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) üçucu yağının kimyasal bileşeni ve Thaumetopoea pityocampa (Denis & Schiffermüller, 1775) (Lepidoptera: Notodontidae) larvaları üzerindeki larvasidal etkinliği

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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 processary 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. 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 L₁, 80.0% for L₂ and 96.6% for L₃ and L₄ 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 processorary moth, toxicity

Öz

Bu çalışmada, hayat (Vitex agnus-castus L.) (Verbenaceae) bitkisinden elde edilen üçucu yağın gaz kromatografi-kütüle spektrometresi (GC-MS) yöntemiyle kimyasal bileşeni analiz edilmiştir ve onun 250, 500 ve 1000 μL/L dozlardında 24., 48., 72. ve 96. saatlerde uygulanmasıyla, çam kesebeciğinin, 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 etkinlik testi üç kez tekrarlanmıştır. β-caryophyllene (%16,8), germacrene D (%14,7), 1,8-cineole (%14,5) ve ζ-gurjunene (%11,8) bu üçucu yağın ana bileşenleri olarak kaydedilmiştir. Çalışmanın 96. saatinde, üçucu yağ %13,30 ile %96,6 arasında olumlere sebep olmuştur. 250, 500 ve 1000 μL/L dozlardaki en yüksek ölüm oranları sırasıyla, L₁ dönemi için %70,0, L₃ dönemi için %80,0 ve L₂, L₃, L₄ dönemleri için ise %96,6 olarak kaydedilmiştir. Bütün bunlar göz önüne alınırdığında, V. agnus-castus üçucu yağının kontrolörle kıyaslandığında, T. pityocampa larvalarının kontrolü için potansiyel bir alternatif olduğu sonucuna varılabilir.

Anahtar sözcükler: Hayat ağacı, üçucu yağ, çam kesebeciğe, toksite

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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 Richeri (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. Yelekiç (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* (Fabricius, 1792) (Coleoptera: Bostrichidae) adults (Ebadollahi et al., 2010). Haydarzade & Moravjev (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). Kesk et al. (2014) reported that *Achillea wilhelmsii* Koch (Asteraceae), *Nepeuta 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. Germiña 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±2°C 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 Sitki Koçman University.

**Essential oil extraction**

Extraction of *V. agnus-castus* essential oil was performed as described by Çakir 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 +4°C until used in experiments.
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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 290°C, 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 60°C for 5 min, then raised to 240°C with 4°C/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 (Küçükaydin 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±2°C, 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) LD₅₀ and LD₉₀ 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 verticillil (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-ocimene                            | 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               | ocimeno                             | 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               | germacrone 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               | verticilloïl                        | 1.91              | GC/MS/RI               |
| **Total identified (%)** |                                     | **100.00**         |                        |

*Calculated retention index to n-alkanes (C₈-C₂₈) 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 L₁, L₂, L₃, L₄ and L₅ instars was established to be 1000 µL/L. While the highest mortality rates were found between 70.0 and 96.6% for L₁ instar 96 h after treatment, the lowest mortality for L₂ 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 L₁ instar. However, the lowest larvicidal toxicity was with 250 µL/L in L₂ instar. Similarly, 48 h after treatment, the highest mortality was with 1000 µL/L, 76.6% in L₁ instar. With the same treatment time, the lowest mortality was with 250 and 500 µL/L, 10.0 and 23.3% in L₂ and L₅ 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 L₁, L₂, L₃, L₄ and L₅ instars. The highest mortality was with 1000 µL/Petri dish, 90.0% and 80.0% in L₁ and L₃ instars, respectively. However, the lowest mortality was with 250 and 500 µL/L, 13.3% and 16.6% in L₂ instar, respectively. In general, while most mortality was in L₁; instar 72 h after treatment, the lowest mortality was for L₂ instar. Ninety-six h after treatment, mortality was from 13.3 to 96.6% in L₁, L₂, L₃, L₄ and L₅ instars. However, differences between mortality were not significant 96 h after treatment period for 250, 500 and 1000 µL/L in L₁, L₃ and L₄. 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 L₂) 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 (LD₉₀ and LD₉₀) of the study are summarized in Table 3. When LD values 96 h after treatment were compared the highest toxicity based on the LD₉₀ and LD₉₀ were in L₁ instar (0.53 and 1.25 µL/larva), respectively. However, the lowest toxicity based on the LD₉₀ and LD₉₀ 96 h after treatment were 2.57 and 22.09 µL/larva in L₅ instar, respectively. Similarly, LD₉₀ 96 h after treatment were 0.62, 0.97, 1.03 and 2.57 µL/larva for L₂, L₃, L₄ and L₅ instars, respectively (Table 3). These results indicated that the larvicidal activity increased with increasing dose and exposure time. Vitex agnus-caspos 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, L₂ instar of T. pityocampa was the most resistant (due to mortalities between 13.3 and 40.0%), while L₁ 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-caspos. Senatore et al. (1996) recorded 1.8 cineole (flowers 14.1%, leaves 15.6%), α-terpinene (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-caspos. 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-caspos essential oil in Nigeria. Erığııt 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-caspos 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-caspos 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-caspos 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-caspos 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 |
| L₁ 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  |
| L₂ 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  |
| L₃ 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  |
| L₄ 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  |
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 Origanum onites L. (Lamiaceae) and Citrus aurantium L. (Rutaceae) essential oils caused mortality between 72.5 and 97.5% in L4 and L5 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 L4 and L5 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) Tzvel. (Asteraceae) caused the highest mortality in L2, L3 and L4 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 L1, L3 and L4 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 (Lamiaceae) 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 L2, L3 and L4 instars 48 h after treatment were 73.3, 73.3 and 83.3% in L2 instar, 43.3, 50.0 and 73.3% in L3 instar, 36.6, 43.3 and 53.0% in L4 instar, respectively (Kesdek et al., 2020). Usanmaz Bozhüyük et al. (2018) found that the essential oils of Seriphidium santonicum (L.) Sojak and Artemisia absinthium L. (Asteraceae) caused 6.66 to 100% mortality 48 h after application of three different

### Table 2. Continued

| Essential Oil       | Dose (µL/L) | 24       | 48       | 72       | 96       |
|---------------------|-------------|----------|----------|----------|----------|
| 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 |
| Vitex agnus-castus  | 250         | 100±0.0 d | 100±0.0 d | 100±0.0 d | 100±0.0 d |
| Positive Control    | 500         | 100±0.0 d | 100±0.0 d | 100±0.0 d | 100±0.0 d |
| (Kormilin)          | 1000        | 100±0.0 d | 100±0.0 d | 100±0.0 d | 100±0.0 d |
| Control             | 250         | 0.0±0.0 a | 0.0±0.0 a | 0.0±0.0 a | 0.0±0.0 a |
| (Steril Water+Ethanol) | 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.

d2

d2

### Table 3. LD50 and LD90 values (µL/L) of Vitex agnus-castus essential oil on the five instars of Thaumetopoea pityocampa

| Vitex agnus-castus | LD50 (Limits) | LD90 (Limits) | X2 | Slope±SE (Limits) | Probability |
|--------------------|---------------|---------------|----|-------------------|-------------|
| L1 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 |
| L2 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 |
| L3 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 |
| L4 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 |
| L5 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.
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 L1, L3 and L4 instars (46.6, 63.6 and 76.6% for L1 instar; 40.0, 50.0 and 56.6% in L3 instar; 36.6, 40.0 and 60.0% in L4 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 sipsileus* 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 L2, L3, and L4 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 L2, L3, and L4 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 L2, L3 and L4 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*, LD50 values were 0.53, 0.62, 0.97, 1.03 and 2.57 µL/larva in L1, L2, L3, L4 and L5 instars, respectively. However, the highest toxicity was with 0.53 µL/larva in L1 instar, the least toxicity was with 2.57 µL/larva in L5 instar. For LD50 and LD90 96 h after treatment, the highest toxicity was 0.53 and 1.25 µL/larva in L1 instar, respectively. However, at the same time, the lowest toxicity for LD50 and LD90 were 2.57 and 22.09 µL/larva in L5 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 L1, L3 and L4 instars. The lowest toxicity was in L2 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.

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