of these enzymes may simply or likely be associated with the drug administration. By the less, no significant difference in transaminase levels were observed across sex regardless of whether the studied rats were treated (test groups) with extracts or not (control groups). The non-significant difference in the levels of these two enzymes in the sub-chronic toxicity study indicates that there is no AECVL-induced toxicity at administered dose levels on the liver function and to some extent on the heart function. Creatinine is a biochemical parameter indicating an effect on the renal function [22,23]. An increase in the level of creatinine in the serum or urine is an indicator of kidney dysfunction or damage on the nephrons of the kidneys [18,24]. In the present study, there was no significant difference in creatinine levels between male and female rats of different groups, therefore, speculating that AECVL at administered dose levels are relatively safe on the renal function of the studied rats. Another important biochemical parameter is the total serum or liver proteins. A change in the quantity of this parameter is a sign of non-specific tissue damage or particularly liver toxicity [22]. Generally, endogenous proteins are implicated not only in the transport of xenobiotics into the bloodstream through the organs, but also their biotransformation in the liver for their activation, detoxification, or excretion [25,26]. No significant change was observed in the levels of total serum and liver protein suggesting that AECVL did not induce any toxic effect in the various organs of the treated rats compared to the organs in rats of the control groups. However, there is a need to perform histopathological analysis for each of the targeted organs.

Conclusions

Based on the aforementioned discussions, it can be concluded that, no observable adverse effect was noticed following the administration of the AECVL up to 24 g.Kg^{-1} b.w. in the acute toxicity study; and up to 200 mg.Kg^{-1} b.w. in the sub-chronic study on mice and rats of both sexes respectively. This speculated that the AECVL is relatively safe and

can be used at therapeutic doses without observable effects in traditional healthcare system for the treatment of amoebic dysentery.

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