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RESEARCH ARTICLE

Ameliorative Potential of Natural Antioxidants Against Paraquat-Induced Oxidative Stress and Locomotor Impairment in *Drosophila melanogaster*: A Comparative Study

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Abstract:

Background:

Natural antioxidants show neuroprotective potential to protect against neurodegenerative disorders in experimental animals. There is a need to characterize newer promising neuroprotective natural molecules.

Objective:

In the present study, we have compared the neuroprotective activity of 4hydroxyisophthalic acid (DHA-I), a novel natural antioxidant from the roots of *Decalepis hamiltonii,* with the other natural neuroprotective antioxidants, ellagic acid, quercetin and nicotinamide, against paraquat (PQ) neurotoxicity in *D. melanogaster*.

Results:

Flies exposed to multiple (sub-lethal) dose of PQ showed movement disorder characteristic of Parkinson's disease (PD). The four natural antioxidants showed ameliorative effects against PQ neurotoxicity in the sub-acute model as seen in survivability, locomotor activity as well as oxidative stress markers including reactive oxygen species (ROS), lipid peroxidation and the endogenous antioxidant defenses.

Conclusion:

Our study shows that the antioxidant compounds exhibit varying degrees of protection against PQ-induced oxidative stress and neurotoxicity with DHA-I, quercetin, and nicotinamide being the most effective and ellagic acid, the least potent in *Drosophila*. Our results show that mitochondrial Mn-SOD is a critical target for PQ neurotoxicity and the neuroprotection by the antioxidants involves the attenuation of mitochondrial ROS production and oxidative damage.

Keywords: Parkinson's disease, Free radicals, Antioxidant enzymes, Mitochondria, *Drosophila melanogaster*, Neurodegenerative disorders.

1. INTRODUCTION

Oxidative stress occurs when there is an imbalance between the generation of free radicals and cellular antioxidant defenses [1], which is implicated in the pathophysiology of certain neurodegenerative diseases [2]. Paraquat (PQ), a widely used herbicide, induces Parkinson's disease (PD) phenotype in experimental models including laboratory rats, mice and *Drosophila* [3 - 6]. PQ can undergo redox cycling wherein, a divalent cation of PQ (PQ^{2+}) is reduced to monocation radical (PQ^{+}), which reacts with the oxygen molecule to give rise to free radical generation [7].

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Although the mechanisms underlying PQ induced PD are not fully understood, it is believed to be mediated by free radical-induced oxidative stress that causes mitochondrial dysfunction leading to neuronal cell death [8, 9]. Neurotoxic compounds that target complex-I of mitochondria can induce dopaminergic cell death to produce PD condition. Defects in mitochondrial function in substantia nigra of the brain in PD patients have been observed which gives a compelling evidence for the role of mitochondrial dysfunction in the pathogenesis of PD [10].

Drosophila melanogaster is widely used as a model to study neurodegenerative diseases including PD to elucidate the mechanisms involved in the disease [11 - 13]. Genetic and pesticide induced PD models of *Drosophila* are useful to screen natural bioactive compounds for their neuroprotective potential. The acute PQ model of PD in *Drosophila* is widely used to evaluate the neuroprotective potential of bioactive compounds [14 - 16]. However, the acute PQ model of *Drosophila* to induce PD is inadequate because of high mortality, reversible symptoms of motor impairment and neurodegeneration [17, 18]. We have used a revised sub-acute model wherein flies exposed to multiple-dose (sub-lethal) of PQ replicates PD-like phenotype showing irreversible neurodegeneration in the brain and therefore, a more appropriate model to test the efficacy of neuroprotective agents [18].

A large body of evidence suggests that phytochemicals possess health-promoting properties attributed to their ability to alleviate oxidative stress [15, 19 - 21]. Since oxidative stress-induced mitochondrial dysfunction, is implicated in the pathogenesis of PD, treatment with antioxidants could be a useful strategy in ameliorating neurodegeneration, and slowing down the disease progression [22].

Many bioactive compounds have been tested for their ability to protect against neurodegeneration, and those possessing antioxidant properties such as vitamins and flavonoids are of particular interest. Quercetin, a natural flavonoid antioxidant, showed neuroprotective activity in several neurodegenerative disease models including PD [23 - 25]. Nicotinamide, the amide form of niacin, and a precursor of the cofactor, nicotinamide adenine dinucleotide (NAD^+), acts as an endogenous antioxidant [26] and reported to possess neuroprotective activity in several PD models [27, 28].

The edible roots of *Decalepis hamiltonii (Dh)* are a source of potent cocktail of natural antioxidants [29] and, several novel antioxidant molecules have been isolated and characterized [30, 31]. The natural bioactive compounds from *Dh* have been shown to exhibit cytoprotective potential against oxidative stress-induced cell injury [32 - 35] Furthermore, hepatoprotective [36] and neuroprotective [37 - 39] activity of the roots of *Dh* against hepatotoxic and neurotoxic chemicals *in vivo* has been reported. Ellagic acid, a polyphenolic antioxidant from *Dh*, exhibits *in vitro* free radical scavenging activity as well as cytoprotective activity against xenobiotic-induced oxidative stress in primary hepatocytes and Ehrlich Ascites tumor cells [32]. Recent reports suggest that ellagic acid mitigates neurodegeneration possibly *via* free radical scavenging activity [40 - 42]. 4Hydroxyisophthalic acid (4- HIPA or DHA-I), a novel bioactive molecule isolated from the aqueous extract of *Dh* roots, shows potent *in vitro* free radical scavenging activity and cytoprotective activity against xenobiotic-induced oxidative stress in primary hepatocytes and Ehrlich Ascites tumor cells [34]. Furthermore, we have shown that DHA-I exhibits neuroprotective potential by attenuating tau neuropathy in transgenic *Drosophila* model [43]. The objective of this study was to compare the antioxidant and neuroprotective activity of DHA-I with the three natural antioxidants, ellagic acid, quercetin and nicotinamide, in the *Drosophila* model of PQ neurotoxicity and motor impairment.

2. MATERIALS AND METHODS

2.1. Chemicals and Antioxidant Compounds

Paraquat dichloride (PQ), 2'7'- dichlorofluorescin diacetate (DCFH-DA), acetylthiocholine iodide (ATCI), pyrogallol, bovine serum albumin, thiobarbituric acid (TBA), sodium lauryl sulphate (SDS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Nicotinamide (NA), ellagic acid (EA) and quercetin (Que) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

5, 5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), hydrogen peroxide (H_2O_2), trichloroacetic acid (TCA) and other chemicals were purchased from Sisco Research Laboratories, Mumbai, India.

The isolation of DHA-I from the aqueous extract of *Dh* roots was done by silica gel column chromatography and RP-HPLC, and characterized by UV, IR, LC-MS, and NMR spectroscopic techniques as described earlier [31, 34].

2.2. *Drosophila* Culture

Wild-type *D. melanogaster* (Oregon K) adult flies were obtained from Drosophila Stock centre, Department of Zoology, Manasagangotri, University of Mysore, Karnataka, India. All flies were maintained in a 150 ml culture bottle containing 30 ml of standard wheat cream–agar medium seeded with yeast. Flies were reared at 22±1°C and 70–80% relative humidity for all the experiments.

2.3. Treatment

The experimental diets containing DHA-I (dissolved in phosphate buffered saline), nicotinamide (dissolved in distilled water), ellagic acid (dissolved in 0.6% DMSO) and quercetin (dissolved in distilled water) were prepared at a final concentration of 0.01%, 0.02% and 0.05% (w/v) in wheat cream-agar diet. Newly eclosed flies fed with control diet (without antioxidants) and experimental diets for five days were transferred to the vials containing filter paper soaked with 5% sucrose solution and acute dose of PQ (20 mM). Exposure to the acute dose of PQ is associated with high mortality in adult flies and the number of dead flies was scored after 24h of exposure. Based on survival against acute PQ toxicity, effective doses of each of the antioxidant compounds were determined. For each tested group, six replicates of ten flies each per sex were tested and expressed as percentage survival.

Flies were exposed to multiple sub-lethal dose of PQ (5.375mM - one-fourth of LC_{50}) to induce oxidative stress and PD-like symptoms. Briefly, six-day-old flies were transferred to the vials containing filter paper soaked with 5% sucrose solution and 5.375 mM PQ for 24h on every alternate day for 8 days [18]. Flies were transferred back to normal wheat-cream agar medium after every PQ exposure to minimize any profound effects of PQ as well as sucrose diet on the survivability of flies [44].

For neuroprotection studies, newly eclosed flies were fed with control diet or experimental diets containing following compounds: DHA-I, nicotinamide, ellagic acid and quercetin at a final concentration of 0.02% (effective dose) for next five days. Following feeding process, flies were exposed to 5.375 mM of PQ for 24h on alternative day for 8 days and transferred to the vials containing respective diets after each PQ exposure.

2.4. Climbing Activity (Negative Geotaxis Assay)

To determine the effect of PQ on locomotion pattern, the climbing ability of flies was monitored by negative geotaxis assay. Briefly, ten flies were introduced into a vertical glass column (standard length, 25 cm). After a brief recovery period, flies were gently tapped to the bottom and then allowed to climb [11]. Effect of antioxidant compounds on PQ-induced locomotor impairment was monitored from the climbing response. The number of flies climbed beyond the minimum distance of 12 cm in 20s of interval was recorded. For each tested group, six replicates of ten flies each per sex were tested and expressed as an average of six replicates.

2.5. Biochemical Assays

Both control and treated flies were subjected to cold anesthesia for 10min. 50 fly heads were homogenized in 500 µl of respective assay buffers and centrifuged at 2500×g for 10min at 4°C. The supernatant was used to determine the levels of ROS, lipid peroxidation (LPO), reduced glutathione (GSH), the activity of antioxidant enzymes (SOD and catalase) and acetylcholinesterase (AChE).

2.5.1. Reactive Oxygen Species

ROS was quantified by fluorimetric method as described previously [45]. The reaction involves ROS-mediated conversion of DCFH-DA into a fluorescent product, 2'7'- dichlorofluorescin (DCF) which was measured using multimode plate reader with an excitation wavelength of 488 nm and emission at 525 nm. ROS was quantified using DCF standard curve and expressed as µmoles of DCF formed/min/mg protein. For each tested group, three replicates of 50 flies each per sex were used.

2.5.2. Lipid Peroxidation

LPO was assayed by measuring the thiobarbituric acid reactive substances. Briefly, 500 µl of tissue homogenate was heated with the reaction mixture containing 1.5 ml of 20% (v/v) acetic acid (pH 3.5), 1.5 ml of 0.8% (w/v) TBA, and 200 µl of 8% (w/v) SDS in boiling water bath for 1h. After cooling, adducts were extracted in 3 ml of 1-butanol. Following the centrifugation, the absorbance of the supernatant was determined at 532 nm and expressed as

malondialdehyde equivalents [46]. For each tested group, three replicates of 50 flies each per sex were used.

2.5.3. Reduced Glutathione

Fly head-homogenate was prepared in 1ml of 5% (w/v) TCA, centrifuged at 2,500×g for 10min at 4°C. The deproteinized supernatant was subjected to GSH estimation by using Ellman's reagent. Briefly, 200 µl of tissue homogenate was incubated with 2.8 ml of 0.2M tris-HCl buffer (pH 8) and 50 µl of 10 mM DTNB at room temperature for 5 min. The yellow colored product was measured at 412 nm. GSH content was quantified using GSH standard curve and expressed as µg of GSH/mg protein [47]. For each tested group, three replicates of 50 flies each per sex were used.

2.5.4. Antioxidant Enzymes

SOD activity was determined by measuring the inhibition of pyrogallol autoxidation. Briefly, the reaction was started by adding 0.5 ml of 2 mM pyrogallol to the reaction mixture containing 500 µl of tissue homogenate, 0.5 ml of distilled water and 2 ml of 0.1 M tris-HCl buffer (pH 8.2). The change in absorbance was monitored for 3 min using spectrophotometer at 420 nm. The activity was expressed as enzyme units required to inhibit 50% pyrogallol autooxidation. Cyanide selectively inhibits the activity of Cu, Zn-SOD and allows the measurement of Mn-SOD activity. By using Potassium Cyanide (1mM), activities of both Cu, Zn- SOD and Mn-SOD were determined [48]. For each tested group, three replicates of 50 flies each per sex were used.

Catalase activity was determined by measuring the rate of H_2O_2 decomposition as described previously [49]. Briefly, 50 µl of 1% (v/v) H_2O_2 was added to 1 ml reaction mixture containing 50 µl homogenate and 950 µl of 0.05M phosphate buffer (pH 7). The change in absorbance was monitored for 3 min using spectrophotometer at 240 nm and expressed as µmoles of H_2O_2 decomposed/min/mg protein.

For each tested group, three replicates of 50 flies each per sex were used.

2.5.5. Acetylcholinesterase

AChE activity was assayed by measuring the rate of hydrolysis of ATCI in 0.1 M phosphate buffer (pH 8). The reaction was started by adding 200 µl of tissue homogenate to 2.8 ml reaction mixture containing 250 µl 10 mM DTNB, 100 µl 30 mM ATCI and 2.45 ml of phosphate buffer. The change in absorbance was monitored for 3mins using spectrophotometer at 412nm and expressed as µmoles of ATCI hydrolyzed/min/mg protein [50]. For each tested group, three replicates of 50 flies each per sex were used.

2.5.6. Protein Estimation

Protein concentration in the tissue homogenate was determined by Lowry's method [51].

2.6. Statistical Analysis

All data were analyzed by one-way analysis of variance (ANOVA) followed by post hoc 'Tukey' test using SPSS software. Data were expressed as mean±SE and the significant difference ($p<0.05$) was determined.

3. RESULTS

3.1. Paraquat-induced Mortality

In flies exposed to the acute dose of PQ (20 mM), a significant mortality was observed after 24h. Treatment with different antioxidant compounds improved the survival of adult flies against PQ toxicity than control flies (Fig. **1**). Both male and female flies fed with different antioxidant compounds exhibited dose-dependent survival against PQ- toxicity. The percentage survival of males fed with 0.02% and 0.05% of DHA-I, nicotinamide or quercetin was significantly higher compared to other doses whereas ellagic acid was effective against PQ toxicity only at 0.02%. Also, the survival of females fed with 0.02% of DHA-I or ellagic acid, 0.02% and 0.05% of nicotinamide or quercetin was significantly higher compared to other doses of respective compounds. Based on the effective doses against acute PQ-toxicity, 0.02% was used as an optimal concentration to compare the neuroprotective action of these compounds against PQ-induced oxidative stress and neural dysfunctions.

After 24h of PQ exposure, flies fed with 0.02% of DHA-I, quercetin and nicotinamide showed better survival than the fly group fed with ellagic acid. However, there were sex differences in the protective action of antioxidants against

PQ toxicity. DHA-I and quercetin showed protective action against PQ-induced mortality in both the sexes; whereas, in females, the protective effect of nicotinamide on survival was higher compared to DHA-I and quercetin (Fig. **2**).

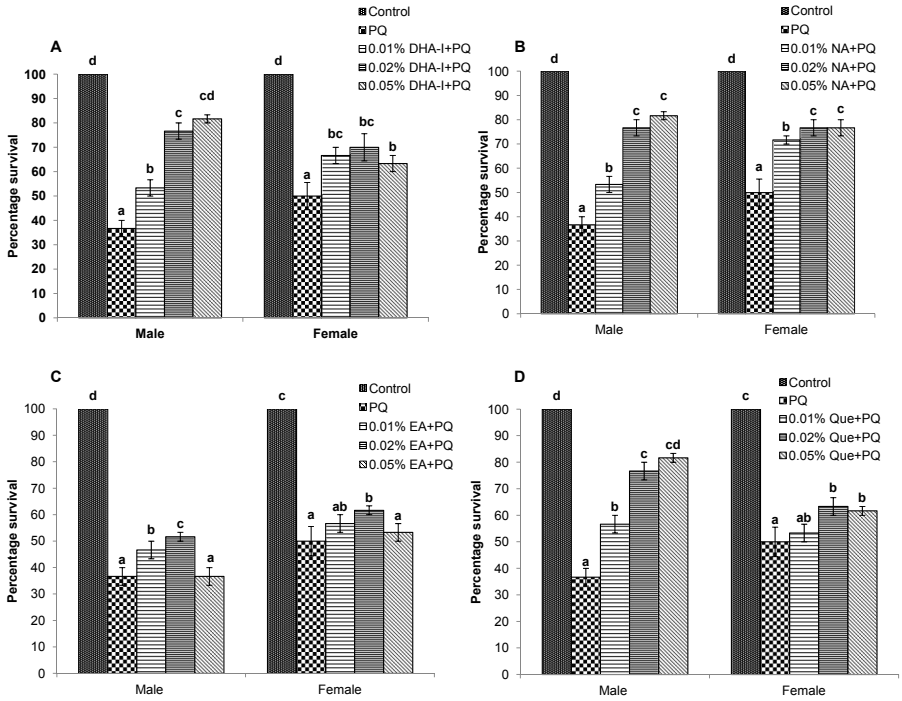

Fig. (1). Dose-dependent effect of the antioxidant compounds on PQ toxicity. Flies fed with different concentrations of antioxidants (4-Hydroxyisophthalic acid-DHA-I, Nicotinamide-NA, Ellagic AcidEA, Quercetin-Que) were exposed to 20mM PQ (**A-D**) and mortality was recorded after 24h. All the antioxidant compounds exhibited dose-dependent protection against PQ toxicity. Values are mean±S.E. **(**n=6; 10 flies per replicate) and bars denoted by identical lowercase alphabets (a–c) indicate no significant difference, whereas, values denoted by different alphabets indicate a statistically significant difference ($p<0.05$).

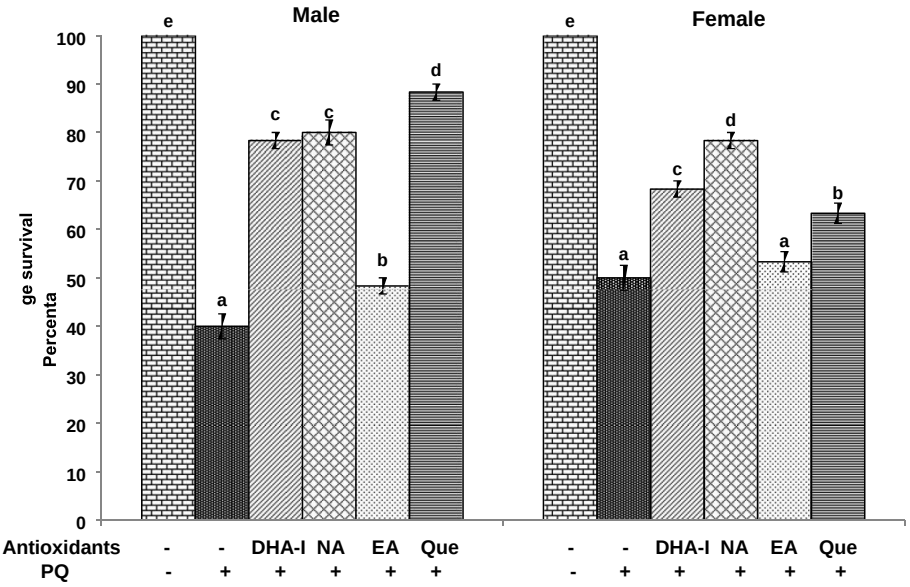

Fig. (2). Comparison of the protective effect of the antioxidant compounds on PQ-induced mortality.
Flies fed with effective concentration (0.02%) of different antioxidants (4-Hydroxyisophthalic acidDHA-I, Nicotinamide-NA, Ellagic Acid-EA, Quercetin-Que) were subjected to PQ (20mM) treatment and mortality was recorded after 24 of exposure. Values are mean±S.E. (n=6; 10 flies per replicate) and bars denoted by identical lowercase alphabets (a–c) indicate no significant difference, whereas, values denoted by different alphabets indicate a statistically significant difference ($p<0.05$).

3.2. Locomotor Deficits

Flies exposed to multiple-dose of PQ resulted in severe locomotor impairment as evident from the negative geotaxis assay. Flies tend to stay at the bottom of the column which indicates deleterious effects of PQ on the climbing ability, characteristic of PD. Dietary supplementation of the antioxidant compounds was able to alleviate the locomotor deficits caused by PQ exposure (Fig. **3**). Protective action of DHA-I, quercetin and nicotinamide on climbing ability was markedly higher compared to ellagic acid in both the sexes. However, in females, nicotinamide provided better protection against PQ-induced locomotor impairment compared to quercetin and DHA-I.

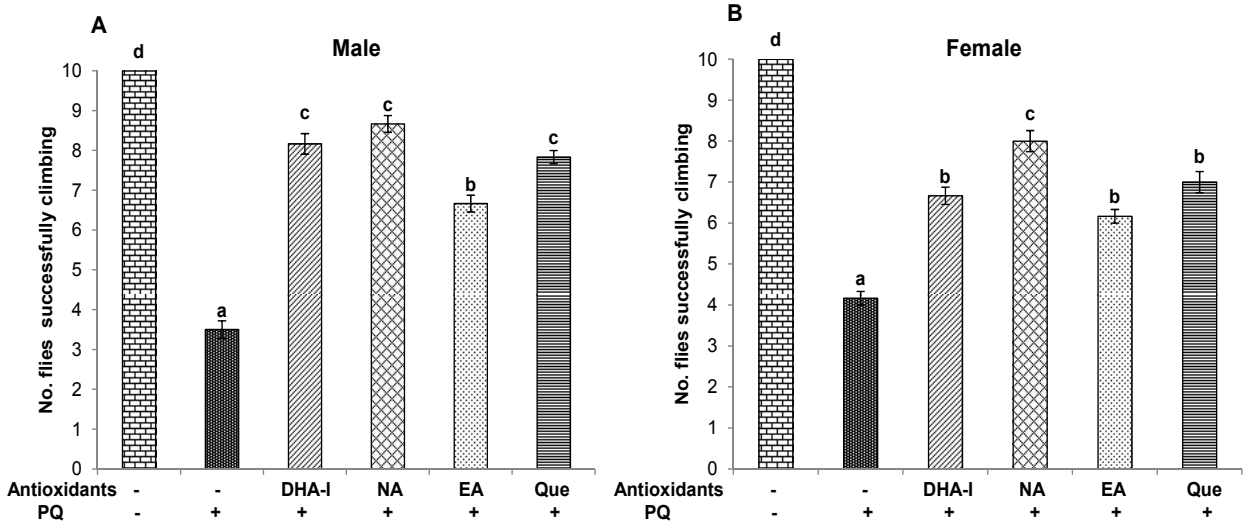

Fig. (3). Modulatory effect of the antioxidant compounds on PQ-induced locomotor deficits measured by negative geotaxis assay. Flies exposed to multiple dose of PQ showed a significant reduction in the climbing activity in males **(A)** and females **(B)**. Treatment with antioxidants (4-Hydroxyisophthalic acid-DHA-I, Nicotinamide-NA, Ellagic Acid-EA, Quercetin-Que) ameliorated the PQ-induced locomotor deficits in both sexes. Values are mean±S.E. (n=6; 10 flies per replicate) and bars denoted by identical lowercase alphabets (a–c) indicate no significant difference, whereas, values denoted by different alphabets indicate a statistically significant difference ($p<0.05$).

3.3. Oxidative Stress Markers

Flies exposed to multiple dose of PQ showed a significant increase in ROS levels in both sexes (Figs. **4A** and **4B**). There was also a marked increase in the level of LPO in PQ-treated flies (Figs. **4C** and **4D**). All four fly groups fed with the antioxidant compounds showed decreased levels of ROS and LPO when compared to the PQ-only treated flies. Among the antioxidant compounds, nicotinamide caused a greater reduction in ROS levels followed by quercetin, DHA-I, and ellagic acid. Also, administration of DHA-I, nicotinamide and quercetin caused a significant decrease in LPO level compared to ellagic acid in both the sexes.

Exposure to PQ resulted in decreased level of GSH compared to control. Treatment with DHA-I, nicotinamide and quercetin showed an increase in GSH content; whereas, ellagic acid did not exert any influence on GSH level (Fig. **5**). Interestingly, in females, the ameliorative effect of nicotinamide against PQ-induced GSH depletion was higher compared to DHA-I and quercetin.

DHA-I treatment prevented the PQ-induced changes in SOD activity in both sexes. The protective effect of DHA-I on Mn-SOD was clearly higher than that of Cu-Zn SOD. Similar protective effect of quercetin on PQ-induced SOD activity was seen. Interestingly, there were sex differences in the efficacy of the antioxidants. DHA-I and quercetin showed protective action on SOD activity in both the sexes; however, in females, the protective effect of nicotinamide against PQ-induced changes in SOD activity was higher compared to DHA-I and quercetin (Fig. **6**). Catalase, on the other hand, was significantly increased in flies exposed to PQ and administration of antioxidant compounds prevented these alterations to varying degree. DHA-I and quercetin were more effective in prevention of changes in catalase activity than nicotinamide and ellagic acid (Fig. **7**).

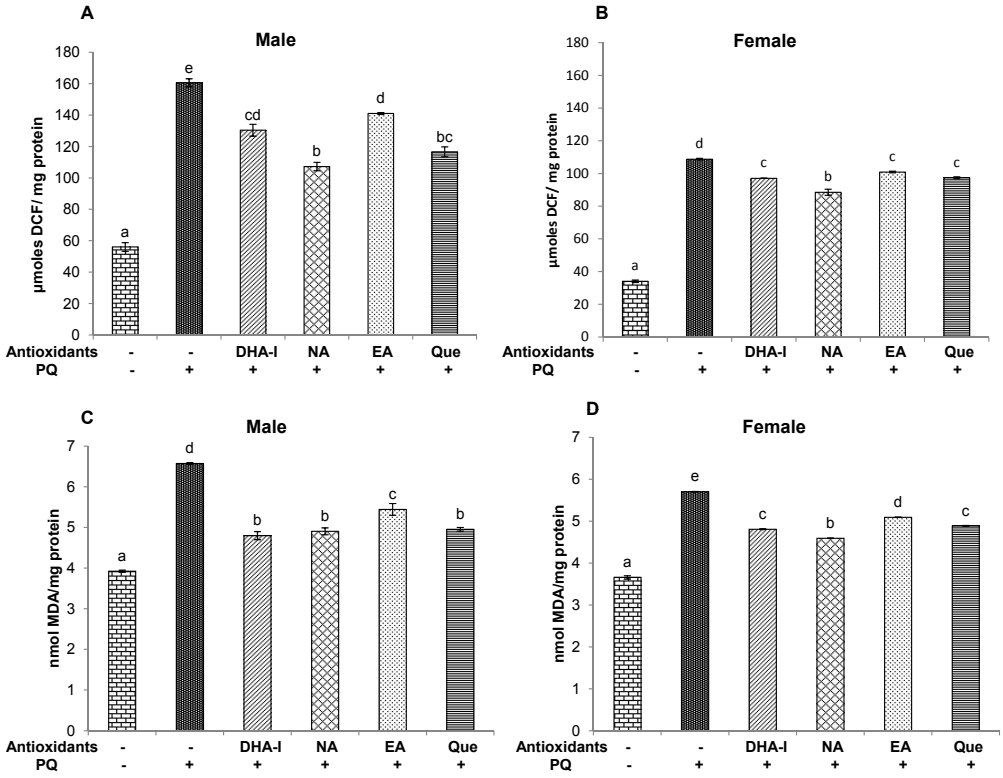

Fig. (4). Protective action of the antioxidant compounds on PQ-induced oxidative stress. Treatment with multiple-dose of PQ showed significant induction of ROS in males **(A)** and females **(B)** with the concomitant increase of LPO level in males **(C)** and females **(D)**. Treatment with antioxidant compounds (4-Hydroxyisophthalic acid-DHA-I, Nicotinamide-NA, Ellagic Acid-EA, Quercetin-Que) markedly reduced the level of ROS and LPO in both sexes. Values are mean±S.E. and bars denoted by identical lowercase alphabets (a–c) indicate no significant difference, whereas, values denoted by different alphabets indicate a statistically significant difference ($p<0.05$).

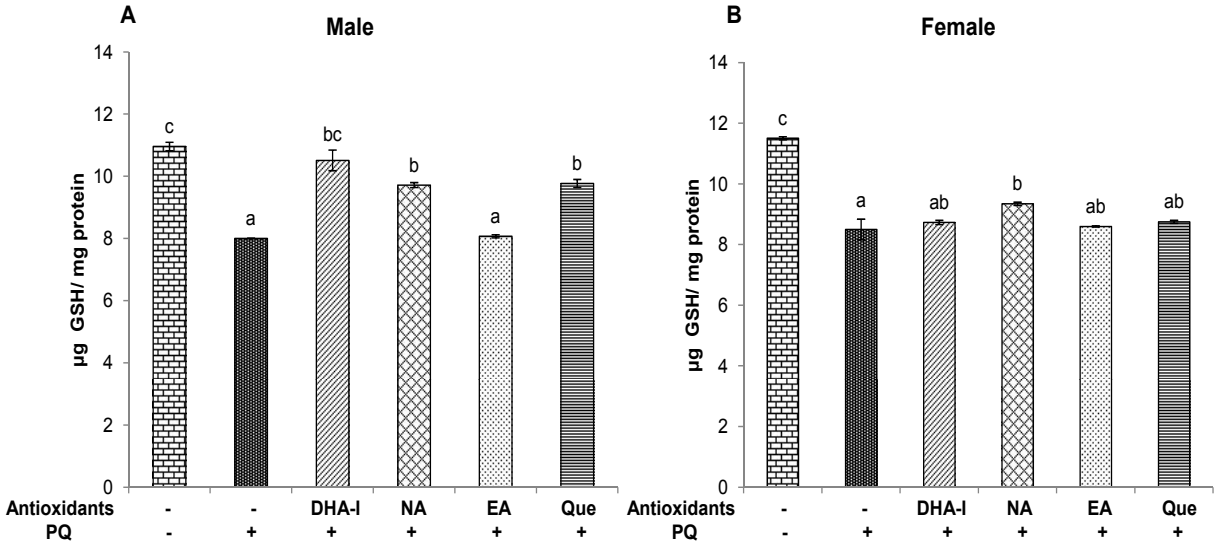

Fig. (5). PQ-induced GSH depletion and protective effect of the antioxidant compounds. Both males **(A)** and females **(B)** showed a significant decrease in GSH content when exposed to multiple dose of PQ. Treatment with antioxidant compounds (4-Hydroxyisophthalic acid-DHA-I, Nicotinamide-NA, Ellagic Acid-EA, Quercetin-Que) protected against GSH depletion. Values are mean±S.E. and bars denoted by identical lowercase alphabets (a–c) indicate no significant difference, whereas, values denoted by different alphabets indicate a statistically significant difference ($p<0.05$).

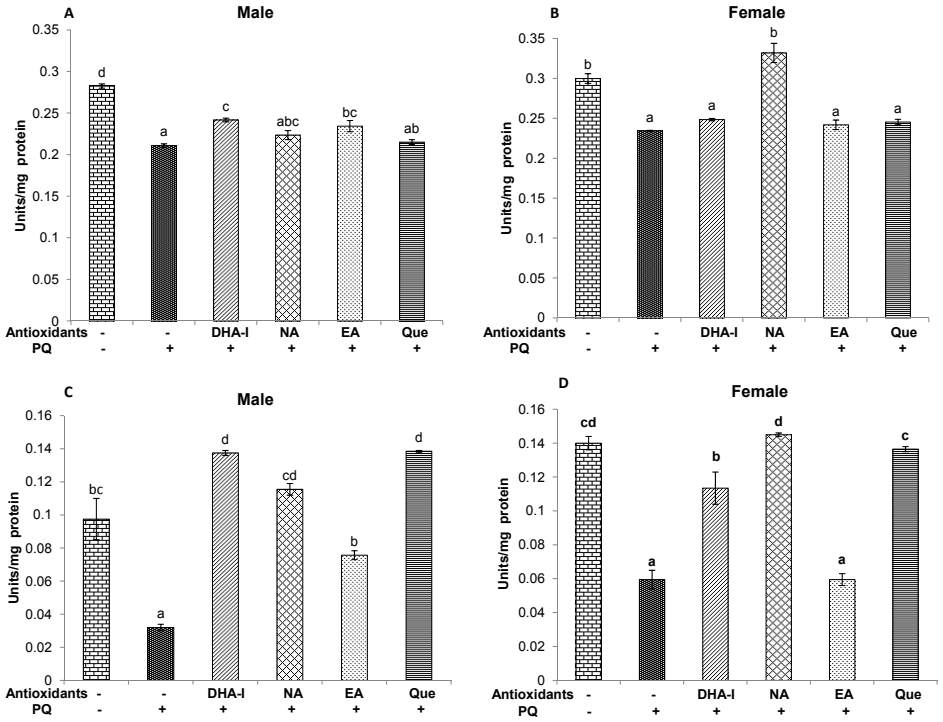

Fig. (6). Modulatory effect of the antioxidant compounds on SOD activity upon PQ exposure. Both males (**A)** and females (**B**) showed a significant decrease in the activity of Cu/Zn-SOD when exposed to multiple dose of PQ while the activity of Mn-SOD was markedly decreased in males (**C**) and females (**D**). Antioxidant compounds (4-Hydroxyisophthalic acid-DHA-I, Nicotinamide-NA, Ellagic Acid-EA, Quercetin-Que) prevented the PQ-induced changes in SOD activity. Values are mean±S.E. and bars denoted by identical lowercase alphabets (a–c) indicate no significant difference, whereas, values denoted by different alphabets indicate a statistically significant difference ($p<0.05$).

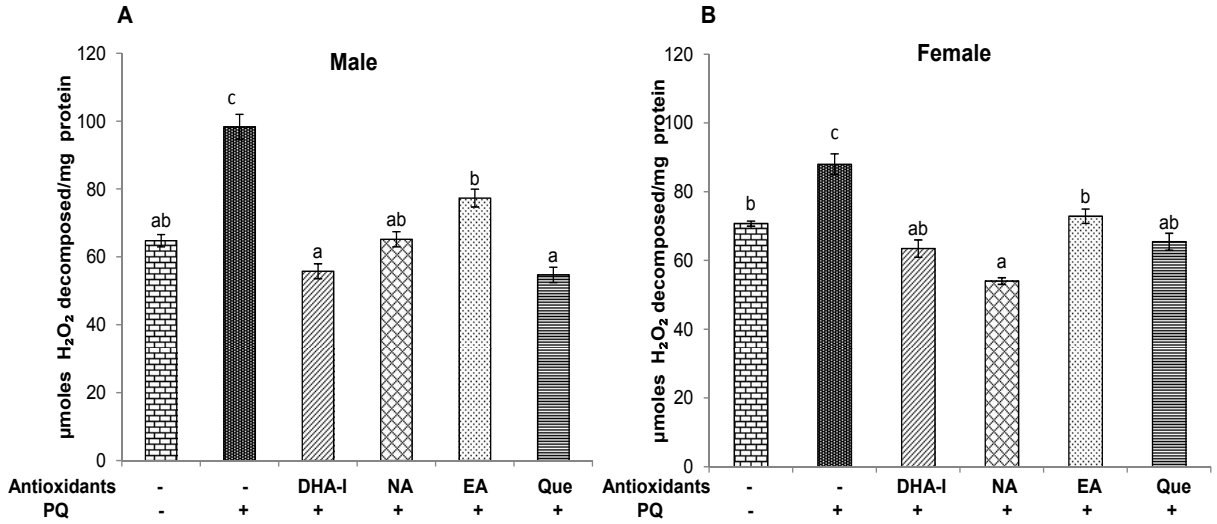

Fig. (7). Modulatory effect of the antioxidant compounds on PQ-induced catalase activity. Both males (**A)** and females (**B**) showed a marked increase in the activity of catalase when exposed to multipledose of PQ. Antioxidant compounds (4-Hydroxyisophthalic acid-DHA-I, Nicotinamide-NA, Ellagic Acid-EA, Quercetin-Que) prevented the PQ-induced changes in the catalase activity. Values are mean±S.E. and bars denoted by identical lowercase alphabets (a–c) indicate no significant difference, whereas, values denoted by different alphabets indicate a statistically significant difference ($p<0.05$).

3.4. Acetylcholinesterase

Flies exhibited a significant increase in the activity of AChE when exposed to PQ and dietary supplementation of antioxidant compounds prevented these alterations. Among the antioxidants, DHA-I, quercetin and nicotinamide were more effective in the prevention of changes in AChE activity than ellagic acid (Fig. **8**).

Flies treated with the natural antioxidants alone did not show significant differences in toxicity and biochemical markers compared to that of controls (Data not shown).

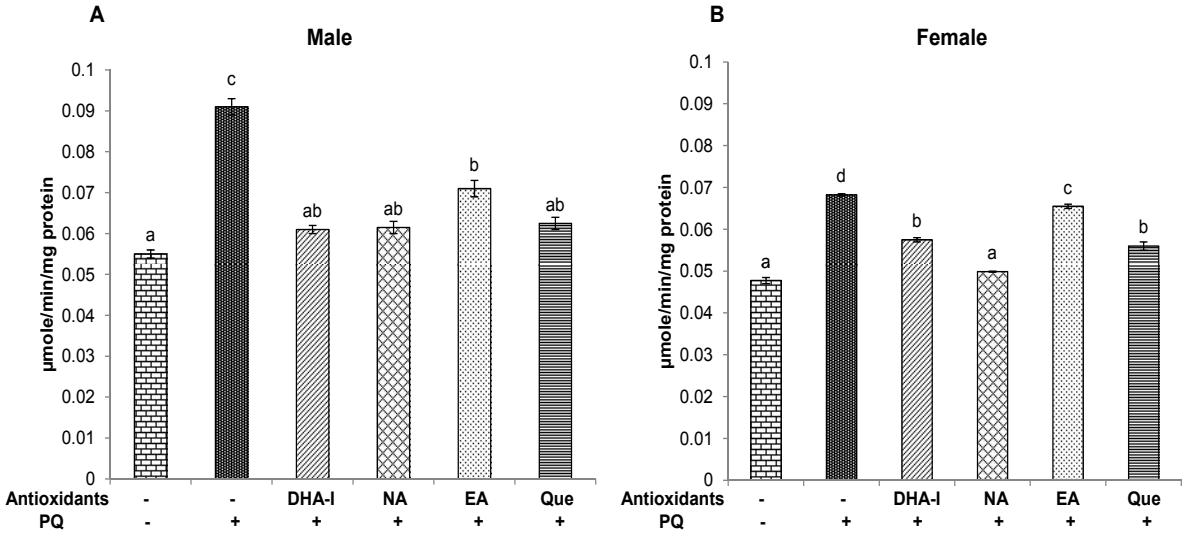

Fig. (8). Protective effect of the antioxidant compounds on PQ-induced changes in the activity of AChE.
Exposure to multiple-dose of PQ increased the activity of AChE in males **A)** and females **(B)** 556 whereas antioxidant compounds (4-Hydroxyisophthalic acid-DHA-I, Nicotinamide-NA, Ellagic Acid-EA, Quercetin-Que) significantly restored these changes. Values are mean±S.E. and bars denoted by identical lowercase alphabets (a–c) indicate no significant difference, whereas, values 559 denoted by different alphabets indicate a statistically significant difference ($p<0.05$).

4. DISCUSSION

The ability of antioxidants to quench the oxidative stress-mediated damage is believed to contribute to their therapeutic potential in preventing or slowing down neurodegeneration. Several studies have demonstrated that the antioxidant compounds protect neuronal cells by neutralizing the excessive free radicals and/or by enhancing the antioxidant defenses [52, 53]. The involvement of oxidative stress in the initiation or progression of neurodegeneration provides the basis for considering antioxidant therapy as a prophylactic treatment for neurodegenerative diseases including PD.

Previous studies from our laboratory have shown that the antioxidant-rich root extract of *Dh* exhibits neuroprotective action against acute PQ neurotoxicity and enhance the cognitive ability in *Drosophila* [54, 55]. The root extract of *Dh* also showed neuroprotective effects against PQ toxicity in αsynuclein transgenic flies and delayed the onset of PD-like symptoms, which could be attributed to its antioxidant constituents [56]. DHA-I, the bioactive compound from *Dh*, showed neuroprotective activity against taupathy in *Drosophila* [43]. In the present study, the neuroprotective efficacy of DHA-I was compared with the three natural antioxidants against multiple-dose PQ neurotoxicity that induces locomotor impairment and oxidative stress. We have earlier shown that multiple-dose of PQ treatment causes oxidative stress-induced neurodegeneration and movement disorder typical of PD [18] suggesting the association between oxidative stress and PQ neurotoxicity. Our results show that the natural antioxidants suppressed PQ-induced oxidative stress as evident from decreased ROS and LPO with augmented GSH level, and improved survival and climbing ability. PQ treated flies showed overall decrease in the activity of total SOD. Interestingly, the activity of mitochondrial Mn-SOD, an enzyme involved in the detoxification of mitochondrial ROS, was markedly decreased, which could be associated with mitochondrial damage and neurodegeneration as reported in our previous study [18]. Catalase activity, on the other hand, was increased upon PQ exposure. The activity of AChE, a general biochemical marker of neural function, is also elevated in PQ-treated flies. Administration of the antioxidant compounds showed varying effects on SOD, catalase and AChE activities in the fly head. Flies fed with the antioxidant compounds prevented the PQ-induced changes in the activity of Mn-SOD, indicating their ameliorative potential against oxidative stress-mediated mitochondrial damage involved in neurodegeneration. Also, antioxidant compounds attenuated the PQ-induced changes in the activity of catalase and AChE, suggesting the ameliorative effect of natural antioxidants in improving the antioxidant defenses and AChE activity involved in the cholinergic function.

Overall, the antioxidant compounds showed protective effect against PQ-induced oxidative stress and neurotoxicity

in *Drosophila* as evident from better survivability, improved locomotor activity and enhanced antioxidant defenses. Our results are consistent with the previous studies which have reported that quercetin, ellagic acid and nicotinamide show neuroprotective activity in several models [27, 28, 40 - 42, 57]. Nicotinamide, a precursor of NAD is vital to cellular oxidation-reduction reactions and acts as a cofactor for mitochondrial enzymes involved in ATP production [58]. Nicotinamide is known to inhibit the activity of poly ADP-ribose polymerase-1 (PARP-1), an enzyme activated by DNA damage that catalyzes the cleavage of NAD^+ leading to ATP depletion and cell death [59, 60]. Furthermore, depletion of NAD^+ leads to decreased activity of silent information regulator 2 (Sir2) family (sirtuins) involved in the regulation of aging/longevity. Sirtuins are NAD-dependent protein deacylases, which acts on a variety of proteins including transcription factors involved in the activation of antioxidant genes [61]. Interestingly, supplementation of nicotinamide is associated with exogenous replenishment of NAD^+, which regulates sirtuin activity in the nucleus and mitochondria [62]. Nicotinamide decreases oxidative stress and improves mitochondrial functions as well as motor deficits in *Drosophila* model of PD [28]. Nicotinamide has also been shown to attenuate dopaminergic neurodegeneration in a mouse model of PD, possibly by PARP-1 inhibition, increased NAD^+ levels or both [60]. Quercetin, on the other hand, exhibits diverse pharmacological effects including anti-inflammatory and antioxidant activity with the ability to enhance antioxidant defenses [63]. The ability of quercetin to modulate GSH redox system has been attributed to its protective action against oxidative stress-induced neuronal cell death in cell cultures [64]. Quercetin rescues mitochondrial dysfunction possibly through the activation of AMPactivated protein kinase (AMPK) pathway [65]. Similarly, ellagic acid has been reported to inhibit neuroinflammation and associated cell death in the brain, which could be related to its anti-inflammatory as well as antioxidant activity [41]. Ellagic acid mitigates mitochondrial dysfunction by suppressing mitochondrial ROS production and loss of membrane potential [42]. Mitochondrial dysfunction due to increased production of ROS is believed to contribute to neurodegenerative events in PD, and maintenance of the integrity of mitochondria is considered to be a promising therapeutic strategy in PD [66, 67]. The natural antioxidants employed in our study showed varying degrees of protection against PQ neurotoxicity and, also exhibited sex differences in their action. The protective effect of DHA-I was comparable to that of nicotinamide as well as quercetin and was better than ellagic acid against PQinduced oxidative stress. Our results show that neuroprotective action of DHA-I and the natural antioxidants may act *via* attenuation of mitochondrial oxidative stress which is implicated in neuronal dysfunction.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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