Türk. entomol. derg., 2024, 48 (4): 383-398
DOI: http://dx.doi.org/10.16970/entoted.1538480

ISSN 1010-6960
E-ISSN 2536-491X

Original article (Orijinal araştırma)

Investigation of biodegradable insecticidal properties of different plant lectins on *Tropinota* (*Epicometis*) *hirta* (Poda, 1761) (Coleoptera: Cetoniidae)[1]

Tropinota (*Epicometis*) *hirta* (Poda, 1761) (Coleoptera: Cetoniidae) üzerinde farklı bitki lektinlerinin biyolojik olarak parçalanabilir insektisit özelliklerinin araştırılması

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Abstract

Tropinota (*Epicometis*) *hirta* (Poda, 1761) (Coleoptera: Cetoniidae), a polyphagous agricultural pest that causes significant economic damage to fruits, vegetables and cereals. Due to its presence during the same period as bees, chemical control methods are not recommended. This study aims to evaluate the effectiveness of plant lectins as bioinsecticides in comparison to synthetic insecticides. Adult insects were collected from various locations in İvrindi district of Balıkesir, Türkiye, between March 25 and June 6, 2024. The insects were fed *in vivo* with lectins from *Wisteria floribunda* (Willd.) DC. (Fabales: Fabaceae*)* (WF), *Phaseolus vulgaris* L. (Fabales: Fabaceae) (PV), *Triticum vulgaris* L. (Poales: Poaceae) (TV) and *Phytolacca americana* L. (Caryophyllales: Phytolaccaceae) (PA) at concentrations of 200, 250 and 300 mg/g for 7 days. The highest lethal effect among the applied lectin doses was detected at the PA300 dose. Upon examining the insect gut tissues, it was found that the TV300 lectin dose contained higher levels of oxidants compared to the synthetic insecticide. Additionally, the applied lectins exhibited inhibitory effects on the activity of various digestive enzyme in insects. Our findings suggest that lectins can be used as a biodegradable alternative to chemical insecticides for controlling *T. hirta*.

Keywords: Bioinsecticides, digestive enzymes, lectins, oxidative stress, apple blossom beetle

Öz

Tropinota (*Epicometis*) *hirta* (Poda, 1761) (Coleoptera: Cetoniidae), meyve, sebze ve tahıllara önemli ekonomik zararlar veren polifag bir tarımsal zararlıdır. Arılarla aynı dönemde bulunması nedeniyle kimyasal kontrol yöntemleri önerilmemektedir. Bu çalışma, sentetik insektisitlere kıyasla biyoinsektisit olarak bitki lektinlerinin etkinliğini değerlendirmeyi amaçlamaktadır. Ergin böcekler, 25 Mart-06 Haziran 2024 tarihleri arasında Türkiye'nin İvrindi (Balıkesir) ilçesinin çeşitli lokasyonlarından toplanmıştır. Böcekler, 7 gün boyunca *Wisteria floribunda* (Willd.) DC. (Fabales: Fabaceae*)* (WF), *Phaseolus vulgaris* L. (Fabales: Fabaceae) (PV), *Triticum vulgaris* L. (Poales: Poaceae) (TV) ve *Phytolacca americana* L. (Caryophyllales: Phytolaccaceae) (PA) lektinlerin 200, 250 ve 300 mg/g konsantrasyonuyla *in vivo* olarak beslenmiştir. Uygulanan lektin dozları arasında en yüksek öldürücü etki PA300 dozunda tespit edilmiştir. Böcek bağırsak dokuları incelendiğinde, TV300 lektin dozunun sentetik insektisit ile karşılaştırıldığında daha yüksek düzeyde oksidan içerdiği tespit edilmiştir. Ek olarak, uygulanan lektinler böceklerdeki çeşitli sindirim enzimlerinin aktivitesi üzerinde inhibitör etki göstermiştir. Bulgularımız, lektinlerin *T. hirta*'nın kontrolünde kimyasal pestisitlere alternatif biyolojik olarak parçalanabilen bir insektisit olarak kullanılabileceğini göstermektedir.

Anahtar sözcükler: Biyo-insektisit, sindirim enzimleri, lektinler, oksidatif stres, çiçek zınnı

[1] This study is derived from the PhD thesis of the first author. This study was supported by TUBITAK (Scientific and Technological Research Council of Turkey) 2211-C 'Biotechnological Pharmaceutical Technologies' Category PhD Support Grant and by Balıkesir University, Scientific Research Projects Unit (BAP) with Project Number 2022/095.
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Received (Alınış): 25.08.2024 Accepted (Kabul ediliş): 07.01.2025 Published Online (Çevrimiçi Yayın Tarihi): 09.01.2025

Introduction

Tropinota (*Epicometis*) *hirta* (Poda, 1761) (Coleoptera: Cetoniidae), commonly known as the Apple Blossom Beetle, is a polyphagous agricultural pest (Kara, 1995). This species, which has a widespread global distribution, has been reported primarily from Europe, North Africa and a large part of Northern Asia (Çelik & Yaşar, 2021). It also has a widespread distribution across Turkey (Lodos et al., 1999; Şenyüz et al., 2015). It has been reported to damage over 70 plant species, including fruits such as apples, cherries, quinces, strawberries and peaches, as well as grains, ornamental plants and various weeds (Buşmachiu & Toderaş, 2014). Adult insects attack the pistils, leaves, buds and fruits of plants, leading to economic losses (Kara, 1995). It has been reported to cause a 70% reduction in flowers in Bulgarian cherry orchards (Kutinkova & Andreev, 2004), 90%-100% damage in pear orchards around Tokat (Kara, 1995), and 50% damage in blueberry orchards (Slav et al., 2018). Adult insects, which begin flying in April due to rising temperature, remain active until mid-July (Oltean et al., 2015).

Chemical control is not recommended because the insects, whose populations increase during the flowering period, coexist with bees and other beneficial insects. This drawback of chemical control has prompted researchers to explore biological and biotechnical control methods (Yaşar & Dahham, 2019). Traps of different colors and various attractants added to them are frequently used in the control of *T. hirta* (Schmera et al., 2004). Efforts are ongoing to develop biological agents against *T. hirta* using entomopathogenic nematodes (Akpınar et al., 2020) and entomopathogenic fungus isolates (Atmaca et al., 2018). While these studies have been effective in reducing the population, they are not yet sufficient for definitive control. As a result, local producers continue to rely on chemical pesticides to manage the increasing population.

Lectins are essential carbohydrate-binding glycoproteins that serve as one of the defense mechanisms of insect-resistant plants (Upadhyay & Singh, 2012). The fact that lectins are generally destructive in the digestive tract is because it is the first site of action of orally ingested nutrients. Therefore, it is quite possible that lectins with specific carbohydrate-binding properties bind to these sites where activities such as enzyme production, secretion, and nutrient absorption take place. In addition, lectins deform epithelial cells and cause them to swell. This leads to the absorption of toxic substances in the gastrointestinal system. As a result, toxic substances that enter the insect's circulatory system bind to various sites and exert harmful effects (Gatehouse et al., 1984; Peumans & Van Damme, 1995; Jaber et al., 2010; Vandenborre et al., 2011; Douglas, 2012). Therefore, in this study the effects of the lectins on important digestive enzymes (protease, trypsin, acid phosphatase, alkaline phosphatase, exoglucanase, endoglucanase, β-glucosidase, and α-amylase) were examined to be able to correlate the mechanisms by which lectins kill *T. hirta. In addition,* it has been reported that antioxidant enzyme activities increase due to lectin intake in insects and this increase leads to hydrogen peroxide accumulation. Thus, free radicals can be produced, and oxidative stress can be induced in insects (Lima et al., 2016; Rahimi et al., 2018; Khoobdel et al., 2022). *Phaseolus vulgaris* L. (Fabales: Fabaceae) lectin showed toxic effects on *Callosobruchus maculatus* (Fabricius, 1775) (Coleoptera: Bruchidae). Lectins isolated from *P. vulgaris* and *Triticum vulgaris* L. (Poales: Poaceae) have shown strong effects on Coleoptera and Lepidoptera orders (Czapla & Lang, 1990; Sauvion et al., 2004). Lima et al. (2016) found that the lectin they applied caused a decrease in the number of digestive cells and that this lectin caused cell death. Additionally, the findings observed in the peroxidase staining analysis indicated the presence of oxidative stress. Thus, the total oxidant status (TOS) values in the intestinal tissues were measured in this study to assess the oxidative stress by lectins in *T. hirta.*

A number of entomotoxic effects of plant lectins on insects have been demonstrated in the literature. These include food deterrence, enzyme inhibition due to deformation of the digestive tract, reduced oviposition, impaired larval development, induction of oxidative stress and death. These effects provide important data in the field of pest control (Czapla & Lang, 1990; Macedo et al., 2002; Sauvion et al., 2004; Napoleão et al., 2013; Lima et al., 2016). Khoobdel et al. (2022), noted that some digestive enzymes were

inhibited in adult *Sitophilus oryzae* (L., 1763) (Coleoptera: Curculionidae), fed lectin. It has been reported that lectin binding to epithelial cells may result in cytotoxicity, decrease the number of cells secreting digestive enzymes, or inhibit enzymes by binding to enzyme receptors. There are no study investigating the use of plant lectins for the control of *T. hirta.* This study aims to explore alternative methods to reduce the reliance on chemical pesticides in managing *T. hirta*. Additionally, it seeks to determine the entomotoxic effects of plant lectins on this pest and to provide preliminary data for their potential use as biopesticides in future research. Therefore, mortality rates of *T. hirta* fed with lectins extracted from *Wisteria floribunda* (Willd.) DC. (Fabales: Fabaceae), *P. vulgaris*, *T. vulgaris* and *Phytolacca americana* L. (Caryophyllales: Phytolaccaceae) were calculated. Furthermore, the total oxidant status (TOS) values in the intestinal tissues were measured to assess the oxidative stress by lectins, given the known effects of insecticides on insects' oxidative balance. Many of the natural plant compounds and organic compounds used in the control of insect pests are known to affect digestive enzymes. To evaluate how lectins influence digestive physiology, their effects on the activity of key enzymes -protease, trypsin, acid phosphatase, alkaline phosphatase, exoglucanase, endoglucanase, β-glucosidase and α-amylase- within insect intestines were also investigated.

Materials and Methods

Insects

Adults of *T. hirta* were manually collected from wild mustard, *Sinapis arvensis* L. (Brassicales: Brassicaceae) plants at different locations in Balıkesir/İvrindi at regular intervals between March 25 and June 6, 2024. The collected insects were transported to the laboratory in 1.2 liter plastic containers. Fresh wild mustard plants were added to the containers and the insects fed on them until the study was carried out. To ensure that the insects remained alive for the experiments, the containers were covered with a tulle to allow airflow. The insects were kept at 28±2ºC, %65±5 humidity and 16:8 photoperiod in the rearing room for seven days. Each experimental group consisted of 12 adults that were in the colony for 1-2 months of mixed sex. The study was conducted in 3 replications and 6 male- 6 female insects were used for each experimental group. In each set of experiments, 168 individuals were used, totaling 504 individuals for 3 replicates.

Lectins

Lectins from *Wisteria floribunda* (Sigma L8258-1MG), *Phaseolus vulgaris* (Sigma L2646-25MG), *Triticum vulgaris* (Sigma 61767-5MG), and *Phytolacca americana* (pokeweed) (Sigma L9379-5MG) were purchased from Sigma-Aldrich.

Chemicals

P-nitrophenyl phosphate, azocasein, trichloroacetic acid solution, Triton X-100, Nα-Benzoyl-L-arginine 4-nitroanilide hydrochloride (BApNA) and avicel were purchased from Sigma-Aldrich. *P*-nitrophenyl-β-D-glucopyranoside and 3,5-dinitrosalicylic acid (DNS) were purchased from Bostonchem. Carboxymethyl cellulose was purchased from BLDpharm, while cellulose was acquired from Central Drug House. Starch was purchased from ISOLAB chemicals. Other chemicals were procured from local vendors and met the required purity standards.

Growth media preparation

100 g of wild mustard flowers were blended with 100 mL of water creating a porridge mixture (Napoleão et al., 2013). This mixture was distributed equally among 14 experimental groups. The negative control group received only the water and flowers while the positive control group was treated with 5 mg/mL Bayer K-othrine SC50 (Deltamethrin) insecticide. Four distinct lectins were introduced in the nutrient medium of the other experimental groups, each applied *in vivo* at three different doses (200, 250 and 300 mg/g). Lectins, isolated from Wisteria plant (*W. floribunda*), kidney bean (*P. vulgaris*), wheat (*T. vulgaris*) and sugar cane plant (*P. americana*), were coded as outlined in Table 1.

Table 1. The types of plant lectins and their respective doses used in the experiments

Code	Plant Source	Dose (mg/g)
PA200	*Phytolacca americana*	200
PA250	*Phytolacca americana*	250
PA300	*Phytolacca americana*	300
PV200	*Phaseolus vulgaris*	200
PV250	*Phaseolus vulgaris*	250
PV300	*Phaseolus vulgaris*	300
TV200	*Triticum vulgaris*	200
TV250	*Triticum vulgaris*	250
TV300	*Triticum vulgaris*	300
WF200	*Wisteria floribunda*	200
WF250	*Wisteria floribunda*	250
WF300	*Wisteria floribunda*	300

Death rates

Experiments were conducted at 28±2ºC over a period of seven days with daily monitoring of the insects. At the end of the 7th day, live and dead insects were counted and placed in separate tubes. The insects were then prepared for intestinal dissection.

At the end of the experiments, the mortality rate (%) was calculated using the following formula.

Mortality rate (%) = (Survivors / Total Number of Individuals tested) * 100

Intestinal extracts

Insects fed for 7 days with growth media containing lectin extracts (200-250-300 mg/g), insecticide (Bayer K-othrine SC 50) and pure water (control), then kept inactive at -20ºC for 10 minutes (Napoleão et al., 2013). The intestines of each insect were manually separated and homogenized in appropriate buffer solutions (1 mL tris buffer (0.1 M tris HCl, pH 8.0, 0.02 M $CaCl_2$ and 0.15 M NaCl), 1 mL acetate buffer (0.1 M sodium acetate, pH 5.5, 0.02 M $CaCl_2$ and 0.15 M NaCl) or 1 mL sodium phosphate buffer (0.02 M sodium phosphate, pH 7.0, 0.15 M NaCl). Intestinal samples were centrifuged at 9157 rpm for 15 minutes at 4ºC. Extracts were sampled and assayed for enzyme activity.

Total oxidant status (TOS) analysis in intestinal extract

Total oxidant levels (TOS) were measured in the intestinal tissues of *T. hirta* adults after a seven-day interaction with different lectin proteins *in vivo*. TOS assay kits (Rel Assay Diagnostics, Türkiye) were used, which operate on the principle of Fe^{+2} the conversion to Fe^{+3} by different oxygen species in an acidic environment. This reaction results in the color change due to the xylenol orange and Fe^{+3}, measured at 530 nm. Measurements were taken in a microplate spectrophotometer. The standard concentration was 10 µmol H_2O_2 equivalent/L with results expressed as µmol H_2O_2 equivalent/L (Erdem et al., 2021).

Enzyme experiments

Protease enzyme activity

The protease activity of the gut extract in Tris buffer was assayed following the method of Napoleão et al. (2013), with azocasein as the substrate. The assay mixture consisted of 50 µL of gut extract, 300 µL of 0.1 M sodium phosphate (pH 7.5) containing 50 µL of 0.6% (w/v) azocasein and 100 µL of 0.1% (w/v) Triton X-100. The mixture was incubated at 37ºC for 3 hours, after which the reaction was stopped by the addition of 200 µL of 10% (v/v) trichloroacetic acid. The sample was then incubated at 4ºC for 30 minutes, followed by centrifugation at 9157 rpm for 10 minutes. The absorbance of the supernatant was measured at 366 nm using a spectrophotometer (Napoleão et al., 2013).

Trypsin-like enzyme activity

Enzyme activity in the intestinal samples in Tris buffer was measured in a 96-well microplate using BApNA as the substrate (Napoleão et al., 2013). Intestinal homogenate (30 µL), BApNA (15 µL), Tris buffer (55 µL) were mixed in a microplate (100 µL in total). This mixture was incubated at 37ºC for 30 minutes, and enzyme activity was measured at 405 nm using a microplate reader.

Acid and alkaline phosphase activity

In accordance with the methodology proposed by Napoleão et al. (2013), 50 µL of intestinal extract in sodium phosphate buffer was combined with 450 µL of either 0.05 M sodium acetate buffer (pH 4.0) for an acid phosphatase assay, or 0.05 M Tris-HCl buffer (pH 8.0) for an alkaline phosphatase assay. To each mixture, 500 µL of 12.5 mM p-nitrophenyl phosphate, prepared separately in the respective buffers, was added. The mixture was incubated at 37ºC for 15 minutes in a water bath. To complete the reaction, 100 µL of 0.5 M sodium hydroxide was added to the mixture. Intestinal samples were centrifuged at 6105 rpm for 5 minutes, and the absorbance of the resulting supernatant was measured at 410 nm using a spectrophotometer (Thermo Scientific Multiskan Go, Type: 1510, Finland).

Endoglucanase and exoglucanase activity

To determine endoglucanase enzyme activity, 100 µl of intestinal extract in acetate buffer was incubated with a 1% (w/v) carboxymethylcellulose solution sodium acetate buffer (pH 5.5) containing 400 µl of 0.15 M NaCl at 50ºC for 10 minutes. For exoglucanase activity, 1% (w/v) sodium hydroxide solution was used. After the incubation period, 500 µL of DNS was added to terminate the reaction. The mixture was heated at 100ºC for six minutes and subsequently cooled on ice for 15 minutes. Subsequently, the absorbance was measured at 540 nm using a spectrophotometer (Napoleão et al., 2013).

Betaglucosidase enzyme activity

Intestinal homogenate (50 µL) in acetate buffer and 400 µl of *p*-nitrophenyl-b-D-glucopyranoside were incubated in an eppendorf tube at 50ºC for 10 minutes. To stop the reaction, 500 µL of 10% sodium bicarbonate was added to the tubes. 300 µL of mixture was added to each well for measurements, and absorbance was read 410 nm using a spectrophotometer (Napoleão et al., 2013).

Alpha amylase enzyme activity

Enzyme activity was determined following the method of Napoleão et al. (2013). A mixture of 100 µL of intestine in acetate buffer and 400 µL of starch solution were incubated at 50ºC for 10 minutes. The reaction was stopped by adding 500 µL of DNS solution. The mixture was heated at 100ºC water for 6 minutes in a water bath and then cooled on ice for 15 minutes. Afterward, 300 µL of the mixture was added to each well in the microplate in triplicate. Absorbance measurements were taken at 540 nm using a spectrophotometer (Napoleão et al., 2013).

Statistical analysis

All experiments were performed in three biological replicates. Standard error values and statistical analysis for mortality rates and survival rates were calculated using the one-way Anova and Tukey's tests in SPSS. One-way Anova and Dunnett's post-hoc tests were used to calculate total oxidant status and enzyme activity experiments. Values of $p < 0.05$ were considered statistically significant. Enzyme unit was defined as the change of 0.001 in absorbance in 1 min for 1 mL of enzyme solution.

Results and Discussion

Mortality and survival rates

The toxic effects of plant lectins on insects suggest they are effective control proteins that can be used as biopesticides (Mantzoukas et al., 2020). Lectins targeting coleopterans, flies, butterflies, hemipterans, termites, bees and neuropterans decrease the number of egg laying, act as feeding deterrents, inhibit or stimulate enzyme activities in the digestive system, and cause mortality, highlighting their potential of lectins in combating pests (Powell, 2001; Carlini & Grossi-De-Sá, 2002; Jaber et al., 2010; Napoleão et al., 2013; Mishra et al., 2019).

The aim of this study was to evaluate the insecticidal activity of plant lectins against *T. hirta* and to provide preliminary data for the use of lectins as biopesticides. Developing effective toxicokinetic and toxicodynamic models needs comprehensive understanding of the processes that can cause mortality of individuals when exposed to toxic chemicals (Sowa et al., 2024). Mortality studies on insects provide important insights into the nature of the vital question whether the physiological changes at the individual level influence the limited time/lifetime patterns of cohort mortality (Carey & Liedo, 1999). Therefore, in this study, over a 7-day study period, daily and cumulative mortality rates were calculated, with daily survival rates illustrated in Figure 1 and cumulative mortality at 7 days detailed in Table 2.

Table 2. Post-experimental mortality rates of *Tropinota (Epicometis) hirta* adults fed with various plant lectins at varying concentrations

Experimental groups	Mean Mortality rate (%)±S. error	p values
NC	0.0 [a]	
TV200	47.8±7.8 [ab]	,172
TV250	51.1±10.6 [ab]	,113
TV300	60.6±16.4 [b]	,029
PA200	52.2±6.1 [ab]	,097
PA250	61.1±5.6 [b]	,027
PA300	79.4±2.4 [b]	,001
PV200	65.0±12.3 [b]	,015
PV250	57.8±13.1 [b]	,044
PV300	67.2±17.2 [b]	,010
WF200	51.1±10.6 [ab]	,113
WF250	66.7±17.4 [b]	,011
WF300	77.8±12.1 [b]	,002
PC	100.0±0.0 [b]	,000

NC: Negative control, PC: Positive control. $p < 0.05$ values are significant for one-way ANOVA and Tukey's tests. a,b : Different letters in the same column indicate differences between groups.

Our findings indicate that there was no mortality in the negative control (NC) group, while all insects in the positive control (PC) group died. In the lectin-fed experimental groups, mortality occurred in relation to the dose applied, with higher lectin doses leading to proportionally higher mortality rates ($p<0.05$) (Table 2). Additionally, an independent experimental group was established where the insects were starved and given no food. In this experimental group, no mortality was observed by day 7; the first mortality occurred on day 12. These results demonstrate that the insect mortality observed in our study was due to the toxic effects of the lectins rather than starvation alone. The highest dose of *T. vulgaris* lectin used increased insect mortality by 60.6% compared to the control group (Table 2). In previous studies, *T. vulgaris* lectin added in a 2% solution showed a 100% lethal effect on *Ostrinia nubilalis* (Hübner, 1796) (Lepidoptera: Crambidae) larvae. Additionally, *P. americana* lectin resulted in 25% mortality of *Diabrotica undecimpunctata* howardi Barber, 1947 (Coleoptera: Chrysomelidae) larvae (Czapla & Lang, 1990). In our study, this lectin was found to be the most lethal lectin, causing 79.4% mortality in adults at the highest dose tested (Table 2). Adult mortality

rates for PV lectin ranged from 57.8% to 67.2% (Table 2). For *C. maculatus* larvae treated with *P. vulgaris* lectin, a mortality rate of 40% was reported (De Sá et al., 2014). In a study conducted with *Trogoderma granarium* Everts, 1898 (Coleoptera: Dermestidae) larvae, *P. vulgaris* caused 40% and 86.7% mortality at the PV25 and PV100 dose respectively (Mantzoukas et al., 2020). Wheat germ agglutinin (WGA) has been reported as the lectin with the highest entomotoxic property against *C. maculatus* with insect mortality increasing proportionally with dose (Murdock et al., 1990). Additionally, *Myracrodruon urundeuva* Allemão (Sapindales: Anacardiaceae) extract has been shown to cause significant insect mortality in *Sitophilus zeamais* Motchulsky, 1855 (Coleoptera: Curculionidae) adults at the highest doses (100-150mg/g). Lectins not only cause food rejection, but also release the toxic substances that can be lethal to insects. High doses of extracts and lectins cause deformation in the digestive and absorption activities of target organism (Napoleão et al., 2013). Studies indicate that plant-derived pesticides, when applied at appropriate doses, exhibit a high lethal potential against insects at both adult and larval stages.

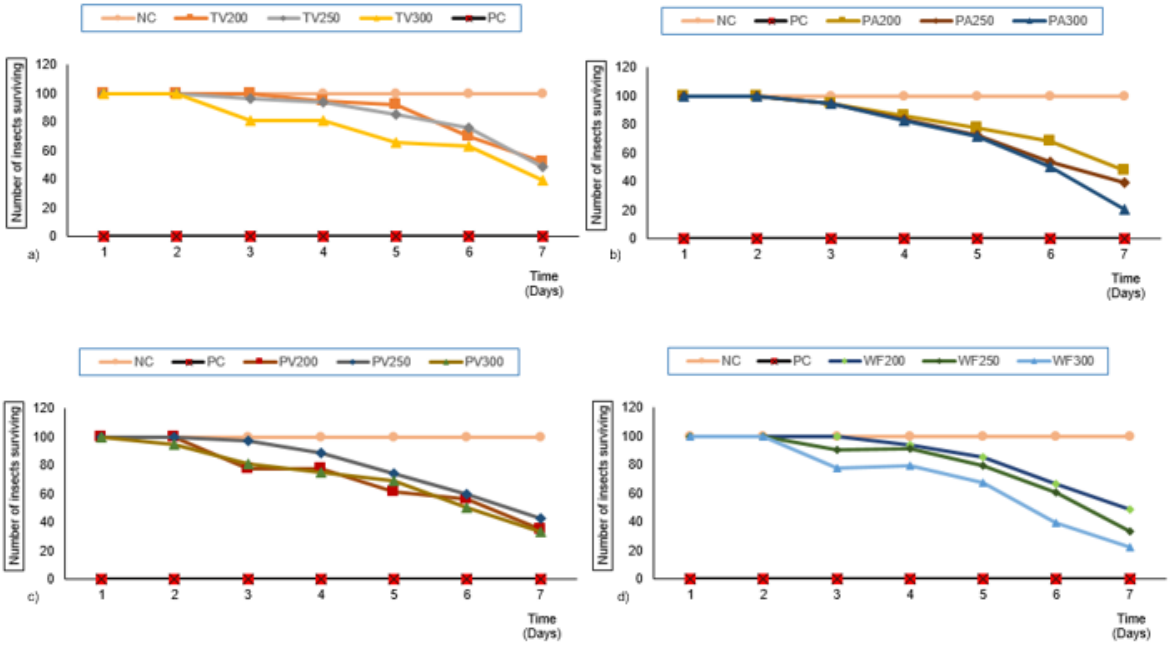

Figure 1. The mean number of insects surviving observed over to the incubation days for each lectin type: a) *Triticum vulgaris* lectin, b) *Phytolacca americana* lectin, c) *Phaseolus vulgaris* lectin, d) *Wisteria floribunda* lectin. Each lectin dose, along with the negative control (NC) and positive control (PC) groups, is represented in the graph.

Insect mortality was recorded daily. After 7 days of feeding, the first lethal effect was noted on day 2 for the PV300 dose and on day 3 for the other lectins (Figure 1). The PV300 dose caused 5.6% mortality of the insects on day 2, with mortality rates continuing to rise each day. On day 3, the WF300, PA300, and TV300 dose killed 22.8%, 5.6%, and 19.4% of the insects, respectively Although the first insect death was caused by the PV300 dose, the PA300 dose produced the highest statistically significant lethal effect by the end of day 7 (Figure 1). It has been reported that *P. vulgaris* increased aphid mortality within 24 hours of the experiment's start (Sprawka, 2008). In another study, *P. vulgaris* was observed to cause the earliest insect mortality among the lectins tested at the lowest dose. With a higher dose, this lectin was reported to kill the majority of insects in 2 days (Habibi et al., 1993). The effectiveness of lectins in insect mortality may be linked to their ability to spread early in the digestive tract. Indeed, the results of this study align with this hypothesis.

Total oxidant status (TOS) analysis

Pesticides are known to cause significant oxidative stress across a wide variety of animal species, including insects (Chakrabarti et al., 2015). In our study, as expected, the negative control group had the lowest oxidant levels, while the positive insecticide-treated group had higher TOS levels ($p<0.05$) (Tables 3). The dose of TV300 lectin used in this study induced oxidative stress in insects by increasing oxidant levels.

Table 3. Mean TOS values determined after 7 days of feeding with growth media containing lectins and pesticides

Lectin dose (mg/g)	Mean H_2O_2 concentration±. error (mmol/L)	P values
NC	0.82±0.5	
TV200	6.10±1.6	,181
TV250	6.70±1.7	,107
TV300	7.51±2.9*	,049
PA200	4.80±0.2	,475
PA250	4.72±0.6	,630
PA300	5.49±1.7	,296
PV200	5.47±1.4	,300
PV250	6.51±2.2	,127
PV300	6.09±1.5	,183
WF200	4.94±1.0	,436
WF250	5.13±0.4	,383
WF300	4.92±1.4	,440
PC	5.65±2.2	,261

* $p < 0.05$ values are significant for one-way ANOVA and Dunnett's post-hoc tests (*), NC: Negative control, PC: Positive control

Many phytophagous insects exhibit detoxification enzymes that reduce oxidative radicals generated by plant pesticides. Research has shown that both leaf extract and synthetic insecticides used in studies on aphids yield similar lethal effects, resulting in peroxide and malondialdehyde accumulation. Elevated levels of oxidative damage have been linked to increased insect mortality (Quandahor et al., 2022). Furthermore, *M. urundeuva* lectin has been found to causes more intense peroxidase staining in the midgut epithelium of termites compared to control groups (Lima et al., 2016). Another study demonstrated that the lectin form *Polygonum persicaria* L. (Caryophyllales: Polygonaceae) (PPA) induced physiological disorders in *Sitophilus oryzae* leading to digestive system deformation and oxidative stress (Khoobdel et al., 2022). Literature findings also suggest that a varied diet can trigger oxidative stress by increasing oxidative radicals in insects (Krishnan & Sehnal, 2006). The findings in our study show that changes in insect feeding and exposure to lectins may contribute to heightened oxidative stress.

Enzyme Activity Results

It is a well-known fact that many of the natural compounds used in the control of insect pests are known to affect digestive enzymes (Senthil-Nathan et al., 2006). One of those compounds are the lectin glycoproteins having globular protein subunits which contain one or more carbohydrate-binding sites. As a result of their molecular structure, when they are consumed frequent exposure begins in the digestive tract, because they are relatively stable against heat denaturation and proteolytic digestion (Muramoto, 2017). Considering the economic and environmental advantages compared to the traditional chemical insecticides, it is clear that new control methods are needed such as digestive enzyme inhibitors (Mehrabadi et al., 2012).Therefore, in this study the effects of different plant lectins on the digestive enzymes were analyzed.

Protease enzyme activity

The protease activity determined in the course of this study is illustrated in Figure 2a. Our results show that the dose with the highest effectiveness in reducing protease enzyme activity compared to the control group was TV200 (90.1%) ($p<0.05$) (Figure 2a). In the PC group, activity decreased by 77.3%. TV200 and TV300 doses were more effective at reducing enzyme activity than the synthetic insecticide. Some of the lectin doses used in the study showed inhibitory effects on protease enzyme activity.

The binding of plant lectins to different regions of the digestive system of insects can inhibit digestive enzymes (Macedo et al., 2002; Camaroti et al., 2018; Lima et al., 2018). Amino acids, released by the breakdown of proteins by proteases, play vital roles in insect growth, development and energy production (Gholamzadeh Chitgar et al., 2013). Studies have shown that the protease activity in *S. zeamais* adults exposed to *M. urundeuva* lectin (MuLL) decreased by 69.7% compared to the control, with lectin proving effective in reducing the activity of enzymes involved in insect protein metabolism (Napoleão et al., 2013). Lectins used in our study showed dose-dependent effects on protease activity, reducing it by 70.7% to 90.1% (Figure 2a). Additionally, the protease-inhibiting effects of *P. vulgaris* lectin were also observed in *C. maculatus* larvae (De Sá et al., 2014), and similiar inhibitory effects were comfirmed in our study (Figure 2a). Two different lectins from *M. urundeuva*, MuBL and MuLL,), have been reported to reduce protease activity in insects by 40.4% and 27%, respectively (Lima et al., 2018). Additionally, *Schinus terebinthifolius* Raddi (Sapindales: Anacardiaceae) leaf lectin (SteLL), when applied to *S. zeamais* adults, significantly reduces protease activity due to its interaction with the peritrophic membrane, which suppresses nutrient absorption (Camaroti et al., 2018). In contrast, protease activity is stimulated in insects exposed to *Opuntia ficus - indica* L. (Caryophyllales: Cactaceae) lectin, potentially leading to unbalanced hydrolysis of proteins during digestion (Souza et al., 2018). A reduction in protease activity can hinder the conversion of dietary proteins into amino acids, thus impairing the insect's ability to perform essential activities. Consequently, targeting digestive enzymes in insects could be valuable strategy for pest control.

Trypsin-like enzyme activity

Trypsin-like enzymes play an essential role in physiological processes in insects, including molting, diapause, tissue regeneration, reproduction and development. Control methods targeting the trypsin enzyme have been promise in pest management (Lazarević & Janković-Tomanić, 2015). As shown in Figure 2b, most doses tested in the trypsin-like enzyme activity experiment were highly effective in reducing the enzyme activity. Compared with the negative control, the most effective dose in reducing the activity was PA300 (81.13 %) ($p<0.05$) (Figure 2b). In this study, lectin applications resılted in strong inhibitory effects on trypsin-like activity than chemical pesticides, pronounced disruptions.

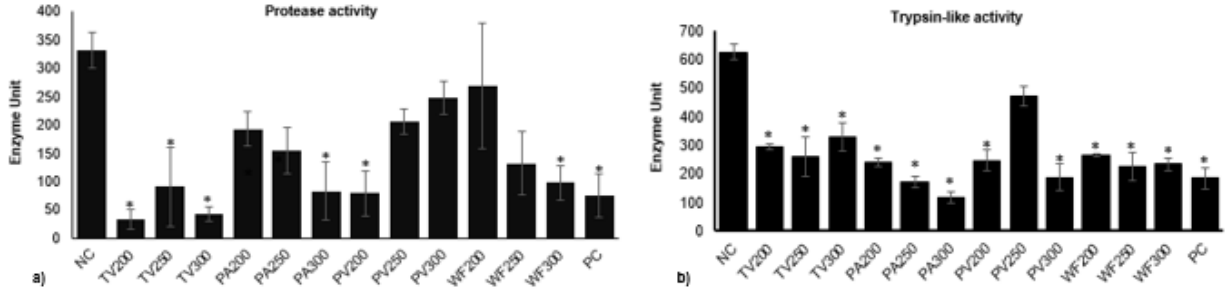

Figure 2. a) Protease and b) trypsin-like enzyme activities after 7 days of feeding with growth media containing various lectins and insecticide. $p < 0.05$ values are significant for one-way ANOVA and Dunnett's post-hoc tests (*).

The binding of lectin to the peritrophic membrane, which is composed of protein, proteoglycan and chitin, may disrupt the porous structure of this membrane, consequently affecting the enzyme regulatory

system (Martinez et al., 2012). Studies have shown that the activity of adult *Sitophilus zeamais* (Motschulsky, 1855) (Coleoptera: Curculionidae) treated with MuLL decreased by 55% (Napoleão et al., 2013). Furthermore, *Annona coriacea* Mart. (Magnoliales: Annonaceae) lectin reduced trypsin activity in *Anagasta kuehniella* (Zeller, 1879) (Lepidoptera: Pyralidae) larvae by 34%. The disruption of the peritrophic membrane caused by lectins is thought to result in the reduction in trypsin activity (Coelho et al., 2007; Camaroti et al., 2018). In our study, the lowest reduction in trypsin-like activity (47.5%) was observed at the TV300 dose, while the highest (81.1%) occurred at the PA300 dose (Figure 2b). Variations in these reduction ratios may arise from the specific binding sites and enzyme inhibitory properties of lectins. Lectins bind to sugars on glycosylated enzymes, while, in non-glycosylated enzymes, they attach to areas outside the substrate-binding site, thereby inhibiting enzyme activity. Lectin binding action hinders digestion and limits the metabolic activities essential for the insect (Macedo et al. 2007).

Acid phosphatase and alkaline phosphatase enzyme activities

Acid phosphatase (ACP) and alkaline phosphatase (ALP) are hydrolase enzymes that play crucial roles in insect physiological processes, including reproduction and growth, tissue cytolysis, the last stage of digestion, molting and maturation of reproductive cells (Zibaee & Bandani, 2010; Hamadah et al., 2016). ACP and ALP enzyme activities observed in this study are shown in Figure 3. According to our results, all doses, except PA200 and PV200, inhibited ACP activity by 43.63% to 82.51% compared to the negative control. In positive control, ACP activity decreased by 57.84% ($p<0.05$) (Figure 3a). Similarly, ALP activity was affected by some of the lectin doses applied, with significant reductions observed at the highest doses (PA300: 73.73%, TV300: 73.31%, WF300: 77.60%). In the positive control group, ALP activity decreased by 38.77%, but this result was not statistically significant (Figure 3b).

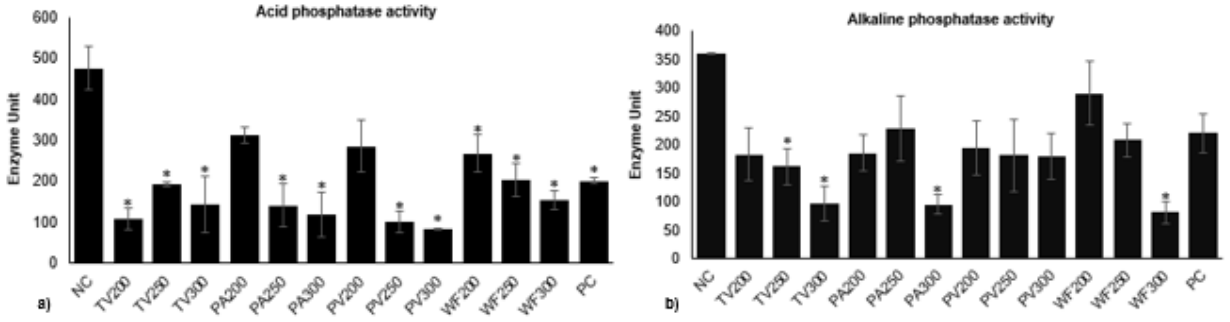

Figure 3. a) Acid and b) alkaline phosphatase enzyme activities after 7 days of feeding with growth media containing various lectins and insecticide. $p < 0.05$ values are significant for one-way ANOVA and Dunnett's post-hoc tests (*).

A significant decrease in acid and alkaline phosphatase activities was reported in *Pieris brassicae* (L., 1758) (Lepidoptera: Pieridae) larvae fed high concentrations of PPA. It was reported that the administered lectin was reported to affect enzymes involved in lipid digestion (Zibaee et al., 2014). Similarly, 2% dose of *Melia azedarach* L. (Rutales: Meliaceae) seed extract caused 69% and 71% decrease in acid and alkaline phosphatase activity in the insect, respectively. Changes in ALP and ACP activities adversely affect the intestinal physiology of insects (Senthil-Nathan, 2006). Kaur et al. (2006) reported that *Arisaema helleborifolium* Schott (Alismatales: Araceae) lectin used against *Bactrocera cucurbitae* (Coquillett, 1899) (Diptera: Tephritidae) decreased acid and alkaline phosphatase activities, findings that were similarly noted by Sprawka et al. (2011). The inhibitory effect of lectin on phosphatase activities was observed in aphids treated with *P. vulgaris* lectin. Our study's results align with these findings, indicating that most of the lectins we used have an inhibitory effect on phosphatase enzyme activities. This is because plant-derived substances can inhibit these enzymes, limiting nutrient utilization, and the inability to convert substances essential to insect life leads to mortality (Senthil-Nathan, 2006).

Endoglucanase and exoglucanase enzyme activities

When cellulose is broken down, large amounts of free glucose molecules are released, making cellulose a significant of carbohydrate source for phytophagous insects. The enzymes endoglucanase, exoglucanase and betaglucosidase are involved in the hydrolysis of cellulose (Douglas, 2012). The enzyme activities for endoglucanase, exoglucanase and β-glucosidase observed in this study are shown in Figure 4. Results indicate that in the positive group the endoglucanase activity decreased by 73.9%. This effect was not significant in exoglucanase activity. Among the TV lectin doses, TV300 significantly reduced endoglucanase and TV200 significantly reduced exoglucanase enzyme activity ($p<0.05$) (Figure 4a, 4b). Compared to the negative group, lectin uptake reduced insect endoglucanase and exoglucanase activity, depending on the dose and specificity of the lectin.

A study reported no change in endoglucanase activity observed in *Nasutitermes corniger* (Motschulsky, 1855) (Blattodea: Termitidae) insects following exposure to MuBL and MuLL although exoglucanase enzyme activity was stimulated (Lima et al., 2018). In contrast, *S. zeamais* treated with MuLL showed no change in exoglucanase activity, but endoglucanase activity was decreased (Napoleão et al., 2013). Administration of *Moringa oleifera* Lam. (Brassicales: Moringaceae) lectin (WSMoLc) to *N. corniger* workers and soldiers, beginning with a low dose, led to decreased exogluconase and endogluconase activities (Oliveira et al., 2023). The lectins used in this study also varied in their effects on the enzymes involved in cellulose digestion. Our results suggest that the specificity of each lectin arises from the variability of their mechanisms of action across different insect species. The enzymes involved in cellulose digestion work in a delicate balance; any alteration within this system disrupts the mechanism, thereby hindering digestion (Lima et al., 2018).

Betaglucosidase enzyme activity

The enzyme β-glucosidase is involved in the hydrolysis of cellobiose to glucose in insect digestion, aiding in the breakdown of cellulose and hemicellulose (Huber et al., 2021). The highest glucosidase activity has been observed in phytophagous insects (Douglas, 2012). In our study, β-glucosidase activity is shown in Figure 4c where most lectin doses significantly reduced this enzyme activity, ranging from 55.3% to 84.0% (Figure 4c).

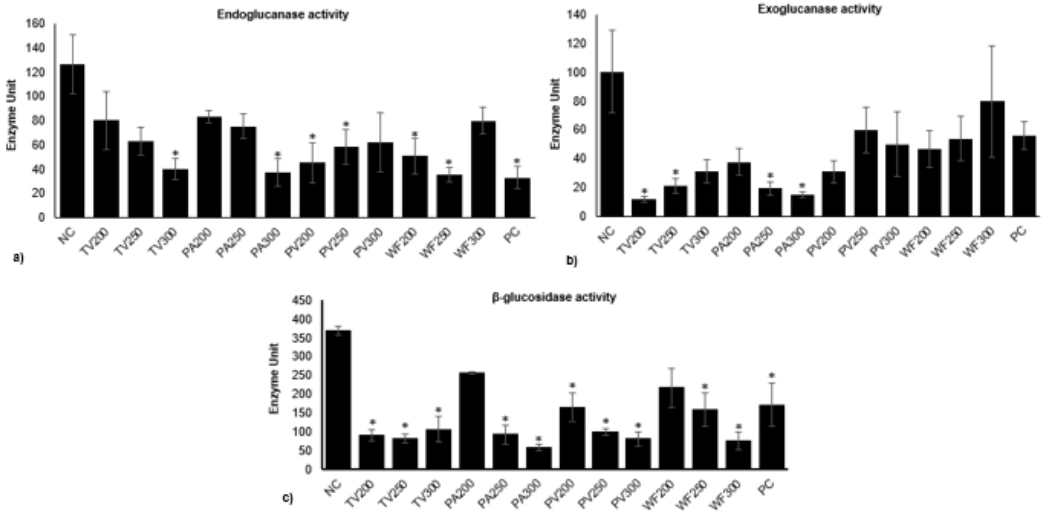

Figure 4. a) Endoglucanase, b) exoglucanase and c) β-glucosidase enzyme activities after 7 days of feeding with growth media containing various lectins and insecticide. $p < 0.05$ values are significant for one-way ANOVA and Dunnett's post-hoc tests (*).

The results demonstrated that significant lectin doses were more effective in reducing enzyme activity than the PC. Numerous studies have also reported that lectins affect β-glucosidase activity, with changes in this enzyme activity leading to impaired digestion. When *N. corniger* termites were fed *Microgramma vacciniifolia* (Langsd. & Fisch.) Copel. (Polypodiales: Polypodiaceae) rhizome (MvRL), β-glucosidase activity was inhibited (Lima et al., 2018). The similar results were observed in *S. zeamais* adults, where MvRL was reported to reduce β-glucosidase activity (Albuquerque et al., 2020). Additionally, Sprawka et al. (2011) reported that phytohemagglutinin (PHA) suppressed β-glucosidase enzyme at high doses, while at low doses, it stimulated enzyme activity. According to the researchers, this stimulation may suggest that the lectin used plays a role in detoxification.

Alpha-amylase enzyme activity

Amylase enzyme activity observed in this study is shown in Figure 5. Some of the doses used in the study significantly inhibited α-amylase activity, with rates ranging from 61.74% to 87.52% compared to the control ($p<0.05$).

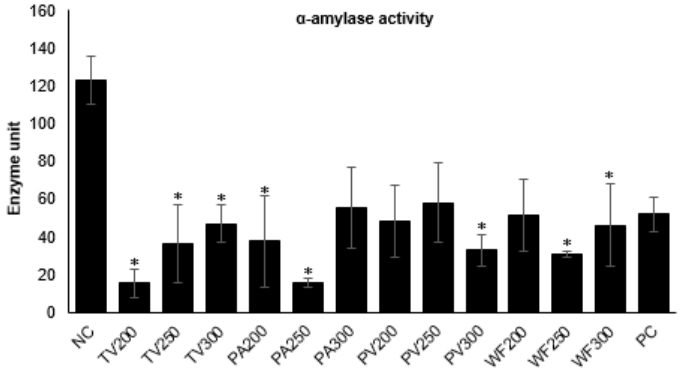

Figure 5. α-amylase enzyme activity after 7 days of feeding with growth media containing various lectins and insecticide. $p < 0.05$ values are significant for one-way ANOVA and Dunnett's post-hoc tests (*).

The α-amylase enzyme is particularly sensitive to grain-feeding insects (Terra & Ferreira, 1994). The result of our study supports the idea that lectins, which can bind to cells in the digestive system, cause cellular damage and reduce enzymes-secreting cells, effectively reducing enzyme activity (Zibaee et al., 2014). *Microgramma vacciniifolia* rhizome lectin (MvRL) inhibited α-amylase activity in *S. zeamais*, likely due to lectins interfering with carbohydrate digestion and resulting in inefficient biomass conversion (Albuquerque et al., 2020). *Moringa oleifera* lectin inhibited amylase activity in worker termites while stimulating it in soldier termites. Enzymatic differences, such as inhibition or stimulation have negative effects on carbohydrate digestion (Oliveira et al., 2023). *Polygonum persicaria* lectin reduced alpha-amylase activity in adult *S. oryzae* by 63% (alpha-amylase in PPA: 1.58, U/mg protein, control 4.28, U/mg protein) (Khoobdel et al., 2022). Similarly, lectins from *Glycine max* (L.) Merr. (Fabales: Fabaceae) and *P. vulgaris* plants caused a 58.8% to 66% reduction in amylase activity in *Earias insulana* (Boisduval, 1833) (Lepidoptera: Nolidae) larvae (Metayi et al., 2024). Conversely, lectin has been reported to stimulate amylase activity, increasing enzyme levels in *S. zeamais* adults fed with SteLL (Camaroti et al., 2018). These effects show that while lectin effects vary among insect species, they ultimately disrupt the normal function of digestive enzymes.

This study found that mortality rates in *T. hirta* increased significantly with higher lectin doses. Additionally, the inhibitory effect of plant lectins on digestive enzymes restricts developmental and reproductive activities of insects. The increase in oxidant levels, leading to oxidative stress, further supports these entomotoxic effects. By highlighting lectins as environmentally safe biopesticides, this study contributes to agricultural pest management. To maximize the practical application of these findings, field testing is recommended, with a particular focus on controlling insects that undergo their larval and pupal stages underground.

Acknowledgements

This article was produced from the PhD thesis of the corresponding author. This study was supported by Balıkesir University Scientific Research Projects Unit (BAP) with project number 2022/095. I would like to express my gratitude to TUBITAK (The Scientific and Technological Research Council of Türkiye) for the doctoral support scholarship titled 2211/C Priority Areas 'Biotechnological Pharmaceutical Technologies'. I would like to thank Prof. Dr. Yakup Şenyüz for his help in identifying the insects and Dr. Guido Sabatinelli for his help in identifying the aedeagus of male individuals.

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