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Potential of whey protein as a nutritional intervention in alleviating manganese and paraquat-mediated oxidative stress in *Apis mellifera meda*

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Abstract – The role of nutrition in alleviating the physiological disorders caused by toxicants in honeybees is currently receiving considerable attention. This study aimed to determine whether dietary supplementation with whey protein (WP) could protect *Apis mellifera* from manganese and paraquat-induced disorders. In this study, honeybees were exposed to sublethal concentrations of paraquat and manganese ($LC_{10} = 55.691, 106.189$ mg/L; $LC_{25} = 106.867, 346.410$ mg/L) alone or in combination with WP (1% w/v) added to sucrose solution. Several biomarkers of oxidative stress and acetylcholinesterase activity were evaluated in the treated honeybees. The results indicated that the activities of catalase, superoxide dismutase, ascorbate peroxidase, and glutathione S-transferase were significantly elevated in honeybees treated with either paraquat or manganese compared to controls. Malondialdehyde levels varied statistically between control and treated honeybees. Additionally, a significant increase revealed in acetylcholinesterase activity in treated honeybees. In the co-exposure paradigm, WP substantially reduced oxidative stress induced by manganese or paraquat and restored the activity of acetylcholinesterase and antioxidant enzymes. These findings suggest that WP may be neuroprotective against paraquat and manganese. Furthermore, we propose that supplementing honeybees' diets with WP is an effective strategy for reducing manganese or paraquat-induced neurotoxicity.

Apis mellifera meda / manganese / neurotoxicity / oxidative stress / paraquat / whey protein

1. INTRODUCTION

In alarming reports, the negative impacts of pesticides and heavy metals on insect pollinators are well documented (Søvik et al. 2015). Due to their pollination role, insect pollinators such as honeybees are invaluable to commercial agriculture (Hung et al. 2018). There is growing concern about the loss of honeybee colonies worldwide. In other words, many factors have been linked to colony declines, including releasing toxins into

the environment, such as herbicides and heavy metals, which are detrimental to the colony's health and survival (Burden et al. 2016; O'Neal et al. 2018). The accumulation of sublethal concentrations of toxins in the hive may compromise the colony's health and increase its susceptibility to other toxins, diseases, and pest or parasite infestation (Burden et al. 2016). Environmental chemicals (such as pesticides, herbicides, heavy metals, and botanicals) act as stressors and lead to oxidative stress in honeybees (Li-Byarlay et al. 2016). An imbalance between ROS (reactive oxygen species), and neutralization of either the resulting damage or the reactive molecules

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by a living system, causes oxidative stress in the organism (Kojić et al. 2020).

Among environmental chemicals, paraquat (PQ) has the most profound impact on the survival of newly emerged bee workers and mature drones (Li-Byarlay et al. 2016). During detoxification, PQ generates free radicals and is frequently used to induce oxidative damage in animals (Kojić et al. 2020). The accumulation of free radicals induced by PQ is caused by the decreased activity of antioxidant defense enzymes such as catalase (CAT) and superoxide dismutase (SOD). Another study found significant changes in the expression of detoxification and antioxidant genes in *Apis mellifera* when exposed to PQ (de Mattos et al. 2018).

As with pesticides, heavy metals pose significant threats to honeybee survival and their extensive foraging flights (Søvik et al. 2015; Burden et al. 2019). Manganese, iron, cobalt, copper, nickel, arsenic, chromium, titanium, and vanadium are the most common heavy metals found in ecosystems (Briffa et al. 2020). Manganese (Mn) is a crucial cofactor in the biochemical processes of organisms such as *Drosophila melanogaster* (Vásquez-Procopio et al. 2020). Excessive Mn exposure can induce neurodegenerative diseases like manganism in animal models such as *D. melanogaster* (Ben-shahar 2018). Moreover, excessive Mn contributes to dopaminergic neurodegeneration and modifies antioxidant defense mechanisms (Farina et al. 2013).

Recent research has placed significant emphasis on the nutrition-modulatory role in reducing neurotoxicity in the animal model (Kumar and Muralidhara 2015). This approach implies that a diet supplemented with additional proteins is essential for improving animals' malnutrition and immune systems. Doug (2005) observed that whey protein (WP) enhances the activity of bees among supplemental proteins. As a milk protein component, WP is a rich source of amino acids. Evidence suggests that WP may influence oxidative stress and longevity in animal models (Shertzer et al. 2013). Moreover, it has been demonstrated that WP has therapeutic potential in neurodegenerative diseases (Aslanidis et al. 2015). Mohandas et al. (2017) demonstrated WP's potential in mitigating Mn-induced neurotoxicity in *Drosophila*.

Additionally, they observed that WP ameliorates Mn-induced locomotor phenotype and oxidative stress in *Drosophila* (Mohandas et al. 2017).

Based on the above, it is proposed that a WP-enriched diet could improve the responses of honeybees to environmental toxins that cause oxidative stress. Therefore, the present study evaluated the neuroprotective effects of WP against PQ- and Mn-induced toxicity in honeybees with no prior exposure to these chemicals. To determine the protective mechanism of WP, we measured the activities of acetylcholinesterase (AChE) and antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione s-transferase (GST), as well as lipid peroxidation in honeybees exposed to sublethal concentrations of PQ and Mn. Based on toxicology principles, we anticipated significant decreases in bee enzymatic activity in response to either PQ or Mn. In addition, we hypothesized that supplementing diets with WP would alleviate the oxidative stress caused by PQ or Mn in the treated honeybees.

2. MATERIALS AND METHODS

2.1. Chemicals

Paraquat (20% SL), manganese chloride (purity 99.99%), acetylcholine iodide, DTNB (5,5'-dithiobis (2-nitrobenzoic acid)), thiobarbituric acid (TBA), and reduced glutathione were purchased from Sigma-Aldrich (Madrid, Spain). Whey isolate protein was procured from Axier Nutrition Group (Isoject Evogen, Iran). All other chemicals used were of analytical grade (Merck) and were utilized in their received form.

2.2. Insects and exposure protocols

Frames containing sealed broods were collected from healthy *Apis mellifera meda* colonies at the University of Zabol (Sistan and Baluchestan, Iran). The randomly selected frames were kept in the incubator (35 ± 1 °C, $50 \pm 10\%$ relative humidity). The new workers were caught on the brood frames when

emerging from the cells and were caged in groups of 100 and maintained in the incubator under the mentioned conditions. In preliminary experiments, young honeybees (1-day-old) were exposed to a range of PQ (100–1000 mg/L) and Mn (50–5000 mg/L) concentrations diluted in a syrup solution (50% w/v) for 4 days. Each treatment was repeated five times. The LC₁₀ concentrations for PQ and Mn were 55.691 mg/L and 106.189 mg/L, respectively. The LC₂₅ values for PQ and Mn were also determined to be 106.867 mg/L and 346.410 mg/L, respectively. To determine the effects of sub-lethal concentrations on physiological responses, honeybees received 20 mL of sucrose solution daily containing LC₁₀s and LC₂₅s of PQ and Mn individually.

2.3. Effect of whey protein (WP) on Mn and PQ-induced toxicity in honeybees

In a preliminary test to assess the modulatory effect of WP on Mn and PQ-induced oxidative stress, young honeybees (1-day-old) were exposed to sublethal concentrations of either Mn or PQ (LC₁₀ and LC₂₅ values) added in syrup solution (50% w/v) containing different concentrations of WP (0.5%, 1%, 2% w/v) until death.

The mortality rate of honeybees was monitored daily. The optimal concentration of WP that exhibited a modulatory effect in bees treated with PQ and Mn was selected as the final protein concentration (1% w/v). The final experiments were then conducted with mixtures of WP (1% w/v) and sublethal concentrations of either PQ or Mn for 7 days. Daily administration of 20 mL of a 50% sucrose solution containing 1% WP and one of the sublethal concentrations (LC₁₀ and LC₂₅) of either PQ or Mn to honeybees. The control groups received WP (1% w/v) diluted in a 50% sucrose solution. Each treatment (~100 bees) was repeated five times (= 500 bees).

2.4. Biochemical tests

2.4.1. Tissue homogenization of honeybees

Alive honeybees of both treatment groups and control were anesthetized. Afterward, 50 bees (for each repetition) were randomly selected

and their head and thorax were separated using a sharp blade and homogenized using a micro pestle in an ice-cold isolation buffer (0.01 M Tris-HCl buffer). Then, the samples were centrifuged at 13,000 g. The supernatants were collected and stored at -80 °C for the following experiments (Chicas-Mosier et al. 2022).

2.4.2. Superoxide dismutase assay

To determine the activity of SOD, xanthine oxidase solution was initially prepared and added to 2 ml of phosphate buffer (0.1 M, pH 7). Then, 60 µl of the current mixture was added to a solution containing nitro blue tetrazolium and xanthine, both dissolved in phosphate-buffered saline (0.1 M, pH 7). Finally, 20 µl of tissue homogenization of honeybees was added to the whole mixture, and incubation was initiated in the darkness (20 min). The absorbance was read at 560 nm and reported as ΔA₅₆₀ nm/min/mg protein (McCord and Fridovich 1969).

2.4.3. Catalase assay

To assay the activity of CAT, as described by Wang et al. (2001), a reaction mixture containing tissue homogenization of honeybees (50 µl) and 500 µl of hydrogen peroxide (1%) were provided and incubated at 28 °C (10 min) prior reading the absorbance at 240 nm finally reported as ΔA₂₄₀ nm/min/mg protein.

2.4.4. Ascorbate peroxidase assay

To assay the activity of APX, as described by Asada (1984), 50 µl of tissue homogenization of honeybees was added into a mixture containing 150 µl of phosphate buffer (100 mM) and ascorbic acid (2.5 mM). Finally, 200 µl of hydrogen peroxide was added and the absorbance was recorded at 290 nm for 5 min and reported as ΔA₂₉₀ nm/min/mg protein.

2.4.5. Glutathione S-transferase assay

The activity of GST was measured base on Oppenoorth et al. (1979). Twenty microliters of

1-chloro-2,4-dinitrobenzene and 10 μl of reduced glutathione were added to microplate wells and incubated with 40 μl of tissue homogenization of honeybees. The absorbance was measured at 340 nm after 5 min of incubation. The activity of GST was reported as ΔA at 340 nm/min/mg protein.

2.4.6. Malondialdehyde assay

Malondialdehyde (MDA) was assayed using the method of Bar-Or et al. (2001). Initially, a solution containing 80 μl of trichloroacetic acid (20%) and 20 μl of hemolymph sample were prepared and centrifuged at 12,000 g. Then, the obtained supernatant was added to 100 μl of TBA reagent and incubated at 100 °C for 60 min. The absorbance was read at 535 nm. The concentration of MDA was calculated using the molar extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$.

2.4.7. Acetylcholinesterase activity

The AChE activity was assayed using acetylcholine iodide based on the method of Ellman et al. (1961). As instructed, 80 μl of phosphate buffer (100 mM, pH 7), 50 μl of acetylcholine iodide, and 50 μl of DTNB (10 mM) were incubated for 5 min at 27 °C. Then, 20 μl of homogenates of honeybees (mixture of head and thorax of each sample) were added to the whole mixture and re-incubated for 30 min at 25 °C. Finally, the absorbance was recorded at 405 nm.

2.5. Statistical analysis

Probit analysis (Finney 1971) was used to determine LC_{10} and LC_{25} values and 95% CI values using POLO-PC software. Biochemical data (the activities of CAT, SOD, APX, GST, AChE, and amount of MDA) from a complete randomized design were compared by a one-way analysis of variance followed by Tukey's test at a probability of less than 0.05% using SPSS software version 23.

3. RESULTS

3.1. Mortality rate of honeybees

Table I shows the different lethal concentrations (LC_{10} , LC_{25} , LC_{50} , and LC_{90}) of Mn and PQ in honeybees before the fortification of the nutrient with WP (1% w/v). To assay the meditative effects of whey protein on oxidative stress of Mn and PQ, the detrimental effects of the LC_{10} and LC_{25} values of these chemicals were evaluated. When honeybees were exposed to sublethal concentrations of either Mn or PQ (LC_{10} and LC_{25} values) added in syrup solution (50% w/v) containing 1% w/v of WP, no mortality was recorded as a result of treatment with any of the treatments (Mn: LC_{10} + WP and LC_{25} + WP; PQ: + WP and LC_{25} + WP) and control groups over the 7 days of monitoring. Less than 10 percent of mortality was recorded 8 to 14 days after treatments (Figure 1), which was hypothesized as a reason for Mn or PQ exposure time on honeybees under laboratory conditions.

3.2. Superoxide dismutase activity

As illustrated in Figure 2a, b, honeybees fed diets containing LC_{10} and LC_{25} values of PQ and Mn exhibited higher SOD activities than control bees (PQ: $F_{5,12} = 50.498$, $P < 0.0001$; Mn: $F_{5,12} = 142.568$, $P < 0.0001$). Comparing the PQ and PQ + WP groups and the Mn and Mn + WP groups revealed that adding WP to the bees' diet significantly decreased SOD activities ($P < 0.0001$). In other words, our results demonstrated that WP-containing diets increased the activity of SOD in treated honeybees only in the presence of LC_{25} PQ or Mn. No significant differences were observed between honeybees treated with LC_{25} PQ or Mn compared to controls.

3.3. Catalase activity

Similar observations were made regarding CAT activities (PQ: $F_{5,12} = 43.144$, $P < 0.0001$; Mn: $F_{5,12} = 109.593$, $P < 0.0001$). There were

Table I Probit analysis of lethal concentrations of manganese (Mn) and paraquat (PQ) on young honeybees

	Treatments	
	Manganese (Mn)	Paraquat (PQ)
LC ₁₀ (95% CL)	106.189 mg/L (70.621–146.398)	55.691 mg/L (21.543–90.521)
LC ₂₅ (95% CL)	346.410 mg/L (271.408–423.922)	106.867 mg/L (56.872–150.916)
LC ₅₀ (95% CL)	1294 mg/L (1134.810–1474.549)	220.384 mg/L (157.618–282.554)
LC ₉₀ (95% CL)	15,740 mg/L (11,495.317–23,560.111)	872.513 mg/L (615.425–1652.708)
Slope ± SE	1.181 ± 0.080	2.145 ± 0.117
X ² (df)	0.106 (3)	11.986 (3)
P-value	0.991	0.007

LC₁₀, LC₂₅, LC₅₀, and LC₉₀: lethal concentration that kills 10, 25, 50, and 90% of young honeybees after exposure to manganese (Mn) and paraquat (PQ). “CL,” “SE,” and “df” refer to “confidence limits,” “standard error,” and “degrees of freedom,” respectively

no significant differences in CAT activity between the control group and the bees-fed WP (control + WP; Figure 2c, d).

3.4. Ascorbate peroxidase activity

Based on the results, the activity of the APX under the influence of sublethal concentrations of PQ and Mn increased with the addition of concentration compared to the control (Figure 2e, f). The highest activities of this enzyme were observed by LC₂₅ of PQ and Mn. Furthermore, the activity of APX decreased significantly in honeybees fed WP + LC₁₀ PQ relative to controls. When honeybees were fed “WP + LC₂₅ PQ” or “WP + LC₂₅ Mn,” the results showed that GST activities significantly increased in the bees treated with LC₂₅ of PQ compared to the control group (PQ: F_{5,12} = 7.600, P < 0.0001; Mn: F_{5,12} = 12.036, P < 0.0001).

3.5. Glutathione S-transferase activity

The results showed that the activities of GST significantly increased in the bees treated with LC₂₅ of PQ compared to the control group (F_{5,12} = 12.036, P < 0.0001). Similarly, in honeybees treated with Mn, honeybees exposed to LC₂₅ and LC₁₀ of Mn exhibited the highest

and lowest GST activities relative to the control, respectively. In addition, GST activities decreased in bees fed a diet containing LC₁₀ or LC₂₅ of PQ + WP relative to those fed a diet devoid of protein (Figure 2g, h).

3.6. Amount of malondialdehyde

Figure 3a, b displays the significant effects of LC₂₅ values of PQ and Mn on MDA concentration (PQ: F_{5,12} = 106.951, P < 0.0001; Mn: F_{5,12} = 98.56, P < 0.0001). Compared to controls, the presence of WP in the diet of honeybees treated with Mn significantly decreased the amount of MDA at both LC₁₀ and LC₂₅ values (Figure 3b). Similar results were observed in honeybees treated with PQ + WP (Figure 3a), with the lowest levels of MDA found in those fed a diet containing WP + LC₂₅ Mn. A diet containing lethal concentrations of PQ + WP significantly reduced the amount of MDA in honeybees compared to lethal concentrations of PQ alone (PQ + WP: F_{2,6} = 17.787, P = 0.003; PQ: F_{2,6} = 193.933, P < 0.0001).

3.7. Acetylcholinesterase activity

AChE activity was also measured in honeybees treated with PQ. Our results indicated that

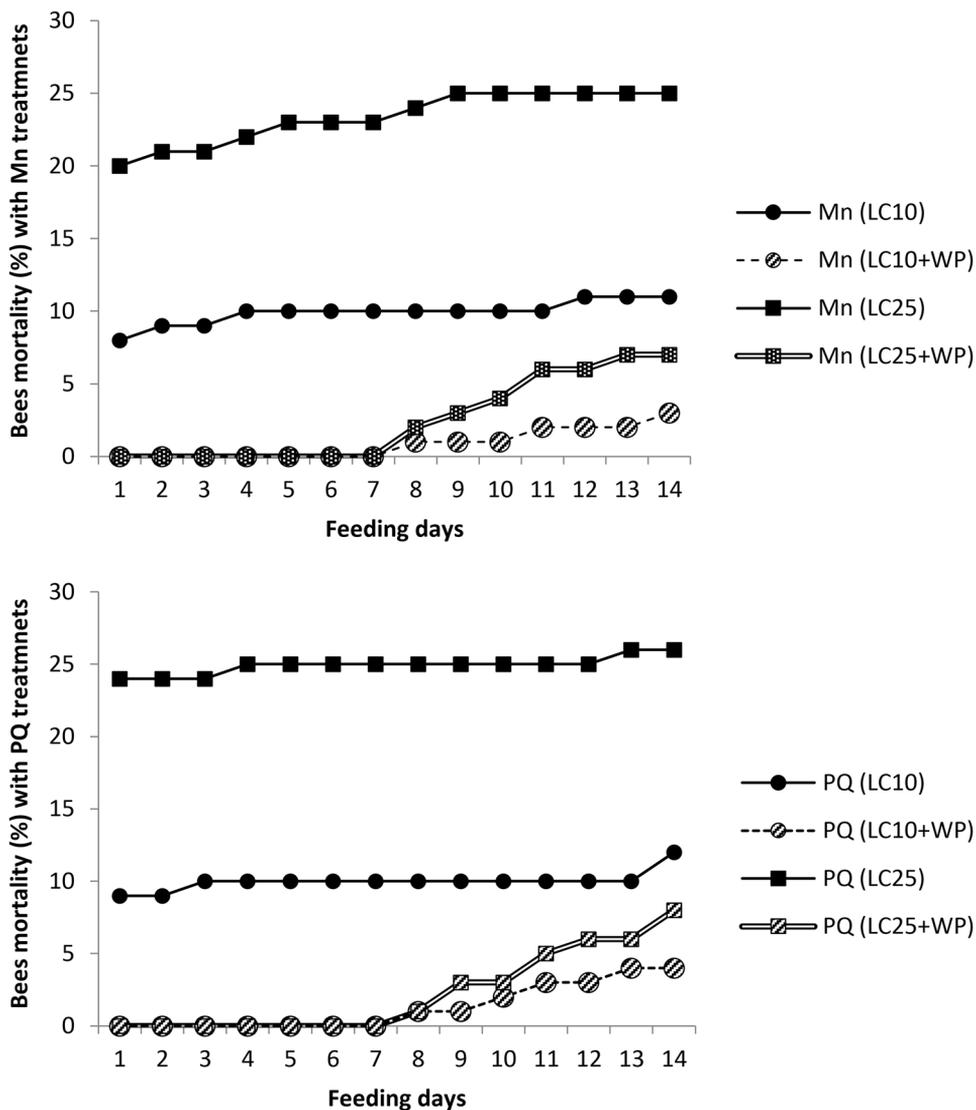


Figure 1. Bee mortalities over 14 days of feeding on sublethal concentrations of either Mn or PQ (LC₁₀ and LC₂₅ values) added in syrup solution (50% w/v) after fortification of the nutrient with WP (1% w/v). Bars indicate mean ± I standard deviation.

PQ exposure caused an increase in the activities of AChE in honeybees fed a diet containing sublethal concentrations of LC₁₀ and LC₂₅ of PQ compared to controls (Figure 4a; for PQ alone: $F_{2,6} = 26.648$, $P = 0.001$), with the highest activity being found in honeybees treated with LC₁₀ of PQ. By adding WP to the bees' diet, AChE activity was significantly reduced

in bees treated with LC₁₀ and LC₂₅ of PQ (Figure 4a; for PQ + WP: $F_{2,6} = 111.06$, $P = 0.001$). Additionally, honeybees treated with sublethal concentrations (LC₁₀ and LC₂₅) of Mn alone exhibited significant increases in AChE activities relative to the control group. The AChE activity of WP-treated honeybees, on the other hand, showed a trend in the opposite

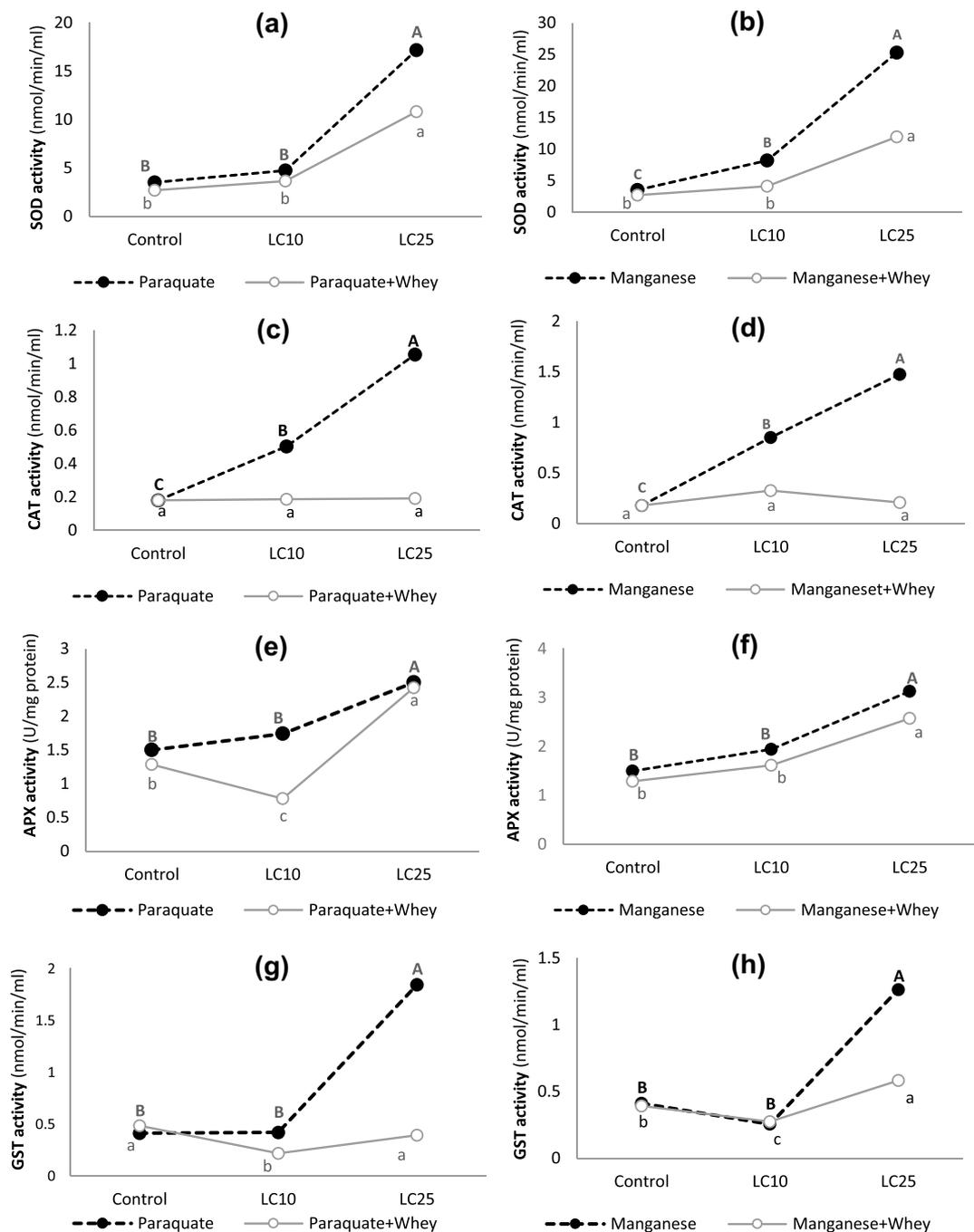


Figure 2. Effects of sublethal concentrations of paraquat (PQ) and manganese (Mn) (before and after fortification of the nutrient with WP) on the activities of antioxidant enzymes in the honeybees. Statistical differences have been marked by different letters in each line (Tukey test, $P < 0.05$).

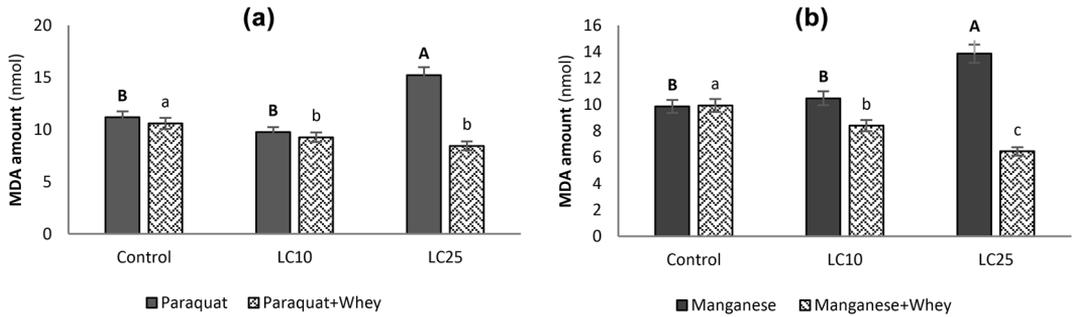


Figure 3. Effects of sublethal concentrations of manganese (Mn) and paraquat (PQ) (before and after fortification with WP) on the MDA amount in the honeybees. Statistical differences have been marked by uppercase and lowercase letters between treatments without and with WP, respectively (Tukey test, $P < 0.05$).

direction. Compared to treatments containing only Mn, honeybees exposed to LC₂₅ manganese co-treatment with WP exhibited significant decreases in AChE activities (Figure 4b; For Mn + WP: $F_{2,6} = 55.072$, $P = < 0.0001$). In contrast, the AChE activities of control honeybees fed the WP diet did not differ significantly from those of control honeybees fed a protein-free diet (Figures 4a and 3b).

4. DISCUSSION

Several experiments have linked Mn or PQ exposure to oxidative stress and neurotoxicity (Jahromi et al. 2013; Peres et al. 2016; Adedara et al. 2016). Due to the correlation between Mn or PQ exposure and oxidative stress, a great deal

of focus has been placed on compounds with antioxidant properties that may mitigate Mn- and PQ-induced toxicity in living organisms. However, there are few studies on the potential modulatory effects of protein-enriched diets on oxidative stress and neurotoxic responses. Prevention, repair, antioxidant production, and uptake of dietary antioxidants or their precursors are the cell protective mechanisms against oxidative stress (Reviewed by Corrochano et al. 2018). Containing bioactive peptides, the possible mechanism of WP’s protective effect against oxidative stress might lie in its potent antioxidant activity via radical-scavenging activity (Yu et al. 2021). WP is rich in aspartic acid, glutamic acid, and leucine and lysine amino acids, which enhance immune functions through their conversion to glutathione (Yu et al. 2021). WP increased the

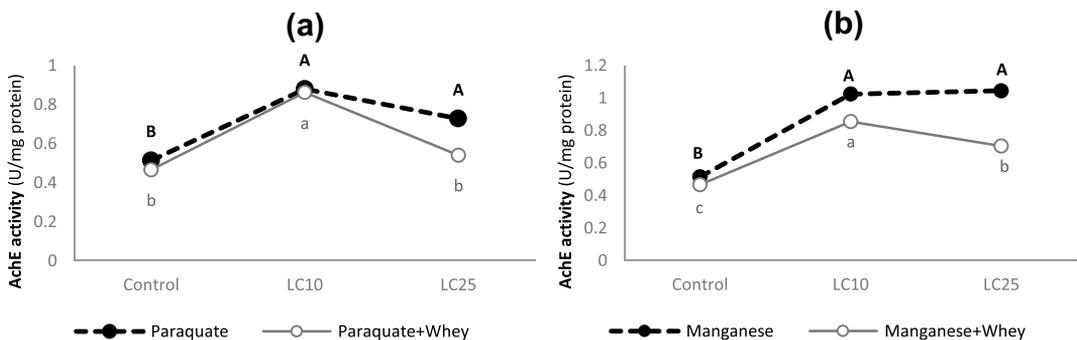


Figure 4. Effects of sublethal concentrations of manganese (Mn) and paraquat (PQ) (before and after fortification with WP) on AChE activity in the honeybees. Statistical differences have been marked by different letters in each line (Tukey test, $P < 0.05$).

branched-chain amino acids and essential amino acid levels and reduced glutathione levels (a key antioxidant in preventing oxidative cell damage) (Peng et al. 2014). Furthermore, dietary WP decreased oxidative stress and improved mitochondrial function in animal models (Shertzer et al. 2013). Mohandas et al. (2017) demonstrated WP's potential to protect against toxicant-induced oxidative stress and neurotoxicity in *Drosophila*.

Since WP's antioxidation activity and regulation of lipid metabolism are well-established (Jin et al. 2013), we tested whether WP could mitigate the adverse effects of PQ and Mn in a honeybee model. First, we determined the toxicity of PQ and Mn to young honeybees to test our hypothesis. Sublethal concentrations of PQ and Mn (LC_{10} = 55.691, 106.189 mg/L; LC_{25} = 106.867, 346.410 mg/L) were associated with 10 and 25 percent mortality rates in honey bees. The results showed that food fortification with WP significantly mitigated bee mortality due to Mn or PQ sublethal concentrations, which is supported by Mohandas et al. (2017), who found that WP attenuates manganese-induced neurotoxicity and oxidative stress in *Drosophila*. They concluded that Mn affects antioxidant defense mechanisms, mitochondrial function, and dopamine transporters, produces ROS, and results in inducing oxidative stress. In addition, it has been demonstrated that PQ exposure induces oxidative damage in *Drosophila* (Rzezniczak et al. 2011; Maitra et al. 2019). Chemical exposure could compromise the health of honey bees and diminish their coping ability with oxidative stress. Honey bee susceptibility to chemicals (e.g., pesticides and heavy metals) is increased with nutritional deprivation, and the fortified diets with essential nutrients can promote honey bees' vitality (survival). WP, a soluble and fast protein, is rapidly hydrolyzed and released into the animal plasma (Boirie et al. 1997) and neutralizes oxidative stress. The present study showed that WP leads to significantly improved survival of the honey bees exposed to Mn and PQ, suggesting that WP supplementation may protect them against oxidative-induced damage due to its antioxidant effects.

Compared to the control groups, the activities of CAT and SOD increased significantly in honeybees treated with Mn or PQ. This observation may refer to the induction of oxidative stress in honeybees primarily through producing O_2^- and H_2O_2 radicals (Kojić et al. 2020). CAT and SOD are essential antioxidant enzymes for removing ROS byproducts from cells. It has been shown that an organism's tissues can respond to oxidative stress by modulating its antioxidant defenses (Duavy et al. 2019). The production of free radicals increases the activity of SOD. The SOD transforms O_2^- to H_2O_2 . The presence of H_2O_2 activates and increases the activity of the CAT. Catalase converts H_2O_2 to H_2O and O_2 to reduce the toxicity of Mn or PQ.

Moreover, it was demonstrated that when PQ enters a bee's body, it cycles from the reduced (PQ^+) to the oxidized (PQ^{2+}) form, producing the superoxide anion free radical (O_2^-) continuously. In animal tissues, O_2^- produces H_2O_2 and OH, particularly OH^- (Vicente et al. 2001).

When the number of superoxide radicals (O_2^-) in the bodies of treated honeybees increases, the antioxidant enzyme SOD is activated to convert the O_2^- to an oxygen molecule (O_2) or H_2O_2 . Additionally, H_2O_2 is harmful to cells, and catalase converts it to water and oxygen. In other words, the SOD and CAT enzymes are functionally interdependent; SOD catalyzes the dismutation of superoxide radical anions into H_2O_2 , a substrate for CAT (Nikolić et al. 2015) and CAT catalyzes H_2O_2 into H_2O and O_2 . Based on our findings, it is plausible that PQ or Mn increases the amount of H_2O_2 and O_2^- in honeybee tissues and increases the activities of SOD and CAT. Further investigation is required.

Mn induces neurotoxicity in honeybees similarly to PQ (Søvik et al. 2015; Peres et al. 2016). Although Mn could activate some enzymes, its low concentrations in cells (such as neurons; $< 10^{-5}$ M) may prevent it from functioning as an enzyme activator in vivo (Prohaska 1987). SOD, which catalyzes the disproportionation of O_2 (Ahmad 1995), exemplifies an essential function of Mn in the central nervous system. Eventually, Mn causes an increase in O_2^- and H_2O_2 production (Farina et al. 2013). H_2O_2 is

a permeable and reactive molecule capable of reacting with metals. Consequently, H_2O_2 may produce hydroxyl radicals, leading to oxidative damage in living cells. Similar results were reported by Adedara et al. (2016) in *D. melanogaster* treated with Mn, where a significant increase in ROS levels was observed. The present study identified a state of oxidative stress in Mn-treated honeybees, as demonstrated by a significant increase in CAT activity.

Increased effects of WP on the activities of SOD in honeybees treated with PQ or Mn have been demonstrated to protect honeybees from oxidative damage. WP had no significant effect on CAT activity in honeybees treated with PQ or Mn. This finding may be due to low levels of H_2O_2 produced by SOD, a hypothesis warranting further research.

The activities of glutathione S-transferase (GST) were evaluated based on our findings. GST groups generally participate in the oxidative defense and detoxification of chemicals, including those of natural and synthetic origin, in insects against toxins (Delkash-Roudsari et al. 2022). The metabolism of toxins generates toxic oxygen-free radical species, and GSTs eliminate these radicals. GSTs metabolize toxins and facilitate their reductive dehydrochlorination or phase II reactions with reduced glutathione, producing water-soluble metabolites that excrete more readily (Enayati et al. 2005). According to the present study, increased GST activity in honeybees exposed to PQ or Mn suggests ongoing oxidative stress in vivo. Several studies have demonstrated the role of oxidative stress in the toxicity of PQ or Mn and the subsequent increase in GST activity in a *Drosophila* and honeybee model. For instance, de Mattos et al. (2018) demonstrated that GSTS4 protects honeybees against xenobiotic and peroxidative damage induced by PQ. In the same study, PQ induced significant changes in the antioxidant/oxidant status of *Drosophila*, including significant increases in levels of ROS and lipid peroxidation and an increase in the activity of GST (Duavy et al. 2019). In addition to the effects of PQ on the induction of oxidative stress, our results regarding the effects of Mn on honeybees are consistent with the findings

of a study on *Aiolopus thalassinus* (Orthoptera: Acrididae) nymphs exposed to heavy metal contamination that had increased GST activities (Yousef et al. 2019). In another study, the effect of Mn on *Lymantria dispar asiatica* (Lepidoptera: Erebididae) revealed a significant increase in GST activities following Mn exposure. Increases in GST activity indicate adaptive mechanisms against oxidative stress (Zeng et al. 2021). In other words, an increase in GST activity could be attributable to PQ or Mn exposure either as a result of a detoxification response induction (Kojić et al. 2020) or antioxidant defenses induction (de Mattos et al. 2018).

Although the activity of GST increases when insects are exposed to chemicals, lipid peroxidation may also contribute to the increased activity of GST. Lipid peroxidation disrupts the structural organization of cell membranes; therefore, GST should be induced to eliminate lipid peroxidation products or hydroperoxides from damaged cells (Huang et al. 2017). The induction of GST activity and increased MDA concentration confirms our conclusion regarding lipid peroxidation-induced oxidative stress in treated honeybees. Our results indicated that sublethal concentrations of Mn or PQ result in greater activity.

In this study, bees fed the WP diet had lower GST activities than those fed the PQ or Mn-containing diets. Similar to our findings, Gad et al. (2011) demonstrated that WP has antioxidant and free radical-scavenging properties. It is widely believed that an increase in the production of free radicals coincides with a decrease in GSH levels. One of the pathways for detoxifying GST is conjugation with GSH. WP aids the GST by stimulating cells to produce GSH and modulating GSH levels (Xu et al. 2011). Consequently, antioxidant effects and cellular protection of WP, such as promoting the production of GSH, can reduce oxidative stress and, subsequently, GST activity (Xu et al. 2011). The current study demonstrated this conclusion.

Malondialdehyde (MDA) levels were determined in honeybees fed sublethal concentrations of Mn or PQ in the presence or absence of WP supplementation. MDA is generated due to lipid peroxidation and subsequent cell

membrane disruption (Jacob and Mason 2005). ROS typically cause the catalysis of polyunsaturated fatty acids, the induction of MDA, and the generation of toxic stress within cells (Wang et al. 2001). Consequently, lipid peroxidation (with MDA as the final product) is a crucial indicator of oxidative damage (Ahmad 1995). We discovered that sublethal concentrations of either Mn or PQ (LC₁₀ and LC₂₅) increased MDA levels in honeybees. In the co-exposure paradigm (WP + Mn or WP + PQ), WP significantly reduced oxidative stress induced by Mn or PQ. In relation to this, a recent study (Mohandas et al. 2017) demonstrated that WP normalized the elevated levels of MDA caused by Mn in *D. melanogaster*. According to the review of the relevant literature, the same study has not been conducted on other insects. However, Yu et al. (2021) demonstrated that WP could significantly reduce MDA accumulation in aging mice models. Ebaid et al. (2011) demonstrated that WP significantly reduces MDA levels in diabetic rat models. In a separate study, Shertzer et al. (2013) investigated the ability of WP to stimulate the energy metabolism of mice. In mice treated with WP, the amount of MDA was 40% lower than in untreated mice. Their findings suggested that dietary supplementation with WP could ameliorate oxidative stress-related conditions.

Ascorbate peroxidase (APX) is an additional oxidative enzyme found in organisms such as insects that eliminates H₂O₂ via simultaneous ascorbate oxidation (Mathews et al. 1997). Ascorbate scavenges singlet oxygen, water-soluble radicals, and nitroxide radicals (Ahmad 1995). In addition to its peroxidase activity, the efficient recycling of ascorbate confers a potent antioxidant function. In the present study, the activity of APX was elevated in honeybees treated with either Mn or PQ compared to the control group. APX could compete with the overproduction of H₂O₂ in response to increased levels of free radicals caused by oxidative stresses after treatment with Mn and PQ; consequently, APX activity increases to eliminate and detoxify H₂O₂ levels. Due to the interactions between H₂O₂ and ascorbate, the results revealed a positive correlation between APX activity and Mn or PQ. In contrast,

APX activity decreased when oxidative stress was reduced by feeding the bees whey protein. Where H₂O₂ concentrations decreased, APX action was no longer required.

The measurement of AChE activity is one of the most commonly used biomarkers for neurotoxicity (Silva et al. 2021). Some of the Mn-induced and PQ alternations in behavioral patterns and the function of living organisms are linked to neurotransmitter metabolism disruptions, as well documented. This study examined AChE activity in PQ- or Mn-treated honeybees with or without a WP-supplemented diet. We discovered that PQ and Mn increased AChE activities in treated honeybees relative to controls (Chicas-Mosier et al. 2022). PQ increased AChE activity, resulting in decreased cholinergic neurotransmission, increased oxidative stress, and neurological dysfunction in *Drosophila* (Jahromi et al. 2013).

Furthermore, there is evidence that Mn impairs the cholinergic system, motor function, locomotion, and cognitive function (Peres et al. 2016). The administration of Mn significantly increased the activities of AChE in rats and *D. melanogaster* (Silva et al. 2021), consistent with our findings. Jahromi et al. (2013) demonstrated that 24 h of exposure to PQ induced elevated levels of AChE in flies compared to controls. In addition, Soares et al. (2017) investigated the neurotoxicity induced by PQ in *Drosophila*. Their research revealed a correlation between PQ neurotoxicity and significant increases in AChE activity. This result may be attributable to the fact that PQ leads to an excess of acetylcholine and an increase in the AChE activity (Aosaki et al. 2010). Additional research is required to understand this mechanism better.

Moreover, our results demonstrated that the presence of WP in a diet containing either PQ or Mn might restore the cholinergic system in bees exposed to Mn and PQ by maintaining AChE activity at the same level as the control. In other words, bees fed a WP diet had lower AChE levels than those fed PQ alone or Mn alone. In addition, acetylcholine has been shown to scavenge superoxide anions, thereby reducing lipid peroxidation (Pillay et al. 2003). Hernández-Ledesma et al.

(2008) demonstrated that WP possesses antioxidant properties and inhibits AChE. Therefore, WP can inhibit AChE activity and reduce AChE activity. Likewise, Yu et al. (2021) demonstrated that WP significantly reduced AChE activity in mouse brain tissue. After treatment with whey protein, oxidative stress induced by Mn or PQ is reduced, and AChE activity is restored in honeybees, demonstrating the neuroprotective effect of whey protein.

In conclusion, it can be claimed that fortified nutrition is an effective strategy for delaying or managing physiological and neural disorders in honeybees caused by toxicants. Seidl et al. (2014) demonstrated the links between protein malnutrition and oxidative stress and the inability to learn and retain, which could be similarly traced in honeybees. In conclusion, the neuroprotective effect of WP is demonstrated by the reduction of oxidative stress induced by Mn and PQ and the restoration of AChE activity in honeybees treated with WP. Understanding the biochemical processes involved in oxidative stress and neurotoxicity caused by pesticides and heavy metals in honeybees could help introduce new strategies in the beekeeping industry, such as WP-fortified diets.

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AUTHOR CONTRIBUTION

ZK and NS designed the study; ZK and NS performed lab work and analysis; ZK wrote the manuscript with contributions from NS, MH, and AK. All authors read and approved the final manuscript.

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DATA AVAILABILITY

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

CODE AVAILABILITY

Not applicable.

DECLARATIONS

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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