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ORIGINAL STUDY

Antioxidant Effect of *Morus Indica* L Against Paraquat-induced Oxidative Stress in *Drosophila Melanogaster*

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Abstract

Background: **Mulberry extracts and active components have numerous good neurological and biological benefits, making them promising candidates in the research for new medications to treat neurological problems.**

Methods: **In the present study we evaluated the neuroprotective role of mulberry fruit extract against the paraquat (PQ) induced oxidative stress in *Drosophila melanogaster*. After the exposure to PQ, Flies were assayed for climbing activity, reactive oxygen species (ROS) and lipid peroxide (LPO) content, acetylcholine esterase activities (AChe), and also the antioxidant defense system such as superoxide dismutase (SOD), catalase activities (CAT) and glutathione synthetase (GSH).**

Results: **In a negative geotaxis assay, MF pre-treated flies exposed to PQ showed a lower incidence of mortality and enhanced climbing activities of flies when compared to untreated flies exposed to PQ. In addition, when exposed to PQ, untreated flies resulted in a significant ($p \leq 0.05$) increase in oxidative stress markers such as ROS, LPO content and AChe and decreased the antioxidant defense system such as SOD, CAT, and GSH. However, flies pre-treated with MF when exposed to PQ ameliorated oxidative stress markers and by restoring the antioxidant defense system, additionally, the pre-treatment of MF flies also reduced AChe activities.**

Conclusion: **The pre-treatment of flies with MF extract has the potential to reduce PQ-induced oxidative stress due to its antioxidative nature and ability to modify the activities of antioxidant defense systems. However, further research is needed to understand the exact mechanism of its activity.**

Keywords: Paraquat, Oxidative stress, Neuroprotection, Mulberry, *Drosophila*

1. Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by the destruction of pigmented dopaminergic neurons in the nigrostriatal pathway, followed by a reduction in dopamine levels. PD symptoms include lethargic motion, muscle rigidity, and resting tremors [1]. According to recent research, there is a growing interest in natural substances, particularly plants, for the treatment of PD. The potential of these natural compounds to control excitotoxicity, mitochondrial function, dopamine degradation, ROS, neuroinflammation, and cellular signaling pathways, all of which are impacted in the PD brain and are assumed to be the cause of their anti-PD effects [2].

In both *drosophila* and mammalian models, PQ has been shown to induce oxidative stress (OS) and mimic pathological symptoms comparable to PD, such as decreased locomotor performance and selective neuronal death. To explore the neuroprotective potential of bioactive compounds, the acute PQ model of PD in *drosophila* is extensively employed [3]. OS caused by an imbalance between endogenous antioxidant defenses and free radical generation is a major mechanism contributing to its

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development. Natural bioactive substances have a strong antioxidant capacity, which may help reduce OS and even repair the damage caused by ROS [4]. Fruits are particularly high in phytochemicals with antioxidant capabilities, and their ability to prevent the development of neurological disorders is of major relevance [5].

Mulberry belongs to the *Moraceae* family and is termed a special plant on earth, because of its wide geographical occurrence across the continents. Mulberry leaves have been reported to have anti-hyperglycemic, antioxidant activity, and anti-glycation [6]. Because of their remarkable medicinal properties, the numerous vegetative parts of the mulberry are a subject of tremendous interest in the current scenario. Mulberry fruits (MF) are currently a major source of concern because of their significant medicinal properties and pharmacological importance. MF is utilized in a variety of forms for human health, including juice, wine, tea, jams, jellies, and other items. MF has also been found to contain useful phytochemicals such as anthocyanins, phloridzin, quercetin, chlorogenic acid, and resveratrol [7]. MF also had the highest resveratrol content, which lowers reactive oxygen species (ROS) levels by inhibiting lipid peroxidation. This activity also reduced the likelihood of DNA damage by lowering the mutation rate [8]. Although a number of studies have carried out the bioactivities of the MF, there is a dearth of knowledge on the specific health benefit components. With this as a context, the current study used *drosophila melanogaster* to induce oxidative stress as a model of PD caused by paraquat and to screen MF for its therapeutic effectiveness in delaying the progression of PD. Furthermore, the therapeutic efficacy of MF will be compared with L-dihydroxyphenylalanine (L-DOPA) for PD. Since L-DOPA was first introduced decades ago, it has continued to be the most successful PD treatment. To our knowledge, this is the first exploration into the antioxidant and neuroprotective properties of MF against PQ-induced oxidative stress in *Drosophila melanogaster.*

2. Materials and Methods

2.1. Chemicals

Acetylthiocholine iodide (ATCI), thiobarbituric acid (TBA), 2′7′- dichlorofluorescein acetate (DCFH-DA), Paraquat dichloride (PQ), and superoxide dismutase (SOD) kit was procured from Sigma Chemical Co. (India). Trichloroacetic acid (TCA), hydrogen peroxide (H_2O_2), and 5, 5′- dithio-bis-(2-nitrobenzoic acid) (DTNB), 3,4 Dihydroxy-L-phenylalanine (L-DOPA) were procured from Himedia, (India). Other chemicals used in the study were of analytical grade.

2.2. Drosophila culture

The National Drosophila Stock, University of Mysore provided the *drosophila* culture. All the flies were maintained in a 150 ml glass bottle vial with 30 ml of nutrient–agar medium seeded with yeast, Flies were reared for all experiments at a temperature of 22 °C with a relative humidity of 70–80%.

2.3. Mulberry fruit extract

Mulberry fruit concentrated extract with an active constituent of 42% anthocyanin was obtained as a gift sample from M/s Bhoopalam Botanicals, Bengaluru, India.

2.4. PQ-induced lethality response

One hundred newly eclosed male flies were fed food for 2 days before being placed to clean glass bottles containing PQ mixed in sucrose solution and placed on filter paper and then treated with PQ at five concentrations of 2,4,6,8, and 10 mM for 48 h. Due to desiccation, filter papers needed to be changed every 24 h. Flies were seen to survive at 12, 24, 36, and 48 h after PQ exposure. The 50% lethality concentration (LC_{50}) was observed 24 h after PQ exposure. The LC_{50} was calculated using dose-dependent mortality data. All experiments were carried out four trails per replicate. The neuroprotective potential of MF on flies was examined using the LC_{50} of PQ (5 mM).

2.5. Behavioral assays: climbing activity assays

A negative geotaxis experiment was used on twenty newly eclosed male adult flies in a glass jar to assess their movement tendencies (10 cm length, 2 cm diameter). The bottom of the jar was gently tapped, and the flies were allowed to fly to the top. Six zones were marked for the distances from the bottom of the glass jar: 0, 0–2, 2–3, 3–5, 5–7, and more than 7 cm. The number of flies moving from the bottom to each zone was counted after 1 min of observation following tapping. Each experiment was carried out ten times at 10-minute intervals.

2.6. Paraquat resistance tests

To study the effect of oxidative stress in flies, adult flies were fed for two days. After 4 h of starvation,

flies were transferred to a glass vial filled with filter paper and soaked in a 5% sucrose solution containing 5 mM PQ. The survival of flies was monitored and noted every 12 h for 2 days.

2.7. Effect of pre-treated MF on fly survival

Groups of 2–3 day-old male flies were fasted for 4 h before being placed into new clean glass vials and allowed to feed on sucrose 5% solution (control group). Male flies were pre-treated with 1% MF extracts and 0.05% L-DOPA considered to be the test group. Each batch of pre-treated flies was then placed in a vial with 500 µl of 5 mM PQ in a 5% sucrose solution soaked on filter paper [9]. Every 24 h, the filter paper in each glass vial was changed without disturbing the flies because the soaking filter paper dried out. After 12, 24, 36, and 48 h, the vial's dead flies were counted.

2.8. Climbing activity

The effect of pre-treated MF on PQ-induced locomotion deficits was observed from the climbing activity at 24 hours after PQ exposure. The climbing activity assay was carried out as described in the above.

2.9. Biochemical parameters

2.9.1. Preparation of homogenates from the heads of flies

Cold anesthesia was treated to both the control and pre-treated flies for 10 min 50 fly heads were homogenized in 1000 µl of the ice-cold sodium phosphate buffer (0.1 M, pH 8.0) and centrifuged at 2,500 g for 10 min at 4 °C. The supernatant filtered through nylon mesh was evaluated for ROS, LPO, SOD, CAT, GSH, and AChE.

2.9.2. The protective action of the pre-treated MF on ROS in PQ-induced oxidative stress

Dichlorodihydrofluorescein diacetate (DCFH-DA) is frequently used to detect ROS [10]. The ROS was quantified in a homogenate sample using a dye, DCFH-DA that was later oxidized ROS producing highly fluorescent 2-dichlorofluorescein (DCF). In a brief, 10.0 M DCFH-DA was incubated with 100 µl of the homogenized sample at RT and it was incubated at dark for 1 hour. After incubation increased relative DCF fluorescence intensity was measured by using a multimode microplate reader (Thermo Scientific, Varioskan flash) with excitation (485 nm) and emission (535 nm).

2.9.3. The protective action of the pre-treated MF on LPO in PQ-induced oxidative stress

The thiobarbituric acid reactive compounds were measured to determine LPO [11]. In brief, 200 µl of homogenate was heated in a boiling water bath for 1 h with a reaction tube consisting of 200 µl of 8% SDS, 1.5 ml of 20% acetic acid (pH 3.5), and 1.5 ml of 0.8% TBA. Adducts were extracted in 3 ml of 1-butanol after cooling. After centrifugation, the absorbance of the supernatant at 532 nm was estimated and reported as malondialdehyde (MDA) equivalents.

2.9.4. Modulatory effect of the MF on SOD in PQ-induced oxidative stress

SOD activity has been measured in biological samples using the SOD Assay kit [12]. The kit includes all of the chemicals and solutions needed for an indirect assay method based on xanthine oxidase and a specific color reagent that was measured at 440 nm absorbance using spectroscopy. The test was performed according to the manufacturer's guidelines.

2.9.5. Modulatory effect of the pre-treated MF on CAT in PQ-induced oxidative stress

Catalase activity was evaluated by measuring the rate of H_2O_2 decomposition [13]. In a brief, 50 µl of 1% H_2O_2 was added to 1 ml of a reaction mixture containing 0.05 ml of homogenate and 0.95 ml of 0.05 M phosphate buffer. A spectrophotometer was used to quantify the change in absorbance over a 3-minute period at 240 nm, and the results were represented as moles of H_2O_2 decomposed/min/mg protein.

2.9.6. PQ-induced GSH depletion and protective effect of pre-treated MF

Ellman's reagent was used to measure the glutathione (GSH) content DTNB [14]. The homogenate was precipitated in a 1:1 ratio with 5% TCA acid. The samples were stored at 4 °C for 1 h before being centrifuged at 5000 rpm for 10 min at 4 °C. 550 µl of 0.1 M phosphate buffer, 100 µl of supernatant, and 100 µl of DTNB were used in the test. The optical density (OD) was measured at 412 nm and the results were represented as micromoles of GSH/mg protein.

2.9.7. Effect of pre-treated MF on AChe activity in flies exposed to PQ

To determine AChe activity. 10 mM of DTNB, 50 µl homogenate, and 1 ml phosphate buffer are added to the reaction mixture (0.1 M, pH 8.0). The acetylthiocholine iodide was added to initiate the reaction, and the change in absorbance at 412 nm

was monitored for 3 min. The amount of substrate hydrolyzed by the enzyme each minute per mg of protein was expressed in nmoles [15].

2.10. Statistical analysis

All data were analyzed using one-way analysis of variance (ANOVA) and the post hoc "Tukey" test using SPSS software. The results were presented as mean ± standard error (SE), and a significant difference ($p \leq 0.05$) was observed.

3. Results

3.1. PQ-induced lethality response

Flies were co-exposed to 5 concentrations of PQ (2, 4, 6, 8, and 10 mM). Concentration-dependent mortality was observed on PQ exposure [Fig. 1A]. The survivorships at the exposed concentrations of 2, 4, 6, 8, and 10 mM after 24 hours were 93%, 63%, 41%, 22%, and 8%, respectively, and lower mortality was observed at concentrations of 2 mM, Further, a significant ($p \leq 0.05$) mortality was observed from concentrations 4–10 mM. Therefore, the LC_{50} value of PQ toxicity to flies was determined after 24 hours of exposure. The LC_{50} was found to be 5 mM at 24-hours time point.

3.2. Effects of pre-treated MF on fly survival in PQ-induced toxicity

Flies pre-treated with L-DOPA (0.05%) and MF (1%) were investigated, to study the survival rate and compared with PQ exposed as well as untreated groups (only sucrose). After 12 hours of PQ exposure, flies pre-treated with L-DOPA and MF survived with 93 ± 1.5%, and 90 ± 0.8% survival rates respectively, which was found to be high when compared with only PQ exposed flies (82 ± 1.8%). Further, the survival rates of flies pre-treated with MF after 24 hours of PQ exposure, demonstrated a better survival rate 64 ± 1.5%, compared to a fly group pre-treated with L-DOPA (61 ± 1.2) and an untreated group (51 ± 1.1%), as shown in Fig. 1B. Flies pre-treated with L-DOPA and MF extracts after 36 hours showed low survival rates of 20 ± 1.3% and 17 ± 1.3%, compared to the untreated group which has zero survival rate. Notably, no survivors were found in any of the flies groups after 48 h [Fig. 1 B].

3.3. Effects of pre-treated MF on PQ-induced locomotion deficits

Flies exposed to PQ resulted in severe locomotor destruction as evident from the negative geotaxis assay. In the present study, the PQ-induced locomotion deficits of the flies were studied by measuring the negative geotaxis. All the flies that were not exposed to PQ showed a movement of over 7 cm from the bottom of the jar. The flies exposed to 5 mM PQ for 12 hours and 24 hours showed completely different patterns of movement. They stayed in the middle or bottom of the jar, implying that PQ may increase toxic effects on fly locomotion and illness as shown in Fig. 2A. Furthermore, the climbing activity of the flies exposed to PQ was determined, which showed that pre-treated L-DOPA had 21% and MF has 20% climbing activity over 7 cm, whereas the PQ untreated group showed only 8% climbing activity as shown in the Fig. 2b

3.4. Effects of pre-treated MF on oxidative stress marker in PQ exposed flies

3.4.1. Alternations of ROS and LPO levels

Untreated flies, when exposed to PQ resulted in a significant ($p \leq 0.05$) increase in the ROS and LPO content. Flies pre-treated with MF exposed to PQ

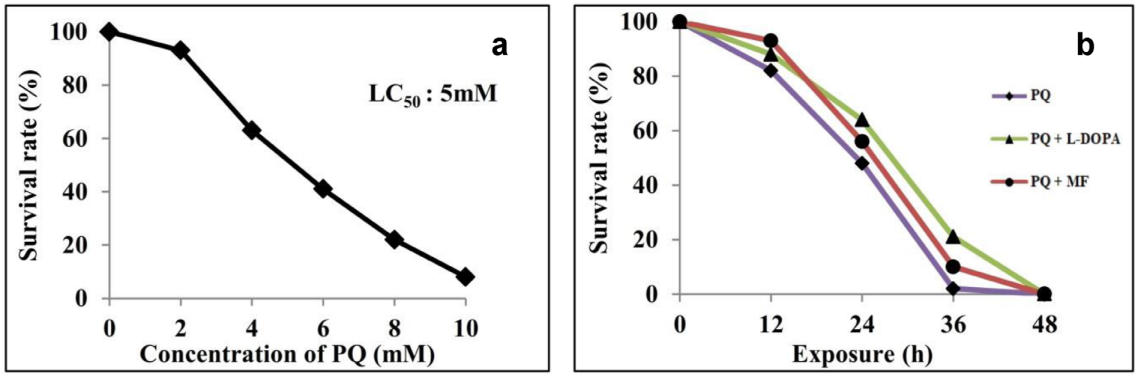

Fig. 1. (a). Effect of different concentrations of PQ exposure on survivability. (b) Effects of pre-treated MF on fly survival in PQ-induced toxicity. LC_{50}: Lethal Concentration, PQ: Paraquat (5 Mm), MF: Muberry fruit (1%).

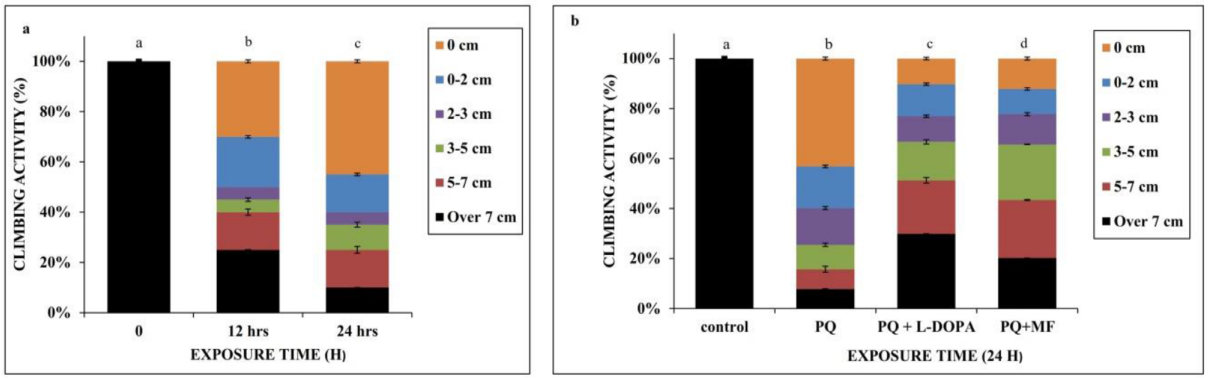

Fig. 2. PQ-induced locomotion deficits. (a) 5 mM paraquat exposed induced the deficits in locomotion of flies after 12 hours and 24 hours. (b) Effects of pre-treated MF on PQ-induced locomotion deficits. According to Tukey's HSD, bars with the same letters are not significantly different ($p \leq 0.05$). PQ: Paraquat (5 Mm), MF: Muberry fruit (1%).

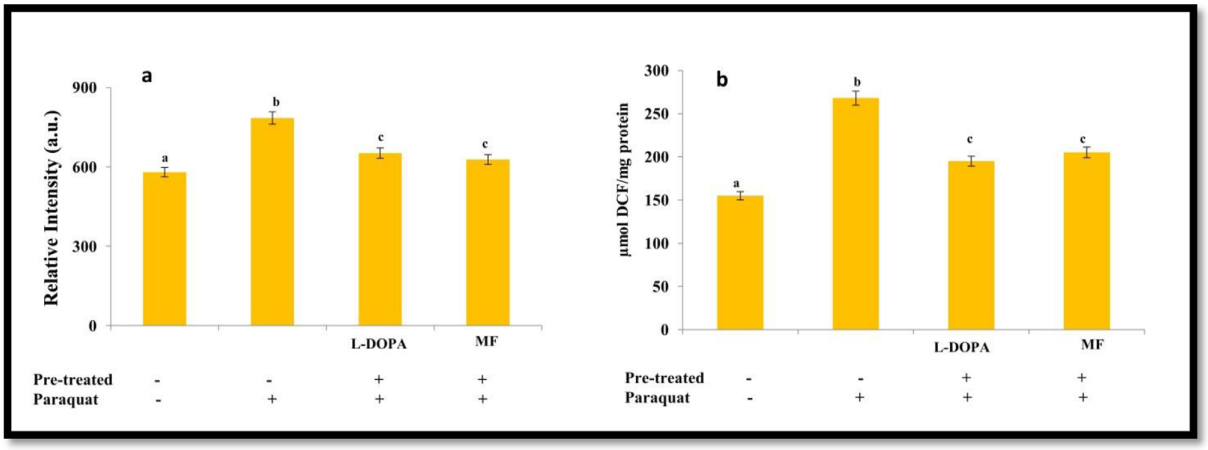

Fig. 3. Effect of MF on oxidative stress marker in PQ exposed flies (a) Protective action of the MF on ROS in PQ-induced oxidative stress (b) Protective action of the MF on LPO in PQ-induced oxidative stress. According to Tukey's HSD, bars with the same letters are not significantly different ($p \leq 0.05$). MF: Muberry fruit (1%).

showed a significant ($p \leq 0.05$) reduction in the levels of ROS and LPO compared to PQ exposed untreated flies as shown in Fig. 3a and b.

3.5. Effects of pre-treated MF on antioxidant enzyme activity in PQ exposed flies

3.5.1. Antioxidant enzyme activity

As shown in Fig. 4 a, b and c. It was observed that SOD, CAT, and GSH levels were elevated in flies pre-treated with L-DOPA and MF compared to the PQ exposed untreated group which was found to be statistically significant ($p \leq 0.05$).

3.6. Effects of pre-treated MF on AChE enzyme activity in PQ exposed flies

Untreated flies exposed to PQ had significantly higher ($p \leq 0.05$) AChE activity levels than flies not exposed to PQ control flies [Fig. 4D]. AChE activity was dramatically reduced in groups of flies pre-treated with L-DOPA and MF. The MF fly groups of AChE activity levels were comparable to L-DOPA groups.

4. Discussion

Humans may be exposed to PQ either directly during cultivation or indirectly through contaminated food. PQ causes developmental and neurological disorders in humans even at very low concentrations when consumed with processed foods. *Drosophila* model been widely used by researchers in the field of neurobiology due to similarities in terms of the human disease gene, protein sequences, and behavioral features of PD, including resting tremors, bradykinesia, and postural instability when Parkinsonism is induced in flies [16,17].

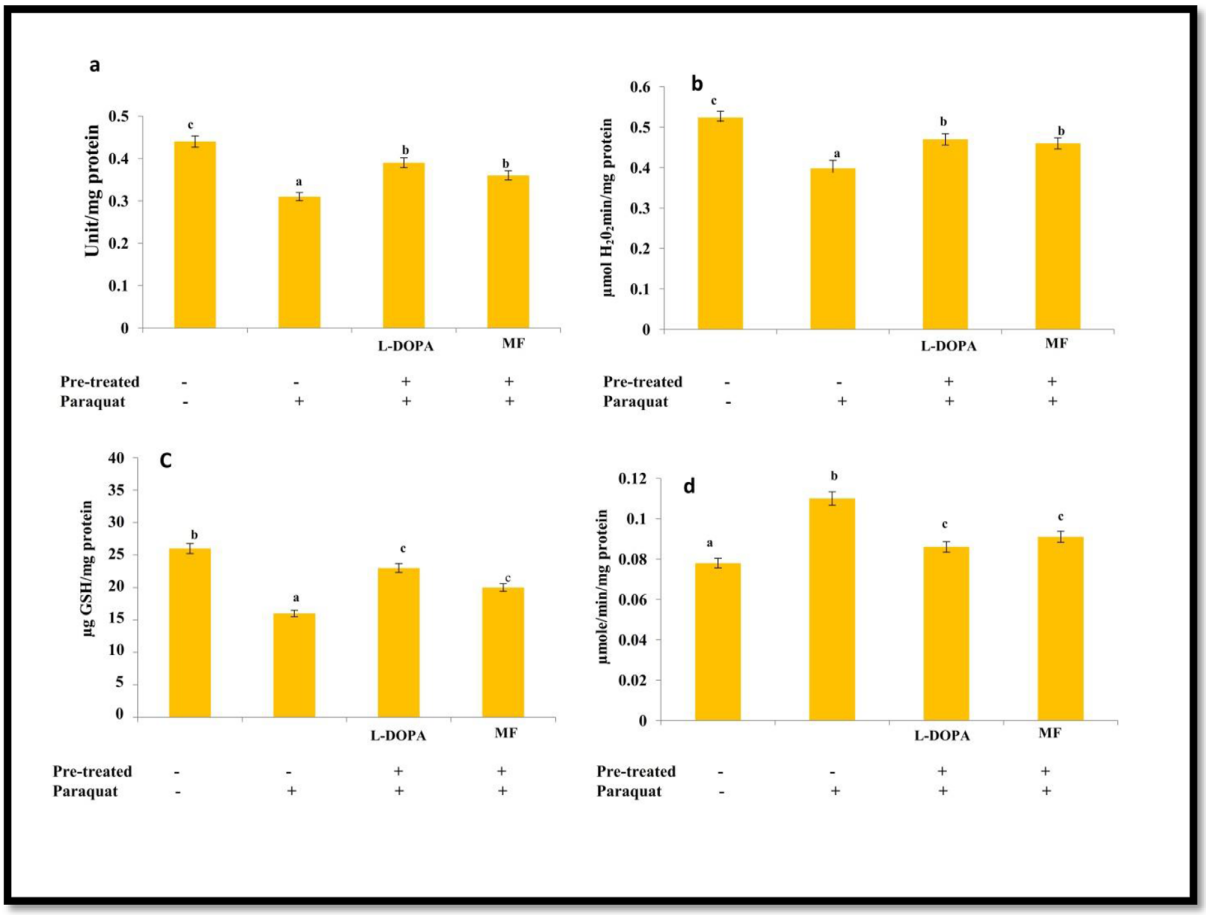

Fig. 4. Effect of MF on antioxidant enzymes and AChe activity in PQ exposed flies (a) Modulatory effect of the MF on SOD in PQ-induced oxidative stress (b) Modulatory effect of the MF on CAT in PQ-induced oxidative stress (c) PQ-induced GSH depletion and protective effect of MF. (d) Effect of MF on Ache activity in flies exposed to PQ. According to Tukey's HSD, bars with the same letters are not significantly different ($p \leq 0.05$). MF: Mulberry fruit (1%).

According to PQ can get through the inner membrane of the mitochondria and block the activity of mitochondrial complex I by generating superoxide radicals and other redox products. Repeated sublethal PQ exposure in flies causes selective destruction of dopaminergic neurons which leads to locomotor abnormalities [3].

Due to safety and efficacy, plants and their bioactive ingredients are of tremendous interest. Indeed, numerous plant-derived substances, like as phenolic acids and flavonoids, have been demonstrated to have anti-disease Alzheimer's (AD) effects against oxidative stress and AchE [18,19] Mulberry fruits have been recognized to be rich in anthocyanin's as members of the flavonoids [6]Therefore, this research aimed to study the neuroprotective properties of MF against paraquat-induced oxidative stress in *Drosophila melanogaster*.

Our findings from the current study indicate that untreated PQ-exposed files showed a deficit in locomotion and remained at the bottom of the jar in the negative geotaxis assay because they were unable to coordinate their legs while rising. As opposed to flies subjected to PQ, interestingly, flies pre-treated with MF extract prior to PQ exposure had better locomotory activity in the negative geotaxis assay, indicating the ameliorative effect of MF on the PQ-induced locomotory deficit. According to our findings, untreated flies exposed to PQ had toxicity which caused a limited chance of surviving. The fact that the MF pre-treated flies showed low mortality, compared to untreated flies exposed to PQ, suggests that the bioactive components in MF had a protective effect against the neurotoxic effects of PQ. PQ can enter dopamine (DA) neurons through DAT in its monovalent cation state through microglia with the help of NADPH oxidase or reducing agent and aggregates in DA neurons, where it generates ROS [20]. Our findings demonstrated that untreated PQ flies elevated ROS and

LPO levels while diminishing SOD, CAT, and GSH levels. The generation and elimination of ROS equilibrium are essential in homeostasis and are maintained by antioxidant enzymes [21].CAT and SOD are commonly regarded as key markers of oxygen species. SOD turns O_2 to hydrogen peroxide (H_2O_2), which is then converted to water by CAT and GPx [22]. The OS has the ability to damage a wide spectrum of macromolecules, including lipids, proteins, and sugars [22]. According to Nikdad et al. [23] the primary source of PQ-induced toxicity in the brain mitochondria is OS, which is supported by the induction of LPO and a decrease in CAT and SOD activity. However, when MF pre-treated flies were exposed to PQ, they significantly diminished ROS and LPO levels by restoring antioxidant enzyme activity such as SOD and CAT.

AChE activity is well-described and has been used to validate the efficacy of therapies for Parkinson's disease and other neurodegenerative disorders in addition to the behavioral parameter [24]. It takes place during cholinergic neurotransmission and is in the hydrolysis of the neurotransmitter acetylcholine, which has an impact on movement and motor function [25]. In our work, untreated flies exposed to PQ, showed that there was an increase in AChE activity. This increase in AChE activity may result in lower levels of acetylcholine in the synaptic cleft and, as a result, a decrease in the effectiveness of that cholinergic neurotransmitter, as shown in reduced climbing activity. Our findings also demonstrate that MF has a protective function in our PQ-induced neurotoxicity model in *Drosophila melanogaster* by preventing excessive AChE activity, maintaining it at regulated levels, and increasing climbing performance. This finding can be explained by the fact that anthocyanin from mulberry fruits serves as an antioxidant and aids in the reduction of AChE activity. Our findings are consistent with Lee et al. [26], who reported that MF extract has antioxidant properties that prevent ROS production, and, lipid peroxidation, and increase GSH levels in cellular systems. Furthermore, decreasing the AChE activity.

5. Conclusion

We employed the *drosophila* model system in this study to screen MF extract for neuropharmacological effects before trying it in mammalian models. Based on our findings, we conclude that pre-treated *drosophila* with MF extract has the potential to reduce PQ-induced oxidative stress due to its antioxidative characteristics and it is able to modify the enzyme activities of antioxidant defense systems which are SOD, CAT, and GSH. Furthermore, the neuroprotective properties of MF were clearly demonstrated by its ability to drastically reduce PQ-induced oxidative damage, increase locomotor performance, and restore AChe levels. However, before advocating for human consumption, the specific mechanism causing mitochondria-targeted neuroprotection by MF must be investigated further.

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Conflict of interest

There are no conflicts of interest.

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