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Research Article

Olive Leaf Extract-Induced Changes in Phenoloxidase Activity of *Galleria mellonella* (Lepidoptera: Pyralidae) Hemolmyph

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Abstract

Since the fruits and leaves of the Olive (*Olea europea*) tree, which grows naturally in Mediterranean countries, are thought to be beneficial for many diseases, it is widely used in folk medicine in these countries. Olive leaf extract has been used in the treatment of many diseases such as malaria, high fever, heart diseases and cancer. The greater wax moth *Galleria mellonella* (Lepidoptera: Pyralidae) is frequently used in invertebrate immunity studies due to its similarity to the mammalian natural immune system. Melanization occurs when quinone precursors are converted to melanin and inactive profenoloxidase to phenoloxidase enzyme. In this study, the phenoloxidase activity of *G. mellonella* hemolymph larvae injected with olive leaf extract in different doses was determined by ELISA microplate reader at 492 nm absorbance. The findings obtained from this study showed that olive leaf extract increased phenoloxidase activity at a dose of 0.001 mg mL^{-1}. Lower doses of 0.001 mg mL^{-1} did not cause changes in phenoloxidase activity compared to the untreated and Dimethyl Sulfoxide (DMSO) groups. The highest olive leaf extract dose of our study is 0.01 mg mL^{-1} and the larvae did not survive for 24 hours over those doses. The results of our study show that olive leaf supports humoral immune responses when used in low doses.

Key words: *Olea europea, Galleria mellonella, hemolymph, phenoloxidase*

Galleria mellonella Hemolenfinde Zeytin Yaprağı Ekstresi Kaynaklı Fenoloksidaz Aktivitesindeki Değişimler

Öz

Akdeniz ülkelerinde doğal olarak yetişen Zeytin (*Olea europea*) ağacının meyve ve yapraklarının birçok hastalığa faydalı olduğu düşünüldüğünden bu ülkelerde halk hekimliğinde yaygın olarak kullanılmaktadır. Zeytin yaprağı ekstresi sıtma, yüksek ateş, kalp hastalıkları ve kanser gibi birçok hastalığın tedavisinde kullanılmıştır. Büyük balmumu güvesi *Galleria mellonella* (Lepidoptera: Pyralidae), memelilerinnin doğal bağışıklık sistemine benzerliği nedeniyle omurgasız bağışıklık araştırmalarında sıklıkla kullanılmaktadır. Melanizasyon, kinon öncüleri melanin ve inaktif profenoloksidaz fenoloksidaz enzimine dönüştürüldüğünde meydana gelir. Bu çalışmada, zeytin yaprağı ekstresi ile farklı dozlarda enjekte edilen *G. mellonella* larvalarının hemolenf fenoloksidaz aktivitesi 492 nm absorbansında ELISA mikroplaka okuyucu ile belirlenmiştir. Bu çalışmadan elde edilen bulgular, zeytin yaprağı ekstresinin 0,001 mg mL^{-1} dozunda fenoloksidaz aktivitesini artırdığını göstermiştir. Daha düşük 0.001 mg mL^{-1} dozları, işlem görmemiş ve Dimetil Sülfoksit (DMSO) gruplarına kıyasla fenoloksidaz aktivitesinde değişikliklere neden olmamıştır. Çalışmamızın en yüksek zeytin yaprağı ekstresi dozu 0.01 mg mL^{-1}'dir ve bu doz üzerinde 24 saatlik gözlemlerde larvaların hayatta kalamadığı tespit edilmiştir. Çalışmamızın sonuçları, zeytin yaprağının düşük dozlarda kullanıldığında humoral bağışıklık tepkilerini desteklediğini göstermektedir.

Anahtar kelimeler: *Olea europea, Galleria mellonella, hemolenf, fenoloksidaz.*

Introduction

The main component of the extract obtained from olive leaf, which is also marketed as a medicine and is thought to have many benefits for human health, is oleuropein. Its color is dark brown, while its taste is bitter (Sudjana et al., 2009). It was used in the past to treat malaria and associated fever (Lee, 2010). Olive leaf is resistant to many pathogens and insect attack in nature, and many researchers have studied the components of this plant (Furneri et al., 2002; Li et al., 2016). Olive and olive oil contain many bioactive ingredients. Among these compounds, oleuropein has various biological properties, especially antioxidant and anti-inflammatory activities (Visioli et al., 1995; Ghisalberti, 1998; Saija et al., 2001). Phenolic compounds with broad microbial action in olive include phenolic glucoside, oleuropein and hydrolysis products such as 3,4-dihydroxy-phenylethanol (hydroxytyrosol), elenolic acid and aglycone (Furneri et al., 2002). Oleuropein, the most studied phenolic compound, is a 3,4-dihydroxy phenylethanol ester containing a beta-glucosylated elenolic acid (Furneri et al., 2002). The bioactive components of olives can change the composition of the extract due to physical changes such as the nutrition of the plant and geographical location (Sudjana et al., 2009). While oleuropein is considered to be a promising antimicrobial agent for the treatment of respiratory tract infections in humans, it has also been reported to have an antioxidant structure that reduces the risk of cancer and heart disease and to show not only antioxidant but also hypolipidemic and hypoglycemic effects (Rey et al., 2020). However, olive leaf has been observed to reduce blood pressure and increase blood flow in coronary arteries (Lee, 2010). Studies have shown that Oleuropein increases nitric oxide (NO) production in macrophage members and eliminates pathogens in this way (Li et al., 2016). It has been observed that oleuropein, if taken as food, will help the treatment of many infectious diseases and that olive oil phenols also have protective effects against brain damage and aging (Sudjana et al., 2009; Khalatbary and Ahmadvand, 2012). In fact, the low rate of heart diseases, neurodegenerative diseases, diabetes and cancer in the Mediterranean region in the studies conducted suggests that it is due to the high consumption of these phenols (Samara et al., 2017; Maruca et al., 2019).

In insects, melanin pigments and their precursors are important as structural and protective components of the cuticle (Dubovskiy et al., 2013). The concept of melanogenesis is the process of formation of melanin pigment and toxic byproducts as a result of the interaction of phenoloxidase with quinone precursors. Melanogenesis mainly occurs in cuticle structures, midgut epithelium, and hemolymph, where it plays dual roles in hardening-thickening the cuticle and immune defense (Cerenius and Söderhäll., 2004; Andersen, 2010). Phenoloxidase is normally found in eukaryotes and prokaryotes as its inactive form, prophenoloxidase (Ratcliffe, 1985).

Compared to many mammalian model organisms, *G. mellonella* larvae are easy to maintain and to be reared in large numbers in a limited area. Those organisms can manufacture without special equipment, do not require ethical approval for their use, and are close to the mammalian innate immune system. Their similarity makes them ideal for large-scale studies (Ignasiak and Maxwell, 2017). *G. mellonella* larvae could live between 25 and 37 °C, which makes this model organism a suitable option for studying microorganism pathogenicity at mammalian temperature and also for studying temperature-dependent virulence factors (Desalermos et al., 2012; Smoot et al., 2001). By targeting potential sites of infection or damage to deal with *G. mellonella*, fungal pathogens, they prioritize immediate defenses and sacrifice fertility, size, and longevity (Dubovskiy et al., 2013).

The effects of oleuropein, which is taken into the body in various ways, on the immune responses of living things have been tried to be determined by the model organism. It was determined in a previous study that a certain dose of olive leaf extract supported hemocyte-mediated immunity in *G. mellonella* (Kaya and Demir, 2020). This study aimed to determine the effect of olive leaf extract on the hemolymph phenoloxidase activity of the model organism *G. mellonella*.

Materials and Methods

Insect rearing

The *G. mellonella* samples used in our study were fed with artificial food (natural blackened honeycomb, wheat bran, honey, water and glycerin) developed by Bronskill (1961) and modified by Sak et al (2006). Larvae reaching the last stage (0.18 ± 0.02 g) were selected for use in the experiment.

Collection of plant material, extraction and preparation of test doses

The collected olive leaf samples were dried at room temperature and in the shade and then pulverized with a grinder. The powdered

material was dissolved in 80% Ethyl alcohol in the Soxhlet device. Dry matter was obtained by removing the alcohol from the solvent at room temperature. This dry substance was stored at + 4 °C in a light-proof bottle until its use. The dry matter obtained from olive leaf extraction was dosed 0.001, 0.0025, 0.005 and 0.01 mg mL^{-1} in 40% (v/w) DMSO.

Injection of doses

The selected larvae of *G. mellonella* were used for the experiment. The surface of the larvae was sprayed with 70% ethyl alcohol for sterilization. After the sterilization process, the larvae were anesthetized until they remained immobilized on ice cassettes. The immobilized samples were injected into each larvae under a stereo microscope (Olympus, SD30, Japan) with a microinjector (Hamilton, USA) from the last of the prolegs at the experimental dose of 5 µl. Four replicates were performed for each dose and four samples were used in each replicate (n = 16). After the dosing applications, 24 hours were waited for the applied doses to take effect before starting the experiments.

Measuring phenoloxidase activity

The method stated by Kaya (2020) was used to determine the phenoloxidase activity. From each sample injected with oleuropein, 20 µl of hemolymph leaking from the anterior segment of the prolegs through a hole opened with a sterile needle was collected. The collected hemolymph fluid was then placed in microcentrifuge tubes containing 180 µl of phosphate buffer solution under 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 for 5 minutes at 10,000 g in a refrigerated centrifuge (Hettich, Germany) at +4 °C, and the supernatant was collected. 40 µl of these superntants were taken and placed in a 96-well microplate. Then, 160 µl of 3,4-Dihydroxy-L-phenylalanine (L-DOPA-Sigma-Aldrich, St Louis, MO) dissolved in 3 mg ml^{-1} in phosphate buffer solution was added to each well as a substrate. The prepared microplate was read in an ELISA microplate reader (Thermo Scientific Multiskan FC) at an absorbance of 490 nm at intervals of 5 minutes for 30 minutes. The data obtained for each subject were determined as U / mg protein / minute (Brookman et al., 1989). The molar extinction coefficient of 3,56 M^{-1} cm^{-1} were used to determinate the rate of DOPA chrome formation.

Total protein (TP)

TP determination in the study was made using the method of Bradford (1976). For TP determination in each larvae, 5 µl of the supernatant collected was taken and placed in a 96-well microplate. 40 µl of Bradford reagent (Sigma, Germany) and 155 µl of deionized water were added to the supernatant. The prepared microplate was read at 595 nm (A°595) in an ELISA microplate reader. The data obtained were calculated as mg protein ml^{-1}.

Statistics

The data obtained after the experiments were evaluated in terms of differences between the groups by performing a one-way ANOVA with the SPSS v.20 program, using Tamhane's T2 test.

Results and Discussions

The changes in the total protein amount according to the doses applied are shown in Table 1. According to the data obtained, total protein amount was determined the highest in the 0.01 mg ml^{-1} injection group and the lowest in the 0.001 mg ml^{-1} injection group. According to the statistical evaluation, the difference between the 0.01 mg ml^{-1} injection group and the other groups is statistically significant, but the difference between the other groups is insignificant (F: 7.510; df: 5; Sig: 0.000< p 0.05).

Table 1. The effect of olive leaf extract on the total protein amount of *G. mellonella* hemolymph.

Total Protein	Mean ± SE*
Untreated	0,915 ± 0,013[a]
DMSO	0,908 ± 0,015 [a]
0,0010 mg mL^{-1}	0,930 ± 0,019 [a]
0,0025 mg mL^{-1}	0,950 ± 0,019 [a]
0,0050 mg mL^{-1}	0,936 ± 0,024 [a]
0,0100 mg mL^{-1}	1,032 ± 0,012 [b]

*SE: Standart error

** Different letters (a, b, c, and d) indicate statistical differences between groups at the P <0.05 level at the same column

The data obtained as a result of studies aimed at determining phenoloxidase activity according to dose are shown in Figure 1. Accordingly, the highest enzyme activity among the groups with olive leaf injected was determined as 0.07876 U / mg protein / min at a dose of 0.001 mg mL^{-1}, and the lowest as 0.05853 U / mg protein / min at a dose of 0.01 mg mL^{-1}. The average of the control group was determined as 0.03680 U / mg protein / minute. The difference between the groups is insignificant (F: 45,557; df: 5; sig: 0,000 <p: 0.05).

When our results are evaluated together, it is seen that olive leaf extract can support immunity at a dose of 0.001 mg mL^{-1} and that its effectiveness will decrease in higher doses.

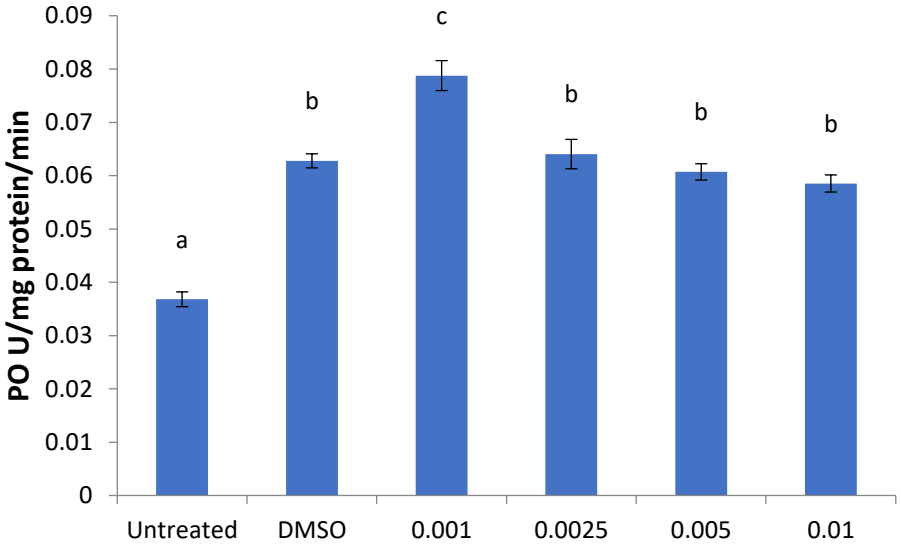

Figure 1. *G. mellonella* hemolymph phenoloxidase activity changes by olive leaf extract injection (U / mg protein / min). Different letters (a, b and c) indicate statistical differences between groups at the P <0.05 level at the same column. Each bar represents 30 min changes in enzyme activity at 16 individual samples

An important step of encapsulation and nodulation immune responses is hemocyte degranulation (Dubovskiy et al., 2016), which releases activators of prophenoloxidase (proPO) and cell aggregation, which usually destroy cells. The ProPO cascade plays a role in the melanization process (Chain and Anderson, 1982; Takahashi and Enomoto, 1987; Pech and Strand, 2000) and is an important marker in studies on immunity. In general, the increase in phenoloxidase has been interpreted as supporting immunity in the literature. As a result of the obtained data in the current study, it was observed that oleuropein increased the phenoloxidase activity at low doses however this increase declined with increasing doses (Figure 1). In their study on shrimps for the treatment of white spot virus, Gholamhosseini et al. (2020) showed that the survival rate of those fed with oleuropein increased up to 65%. In their current findings, they found that white spot virus infection caused a significant decrease in oleuropein doses compared to control groups. They suggested that therapeutic nutrition with oleuropein could significantly reduce phenoloxidase activities and hemolymph coagulation time. Similarly, in our study, it was found that phenoloxidase activity decreased at high doses.

The prophenoloxidase (PPO) is found mainly in oenocytoids in *G. mellonella* hemocytes, and these cells originate from an immature prohemocyte and are known to play a role in the immune response (Kavanagh and Reeves, 2004). Kaya and Demir (2020) found in their study that olive leaf extract increased the number of hemocytes at certain doses (1000 ppm - 2500 ppm), while this increase decreased at the highest doses. Since hemocytes are seen as the sole source of phenoloxidase, changes in hemocyte count help to understand changes in phenoloxidase activity. In our study, phenoloxidase activity showed a higher activity at the rate of 0.001 mg ml^{-1} compared to other doses, which corresponds to 1000 ppm in the study of Kaya and Demir (2020). In both our study and the study of Kaya and Demir (2020), the same dose showed a positive effect on *G. mellonella* immunity.

After phenoloxidase activation, melanization occurs in *G. mellonella* as a result of activated immune responses. Kaya and Demir (2020) examined the melanization response in two periods of 4 and 24 hours in their study and found

that the melanization response did not change at doses other than 1000 ppm in 24 hours compared to the control. Phenoloxidase, the subject of our study, is the key element for the occurrence of the melanization response. In the results of our study, there was a significant increase in phenoloxidase activity at the rate of 0.001 mg mL^{-1}, while the difference between the DMSO injection group at other doses was insignificant. Accordingly, although an increase in phenoloxidase activity was observed, the reason why there was no significant change in the melanization response in the study of Kaya and Demir (2020) may be that the secondary metabolites in olive leaf affected the enzyme pathways to a certain extent, blocking the enzyme from reaching the required end product.

Conclusions

Further research is required to find out the ways in which olive leaf extract affects immune responses and to determine the genotoxic and enzymatic effects. In this way, it will be possible to fully evaluate which mechanism is affected, and its possible consequences.

As seen in previous studies on *G. mellonella*, there is much in common between innate insect immunity and innate mammalian immunity. For this reason, based on the results of our and previous study (Kaya and Demir, 2020), our opinion that the consumption of certain doses of olive leaves will support the immunity of certain organisms, including humans.

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References

Andersen, S.O. 2010. Insect cuticular sclerotization: a review. Insect biochemistry and molecular biology, 40 (3): 166-178.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical biochemistry, 72 (1-2): 248-254.

Bronskill, J. 1961. A cage to simplify the rearing of the greater wax moth, *Galleria mellonella* (Pyralidae). J. Lep. Soc, 15 (2): 102-104.

Brookman, J. L., Ratcliffe, N. A. and Rowley, A. F. 1989. Studies on the activation of the prophenoloxidase system of insects by bacterial cell wall components. Insect Biochemistry, 19 (1): 47-57.

Cerenius, L. and Söderhäll, K. 2004. The prophenoloxidase-activating system in invertebrates. Immunological reviews, 198 (1): 116-126.

Chain, B.M. and Anderson, R. S. 1982. Selective depletion of the plasmatocytes in *Galleria mellonella* following injection of bacteria. Journal of insect physiology, 28 (4): 377-384.

Desalermos, A., Fuchs, B.B. and Mylonakis, E. 2012. Selecting an invertebrate model host for the study of fungal pathogenesis. PLoS pathog, 8 (2): e1002451.

Dubovskiy, I.M., Whitten, M.M.A., Kryukov, V.Y., Yaroslavtseva, O.N., Grizanova, E. V., Greig, C. and Butt, T.M. 2013. More than a colour change: insect melanism, disease resistance and fecundity. Proceedings of the Royal Society B: Biological Sciences, 280 (1763): 20130584.

Dubovskiy, I.M., Kryukova, N.A., Glupov, V.V. and Ratcliffe, N.A. 2016. Encapsulation and nodulation in insects. Invertebrate Survival Journal, 13 (1): 229-246.

Furneri, P.M., Marino, A., Saija, A., Uccella, N. and Bisignano, G. 2002. In vitro antimycoplasmal activity of oleuropein. International journal of antimicrobial agents, 20 (4): 293-296.

Ghisalberti, E.L. 1998. Biological and pharmacological activity of naturally occurring iridoids and secoiridoids. Phytomedicine, 5 (2): 147-163.

Gholamhosseini, A., Kheirandish, M.R., Shiry, N., Akhlaghi, M., Soltanian, S., Roshanpour, H. and Banaee, M. 2020. Use of a methanolic olive leaf extract (*Olea europaea*) against white spot virus syndrome in Penaeus vannamei: Comparing the biochemical, hematological and immunological changes. Aquaculture, 735556.

Ignasiak, K. and Maxwell, A. 2017. *Galleria mellonella* (greater wax moth) larvae as a model for antibiotic susceptibility testing and acute toxicity trials. BMC research notes, 10 (1): 1-8.

Kavanagh, K. and Reeves, E. P. 2004. Exploiting the potential of insects for in vivo pathogenicity testing of microbial pathogens. FEMS microbiology reviews,28 (1): 101-112.

Kaya, S. 2020. The Effects of Pyrethrum Extract on *Galleria mellonella* Hemolymph Phenoloxıdase Enzyme. Journal of Scientific Perspectives, 4(4), 269-280.

Kaya, S. and Demir, N. 2020. Zeytin (*Olea europaea*) yaprağı ekstraktlarının model organizma *Galleria mellonella* hemosit ve hemosit aracılı bağışıklık tepkileri üzerine etkileri. Türk Tarım ve Doğa Bilimleri Dergisi, 7 (3): 646-653.

Khalatbary, A.R. and Ahmadvand, H. 2012. Neuroprotective effect of oleuropein following spinal cord injury in rats. Neurological research, 34 (1): 44-51.

Lee, O.H. and Lee, B.Y. 2010. Antioxidant and antimicrobial activities of individual and combined phenolics in Olea europaea leaf extract. Bioresource technology, 101 (10): 3751-3754.

Li, X., Liu, Y., Jia, Q., LaMacchia, V., O'Donoghue, K. and Huang, Z. 2016. A systems biology approach to investigate the antimicrobial activity of oleuropein. Journal of industrial microbiology and biotechnology, 43 (12): 1705-1717.

Maruca, A., Catalano, R., Bagetta, D., Mesiti, F., Ambrosio, F.A., Romeo, I. and Costa, G. 2019. The Mediterranean Diet as source of bioactive compounds with multi-targeting anti-cancer profile. European journal of medicinal chemistry, 181: 111579.

Pech, L.L. and Strand, M. R. 2000. Plasmatocytes from the moth *Pseudoplusia includens* induce apoptosis of granular cells. Journal of Insect Physiology, 46 (12): 1565-1573.

Ratcliffe, N.A. 1985. Invertebrate immunity a primer for the non-specialist. Immunology letters, 10 (5): 253-270.

Rey, A. I., de-Cara, A., Calvo, L., Puig, P. and Hechavarría, T. 2020. Changes in Plasma Fatty Acids, Free Amino Acids, Antioxidant Defense, and Physiological Stress by Oleuropein Supplementation in Pigs Prior to Slaughter. Antioxidants, 9 (1): 56.

Saija, A., Tomaino, A., Pellegrino, M.L., Giuffrida, N., Trombetta, D. and Castelli, F. 2001. In vitro evaluation of the antioxidant activity and biomembrane interaction of the lazaroid U-74389G. Life Sciences, 68 (12): 1351-1366.

Sak, O., Uçkan, F. and Ergin, E. 2006. Effects of cypermethrin on total body weight, glycogen, protein, and lipid contents of *Pimpla turionellae* (L.) (Hymenoptera: Ichneumonidae).

Samara, P., Christoforidou, N., Lemus, C., Argyropoulou, A., Ioannou, K., Vougogiannopoulou, K. and Skaltsounis, A. L. 2017. New semi-synthetic analogs of oleuropein show improved anticancer activity in vitro and in vivo. European Journal of Medicinal Chemistry, 137: 11-29.

Smoot, L.M., Smoot, J.C., Graham, M.R., Somerville, G.A., Sturdevant, D.E., Migliaccio, C.A.L. and Musser, J. M. 2001. Global differential gene expression in response to growth temperature alteration in group A Streptococcus. Proceedings of the National Academy of Sciences, 98 (18): 10416-10421.

Sudjana, A. N., D'Orazio, C., Ryan, V., Rasool, N., Ng, J., Islam, N. and Hammer, K.A. 2009. Antimicrobial activity of commercial *Olea europaea* (olive) leaf extract. International journal of antimicrobial agents, 33 (5): 461-463.

Takahashi, S. and Enomoto, G. 1987. Scanning Electron Microscopic Study of the Initial Phase of Encapsulation in Samia cynthia ricini: (encapsulation/ haemocyte lysis/ plasma coagulation/ Samia silkmoth). Development, growth and differentiation, 29 (3): 249-256.

Visioli, F., Bellomo, G., Montedoro, G. and Galli, C. 1995. Low density lipoprotein oxidation is inhibited in vitro by olive oil constituents. Atherosclerosis, 117 (1): 25-32.