Türk. entomol. derg., 2020, 44 (4): 437-447
DOI: http://dx.doi.org/10.16970/entoted.728613

ISSN 1010-6960
E-ISSN 2536-491X

Original article (Orijinal araştırma)

Chemical composition of *Vitex agnus-castus* L. (Verbenaceae) essential oil and its larvicidal effectiveness on *Thaumetopoea pityocampa* (Denis & Schiffermüller, 1775) (Lepidoptera: Notodontidae) larvae[1]

Vitex agnus-castus L. (Verbenaceae) uçucu yağının kimyasal bileşeni ve *Thaumetopoea pityocampa* (Denis & Schiffermüller, 1775) (Lepidoptera: Notodontidae) larvaları üzerindeki larvasidal etkinliği

Murat VARÇİN[2] **Memiş KESDEK[3*]**

Abstract

In this study, the chemical composition of the essential oil obtained from chaste tree (*Vitex agnus-castus* L.) (Verbenaceae) was analyzed by gas chromatography-mass spectrometry (GC-MS) and its larvicidal effectiveness at 250, 500 and 1000 µL/L, and 24, 48, 72 and 96 h against the five instars of the pine processionary moth, *Thaumetopoea pityocampa* (Denis & Schiffermüller, 1775) (Lepidoptera: Notodontidae) investigated. The study was conducted at Muğla Sıtkı Koçman University (Fethiye A.S.M.K. Vocational High School) under laboratory conditions (25±2°C, 65±5% RH and 14:10 h L:D photoperiod) in 2015-2017. Each larvicidal effectiveness test was repeated three times. β-caryophyllene (16.8%), germacrene D (14.7%), 1,8-cineole (14.5%) and ζ-gurjunene (11.8%) were identified as main compounds of the essential oil. Ninety-six h after treatment, the essential oil caused between 13.3 and 96.6% mortality. The highest mortality at 250, 500 and 1000 µL/L doses were 70.0% for L_1, 80.0% for L_3 and 96.6% for L_1, L_3 and L_4 instars after 96 h, respectively. It was concluded that the *V. agnus-castus* essential oil has potential as an alternative for control of *T. pityocampa* larvae.

Keywords: Chaste tree, essential oil, pine processionary moth, toxicity

Öz

Bu çalışmada, hayıt (*Vitex agnus-castus* L.) (Verbenaceae) bitkisinden elde edilen uçucu yağın gaz kromatografisi-kütle spektrometresi (GC-MS) yöntemiyle kimyasal bileşeni analiz edilmiştir ve onun 250, 500 ve 1000 µL/L dozlarında 24., 48., 72. ve 96. saatlerde uygulanmasıyla, çam keseböceğinin, *Thaumetopoea pityocampa* (Denis & Schiffermüller, 1775) (Lepidoptera: Notodontidae) beş farklı dönem larvalarına karşı larvasidal etkinliği araştırılmıştır. Bu çalışma, 2015-2017 yılları arasında Muğla Sıtkı Koçman Üniversitesi'nde (Fethiye A.S.M.K. Meslek Yüksekokulu'nda) laboratuvar şartlarında (25±2°C), %65±5 ON ve 14/10 A/K) yürütülmüştür. Her bir larvasidal etkinlilik testi üç kez tekrarlanmıştır. β-caryophyllene (%16,8), germacrene D (%14,7), 1,8-cineole (%14,5) ve ζ-gurjunene (%11,8) bu uçucu yağın ana bileşenleri olarak kaydedilmiştir. Çalışmanın 96. saatinde, uçucu yağ %13,30 ile %96,6 arasında ölümlere sebep olmuştur. 250, 500 ve 1000 µL/L dozlarındaki en yüksek ölüm oranları sırasıyla, L_1 dönemi için %70,0, L_3 dönemi için %80,0 ve L_2, L_3, L_4 dönemleri için ise %96,6 olarak kaydedilmiştir. Bütün bunlar göz önüne alındığında, *V. agnus-castus* uçucu yağının kontrollerle kıyaslandığında, *T. pityocampa* larvalarının kontrolü için potansiyel bir alternatif olduğu sonucuna varılabilir.

Anahtar sözcükler: Hayıt ağacı, uçucu yağ, çam keseböceği, toksisite

[1] This study was conducted as a Master thesis of first author.

[2] Muğla Sıtkı Koçman University, Environmental Sciences Basic Science, Graduate School of Natural and Applied Sciences, 48300, Muğla, Turkey
[3] Muğla Sıtkı Koçman University, Fethiye Ali Sıtkı Mefharet Koçman Vocational High School A.S.M.K. Vocational High School, Department of Environmental Protection Technologies, 48300, Fethiye, Muğla, Turkey
* Corresponding author (Sorumlu yazar) e-mail: memiskesdek@mu.edu.tr
Received (Alınış): 28.04.2020 Accepted (Kabul ediliş): 13.09.2020 Published Online (Çevrimiçi Yayın Tarihi): 05.10.2020

Introduction

Forests, which protect the natural balance of the world we live in, make a significant contribution to meeting the various needs of people, and are continually evolving with unique life forms and ecological environment. About 28% of Turkey is covered with forests and there are more than 50 tree species adapted to these areas. Among the trees, red pine (*Pinus brutia* Tenellus) (Pinaceae) is widespread in the Mediterranean, Aegean and Marmara Regions of Turkey and supports diverse wildlife. It is used economically in various ways such as firewood, timber with its high quality as wood, and has become the most essential plant in the forestry industry (Oktem, 1987). Red pine represents nearly half of the forest area in the Mediterranean Region, 40% in the Aegean Region and 10% in the Marmara Region of Turkey. In addition, this species is also found in some areas of the Western Black Sea Region. However, many factors affect the red pine population (Neyişci, 1987). Among these factors, the most important ones are illegal and uncontrolled cutting, land clearance, forest fires, unplanned and improper zoning permits, road construction and human migration. In addition to these, insects known as smokeless fire threaten the red pine trees. Among the insects, the pine processionary moth (*Thaumetopoea pityocampa* (Denis & Schiffermüller, 1775) (Lepidoptera: Notodontidae) is one of the most important insect pests of the red pine trees. *Thaumetopoea pityocampa* adults are not harmful, but the larvae feed on conifers such as *Pinus halepensis* Miller*, Pinus sylvestris* L.*, Pinus pinea* L.*, Pinus nigra* Arnold with *Cedrus libani* Richert (Pinaceae) as well as red pine (*P. brutia*) in Turkey and constantly cause economic losses. They cause between 22 and 65% growth reduction in the conifers and also decrease their diameter and height. As a result, weakened trees are invaded by other pests that cause more rapid transmission of diseases (Çanakçıoğlu, 1993; Kanat et al., 2002; Köse, 2007). When the population of *T. pityocampa* is intense, it can cause the death of the trees.

Different methods (mechanical, biological and chemical control) have been used to control *T. pityocampa* larvae in the past, but damage was not fully prevented and a permanent solution has not been found. Also, this pest remains as a significant problem in the coniferous forests of Turkey and the world. In chemical control, the excessive and random use of synthetic chemicals in agricultural and forest areas caused many adverse effects on human and environmental health (Breuer & Devkota, 1990). In particular, they negatively affect beneficial organisms that protect natural balance (Güncan & Durmuşoğlu, 2004). Given all these negativities and the continuing loss by *T. pityocampa* larvae, there is a need to develop alternative control methods against this pest that are eco-friendly and protect the natural balance.

In this regard, plant-derived compounds should be considered for the control of *T. pityocampa* larvae. Many plant species that contain phenolic compounds and essential oils with potent biological activity are used as critical natural bio-agents (Mokbel & Fumio, 2006; Batish et al., 2008). According to recent studies around the world, more than 200,000 plant species have been found to contain insecticidal compounds, but only 1% of them have considered useful. Among these compounds, pyrethrum, rotenone, nicotine and azadirachtin were reported to be the most important plant-derived compounds that have pesticidal effects as a result of studies on many insect pests (Isman, 2006). Essential oils are volatile and fragrant natural compounds extracted from various parts of plants (e.g., flowers, seeds, leaves, fruits and husks), which are usually in the form of liquid at room temperature, quickly crystallized, colorless or light yellow. Plant-derived compounds, especially essential oils, when used against pests the agricultural field, they can help to reduce insect resistance and environmental pollution, with no residual (permanent) effect on the environment. From this perspective, natural insecticides do not pose much threat to human and environmental health (Aksoy, 1982; Güncan & Durmuşoğlu, 2004; Isman, 2006).

The genus *Vitex* L. belongs to Verbenaceae family and has approximately 300 species in tropical regions of the world. Its origin is Mediterranean countries and there are two species (*Vitex agnus-castus* L. and *Vitex pseudo negundo* Haussknecht) grown in natural areas of Aegean and Mediterranean Regions in Turkey (Eryiğit et al., 2015; Tin et al., 2017). Among these species, *V. agnus-castus* was well known by ancient herbalists in the Middle Ages due to its medicinal properties, and also used as a contraceptive and

an antiaphrodisiac drug (Cambie & Brewis, 1997; De Kok, 2007). The species has a great importance because of its specific taste, aroma and medicinal use, and contains large amounts of essential oil. It has been found that a methanol extract of *V. agnus-castus* has an antibacterial effect (Karaman et al., 2008).

Essential oils, extracts and compounds obtained from various plants have been found to have insecticidal, ovicidal, larvicidal, repellent, attractant and antifeedant effects in many studies on harmful insects. Yelekçi (1981) reported that an extract of fruits of *Melia azedarach* L. (Meliaceae) had larvicidal effect on *T. pityocampa*. Regnault-Roger et al. (1993) reported that 22 different essential oils cause mortality at different rates in *Acanthoscelides obtectus* (Say, 1831) (Coleoptera: Bruchidae) and among them, *Origanum majorana* L. and *Thymus serpyllum* L. (Lamiaceae) oils were the most toxic. Shaaya et al. (1993) determined that different plant essential oils were insecticidal to some stored product pest adults. In another study, it was found that the essential oil of *Lavandula stoechas* L. (Lamiaceae) had very toxic effect on *Lasioderma serricorne* Fabricus, 1972 (Coleoptera: Anobiidae) and *Rhyzopertha dominica* (Fabricus, 1792) (Coleoptera: Bostrichidae) adults (Ebadollahi et al., 2010). Heydarzade & Moravvej (2012) determined that *Foeniculum vulgare* Miller (Apiaceae), *Teucrium polium* L. (Lamiaceae) and *Satureja hortensis* L. (Lamiaceae) oils were highly toxic to *Callosobruchus maculatus* (Coleoptera: Fabricius, 1775) (Coleoptera: Bruchidae). Kesdek et al. (2014) reported that *Achillea wilhelmsii* C. Koch (Asteraceae), *Nepeta meyeri* Benth., *S. hortensis*., *Origanum onites* L., *Origanum rotundifolium* Boiss. and *Tanacetum argyrophyllum* (C. Koch) (Lamiaceae) plant extracts had very toxic effects (between 3.33 and 100%) on four larvae instars at 0.25, 0.5 and 1 µl/dish doses. Germinara et al. (2017) determined that *Lavandula angustifolia* Miller (Lamiaceae) oil caused different rates mortality. In another study, five different commercial volatile oils (thyme, sage, poppy, garlic and rosemary) were found to be 70-100% effective against *T. pityocampa* larvae (Yiğit et al., 2019).

No long-term solution has been found against *T. pityocampa* in studies conducted so far. The main objective of the study was to investigate the chemical components of the essential oil obtained from chaste trees (*V. agnus-castus*) growing in natural areas and its larvicidal effectiveness under laboratory conditions against the five instars larvae of *T. pityocampa*, which is a very important coniferous tree pest.

Materials and Methods

Biological material

Thaumetopoea pityocampa larvae used in the study were collected from Esenköy (Dont), Fethiye, Muğla Province. Pouches (nests) on branches of red pine trees were cut with the help of gloves and pruning shears and placed into 30 x 45 x 30 cm-size cardboard boxes, under wrapped filter paper. The larvae were fed adding fresh leafy shoots cut from non-infected shoots. The larvae were removed from pouches using forceps and were placed in Petri dishes at 25±2ºC and 65±5% RH under laboratory conditions. This process was performed separately for each larval instar (between October 2016 and March 2017).

Plant Material

The chaste tree (*V. agnus-castus*) samples used in this study was collected at the flowering stage from Babadağ and Mendos Mountains, Fethiye, Muğla Province in June and September 2015, and June 2016. The aerial parts (flowers and leaves) of the plant were dried in shade before processing with a grinder. Dried plant herbarium samples have been stored in the Department of Environmental Protection Technologies, Fethiye A.S.M.K. Vocational High School, Muğla Sıtkı Koçman University.

Essential oil extraction

Extraction of *V. agnus-castus* essential oil was performed as described by Çakır et al. (2016). The aerial parts of the plant were dried in shaded environmental and milled in a grinder. Then this material (300 g) was subjected to hydro-distillation in a Clevenger-type apparatus for 4 h yielding 0.31% (v/w) of oil. The yields were determined for dry plant samples. The essential oil was stored in the refrigerator at +4ºC until used in experiments.

Gas chromatography-mass spectrometry analysis

The essential oil of *V. agnus-castus* chemical composition was determined by gas chromatography-mass spectrometry (GC-MS). A DB-1 united silica non-polar very thin column (30 m x 0.25 mm I.D., film denseness 0.25 µm) was used for the analysis. Helium was used as the carrier gas with flow rate of 1.4 mL/min. The ion source, injector and MS. transfer line heats were 200, 220 and 290ºC, respectively. Diluted samples (1/100 v/v in ethanol) of 1 µL were injected manually and in the splitless mode. The ionization energy of EI-MS measurements was attained at 70 eV. Mass. range was from m/z 50 to 650 amu. Scan time was 0.5 s with 0.1 s interscan delays. The oven temperature was maintained at 60ºC for 5 min, then raised to 240ºC with 4ºC/min increases and held at this temperature for 10 min. Identification of components of the essential oil was based on GC retention indices and computer matching with the libraries of Wiley, NIST-2008 and TRLIB, as well as by comparison of the segmentation models of the mass spectra (Küçükaydın et al., 2020).

Bioassay of the larvicidal effectiveness of the essential oil

In order to determine the larvicidal effectiveness of the *V. agnus-castus* essential oil against *T. pityocampa* larvae, the essential oil was dissolved at 1:2 in ethanol and the final concentration was prepared as 250, 500 and 1000 µL/L doses (Kesdek et al., 2013). Two layers of sterilized filter paper were placed in Petri dishes (according to 120 ml volume, 9 cm width x 1.5 cm depth). Ten larvae were placed in each Petri dish and 1 mL from the essential oil and positive control (25% diflubenzuron) solutions prepared as the stock were sprayed with a hand spray (Manual Potter Spray Tower-Burkard Scientific Limited, Uxbridge, UK) to contact the larvae. Ethanol in the essential oil was evaporated under ambient conditions for 1 min. To feed the larvae, non-contaminated fresh pine leaves were added in sufficient amounts (according to larvae instar, 5-10 g; by increasing each larval stage) and Parafilm was wrapped around the Petri dishes. Previously prepared essential oil solutions were mixed using vortex in 1 min. before application. Pure water plus ethanol was used as the negative control while the commercial chemical, Kormilin 25 WP (25% diflubenzuron) was used as a positive control. The experiments were conducted at 25±2ºC, 65±5% RH and 14:10 h L:D photoperiod, each test was repeated with three times for each larval stage and dose. Dead larvae were counted 24, 48, 72 and 96 h after treatment (Kesdek et al., 2014, 2020).

Data analysis

To determine whether there was a statistically significant difference between the results obtained, two-way analysis of variance was performed using SPSS (Statistical Package for Social Sciences 17.0). The differences between means were determined by Duncan's multiple range test. Median lethal dose (LD) LD_{50} and LD_{90} values after 24, 48, 72 and 96 h were calculated using the Finney method (Finney, 1971). To determine LD values at 95% confidence limits of each application EPA probit analysis program was used. Significant differences were tested at $P< 0.05$.

Results and Discussion

Chemical compositions of the essential oil

The essential oil of *V. agnus-castus* was analyzed by GC-MS and its chemical components are given in Table 1. In the essential oil, 32 components were determined which was represent 100% of the composition. The major components were β-caryophyllene (16.76%), germacrene D (14.71%), 1,8-cineole (14.52%), ζ-gurjunene (11.82%), α-pinene (9.88%), α-terpineol acetate (5.29%), Tau-cadinol (5.23%), terpinene-4-ol (2.76%), spathulenol (2.02%) and verticillol (1.91%).

Table 1. Chemical components (%) of the oil obtained from aerial parts of *Vitex agnus-castus*

Retention time (min)	Compound name	Essential oil (%)	Identification methods
4.430	3-methyl-2-heptanone	0.08	GC/MS/RI
4.482	α-thujene	0.23	GC/MS/RI
4.667	α-pinene	9.88	GC/MS/RI
6.572	β-pinene	1.11	GC/MS/RI
6.98	α-phellandrene	0.23	GC/MS/RI
7.435	α-terpinene	0.71	GC/MS/RI
7.734	α-camphene	0.26	GC/MS/RI
7.956	1,8-cineole	14.52	GC/MS/RI
8.711	β-ociimene	1.05	GC/MS/RI
9.050	γ-terpinene	1.35	GC/MS/RI
10.183	terpinolene	0.40	GC/MS/RI
10.717	linalool	0.20	GC/MS/RI
11.456	4-isopropyl-1-methyl-2 cyclohexene-1-ol	0.10	GC/MS/RI
13.232	ocimenol	0.36	GC/MS/RI
13.610	terpinene-4-ol	2.76	GC/MS/RI
14.148	α-terpineol	1.38	GC/MS/RI
19.511	ζ-elemene	0.79	GC/MS/RI
20.009	α-terpineol acetate	5.29	GC/MS/RI
21.982	α-gurjunene	1.79	GC/MS/RI
22.303	β-caryophyllene	16.76	GC/MS/RI
23.408	α-caryophyllene	0.15	GC/MS/RI
23.691	germacrene D	14.71	GC/MS/RI
24.855	ζ-gurjunene	11.82	GC/MS/RI
25.408	α-amorphene	0.44	GC/MS/RI
25.739	ζ-cadinene	0.41	GC/MS/RI
27.031	globulol	0.56	GC/MS/RI
27.347	spathulenol	2.02	GC/MS/RI
27.522	caryophyllene oxide	1.46	GC/MS/RI
28.125	ledol	1.27	GC/MS/RI
29.291	Tau-cadinol	5.23	GC/MS/RI
36.011	biformene	0.76	GC/MS/RI
37.777	verticillol	1.91	GC/MS/RI
	Total identified (%)	**100.00**	

[a] Calculated retention index to *n*-alkanes (C_8-C_{28}) on SGE-BPX5 capillary column. GC: co-injection with.standards; MS; previously identified based on computer coordination of the.mass spectra of peaks with Wiley 7N and TRLIB libraries and according to Adams, (2007); RI:.Identification based on comparing of repression index with Adams' data. t, trace (<0.1%).

Larvicidal effect of the essential oil

The application of three different doses (250, 500 and 1000 µL/L) of *V. agnus-castus* essential oil caused mortality at different rates in *T. pityocampa* larvae compared to the control. The mortality rates were between 13.3 and 96.6% for all larval stages 96 h after treatment. Depending on the application dose and time, the mortality increased. When the mortality of the essential oil was compared for 24, 48, 72 and 96 h

after treatment there were statistical differences between the treatments for each larval stage. The most effective dose for L_1, L_2, L_3, L_4 and L_5 instars was established to be 1000 µL/L. While the highest mortality rates were found between 70.0 and 96.6% for L_1 instar 96 h after treatment, the lowest mortality for L_2 instar (between 13.3 and 40.0%) (Table 2; $P<0.05$).

Twenty-four h after treatment, the highest larvicidal toxicity with1000 µL/L was 70.0% in L_1 instar. However, the lowest larvicidal toxicity was with 250 µL/L, 6.7% in L_2 instar. However, there was no mortality with 250 µL/L in L_5 instar. Similarly, 48 h after treatment, the highest mortality was with 1000 µL/L, 76.6% in L_1 instar. With the same treatment time, the lowest mortality was with 250 and 500 µL/L, 10.0 and 23.3% in L_2 and L_5 instars, respectively (Table 2; $P<0.05$). Seventy-two h after treatment with 250 and 500 µL/L the mortality was 13.3 to 90.0% in L_1, L_2, L_3, L_4 and L_5 instars. The highest mortality was with 1000 µL/Petri dish, 90.0% and 80.0% in L_1 and L_3 instars, respectively. However, the lowest mortality was with 250 and 500 µL/L, 13.3% and 16.6% in L_2 instar, respectively. In general, while most mortality was in L_1 instar 72 h after treatment, the lowest mortality was for L_2 instar. Ninety-six h after treatment, mortality was from 13.3 to 96.6% in L_1, L_2, L_3, L_4 and L_5 instars. However, differences between mortality were not significant 96 h after treatment period for 250, 500 and 1000 µL/L in L_1, L_3 and L_4. Overall, the most effective dose was detected to be 1000 µL/L (Table 2; $P < 0.05$). Kormilin 25 WP (25% Diflubenzuron) is one of the most widely used commercial insecticides for *T. pityocampa* larvae. In this study, 100% toxicity (except 250 µL/L dose, 83.3% for L_2) was obtained 96 h after treatment with all doses of Kormilin (250, 500 and 1000 µL/L) (Table 2; $P < 0.05$).

The LD values (LD_{50} and LD_{90}) of the study are summarized in Table 3. When LD values 96 h after treatment were compared the highest toxicity based on the LD_{50} and LD_{90} were in L_1 instar (0.53 and 1.25 µL/larva), respectively. However, the lowest toxicity based on the LD_{50} and LD_{90} 96 h after treatment were 2.57 and 22.09 µL/larva in L_5 instar, respectively. Similarly, LD_{50} 96 h after treatment were 0.62, 0.97, 1.03 and 2.57 µL/larva for L_2, L_3, L_4 and L_5 instars, respectively (Table 3). These results indicated that the larvicidal activity increased with increasing dose and exposure time. *Vitex agnus-caspus* essential oil gave potentially useful toxicity to the larvae of *T. pityocampa* (Tables 2 to 3).

Consequently, 96 h after treatment, when mortality of the five instars of *T. pityocampa* were compared, L_2 instar of *T. pityocampa* was the most resistant (due to mortalities between 13.3 and 40.0%), while L_1 instar was the most susceptible (in varying rates between 70.0 and 96.6%) (Table 2).

There were many studies relating to the chemical composition of the essential oil obtained from different parts of *V. agnus-castus*. Senatore et al. (1996) recorded 1.8 cineole (flowers 14.1%, leaves 15.6%), α-terpineol (flowers 6.4%, leaves 8.5%) and sabinene (flowers 6.3%, leaves 6.9%) as the main components in the essential oil of *V. agnus-castus*. Hamid et al. (2010) determined β-pinene (20.0%), viridiflorol (9.8%), α-pinene (9.1%), cis-ocimene (8.4%), 1.8 cineole (6.7%) and β-farnesene (5.4%) as dominant constituents in the *V. agnus-castus* essential oil in Nigeria. Eryiğit et al. (2015) found *trans*-caryophyllene (19.17%), sabinene (18.05%) and 1.8- cineole (16.13%) as the main compositions in the *V. agnus-castus* plant oil in Turkey. Similarly, in another study from Turkey, Tin et al. (2017) detected 1.8 cineole (8.24%), propenamide (6.07%), caryophyllene (5.56%), bicyclogermacrene (5.51%), sabinene (5.37%), maleimide (5.28%), *trans-β*-farnesene (4.45%) and α-pinene (3.98%) as the main components the *V. agnus-castus* essential oil. In the present study, the main compounds were β-caryophyllene (16.8%), germacrene D (14.7%) and 1.8-cineole (14.5%), respectively (Table 1). When the previous studies and our study are examined carefully, constituents of *V. agnus-castus* essential oil are similar to each other, but rates of components are different. These differences may be due to the fact that the composition of *V. agnus-castus* oil may depend on geographical conditions, such as soil and climatic conditions and environmental factors.

Table 2. Larvicidal effectiveness of *Vitex agnus-castus* essential oil against five instars of *Thaumetopoea pityocampa*

Essential Oil	Dose (µL/L)	Mortality (%)			
		Exposure Time (h)			
		24	48	72	96
		L_1 Instar Larvae			
Vitex agnus-castus	250	33.3±5.74 b	46.6±3.33 b	53.3±6.66 b	70.0±10.0 b
	500	50.0±26.4 c	63.3±8.81 c	70.0±11.5 c	76.6±14.5 b
	1000	70.0±17.4 d	76.6±12.0 d	90.0 ±5.77 d	96.6±3.33 c
Positive Control (Kormilin)	250	100±0.0 e	100±0.0 e	100±0.0 d	100±0.0 c
	500	100±0.0 e	100±0.0 e	100±0.0 d	100±0.0 c
	1000	100±0.0 e	100±0.0 e	100±0.0 d	100±0.0 c
Control (Steril Water+Etanol)	250	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a
	500	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a
	1000	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a
		L_2 Instar Larvae			
Vitex agnus-castus	250	6.7±3.33 ab	10.0±5.77 ab	13.3±3.33 a	13.3±3.33 a
	500	10.0±10.0 ab	10.0±10.0 ab	16.6±12.0 a	26.6±21.8 a
	1000	26.6±17.6 b	36.6±27.2 b	36.6±12.0 a	40.0±30.5 a
Positive Control (Kormilin)	250	70.0±5.77 c	76.6±3.33 c	80.0±5.77 b	83.3±8.81 b
	500	96.6±3.33 d	96.6±3.33 c	96.6±3.33 b	100±0.0 b
	1000	100±0.0 d	100±0.0 c	100±0.0 b	100±0.0 b
Control (Steril Water+Etanol)	250	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a
	500	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a
	1000	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a
		L_3 Instar Larvae			
Vitex agnus-castus	250	10.0±5.77 b	40.0±10.0 b	53.3±12.0 b	53.3±12.0 b
	500	33.3±3.33 c	50.0±10.0 bc	63.3±8.81 b	80.0±11.5 c
	1000	46.6±3.33 d	56.6±6.66 c	80.0±0.0 c	96.6±3.33 cd
Positive Control (Kormilin)	250	100±0.0 e	100±0.0 d	100±0.0 d	100±0.0 d
	500	100±0.0 e	100±0.0 d	100±0.0 d	100±0.0 d
	1000	100±0.0 e	100±0.0 d	100±0.0 d	100±0.0 d
Control (Steril Water+Etanol)	250	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a
	500	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a
	1000	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a
		L_4 Instar Larvae			
Vitex agnus-castus	250	16.6 ±3.33 b	36.6±3.33 b	53.3±12.0 b	53.3±12.0 b
	500	26.6±3.33 c	40.0±15.2 b	56.6±8.81 b	56.6±8.81 b
	1000	30.0±0.0 c	60.0±0.0 c	76.6±3.33 c	96.6±3.33 c
Positive Control (Kormilin)	250	100±0.0 d	100±0.0 d	100±0.0 d	100±0.0 d
	500	100±0.0 d	100±0.0 d	100±0.0 d	100±0.0 d
	1000	100±0.0 d	100±0.0 d	100±0.0 d	100±0.0 d
Control (Steril Water+Etanol)	250	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a
	500	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a
	1000	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a

Table 2. Continued

Essential Oil	Dose (µL/L)	Mortality (%)			
		Exposure Time (h)			
		24	48	72	96
		L_5 Instar Larvae			
Vitex agnus-castus	250	0.0±0.0 a	13.3±3.33 b	20.0±0.0 b	26.6±3.33 b
	500	11.1±6.08 b	23.3±3.33 c	33.3±3.33 c	50.0±5.77 c
	1000	20.0±5.77 c	26.6±3.33 c	33.3±3.33 c	50.0±5.77 c
Positive Control (Kormilin)	250	100±0.0 d	100±0.0 d	100±0.0 d	100±0.0 d
	500	100±0.0 d	100±0.0 d	100±0.0 d	100±0.0 d
	1000	100±0.0 d	100±0.0 d	100±0.0 d	100±0.0 d
Control (Steril Water+Etanol)	250	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a
	500	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a
	1000	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a

[a] Values followed by the letter within columns do not differ significantly at P ≤ 0.05 according to Duncan Multiple test;
[b] Mean±SE of three replicates, each set up with 10 larvae.

Table 3. LD_{50} and LD_{90} values (µL/L) of *Vitex agnus-castus* essential oil on the five instars of *Thaumetopoea pityocampa*

	Vitex agnus-castus				
	LD_{50} (Limits)	LD_{90} (Limits)	X^2	Slope±SE (Limits)	Probability
L_1 Instar Larvae	0.53 (0.01-0.83)	1.25 (0.72-2.20)	6.41	3.42±1.46 (0.56-6.29)	0.83
L_2 Instar Larvae	0.62 (0.10-1.03)	2.64 (1.70-6.63)	16.29	2.03±0.82 (0.74-4.15	0.66
L_3 Instar Larvae	0.97 (0.15-1.39)	2.43 (1.70-15.58)	11.58	3.23±0.84 (1.59-4.88)	0.52
L_4 Instar Larvae	1.03 (0.23-1.44)	3.91 (2.79-65.90)	17.38	2.22±0.75 (0.54-3.41)	0.50
L_5 Instar Larvae	2.57 (1.61-1.11)	22.09 (6.18-7.89)	2.59	1.37±0.70 (0.01-2.73)	0.29

[a] Lethal concentration causing 50% mortality 96 h after treatment;
[b] Lethal concentration causing 90% mortality 96 h after treatment;
[c] Chi square value.

There are some studies reported by a few researchers on the toxicity of essential oils to *T. pityocampa* larvae. Çetin et al. (2006) recorded that O*riganum onites* L. (Lamiaceae) and *Citrus aurantium* L. (Rutaceae) essential oils caused mortality between 72.5 and 97.5% in L_4 and L_5 instars of pine processionary moth, *Thaumetopoea wilkinsoni* Tams, 1926 (Lepidoptera: Notodontidae) at 0.1, 0.5 and 1% doses 24 h after treatment. In the present study, mortality rates were between 11.1 and 30.0% in L_4 and L_5 instars of *T. pityocampa* with *V. agnus-castus* oil at 250, 500 and 1000 µL/L doses 24 h after treatment. In another study, 24 h after treatment the essentials oil obtained from *Satureja hortensis* L., *Origanum onites* L., *O. rotundifolium* Boss. (Lamiaceae) and *Tanacetum argyrophyllum* (C. Koch) Tvzel. (Asteraceae) caused the highest mortality in L_2, L_3 and L_4 instars (100% at 20 µL/dish) (Kesdek et al., 2013). In the present study, 24 h after treatment with 1000 µL/L of *V. agnus-castus* essential oil, the highest mortality was 70.0, 46.6 and 30.0% in L_1, L_3 and L_4 instars (Table 2). When these studies are compared, it was concluded that they support each other. Germinara et al. (2017) found that *Lavandula angustifolia* Miller (Lamicaeae) oil caused different rates mortality (from 91.7 to 100%) at 0.449 mg/adult 24 and 48 h after exposure, respectively. In another study, the mortality with 10, 15 and 20 µL/Petri dish doses of *Achillea biebersteinii* Afan (Asteraceae) oil against L_2, L_3 and L_4 instars 48 h after treatment were 73.3, 73.3 and 83.3% in L_2 instar, 43.3, 50.0 and 73.3% in L_3 instar, 36.6, 43.3 and 53.0% in L_4 instar, respectively (Kesdek et al., 2020). Usanmaz Bozhüyük et al. (2018) found that the essential oils of *Seriphidium santonicum* (L.) Soják and *Artemisia absinthium* L. (Asteraceae) caused 6.66 to 100% mortality 48 h after application of three different

doses (10, 15 and 20 µL/dish) in five instars of *T. pityocampa*. In the present study, we determined that *V. agnus-castus* essential oil caused 36.6 to 76.6% mortality in three different doses (250, 500 and 1000 µL/L) in L_1, L_3 and L_4 instars (46.6, 63.6 and 76.6% for L_1 instar; 40.0, 50.0 and 56.6% in L_3 instar; 36.6, 40.0 and 60.0% in L_4 instar) 48 h after treatment, respectively. In addition, we found that *V. agnus-castus* oil gave the lowest mortality of 10.0% and the highest mortality with 76.6% 48 h after treatment at 250, 500 and 1000 µL/L against five instars of *T. pityocampa* larvae (Table 2). Kesdek et al. (2013) determined that *Origanum acutidens*, *O. onites*, *O. rotundifolium*, *S. hortensis*, *Satureja spicigera* (C. Koch) (Lamiaceae), *Thymus sipyleus* Boiss., *T. argyrophyllum* and *Achillea gypsicola* Hub.-Mor. (Asteraceae) plant essential oils caused the mortality from 73.3 to 100% 72 h after treatment with 10 and 20 µL/Petri dish in L_2, L_3, and L_4 instars of *T. pityocampa*. In the present study, the essential oil of *V. agnus-castus* gave mortality from 13.3 to 90.0% 72 h after treatment with 250, 500 and 1000 µL/L in L_2, L_3, and L_4 instars of *T. pityocampa.* These studies support each other. Kesdek et al. (2014) found that extracts of six different plant species [*Nepeta meyeri* Benth, *S. hortensis*, *O. onites*, *O. rotundifolium* (Lamiaceae), *Achillea santolinoides* (C. Koch) Lag. and *T. argyrophyllum* (Asteraceae)] had larvicidal effect between 3.33 and 100% in L_2, L_3 and L_4 instars of *T. pityocampa* 96 h after treatment. In another study, five different commercial volatile oils (thyme, sage, poppy, garlic and rosemary) were found to be 70-100% effective against *T. pityocampa* larvae (Yiğit et al., 2019). The results of this study showed that *V. agnus-castus* oil caused mortality from 13.3 to 96.6% 96 h after treatment with three different doses (250, 500 and 1000 µL/L) in the five instars of *T. pityocampa* (Table 2).

Considering the lethal dose (LD) 96 h after treatment with *V. agnus-castus* oil in larvae of *T. pityocampa*, LD_{50} values were 0.53, 0.62, 0.97, 1.03 and 2.57 µL/larva in L_1, L_2, L_3, L_4 and L_5 instars, respectively. However, the highest toxicity was with 0.53 µL/larva in L_1 instar, the least toxicity was with 2.57 µL/larva in L_5 instar. For LD_{50} and LD_{90} 96 h after treatment, the highest toxicity was 0.53 and 1.25 µL/larva in L_1 instar, respectively. However, at the same time, the lowest toxicity for LD_{50} and LD_{90} were 2.57 and 22.09 µL/larva in L_5 instar (Table 3).

In conclusion, many studies by different researchers show that chemicals used against diseases and pests in the forest and agricultural areas are very dangerous for human and environmental health and also ecological balance. Therefore, there is a need for alternative methods to protect the environment and human health and ecological balance. For these reasons, components derived from plants are potentially useful. In this study, larvicidal toxicity of *V. agnus-castus* essential oil to pine processionary moth (*T. pityocampa*) larvae was investigated. According to the results, we determined that as the application doses and time of *V. agnus-castus* oil increased, larvicidal toxicities also increased. When the toxicities were compared according to the application dose, the highest toxicity (96.6%) was with the highest dose of the essential oil (1000 µL/L) in L_1, L_3 and L_4 instars. The lowest toxicity was in L_2 instar larvae. In the light of these data, when the larvicidal toxicity of the *V. agnus-castus* essential oil to the five larval instars of *T. pityocampa* is carefully examined, it is concluded that this essential oil could be an alternative for the control of *T. pityocampa*, which is one of the most damaging pests for coniferous trees. Finally, we consider that this study will provide a useful basis for further studies.

Acknowledgements

Authors thank Mohamed Said Omar (Ondokuz Mayıs University), translator and native English speaker, for his contribution in revising the English of this article; and also, Yahya Nas (Muğla Sıtkı Koçman University) for his technical assistance and contributions to the study.

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