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Phytochemical Composition, Antioxidant Activity and Toxicity of Aqueous Extract of *Picralima nitida* in *Drosophila melanogaster*

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ABSTRACT

Picralima nitida is a rainforest plant used for the treatment and management of diabetes and some other diseases in folklore medicine. In recent years, *Drosophila melanogaster* has served as an excellent model organism for toxicity studies of plants and also for the study of some diseases. This study focused on the antioxidant activity, phytochemical composition, and toxicity of aqueous seed extract of *P. nitida* in *D. melanogaster*. Phytochemical and antioxidant analyses of the extract were assessed using standard methods. The toxicity of the aqueous seed extract of *P. nitida* (APN) was also assessed, after seven days of exposure to APN (1-32 mg/mL), based on the rate of survival, locomotive performance and antioxidant effect in flies. Quantitative phytochemical analyses of APN showed the total flavonoid content to be 58.23 ± 0.79 mg quercetin equivalent/g dry weight (DW). The phenolic content was estimated to be 45.02 ± 0.27 mg gallic acid equivalent/g DW while β-carotene and lycopene contents, were 0.039 ± 0.002 and 0.047 ± 0.001 µg/mg, respectively. The anthocyanin content was 3.98 ± 1.28 µg/mL of cyanidin-3-glucoside equivalents. The result of the *in vivo* study showed that APN caused a reduction in the survival and locomotive behaviour of flies at 32 mg/mL. There was also an increase in the concentration of malondialdehyde and a reduction in the activity of acetylcholinesterase, glutathione-S-transferase and superoxide dismutase at high concentration of APN. The study showed that *P. nitida* seeds extract, despite its medicinal benefits, is toxic at high concentrations in *D. melanogaster*.

Keywords: Antioxidant activity, *D. melanogaster*, Survival rate, Locomotive behaviour, *P. nitida*.

Introduction

Plants with medicinal value are used to manage and treat various disease conditions since ancient times.[1] Though advances in the field of medicine and pharmacology had led to the discovery of some therapeutics, medicinal plants in the form of decoction, teas, powders, poultices and several other formulations are still in continuous demand and use today.[2] The continuous use of medicinal plants can be as a result of their accessibility, perceived safety, and reduced cost. The therapeutic effect of these plants is due to the presence of some phytochemicals that occur naturally in plants and serve protective roles against biological and environmental-related hazards.[3–5] These compounds which are known to protect plants have specific therapeutic roles in humans.[4]

Picralima nitida is a rainforest plant from the *Apocynaceae* plant family locally known in the Southwestern part of Nigeria as "*Abeere*".[6,7] Virtually all the parts of the plant have various ethnomedicinal uses. The seeds are known to serve various medicinal purposes especially in Nigeria, Ghana, Gabon, Cameroon and Cote d'Ivoire.[8–11] It is used to treat and manage pneumonia, abscesses, chest condition, as an antipyretic and aphrodisiac. It is also used for the management and treatment of diabetes and hypertension.[10–14] The seeds and bark are often ground to a fine powder and added to foods such as fermented maize pudding, locally called *ogi*.[12] The fruits are also used for the treatment of dysmenorrhea and gastrointestinal disorder.[6,15] In some parts of Sub-Saharan Africa, the fruit shell is usually filled with palm-wine and taken after it has absorbed the bitter taste present in the fruit.[6] It is also used for treating fever. The leaves are used as an anti-parasitic drug for expelling worms and other parasites from the body.[6] The leaf saps are sometimes used for the treatment of middle ear infection by dripping them into the ear.[6] All parts of the plant including the bark, root, seed have fruits have several phytochemicals. The phytochemicals reported to be present in the different parts include alkaloids, flavonoids, saponins, and terpenoids.[6] A number of these bioactive compounds, particularly alkaloids, have been isolated from the aerial part of the plant.[16–19] Some of the isolated alkaloids include picranitine, akuammine, picratidine, akuammicine pseudoakuammine, and akuammidine.[6,16,19] One of the alkaloids, akuammicine, isolated from the seeds of *P. nitidia* stimulated glucose uptake *in vitro*.[16] Despite the beneficial potentials of *P. nitida*, like most other medicinal plants, there is substantial proof of its toxic effects. Although several studies have reported the toxic effect of this plant at specific doses in experimental mammals[7,20,21] there is however no information on the toxic effect of this plant in *D. melanogaster*. In recent years, *D. melanogaster* has served as an excellent model organism for toxicity studies of plants and also for the study of several diseases.[22–26] This study sought to evaluate the phytochemical composition, *in vitro* antioxidant activity of the aqueous seed extract of *P. nitida*. The toxicity of the aqueous seed extract of *P. nitida* (APN) was also assessed, after seven days of exposure to APN (1-32 mg/mL), based on the rate of survival, locomotive performance and antioxidant effect in *D. melanogaster*.

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Materials and Methods

Chemicals and reagents

Gallic acid and quercetin were purchased from Solarbio Life Science, Beijing, China. All other chemicals are of analytical grade and were purchased from Sigma–Aldrich, Germany unless stated otherwise.

Identification and preparation of APN

Seeds of the plant were harvested fresh from local farms in Ota, Ogun State, Nigeria. The seeds were identified and validated by a Botanist at the Department of Biological Sciences, Covenant University and a herbarium specimen of the plant was deposited at the Forestry Research Institute of Nigeria, Ibadan with voucher number FHI 111159. The outer layer of the seeds was removed while the seeds were rinsed, air dried, ground to powder and extracted in distilled water with a mass to volume ratio of 1:10 (200 g/2 L) for 72 h. The extract was dried under vacuum in a rotary evaporator at 55^{o}C.

Phytochemical screening

Preliminary *in vitro* phytochemical screening of aqueous seed extract of *P. nitida* (APN) was carried out by making a stock solution of the extract (1.0 g in 100 mL of distilled water). Standard methods were used to determine the presence or absence of saponins, tannins, alkaloids, flavonoids, coumarins, phenol, glycosides, quinones, and anthocyanin in APN.[27,28]

Quantitative phytochemical analyses

The total flavonoid, anthocyanin, phenolic, lycopene, and β- carotene content in the sample was assessed in order to estimate the amount of some phytochemicals present in APN. The Folin–Ciocalteu (FC) method described previously was used to quantify the total phenolic content of APN.[29] The total flavonoid content of APN was estimated spectrophotometrically based on the aluminium chloride method described previously.[30] Lycopene and β- carotene concentrations in APN were estimated as described previously while total anthocyanin content in APN was determined using pH differential method as previously described.[31]

In vitro antioxidant assessment

DPPH (2,2-Diphenyl-1-Picryhydrazyl) Scavenging Activity

The ability of APN to decolourize DPPH was determined based on the method described previously with modifications.[30,31]DPPH 100 µM) was dissolved in 100 mL of 95% ethanol. Different concentrations of extract (0-1000 µg/mL) were mixed with DPPH (1:9), and a control DPPH sample was prepared without the APN sample. The mixtures were incubated for 30 mins at 25^{o}C, and their absorbance was determined at a wavelength of 515 nm. The percentage of DPPH scavenged was determined using the formula below;

$$\%\ DPPH\ Scavenged = [(Control\ absorbance - Sample\ absorbance) \div (Control\ absorbance\ )] \times 100$$

Total antioxidant capacity (TAC)

Phosphomolybdate method, reported previously, was used to investigate the total antioxidant capacity of APN.[31] Briefly, different concentrations of APN used in the study was mixed with 1.0 mL of phosphomolybdate reagent containing ammonium molybdate (4 mM), phosphate buffer (28 mM) and 0.6 M sulphuric acid. The solution was heated at 95^{o}C for 90 mins, and the absorbance was taken at 695 nm after cooling. The result was represented as µg/mL ascorbic acid equivalent.

Reducing power assay

The ability of APN to act as a reducing agent was assessed by the method reported by Rastogi et al. [32] with little modifications. Briefly, 250 µL of phosphate buffer (pH 6.6), 100 µL of different concentrations of APN (10-1000 µg/mL) and potassium ferricyanide (1%) were mixed. The resulting solution was heated for 20 mins at 50^{o}C in a dry bath. After the incubation time, 250 µL of trichloroacetic acid (10%) was pipetted into the mixture, and the solution was centrifuged for 10 mins at 3000 rpm. Ferric chloride (0.1%) solution (100 µL) that was prepared fresh was added to the supernatant, and the absorbance was taken at 700 nm.

Strain and culture of D. melanogaster

Harwich strain (wild-type) of *D. melanogaster* was obtained from *D. melanogaster* Research laboratory, Department of Biochemistry, College of Medicine, University of Ibadan, Oyo State, Nigeria. The flies were raised at the Department of Biochemistry, Covenant University Ota. Male and female flies were raised in an egg collecting cage and fed with standard brewer's yeast paste on *D. melanogaster* apple agar medium (30 g agar, 700 mL of distilled water, 300 mL apple juice concentrate, 1.2 g of methyl-4-hydroxy benzoate). Eggs (6 hours old) were collected, rinsed in sterile 1x phosphate buffer saline (PBS) and used for further experiment.

Survival assay

The methods described by Abolaji *et al*.; Riaz *et al*. [24,33] were adopted, with modifications, in order to determine how flies survive well on APN. *D. melanogaster* embryos were raised on different concentration of APN (0, 1, 2, 4, 16, 32, 50 and 100 mg/mL). The developmental process was monitored until moulting into adult flies stage. Adults flies, 1-3 days old, 50 per vial, enclosed from embryos raised on different concentration of APN were further exposed to various concentrations (0, 1, 2, 4, 16, 32, 50 and 100 mg/mL) of APN for seven days. The seven days experimental period was chosen based on preliminary studies, which led to the death of all flies at 50 and 100 mg/mL for 14 days. The experiment was carried out in triplicates and repeated twice. The number of flies that were dead and alive was recorded daily throughout the experimental period. The diet mixed with different concentration of APN was changed every 72 hours. Data obtained from the experiment were evaluated, and the result was represented as the percentage of live flies.

Negative geotaxis assay

Locomotive performance of flies treated on different concentration of APN was examined using negative geotaxis assay described previously.[24] After seven days exposure to APN, 10 APN treated flies were made inactive under light anaesthesia on ice and transferred to a vertical column that is labelled and has a length and diameter of 15 and 1.5 cm respectively. All the columns were marked before that time at a 6 cm point. After recovery from the light anaesthesia, flies were moved to the bottom of the column. The total number of flies that moved up to the marked part of the column and those that lingered below the mark in 5 s was recorded. The procedure was performed in three replicates per group and repeated four times at one-minute intervals. Data obtained were analyzed and expressed as a percentage of the average of the number of flies above the 6 cm mark.

Experimental design for determination of antioxidant status in D. melanogaster

Based on the high toxicity of the extract at high concentrations, concentrations of 0, 1, 8 and 32 mg/mL of APN were chosen for further biochemical evaluations to access the effect of APN on the antioxidant status of flies at low to high concentrations for seven days. Adult fruit flies were grouped into four. They were either given normal diet (10 g of agar powder, 50 g of sugar, 15 g of yeast, 50 g of semolina and 1.2 g of methyl-4-hydroxy benzoate in 1 L of distilled water), normal diet + 1 mg/mL of APN, normal diet + 8 mg/mL of APN or normal diet + 32 mg/mL of APN. After the experimental period, 10 flies were made inactive under mild ice anaesthesia. The flies were homogenized in homogenizing buffer containing 1 mM EDTA 0.25 M sucrose and 10 mM HEPES NaOH pH 7.4. The homogenate samples were centrifuged at 4000 rpm for 10 min at 4^{o}C in a refrigerated centrifuge. After centrifugation, the supernatant was stored at -20^{o}C until the time of use.

Estimation of malondialdehyde (MDA) Content

The extent of lipid peroxidation in the homogenate samples was assayed using a spectrophotometric method reported previously.[34] The

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assay relies on the reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA) to give a product whose concentration is proportionate to the concentration of MDA present in the sample and can be monitored at 535 nm and. MDA level in the homogenate samples was determined using 1.56×10^5 $M^{-1}cm^{-1,}$ which is the extinction coefficient of the TBA-MDA complex at 535 nm.

In vivo assessment of antioxidant and acetylcholinesterase activity

The activity of superoxide dismutase (SOD) was assayed, according to the ability of the enzyme to prevent autoxidation of pyrogallol.[35] Briefly, homogenate (50 µL) was diluted with distilled water (830 µL), mixed with phosphate buffer (pH 7.0) and left at room temperature for 10 min. After the incubation time, 20 µL of pyrogallol (10 mM prepared in 10 mM HCL) was added, and an increase in the absorbance was monitored at 420 nm for 3 min at every 30 s. The enzyme activity was estimated based on the % inhibition which was calculated as follows;

$$\%\ inhibition = (\Delta A420\ nm/of\ blank) - (\Delta A420\ nm/minute\ of\ sample)/(\Delta A420\ nm/minute\ of\ blank) \times 100$$

Glutathione-s-transferase (GST) activity, in homogenate samples of adult flies, was evaluated based on a previously described method.[36] The method involves the reaction between 1-chloro-2,4-dinitrobenzene (CDNB) and reduced glutathione (GSH) with to give a thioether based product (S-2,4-dinitrophenyl glutathione) which can be monitored based on an increasing change in absorbance at 340 nm. The enzyme activity was expressed as unit/mg protein using 9.6 mM^{-1} cm^{-1} as the molar extinction coefficient for CDNB.

Glutathione level in homogenates was estimated according to the method described previously.[37] On the other hand, the method reported by Ellman et al. was used to investigate acetylcholinesterase activity in homogenate samples. [38] The total protein level in homogenates was examined by Lowry's method of assessing protein levels in biological samples. Protein levels were extrapolated from the bovine serum albumin standard curve.[39]

Statistical analysis

Statistical Package for the Social Sciences (SPSS) (version 20.0, SPSS Inc., Chicago, IL, USA) was used to analyze data. Means were compared, at $p < 0.05$, using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. IC_{50} values of DPPH scavenging activity were generated using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA). All graphical illustrations were made using the same software.

Results and Discussion

In vitro phytochemical screening of APN showed that the plant extract contains flavonoids, anthocyanin, phenol, quinones, saponin, and coumarins (Table 1). Quantitative phytochemical analyses of APN showed the total flavonoid content to be 58.23 ± 0.79 mg quercetin equivalent/g dry weight (DW). The phenolic content was estimated to be 45.02 ± 0.27 mg gallic acid equivalent (GAE)/g DW while β-carotene and lycopene content, were 0.039 ± 0.002 and 0.047 ± 0.001 µg/mg, respectively. The anthocyanin content was 3.98 ± 1.28 µg/mL of cyanidin-3-glucoside equivalents (Table 2). The study showed that APN, like most medicinal plants, is rich in several phytochemicals, particularly alkaloids and phenolic compounds. Previous studies have shown that various extracts of the plant are good sources of these compounds.[6,7,40,41]

The result obtained from the study also showed that APN could scavenge DPPH. The ascorbic acid, however, was able to scavenge DPPH more effectively than APN. The observation was based on the IC_{50} values, which were extrapolated to be 91.62 µg/mL ± 1.027 and 12.39 µg/mL ± 1.015 for APN and ascorbic acid, respectively (Figure 1). The aqueous extract of the plant has rich hydrogen donating compounds, which donates hydrogen ions to the free radical chemical compound, DPPH. This subsequently leads to the decolourization of the purple colour of the compound. The reducing power assay showed that the reducing power of the extract increased in a dose-dependent manner (Figure 2). The reducing power assay relied on the reductive conversion of potassium ferricyanide to its ferrous form via the action of a substance or a biological material.[35] Although the reductive potential of the plant is low when compared to that of ascorbic acid, the antioxidants present in the plant were able to reduce these chemicals as evident in the colour change observed in the reducing power and DPPH scavenging assay. The total antioxidant capacity of APN based on phosphomolybdate assay showed that the higher the concentration of APN, the higher the antioxidant properties (Figure 3). These results collaborated those observed in earlier studies [6,40,42,43,] which showed that *P. nitida* has good *in vitro* antioxidant properties.

Result of the survival assay showed that flies exposed to APN (0-100 mg/mL) caused 100 % mortality at 50 mg/mL and 100 mg/mL. Flies fed 1, 2 and 4 mg/mL of APN did not cause any substantial effect ($p > 0.05$) in the survival rate of flies. However, APN cause a significant reduction ($p < 0.05$) in the survival of flies fed 32 mg/mL. Besides, flies fed 1 and 8 mg/mL of APN did not show any significant difference in climbing activity when compared to the control flies ($p > 0.05$). APN however significantly reduced ($p < 0.05$) the climbing activity of flies at 32 mg/mL indicating that at high concentration, APN could cause a locomotive deficit in *D. melanogaster*. There was, however, no treatment-related change in locomotive behaviour of flies treated with 1 and 8 mg/mL of APN. Although this is the first study reporting effect of *P. nitida* in *D. melanogaster*, several studies have reported that at high concentration, some medicinal plants affect the survival rate and locomotive behaviour of adult fruit flies.[24,44]

Finding from the study also showed that there was a significant increase ($p < 0.05$) in the concentration of malondialdehyde (MDA) at 32 mg/mL of APN when compared to the control. MDA is an important biomarker for accessing the level of lipid peroxidation. This suggests that APN, at 32 mg/mL, caused an increase in reactive oxygen species, which in turn could lead to lipid peroxidation. Also, findings from this study showed that there was a dose-dependent increase in GST activity in the group fed 1 and 8 mg/mL of APN when compared to the control. GST activity however reduced significantly ($p < 0.05$) in the group fed 32 mg/mL of APN when compared to the control (Figure 7A). SOD activity also increased significantly ($p < 0.05$) at 8 mg/mL of APN when compared to the control however at 32 mg/mL, there was a significant decrease ($p < 0.05$) when compared to the control (Figure 7B). Also, there was a marked decrease in reduced glutathione level in the group fed 32 mg/mL of APN. However, in comparison to the control, the decrease was not significant (Figure 7D). Macromolecules present in cells are protected from the harmful effects of reactive oxygen species via the action of the antioxidant defence system which can either be enzymatic (e.g. SOD, GST) or non-enzymatic (e.g. GSH, β-carotene).

Table 1: *In vitro* qualitative analyses of APN

Phytochemicals	APN
Alkaloids	+
Tannins	+
Anthocyanin and betacyanin	+
Flavonoids	+
Saponins	+
Glycosides	-
Quinones	+
Phenol	+
Coumarins	+

+ = Present; - = Absent

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Table 2: Anthocyanin, β carotene, lycopene, total phenolic and flavonoid content of APN

Phytochemical	Quantity
Total Flavonoid content	58.23 ± 0.79[b]
Total Phenolic content	45.02 ± 0.27[a]
β-carotene (µg/mg)	0.039 ± 0.002
Lycopene content (µg/mg)	0.047 ± 0.001
Anthocyanin (µg /mL)	3.98 ± 1.28

Values with superscript a and b are expressed as mg gallic acid equivalent (GAE)/g DW and mg quercetin equivalent/g DW respectively. Values are means ± SEM of at least three replicates performed in duplicates.

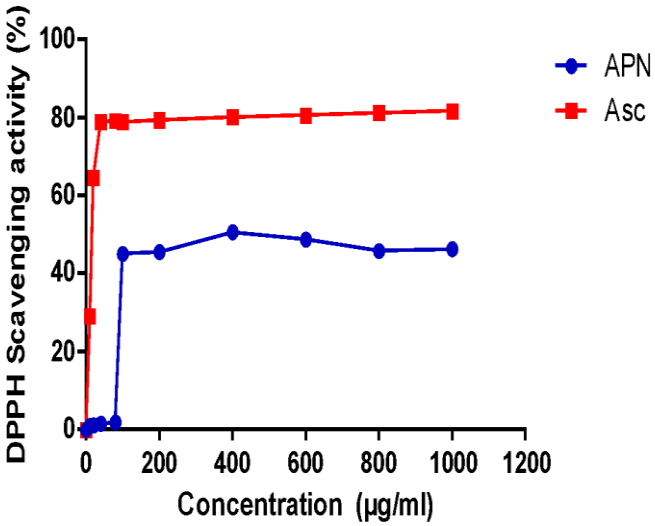

Figure 1: DPPH scavenging activity of APN in comparison to that of ascorbic acid. Values are means ± SEM of at least three biological replicates.

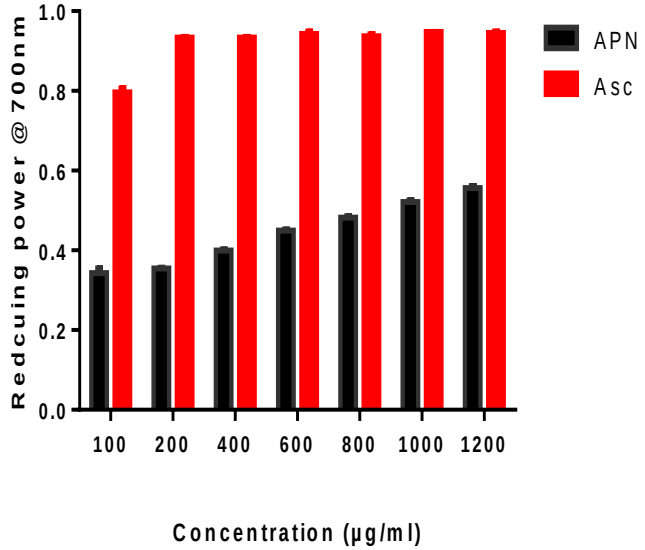

Figure 2: Reducing power potential of APN in comparison to that of ascorbic acid. Values are means ± SEM of three biological replicates.

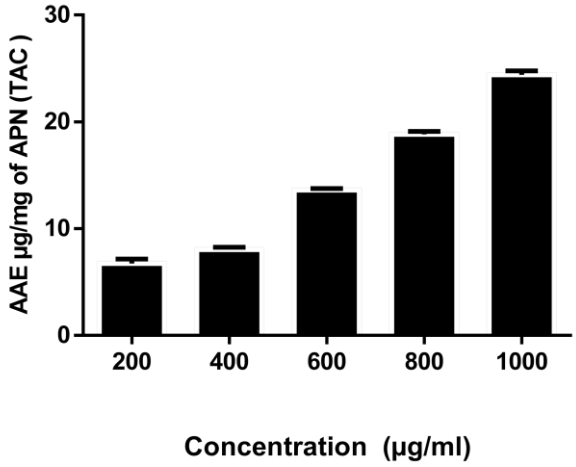

Figure 3: Total antioxidant capacity of APN. All values are mean ± SEM of three biological replicates.

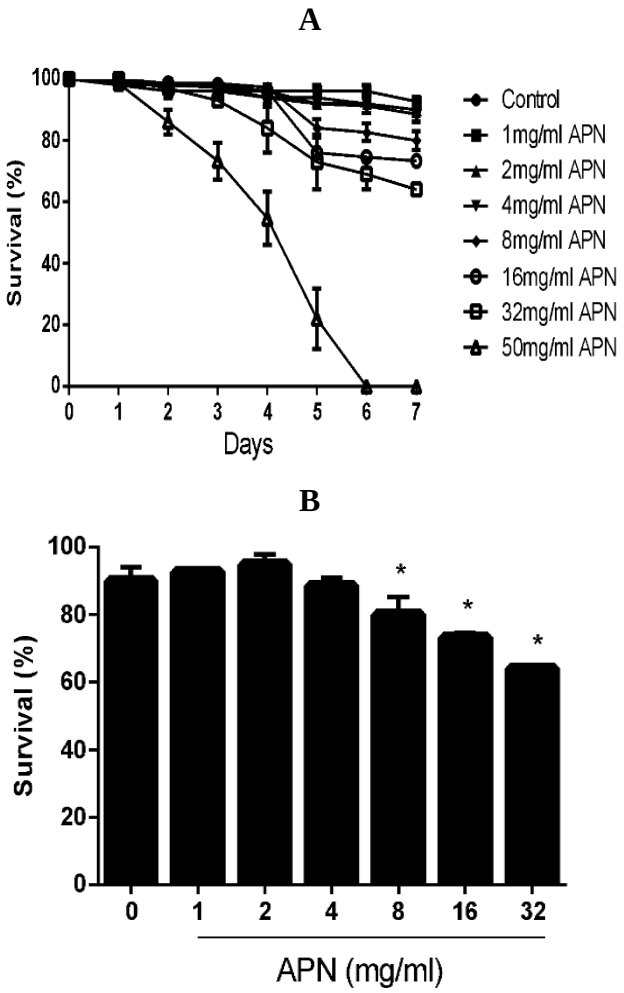

Figure 4: Effect of aqueous seed extract of *Picralima nitida* (APN) on survival rate in *D. melanogaster* .
(A) survival rate curve (B) survival rate (%) of 50 flies (male and female) after 7 days exposure of *Drosophila melanogaster* to APN. Values are means ± SEM of three biological replicates performed in duplicates. * represent $p < 0.05$ vs control.

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Superoxide dismutase (SOD) is an important antioxidant enzyme that helps to protect cells from the harmful effects of the highly reactive superoxide ion and peroxynitrite. It helps in the simultaneous oxidation and reduction of superoxide ion into oxygen and hydrogen peroxide.[45,46] Glutathione-s-transferase (GST) on the other hand are phase II detoxification enzymes that have an important role in promoting the binding of xenobiotics and reactive oxygen species to the reduced form of glutathione for proper detoxification.[47,48] The *in vivo* antioxidant effect of APN observed in the study may be attributed to the secondary metabolites present in the plant. Findings from the study showed that the aqueous extract of *P. nitida* is rich in phenolics, flavonoids, anthocyanin, lycopene and β-carotene. These compounds are secondary metabolites in plants and important exogenous non-enzymatic antioxidants that are responsible for the antioxidant activity of most medicinal plants.[4,5,49] Among several other mechanisms, the mode of action of theses group of compounds is largely dependent on their structure [50]. They have hydroxyl groups, which allow them to be good hydrogen donors. The hydrogen atom binds to the highly unstable reactive oxygen and nitrogen species and thus prevents the generation of new free radicals. It is possible that these compounds act synergistically or additively to confer the beneficial effect of the plant.

Another important finding from the study was that acetylcholinesterase activity increased ($p < 0.05$) significantly in the group fed 8 mg/mL of APN when compared to the control group (Figure 7C). This suggests that APN can increase acetylcholinesterase activity at a moderate dose. However at higher concentration (32 mg/ml), acetylcholinesterase activity was significantly reduced. Acetylcholinesterase is a key enzyme involved in the metabolism of acetylcholine. Acetylcholine plays a crucial role in cognitive function and muscle contraction, however, inhibition of acetylcholinesterase causes acetylcholine levels to rise and ultimately leads to muscular weakness and paralysis.[33,51,52]Inhibition of acetylcholinesterase activity at 32 mg/ml may be responsible for the marked reduction in climbing rate of flies at that concentration.

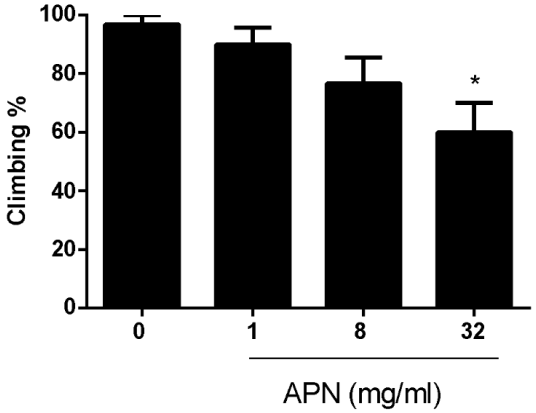

Figure 5: Climbing rate (%) after 7 days exposure of *Drosophila melanogaster* to APN. Values are means ± SEM of three biological replicates performed in duplicates. * represent $p < 0.05$ vs control.

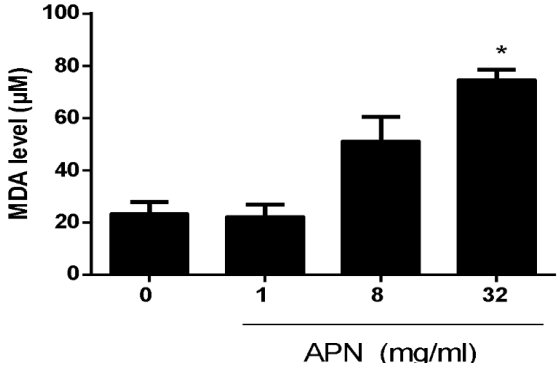

Figure 6: Malondialdehyde concentration of flies exposed to APN after 7 days.
Values are means ± SEM of three biological replicates performed in duplicates. * represent $p < 0.05$ vs control.

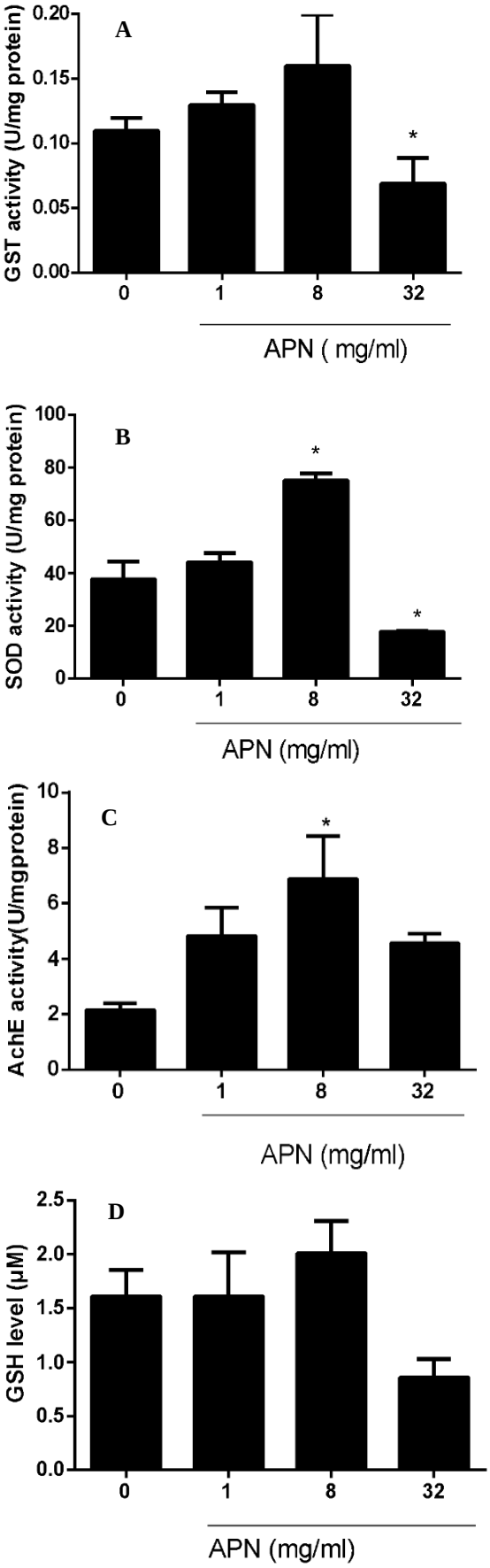

Figure 7: GST, SOD, AchE activities and GSH level of flies exposed to various concentrations of APN (1–32 mg/mL).
(A) GST, (B) SOD, and (C) AchE activities are expressed as U/mg of protein (D) GSH concentration expressed as µM. Values are means ± SEM of three biological replicates performed in duplicates. * represent $p < 0.05$ vs control.

Conclusion

The study showed that *P. nitida* seeds extract, despite its medicinal benefits, caused toxic effects at high concentrations in *D. melanogaster*

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that they will bear any liability for claims relating to the content of this article.

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