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Toxicity of *Azadirachta indica* Hydroethanolic Leaf Extract in Adult *Drosophila melanogaster* (Harwich Strain)

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Authors' contributions

This work was carried out in collaboration among all authors. Author OCT designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors EMA and JMS managed the analyses of the study. Author JMS managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aim: This study is aimed at evaluating the toxic effect of *A. indica* hydroethanolic leaf extracts in *D. melanogaster* (fruit flies) by carrying out a survival study, locomotor, fecundity and biochemical assays.
Place of study: This study was carried out in the *Drosophila* laboratory of Africa Centre of Excellence in Phytomedicine Research and Development (ACEPRD), University of Jos.
Methods: Extraction of *A. indica* extract was carried using hydroethanolic solvent (70:30 v/v ethanol: water). Flies were treated with 5 mg, 10 mg, 20 mg, 50 mg, 100 mg, 250 mg, 500 mg and 5000 mg *A. indica* hydroethanolic leaf extracts per 10 g fly food for 7 days, to determine the lethal concentration (LC_{50}). The survival assay was carried out for 28 days by treating flies with 5 mg, 10 mg, and 25 mg/10 g fly food of the extract. Young flies were treated with several concentrations of

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the extract for 7 days, to determine the effect of the extract on the fecundity and locomotion. Thereafter, flies exposed to the extracts for 7 days were immobilized, weighed, homogenized, and centrifuged. The supernatant was used to assay for acetylcholinesterase and catalase activities. The experiment was replicated 3 times and data was presented as mean ± SEM with statistical value at "$P < 0.05$" considered significant.

Results: The percentage yield was calculated to be 12.7 % and the phytochemicals present in *A. indica* hydroethanolic leaf extract included alkaloids, flavonoids, saponins, tannins, steroids, phenols, and glycosides. The LC_{50} was determined to be 1499 mg/10 g diet and the result showed a dose-dependent significant decrease ($P < 0.05$) in the survival of the flies, when compared to the control group. Further results showed a non-significant decrease ($P > 0.05$) in the fecundity, as well as the locomotor, acetylcholinesterase, and catalase activities of the flies, compared to the control.

Conclusion: This study concludes that *A. indica* hydroethanolic leaf extract, at certain concentrations, may not be safe for consumption as it showed some level of toxicity in *D. melanogaster.*

Keywords: Toxicity; Azadirachta indica; Drosophila melanogaster; hydroethanolic leaf extract.

1. INTRODUCTION

Medicinal plants contain several bioactive compounds which gives it the ability to treat several diseases and ailments [1]. Because of their easily assessable nature, medicinal plants have become very popular for the treatments and prevention of certain diseases and it has been used with or in place of conventional drugs [2,3]. People assume that all medicinal plants are safe for consumption because they are considered "natural/organic", but studies have shown otherwise [4]. Medicinal plants contain several bioactive compounds that are potentially toxic and many cases have reported acute or chronic effect of these plants when consumed [5,6]. These toxicities could result in cardiovascular, neurological, hematological, gastrointestinal, carcinogenic and reproductive effects [7]. Toxicity in medicinal plants have been attributed to factors like the amount consumed, secondary metabolite constituents, climate, and genetics of the plant [8]. Therefore, in order to use medical plants for the treatment and prevention of diseases, it is important to test these plants for their safety and efficacy by carrying out several *in vivo* and *in vitro* toxicity assays [7,9].

Azadirachta indica (A. Juss.) commonly known as Neem is a member of the Meliaceae family which possess various therapeutic properties for the treatment and prevention of some diseases [10]. The neem leaf has been exploited for a long time because it has proven to exhibit antiviral, antifungal, antibacterial, antimalaria, anticancer, anti-inflammatory activities [11]. The neem leaf contains several phytochemicals that play essential roles in the treatment and prevention of some diseases [12]. Despite the therapeutic effect of the leaf extract of *A. indica*, studies have shown some level of toxicity on animal models which could be harmful to the cells [13]. In line with its toxicity, the neem leaves have also been recorded to cause toxic effects on fertility and nervous system in test animals [11]. For the development of safer drugs in the treatment/prevention of some diseases, it is imperative that the toxicity of *A. indica* be evaluated.

In order to adapt to the environmental stress caused by several factors, some plants have evolved to synthesize bioactive compounds to deter these unfavorable conditions and some of these compounds are potentially toxic to humans and animals [14]. Consumption of these toxic substances cause oxidative stress in the cells of organisms and this stress occurs as a result of the accumulation of free radicals or reactive oxygen and nitrogen species (RONS) in the cells, which are not being scavenged or neutralized by biological system like the antioxidants [15]. Oxidative stress induces some human diseases including cancers, Alzheimer's and Parkinson's, and diabetes [16]. Glutathione-s-transferase (GST), Catalase (CAT), Superoxide dismutase (SOD), ascorbic acid, α-tocopherol, etc., are examples of the enzymatic and nonenzymatic antioxidant defense system that acts on and scavenge these free radicals [17]. Several other enzymes play very crucial role in detoxification of harmful substance in the cells e.g., the enzyme acetylcholinesterase (AChE) breaks down acetylcholine (a neurotransmitter that accumulates to cause symptoms of muscarinic and nicotinic toxicity) to prevent its accumulation, making AChE a main target for therapeutic drugs [18,19].

Drosophila melanogaster, commonly known as fruit fly, is an alternative and useful model in carrying out biological, toxicological and biomedical studies because this model has met the 3Rs (Reduction, Replacement and Refinement of laboratory animal usage) standard of European Centre for Validation of Alternative methods (ECVAM) [20]. Fruit flies are appreciated for studying toxicological effects owing to their short life cycle, highly conserved aging pathway, behavior, and development [21,22]. Approximately 65-70 % of human disease-causing genes are believed to have a functional homolog in *D. melanogaster* and a significant fraction of these homolog are expressed in their tissues, which performs the function of the equivalent human tissue [21]. *D. melanogaster* is an excellent model that has been used to study neurodegenerative diseases, oxidative stress and antioxidant markers, cancer, and other metabolic disorders [23]. These many advantages have been useful for carrying out several assays in *D. melanogaster* including survival, negative geotaxis (locomotor activity), fecundity (reproduction), and biochemical assays to test for the efficacy and toxicity of medicinal plants [23,24].

The aim of this study is to evaluate the toxic effect of *A. indica* hydroethanolic leaf extracts in *D. melanogaster* by carrying out a survival study, locomotor, fecundity and biochemical assays.

2. MATERIALS AND METHODS

2.1 Plant Collection, Identification, and Extraction

A. indica leaves were collected from Maza Village Jos North, Plateau State, Nigeria. The plants were identified and authenticated by the Department of Plant Science and Biotechnology, University of Jos. The extraction process was carried out according to the method described by Etuh et al. [25]. The leaves were washed under running tap water to remove dirt and other particles. It was air dried under room temperature for 7 days, then the leaves were reduced to powdered form using mortar and pestle. 70 g of powdered leaves of *A. indica* was macerated for 72 hours with intermittent shaking, using hydroethanolic solvent (70:30 v/v ethanol:water). The macerate was filtered using Whatman filter paper (No. 1) and the filtrate was concentrated using a water bath at 30^0 C. The percentage total yield of the extract was calculated using the formular below:

$$\%\ \text{extraction yield} = \frac{W_2 - W_1}{W_0}\ \text{x}\ 100$$

Where:

W_0= the weight of the initial dried sample,
W_1= the weight of the container alone, and
W_2= the weight of the extract and the container.

2.2 Phytochemical Screening

Following the method described by Sofowara [26], the phytochemical screening of the extract was carried out. The presence of various secondary metabolites was screened for and recorded.

2.3 Animal Model

The model organism used for this study is the Fruit fly (*Drosophila melanogaster*; Harwich strain). The organisms were obtained from the Drosophila research laboratory of Africa Centre of Excellence in Phytomedicine Research and Development (ACEPRD). Species Stock was maintained at constant temperature and humidity (23 ± 2 °C and 60 % relative humidity, respectively) under 12-hour dark/light cycles. The flies were fed on standard *Drosophila* medium composed of yellow cornmeal (100 g), brewer's yeast (20 g), agar (16 g), and methyl paraben (1 g) [24].

2.4 Median Lethal concentration (LC_{50}) Determination

A 7-day acute toxicity study (LC_{50}) was carried out following the method described by Etuh et al. [25]. This was to determine the concentration of the extract that kills 50 % of the flies in seven (7) days [27]. Fifty (50) young fruit flies (1-5 day old) each, were anesthetized under ice and exposed to eight (8) different groups of *A. indica* leaf extract treatments (5 mg, 10 mg, 20 mg, 50 mg, 100 mg, 250 mg, 500 mg and 5000 mg per 10 g fly food) and control (10 g of fly food mixed with 1 ml of distilled water). The experiment was replicated three (3) times. The mortality rate was scored every 24-hour interval for seven (7) days. The survival percentage was plotted against the logarithm concentration in a dose-response simulation using the Graphpad Prism (8.0.2).

2.5 Survival Assay

As described by Abolaji et al. [24], 50 flies of both genders (1-5 day old) were exposed to 5 mg, 10 mg, and 25 mg of *A. indica* hydroethanolic leaf extract per 10 g diet each, and a control (10 g of fly food mixed with 1 ml of distilled water only) for twenty-eight (28) days. The experiment was replicated three (3) times and the number of live and dead flies was scored daily till the end of the experiment. The survival rate was expressed as percentage of live flies.

2.6 Negative Geotaxis Assay (Locomotor Activity)

Negative geotaxis (locomotor activity) was conducted employing the technique demonstrated by Abolaji et al. [24]. Ten (10) flies (both gender) each, were treated with *A. indica* hydroethanolic leaf extract of different concentrations (5mg, 10mg, and 25 mg/10 g fly food), and plain fly food (control) for seven (7) days. The flies from the different groups were anaesthetized under ice and collected into an empty vial. They were then introduced into an empty vertical glass column of diameter and length 1.5 cm and 15 cm respectively. The glass column was initially labeled at 6 cm above the bottom. After a 20-minute recovery phase, the flies were gently tapped to the bottom of the glass column. The number of flies that went up to the column's 6 cm mark in 6 seconds was recorded. This experiment was replicated three (3) times and the data was represented as the proportion of flies that moved beyond the 6 cm limit in 6 seconds (% locomotor activity).

2.7 Fecundity (Reproduction/ Emergence) Assay

This was achieved using the procedure described by Patlolla et al. [28], with slight modification. Ten (10) virgin male and female flies each were treated separately with different concentration of *A. indica* hydroethanolic leaf extract (5mg, 10mg, and 25 mg/10 g fly food) for seven (7) days. Five (5) virgin males and females each from the same treatment groups were placed together into a vial containing a normal diet, and allowed to mate and lay eggs for 24 hours. The flies were transferred out of the diet and the empty vials were examined for laid eggs under a light microscope. This was held at a constant room temperature and relative humidity for 14 days. The number of flies that emerged from each treatment and control group were recorded daily. The score of each group was recorded as an average of the three (3) independent trials.

2.8 Biochemical Assay

Following the method described by Abolaji et al. [24], fifty (50) healthy flies were treated with various concentration of *A. indica* hydroethanolic leaf extract (5mg, 10mg, and 25 mg/10 g fly food) and plain fly food (control) for seven (7) days. The alive flies from this 7-day treatment were anesthetized on ice, weighed, and homogenized in phosphate buffer saline (PBS) at pH 7.4. Homogenates were centrifuged for 10 minutes at 4000 rpm. The supernatant was collected and used for the determination of acetylcholinesterase and catalase activity.

2.8.1 Acetylcholinesterase (AChE) activity

Acetylcholinesterase (AChE) activity was evaluated (with minor modifications) based on Ellman et al. [29] method. 60 µL of 8 mM acetylthiocholine, 180 µL of 100 mM phosphate buffer saline (pH 7.4), 60 µL of 10 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), 285 µL distilled water, and 15 µL of the sample was mixed together. The experimental data was calculated against blank and sample blank. Using a spectrophotometer (Jenway), the absorbance was read at 412 nm for 2 minutes (at 10 seconds intervals). Acetylcholinesterase activity was expressed as µmol/min/mg protein.

2.8.2 Catalase (CAT) activity

A method described by Aebi [30] was employed to determine the catalase activity of the sample. Solution A was prepared using 194 mL of 300 mM H_2O_2 and 100 µL of potassium phosphate buffer (pH 7.0). 10 µL of the sample was mixed with 590 µL of solution A and H_2O_2 clearance was monitored at 240 nm at 25°C. The rate of dissociation of H_2O_2 was monitored by a UV-visible spectrophotometer (Jenway) for 2 minutes at 240 nm and 10-second intervals. The data was expressed as mmol of H_2O_2 consumed/min/mg protein.

2.9 Statistical Analysis

All data were presented as Mean ± SEM. Analysis of statistics was conducted applying a one-way analysis of variance (ANOVA) succeeded by Turkey's posthoc test. Analysis to determine the survival curves was performed

employing the Log-rank (Mentel-cox) test with the aid of GraphPad Prism statistical software (version 7.04). All results at $P < 0.05$ were held statistically significant.

3. RESULTS AND DISCUSSION

3.1 Percentage Total Yield and Phytochemical Contents

The percentage total yield of *A. indica* hydroethanolic leaf extracts was calculated to be 12.7 %. The phytochemicals that were found present include alkaloids, flavonoids, saponins, tannins, steroids, phenols, and glycosides.

3.2 Median Lethal Concentration (LC_{50})

The mortality rate when exposed to *A. indica* hydroethanolic extract showed an 86.56 % mortality of *D. melanogaster* when exposed to 5000 mg/10 g for seven (7) days. The other concentrations showed less mortality as shown in Fig. 1. The 7-day lethal concentration (LC_{50}) was determined to be 1499 mg/10 g diet.

3.3 28-day Survival

The survival assay revealed a significant decrease ($P < 0.05$) in survival of *D. melanogaster* exposed to 5 mg, 10 mg, and 25 mg *A. indica* hydroethanolic leaf extract per 10 g food (in a dose-dependent manner), when compared to the control group (Fig. 2).

3.4 Negative Geotaxis (Locomotor Activity)

The negative geotaxis result revealed a slight and non-significant decrease ($P > 0.05$) in the locomotor activity of fruit flies treated with *A. indica* hydroethanolic leaf extracts when compared to the control group (Fig. 3). The percentage locomotor activity in 5 mg, 10 mg, and 25 mg-treated flies were 86.33 ± 6.52, 88 ± 3.23, and 86.5 ± 3.78 respectively, compared to that of the control (93.33 ± 4.72).

3.5 Fecundity (Emergence Assay)

The assay revealed a sharp but non-significant reduction ($P > 0.05$) in the number of young fruit flies that emerged out of the treated groups, when compared to control (Fig. 4). 5 mg, 10 mg, and 25 mg-treated flies showed an emergence of 17.75 ± 3.98, 24.25 ± 7.47, and 17 ± 5.67 respectively, when compared to the control (39.5 ± 10.07).

3.6 Biochemical Assays

3.6.1 Acetylcholinesterase (AChE) Activity

After the exposure of fruit flies to *A. indica* ethanolic leaf extract, result showed a non-significant reduction ($P > 0.05$) in AChE activities in all treatment groups, compared to the control (Fig. 5). AChE activities in 5 mg, 10 mg, and 25 mg treated flies were quantified as 0.027 ± 0.011, 0.033 ± 0.009, and 0.02 ± 0.017 respectively. The control showed an activity calculated to be 0.04 ± 0.016.

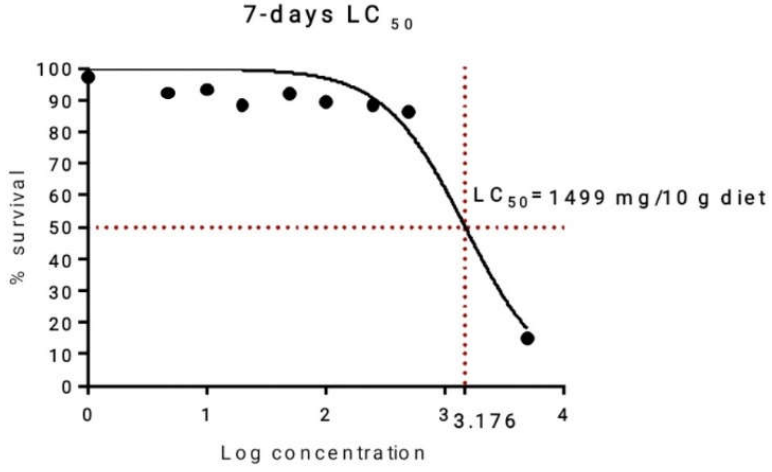

Fig. 1. LC_{50} of *A. indica* hydroethanolic leaf extract in *D. melanogaster* = 1499 mg/10 g diet

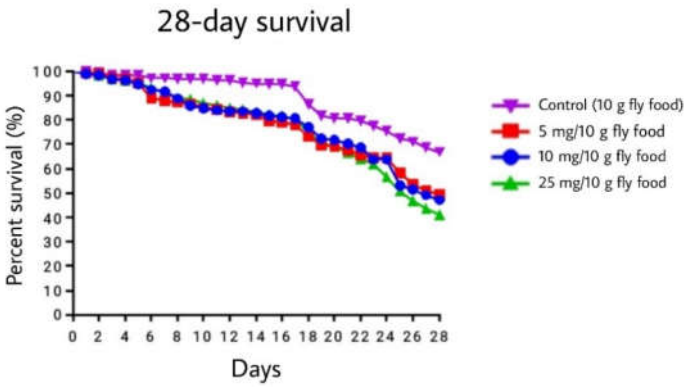

Fig. 2. 28-day survival of *D. melanogaster* exposed to food containing *A. indica* hydroethanolic leaf extract.

(Data are presented as mean ± SEM of three (3) independent biological replicates. P < 0.05 vs control.)

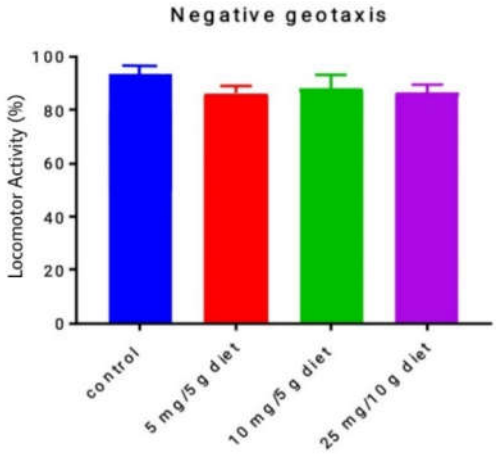

Fig. 3. Locomotor Activity of *D. melanogaster* treated with various concentration of *A. indica* hydroethanolic leaf extract.

(Data are presented as mean ± SEM of three (3) independent biological replicates. P > 0.05 vs control.)

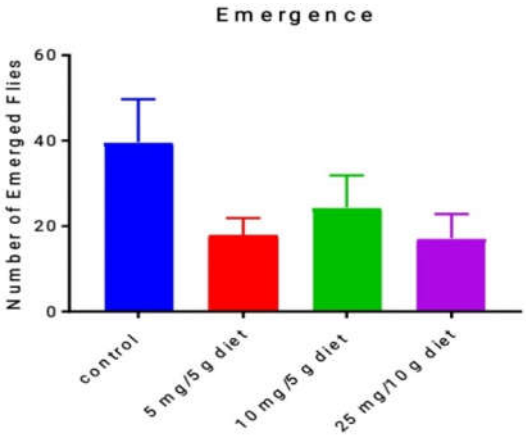

Fig. 4. Emergence of *D. melanogaster* treated with various concentrations of A. *indica* hydroethanolic leaf extract

(Data are presented as mean ± SEM of three (3) independent biological replicates. P > 0.05 vs control.)

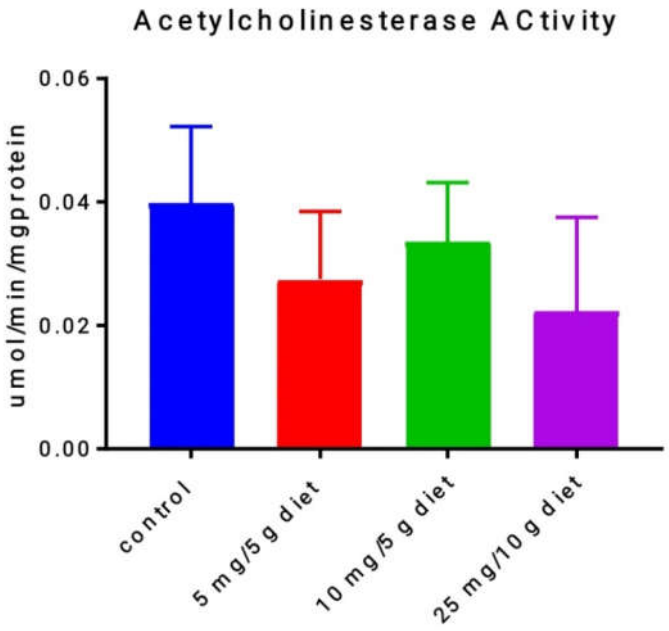

Fig. 5. Acetylcholinesterase Activities of *D. melanogaster* treated with several concentration of *A. indica* hydroethanolic leaf extract.

Data are presented as mean ± SEM of three (3) independent biological replicates. P > 0.05 vs control.

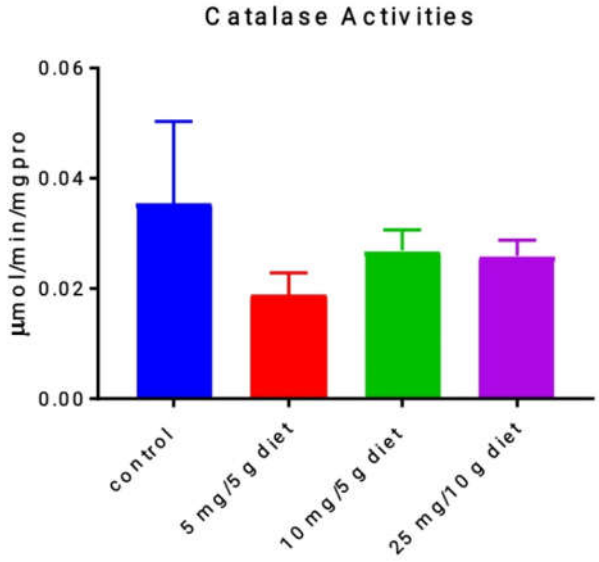

Fig. 6. Catalase Activities of *D. melanogaster* treated with several concentration *A. indica* hydroethanolic Leaf extract

(Data are presented as mean ± SEM of three (3) independent biological replicates. P > 0.05 vs control.)

3.6.2 Catalase (CAT) activity

Fig. 6 shows the catalase (CAT) activity of *D. melanogaster* after the 7-day exposure. The result revealed a non-significant reduction (*P* > 0.05) in the catalase activities of fruit flies treated with 5 mg (0.019 ± 0,004), 10 mg (0.027 ± 0.004), and 25 mg (0.026 ± 0.003) *A. indica*

hydroethanolic leaf extract compared to the control (0.035 ± 0.015).

Although secondary metabolites serve as good sources for the discovery of new drugs and for the treatment/prevention of disease, but some could be toxic when present [31]. The phytochemical analysis of *A. indica* hydroethanolic leaf extracts revealed the presence of alkaloids, flavonoids, saponins, tannins, steroids, phenol and glycoside which is in agreement with the study carried out by Dash et al. [32] and Pokhrel et al. [33], who also evaluated the phytochemical content of *A. indica* leaf extracts. Alkaloids are said to possess several pharmacological activities including antibacterial, anticancer, and antimalaria, yet, some are potentially toxic (e.g., atropine) [34,35]. Also, there are reports of toxic flavonoid and phenolic drugs interactions despite their medicinal properties [36].

The 7-day lethal concentration (LC_{50}) was determined to be 1499 mg/10 g diet. Rapid death was observed in the 5000 mg-treated fly group, while 5 mg, 10 mg, 20 mg, 50 mg, 100 mg, 250 mg, 500 mg-treated flies were observed to have less than 20 % deaths only. Riaz et al. [37] determined the LC_{50} of *A. indica* petroleum ether extract on third instar larvae of *D. melanogaster* to be 39.50 mg per fly food, while Dorababu et al. [38] and Kingsley et al. [39] also determined the LD_{50} of *A. indica* aqueous extract in mice to be 2500 mg/kg and 5000 mg/kg respectively.

Flies treated with the three (3) concentration of *A. indica* hydroethanolic leaf extract for 28 days showed a significant decrease ($P < 0.05$) in their survival when compared to the control. The decrease was in a dose-dependent manner, i.e., the decrease followed a patterned manner in such a way that the highest concentration had the highest mortality while the least concentration revealed the least mortality of flies. This decrease in survival may be attributed to the toxicity of the plant extract at those concentrations. This study agrees with Sani et al. [40] who studied the sub-chronic toxicity profile of *A. indica* hexane and ethyl acetate extract in albino rats for 28 days, and concluded that the extract showed some level of toxicity which affected some hematological parameters.

Negative geotaxis (locomotor activity) of *D. melanogaster* is an assay of how competent the flies are to go up vertically, after being tapped to the base of a vessel as part of its innate escape response. Negative geotaxis is calculated by the distance the flies are able to climb in a set period of time and it has been shown to be affected by oxidative stress, age, and detriment exposures [41,42]. The findings on the climbing activities of *D. melanogaster* fed with diet containing *A. indica* hydroethanolic leaf extract showed a non-significant decrease when compared to the control. Study has shown that *A. indica* has shown neurotoxic properties that affects the nervous system [11]. This neurotoxicity could be the cause of reduced climbing activities in the treated *D. melanogaster.* Toxic plants used as medicines have shown potential neurotoxic effects that has affected the central nervous system, therefore, affecting the locomotion of organisms [43].

Findings of the emergence of offspring from the eggs treated with *A. indica* hydroethanolic leaf extract revealed a sharp and non-significant decline ($P > 0.05$) in the offspring emergence compared to the untreated control. This decline was in a non-dose-dependent manner. These findings agree with Deshpande et al. [44] and Upadhyay et al. [45] who reported that *A. indica* possesses male and female antifertility activities. Infertility caused by medicinal plants have been linked to the toxicity of their phytochemicals, which adversely hinders the normal functioning of the reproductive organs [46]. The emergence of treated *Drosophila* ranged from 17 ± 5.67 to 24.25 ± 7.47, with 10 mg-treated flies having the highest emergence and 25 mg-treated flies having the lowest.

There was a non-significant decline ($P > 0.05$) in acetylcholinesterase (AChE) activity in *D. melanogaster* when exposed to all concentration of the extract compared to the control. AChE is an enzyme that terminates nerve impulses by catalyzing the hydrolysis of acetylcholine (a neurotransmitter) [47]. Once the enzyme is inhibited, it leads to the accumulation of acetylcholine (a neurotransmitter that accumulates to cause symptoms of muscarinic and nicotinic toxicity) and disrupted neurotransmission [48,19]. Acetylcholinesterase activity has been reported to be inhibited by *A. indica*; which has made it a plant of choice for studying neurodegenerative disorder [49]. This study suggests that hydroethanolic extract of *A. indica* induced some level of toxicity (although not significant) that impaired the neurological functions *of D. melanogaster.* The slight

reduction in AChE activities may relate to the slight decrease in the climbing activity of the treated flies because studies have shown that decrease in AChE activities is directly proportional to decrease in climbing activities [24,50,3]. Other studies have linked AChE activities to reproduction, whereby, decrease in AChE affects the male and female fertility in a negative manner [51,23]. The slight decrease in fecundity of *D. melanogaster* treated with *A. indica* hydroethanolic leaf extract may be due to the decrease in AChE activities.

There was a non-significant decrease ($P > 0.05$) in catalase activities of fruit flies when treated with *A. indica* hydroethanolic leaf extract. The catalase activities of treated flies ranged from 0.019 ± 0,004 to 0.027 ± 0.004 µmol/min/mg protein with 10 mg-treated flies having the highest catalase activity and 5 mg-treated flies having the lowest. The slight decrease in CAT activities suggest that the extract may impede the production of catalase, therefore reducing the scavenging of free radicals. Catalase hinders the deleterious effects of hydrogen peroxide (H_2O_2) by reducing it to water (H_2O) and oxygen (O_2), thus avoiding the accumulation of free radicals [52]. Toxicity of plant extracts has led to the reduction of antioxidant enzymes like catalase [53]; hence, the slight reduction of catalase activity may be due to the toxic substance in *A. indica* hydroethanolic extract.

4. CONCLUSION

This study concludes that *A. indica* hydroethanolic leaf extract, at certain concentrations, may not be safe for consumption as it showed some level of toxicity in *D. melanogaster.* The toxicity was evident when the mortality rate of treated flies increased significantly in a dose-dependent manner. Although there was a non-significant decrease on the reproductive performance, locomotor activity, acetylcholinesterase and catalase activities in the flies, but the reduction in these parameters entails toxicity.

RECOMMENDATION

It is recommended that more research be carried out on molecular basis, to test and confirm the toxicity of *A. indica* hydroethanolic leaf extract in *D. melanogaster.*

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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