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Effects of Ni (II) p-hydroxybenzoate with caffeine on metabolic, antioxidant, and biochemical parameters of model insect *Galleria mellonella* L. (Lepidoptera: Pyralidae)

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Abstract: Many insecticides used against pests cause environmental pollution. Therefore, it is very important to develop new alternative strategies to toxic insecticides. In this study, the physiological and biochemical effects of Ni (II) p-hydroxybenzoate with caffeine on the model insect *Galleria mellonella* were investigated. In the present study, transferase enzyme activities such as alanine transferase, aspartate transferase and gamma-glutamyl transferase, which regulate amino acid metabolism, were altered in hemolymph of *G. mellonella* larvae exposed to Ni (II) p-hydroxybenzoate with caffeine. In addition, the activities of metabolic enzymes such as creatine kinase, lactate dehydrogenase, and amylase increased in hemolymph of *G. mellonella* larvae. Amounts of the biochemical parameters such as uric acid, bilirubin, albumin, cholesterol, and total protein were increased exposed to Ni (II) p-hydroxybenzoate with caffeine. Based on these results, we can suggest a well-adjusted Ni (II) p-hydroxybenzoate with caffeine concentrations as a potential alternative chemical for pest control.

Key words: Nonenzymatic antioxidants, transferase enzyme, *Galleria mellonella*, model organism

1. Introduction

A range of physical, chemical, and biological techniques are used to manage pests and increase crop yields. Conventional insecticides used in chemical control with pests cause environmental pollution and have toxic effects on nontarget organisms. In recent years, anthelmintic, antifungal, antibacterial drugs, and some metal complexes with low toxic effects have been used as alternative chemicals to insecticides in the management of pests (Sertçelik et al., 2018; Aslan et al., 2019, Harmancı et al., 2019; Kastamonuluoğlu et al., 2020). Metal complexes show different biological activities such as antioxidant, antibacterial, antifungal, cytotoxic, and insecticide according to the organic molecules in their structure (Liu et al., 2018; Sertçelik et al., 2018; Piri et al., 2019; Schattschneider et al., 2019; Akbaba, 2020; Venugopal et al., 2021; Sertçelik et al., 2021). Hydroxybenzoates and Ni (II) complex are environmentally friendly chemicals with their biological and chemical properties (Sertçelik et al., 2020). In addition, new chemicals with low toxicity are synthesized by binding auxiliary ligands such as imidazole and caffeine to these metal complexes (Agotegaray et al., 2012). Therefore, these metal complexes have the potential to be an alternative new chemical to insecticides used extensively in agricultural areas.

Oxidative damage occurs in organisms as a result of infection and exposure to chemicals. Metabolic enzymes such as alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), creatine kinase (CK), and antioxidants such as superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) eliminate oxidative damage (Sugeçti and Büyükgüzel, 2018; Pisoschi and Pop, 2015; Coskun et al., 2020). In addition, metabolic enzymes and amount of ions have important roles in the regulation of energy metabolism and homeostasis (Kayis et al., 2015; Sugeçti, 2021a)

Galleria mellonella is an important pest belonging to the order Lepidoptera. *G. mellonella* is a pest that causes significant damage to beehives (Kwadha et al., 2017). In addition, in recent years, *G. mellonella* larvae have been used as an invertebrate experimental model (Zorlu et al., 2018; Staniszewska et al., 2020). *G. mellonella* can be easily mass-produced in artificial diet under laboratory conditions. The large surface area of last instar *G. mellonella* larvae and easy isolation of hemolymph provide advantages for investigating toxicological studies. In addition, ethical

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concerns are absent for invertebrate model organisms such as insects. For this reason, it is used as a model insect in the development of pest control methods (Dere et al., 2015; Sugeçti and Büyükgüzel, 2018; Aslan et al., 2019).

Lepidopteran pests cause the most economic loss in agricultural areas. *G. mellonella* is used as a model organism in the management of Lepidopteran pests (Sugeçti and Büyükgüzel, 2018; Kastamonuluoğlu et al., 2020). Therefore, determining the oxidative effects of low toxicity chemicals on *G. mellonella* can enable the development of new strategies in the management of pests. In the present study, the effects of Ni (II) p-hydroxybenzoate with caffeine on the enzyme levels, biochemical parameters and amount of ions in hemolymph of *G. mellonella* larvae were investigated.

2. Materials and methods

2.1. Insect rearing

G. mellonella was reared on the artificial diet in the Biochemistry and Animal Physiology Laboratory at Zonguldak Bülent Ecevit University. Artificial diet content; 420 g wheat bran, 150 mL filtered honey, 150 mL glycerol, 20 g dark old honeycomb and 30 mL pure water (Bronskill, 1961). *G. mellonella* was mass-produced in an incubator (Nüve, ES 500) set at 29 ± 2 °C and 65 ± 5% relative humidity.

2.2. Experimental design and biochemical analyses

Ni (II) p-hydroxybenzoate with caffeine was synthesized according to the method of Taşdemir et al. (2016). Ni (II) p-hydroxybenzoate with caffeine was homogeneously dissolved in ultrapure water. Different concentrations (0.03%, 0.3%, and 3%) of Ni (II) p-hydroxybenzoate with caffeine was injected into the abdomen of *G. mellonella* larvae. Only distilled water was injected into the control group. Hemolymph was collected 24 h after treatment. Last instar larvae of *G. mellonella* were kept on ice for 5 min for anesthesia. After anesthesia, the surface of the seventh instar larvae of *G. mellonella* was disinfected with 95% ethanol. In the present study, hemolymph was collected from the abdomen of the *G. mellonella*. The hemolymph was collected in Eppendorf tubes (1.5 mL) that were kept on ice. A few phenylthiourea (PTU) (Sigma Aldrich, Missouri, USA) crystals were placed in the Eppendorf tubes to prevent melanization. Samples were stored at –80 °C until analysis.

Metabolic enzymes and biochemical analyses were performed with the Beckman Coulter AU5800 (Brea, CA, USA) using appropriate kits. Alanine transferase (ALT) (Kit no: OSR6107), aspartate transferase (AST) (Kit no: OSR6209), CK (Kit no: OSR6279), GGT (Kit no: OSR6120), lactate dehydrogenase (LDH) (Kit no: OSR6128), amylase (AMYL) (Kit No: OSR6182), albumin (ALB) (Kit no: OSR6202), bilirubin (BIL) (Kit no: OSR6212), total protein (TP) (Kit no: OSR6232), uric acid (UA) (Kit no: OSR6298), cholesterol (CHOL) (Kit no: OSR6212), Calcium (Ca) (Kit no: OSR61117), potassium (K) (Kit no: OSR66320), magnesium (Mg) (Kit no: OSR6189), and phosphorus (Phos) (Kit no: OSR6222).

2.3. Statistical analysis

One-way analysis of variance (ANOVA) was used to analyze data on transferase enzymes, metabolic enzymes and biochemical parameters in the hemolymph of *G. mellonella* larvae. Tukey's HSD test was used to determine the significance of the difference between the means. All analyses were performed in SPSS v.15.0 (SPSS, Chicago, IL, USA). A probability level of 0.05 was used to check the significance of the difference between the averages.

3. Results

3.1. Effects of Ni (II) p-hydroxybenzoate with caffeine on metabolic enzyme activity of *Galleria mellonella*

AST level was increased at all concentrations of Ni (II) p-hydroxybenzoate with caffeine ($F_{3,12}$: 102.767, $p < 0.05$) (Figure 1a). However, other transferase enzymes ALT ($F_{3,12}$: 37.553, $p < 0.05$) (Figure 1b) and GGT (only for 3% Ni (II) p-hydroxybenzoate with caffeine concentration) ($F_{3,12}$: 91.723, $p < 0.05$) levels were significantly decreased when compared to the control group (Figure 1c). While there was no statistical difference in cell damage indicator LDH level at 0.03% and 0.3% Ni (II) p-hydroxybenzoate with caffeine concentrations, LDH level significantly increased at 3% Ni (II) p-hydroxybenzoate with caffeine concentration ($F_{3,12}$: 66.806, $p < 0.05$) (Figure 1d). AMYL activity increased approximately 2-fold when compared to the control group at 3% Ni (II) p-hydroxybenzoate with caffeine concentration ($F_{3,12}$: 113.475, $p < 0.05$) (Figure 1e). CK activity in hemolymph significantly increased after application of all treatments ($F_{3,12}$: 557.592, $p < 0.05$) (Figure 1f).

3.2. Effects of Ni (II) p-hydroxybenzoate with caffeine on biochemical parameters of *Galleria mellonella*

The amount of nonenzymatic antioxidants ALB ($F_{3,12}$: 25.542, $p < 0.05$) (Figure 2a), BIL ($F_{3,12}$: 46.708, $p < 0.05$) (Figure 2b) and UA ($F_{3,12}$: 80.504, $p < 0.05$) (Figure 2c) significantly increased at the highest Ni (II) p-hydroxybenzoate with caffeine concentration compared to the control group. The amount of CHOL increased approximately 2-fold when compared to the control group at 3% Ni (II) p-hydroxybenzoate with caffeine concentration ($F_{3,12}$: 50.591, $p < 0.05$) (Figure 2d). Similarly, the amount of TP significantly increased at the highest concentration when compared to the control group ($F_{3,12}$: 60.828, $p < 0.05$) (Figure 2e).

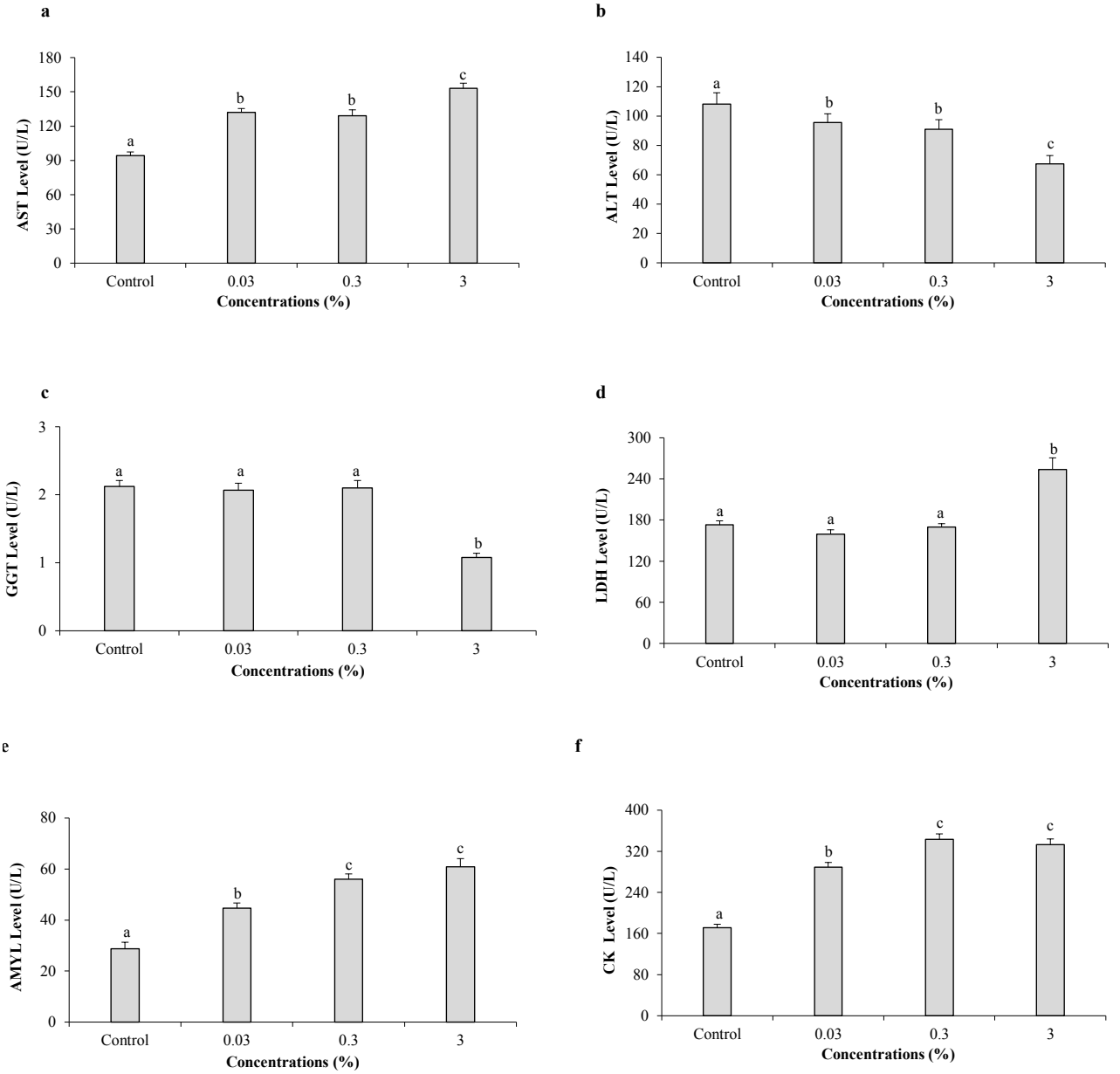

Figure 1. Effects of Ni (II) p-hydroxybenzoate with caffeine on metabolic enzyme activity of *Galleria mellonella.* Bars represent the means (±SD) of four replicates. Means followed by the same letter are not significantly different ($p > 0.05$).

3.3. Effects of Ni (II) p-hydroxybenzoate with caffeine on ion levels of *Galleria mellonella*

In the present study, the amount of Na decreased in the hemolymph of larvae exposed to the highest Ni (II) p-hydroxybenzoate with caffeine concentration; however, there was no statistically significant difference ($p > 0.05$). However, the amount of Na decreased significantly at 0.03% Ni (II) p-hydroxybenzoate with caffeine concentration. ($F_{3,12}$: 24.993, $p < 0.05$) (Figure 3a). The amounts of K ($F_{3,12}$: 160.326, $p < 0.05$) (Figure 3b), Mg ($F_{3,12}$: 8.339, $p < 0.05$) (Figure 3c), Phos ($F_{3,12}$: 6.162, $p < 0.05$) (Figure 3d), and Ca ($F_{3,12}$: 75.851, $p < 0.05$) (Figure 3e) increased significantly at the highest Ni (II) p-hydroxybenzoate with caffeine concentration compared to the control group. The amount of Cl increased at 0.3% and 3% Ni (II) p-hydroxybenzoate with caffeine concentrations, but there was no statistical difference ($F_{3,12}$: 1.129, $p > 0.05$) (Figure 3f).

4. Discussion

This paper demonstrated that cellular stress indicators, antioxidant systems and metabolic enzymes altered in

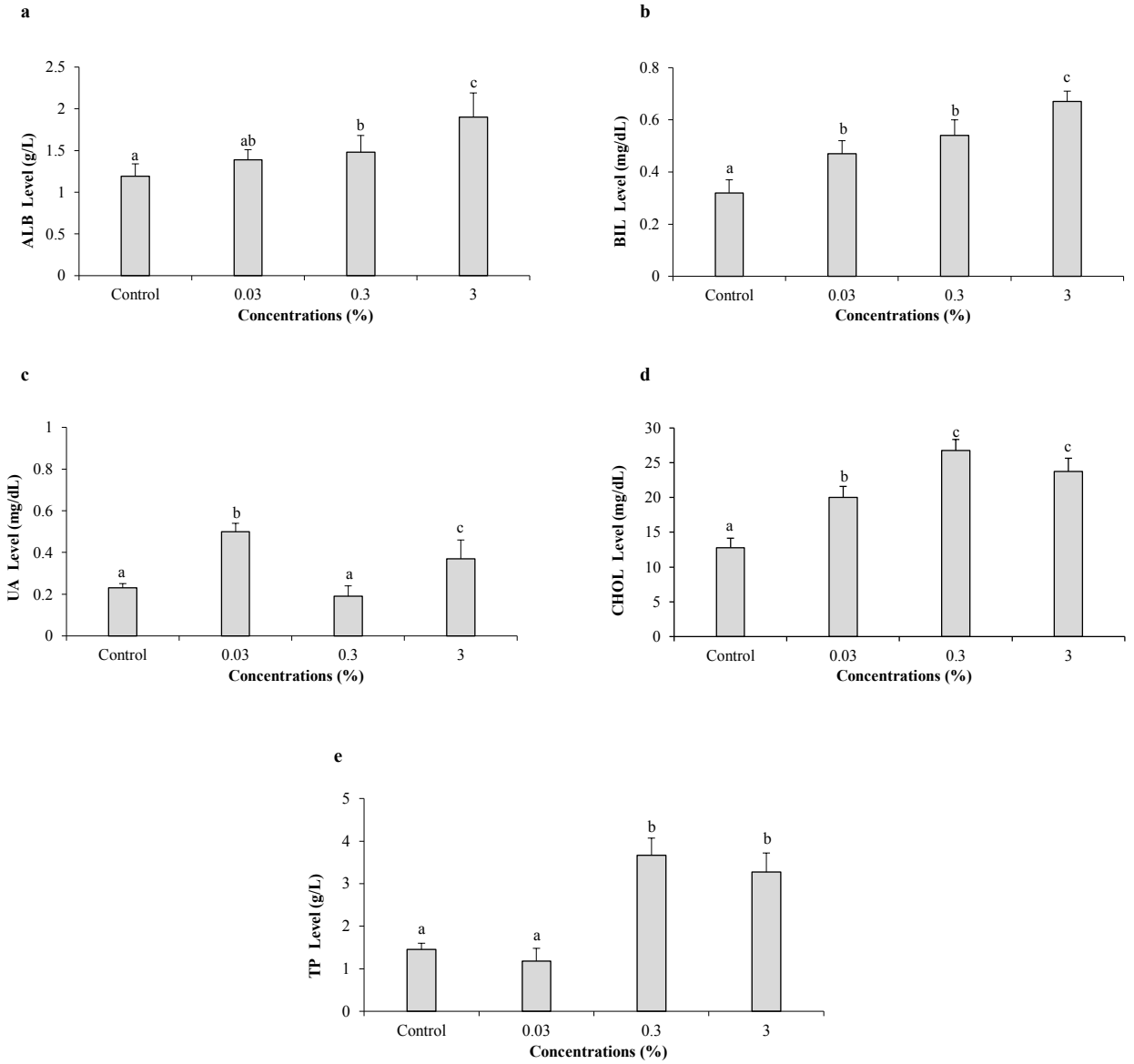

Figure 2. Effects of Ni (II) p-hydroxybenzoate with caffeine on biochemical parameters of *Galleria mellonella*. Bars represent the means (± SD) of four replicates. Means followed by the same letter are not significantly different ($p > 0.05$).

G. mellonella larvae due to the oxidative effect of Ni (II) p-hydroxybenzoate with caffeine.

Metabolic enzymes play an important role in the elimination of oxidative damage due to chemical and biological agents. Transferase enzymes such as AST, ALT, and GGT provide regulation of amino acid metabolism (Hakkak et al., 2018; Sugeçti et al., 2021). CK and AMYL enzymes are involved in the regulation of energy metabolism. The other metabolic enzyme LDH activity is used as an indicator of cell damage (Ciesielczuk et al., 2015). In the present study, ALT and GGT activity significantly decreased while AST activity increased at the highest concentration of Ni (II) p-hydroxybenzoate with caffeine. This change in transferase enzyme activities may be due to disruption of amino acid metabolism. CK, AMYL, and LDH levels were significantly increased in the hemolymph of *G. mellonella* larvae. In another study, it was reported that transferase enzymes such as AST, ALT, and GGT activities and metabolic enzymes such as CK, AMYL, and LDH levels increased due to the oxidative effect of oxfendazole, an anthelmintic drug (Sugeçti and Büyükgüzel, 2018). Tunçsoy et al. (2021) reported that metabolic enzymes such as AST, ALT, GGT, LDH, CK, and AMYL levels significantly increased in hemolymph of *G. mellonella* larvae exposed to copper oxide nanoparticles. In another study, biochemical effects of *Escherichia coli*

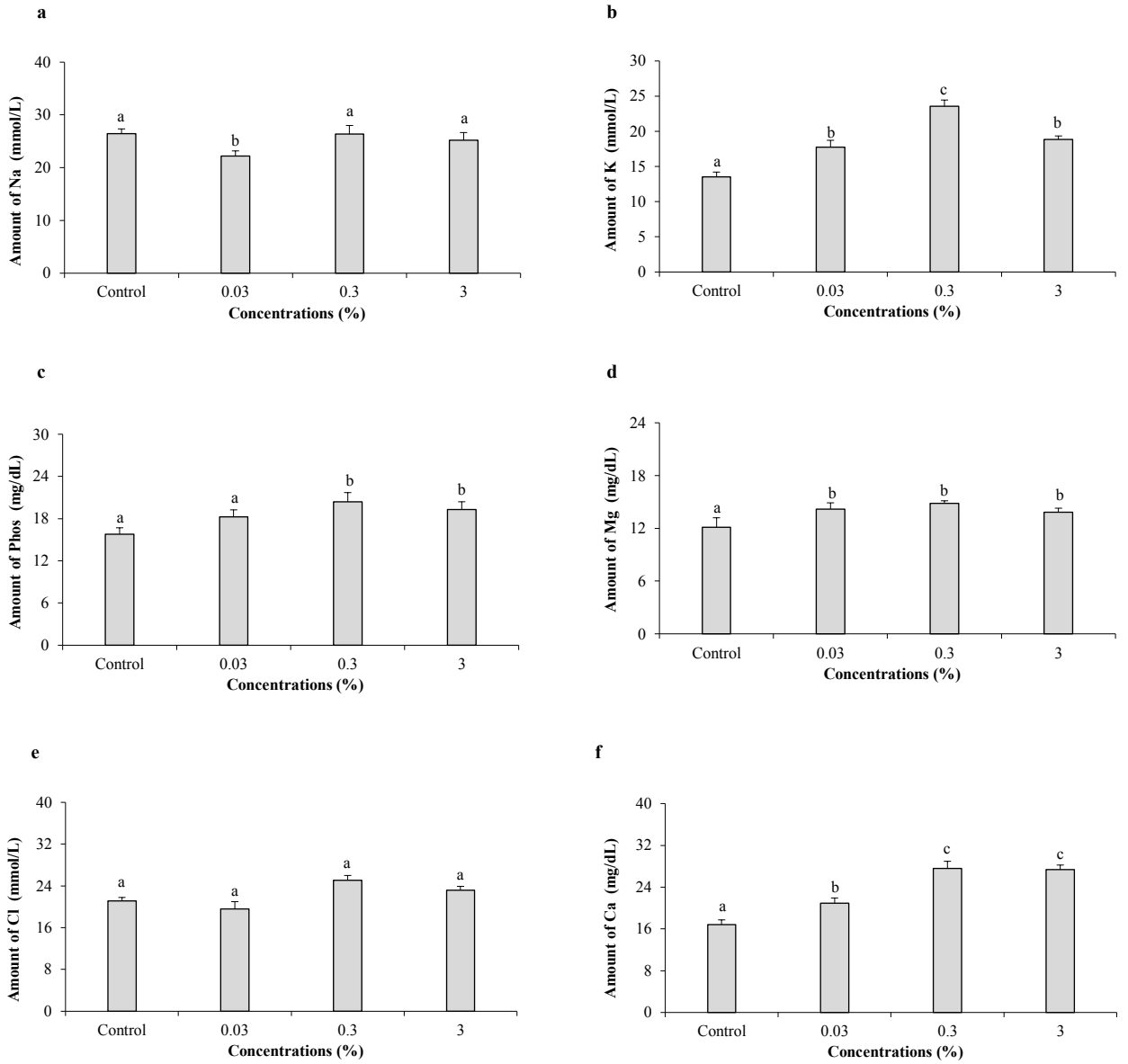

Figure 3. Effects of Ni (II) p-hydroxybenzoate with caffeine on ion levels of *Galleria mellonella.* Bars represent the means (± SD) of four replicates. Means followed by the same letter are not significantly different ($p > 0.05$).

infection on great wax moth were investigated. The authors found that the biochemical and immune responses of the insect was adversely affected in *E. coli* infection, and CK, GGT, AMYL, and ALP levels were increased, especially 8 h after with *E. coli* infection, as a result of oxidative damage in the hemolymph of *G. mellonella* larvae (Sugeçti, 2021b). Data from these studies demonstrated that biological and chemical agents cause similar results on the same insect.

Enzymatic and nonenzymatic antioxidants play a key role in the elimination of oxidative damage caused by physiological and biochemical stresses (Büyükgüzel and Kalender 2007, 2009; Upadhyay et al., 2014; Mirończuk-Chodakowska et al., 2018; Kaur et al., 2021). In addition, amounts of some biomolecules such as protein and lipid in insects increase as an adaptation to oxidative damage (Sak et al., 2011; Emre et al., 2013; Tunçsoy, 2020). In the current study, the levels of nonenzymatic antioxidants such as ALB, BIL, and UA were increased when compared to the control group. Nonenzymatic antioxidants may have been increased to prevent oxidative damage due to Ni (II) p-hydroxybenzoate with caffeine. In this study, the increase in amounts of TP and CHOL may be an adaptation developed against the oxidative damage. In another study, it was reported that antioxidant system

was altered due to oxidative damage in the midgut of *G. mellonella* larvae exposed to a widely used biorational pesticide *Bacillus thuringiensis* (Dubovskiy et al., 2008). In a study by Tunçsoy and Meşe (2021), four different concentrations (5, 50, 250, and 1250 µg/mL) of titanium dioxide nanoparticles were added to diet of model organism *G. mellonella* larvae. The authors reported that antioxidant enzymes levels significantly increased in *G. mellonella* larvae. In another study, the oxidative effects of juglone on *G. mellonella* were investigated. The authors found that antioxidant enzymes were adversely affected and induced lipid peroxidation due to juglone in a concentration-dependent manner (Altuntaş et al., 2020). Our findings show that the oxidative stress caused by Ni (II) p-hydroxybenzoate with caffeine can be eliminated by nonenzymatic antioxidants.

Ions in insects are involved in the regulation of cellular functions, which is deteriorated due to stress caused by biological and chemical agents (Southall et al., 2006). In this study, hemolymph ion levels such as K, Phos, Mg, and Ca increased at the highest Ni (II) p-hydroxybenzoate with caffeine concentration. The change in the amount of ions may be for the regulation of the disturbed cellular functions. In another study, it was determined that cell damage occurred due to *Klebsiella pneumoniae* infection. The author reported that ions such as Mg, Ca, and K due to this damage were suppressed (Sugeçti 2021a). In another study, the effects of organophosphate insecticide dichlorvos (at concentrations of 2, 4, 6, and 8 µg per 100 g diet) on *G. mellonella* investigated. The authors reported that ions such as Na and K significantly increased due to the oxidative effect of dichlorvos (Kayis et al., 2015).

In this study, it was determined that Ni (II) p-hydroxybenzoate with caffeine caused physiological and biochemical stress on the model insect *G. mellonella*. Cell damage, disruption of amino acid metabolism and changes in the amount of nonenzymatic antioxidants may be due to the oxidative effect of Ni (II) p-hydroxybenzoate with caffeine. These results demonstrated that Ni (II) p-hydroxybenzoate with caffeine may be used an alternative chemical for insect pest control.

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