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Toxicity assessment of methanol extract of *Combretum micranthum* in *Drosophila melanogaster*

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Combretum micranthum leaves have been used traditionally in West Africa to manage pyrexia, cough, bronchitis, diabetes, malaria and other related ailments. It has also been demonstrated to alleviate high blood pressure and skin inflammation. *Drosophila melanogaster* has been gaining acceptability in biomedical research because of homologs of its genes present in humans and the relative cost of research using the flies. The purpose of the study is to investigate the acute and sub-acute toxicity of *Combretum micranthum* leaves in *Drosophila melanogaster*. The leaves were extracted using 90%v/v methanol. The extract was screened for presence of phytochemicals, acute and sub-acute toxicity using fruit flies. Doses between 5- and 100 mg/10 g diet for the acute toxicity study while 0 (Control), 1.5, 3 and 6 mg/10 g diet for the sub-acute toxicity study. The phytochemical analysis of the extract revealed the presence of alkaloids, glycosides, saponins, flavonoids and tannins. The acute toxicity study revealed an LD_{50} of 26.42 mg/10 g diet. The methanol extract of *Combretum micranthum* have no effect on the weight of fruit flies ($p<0.05$) among the different interventions. It also did not affect negative geotaxis ability of the fruit flies irrespective of the dose. At 6 mg/10 g diet, the extract raised triglyceride and trehalose levels when compared to the other doses ($p<0.05$). Glucose and glycogen levels were not different among groups. Overall, the results indicated that the methanol extract of *Combretum micranthum* at a dose of 6 mg/ 10 g diet is tolerable in *Drosophila melanogaster*.

Keywords: Acute toxicity; Subacute toxicity, *Drosophila melanogaster*; *Combretum micranthum.*

1. Introduction

Since ancient times, people have been using medicinal plants to address a variety of pathologies (Hosseinzadeh *et al*., 2015). They play a significant role in our everyday lives including those that are natural, conventional, societal, and economic. The World Health Organization estimates that up to 60% of people worldwide use medicinal plants for health treatment (WHO, 2022). This scenario would be influenced by the effectiveness, availability, affordability, and minimization of side effects. Studies of medicinal plants are logical search strategies for finding novel drugs and can play a significant role in drug discovery. The effectiveness of medicinal plants in treating variety of diseases has been documented in numerous studies (Sofowora *et al*., 2013). It is perceived that toxicity is no longer a criterion in ethno-pharmacological methods because the orientation is given to consumers by traditional medicine practitioners based on secular knowledge in choosing interesting species. According to these viewpoints, the initial goal of the search for toxicity is not deemed necessary because the vast majority of known plant extracts are not toxic. The primary issue with using medicinal plants is the lack of evidence-based approaches, such as the legal and regulatory framework, pharmacovigilance, non-standardization, and lack of toxicological profiling of herbal preparations (Ekor, 2014). A wide range of metabolites that plants produce combines to create complex substances that can either be beneficial or detrimental for consumers (Kennedy & Wightman, 2011). Nevertheless, despite the fact that some medicinal plants have therapeutic advantages, some of their constituents have been shown to be possibly toxic, carcinogenic, and teratogenic. It has been shown in some instances that the widespread belief that natural products derived from plants are non-toxic and free of side effects is incorrect

(Mensah *et al*., 2019). Studies on more than 1,500,000 plants have revealed that majority of the plants contain poisonous components. Additionally, it is a well-known fact that using medicinal plants without first assessing their effectiveness and safety can have unanticipated or toxic effects that can alter the physiology of various organs (Ekor, 2014).

Typically, the study of toxicity and, in particular, the assessment of the lethal dose 50, is the first step in the search for pharmacological activity when an unknown compound is investigated. Additionally, a good criterion for directing the search for pharmacological activity is a toxicity study. Despite *Combretum micranthum*'s frequent use in conventional medicine, there is a dearth of experimental evidence regarding its potential toxicity in *Drosophila melanogaster*. The current investigation therefore focuses on the acute and subchronic toxicity of a hydro-methanolic leave extract of *Combretum micr*anthum administered to Drosophila melanogaster over a 14-day period.

2. Materials and Methods

2.1 Plant collection and identification

Fresh leaves of *Combretum micranthum* were obtained from herbal market in Sokoto State of Nigeria. The leaves were identified at the Herbarium section of the Department of Pharmacognosy and Ethnomedicine, Usmanu Danfodiyo University Sokoto. It was assigned a voucher number of PCG/UDUS/COMB/0401. The leaves were air-dried, pulverized and soaked in 90% w/w methanol for 72 hours. Using a rotary evaporator, the extract was collected, filtered, and dried at 40 °C. The extraction process was repeated three times, and the various extracts were combined.

Wild-type (w^{1118} strain) Drosophila melanogaster were acquired from the Drosophila Laboratory of the Centre for Advanced Medical Research and Training (CAMRET), Usmanu Danfodiyo University, Sokoto. They were maintained at room temperature (22 to 25 °C) on a standard diet in media bottles in a natural light/dark cycle.

2.2 Phytochemical screening of the extract

Phytochemical screening of the extract was performed in order to determine the presence of secondary metabolites using standard phytochemical methods as described by Trease & Evans (2002).

2.3 Acute toxicity test

Young male adult fruit flies of about a day old into adulthood were separated into four groups. Each group had five replicates with 10 flies per replicate. For phase one, the first group were fed on the normal fly diet (Table 1), the second group were fed with a diet containing 25 mg of the extract per 10 g of the diet. The third group were fed with a diet containing 50 mg of the extract per 10 g of the diet. The fourth group were fed with a diet containing 100 mg of the extract per 10 g of the diet. The extract was dissolved prior to addition into the respective diets. Ten flies each were subsequently transferred into their respective 50ml centrifuge tube containing their diet mixed the extract at the base of the tube. The flies were monitored for four days for death or signs of toxicity. After the elapse of the phase one, the experiment was repeated for another four days with reduced doses of the extract for a second phase. Groups 2, 3, and 4 received 5mg/10 g diet, 10 mg/10 g diet and 20 mg/10 g diet respectively. Group one was the control group that received the normal diet.

2.4 Median lethal dose determination (LD50)

The median lethal dose was determined using probit regression analysis using Finney`s probit table (Finney & Stephens, 1948).

2.5 Sub-acute toxicity study

The flies were grouped into four groups with 30 flies per group. The flies in group 1 represented the control group and received normal fly diet. Flies in groups 2, 3 and 4 received 1.5 mg, 3.0 mg and 6 mg per 10g diet of the extract respectively. Their respective diets were changed after every three days. Flies were observed for any signs of toxicity and/or death for a period 28 days

2.6 Negative geotaxis Assay and weight changes

Ten flies per group of the second phase were assessed for locomotor activity. The flies were anaesthetized on an ice-cold glass surface and placed at the base of an empty 50 mL measuring cylinder. The cylinder was marked at 6 cm from the base. The anaesthetized flies were transferred into cylinder and allowed five minutes to acclimatize to the new environment. The cylinder was subsequently tapped such that all the flies were displaced to the base of the cylinder. The number of flies that crossed the 6 cm mark in ten seconds were observed and recorded. This was repeated thrice for each group to determine the average pass rate per group with 2 min resting time. The weights of the fruit flies were taken before and after the intervention (14 days) using a Kern analytical weighing scale (Kern & Sohn Ltd., Balingen, Germany). Thirty (30) flies per group were anaesthetized on ice and then placed inside a pre-weighed empty microtube (Wuhan Service bio Technology Co., Ltd., Wuhan, China) and re-

weighed. The differences in weights were recorded in milligrams (mg).

2.7 Biochemical Analyses

Twenty flies per group diet were fasted for 3 h and anaesthetized on ice. They were placed in a dish and rinsed with 100 µL of cold Phosphate-buffered saline (PBS). The flies were then homogenized using 200 µL of cold PBS on ice. The homogenates were centrifuged for 3 min at 14,000× *g* in a floor model refrigerated centrifuge (MX-301 Highspeed, Tomy Kogyo Co., Ltd., Tagara, Japan) at 4 °C. The supernatant containing the haemolymph was collected for biochemical analyses.

2.7.1 Glucose Assay

Fasting glucose levels of haemolymph were quantified using the Glucose Oxidase (GO) enzymatic assay kit (Spinreact, Girona, Spain) following manufacturer instructions, and the results were expressed as mg/dL.

2.7.2 Trehalose Assay

The trehalose level of haemolymph was quantified using the Anthrone colorimetric kit (Solarbio Life Science, Beijing, China) in accordance with manufacturer instructions and the results were expressed as mg/g sample.

2.7.3 Triglyceride Assay

The triglyceride level of the haemolymph was quantified using a colorimetric kit (Spinreact, Girona, Spain) according to the manufacturer's instructions.

2.8 Statistical analysis

The results obtained were expressed as mean and standard deviation (SD). SPSS version 19 was used to conduct statistical analysis of the data. One-way analysis of variance (ANOVA) was utilized. For post hoc examination of differences found using one-way ANOVA, Tukey's test was utilized. The $p < 0.05$ significance level was chosen.

3. Results and Discussion

3.1 Results

a) Phytochemical Screening

Phytochemical screening of the methanol extract shows the presence of alkaloids, glycosides, saponins, flavonoids and tannins (Table 1).

Table 1: Phytochemical screening of methanolic extract of *Combretum micrathum*

Phytochemical	Inference
Alkaloids	+
Glycosides	+
Anthracene	-
Triterpenes	-
Saponins	+
Tannins	+
Flavonoids	+

+ = present; - = absent

b) Mean Lethal Dose (LD_{50})

Table 2 shows the Logarithm concentration and probit value for the fly diet and death respectively. The highest dose employed (100 mg/10 g diet) caused a 100% death while the lowest dose (5 mg/10 g diet) did not cause any death. The LD_{50} was determined to be 27.38 mg/10 g diet.

Table 2: Acute exposure of flies to graded doses of *Combretum micrathum* extract and corresponding probit analysis

Concentration (mg/10 g diet)	Death (%)	Log conc.	Probits
100	100	2.000	8.0900
50	100	1.699	8.0900
25	43.3	1.397	4.6700
20	3.3	1.301	3.1200
10	0.0	1.000	0.0000
5	0.0	0.698	0.0000

c) Change in body weight

Table 3 describes the average change in weight among the groups of flies after a 14-day exposure to graded doses of *Combretum micrathum* extract. There is a significant increase in average weight for each group over the experimental period but there was no difference in average weight among the groups after day 14. The Table also describes the effect of the extract on negative geotaxis performance. All the groups did extremely well in crossing the 6 cm mark within the stipulated time.

Table 3. Body weight changes and negative geotaxis of flies after 14 days of diet intervention.

Dose (mg/10 g diet)	Average weight at day 0 (mg) n=10	Average weight at day 14 (mg) n=10	Negative geotaxis (no. flies that crossed after 6 sec) n=10
Negative control	25.0 ± 1.9	30.5 ± 2.2[a]	9.0 ± 0.4[a]
1.5	25.5 ± 2.1	31.1 ± 1.6 [a]	9.3 ± 0.7[a]
3.0	26.6 ± 1.5	31.0 ± 1.9[a]	9.6 ± 0.6[a]
6.0	25.8 ± 1.4	29.2 ± 2.3[a]	10.0 ± 0.8[a]

Mean ± SD values within a column denoted by the same letters in superscript are not significantly different ($p > 0.05$).

d) Biochemical Analyses

The Triglyceride levels

Figure 1 shows the triglyceride levels in flies exposed to graded doses of the methanol extract of *Combretum micranthum*. The flies exposed to 6 mg/10 g diet of the extract show an elevated level of triglyceride when compared to other groups. The lower doses (1.5 and 3 mg/10 g diet) did not significantly elevate heamolymph tryglyceride levels when compared to that of the vehicle control group.

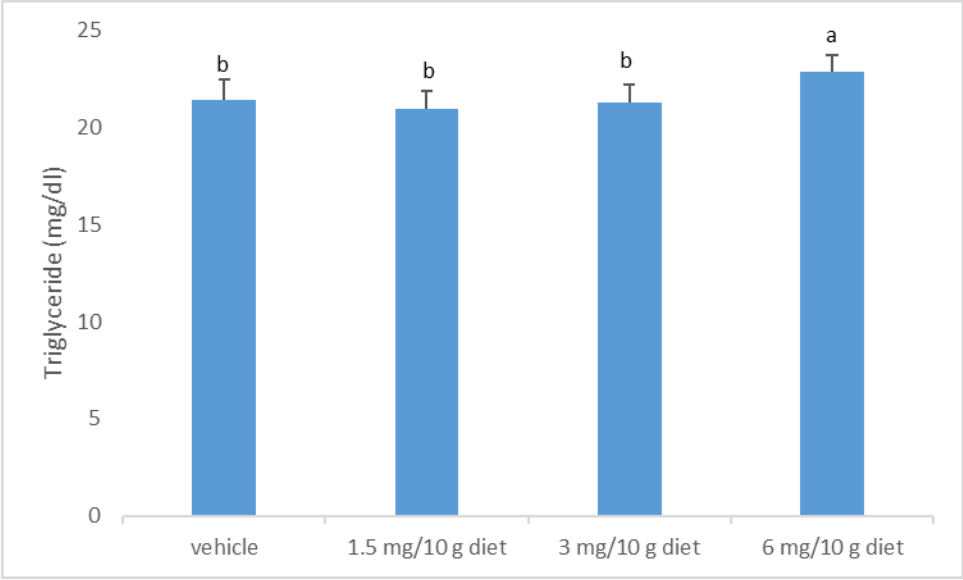

Figure 1: Effects of methanol extract of *Combretum micranthum* on triglyceride levels in flies. Bars represent mean ± SD. Bars denoted by the same letters are not significantly different ($p > 0.05$). ($n = 30$).

The Trehalose levels

Figure 2 shows the trehalose levels in flies exposed to graded doses of the methanol extract of *Combretum micranthum*. The flies exposed to 6 mg/10 g diet of the extract show an elevated level of trehalose when compare tod other groups. The lower doses (1.5 and 3 mg/10 g diet) did not significantly elevate heamolymph trehalose levels when compared to that of the vehicle control group.

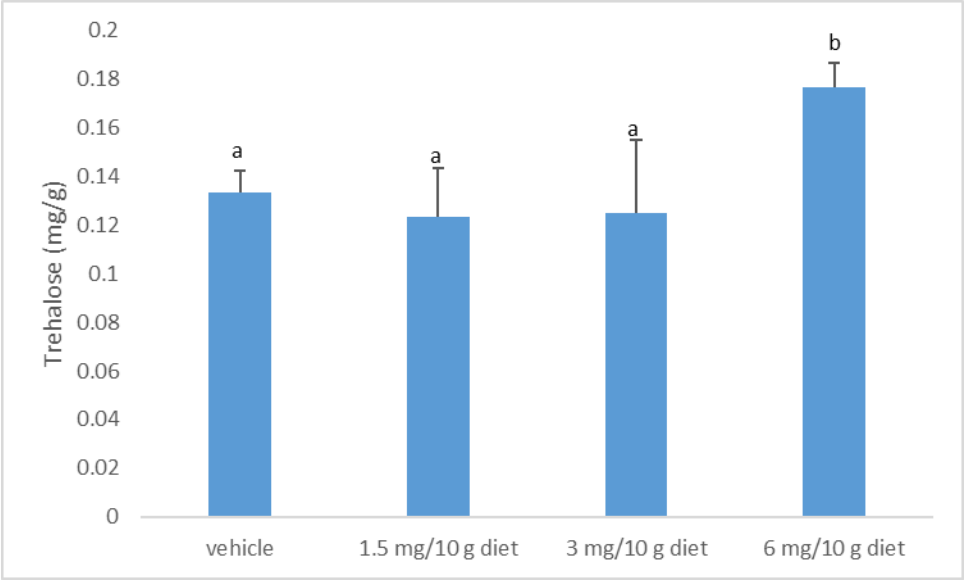

Figure 2: Effects of methanol extract of *Combretum micranthum* on fasting trehalose levels in flies. Bars represent mean ± SD. Barsdenoted by the same letters are not significantly different ($p > 0.05$). ($n = 30$).

The glycogen levels

Figure 3 shows the glycogen levels in flies exposed to graded doses of the methanol extract of *Combretum micranthum*. All the doses employed during the sub-acute toxicity study did not alter heamolymph glycogen levels when compared to that of the vehicle control group.

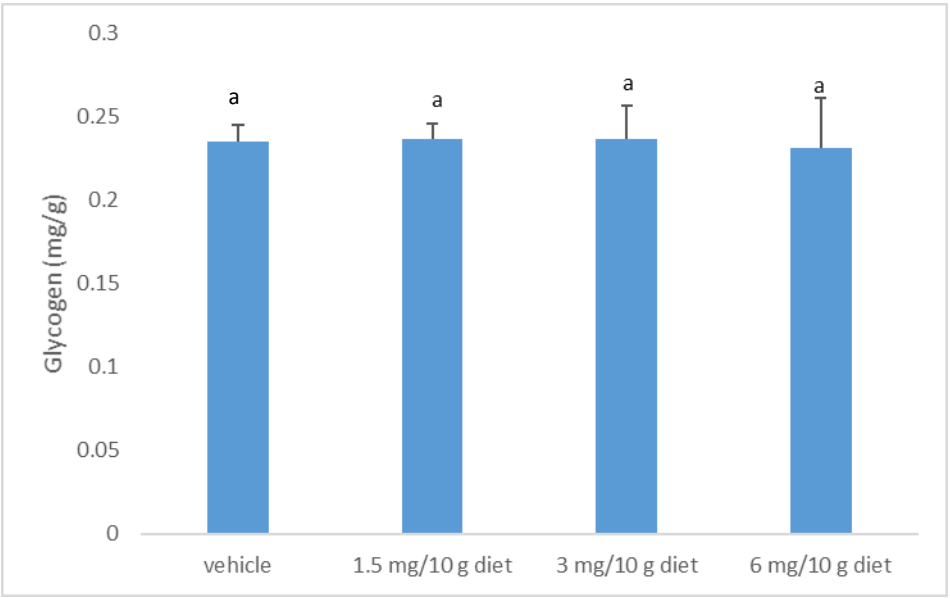

Figure 3: Effects of methanol extract of *Combretum micranthum* on fasting glycogen levels in flies. Bars represent mean ± SD. Bars denoted by the same letters are not significantly different ($p > 0.05$). ($n = 30$).

The glucose levels

Figure 4 shows glucose levels in flies exposed to graded doses of the methanol extract of *Combretum micranthum*. All the doses employed during the sub-acute toxicity study did not alter heamolymph glucose levels when compared to that of the vehicle control group.

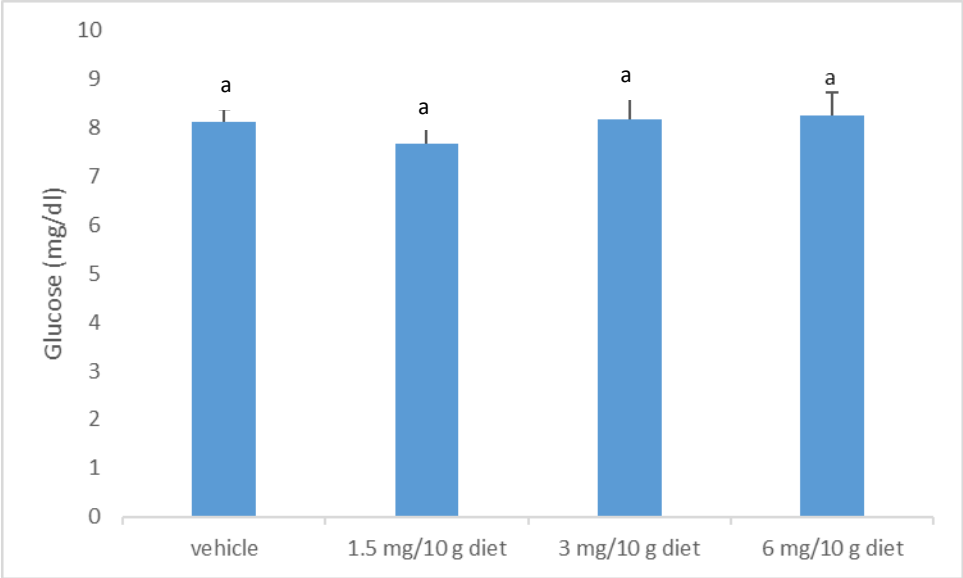

Figure 4: Effects of methanol extract of *Combretum micranthum* on fasting glucose levels in flies. Bars represent mean ± SD. Bars denoted by the same letters are not significantly different ($p > 0.05$). ($n = 30$).

3.2 Discussion

Although acute toxicity studies of *Combretum micranthum* on laboratory rats has been explored (Kpemissi *et al.,* 2020), its toxicity study on *Drosophila melanogaster,* which shares various basic biological and physiological mechanisms and molecular pathways with humans (Pandey & Nichols, 2011; Wang et al., 2012), is lacking. More so, comparison of complete-genome sequences of various species has demonstrated that humans share a substantial number of genes with the fruit flies. Comparative genomic research involving side-by-side analysis estimates that approximately 60% of Drosophila DNA is identical to that of humans, and almost 75% of the genes associated with human diseases, such as autism, diabetes, and cancer, have functional homologs in D. melanogaster (Lloyd & Taylor, 2010). This strongly suggests that phenotypic and genotypic expressions of toxicity or safety profile of the phytochemicals in *Combretum micranthum* on *Drosophila melanogaster* are likely reproducible in Humans.

The methanol extract of *Combretum micranthum* exhibited an LD_{50} of 27.38 mg/10 g diet. This provides a broadband of dosage selection and optimization for further therapeutic and toxicity studies. It also gives a preliminary insight into the short term safety of the extract.

The phytochemical composition result is unremarkable. Phytochemicals like alkaloid, saponins, flavonoids and tannins have been demonstrated to include both toxic and safe varieties (Diaz, 2015). The overall safety of the methanol extract as a whole alludes to the safety of each of the present phytochemical in the plant. The phytochemicals found in this study is similar to what was demonstrated by Kpemissi *et al*. (2019). Body weight loss is an objective measurement and is often used as a primary endpoint in acute and chronic toxicity studies (Wang *et al*., 2019). Body weight loss due to long term exposure has been a serious challenge during development of anticancer drugs and other compounds of therapeutic importance. The absence of effect on body weight by the extract further demonstrates the long term tolerability of the extract. In *Drosophila* and mammals, physiological activity is regulated by the central nervous system through motor neurons. Negative geotaxis is a technique employed to measure fly locomotion and activity. The technique measures a fly's ability to drift against gravity and it also employed as an indicator of Alzheimer's and Parkinson's disease in *Drosophila* models (Rivera *et al*., 2019). *Combretum micranthum* methanol did not affect negative geotaxis ability in the flies.

The circulating energy sources for *Drosophila* heamolymph are trehalose and glucose (Kim & Rulifson, 2004). The highest dose of the extract after a 14-day exposure did increase heamolymph trehalose concentration in the flies when compared to control group. This could be due to the fact that trehalose is tolerated in high concentration in *Drosophila* heamolymph due to its nonreducing nature. Also because trehalose is required by *Drosophila* brain and it is the main source of energy for *Drosophila* flight muscles (Becker *et al*., 1996). The highest dose of the extract could have increased trehalose heamolymph concentration by enhancing the effect glucagon-like adipokinetic hormone or by increasing its secretion. The triglyceride level unlike glycogen and glucose levels were elevated in the flies that were treated with 6 mg/10 g diet of the extract when compared to the

control group. Since *Drosophila* stores energy in form of triglycerides, the elevated triglyceride levels could be due to excess calorie provision by the extract as the plant has been documented to as an energy providing source (Kpemissi *et al*., 2022). Overall, the tolerability of the methanol extract of *Combretum micranthum* in fruit flies over the two weeks' exposure period demonstrates that the extract could be safe in humans. Although further toxicity studies are required, the present study provides a stepping stone for more critical and specific toxicity studies.

4. Conclusion

Based on the acute toxicity study, the LD50 of methanol extract of *Combretum micranthum* was determined to be 27.38 mg/10 g diet. The 14-day sub-acute toxicity study revealed that the compound is well tolerated up to a dose of 6 mg/10 g diet. This study therefore demonstrates the relative tolerability of *Combretum micranthum*.

Conflict of Interest

The author declares that there is no conflict of interest.

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