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Safety Assessment and Antioxidant Enhancing Activity of Polyphenolic-rich Extract of *Illicium verum* (Star Anise) Fruit in *Drosophila melanogaster*

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ABSTRACT

Polyphenols are organic compounds found in plants and are beneficial to health. They have great pharmacological properties; however, they can be harmful in high doses. *Illicium verum* Hook.f ((Schisandraceae), star anise) is an oriental spice used in culinary and therapeutics. It is rich in polyphenols and has been used to manage several diseases, including obesity and diabetes. This study assessed the toxicological effect of the polyphenolic-rich extract of *Illicium verum* (PEIV) fruit on *Drosophila melanogaster* (fruit flies). Flies (n = 5 vials; 50 flies/vial) were distributed into five groups based on a graded dose (0, 0.25, 0.5, 1 and 2 mg/g) of a PEIV-supplemented diet for 21 days. On the 21st day, the flies' behavioural activity (geotaxis test) was assessed for the safety of PEIV on the flies, and thereafter, the whole flies were homogenised for biochemical functions (glucose (GLU), reduced glutathione (GSH), total thiol (TSH), glutathione-S-transferase (GST) and acetylcholinesterase (AChE. This study shows that PEIV has no significant effect on the locomotive ability of the flies. Also, when compared with the control, no significant difference ($p > 0.05$) was observed in the levels of GLU, GSH and TSH. In addition, compared with the control, the activities of GST and AChE were not affected. This study suggests that PEIV, at the tested doses, did not affect the behavioural and biochemical parameters in the flies and could be considered safe for therapeutic purposes and human consumption. However, a chronic evaluation study may be considered to confirm the findings of this study.

Keywords: Behavioural, *Drosophila melanogaster*, *Illicium verum*, Polyphenols, Toxicological.

Introduction

Medicinal plants have long been utilised to enhance the quality of life and prevent or treat diseases.[1] The use of herbal medicine has increased significantly worldwide. Approximately 80% of inhabitants of developing countries rely on self-medication using medicinal herbs to improve their health. Medicinal plants are efficacious, safe, readily available, and affordable.[2, 3] Plants contain rich phytochemicals, such as polyphenols, saponins, flavonoids, alkaloids, and terpenoids, which are responsible for their biological activities.[4] Plant phytochemicals are safer when compared with synthetic drugs in managing various diseases.[5] Unfortunately, the safety profile of most medicinal plants are not known. Therefore, safety evaluation studies are required to provide information on the potential adverse effects of medicinal plants under investigation and to identify the treatment doses needed for experimental studies. Polyphenols are a large group of compounds comprising one or more hydroxyl groups attached to one or more aromatic rings.[6] The hydroxyl groups are responsible for their biological and pharmacological activities.[7] Polyphenols are primarily found in fruits, herbs and vegetables and are generally classified as flavonoids (e.g., flavanols, flavanones, isoflavones, flavan-3-ol and anthocyanins) and non-flavonoids (e.g., phenolic acids, stilbenes, tannins, lignans, and xanthones). They serve a defensive role, protecting against reactive oxygen and nitrogen species. [8]

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Also, they negate free radicals by forming stabilised chemical complexes, which prevent further reactions. [9] Studies have shown that these compounds play a crucial role in health by regulating metabolism and cell proliferation and attenuating several disease pathologies.[10, 11] Over 8,000 polyphenolic compounds have been identified. Even though many of these compounds have demonstrated pharmacological properties, some have yet to be fully explored for their biomedical applications.[12] In recent times, the use of polyphenolic compounds have increased in managing diseases such as diabetes, cancer, hepatic, cardiovascular and neurodegenerative diseases because of the general belief that they are safe (from natural sources) and possess great antioxidant and anti-inflammatory properties.[13, 14] Some of these compounds act as good protective or therapeutic agents, while a few others, such as quercetin and tannic acids, act as pro-oxidants (phytotoxins) when ingested in high amounts, and can alter functional metabolic activities and enhance disease pathogenesis.[15, 16] Certain polyphenolic-rich beverages have been reported to cause high blood pressure, thyroid disease, heart and degenerative diseases.[17] In the pre-absorptive phase, dietary polyphenols reduce the transport of thiamine and folic acid, thus altering the activity of drugs via the drug transporters or enzymes.[18] For example, consuming an iron-rich diet with polyphenols can inhibit iron absorption, therefore limiting the iron-chelating effects of the diet.[19] A previous study reported that isoflavones, found in soy products, could adversely affect women with endometrial and oestrogen-sensitive breast cancers.[20] These findings have raised safety concerns about their short- and long-term use.[11, 18] *Illicium verum* (*I. verum* star anise) is an evergreen tree belonging to the plant family Schisandraceae. It is an aromatic plant found in China, Vietnam and some Southeast Asian countries. The fruit is used as a spice in Chinese cuisine and for medicinal purposes. [21] Unlike its other genus, Japanese star anise (*I. anisatum*), which is poisonous to humans, *I. verum* is non-toxic. It possesses excellent pharmacological properties.[22] Several studies have reported that the flower and seeds of the plant exhibit antiviral, anti-inflammatory, antioxidant, antimicrobial, immune-boosting and reproductive properties. [22-24] The plant is rich in polyphenolic compounds, especially flavonoids and phenolic acids, among which are shikimic acid, linalool, rosmaric acid,

quercetin, monoterpenoids, ferulic, phenylpropanoids (a derivative of anethole found in its essential oil) and sesquiterpenoids. [25, 26] The seeds contain shikimic acid, an active compound in oseltamivir (Tamiflu), a clinical drug used in treating H5N1 avian flu virus.[23] These compounds are responsible for the pharmacological potentials of the plant. Since a high amount of polyphenolic compounds in plants could result in phytotoxins, it is necessary to screen the polyphenolic-rich extract of this plant for its safety.

Drosophila melanogaster (*D. melanogaster* (fruit flies) from the Drosophilae family are animal models used in genetic research because their genome composition is 60% similar to humans.[27] About 75% of the genes causing human diseases have been reported for *D. melanogaster*.[28,29] In addition, fruit flies have fat bodies similar to the liver and adipose tissue in mammals, which are responsible for storing and mobilising energy as glycogen and fat which are used for metabolic processes.[30] Recently, concerns have been raised about using mammals like mice and rats in experimental research because of animal ethics welfare and cost of animal maintenance. Therefore, alternative models that adhere to the European Centre for the Validation of Alternative Methods 3Rs standard (replacement, refinement, and reduction) are considered to circumvent these concerns. This study investigated the toxicological profile of the polyphenolic-rich *I. verum* (PEIV) fruit on *D. melanogaster*.

Materials and Methods

Chemicals and reagents
Acetylcholine iodine and 1-choro-2,4-dinitrobenezene were products of Sigma Aldrich Chemicals, Co. (St. Louis, MO, USA). Sodium hydroxide, trichloroacetic acid, potassium chloride and sodium chloride were products of BDH Chemicals Ltd. (Poole, England). All reagents used in this study were of analytical grade.

Collection and identification of plant materials
The fruits of *I. verum* were obtained in October 2024 from Kaguru, Birnin Gwari Local Government, Kaduna State, Nigeria. Authentication and identification were done at the Department of Plant Science and Biotechnology at the Ekiti State University, Ado-Ekiti, Nigeria, by Mr F. O. Omotayo. A specimen of the plant was deposited under the reference number UHAE 2025010. The fruits were washed and dried in an aerated space away from direct sunlight for five days.

Extraction of plant material
The plant materials were blended to a fine powder. The polyphenolic-rich extract of the plant was produced using the method described by Ajila *et al.* [31] Briefly, 100 g of powdered material was extracted with 80% acetone for 24 h. The mixture was filtered, and the supernatant was concentrated in a rotary evaporator at 40 °C. Then, it was dried using a water bath at 40 °C. The sample was then kept at -20 °C until it was needed.

Drosophila melanogaster stock source and culture
The wild-type (Harwich strain) flies were obtained from Prof. A. Abolaji, Department of Biochemistry, University of Ibadan, Nigeria, October 2023. The flies were reared in Drosophila Laboratory, Department of Chemical Science, Afe Babalola University Ado-Ekiti, Nigeria, on corn meal medium (1% w/v brewer's yeast, 1% w/v agar, and 0.08% v/w methylparaben), maintaining a 12h dark/light cycle conditions, and at room temperature (25 ± 2 °C) and 60-70% relative humidity.

Flies grouping and drug dosing
Flies (both genders, 3-5 days old) were divided into five groups, each containing 50 flies per vial of 5 replicates. The flies were exposed to the diets fortified with the different doses of PEIV as follows: Group 1: Control (non-PEIV-supplemented diet), flies in groups 2, 3, 4 and 5 were exposed to 0.25-, 0.5-, 1- and 2 mg/g diet PEIV-supplemented diet, respectively for 21 days.

Longevity study
This study assessed the dietary inclusion of PEIV on flies' longevity. Flies in each vial were exposed to different dietary inclusions of PEIV as described above (flies grouping). The flies were observed daily for 21 days for behavioural changes and mortality. The longevity rate was determined by counting the number of dead flies, and the data were subsequently analysed and plotted.[32]

Locomotor (geotaxis) activity
The method of Adedara *et al.*[32] was used to assess the locomotor activity (negative geotaxis) of the control and PEIV-treated flies. The immobilised (in ice) flies were transferred to a clean and sterilised (70% alcohol) flat bottom 50 mL tube. The tubes were placed on a table for 10 min to allow the flies to recover from immobility. Flies were awakened from an inactive state by tapping the bottom of the tube. The locomotor activity of flies was recorded by counting the number of flies that passed a 6 cm mark on the tube within 6 s passed. Elevated values indicate the intermediate percentage of flies that cross the 6 cm barrier out of the total number of flies per experiment. Results are expressed as the percentage of flies that escaped from a minimum distance of 6 cm in 6 s.

Preparation of homogenate
On the 21st day of the experiment, flies were immobilised on ice and homogenised in 0.1 M phosphate buffer, pH 7.4, following the method described by Anadozie *et al*.[29] The homogenates were centrifuged at 4000 x *g* at 4°C for 10 min. The resultant supernatant was transferred into new Eppendorf tubes and stored until needed for biochemical assays.

Biochemical assays
Standardised methods were used to measure the following biochemical parameters: acetylcholinesterase (AChE) Ellman *et al.*[33], glucose Galant *et al*.[34], nitric oxide Green *et al.*[35], total thiol (TSH) and reduced glutathione (GSH) Ellman[36], glutathione-s-transferase (GST) Salinas and Wong[37] and catalase (CAT) Claiborne.[38]

Statistical analysis
A one-way analysis of variance was used to analyse data, followed by Tukey's post hoc test on GraphPad Prism 8.3 (GraphPad Software, Inc., San Diego, USA) for multiple comparisons across groups. Results were expressed as mean ± standard deviation. Statistical significance ($p < 0.05$) was considered in all cases.

Results and Discussion

Herbal medicines have a long history of use in managing various health challenges. They possess secondary metabolites responsible for their biological activities.[4] Although herbal-based products have therapeutic benefits, their constituents and safety remain a major concern.[39] Chemical compositions of most plants are altered during plant extraction, thus releasing toxic effects.[40] Medicinal plants or plant-based phytochemicals are often used without exploring their safety profiles; hence, evaluating their safety is important. *Illicium verum* is an ancient Chinese spice that has been used in both culinary and medicinal applications for centuries. The seeds and fruits of the plant have demonstrated great pharmacological properties, including cytotoxic, anti-inflammatory, sedative, antioxidant, and antimicrobial activities.[21, 24, 41, 42] To determine its safety in clinical applications, it was necessary to investigate the effect of repeated graded doses of PEIV on the flies. Several behavioural and biochemical parameters were investigated to assess the effect of the 21-day PEIV-supplemented diet on the flies.

A mortality test is essential in toxicity studies to study a substance's lethal potential. In this study, a longevity test was conducted on the flies to assess the effect of PEIV on their lifespan. Figure 1 shows the effect of graded doses of PEIV (0.25, 0.5, 1 and 2 mg/g of diet) on the survival rate of flies. Statistically, compared with the control, no significant difference ($p > 0.05$) in the mortality rate was observed in the PEIV-treated flies. The doses used in this study suggest the possibility of reducing mortality in humans with certain medical conditions and

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improving their quality of life. *Drosophila melanogaster* has a well-developed nervous system similar to other higher animals and, therefore, is suitable for studying higher animals, including humans' behavioural characteristics and endpoint genomic and ecological factors.[43] Locomotion is an important parameter used to measure the cognitive and motor functions of the central or peripheral nervous system.[44] This study examined the climbing ability of the *D. melanogaster* exposed to a PEIV-supplemented diet after 21 days (Figure 2). Compared to the control, no significant ($p > 0.05$) difference was observed in the %climbing ability of flies exposed to all doses of PEIV. The non-significant changes in the climbing pattern suggest that the PEIV at all doses tested did not alter the neuronal functions of the flies or the permeability of the blood-brain barrier (BBB). A previous study reported that *I. verum* contains anethole, which can reduce BBB permeability, motor dysfunction, and neuronal activity deficiency.[45]

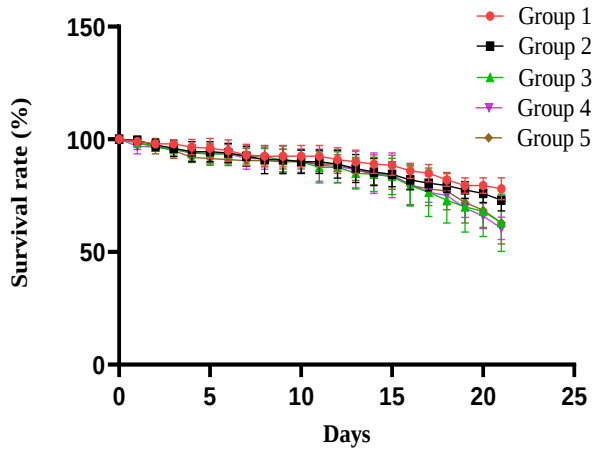

Figure 1: Effect of PEIV on the longevity of *D. melanogaster*

Group 1 = control, group 2 = 0.25 mg/g PEIV diet, group 3 = 0.5 mg/g PEIV diet, group 4 = 1 mg/g PEIV diet, group 5 = 2 mg/g PEIV diet.

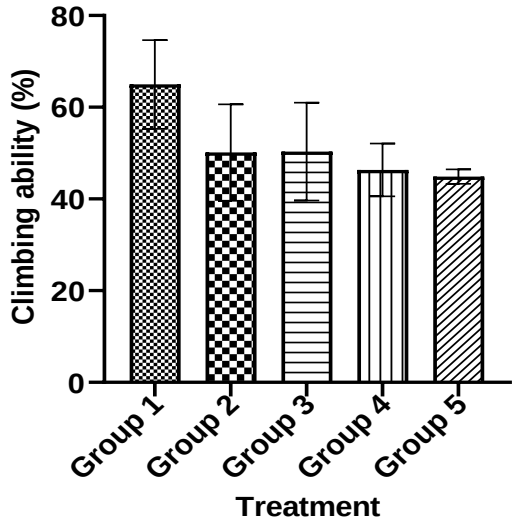

Figure 2: Effect of PEIV on the climbing ability of *D. melanogaster*

Bars represent mean ± standard deviation (n = 5). Group 1 = control, group 2 = 0.25 mg/g PEIV diet, group 3 = 0.5 mg/g PEIV diet, group 4 = 1 mg/g PEIV diet, group 5 = 2 mg/g PEIV diet.

Acetylcholinesterase is a cholinergic enzyme that breaks down acetylcholine, a neurotransmitter that regulates motor function and neuronal activities of the brain, to acetic acid and choline.[46, 47] In this study, all doses of PEIV tested also did not significantly ($p > 0.05$) affect the activity of AChE in the *D melanogaster* when compared with the control group (Figure 3). Thus, this suggests that PEIV possesses neuroprotective abilities. This also complements the result of the locomotor activity of this study, where PEIV did not affect the motor functions of the flies. Poor glycaemic control is relatively linked to high serum sugar levels.[48] Abnormal insulin metabolism could result in type II diabetes and disrupt antioxidant homeostasis.[49] Figure 4 shows the effect of PEIV on glucose levels in *D. melanogaster*. Compared with the control group, no significant ($p > 0.05$) increase in glucose level was observed in the PEIV-treated flies. This result suggests that PEIV at the doses tested could maintain glucose metabolism and protect antioxidant homeostasis. Khan *et al.*[50] reported that *I. verum* has anti-glycation activity, which could be the reason PEIV maintained the blood sugar level in this study.

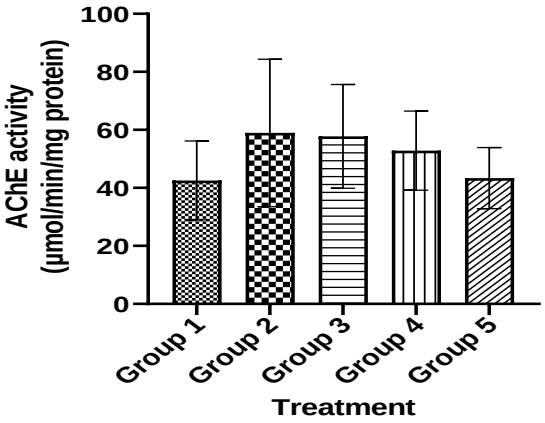

Figure 3: Effect of PEIV on the activity of acetylcholinesterase in *D. melanogaster*

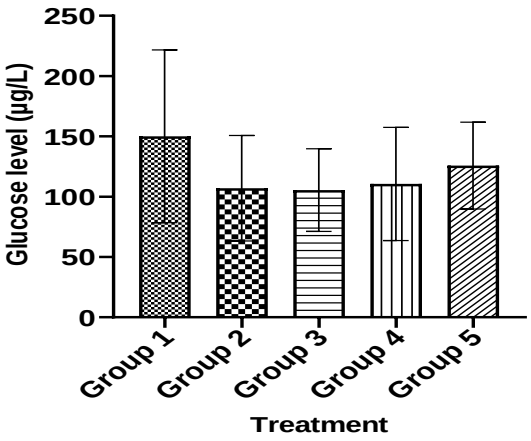

Figure 4: Effect of polyphenolic-rich extract of *Illicium verum* fruit on glucose levels in *D. melanogaster*

Bars represent mean ± standard deviation (n = 5). Group 1 = control, group 2 = 0.25 mg/g PEIV diet, group 3 = 0.5 mg/g PEIV diet, group 4 = 1 mg/g PEIV diet, group 5 = 2 mg/g PEIV diet.

Nitric oxide is a natural gas produced by the human body. Its diffusion rate is very high and has a short half-life; therefore, it can easily be converted to more stable metabolites, nitrite, and nitrate.[51] As a chemical messenger that promotes blood flow, nitric oxide regulates

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communication across nerve cells.[52] In this study, no significant difference ($p > 0.05$) was observed in the nitrite level of flies exposed to a PEIV-supplemented diet compared with the control (Figure 5). This indicates that the PEIV at the tested doses did not produce changes in the nitric oxide cascade, suggesting that PEIV could enhance the relaxation of the vascular smooth muscle and the cellular pathway responsible for metabolic processes.[53, 54] Thiols are organic compounds that comprise a sulfhydryl (-SH) group. They bind to mercury compounds (mercaptans) and can rapidly be oxidised to disulphides during oxidative stress (OS). Thiols have antioxidant properties which scavenge radical species and maintain cellular redox homeostasis. In this study, no substantial difference was observed in the total thiol level in flies exposed to a PEIV-supplemented diet compared to the control (Figure 6).

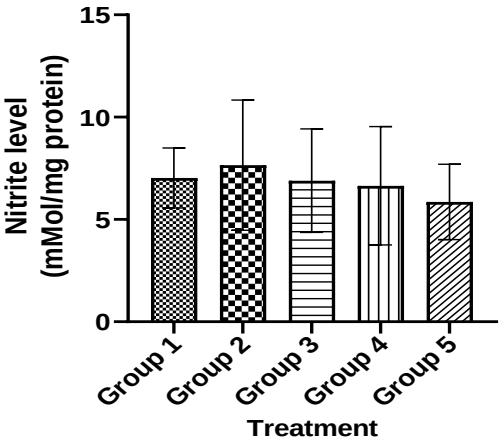

Figure 5: Effect of PEIV on nitrite level in *D. melanogaster*

Bars represent mean ± standard deviation (n = 5). Group 1 = control, group 2 = 0.25 mg/g PEIV diet, group 3 = 0.5 mg/g PEIV diet, group 4 = 1 mg/g PEIV diet, group 5 = 2 mg/g PEIV diet.

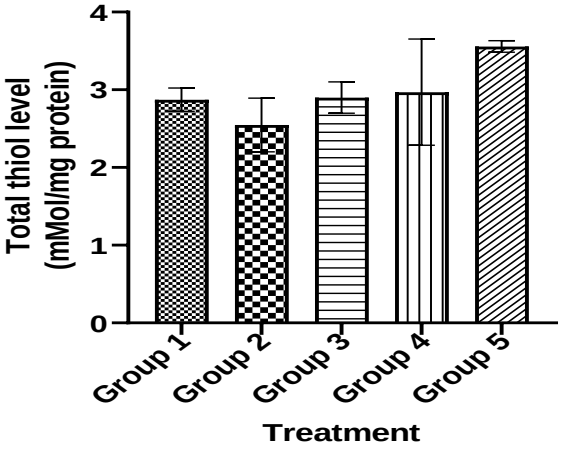

Figure 6: Effect of PEIV on total thiol levels in *D. melanogaster*

Bars represent mean ± standard deviation (n = 5). Group 1 = control, group 2 = 0.25 mg/g PEIV diet, group 3 = 0.5 mg/g PEIV diet, group 4 = 1 mg/g PEIV diet, group 5 = 2 mg/g PEIV diet.

The elevated, although non-significant, thiol level observed in the PEIV (2 mg/g diet) suggests that the PEIV has a rich antioxidant potential. This validates the previous study by Majali[55], which found that *I. verum* is rich in antioxidants. Reduced glutathione (GSH) is a non-enzymatic antioxidant found in high concentrations in the cellular system. It plays a role in detoxifying xenobiotics via electrophilic molecules.[56] A low level of GSH exposes cells to OS attack. In this study, no substantial difference was observed in reduced glutathione levels in flies exposed to a PEIV-supplemented diet at all doses when compared with the control group (Figure 7), suggesting that PEIV could serve as a protective agent against free radicals. A previous study by Hussein and Ahmed [57] reported that *I. verum* enhanced the GSH level in rats exposed to alcohol-induced gastric mucosal injury. Glutathione-S-transferases are a group of antioxidant enzymes involved in the cellular detoxification of xenobiotics. They are ubiquitously found in nearly all living cells, providing defense against various toxic molecules.[56] Additionally, they are involved in cell signaling and post-translational modifications.[58] In this study, no significant ($p > 0.05$) difference was observed in the GST activity in the flies treated with PEIV doses when compared with the control (Figure 8).

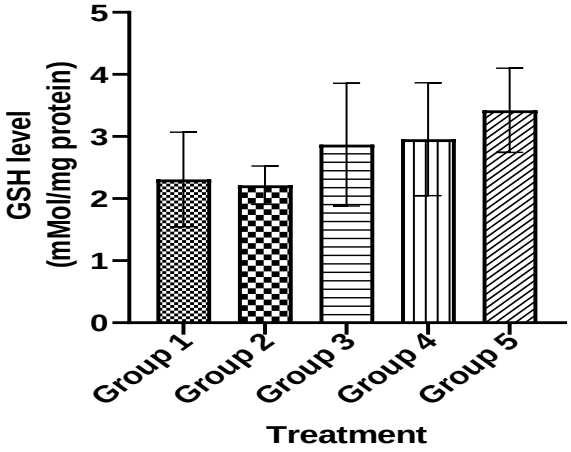

Figure 7: Effect of PEIV on reduced glutathione level in *Drosophila melanogaster*

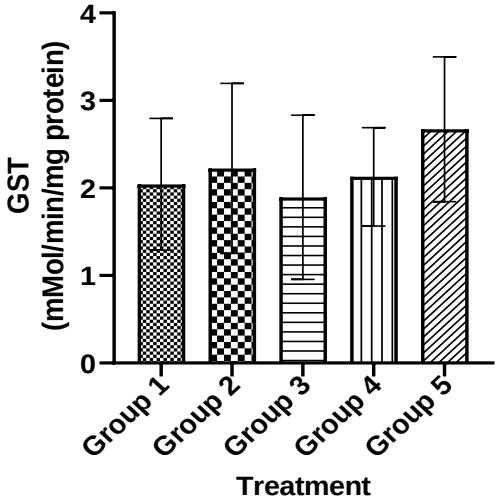

Figure 8: Effect of PEIV on glutathione-S-transferase activity in *D. melanogaster*

Bars represent mean ± standard deviation (n = 5). Group 1 = Control, Group 2 = 0.25 mg/g diet PEIV, Group 3 = 0.5 mg/g diet PEIV, Group 4 =1 mg/g diet PEIV, Group 5 = 2 mg/g diet PEIV.

The PEIV did not change the GST activity in the flies, suggesting its antioxidant capacity. Catalase, a haem-containing enzyme, catalyses hydrogen peroxide to oxygen and water.[52] Compared with the control group, the doses of PEIV caused no significant difference in the catalase activity of the *D. melanogaster* (Figure 9). This indicates a non-compromised antioxidant status at the tested doses of PEIV. Thus, this suggests that the PEIV exerts free radical scavenging ability. A previous study by Majali [55] reported that *Illicium verum* possesses antioxidant activity.

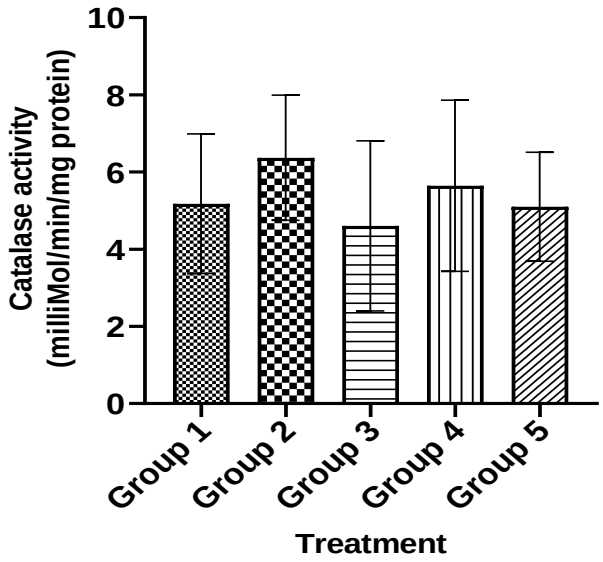

Figure 9: Effect of PEIV on catalase activity in *D. melanogaster*

Bars represent mean ± standard deviation (n = 5). Group 1 = Control, Group 2 = 0.25 mg/g diet PEIV, Group 3 = 0.5 mg/g diet PEIV, Group 4 =1 mg/g diet PEIV, Group 5 = 2 mg/g diet PEIV.

Conclusion

The outcome of this study revealed that the extract has an LD_{50} > 2 mg/g, suggesting that the polyphenolic-rich extract of *Illicium verum* may not be toxic at doses up to 2 mg/g. The extract's lower doses can serve as a potential alternative therapy or/and be developed into a therapeutic agent for managing diseases. Further studies on the long-term (chronic) toxicological effects of the polyphenolic-rich extract of *Illicium verum* on flies at high doses (0.5 - 2 mg/g diet) could be performed to ascertain its safety for clinical applications.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article are original and that any liability for claims relating to the content of this article will be borne by them.

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