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ORIGINAL PAPER

Diphenyl Diselenide Protects Against Mortality, Locomotor Deficits and Oxidative Stress in *Drosophila melanogaster* Model of Manganese-Induced Neurotoxicity

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Abstract Several experimental and epidemiological reports have associated manganese exposure with induction of oxidative stress and locomotor dysfunctions. Diphenyl diselenide (DPDS) is widely reported to exhibit antioxidant, anti-inflammatory and neuroprotective effects in in vitro and in vivo studies via multiple biochemical mechanisms. The present study investigated the protective effect of DPDS on manganese-induced toxicity in *Drosophila melanogaster*. The flies were exposed, in a dietary regimen, to manganese alone (30 mmol per kg) or in combination with DPDS (10 and 20 µmol per kg) for 7 consecutive days. Exposure to manganese significantly ($p < 0.05$) increased flies mortality, whereas the survivors exhibited significant locomotor deficits with increased acetylcholinesterase (AChE) activity. However, dietary supplementation with DPDS caused a significant decrease in mortality, improvement in locomotor activity and restoration of AChE activity in manganese-exposed flies. Additionally, the significant decreases in the total thiol level, activities of catalase and glutathione-*S*-transferase were accompanied with significant increases in the generation of reactive oxygen and nitrogen species and thiobarbituric acid reactive substances in flies exposed to manganese alone. Dietary supplementation with DPDS significantly augmented the antioxidant status and prevented manganese-induced oxidative stress in the treated flies. Collectively, the present data highlight that DPDS may be a promising chemopreventive drug candidate against neurotoxicity resulting from acute manganese exposure.

Keywords Manganese · Diphenyl diselenide (DPDS) · Neurotoxicity · Oxidative stress · *Drosophila melanogaster*

Introduction

Manganese (Mn) is a naturally occurring trace element widely distributed in the earth crust. Besides, manganese compounds are components of some fertilizers, pesticides and gasoline additive [1, 2]. These compounds are reportedly released to the environment as combustion products from automobiles and industrial processes. The two major routes of exposure to manganese are inhalation and ingestion. Absorption of manganese following exposure is often affected by exposure route, the chemical form, the age of individual and dietary factors. Although manganese is an essential biometal which is required in trace amounts in animals and humans, several experimental and epidemiological reports have demonstrated serious health deficits associated with elevated manganese level [3–6].

The brain is the principal target during manganese toxicity. Several investigations with different animal models have associated manganese exposure with loss of cognitive and motor functions [7–9]. Similarly, numerous reports from occupational studies have associated elevated manganese exposure with permanent damage including impaired neurological and neuromuscular control, mental disorders, as well as difficulties with breathing or

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swallowing [10]. Moreover, several previous investigations have shown that induction of oxidative stress, diminution of endogenous antioxidants, alteration in calcium metabolism and dopaminergic regulation of neuronal activity represent the molecular mechanisms involved in the neurotoxicity of manganese [4, 7, 11].

Selenium is an essential trace element with a long history of health benefits in several living organisms, including humans. Selenium is necessary for the expression of about 25 selenium-dependent proteins (selenoproteins) which modulate the cellular redox status [12, 13]. Most of the biological functions of selenium are associated with the antioxidant properties of selenoenzymes. These enzymes play a significant role in the protection of brain tissues against oxidative damage. Furthermore, the interest in organoselenium compounds has increased due to their pharmacological potential. Diphenyl diselenide (DPDS) is a highly lipophilic organic selenium compound widely reported to exhibit antioxidant, anti-inflammatory, anti-nociceptive, hepatoprotective and neuroprotective effects in vitro and in vivo [14–19]. However, there is dearth of information on the possible modulatory effect of DPDS on manganese-induced neurotoxicity in experimental animals.

One of the major concerns of researchers is the reduction in the number of higher laboratory animals for research and testing due to ethical issues. The fruit fly, *Drosophila melanogaster*, has been recommended by the European Centre for the Validation of Alternative Methods (ECVAM) for promoting the 3Rs (reduction, refinement and replacement) of laboratory animal usage in toxicity studies [20]. *Drosophila* possesses systems which control nutrient uptake, storage and metabolism and these systems have been reported to be analogous to those of humans [21–24]. *Drosophila* is well known for its high sensitivity to toxic substances and is being considered as a useful model for toxicity studies as well as evaluating the biological action of pharmacological agents [25]. Indeed, *Drosophila* has been previously demonstrated to be a useful model for elucidating the mechanisms underlying manganese-induced neurotoxicity [26, 27].

The present study assessed the neuroprotective effects of DPDS on manganese-induced toxicity in newly emerged flies, by evaluating the mortality and locomotor deficit following short-term dietary regimen. Furthermore, using the head region of flies, we evaluated some biochemical markers of toxicity such as acetylcholinesterase (AChE) activity, antioxidant enzymes activities, reactive oxygen and nitrogen species (RONS) level, as well as levels of total thiol and thiobarbituric acid reactive substances (TBARS), subsequent to manganese exposure in the flies, in order to understand the protective mechanism of DPDS.

Materials and Methods

Chemicals

Manganese chloride ($MnCl_2$), diphenyl diselenide (DPDS), acetylthiocholine iodide, glutathione (GSH), 2′,7′-dichlorofluorescein diacetate (DCFH-DA), thiobarbituric acid (TBA), 5′,5′-dithiobis (2-nitrobenzoic acid) (DTNB) and 1-chloro-2,4-dinitrobenzene (CDNB) were purchased from Sigma Aldrich (St. Louis, MO, USA).

Drosophila melanogaster Stock and Culture

Drosophila melanogaster (Harwich strain) used in the present investigation were obtained from the National Species Stock Center (Bowling Green, OH, USA). The flies were maintained and reared on corn meal medium containing 2 % w/v sucrose, 1 % w/v brewer's yeast, 1 % w/v agar, 1 % w/v powdered milk and 0.08 % v/w nipagin at constant temperature and humidity (23 ± 1 °C; 60 % relative humidity, respectively) under 12 h light/dark cycle conditions. All the experiments were performed using the same fly strain.

Experimental Design

The assessment of the effects of DPDS on manganese-induced mortality, locomotor deficits, neurotoxicity and oxidative stress was carried out by employing a short-term dietary regimen. Newly emerged flies (2- to 3-day-old) were divided into five groups consisting of 50 flies each in three replicates per group were exposed to manganese (30 mmol per kg) with or without DPDS (10 and 20 µmol per kg) for 7 consecutive days. The doses of manganese and DPDS as well as the duration for the present study were selected based on the pilot study and previously published data [25, 27]. The average life span of *Drosophila melanogaster* in optimal temperatures is 60–70 days [25, 26]. The newly emerged flies were used for the present study because it represents an important stage in which most organisms undergo dramatic changes in shape as they grow and mature as well as when they exhibit a variety of ontogenetic changes in morphology and physiology. Thus, the developing organism is commonly more susceptible to toxic insult than the adult. The 7 days treatment period chosen for this model organism would correspond with young age of humans.

Group I Control flies fed with normal diet.
Group II Flies fed with diet containing DPDS (20 µmol per kg).
Group III Flies fed with diet containing manganese (30 mmol per kg).

Group IV Flies fed with diet containing manganese (30 mmol per kg) and DPDS (10 μmol per kg).
Group V Flies fed with diet containing manganese (30 mmol per kg) and DPDS (20 μmol per kg).

Lethality Response

The flies were observed daily for the incidence of mortality. The survival rate was determined by counting the number of dead flies, while the survivors were transferred to freshly prepared diet. The data were subsequently analyzed and plotted as cumulative mortality and percentage of live flies after the treatment period.

Measurement of Locomotor Performance by Negative Geotaxis Assay

Evaluation of locomotor performance of the control and treated flies was carried out after the treatment period using the negative geotaxis assay [28]. Briefly, ten flies from control and the treated groups were separately immobilized in ice anesthesia using labeled vertical glass columns (15 cm in length × 1.5 cm in diameter). Following a 10 min of recovery, the flies were tapped at the bottom of the column and the number of flies that crossed the 6 cm line within 6 s was recorded. Normally, flies without locomotor deficit move very fast to the top, whereas those with motor defect are slow in movement and may remain near the bottom. The climbing scores denote the average percentage of flies that crossed the 6 cm line among the total number of flies per experiment. The results are expressed as percentage of flies that escaped beyond a minimum distance of 6 cm in 6 s during three independent experiments.

Preparation of Sample for Biochemical Assays

Following the negative geotaxis assay, the flies from control and the treatment groups were anaesthetized in ice and weighed. The head region was carefully separated, homogenized in 10 volumes 0.1 M phosphate buffer, pH 7.4, and centrifuged at 6000*g* for 10 min at 4 °C in a Biofuge Sorvall Fresco centrifuge (Kendro Laboratory Products, Germany). The supernatant was collected and subsequently used for the determination of biochemical parameters. The protein concentration of head homogenates was determined using the Lowry method [29]. All biochemical determinations were performed in duplicates in three independent experiments.

Estimation of Acetylcholinesterase (AChE) Activity

AChE activity was determined according to the method of Ellman et al. [30]. Briefly, the assay medium consisted of 135 μL of distilled water, 20 μL of 100 mM potassium phosphate buffer (pH 7.4), 20 μL of 10 mM DTNB, 5 μL of sample, and 20 μL of 8 mM acetylthiocholine as substrate. The degradation of acetylthiocholine iodide was analyzed for 5 min (30 s intervals) at 412 nm using a SpectraMax plate reader (Molecular Devices, CA, USA) and the results were expressed as μmol/min/mg protein.

Measurement of Reactive Oxygen and Nitrogen Species (RONS) Generation

The level of intracellular RONS generation as an index of oxidative stress was determined by quantifying 2′,7′-dichlorofluorescein (DCFH) oxidation according to established procedure [31]. The assay reaction mixture contained 150 μL of 0.1 M potassium phosphate buffer (pH 7.4), 40 μL of distilled water, 5 μL of DCFH-DA (200 μM, final concentration 5 μM), and 5 μL of the sample (1:10 dilution). The fluorescence emission of DCF resulting from DCFH oxidation was analyzed for 10 min (30 s intervals) at 488 and 525 nm, excitation and emission wavelengths, respectively, using a SpectraMax plate reader (Molecular Devices, CA, USA). The rate of DCF formation was expressed as percentage of control group.

Determination of Thiobarbituric Acid Reactive Substances

The lipid peroxidation end products were quantified as thiobarbituric acid reactive substances (TBARS) according to established procedure [32]. Briefly, the stock reagent consisted of equal volumes of trichloroacetic acid (10 %, w/v) and 2-thiobarbituric acid (0.75 %, w/v) in 0.1 M HCl. One volume (100 μL) of tissue supernatant and two volumes (200 μL) of stock reagent were incubated at 95 °C for 60 min for color development. The reaction mixture was subsequently centrifuged at 8000×*g* for 10 min and the absorbance of the supernatant measured at 532 nm using a SpectraMax plate reader (Molecular Devices, CA, USA). The TBARS values were expressed as nMol MDA/mg protein.

Estimation of the Total Thiol Level

The total thiol level was determined in the control and treated flies according to the method previously described by Ellman [33]. The reaction system was made up of 170 μL of 0.1 M potassium phosphate buffer (pH 7.4), 20 μL of sample, and 10 μL of 10 mM DTNB. At the end of 30 min incubation at room temperature (25 °C), the absorbance was measured at 412 nm. A standard curve was plotted for each measurement using GSH as a standard and the results expressed as μMol/mg protein.

Estimation of Catalase (CAT) Activity

Catalase activity was assayed spectrophotometrically according to the method of Aebi [34] by monitoring the disappearance of H_2O_2. Briefly, the reaction medium contained 1800 µL of 50 mM phosphate buffer (pH 7.0), 180 µL of 300 mM H_2O_2, and 20 µL of sample (1:10 dilution). The reaction was analyzed for 2 min (10 s intervals) at 240 nm with a UV–visible spectrophotometer (Shimadzu, Japan). Activity of CAT is expressed as µmol of H_2O_2 consumed/min/mg protein.

Estimation of Glutathione-*S*-Transferase (GST) Activity

Glutathione-*S*-transferase activity was assayed according to the method of Habig and Jakoby [35] with slight modifications [36] using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate. The reaction mixture consisted of 270 µL of a solution containing (20 mL of 0.25 M potassium phosphate buffer, pH 7.0, 10.5 mL of distilled water, and 500 µL of 0.1 M GSH at 25 °C), 20 µL of sample (1:5 dilution), and 10 µL of 25 mM CDNB. The reaction was analyzed for 5 min (30 s intervals) at 340 nm in a SpectraMax plate reader (Molecular Devices, CA, USA) and GST activity expressed as µMol/min/mg protein using the molar extinction coefficient (ε) of 9.6 mM^{-1} cm^{-1} for CDNB conjugate.

Statistical Analyses

Statistical analyses were carried out using one-way analysis of variance (ANOVA) to compare the experimental groups followed by Bonferroni's post hoc test to identify significantly different groups (SPSS for Windows, version 17; SPSS Inc., Chicago, IL). The level of statistical significance was set as $p < 0.05$.

Results

Mortality of Flies During Co-Exposure to Manganese and DPDS

The cumulative mortality and percent live flies in the control and flies exposed to manganese alone or in combination with DPDS for 7 consecutive days are presented in Fig. 1. There was no mortality of flies in control and those treated with 20 µmol per kg of DPDS alone. There was a significant ($p < 0.05$) increase in the mortality of flies exposed to manganese when compared with the control. Conversely, co-exposure with DPDS significantly decreased flies mortality when compared with the manganese-treated flies. The cumulative percent live flies at the end of 7 days was 100 % for both control and flies exposed to DPDS alone. Acute exposure to manganese significantly decreased the percent live flies to 72 %. Interestingly, co-exposure with DPDS significantly increased the percentage of live flies when compared with the group treated with manganese alone. The percentages of live flies were 90 and 92 % for groups co-treated with 10 and 20 µmol per kg DPDS respectively.

Locomotor Performance and Acetylcholinesterase Activity

The locomotor performance and AChE activity of control and flies exposed to manganese alone or in combination with DPDS for 7 consecutive days are presented in Fig. 2. The climbing behavior of control and flies treated with DPDS alone were not significantly different from each other. The percentages of flies that crossed the 6 cm mark within 6 s were 94 and 95 % for control and flies treated with DPDS alone at 20 µmol per kg respectively. Conversely, the flies exposed to manganese alone showed significant impairment in the climbing behavior with 47 % of flies crossing the 6 cm mark within 6 s when compared with control. Interestingly, the flies co-exposed to manganese and DPDS demonstrated significant improvement in the locomotor performance when compared with those inmanganese alone group. The percentage of flies that crossed the 6 cm mark within 6 s were 76 and 82 % for groups co-treated with DPDS at 10 and 20 µmol per kg respectively. However, a contrary trend of result was observed in the AChE activity in the control and treated flies. There was a significant increase in AChE activity in flies exposed to manganese alone, whereas co-treatment with DPDS resulted in a significant decrease in AChE activity when compared with flies exposed to manganese alone.

Biomarkers of Oxidative Stress in Control and Treated Flies

The levels of oxidative stress indices determined in control and flies exposed to manganese alone or in combination with DPDS for 7 consecutive days are presented in Fig. 3. There were no treatment-related effects on the oxidative stress indices in flies exposed to DPDS alone when compared with the control. However, manganese exposure alone significantly elevated the levels of TBARS in the treated flies by 300 % when compared with control group. Co-treatment with DPDS at 10 and 20 µmol per kg significantly decreased the levels of TBARS production by 80 and 98 % respectively, when compared with flies treated with manganese alone group. Similarly, manganese

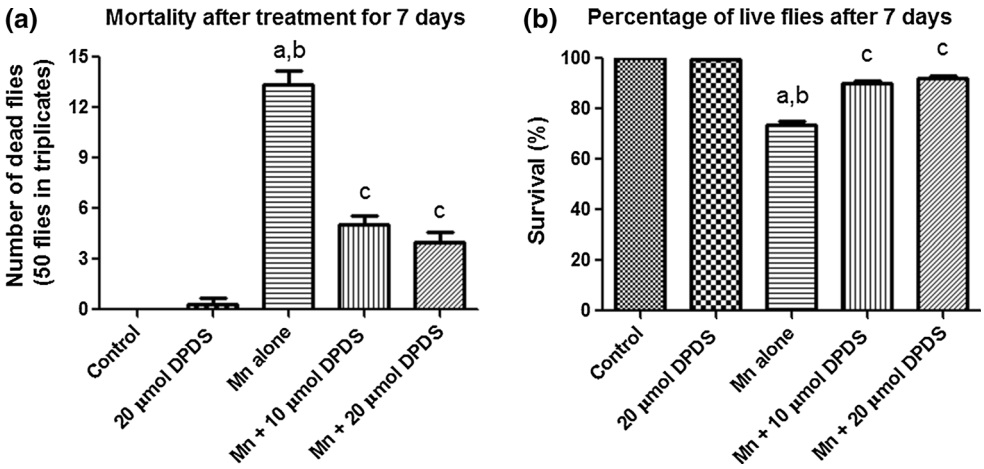

Fig. 1 Cumulative mortality expressed as number of dead flies and the percent live flies expressed as survival (%) of flies during a 7-day co-exposure period to manganese and DPDS. n = 50 flies/three replicates. Values are mean + SD. *a* $p < 0.05$ against control; *b* $p < 0.05$ against 20 µmol DPDS; *c* $p < 0.05$ against manganese alone

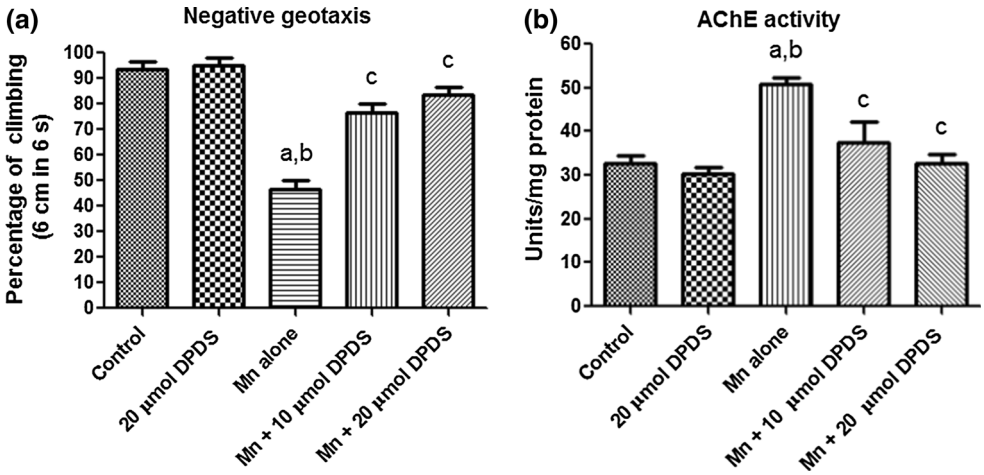

Fig. 2 Incidence of locomotor impairment (expressed as percentage of climbing 6 cm in 6 s) determined in a negative geotaxis assay and acetylcholinesterase (AChE) activity in control and treated flies during a 7-day co-exposure period to manganese and DPDS. n = 50 flies/three replicates. Values are mean + SD. *a* $p < 0.05$ against control; *b* $p < 0.05$ against 20 µmol DPDS; *c* $p < 0.05$ against manganese alone

exposure caused a significant elevation in RONS level, whereas it decreased the total thiol level in the treated flies by 110 and 60 % respectively, when compared with the control group. However, co-treatment with DPDS at 10 and 20 µmol per kg significantly decreased the level of RONS production by 92 and 109 %, whereas it increased the total thiol level by 85 and 91 % respectively, when compared with flies in manganese alone group.

Antioxidant Enzymes Activities

The antioxidant activities of CAT and GST determined in control and flies exposed to manganese alone or in combination with DPDS for 7 consecutive days are presented in Fig. 4. There were no treatment-related effects on CAT and GST activities in flies exposed to DPDS alone when compared with the control. However, manganese exposure alone significantly decreased CAT and GST activities in the treated flies by 50 and 57 % respectively, when compared with the control group. Co-treatment with DPDS at 10 and 20 µmol per kg significantly increased CAT activity by 88 and 96 % while GST activity was increased by 86 and 90 % respectively, when compared with flies in manganese alone group.

Discussion

Manganese is an essential trace element for the regulation of many biochemical processes including the development and maintenance of the central nervous system [8, 37, 38]. However, excessive exposure to manganese is well-known to cause manganism, a neurological disorder characterized by psychological and neurological abnormalities [39]. This is the first report to describe the protective role of DPDS in manganese induced toxicity in *Drosophila melanogaster*. This study demonstrated the neuroprotective potential of DPDS against manganese induced motor dysfunction and mortality in flies which is attributable to the previously reported beneficial effects in different experimental animals. In addition, we report herein the ameliorative effect

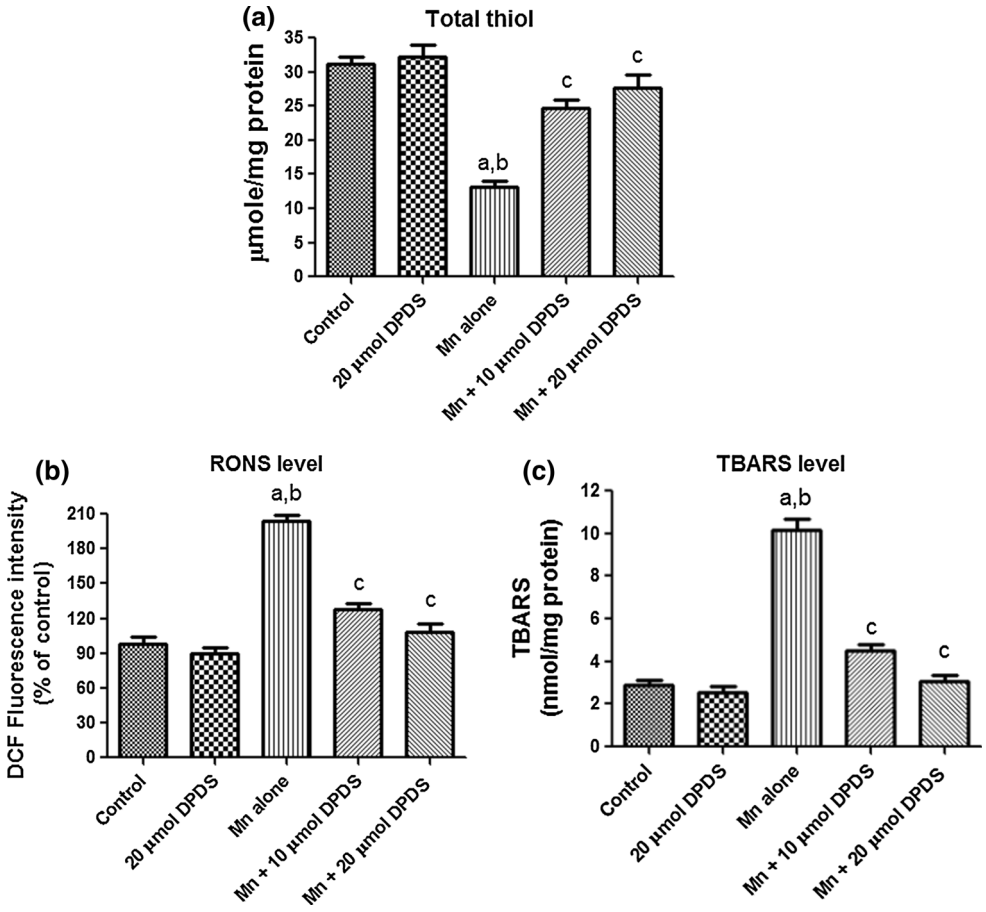

Fig. 3 Levels of total thiol, reactive oxygen and nitrogen species (RONS) and thiobarbituric acid reactive substances (TBARS) in control and treated flies after a 7-day co-exposure period to manganese and DPDS. n = 50 flies/three replicates. Values are mean + SD. *a* $p < 0.05$ against control; *b* $p < 0.05$ against 20 µmol DPDS; *c* $p < 0.05$ against manganese alone

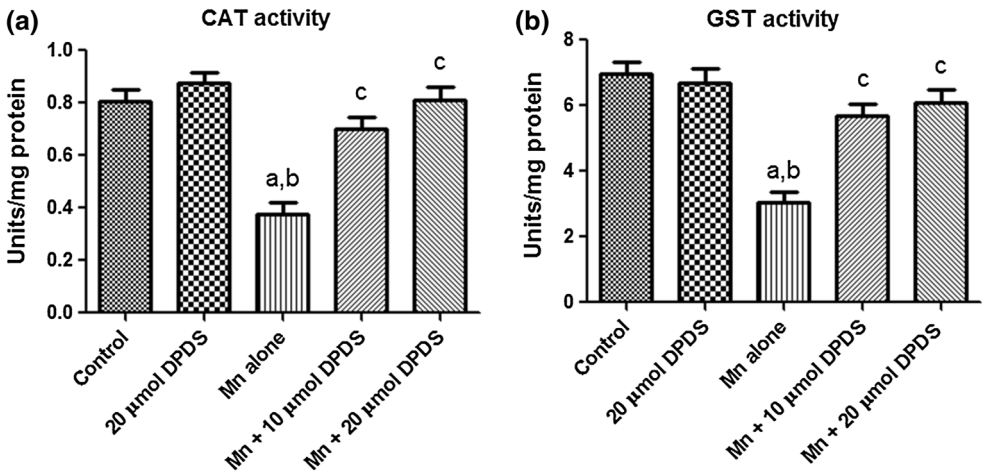

Fig. 4 Catalase (CAT) and glutathione S-transferase (GST) activities in control and treated flies after a 7-day co-exposure period to manganese and DPDS. n = 50 flies/three replicates. Values are mean + SD. *a* $p < 0.05$ against control; *b* $p < 0.05$ against 20 µmol DPDS; *c* $p < 0.05$ against manganese alone

of DPDS against manganese induced oxidative stress in the flies at exposure regimen of 10 and 20 µmol per kg.

The present study demonstrated that dietary exposure to manganese at 30 mmol per kg caused a significant increase in the cumulative number of dead flies and consequently, a significant reduction in the percentage of live *Drosophila melanogaster* following 7 days of treatment regimen. This observation could be attributed to the cytotoxic effect of manganese which has been previously reported. Exposure to manganese significantly and dose-dependently reduced cell viability in astrocyte cultures from cerebral cortices of newborn Sprague–Dawley rats [38]. In addition, manganese reportedly decreased cell viability through increased ROS production and apoptotic cell death in rat

astrocytoma C6 cells [40]. However, dietary supplementation of DPDS significantly prevented the manganese mediated toxicity by decreasing the mortality and consequently increased the percent live flies. The protective effect of DPDS against mortality induced by insecticide chloropyrifos in *Drosophila melanogaster* has been reported [25]. It is worthy of note that the relative safety of DPDS at 0.3, 3.0 and 30 p.p.m during 8 months administration to rabbits has been reported [41]. Moreover, chronic administration of DPDS to diabetic rats reportedly abolished mortality rate without obvious sign of toxicity [42]. Thus, the lack of statistically significant difference in the cumulative number of dead flies and the percent live flies following DPDS treatment in the present investigation possibly suggests its safety benefit at these doses.

Acetylcholinesterase (AChE) is a serine protease that hydrolyses acetylcholine, a neurotransmitter which regulates motor function and locomotion [43]. Acute exposure of newly emerged flies to manganese caused significant decrease in climbing activity with concomitant increase in AChE activity in the present study. These findings are in agreement with previous observations in rodents. Short-term administrations of high-doses of manganese were reported to significantly increased brain AChE activity in rat [44, 45]. Also, exposure to manganese induced a significant increase in AChE activity in both the rat serum and brain [46]. However, the contradictory results from Santos et al. [47] has been attributed to the use of pentobarbital for euthanizing the rats rather than decapitation. Pentobarbital reportedly decreased rat brain AChE activity [48–50]. Thus, the increase in the AChE activity following dietary exposure to manganese in the present study could lead to a decrease in acetylcholine levels in the synaptic cleft and consequently reduce cholinergic neurotransmission efficiency and impair climbing activity in the flies. However, dietary supplementation of DPDS was associated with decreased AChE activity and improved climbing performance in flies exposed to manganese, thus indicating the protective role of DPDS in *Drosophila melanogaster* model of manganese neurotoxicity.

Total thiol level is a well-known indirect oxidative stress biomarker, which is indicative of chemical changes in thiol groups of proteins and peptides [36, 51, 52]. Catalase and GST are antioxidant enzymes which protect cellular macromolecules by neutralizing intracellular peroxides and electrophilic oxidants, respectively. The present study demonstrated a state of oxidative stress in flies treated with manganese alone as evidenced by the significant elevation in RONS and TBARS production with concomitant significant decrease in total thiol level as well as CAT and GST activities. The reduction in these antioxidant enzymes activities could increase the RONS levels and consequently lead to cell death. These findings are in accordance with several previous reports which showed that increased AChE activity is associated with increased neuronal oxidative and nitrosative stress, alterations in energy metabolism in invertebrates and vertebrates [53]. Interestingly, dietary supplementation with DPDS significantly decreased RONS and TBARS production levels, augmented total thiol level and increased antioxidant enzymes activities in the treated flies, thus suggesting the antioxidant effect of DPDS against manganese-induced oxidative stress in the treated flies. The protective mechanism of DPDS on manganese-induced cellular damage is attributed to its ability to maintain antioxidant enzymes, scavenge RONS and consequently inhibit oxidative damage in the treated flies.

Taken together, the findings from this study revealed that DPDS protected against manganese-induced toxicity in flies via reduction of mortality, inhibition of oxidative stress indices and maintenance of endogenous antioxidants. Moreover, dietary DPDS supplementation was associated with neuroprotection characterized by improvement in the AChE activity and locomotor function in *Drosophila melanogaster*. These observations highlight that DPDS may be a promising chemopreventive drug candidate against neurotoxicity resulting from acute manganese exposure.

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Compliance with Ethical Standards

Conflict of interest The authors have no conflicts of interest to declare.

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