THE EFFECTS OF PYRETHRUM EXTRACT ON *Galleria mellonella* HEMOLYMPH PHENOLOXIDASE ENZYME

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**ABSTRACT**

Pyrethrum is a natural neurotoxic insecticide which is obtained from the flowers of *Chrysanthemum cinerariaefolium* plant. Pyrethrum extract causes DNA damage, genotoxic effect, induction of autophagy and apoptosis, mitochondrial dysfunction, oxidative stress, inhibition of biochemical processes. The greater wax moth *Galleria mellonella* L. (Lepidoptera: Pyralidae) is gaining increasing attention in immunity studies as an invertebrate model organism. Melanization, which is the most important response of invertebrate humoral immunity, occurs when inactive prophenoloxidase turns into phenoloxidase enzyme. Changes in phenoloxidase enzyme activity are an important marker for humoral immunity. In our study, the phenoloxidase enzyme activity of hemolymph collected from *G. mellonella* larvae treated with different doses of pyrethrum extract was determined by reading against a certain absorbance in an ELISA microplate reader. The findings obtained from this study showed that 0.6 mg/ml pyrethrum extract increased phenoloxidase enzyme activity. Doses above and below this dose did not cause a significant change in phenoloxidase activity compared to control groups. In the evaluation made in terms of the change of enzyme activity over time, while the enzyme activity increased rapidly in the first 15 minutes, the enzyme activity rate decreased after the 20th minute. The effect of pyrethrum extract on phenoloxidase enzyme activity in *G. mellonella* larval hemolymph at a certain dose is consistent with the literature. The reason for this effect of the extract is closely related to its genotoxic and cytotoxic effects.

**Keywords:** Pyrethrum, *Galleria mellonella*, phenoloxidase, hemolymph, enzyme activity
1. INTRODUCTION

The current upward trend of the human population has brought the problem of food production. The human population shows exponential growth, but agricultural areas show an arithmetic increase. The current structure of agricultural areas and techniques are insufficient to feed the growing population. In this case, pesticides used in the fight against agricultural pests are frequently preferred in order to obtain the highest yield per unit area in existing agricultural lands (Kurutaş and Kılınç, 2003). There are pesticides specific to many species such as herbicides, insecticides, fungicides, rodenticides and acaricides.

The role of insecticides in human society is very important (Pavela, 2016). Insecticides grouped as organophosphorous, carbamates, organochlorine and pyrethroids constitute the largest and most important pesticide group. Among these groups, pyrethroids cause lower toxicity in mammals and less residues in the environment than organochlorines and organophosphates (Kojima et al., 2004; Costa, 2008; Mnif et al., 2011). Pyrethroids, along with insecticide applications, have a wide range of usage areas including agriculture, medical, veterinary, aquatic system and pest control at home. Nevertheless, this widespread use causes people to be more exposed to pesticides (Radovanović et al., 2017; Romero et al., 2017).

Natural pyrethrins are obtained from flowers of the *Chrysanthemum cinerariaefolium* type known as "pyrethrum", which contains six active ingredients (Valentine, 1990; Arslan and Yılmaz, 1993; Anadon, et al., 2009; Palmquist et al., 2012; Yang et al., 2018). This type of flower is also consumed as herbal tea in some countries. The active ingredients found in natural pyrethrins are pyrethrin I-II, synerine I-II and jasmolin I-II. Although these substances show strong activity against many different types of insects, their permanence is very low and easily degrades after contact with air and sunlight (Anadon et al., 2009; Yang et al., 2018). Long-term low-dose exposure to pyrethroids can cause chronic diseases of the nervous system, immune system, cardiovascular system, and produce toxic effects including teratogenicity, carcinogenicity and mutagenicity (Tang et al., 2018).

Pyrethrins pass through the exoskeleton of insect chitin by passive diffusion and cause depolarization by preventing the closure of the sodium channels of the cell membrane in nerve and muscle cells. Their mechanism of action is to inhibit voltage-dependent sodium channels that regulate sodium permeability in the cell membrane, which is involved in the production of neuronal action potentials of insects. In addition, sodium potassium inhibits ATPase channels and blocks reuptake, which stimulates the release of other neurotransmitters by disrupting the sodium gradient (Soderlund et al., 2002; Patel et al., 2007; Gupta et al., 2013). As a result of this change in sodium channels, either repetitive firing or neuronal depolarization is blocked, depending on the length of time the sodium channels stay open (Calderón-Segura et al., 2018).

Studies on cypermethrin (Taju et al., 2014; Huang et al., 2016), cyhalothrin (Deeba et al., 2016) and allethrin (Madhubabu and Yenugu 2014) have revealed that many synthetic pyrethrin types cause oxidative damage.

Insect and mammalian humoral responses include processes such as melanization, coagulation, and secretion of antimicrobial peptides (Sheehan et al., 2018). Among the humoral immune responses in insects, the most effective response is melanization (Lee and Anssee, 1995). The formation of the black pigment called melanin, is catalyzed by the phenoloxidase (PO) enzyme, which is converted into its active form by the serine protease cascade (Vilmos and Kurucz, 1998). Hemocytes in insects are the only source of phenoloxidase (Ashida and Brey, 1998). The inactive phenoloxidase (PPO) that is synthesized in hemocytes, accumulates by cell breakdown in scar tissue or around the encapsulated invader (Vilmos and Kurucz, 1998). The layer formed around the foreign body as a result of melanization completely abstracts the
object from its surroundings and cuts its contact with the outside. Most of the biochemical pathways that cause melanin formation are common in both mammals and insects (Nappi and Christensen, 2005).

*Galleria mellonella* larvae are more likely to be used in experiments for many reasons such as low production cost, rapid breeding without special equipment, survival at 37 °C, 6 weeks of life cycle, no need for large physical areas for breeding, and generally not requiring ethical permits (Ignasiak and Maxwell, 2017). At the same time, the size of *G. mellonella* last instar larvae (250-300 mm) makes it easy for intraperitoneal injection of the compounds to be tested. In addition, the possibility of adding these compounds to food and exposure through the skin makes them stand out as a suitable invertebrate model organism for experiments. In addition, the insect immune system is functionally and structurally similar to the innate immune system of mammals (Browne et al., 2013), therefore invertebrate model organisms are preferred in immunity studies.

In this study, it was aimed to determine the effect of pyrethrum extract, a natural pesticide, on phenoloxidase enzyme activity in the model organism *G. mellonella* hemolymph. Phenoloxidase is the enzyme that carries out melanization, in other words the humoral immune mechanism, so plays a key role in humoral immune responses. The effects of pyrethrum exposed in various ways on immunity have been tried to be determined through the model organism. It is thought that an idea can be obtained about the effect of pyrethrin, which is the main active ingredient of *C. cinerariaefolium* plant that is collected from nature and consumed as tea, on the natural immune mechanism in animals.

2. MATERIAL and METHODS

2.1 Insect Rearing

The *G. mellonella* larvae were grown in 25 ± 1°C temperature, 65 ± 1% relative humidity and 12:12 h. (light:dark) photoperiod conditions in the laboratory of Biology Department of Çanakkale Onsekiz Mart University. Adult male and female moths were placed in a 1 liter glass jar with 2 grams of natural blackened honeycomb. Since the larvae hatched from the eggs, the larvae were fed with 10 g of artificial food (Sak et al., 2006) daily. Last instar larvae (0.18 ± 0.02 g) were selected and used for the experiment. The samples of *G. mellonella* larval surface were sterilized before used in experiments with 70% ethanol.

2.2. Pyrethrum Injection

Preliminary studies were carried out by dissolving Pyrethrum extract (Sigma, Germany) in 10% ethanol (EtOH). The LC50 value for the subjects was determined as 50 mg/kg. According to this value, 2 mg/ml was prepared as a stock solution for late stage larvae. The doses were prepared by diluting the stock solution with 10% EtOH at the rates of 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 and 1.6 mg/ml. Control groups were determined as untreated and 10% EtOH. From these prepared doses, each subject was injected from the last proleg with the help of 5 µl microinjectors (Hamilton, USA). It was expected to act for 24 hours for post-injection experiments. Four replicates at each dose were performed for the experiments and 16 samples were used for each group.

2.3. Phenoloxidase (PO) Enzyme Activity

For the determination of phenoloxidase enzyme activity, 20 µl hemolymph leaking from the anterior segment of the prolegs through the hole opened with a sterile needle were collected from each Pyrethrum injected sample. The collected hemolymph fluid was then placed in microcentrifuge tubes containing 180 µl phosphate buffer solution ice-cold and immediately frozen at -20 °C without allowing it to darken. This hemolymph-phosphate buffer mixture,
which was dissolved before the experiment, was centrifuged at 10,000 g for 5 minutes in a refrigerated centrifuge (Hettich, Germany) at +4 °C and the supernatant was collected. 40 μl of this supernatant was taken and placed in a 96-well microplate. Then, 160 μl 3,4-Dihydroxy-L-phenylalanine (L-DOPA-Sigma-Aldrich, St Louis, MO) dissolved in phosphate buffer solution at a rate of 3 mg/ml was added onto the microplate. The prepared microplate was read in ELISA microplate reader (ThermoScientific Multiscan FC) at 490 nm (A$_{490}$) absorbance at intervals of 5 minutes from 0 to 30 minutes. The data obtained for each subject was determined as U/mg protein/min (Brookman et al., 1989).

2.4. Total Protein (TP)

TP determination in the study was carried out using the method of Bradford (1976). For TP determination in each subject, 5 μl of the collected supernatant was taken and placed in a 96-well microplate. 40 μl Bradford reagent (Sigma, Germany) and 155 μl deionized water were added into the supernatant. The prepared microplate was read at 595 nm (A$_{595}$) in an ELISA microplate reader (Thermo Scientific Multiskan FC). The data obtained were calculated as mg protein/ml.

2.5. Statistics

The data obtained after the experiments were evaluated with Tukey HSD by performing one-way-ANOVA with the SPSS v.20 program, in terms of differences between both duration and doses.

3. RESULTS

The changes in the total amount of protein according to the doses applied at the end of our study are presented in Table 1. According to the data obtained, the total amount of protein was determined the highest in the 0.2 mg/ml injection group and the lowest in the 0.6 mg/ml injection group. The difference between the groups was found to be statistically insignificant (F=1.405; Sig=0.191>0.05).

| Doses   | TP (protein mg/ml) ± SE*       |
|---------|--------------------------------|
| Untreated | 0.923070 ± 0.014               |
| 10% EtOH  | 0.919344 ± 0.018               |
| 0.2 mg/ml | 0.962546 ± 0.013               |
| 0.4 mg/ml | 0.928285 ± 0.011               |
| 0.6 mg/ml | 0.909868 ± 0.016               |
| 0.8 mg/ml | 0.931693 ± 0.009               |
| 1 mg/ml   | 0.937254 ± 0.012               |
| 1.2 mg/ml | 0.929527 ± 0.014               |
| 1.6 mg/ml | 0.912887 ± 0.008               |
| 2 mg/ml   | 0.914693 ± 0.011               |

*SE is Standart Error

Table 1. Total protein values of G. mellonella hemolymph which injected by pyrethrum extract (mg protein/ml).

The data obtained as a result of the studies on determining the phenoloxidase enzyme activity are presented in Figure 1. Accordingly, the highest enzyme activity was determined as 0.081 U/mg protein/min at a dose of 0.6 mg/ml, and the lowest as 0.054 U/mg protein/min at a dose of 1 mg/ml. The mean of the untreated group was determined as 0.064 U/mg protein/min. According to the statistical evaluation, the difference between the 0.6 mg/ml injection group
and the 0.4 mg/ml injection group is statistically insignificant, but the difference between the other groups is significant (F:4.553; df: 9; sig:000<0.05).

**Figure 1.** The effects of Pyrethrum extract on *G. mellonella* hemolymph phenoloxidase enzyme activity (U/mg protein/min). The difference between groups bearing the different letters in the figure is significant (p<0.05).

The change in phenoloxidase enzyme activity over time is presented in Figure 2. Accordingly, the enzyme activity increased linearly between the beginning and the 15th minute. Within this interval, the measurement at every fifth minute revealed a significant difference with the measurements before and after it. In addition, the difference between 15th to 20th, 20th to 25th, and 25th to 30th minutes was still insignificant, while the difference between 15th with 25th, and 20th with 30th minutes measurements was significant (F:108.510; df: 6; sig:000<0.05).

**Figure 2.** The change of *G. mellonella* hemolymph phenoloxidase enzyme activity average by time (U/mg protein/min). The difference between groups bearing the different letters in the figure is significant (p<0.05).
4. DISCUSSION

The biocidal products are preferred more than synthetic pesticides. With the increasing importance of ecological agriculture, natural insecticides are also increasingly met with interest. The primary toxic effects of pyrethrins, one of the natural insects, are related to their direct effects on the nervous system (Yang et al., 2017).

Deltamethrin, a pyrethrin synthesis, reduced the total hemocyte count in *G. mellonella* and showed genotoxic effect by inducing the formation of micronuclei (Kurt and Kayiş, 2015). Deltamethrin even at a very low dose displays harmful effects by disrupting hepatic and renal function and causing DNA damages in pubescent female rats (Chargui et al., 2012). Rats treated with pyrethrin in the early period experience serious heart problems when they become adults. This situation is related to the damage in DNA in the early period (Vadhana et al., 2011). Organophosphorus insecticides cause metabolic and synaptic dysfunction as well as oxidative stress in *G. mellonella* (İçen et al., 2005; Alp and Coşkun 2018).

Studies on cypermethrin, a synthetic pyrethroid type insecticide, have shown that cypermethrin causes a decrease in protein, glycogen and lipid levels on Pimpla turionellae (Sak et al., 2006). Cypermethrin also increases life expectancy of female *P. turionellae* (Sak et al., 2009). It has been determined that as the dose of cypermethrin increases, it delays larval development and pupation time, decreases the pupation percentage and increases the mortality rate at *G. mellonella* (Sak and Uçkan, 2009). Ergin et al. (2007) in their work shown that sublethal doses of Cypermethrin could limit the development, survival, and growth of Apanteles galleriae due to possible metabolic, hormonal, and nutritional deficiencies.

The pyrethrin has a genotoxic effects and lowers the mitotic index (Azab et al., 2017). Yang et al. (2017), using the human liver cancer cell line (HepG2), found out that pyrethrins induce apoptosis, cause mitochondrial dysfunction, cytotoxic effect and DNA damage, induce autophagy, and cause oxidative stress in cells. Natural pyrethrins induce autophagy of HepG2 cells, so activation of the AMPK / mTOR signalling pathway may pose a potential risk to human health (Yang et al., 2018). It has been determined that prophenoloxidase activation is an integral component of the insect defence system, which includes a large number of enzymes (e.g. proteinases, oxidases, and dopachrome conversion enzyme) that immobilize and kill invading microorganisms (Zhao et al., 2007). During melanization, the conversion of inactive PPO to the active form of PO is provided by oxidative processes (Nakhleh et al., 2017); and this causes an increase in oxidative stress in the organism.

According to Chen et al. (2017), fenpropathrin, a type of pyrethrin, also causes an increase in total PO activity in honey bee (*Apis mellifera*). This increase is due to the moderate inhibition of fenpropatrin on the diphenolase activity of tyrosinase (Tang et al., 2009). Our results shown that the pyrethrum extract is effective on phenoloxidase activity at 0.6 mg/ml level (Figure 1). The data obtained from our study confirms the results of Chen et al. (2017). The injection of pyrethrum extract at the level of 0.6 mg/ml is inducing PO formation. The author suggest which is should be because of the try to deal with toxic effects in *G. mellonella* at that dose of pyrethrum. The upper doses of extract must have inhibited the biochemical process of tyrosine by increasing the oxidative stress of the organism as a result of higher exposure. In this way, PO activity decreased at high concentrations of pyrethrum.

5. CONCLUSION

It is understood from the literature that pyrethrum has negative effects on living organisms. These negative effects are such as the decrease in total hemocyte count, DNA damage, genotoxic effect, induction of autophagy and apoptosis, mitochondrial dysfunction, oxidative stress, inhibition of biochemical processes (Yang et al., 2017). The results of our
study are related to changes in hemocyte count and oxidative stress factors. Because hemocytes are the only source of phenoloxidase (Ashida and Brey, 1998) and activation processes of phenoloxidase are closely related to oxidative stress factors. Since the increasing oxidative stress interrupts the biochemical processes in the organism, decreases in PO activity are observed. The effects of pyrethrum on antioxidant enzyme activity will be determined by further studies. This studies; will be clarify changing in oxidative stress by pyrethrum applying. Determination of changes in hemocyte count will also help explain phenoloxidase enzyme activity. As a result of this study, the pyrethrum extract increased activity at a certain dose.

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KAYA / The Effects of Pyrethrum Extract on Galleria mellonella Hemolymph Phenoloxidase Enzyme Activity

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