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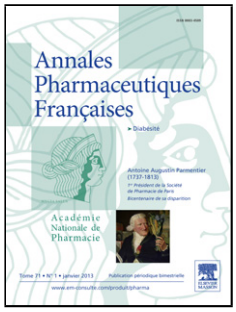

TOXICITY OF MANGIFERA INDICA AQUEOUS STEM BARK EXTRACT EVALUATED IN DROSOPHILA MELANOGASTER USED AS MODEL ORGANISM

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Running Title: Toxic Effect of *Mangifera indica* on *Drosophila melanogaster*

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Running Title: Toxic Effect of *Mangifera indica* on *Drosophila melanogaster*

Summary

Background: *Mangifera indica* has been used for treating health complications with little data on its toxicological impact on survival, geotaxis, reproduction, and the antioxidant system.

Methods: Total phenol and flavonoid contents were estimated. The ingestion method of exposure (extract was mixed in flies' food) was used. Each concentration was administered per 10 g fruit flies diet. 7-day LC_{50} was determined by exposing 50 flies for 7 days to *Mangifera indica* concentration ranging from 100 mg extract /10 g diet to 2000 mg extract/10 g diet. 28 days survival assay was performed by exposing 50 fruit flies each to 25 mg extract/10 g diet, 50 mg extract/10 diet g, and 100 mg extract/10 g diet for 28 days. A 6-day short term exposure was also conducted to assess *Mangifera indica* toxic effect on climbing activity, survival, reproduction, and antioxidant system in *Drosophila melanogaster*.

Results: Total phenol and flavonoid content were 0.226±0.02 and 0.027±0.05mg/g dry weight of the extract, respectively. There was a significant mortality rate ($P<0.05$), and the 7-day LC_{50} was 353 mg extract/10 g diet. At 25 mg extract/10 g diet 50 mg extract/10 g diet and 100 mg extract/10 g diet, the survival-rate of fruit flies significantly dropped ($P<0.05$) with arise in *Mangifera indica* concentration. Short-term exposure also showed a significant reduction ($P<0.05$) in GST-activity, survival-rate, and emergence of young fruit flies with an increase in concentration. Total thiol, locomotor, AChE, and CAT activities decreased non-significantly ($P>0.05$).

Conclusion: The significant adverse effect of *Mangifera indica* extract as seen in the decrease in survival rate, the emergence of young flies, climbing, and antioxidant activities of fruit flies suggests its cautious application and use in herbal medicine.

Keywords: *Mangifera indica;* Toxicity; *Drosophila melanogaster*; Survival; Reproduction; Antioxidant System

Résumé

Contexte: Mangifera indica a été utilisé pour traiter les complications de santé avec peu de données sur son impact toxicologique sur la survie, la géotaxie, la reproduction et le système antioxydant.

Méthodes: Les teneurs totales en phénol et en flavonoïdes ont été estimées. La méthode d'exposition par ingestion (l'extrait était mélangé dans la nourriture des mouches) a été utilisée. Chaque concentration a été administrée par régime alimentaire de 10 g de mouches des fruits. La CL50 sur 7 jours a été déterminée en exposant 50 mouches pendant 7 jours à une concentration de Mangifera indica allant de 100 mg d'extrait / 10 g de régime à 2000 mg d'extrait / 10 g de régime. Un test de survie de 28 jours a été réalisé en exposant 50 mouches des fruits chacune à 25 mg d'extrait / 10 g de régime, 50 mg d'extrait / 10 g de régime et 100 mg d'extrait / 10 g de régime pendant 28 jours. Une exposition à court terme de 6 jours a également été menée pour évaluer l'effet toxique de Mangifera indica sur l'activité d'escalade, la survie, la reproduction et le système antioxydant chez Drosophila melanogaster.

Résultats: La teneur totale en phénol et en flavonoïdes était de 0,226 ± 0,02 et de 0,027 ± 0,05 mg / g de poids sec de l'extrait, respectivement. Il y avait un taux de mortalité significatif (P <0,05) et la CL50 à 7 jours était de 353 mg d'extrait / 10 g de régime. À 25 mg d'extrait / 10 g de régime, 50 mg d'extrait / 10 g de régime et 100 mg d'extrait / 10 g de régime, le taux de survie des mouches des fruits a chuté de manière significative (P <0,05) avec la concentration de Mangifera indica. Une exposition à court terme a également montré une réduction significative (P<0,05) de l'activité de la GST, du taux de survie et de l'émergence de jeunes mouches des fruits avec une augmentation de la concentration. Les activités totales de thiol, locomoteur, AChE et CAT ont diminué de manière non significative (P> 0,05).

Conclusion:L'effetindésirablesignificatif de l'extrait de Mangiferaindicatelque vu dans la diminution du taux de survie, l'émergence de jeunesmouches, les activitésgrimpantesetantioxydantes des mouches des fruits suggère son application et son utilisationprudentes en phytothérapie.

Mots clés: *Mangifera indica*; Toxicité; *Drosophila melanogaster*; Survie; La reproduction; Système antioxydant

Introduction

Drosophila melanogaster had been validated earlier in the 21st century as a model system for immunity research after studying its genome showed compelling complexity and likeness to the innate immune system in mammals [1,2]. For over a century, the inexpensive, quick propagation period, and awesome genetic resources made the fly central to basic research; and its application with numerous molecular tools allowed it to keep up with the latest advances [3].

D. melanogaster is an arthropod belonging to the family *Drosophilidae*. For about 100 years now, this insect has been an indispensable model in the field of biology. This model has been used in genotoxicity [4] and as a potential model for studying systemic toxicology or an alternative model for studying toxicology [5]. *D. melanogaster* as a model is also widely used in biochemistry, cell biology, and developmental biology. The routes of exposure in toxicology study using adult *D. melanogaster* are inhalation, injection, and ingestion. The ingestion method of exposure is carried out by mixing the test sample with a substrate that tastes well as sucrose or yeast paste [5].

Mangifera indica is a common and well-known plant in the tropical and subtropical regions around the world with many varieties; serve as sources of fruit while its other parts (leaf, stem bark, kennel, and root) are reservoirs of many photochemical compositions with medicinal activities. The traditional application of *M. indica* for therapeutic purposes includes; gastrointestinal (dysentery, piles, constipation), respiratory (bronchitis, asthma), genitourinary (urinary and vaginal discharges), and ophthalmic diseases[6]. It is also globally employed as a

laxative, tonic, aphrodisiac, appetizer, and diuretic [6,7]. Interestingly, this plant has been used for treating several health complications with inadequate information on its toxicological impact on life span and other physiological processes. Hence, this research aims to investigate the toxicity impact of cold aqueous extract of *M. indica* stem bark in *D. melanogaster* antioxidant system, survival, and locomotor activity (AChE and Negative geotaxis).

Materials and methods

Reagents

Analytical grade chemicals were utilized all through this experiment. Acetylcholine iodide, DTNB (5,5′-dithiobis (2-nitro-benzoic acid)),GSH(reduced glutathione), and CDNB (1-chloro-2,4-dinitrobenzene,5,5) were procured from Sigma Aldrich (USA).While ethylenediaminetetraacetic acid (EDTA), potassium hydrogen phosphate (K2HPO4), Randox protein kit, and sodium dihydrogen phosphate (NaH2PO4. 2H2O) were sourced from Chidex Surgical Suppliers Limited, Nigeria.

Plant Collection and Extraction

M. indica stem bark was obtained from the University of Jos, Nigeria. *M. indica stem bark* was collected from the senior staff quarters of the University of Jos, Nigeria, rinse off of the sand and some particles under running tap water. It was further air-dried using room temperature for 7 days, then the stem bark surface areas were increased using mortar and pestle. 360 g of pounded stem bark of *M. indica* was extracted in distilled water for 72 hours, filtered and the filtrate was freeze-dried using a freeze drier as described by Alexander et al. [8]. The percentage total yield was calculated using the formula below [9].

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

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.

D. melanogaster Model

The *D. melanogaster* strain (Harwich) was supplied by the National Species Stock Center (USA). They were grown at the fruit fly laboratory, ACEPRD (University of Jos, Nigeria), and cultured following the technique used by Abolaji et al. [10]. The flies diet is composed of cornmeal medium (1% w/v), brewer's yeast (2% w/v), agar and nipagin (0.08% w/v)[10].

Phytochemical screening of cold aqueous extract of Mangiferaindicastem bark

Estimation of Total Phenolic Content (TPC)

Phenolic concentration in the plant extract was estimated using the Folin- Ciocalteu technique as defined by Behera [11] with little modification. A 20 µL of methanol extract (1mg/ml), 7% of 100 µL of Folin- Ciocalteu reagent, and 1.58 mL distilled water were combined. We waited 8 minutes, and then added 10% of the 300 µL saturated sodium carbonate solution (250 g/L). At room temperature, the solution was incubated for 2 hr, and absorbance was taken at 765 nm in triplicate. As normal, gallic acid was used and the whole phenolic composition was expressed as an equal milligram of gallic acid (mgGAE/g) of plant extract dry weight.

Estimation of Total Flavonoid Content (TFC)

The whole composition of flavonoids was estimated via the spectrophotometric method as described by Behera [11]. The crude extract consists of 1 ml of the extract's methanol solution at

a concentration of 1mg/ml alongside 1 ml of 2% $AlCl_3$ solution melted in methanol. The solutions were covered at room temperature for 1 hour. Furthermore, we estimated the absorbance in triplicate at 415 nm. For the regular solution of rutin, the same protocol was used. The flavonoid content was measured in terms of the dry weight of plant extract at rutin equivalent (mg RUE / g).

7-days LC_{50} and 28-days Survival Assay

Determination of 7-days LC_{50} of the plant extract was tested employing the technique defined by Charpentier et al. [12] via the ingestion method of exposure. 50 flies were counted by anesthetizing on ice chips and treated with 100 mg extract/10 g diet, 250 mg extract/10 g diet, 500 mg extract/10 g diet, 1000 mg extract/10 g diet, and 2000 mg extract per 10 g diet. Each of these concentrations was mixed with a 10 g fruit flies diet. Mortality reading was taken every 24 hours for 7 days.

A survival study was performed employing the method of ingestion as demonstrated by Abolaji et al. [10]. In triplicates, 50 flies were treated with concentrations below 7-day $LC_{50,}$ 25 mg extract/10 g diet, 50 mg extract/10 g diet and 100 mg extract per 10 g fruit flies diet. During this experiment, relative humidity and temperature were maintained, while mortality reading was attained at 28 days every 24 hours.

Six(6)- days Treatment of D. melanogaster with M. indica

Short exposure of *D. melanogaster* was conducted as mentioned by Abolaji et al. [10] via the ingestion method of exposure. Treatment duration was determined from the survival study; which corresponds to a day with a 70 % survival proportion in the highest concentration, 100 mg extract/10 g diet. Fifty flies were counted with mild ice anesthesia and treated via ingestion

method of exposure with 25 mg extract/10 diet, 50 mg extract/10 g diet, and 100 mg extract/10 g diet For 6 days, the reading of mortality was taken every 24 hours.

The number of flies that survived were subjected to negative geotaxis assay and later sacrificed, homogenized utilizing 0.1 M phosphate buffer saline (7.4 pH), centrifuged (using a cold centrifuge, 4^{o}c), and the supernatants were collected and used for total thiol content quantification, acetylcholinesterase, catalase, and glutathione-s-transferase activities.

Locomotor Activity (Negative Geotaxis and Acetylcholinesterase activity)

Negative Geotaxis (measured by the % of climbing activity)

Negative geotaxis is a behavior in *D. melanogaster* that reveals the wellness of the locomotor system of this organism. Negative geotaxis was conducted employing the technique demonstrated by Abolaji et al [10]. 10 flies (both gender) were counted from both the extract-treated flies and the control group for six (6) days. They were then introduced in the labeled vertical glass columns of diameter and length 1.5 cm and 15 cm respectively.

Furthermore, the flies were softly knocked to the base of the glass column after recovery (approx. 20 min). The number of flies that went up to the column's 6 cm mark was registered after 6 s along with those that remain below this level. The data were shown as the proportion of flies that fled in 6s beyond the 6 cm limit(% climbing activity)

Acetylcholinesterase Activity

Acetylcholinesterase, an enzyme that plays a vital role at a neuromuscular junction is highly implicated in *D. melanogaster* locomotor activities. AChE activity was evaluated with minor modifications based on the Ellman method as defined by Alexander et al. [8]. Also, the reaction

mixture, absorbance of the sample, sample blank, and shift in absorbance were all performed using the technique used by Alexander et al. [8]. Data obtained were measured against the blank and sample blank, while protein content was used to correct the results. The enzyme activity had been expressed as protein mmol/min/mg.

In vivo Antioxidant Activities (GST, Total thiol, and Catalase Activities).

Plasma Protein Estimation

The total plasma protein of the samples was determined using the Bradford method [13]. The Randox protein assay kit was used to carry out this assay.

Total thiol determination

This was assessed based on the Ellman method demonstrated by Abolaji et al. [10]. The preparation of the assay reaction mixture and absorbance measurement employed to determine total thiol levels in the sample (in mmol/mg protein) followed the procedure used by Etuh et al. [3].

The activity of Glutathione-S-transferase (GST)

We calculated glutathione-S-transferase activity following Habig and Jacoby's method as defined by Etuh et al. [3]. The preparation of the assay reaction mixture and absorbance measurement used to determine GST activity in the sample followed the procedure used by Etuh et al. [3]. The results were presented in mmol/min/mg protein.

The activity of Catalase (CAT)

A method described by Aebi [14] was employed to test catalase activity. Solution A was prepared using 194 mL of 300 mM H_2O_2 and 100 mL of potassium phosphate buffer (pH 7.0). 10 µL of the sample mixed with solution A of 590 µL and H2O2 clearance was controlled at 240 nm and 25°C. The fall in H_2O_2 was supervised by a UV-visible spectrophotometer (Jenway) for 2 min at 240 nm and 10 s intervals and presented as mmol of H_2O_2 used/min/mg protein.

Reproductive Toxicity Assay (Emergence/Eclosion Assay)

This was achieved using the procedure described by Patlollaet al. [15]. Fifty (50) virgin male and female flies each were treated with the extract for 6 days. Five virgin males and females each were placed into a vial containing a normal diet and allowed for 24 hours of mating. The flies were removed from the diet, and the vials were examined for laid eggs under a light microscope and held at the correct temperature and relative humidity for 14 days so that adult flies could emerge.

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) with the aid of GraphPad Prism statistical software (version 7.04). All results at $P<0.05$ were held statistically significant.

Results

Phytochemical screening and Percentage Total Yield

The percentage total yield of the extraction was determined to be 15.8 %. The total phenol and flavonoid content was determined to be 0.226±0.02 and 0.027±0.05 mg/g dry weight of the extract, respectively

7-day LC_{50} and 28-days Survival curve

The extract significantly killed ($P<0.05$) the flies as compared to control, with 100 % mortality reported in 2000 mg extract/10 g diet. As shown in Figure 1, the LC50 was determined to be obtained for treatment with a 353 mg extract/10 g diet. As seen in Figure 2, the proportion of flies treated with *M. indica* crude stem bark extract for survival decreased significantly ($p<0.05$) for all the treated flies relative to control.

Six(6)- days Treatment of D. melanogaster with M. indica Stem Bark Extract

The short-term exposure or treatment of flies with an aqueous extract of *M. indica* stem bark also presented a significant fall in the survival ratio ($P<0.05$)relative to the control group as shown in Figure 3.

Locomotor (Climbing) and AChE Activities

The extract decreased the climbing activity of flies without a significant difference compared to the control ($P>0.05$), while AChE activity sharply decreased in flies treated with aqueous extract *M. indica* stem bark without significant difference ($P>0.05$) as shown in Figure 4.

In Vivo Antioxidant Activities

The extract decreased the total content of thiol and catalase activity without a significant difference relative to the control ($P>0.05$), while the activity of GST in the extract-treated flies significantly decreased ($P<0.05$) relative to the control (Figure 5).

Reproductive Toxicity Assay

Our findings demonstrated a significant effect ($P<0.05$) of *M. indica* stem bark extract on eggs laid, alongside the growth of these eggs to larva, pupa, and finally to adult. The number of eggs laid by flies in the control group was more than those in the treated group. 100 % of the total eggs laid emerged to adult flies in the control group, while 20 %, 15 %, and 16 % of the eggs laid in 25 mg extract/10 g diet, 50 mg extract/10 g diet, and 100 mg extract/10 g diet emerged to adult flies respectively as shown in Figure 6.

Discussion

Plant extracts can be beneficial or detrimental to a living organism either by ingestion, inhalation, or contact; the detriments of ingesting toxic plants are many ranging from malfunctioning of the system, immune-suppression, and short life span, while the benefits of some plant extracts range from being protective, medicinal (preventive and curative activity) to longevity activity [16]. A plant extract with low LC_{50} is known to be very toxic while that with high LC_{50} is known to be less or non-toxic [17].

Findings from this study show that the 7-days LC_{50} of the extract (concentration that can kill 50 % of the test fruit flies) was 353 mg extract/10 g diet, while 28 days survival result showed a significant fall in survival ratio of *M. indica* extract-treated flies relative to the controlled flies ($P<0.05$). 7-day LC_{50} and 28-day survival results throw new light on the negative impact of the plant extract in *D. melanogaster*. However, this finding disagrees with the findings from studies conducted by Amien et al. [18] and Prado et al. [19] who reported the safety of this plant but agrees with Alexander et al. [8]. Previous studies report incidences of acute poisoning of persons who later died mainly due to ingestion of medicinal plants with toxic substances [20].

Also, we tried to know the impact of the plant extract on the AChE and climbing activities in *D. melanogaster*. AChE and climbing activities of fruit flies are impaired under abnormal conditions such as oxidative stress, cold and toxic substances exposure as reported in a previous study [8]. AChE activity remains a prime marker of neurotoxicity, as its' inhibition may indicate poor locomotive operations and common toxicity [21]. Furthermore, this research demonstrated that *M. indica* stem bark extract non significantly inhibited ($P>0.05$) AChE and climbing activities when compared to control. Few studies reported that climbing activity is directly proportional to AChE activity; and that the higher the AChE activity, the higher the climbing activity of fruit flies [3,22]. Our results agreed with findings obtained from the studies conducted by Alexander et al. [8] and Abolaji et al. [10] which suggested that reduction in AChE activity was responsible for behavioral and locomotor changes in flies exposed to toxic substances.

We further explored the antioxidant activities (*in vivo*) of the plant extract in *D. melanogaster*; our results indicated a decrease in GST, CAT activities, and whole thiol content relative to control. To the best of our knowledge, our finding is the first to propose that the plant extract used in this study interferes with the antioxidant system of fruit flies, and this may be responsible for the fall in fruit flies' survival proportion as indicated in Figure 2. Antioxidants usually combine with free radicals and counteract them, thereby stopping cellular damage in the biological system [10]. Flavonoids and phenols are polyphenolic compounds that express several biological properties including; antioxidant, anti-cancer, anti-inflammatory, anti-viral, and anti-hepatotoxic effects [23].

We also experimented to examine the reproductive ability of fruit flies fed a diet with the plant extract. A good state of reproductive fittingness is a broad, environmentally sound health measure. In this present study, the emergence of young flies from the extract-treated flies was

employed to measure reproductive capability. Our result revealed a significant reduction in the emergence of young flies from the extract-treated flies relative to the control ($p<0.05$). A low concentration of AChE leads to a high concentration of acetylcholine (ACh), which in turn causes conception failure in the uterus [24]. The AChE in the luteal cells of the ovary hydrolyzes acetylcholine in the production of acetic acid, which is used subsequently in the pathways to produces teriodogenes for hormone production [25]. The decrease in the number of emergent flies may also be linked to oxidative stress [8]. It has been reported that immune challenged females not only have a shorter life span but fewer offspring [26]. The decreased AChE activity and the emergence of young flies of extract-treated flies might be an indicator of a lack of steriodogenes.

Conclusion

From our findings, it can be inferred that the aqueous extract of *Mangifera indica* stem bark decreases *D. melanogaster* GST significantly ($p<0.05$), Total thiol, and Catalase non-significantly ($p>0.05$). This extract also decreases the climbing activities (acetylcholinesterase and negative geotaxis), survival rate, and young flies' emergence rate from eggs of adult *D. melanogaster* administered aqueous stem bark extract. Due to the similarities in the conservation of genes and biochemical pathways that exist between humans and fruit flies, we hereby recommend that caution be exercised regarding the general use of this medicinal plant in humans to avoid some adverse conditions.

Conflict of interest

The authors declare no conflict of interest.

Ethical approval

This study obtained approval from the Ethical Review Board, University of Jos, Plateau State, Nigeria.

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