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RESEARCH

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Proanthocyanidins supplemented diet alter anti-aging-markers and improved lifespan in *Drosophila melanogaster* model

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Abstract

Background It is unequivocally believed that phenolics and flavonoids from fruits and vegetables hold robust prevention potentials against age-related disease development through their abundant hydroxyl groups. This study explored the potential neuromuscular enhancement and anti-aging effects of dietary supplemented proanthocyanidins-rich fraction from *Tamarindus indica* on *Drosophila melanogaster* model. One- to three-day-old male and female *D. melanogaster* were fed with a proanthocyanidins-rich fraction-supplemented diet for 7 days at two different concentrations. Following the effective dose determination, longevity assay (rate of survival), behavioral assay (negative geotaxis and eclosion), and biochemical assays (aging and antioxidant enzymes activities) were conducted to assess the fraction's longevity, antioxidant, and anti-aging effects on *D. melanogaster* model.

Result The results showed a significant ($p < 0.05$) improvement in the rate of emergence and lifespan of the flies fed with proanthocyanidins-rich fraction-supplemented diet at both concentrations (1.5 mg/g and 2.5 mg/g) compared to the normal control. A significant decrease in acetylcholinesterase (AChE) activity and the level of caspase-3 and caspase-9 were observed in the *D. melanogaster* flies fed with the fraction-containing diet when compared with the normal control. The supplemented diet also significantly increases the activity of catalase, superoxide dismutase (SOD), and glutathione-s-transferase (GST) in a concentration-dependent manner but not nicotinamide quinone oxidoreductase one (NQO1) in *D. melanogaster* upon comparison with the normal control.

Conclusion The observable changes in the experiment were attributed to the *T. indica*-derived proanthocyanidins, flavonoids with robust biological activities. The flavonoid-rich fraction proved its potential by enhancing the antioxidant system in *D. melanogaster* via the increase in the activities of some of the phase II antioxidant enzymes. The present study provides more insights into the wider perspectives of societies on the use of plant-derived natural compounds as the potential approach toward prevention against aging and age-related morbidities which enhance wellness and the quality of life in humans and animals.

Keywords Age-related diseases, Anti-aging markers, *Drosophila melanogaster*, Neuroprotection, Proanthocyanidins, *Tamarindus indica*

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1 Background

Despite its complexity, aging is considered a gradual accumulation of changes over time that are associated with increased vulnerability to morbidities and mortality [1]. As the global population ages, neurodegenerative diseases like Alzheimer's and Parkinson's become increasingly common, posing a serious threat to human health [2, 3]. Mitochondria play a pivotal role in generating free radicals such as reactive oxygen (ROS) and nitrogen species (RNS) that alter the redox status of the body [3]. Progressive oxidation of macromolecules generates large amounts of ROS to pathological levels that trigger mitochondrial damage through oxidative stress with consequent apoptotic cell death [3, 4]. The production of ROS and the subsequent response to oxidative stress have been established as important factors in the determination of longevity [5, 6].

The process of aging is a multifaceted molecular phenomenon that is influenced by a variety of molecular pathways and biochemical occurrences, which are influenced by both genetic and environmental factors [7]. Aging can be specifically described as a gradual decrease in functional capacity and stress resistance over time, which is accompanied by an increased likelihood of experiencing illness and death [8]. The effects relate to the progressive accumulation of stressors associated with aging, leading to the gradual deterioration of biomolecules and subsequent disruption of cellular homeostasis [9]. However, previous studies demonstrated that genetic or dietary interventions have the potential to extend the lifespan of a wide range of organisms, suggesting that it is possible to delay mortality through such interventional approaches [10–12].

Natural compounds are a vast collection of structurally diverse scaffolds that hold great potential as candidate chemical entities for addressing the significant healthcare challenge of extending health-spans and/or slowing down the aging process [13, 14]. One of the medicinal plants rich in bioactive phytochemicals is *Tamarindus indica* commonly called Tamarin tree. *T. indica* holds significant dietary importance in sub-Saharan Africa and possesses considerable medicinal attributes [15, 16]. The fruit contains a variety of bioactive phytochemicals, such as alkaloids, phenolic, and bioflavonoids [16]. The primary focus of this research has been on proanthocyanidins, the principal bioflavonoid found in the fruit part of the plant. Several studies have investigated its medicinal effect in various model organisms and reported its potential to be considered for clinical trials [17, 18].

Drosophila melanogaster is among the most influential model organism in biomedical research, and it has been used extensively for biochemical research such as molecular mechanisms that underline human diseases [19–21]. The model has revealed a noteworthy resemblance in neurotoxicity between Homo sapiens (humans) and *D. melanogaster* (fruit flies) [22]. Researchers took advantage of the flies' simple neural network for the exploration of antioxidant activities of bioactive compounds with potential neuroprotective activities [23]. These and other reasons strengthen the use of the fly model to unveil the mystery of life at a molecular level and screen the potential therapeutic agents [24]. In this study, we focused on evaluating the anti-aging activity of proanthocyanidins-rich fraction from *T. indica* and its ability to enhance longevity in a *D. melanogaster* model.

2 Methods

2.1 Collection of sample and preparation

A sample of the whole fruit of Tamarin was collected from Zaria main market of Kaduna State Nigeria and was identified by a botanist with a Boucher number 5451. Upon drying and removal of the shelves, the pulp parts were crushed into finely powdered particles using a stainless steel blender. The powdered sample was soaked in ethanol for 72 h. The filtrate was reduced to dryness by a rotary evaporator, and the percentage yield was 8.2% w/w, which was kept in the refrigerator until needed.

2.2 Stocking and culturing of *Drosophila melanogaster*

Drosophila melanogaster (Harwich strain) was donated by the College of Medicine, University of Ibadan, Nigeria. The flies originated from the National Species Stock Center, Bowling Green, Ohio, USA, and were grown in the *Drosophila* Research Laboratory, Department of Biochemistry, Kaduna State University. They were maintained at the respective standard temperature (24 ± 2 °C) and relative humidity (60 – 70%), under 12 h of lightness/darkness cycle conditions on a cornmeal diet containing 0.08% w/v methylparaben, 1% w/v agar–agar, 1% w/v brewer's yeast, and 2% w/v sucrose.

2.3 Experimental design

Two- to three-day-old male and female flies were placed into three separate groups, namely normal control (diet without fraction), treatment group 1 (1.5 mg proanthocyanidins / g diet) and treatment group 2 (2.5 mg proanthocyanidins / g diet) as the most effective doses of the fraction. Each group comprised three replicates containing 100 flies and were fed for seven days.

2.4 Behavioral assays

2.4.1 Longevity assay

To ascertain the impact of proanthocyanidins-rich fraction on the lifespan of experimental *D. melanogaster*, a total of 100 flies per vial in triplicates were subjected to seven days treatment with or without a

proanthocyanidins-rich fraction at 1.5 and 2.5 mg/g diet. Daily mortality of the flies was observed and recorded for seventy-seven (77) days. The survival rate was analyzed using GraphPad Prism and presented in the result section.

2.4.2 Negative geotaxis assay

Locomotor activity of *D. melanogaster* supplemented with proanthocyanidins-rich fraction at two concentrations (1.5 and 2.5 mg/g diet) were evaluated using a negative geotaxis assay as described by [25] with modifications. Briefly, following anesthesia, a total of twenty (20) flies out of the 100 from each experimental vial were picked and placed into a graduated column of 15 cm in height and 1.5 cm in diameter. The 8 cm of the column was considered a threshold and was marked, and the number of flies that traversed the line and those that remained at the bottom within 8 s were recorded. The experiment was conducted three times per vial at the interval of 1 min between readings, and the data were analyzed and presented.

2.4.3 Emergence rate determination

The rate of flies' offspring emergence in the ADFP fraction treatment group was evaluated as previously described by [26].

2.5 Evaluation of aging-related markers in *Drosophila melanogaster*

2.5.1 Determination of total protein and estimation of Caspase-3 and Caspase-9 levels

The levels of caspase-3 and caspase-9 were estimated spectrophotometrically using a GenScript colorimetric assay kit (GenScript, Piscataway, NJ, USA). Upon completion of the experimental period, flies were homogenized in ice-cold PBS in a ratio of 1 fly to 10 µL PBS. To lyse the cells of the flies, about 50 µL cold lysis buffer containing 0.25 µL phenylmethanesulfonyl fluoride (PMSF) and 0.5 µL Dithiothreitol (DTT) was added to tubes containing the homogenate. The tubes were kept on ice for an hour with a thorough vortex at intervals of 8–12 min. The tube content was centrifuged at 10,000 rpm under 4 °C for one minute, and protein concentrations were measured using the Bradford assay in the collected supernatant thereafter as highlighted by [27]. Then, 200 µg of protein was added to a tube containing 50 µL reaction buffer comprising 0.25 µL PMSF and 0.5 µL DTT, and the contents were vortexed and allowed to stay on ice. The suspension was transferred to 96-well plates upon the addition of 5 µL caspases substrates. The plate was covered with aluminum foil and placed in the dark at physiological temperature for four (4) hours. The levels of caspase-3 and caspase-9 were estimated spectrophotometrically at 405 nm using a microplate reader (Universal Microplate Reader; Biotech, Inc).

2.5.2 Estimation of acetylcholinesterase activities

AChE activity was measured using the modified method of [28]. Briefly, a reaction mixture comprising 135 µL dH_2O, 20 µL 10 mM DTNB, 20 µL 100 mM potassium phosphate buffer (pH 7.4), 5 µL homogenate sample, and 20 µL 8 mM ACh substrate was shaken vigorously. The activity of acetylcholinesterase was observed using a UV/visible spectrophotometer for 5 min (at an interval of 15 s) at 412 nm. The resulting data were corrected using protein content upon calculation with blank and sample blank.

2.6 Evaluation of antioxidants related markers *Drosophila melanogaster*

2.6.1 Estimation of catalase activity

A modified method reported by [29] was used to measure catalase activity. A reaction vessel containing 1800 µL of 50 mM phosphate buffer (pH 7.0), 20 µL of homogenate sample (1:50 dilution), and 180 µL of 300 mM H_2O_2 substrate. The disappearance of the substrate was monitored for 2 min at an interval of 10 s using a UV/visible spectrophotometer at 240 nm. The results were expressed as µmol of hydrogen peroxide (H_2O_2) consumed/min/mg of protein.

2.6.2 Estimation of superoxide dismutase (SOD) activity

The SOD activity was evaluated according to [29] methods with slight modification, by reducing nitrite formation in 40 min at 37 °C. The test was based on the SOD-mediated inhibition of nitrite formation from hydroxyl ammonium in the presence of O_2 generators. The activity was measured spectrophotometrically at 550 nm, and the results were presented as the unit of the enzyme's activity/mg of protein.

2.6.3 Estimation of glutathione-s-transferase activity

As demonstrated in [30], the activity of glutathione-s-transferase was determined by careful monitoring of the increase in the absorbance at 340 nm wavelength. The sample (50 µL) was added to the tube containing 20 µM each of 1-chloro-2,4-dinitrobenzene (CDNB) and a reduced form of glutathione. Optical density was taken at 406 nm for three minutes, and the result was expressed as the quantity of protein necessary to inhibit half of the quercetin auto-oxidation.

2.6.4 Estimation of N-quinone oxidoreductase one (NQO1) Activity

The activity of NQO1 enzyme was estimated spectrophotometrically using 2,6-Dichlorophenolindophenol

(DCPIP) reduction method as described by [31] with some modifications. Briefly, the experiment was conducted kinetically on a microplate reader (Universal Microplate Reader; Biotech, Inc) at 600 nm, employing the enzyme's substrate (DCPIP) and its inhibitor (dicumarol). To calculate the activity, the mean value of DCPIP reduction from 0 to 1 min in the presence of dicumarol was subtracted from the mean value of the reduction without the inhibitor. The NQO1 activity was expressed as mole DCPIP reduced/min/mg protein.

2.7 Statistical analysis

The data were presented as means ± standard deviations, and statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey post hoc test on GraphPad Prism (v 6) (San Diego, CA, USA). Differences in the results were considered statistically significant ($p < 0.05$) at 95% confidence level. All experiments were conducted in three replications ($n = 3$).

3 Results

3.1 Effect of proanthocyanidins-rich fraction-supplemented diet on longevity in *D. melanogaster*

The life span in *D. melanogaster* was improved significantly upon supplemented with a proanthocyanidins-rich fraction of *T. indica* for thirty days compared to the normal control that received only diet. The effect was in a concentration-dependent manner as the flies supplemented with 2.5 mg of the fraction per gram of diet showed a higher increase in the lifespan than those that received 1.5 mg/g diet, though the difference between the two concentrations was not remarkable as seen in Fig. 1.

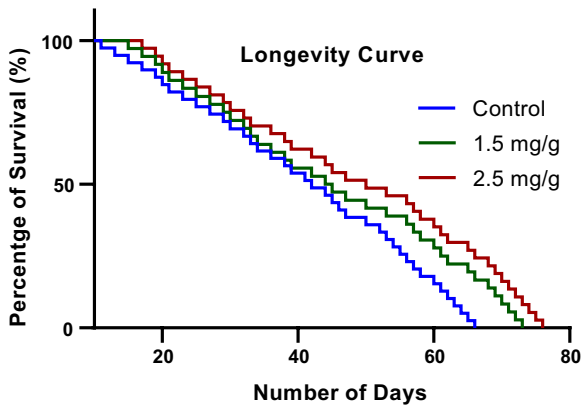

Fig. 1 Effect of proanthocyanidins-rich fraction-supplemented diet on longevity in *D. melanogaster*. All experiments were conducted in three replications ($n = 3$)

3.2 Effect of proanthocyanidins fraction on emergence rate and locomotor function in *D. melanogaster*

Figure 2 shows the effect of proanthocyanidins-rich fraction-supplemented diet on the rate of flies emergence and locomotor activities in *D. melanogaster*. The fraction enhanced significantly ($p < 0.05$) the emergence of new flies supplemented at both concentrations (1.5 and 2.5 mg fraction/g diet) compared to the normal control. The effects seemed to be in concentration-dependent manner as 2.5 mg/g appeared to be more effective than 1.5 mg/g as seen in Fig. 2a. Furthermore, the locomotor activity of the flies supplemented with proanthocyanidins-rich fraction increased significantly ($p < 0.05$) in a dose-dependent manner compared to the normal control group (Fig. 2b).

3.3 Effect of proanthocyanidins-rich fraction dietary supplement on aging-related enzymes' activities and quantity in *D. melanogaster*

The effect of the fraction was further evaluated on aging-related enzymes where the fraction showed inhibitory activity on acetylcholinesterase in *D. melanogaster*. Only 2.5 mg of fraction/ g of diet revealed a significant ($p < 0.05$) difference but not the 1.5 mg/g when compared to the normal control (Fig. 3a). Likewise, Caspase-3 level dropped significantly ($p < 0.05$) in the flies that received proanthocyanidins-rich fraction at both concentrations. Again, the increase in the level of the enzyme occurs in a concentration-dependent manner (Fig. 3b). Similarly,

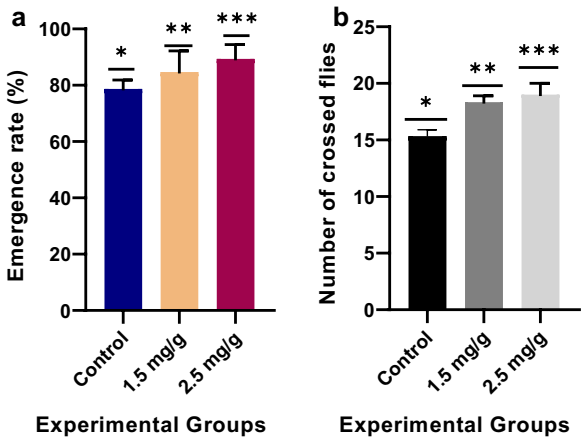

Fig. 2 Effect of proanthocyanidins-rich fraction-supplemented diet on rate of emergence (**a**) and locomotor function (**b**) in *D. melanogaster*. The result for emergence rate was presented as percent pupation of new flies with and without the fraction and also that of negative geotaxis assay was presented as mean ± SD of the number of flies that cross an 8 cm in 8 s within a column. Differences in the results were considered statistically significant ($p < 0.05$) at 95% confidence level. All experiments were conducted in three replications ($n = 3$)

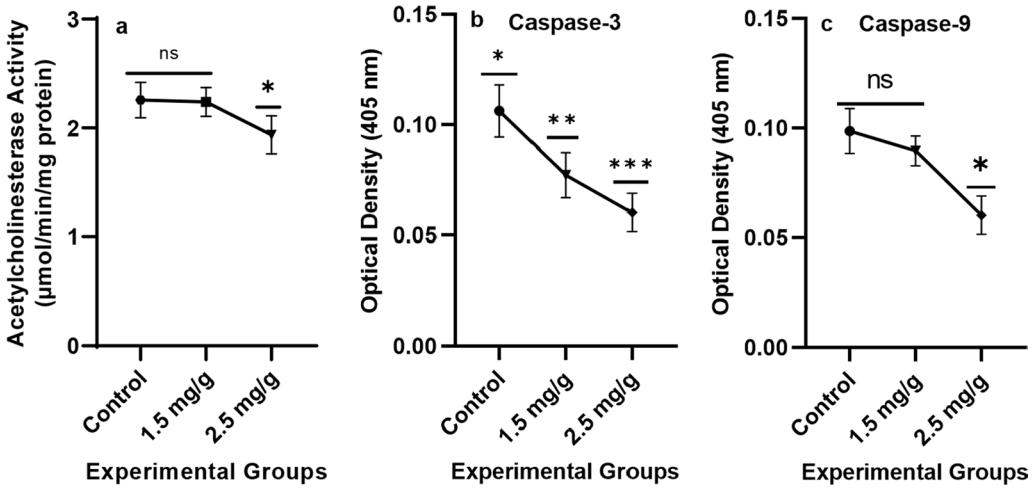

Fig. 3 Effect of proanthocyanidins-rich fraction-supplemented diet on the activity and level of acetylcholinesterase (**a**), Caspase-3 (**b**), and Caspase-9 (**c**) in *D. melanogaster*. The results were expressed as mean ± SD of the activity and quantity of the enzymes. Differences in the results were considered statistically significant ($p < 0.05$) at 95% confidence level. All experiments were conducted in three replications ($n = 3$)

the level of Caspase-9 was lowered by the fraction at 2.5 mg/g when compared to the normal control. However, the 1.5 mg/g fraction did not show any effect on the level of Capspase-9 in the flies fed with the supplemented diet (Fig. 3c).

3.4 Effect of proanthocyanidins-rich fraction supplement on antioxidants and age-related enzymes' activities in D. melanogaster

Figure 4 shows the effect of proanthocyanidins-rich fraction dietary supplemented diet on selected antioxidant markers in *D. melanogaster*. A 2.5 mg/g of the fraction effectuated a significant ($p < 0.05$) increase in catalase activities in the flies when compared with the normal control group (flies that were fed with only diet without the fraction). However, there was no significant effect on the activity when the flies were fed with 1.5 mg/g supplemented diet (Fig. 4a). The result also revealed a significant ($p < 0.05$) increase in the activity of superoxide dismutase (SOD) in the flies upon fed with the fraction-supplemented diet compared to the normal control flies. The two concentrations of the supplemented fraction showed a similar pattern of effect on the SOD activity since there was no observable difference in their means (Fig. 4b). Similarly, we observed a significant ($p < 0.05$) increase in the activity of glutathione-s-transferase by the fraction in a concentration-dependent manner when compared with the normal control group. The

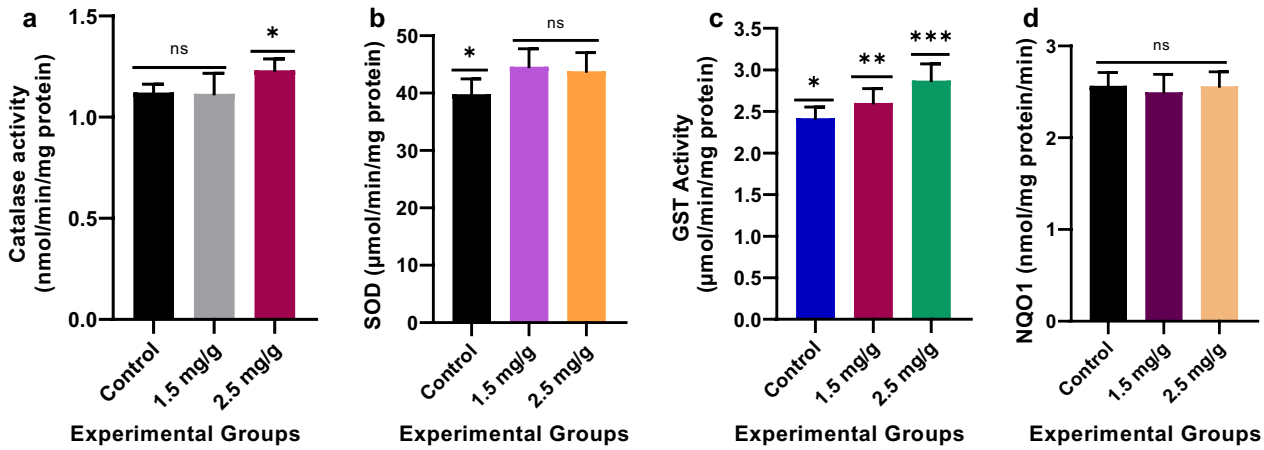

Fig. 4 Effect of proanthocyanidins-rich fraction supplement on some selected antioxidant and anti-aging enzymes activities; Catalase (**a**), superoxide dismutase (**b**), glutathione-s-transferase (**c**) and quinone oxygenase one (**d**) in *D. melanogaster*. Differences in the results were considered statistically significant ($p < 0.05$) at 95% confidence level. All experiments were conducted in three replications ($n = 3$)

increasing effect of 1.5 mg/g fraction on the enzyme's activity was significantly lowered ($p < 0.05$) when the two were compared statistically (Fig. 4c). In contrast, the fraction-supplemented diet had no effect on the activity of nicotinamide quinone oxidoreductase one (NQO1) at both concentrations (Fig. 4d).

4 Discussion

The effects of various flavonoids and polyphenols on lifespan extension, health improvement, and aging-related morbidities have been investigated using *Drosophila melanogaster* model [32–34]. However, the specific impact of proanthocyanidins on longevity, redox status, and aging conditions remained to be elucidated. Proanthocyanidins demonstrated numerous health benefits on various model organisms [18, 35]. The role of oxidative stress has been suggested in the process of aging and the development of various age-related diseases [36]. Oxidative stress is characterized by a disparity between the generation of ROS and RNS, and the ability of the cellular antioxidant defense system to counteract the generated species [37]. This condition arises when there is an elevation in ROS/RNS levels or a decline in antioxidant capacity [37]. Meanwhile, the aging process is influenced by the detrimental effects on lipids, proteins, and DNA in different tissues [38]. According to the findings of this study, the inclusion of proanthocyanidins-rich fraction in the diet has been shown to improve the lifespan of the flies above those that have not received the fraction (Fig. 1). The current findings align with prior research that demonstrated the life-extending effects of a phenolic and other forms of flavonoids from fruits [39–41]. Phenolics and flavonoids are robust bioactive compounds found primarily in vegetables and several other fruits including *T. indica* [42]. Their demonstrated antioxidant properties are attributed to the hydroxyl groups on their aromatic ring structures and the presence of highly activated carbon atom between the two methoxyphenol rings [34, 42].

On the other hand, several studies have shown that the cholinergic system and intrinsic mitochondrial pathway play an important role in the pathophysiology of aging and other neurodegenerative diseases [43–45]. Acetylcholine (ACh), a cholinergic neurotransmitter, plays a crucial role in modulating cholinergic functions such as learning, memory, and locomotor activity [46, 47]. AChE, a serine protease, however, hydrolyses acetylcholine to choline and acetate, affecting cholinergic neurotransmission [47]. The cholinergic marker enzyme (AChE) is specific for the active state of cholinergic neurons, and it is crucial for maintaining acetylcholine levels at cholinergic neurons and responsible for acetylcholine degradation in the synaptic cleft [48]. AChE activities have been linked to other neurodegenerative diseases and the aging process [49]. When compared to the control, the proanthocyanidins-rich fraction-supplemented diet resulted in a significant decrease in AChE activity (Fig. 3a) and an increase in climbing activity (Fig. 2b), translating to neuromuscular strength enhancement in the experimental flies. These findings are consistent with previous reports on in vitro and in vivo findings [50–54]. Thus, in our study, the decrease in AChE activity after dietary proanthocyanidins supplementation could lead to an increase in acetylcholine levels in the synaptic cleft and, as a result, increase cholinergic neurotransmission efficiency in the flies.

Furthermore, the impact of the fraction on caspase-3 and caspase-9 levels in the flies also signified its key role in mitigating the development of age-related diseases. Caspase-3 and -9 are the primary markers for the mitochondrial intrinsic pathway that contribute to cellular senescence with eventual cell death, leading to aging and its associated complications. Study has shown that phenolics enhance the longevity of organisms by decreasing the level of caspases and consequent disruption of the intrinsic pathway [55]. Our findings revealed a significant decreasing effect on the two caspases level by proanthocyanidins-rich fraction-supplemented diet in *D. melanogaster* (Fig. 3b, c). The present findings are in line with documented evidences that reported the inhibitory effects of phenolic on caspases level, activities, and their expression level in pathophysiological age-related morbidities [56–58].

The current investigation demonstrates that the inclusion of proanthocyanidins-rich fraction in the diet also leads to an enhancement in the antioxidant status of *D. melanogaster*, as depicted in Fig. 4. Multiple studies have demonstrated the antioxidative properties of phenolics, flavonoids and other important plant-derived active compounds [55, 59–61]. The effective strategies employed by organisms to mitigate the harmful effects of reactive oxygen and nitrogen species (ROS and RNS) involve the enzymatic activity of numerous markers including catalase, superoxide dismutase (SOD), glutathione-s-transferase, nicotinamide quinone oxidoreductase (NQO1) and others [16, 41, 62]. SOD aids in the transformation of superoxide anion into less harmful compounds, which are subsequently converted into water through the catalytic activity of catalase [63]. The significance of this mechanism in the lifespan of *D. melanogaster* has been described in several reports [63, 64]. Previous studies have demonstrated that the genome of *D. melanogaster* contains individual single regions that exhibit the ability to enhance SOD and catalase activities, along with four regions that possess the capacity to suppress their respective activity as well [65, 66].

It is currently observed that feeding *D. melanogaster* with proanthocyanidins-rich fraction results in a significant alteration in the activities of SOD and catalase in comparison with the control group (Fig. 4a, b). Again, our findings coincide with the results presented in [67], which reported an increase in SOD and catalase activities in fruit flies fed with polyphenolic (curcumin) compared to those on the control diet. Another important antioxidant marker is glutathione-s-transferase (GST) which represents a phase II group of multifunctional enzymes characterized by the presence of cysteine-rich domains [68]. The catalytic activity of GST in the conjugation of glutathione (GSH) with electrophilic molecules is a crucial process in the detoxification of xenobiotics that could disrupt redox status in living organisms [69]. The present findings demonstrated the positive effect of proanthocyanidins-rich fraction on GST activities in flies fed with the fraction compared to the normal control group. GST activity was increased significantly by curcumin even within a toxic environment. It also neutralized the noxious effect of the ecotoxic agent that alters redox status of an organism [67]. However, the present findings revealed the ineffectiveness of the supplemented diet on the activities of NQO1, which is contrary to the previous reports on the impact of the enzyme, where NQO1 and other phase II enzymes demonstrated strong antioxidant activities [70, 71]. Our finding strengthened the existence of isoforms of the protein in some organisms and their respective physiological role in different model organisms.

5 Conclusion

Collectively, our findings indicate that proanthocyanidins-rich fraction slows down aging process in flies. It is posited that the anti-aging capacity of the flavonoid-rich fraction can be attributed to its antioxidative properties, as indicated by the observed increase in the activities of some phase II antioxidant enzymes with the consequent decrease in acetylcholinesterase activity and level of mitochondrial intrinsic pathway caspases in *D. melanogaster*. Therefore, proanthocyanidins-rich fraction of *T. indica* origin could be considered a potential anti-aging intervention and may provide protection against neurological-related disorders particularly those associated with oxidative stress, such as Parkinson's and Alzheimer's diseases. Moreover, the findings of the current study provide additional evidence supporting the effectiveness of *Drosophila melanogaster* as a valuable model organism for exploring potential therapeutic interventions that hold promise in the management of neurodegenerative disorders.

Abbreviations

AChE	Acetylcholinesterase
Ach	Acetylcholine
GST	Glutathione-s-transferase
SOD	Superoxide dismutase
NQO1	Nicotinamide quinone oxidoreductase one
RNS	Reactive oxygen species
ROS	Reactive oxygen species
PMSF	Phenylmethanesulfonyl fluoride
DTT	Dithiothreitol
UV	Ultraviolet
H_2O_2	Hydrogen peroxide
O_2	Oxygen
DCPIP	Dichlorophenolindophenol
ANOVA	Analysis of variance

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Author contributions
JMS, ZKM and SAM conceived and designed the experiment; JMS, ZKM and AY conducted the experiment and analyzed the data; JMS, ASM and AFAR drafted and proofread the manuscript. All the authors read and approved the final draft.

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Availability of data and materials
All data are available in the present manuscript.

Declarations

Ethics approval and consent to participation
Not applicable.

Consent for publication
All the authors have read the manuscript and gave their consent for publication.

Competing interests
The author declared that there is no competing interest in the present study.

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