Original Research Article

The Climbing Performance, Neuromuscular transmitter (AChE) Activity, Reproductive Performance and Survival of *Drosophila melanogaster* Fed Diet with *Mangifera indica* Cold Aqueous Leaf Extract

Abstract

Objective: To screen the toxic effect of *Mangifera indica* aqueous leaf extract in *Drosophila melanogaster.*

Materials and Methods: Phytochemical screening was carried out. 20 Adult flies were exposed to 7.5 mg, 15 mg, 30 mg, 45 mg and 100 mg /10 g diet for acute toxicity (168hrs) while 50 flies were exposed to 2.5 mg, 5 mg and 10 mg/10 g diet for sub chronic (28 days). All concentrations were prepared in 1000µl of distilled water and replicated three (3) times. Diet+1000µl of Distilled water served as control. Fecundity/developmental toxicity, Climbing and AChE activities were carried out by exposing flies to the sub-chronic concentrations for 5 days.

Results: Phytochemical screening revealed the presence of alkaloids, phenols, flavonoids, tannins, saponins and terpenoids. The acute toxicity test showed 100% mortality at 100 mg/ 10 g diet and 168hrs LC_{50} was 72.4 mg/10 g diet. The sub chronic toxicity test showed decrease in flies survival along concentration with a least survival at 10 mg/10 g diet. There was a slight reduction and elevation in the Climbing and AChE activities respectively but not statistically

significant ($p>0.05$) compared to control. At 5 mg and 10 mg/10 g diet there was a delay in the development with few emerged flies.

Conclusion: from the Results, it can be concluded that *Mangifera indica* aqueous leaf extract may be toxic at high dose from 72.4 mg/10g diet and might have an adverse effect on the development and survival of flies at sub chronic concentration as low as 2.5mg/10g diet.

1 Introduction

From time immemorial, nature has been of great help to humans and animals alike, particularly plants that have been a source of new drugs with curative properties to treat various diseases. Plants are used in traditional medicine to treat different infectious and noninfectious diseases [1, 2]. These treatments include body-washes, massages, ingestions, etc. [3]. It has been documented that nearly 80% of the world's population relies on traditional medicines for primary health care, most of which involve the use of plant extracts [4]. Little or no detailed information on the toxicological profile of these medicinal plants has been documented. Despite the medicinal properties of these plant extracts against infections and diseases some plant extracts can be hepato-toxic, neurotoxic, hemotoxic and damaging to the reproductive cells even at low concentration [5]. Reproductive fitness is a broad, ecological measure of health [5]. Many herbs can negatively affect male and female fertility as well health during pregnancy [6]. Some medicine can alter ovulation and endometrial or uterine receptivity to a pregnancy [7]. Medical plant can however be poisonous if wrong plant part or wrong concentrations are used [8]. Nowadays, toxicity and safety of medicinal plants are the most concerning topics, as use of plant

product has become popular worldwide [9]. Toxicity can be acute, sub-chronic and chronic depending on the exposure period and the product concentration. It has been shown that the toxicity of a given plant depends on various factors, including the strength of secondary metabolites, the quantity consumed, the time of exposure, different parts of the plant (root, leaves, stem bark and seeds), individual body chemistry, climate and soil, and genetic differences within the species [10]. Indeed, of about 1,500,000 plants investigated, most of them contain toxic substances [11].

Mangifera indica is a large evergreen and a heavy, dome-shaped crown tree in the family Anacardiaceae [12]. It is distributed all over the tropical regions of the world [12] and has been useful as horticultural and medicinal plant. Fruits contain protein, fat, carbohydrate, minerals, vitamins A, B and C and amino acids. The fruits also yield a resin containing mangiferene, mangiferic acid, resinol, maniferol and others [13, 14]. The leaves have been reported to contain saponins, glycosides, unsaturated sterols, polyphenols, euxanthin acid, mangiferine and tannins etc. Burns, scalds, sores, cough and diarrhoea are treated with the ashes of the leaves of *M. indica* in South America and other parts of the world [13, 15]. The *in vivo* protective antioxidant activity of *M. indica* in *Drosophila melanogaster* has been reported [12]. Antiseptic activity leaf extracts in the treatment of infections, wounds scalds, burns, sores, and abscesses in animals and humans has been documented in a number of ethnobotanical surveys [16].

Toxicity studies of medicinal plants, *in-vitro* and *in-vivo* models are available. Regardless of the type of extract, the parts of plant used, the concentration of the extract, the mode of administration, and the organism under consideration, the lethal dose/concentration 50 (LD/LC50), defined as ; the dose/concentration that can kill 50% of a tested population, is used to establish the toxicity of a certain plant extract [17].

The use of an *in vivo* approach facilitates the crucial understanding of how chemicals affect metabolic complex at the cell and organ level. Mice, rats, rabbits, and other laboratory mammals have been used extensively to study chemical toxicity. However, due to the large amounts of toxic test chemicals, the *in vivo* assays with those species are often expensive and are difficult to use in a moderate-throughput to high-throughput manner. Those drawbacks have led toxicologists to develop alternative animal models for chemical testing. Many alternative test organisms share biological processes with rodents and other mammals, including humans. Three test platforms that can be adapted to high-throughput screening is the use of insect, nematode and zebrafish models [18]

Drosophila melanogaster, known colloquially as fruit fly, remains one of the most commonly used model organisms for biomedical science [12]. Due to the success achieved in the use of *D. melanogaster* in experimental studies, it has met the standard of the European Centre for the Validation of Alternative Methods (ECVAM) for: Reduction, Refinement and Replacement (3Rs) of laboratory animal usage [19]. For more than one hundred years, the low cost, rapid generation time, and excellent genetic tools have made the fly indispensable for basic research [12]. *D. melanogaster* is also a good model for studying toxicological effect on multiple generations owing to their short life cycle, valuable for the analysis of molecular mechanisms underlying genetic phenomena, behavior and development. Approximately 65% of human disease-causing genes are believed to have a functional homolog in flies [20] and a significant fraction of these homolog are expressed in *D. melanogaster* tissues that perform the function of the equivalent human tissue [21]. In addition to similarities in basic cellular structure and function, humans and *D. melanogaster* share pathways for intercellular signaling [22], learning

and behavior [23] etc. Fruit flies have the potential to be used for chemical-toxicity screens [24, 25].

The use of *M. indica* extract in the treatment of human diseases in the western part of Nigeria is on the increase and the dearth of information on the acute and sub-chronic toxicity of this plant is becoming a course of concern. Therefore, it is necessary to evaluate the toxicity of *M. indica* cold aqueous leaf extract by using *D. melanogaster* as an alternative method for studying this plant toxicological effect to humans

2 Materials and Methods

2.1 Plant Collection

The leaf of *M. indica* was collected from University Senior Staff Quarters, Jos, Plateau State, Nigeria. It was identified by a Plant taxonomist in plant science Department University of Jos. To make cold aqueous leaf extract, leaves were air dried using room temperature for 7 days, pulverized using mortar and pestle and extracted using 260g of dried leaf material and distilled water 1:10 w/v, the filtrate was concentrated to dryness using freeze dryer as described in our previous work [12]. The total percentage yield of the extract was calculated to be 11.7% and it was preserved in an airtight container and kept in the refrigerator for further works.

2.2 Animal Model

Drosophila melanogaster Harwich strain was obtained from the Africa Centre of Excellence in Phytomedicine Research and Development. Species Stock was maintained at constant temperature and humidity (23±1 °C; 60% relative humidity, respectively) under 12 h dark/light cycles. We used standard Drosophila medium composed of cornmeal medium (1% w/v),

brewer's yeast (2% w/v), agar and nipagin (0.08% w/v). The maximum average lifespan of flies used here (*D. melanogaster*, Harwich strain) is about 50-58 days, and about 50% of the flies generally die within 41-45 days. However, this can vary depending on a variety of factors such as diet composition and temperature (26).

2.3 Phytochemical Screening

Phytochemical examinations of *M. indica* cold aqueous leaf extract was carried out to determine the presence of metabolites such as alkaloids, flavonoids, phenols, saponins, terpenoids, tannins and glycosides according to standard methods [27]. Any visible change of color or precipitate formation was used as an indicative for the presence (+) or absence (–) of individual metabolites

2.4 Experimental Design

The Leaf extract of *M. indica* concentration, 7.5 mg, 15 mg 30 mg, 45 mg and 100 mg were prepared in 1000 µl of distilled water and 9g of diet given each concentration per 10g diet (7.5 mg/10 g diet, 15 mg/10 g diet, 30 mg/10 g diet 45 mg/10 g diet and 100 mg/10 g diet). Diet with 1000 µl of distilled water served as control. 20 adult flies were exposed to these concentrations for 7 days and 168 hrs LC_{50} acute concentration was evaluated. For sub-chronic (survival) evaluation, 2.5 mg, 5 mg and 10 mg concentration of *M. indica* cold aqueous leaf extract were prepared in 200 µl of distilled water and 9.8 g diet making a total of 10g (200 µl of distilled water + 9.8g diet) and were exposed to flies for 28 days. Five (5) Days-Short term exposure of *D. melanogaster* to the sub-chronic concentration (2.5 mg/10 g diet, 5 mg/10 g diet and 10 mg/10 g diet) was carried out to evaluate the Climbing performance, Acetylcholinesterase activity and fecundity of the exposed flies.

2.4.1 Determination of 168 hrs LC50

This study was carried out following the method described by Sayanti & Sumedha [28], with slight modification. 20 flies of age rang 1- 3 days were anesthetized, counted and exposed to graded concentration of *M. indica* cold aqueous leaf extract. The following Concentrations, 7.5 mg/10 g diet, 15 mg/10 g diet, 30 mg/10 g diet 45 mg/10 g diet and 100 mg/10 g diet for the first phase. The second phase concentration ranging from 500 mg, 1000 mg and 2000 mg/10 g diet. Each phase is for 168 hrs (7 days). Mortality reading was scored every 24 hrs interval for 7 days. The mortality rate was subjected to Probit analysis of Graphpad prism 7.04 for LC_{50} determination.

2.4.2 Survival of *D. melanogaster* Fed on *M. indica* Cold Aqueous Leaf Extract

In this experiment, 50 flies of both genders (1-3 days old) were exposed to 2.5mg/10 g diet, 5mg/10g diet and 10mg/10g diet of *M.* indica aqueous leaf extract in five replicates for 28 days as described by [29]. The number of live and dead flies was scored daily till the end of the experiment and the survival rate was expressed as percentage of live flies. Short term exposure of sub chronic concentration was also set up for 5 days in triplicates, 50 adult flies each and were used for climbing assay activity, fecundity assay and Homogenized in 0.1m PBS pH 7.4, centrifuged using cold centrifuge. The supernatant was used for determining Acetylcholinesterase activity of the exposed flies.

2.4.3 Negative Geotaxis (Behavioral Assay)

The locomotor (Climbing) performance of *Mangifera indica* aqueous leaf extract exposed and control flies were investigated using the negative geotaxis assay [29]. Briefly, ten (10) *M. indica* cold aqueous leave extract exposed and controlled flies were immobilized under mild ice

anesthesia and placed separately in labeled vertical glass columns (length, 15 cm; diameter, 1.5 cm). After the recovery period (about 20 min), the flies were gently tapped to the bottom of the column. Following 6 s, the numbers of flies that climbed up to the 6 cm mark of the column, as well as those that remain below this mark were recorded. Data was expressed as the percentage of flies that escaped beyond the 6 cm mark in 6 s. The score of each group is an average of three trials for each group of treated and controlled flies.

2.4.4 Emergence Assay

Five virgin *D. melanogaster* females with five males were collected as described by Rauser, *et a*l., [30]. They were fed on *M. indica* cold aqueous leaf extract mixed with diet for 5 days. Males and female were treated separately (separate vials) for exposure period of 5 days. They were combined and allowed to mate on a normal diet without an extract for 24 hours. After 24 hours the adult flies were transferred off diet and sacrificed. The vials were maintained at constant temperature and humidity (23±1 °C; 60% relative humidity, respectively) under 12 h dark/light cycles for the development of eggs for 12 days. The number of emerged adults was recorded.

2.4.5 Acetylcholinesterase (AChE) activity

The 5 days *M. indica* cold aqueous leaf extract-treated flies, were anesthetize on ice, homogenized with 100mM phosphate buffer saline pH 7.4 and centrifuged using 4^{o}C Cold centrifuge for 10 min at 3000 rpm. The supernatant was collected and used for the determination of AChE activity following the method described by Ellman *et al*. [31] with slight modification. To the reaction mixture containing 285 µl of distilled water, 180 µl of 100 mM potassium phosphate buffer (pH 7.4), 60 µl of 10 mM DTNB, and 15 µl of sample, 60 µl of 8 mM

acetylcholine was added. The change in absorbance was monitored at 412 nm for 2min at 15 s intervals, using a UV-VIS Spectrophotometer (Jenway). The data were calculated against blank, sand sample blank and the results were corrected by the protein content. The enzyme activity was expressed as mmol/min/mg of protein**.**

2.5 Statistical analysis

The data was expressed as mean±SEM (standard error of mean), and the statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Turkey's post-hoc test and Log-rank (Mantel-Cox) test (curve comparism) and Dose-response-$LC_{50.}$ Graph pad Prism 7.04 statistical package was used. The results were considered statistically significant at $p < 0.05$.

3 Results

3.1 Qualitative Phytochemical Screening

Phytochemical examinations of *M. indica* cold aqueous leaf extract revealed the presence of alkaloids, phenols, flavonoids, tannins, saponins and terpenoids while glycosides were absent.

3.2 168 hrs LC_{50}

The mortality rate of *M. indica* cold aqueous leaf extract was 4% and 95% at 0 mg/10 g diet (control) and 100 mg/10 g diet respectively. The 168 hrs LC_{50} was evaluated to be 72.4 mg/10 g diet (df=8) with 95% confidence limit at 7.561 mg/10 g as shown in figure 1.

3.3 28 Days (Sub-Chronic) Survival

The survival proportion of flies treated with sub-chronic concentrations, 2.5 mg/10 g diet, 5 mg/10 g diet and 10 mg/10 g diet after 28 days ranged from 2-30%. The highest survival proportion was recorded in the controlled flies while the least was recorded in 10 mg/ 10 g diet.

At 2.5 mg/ 10 g diet and 5 mg/10 g diet, the survival proportion was recorded to be 27 % and 7 % respectively. There was significant difference ($p<0.05$, dF=3) comparing the survival curve of the treated flies to the control survival curve. Log-rank (Mantel-Cox) test p value < 0.0001.The result is presented in figure 2.

3.4 Climbing Activity

The negative geotaxis of *M. indica* cold aqueous leaf extract-treated flies ranged from 62-71 %. The highest climbing activity was recorded in the controlled group while the least was recorded in 10 mg/10 g diet. The climbing performance of flies treated with 2.5 mg/10 g diet and 5 mg/10 g diet was recorded to be 70% and 68% respectively. There was no significant difference ($p>0.05$, df= 3) comparing the climbing activity of the treated flies to control flies. The result is presented in figure 3

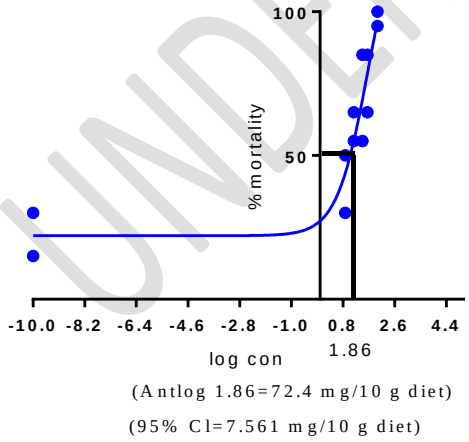

Fig 1: LC_{50} of *M. indica* cold aqueous leaf extract in Flies

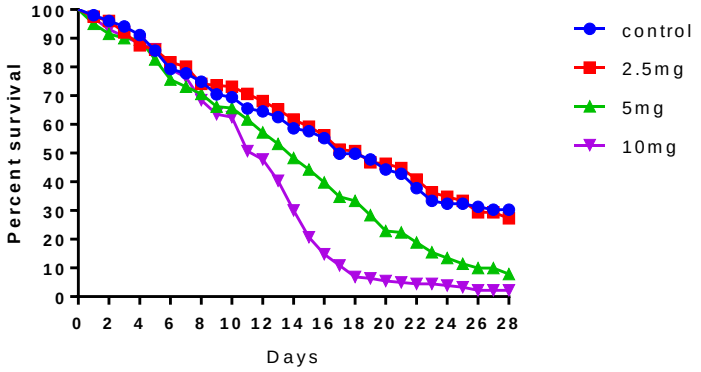

Fig 2: 28 Days (Sub-Chronic) Survival of *M. indica* cold aqueous leaf extract treated flies

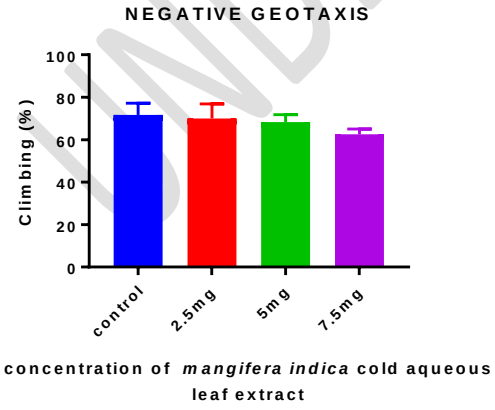

Fig 3: The climbing performance of *M. indica* cold aqueous leaf extract-treated flies.

3.5 Emergence Assay

The number of adult flies emerged from the egg laid by *M. indica* cold aqueous leaf extract-treated flies were used as a measure of reproductive fitness. The number of eggs laid ranged from 8-25 while the emerged flies ranged from 7- 18. The highest number of emergence was recorded in the control while the least number was recorded in 10 mg/10 g diet. 17 and 11 emerged adult flies from 20 and 13 eggs were recorded in 2.5 mg/10 g diet and 5 mg/10 g diet respectively. There was no significant difference ($p>0.05$, $df=3$) comparing the fecundity performance of the treated flies to the controlled flies. The result is presented in figure 4.

3.6 Acetylcholinesterase Activity

Acetylcholinesterase activity of flies treated with *M. indica* cold aqueous leaf extract ranged from 1.3±0.16-2.21±0.64 umol/min/mgprotein. The highest activity was recorded in 2.5 mg/10 g diet while the least activity was recorded in the controlled flies. Acetylcholinesterase activity of 5 mg/10g and 10 mg/10 g diet treated flies was recorded to be 2.21±0.47 and 1.94±0.38 µmol/min/mgprotein respectively. There was no significant difference ($p>0.05$, $df=3$) comparing the AChE activity of the treated flies to the controlled flies. The result is presented in figure 5.

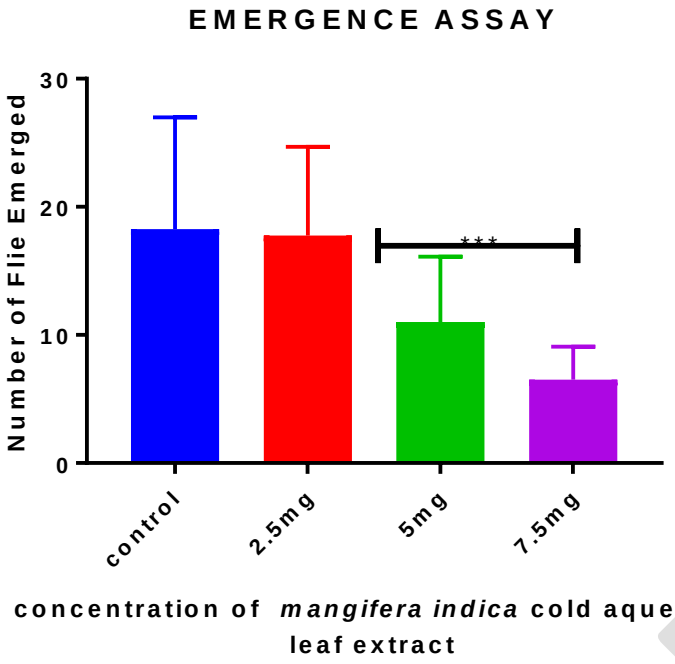

Fig 4: The number of emerged adult flies from the eggs of *M. indica* cold aqueous leaf extract-treated flies.

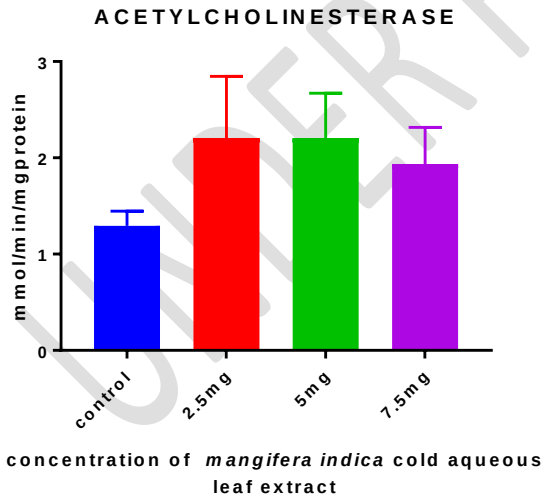

Fig 5: Acetylcholinesterase Activity of *M. indica* cold aqueous leaf extract treated flies

4.0 Discussion

Several processes have been used for the isolation of phytochemicals from the natural products. Some of these processes are milling, grinding, homogenization and extraction. It has been reported that extraction is one of the important processes for separating phytochemicals from natural products [32]. The extraction effectiveness is affected by the chemical nature of phytochemicals, the extraction method used, sample particle size, the solvent used, as well as the presence of interfering substances [33]. It is important to depict the chemical nature of plant materials when their pharmacological activities are evaluated. *M. indica* cold aqueous leaves extract revealed the presence of alkaloids, tannins, saponins, terpenoids, flavonoids and phenols as secondary metabolites. These secondary metabolites are reported to have many pharmacological properties, so this species is expected to have many medicinal uses [34]. Different alkaloids are contained in many plants etc which are soluble in water and toxic to herbivorous insects. Many authors reported that the aqueous extract of plants has significant effect on insect mortality, their growth and reproduction [35-40]. From our result, the 100% acute concentration was found out to be 100 mg/10g diet while the 168 hrs LC_{50} was found to be 72.4 mg/10 g diet, confirming the toxicity of Mango leaves extract toxicity in insect. This might be due to some of these phytochemical components that are known to be toxic even at lowest concentration. The survival study (sub-chronic) of flies in our present study, shows high survival of flies in the control treatment while there was gradual decrease in the survival of fliess along concentration gradient (from 2.5 mg/10 g diet to 10 mg/10 g diet) and this might be traceable to some of these phytochemicals in *M. indica* cold aqueous leaf extract. Fouad *et al.*, [41], reported that the plant extracts possess broad spectrum toxic substances (toxic alkaloids, tannins etc.) that interrupt insect's normal physiology and influence on their feeding and mortality.

Negative geotaxis is the measure of how quickly a fly is able to climb vertically after being tapped to the bottom of a vessel as part of its innate escape response. Negative geotaxis is measured by either the distance an animal is able to climb in a set time or the length of time it takes an animal to climb a set distance. Negative geotactic ability has been shown to be sensitive to oxidative stress, age, and cold exposure [42–45]. The geotactic ability of flies is impaired under abnormal conditions. From the result, there was a slight decrease in the climbing activity of *M. indica* cold aqueous leaf extract fed flies along concentration but statistically, there was no significant difference ($p>0.05$, $df=3$). This may be due to the alteration of the diet compositions with *M. indica* cold aqueous leaf extract. Linderman, *et al*., [46] showed a decline in negative geotactic ability in *L. monocytogenes* infected Oregon-R flies. Impaired climbing and flight behaviour in *D. melanogaster* following carbon dioxide anesthesia was a work carried out by Bartholomew, *et al*.,[47], who reported poor climbing performance of flies at 5 min, 10min and 30 min Co_2 exposure. The climbing performance was worse with increase in exposure time or period. This negative geotactic ability has been useful in toxicological evaluation of toxic or poisonous plants, herbicide and neurotoxin.

Further, we sought to know if *M. indica* cold aqueous leaf extract could affect the acetylcholinesterase (AChE) activity in the flies. Acetylcholinesterase is an enzyme that hydrolyzes acetylcholine at the post synaptic cleft. Acetylcholinesterase (AChE) is a key enzyme in the nervous system [48]. It terminates nerve impulses by catalyzing the hydrolysis of neurotransmitter acetylcholine. As a specific molecular target of organophosphate and carbamate pesticides, acetylcholinesterase activity and its inhibition have been early recognized to be a human biological marker of pesticide poisoning [48-50]. The success of this biomarker arises from the fact that it meets a number of characteristics necessary for the successful application of

a biological response as biomarker in human bio-monitoring: the response is easy to measure, it shows a dose-dependent behavior to pollutant exposure, it is sensitive, and it exhibits a link to health adverse effects [48-52]. AChE activity determination is a sensitive marker of neurotoxicity, since inhibition of its activity can be an indicator of poor locomotor activity [53] and general toxicity [54]. From the result, we noticed significant increase in AChE activity which might have enhanced the climbing performance of the treated flies above 60% but there was no significant difference ($p>0.05$, df=3) compared to the controlled flies. Similar to our result, a high activity of AChE, was reported by Agarwal *et al*., [55], and dePeyster *et al*.,[56], and these authors suggested that it may inhibit the action of acetylcholine in the uterus to protect the pregnancy, thereby supporting fertility in female.

We sought to understand the impact of *M. indica* cold aqueous leaf extract on flies fecundity and development. It has been reported that high activity of acetycholinesterase plays important role in female fertility by catalyzing the hydrolysis of high concentration of acetylcholine in the uterus protecting pregnancy [56]. In environments where nutrients are limited, there is a negative correlation between female fecundity and resistance to bacterial infection in *D. melanogaster* [57]. Immune challenged females not only have fewer offspring, but those offspring also have shorter lifespan compared to the offspring of unchallenged female *D. melanogaster* [58] Reproductive fitness is a broad, ecological measure of health. Our result showed fewer offspring in the 5mg and 10mg *M. indica* cold aqueous leaf extract-treated flies compared to the controlled flies. In these 5 mg/10 g diet and 10 mg/10 g diet concentration some pupa did not emerged even after 12days of life cycle. Many authors reported that the aqueous extract of plants has significant effect on insect mortality, their growth and reproduction [35-40].

This may be due to oxidative stress induced by toxic phytochemicals present in this extract at these concentrations.

4.1 Conclusion

M. indica is commonly used in herbal medicine for one infection or the other and some authors have documented that it is one of the safe medicinal plants with no record of LD/LC_{50}. From our study, was recorded mortality of flies exposed to mango leaves extract. This may be due to the presence of alkaloids and tannins in the aqueous extract. The 168hrs LC_{50} was found to be 72.4 mg/10 g diet in *D. melanogaster*. The sub chronic result showed decrease in fly's survival along concentration of mango cold aqueous leaves extract with a least survival recorded in 10 mg/10 g diet. Were also observed the slight decrease in the climbing activity, elevated AChE activities, the delay in the development and eclosion of flies in 5 mg and 10 mg/10 g diet From this finding, *M. indica* cold aqueous leaf extract might not be safe for long term consumption.

4.2 Recommendation

It is important to carry out the genetic study of *D. melanogaster* fed on diet with *M. indica* cold aqueous leaf extract so as to draw a final conclusion on its toxicity in this species

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