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Larvicidal activity of *Nerium oleander* L. leaf extract against Pine Processionary Moth (*Thaumetopoea wilkinsoni* Tams.)

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Abstract
Pine processionary moth (*Thaumetopoea wilkinsoni* Tams.) is the most important defoliator in the Mediterrenean Basin. Larval defoliation of this pest results in decreasing the annual diameter increment of host trees. Defoliated trees can become highly prone to the incidence of secondary insects. Plant natural products have received a great attention from scientific world as they serve as a rich source for novel compounds possessing insecticidal properties. In this study, the crude aqueous extracts of *Nerium oleander* leaves were reported for larvicidal activity against the *Thaumetopoea wilkinsoni*. Third and fourth instar mortality from eight concentrations (62.5, 125, 250, 500, 1000, 2000, 4000 and 8000 ppm) of plant extract were recorded and mortality was observed in 0, 24, 48, 72 and 96 hours. Aqueous leaf extract exhibited highest larvicidal activity with a LD50 value of 322.50 ppm and 190.00 ppm after 24 and 48 hours respectively. Further detailed investigations are needed to clarify this activity against pest species and also the active ingredient(s) should be identified. This study is the first to report on the larvicidal activity of leaf extracts of *N. oleander* against *T. pityocampa.*

Keywords: *Nerium oleander*, leaf extracts, *Thaumetopoea wilkinsoni*, larvicidal activity.

Introduction
Pine processionary moth (PPM) (*Thaumetopoea wilkinsoni* Tams.) is a major defoliator of the forest land in Turkey. Natural forests and plantation areas are subject to periodic outbreaks. In mature forests, trees are barely killed, but significant losses bring about in volume and radial growths [1]. It is well known that defoliation may increase tree stress and their susceptibility to secondary pests such as bark beetles and pine weevils, particularly in young trees [2]. Fourth- and fifth-instar larvae can consume all the needles of host trees, so that trees have to be scorched. The average decrease in annual diameter increment for *Pinus* species in damaged areas, compared with healthy trees, can reach about 22% [3]. PPM increased its distribution 6 times between 1938 and 2009 in Turkey [4]. *T. wilkinsoni* has urticating hairs which can cause extreme itching, skin irritations and damage to eyes and the respiratory system to humans and domestic animals. For PPM management, mechanical-physical, chemical, bio-technical and biological methods are widely used. Up to now, although several methods have been used in attempting to control this pest, the problem still remains largely unsolved in Turkey.
Nerium oleander L. is evergreen sclerophyllous tree or shrub growing to 6 m in height depending on local conditions. It is distributed in very different geographical places in Mediterranean Basin. *Nerium oleander* is a typical drought-tolerant species and belongs to the Apocynaceae family. The leaves are 6 to 30 cm long, very narrowly elliptic, acute, narrowed at base, shortly petiolate, with a coriaceus dark-green blade. Flowers are about 5 cm in diameter and different colours vary rose to red. The fruit consists of a narrow follicle of 10 to 18 cm long and seeds are about 4 mm. This plant can be found from sea level to 800 m [5]. The poisonous leaves contain the cardiac glycosides, alkaloids and carbohydrates [6-8]. The different parts of *N. oleander* possesses strong larvicidal, insecticidal and anti-feedant [9-12], antibacterial [13, 14], antimicrobial [15], anti-inflammatory, antinociceptive [16] and antitumor [17] activity.
Though chemical insecticides play a crucial role in fighting against pests, these also show a negative impact in areas of beneficial and non-target organisms. In view of an increasing interest in developing plant-derived compounds as larvicides as an alternative to synthetic insecticide, this study was undertaken to determine the larvicidal potential of the extracts from the *N. oleander* against to PPM.

Materials and Methods

Study Materials and Preparation of Aqueous Extracts

Third and fourth instar larvae of PPM were collected from infested *P. brutia* trees in the forest part of Babadağ, Denizli, Turkey. *Nerium oleander* leaves used in the experiments were collected from the Buldan, Denizli. PPM larvae and seeds have been transferred to Pamukkale University, Department of Biology, Chemical Ecology Laboratory and then, they were stored under laboratory conditions until experiments were performed. All tests were carried out at 20ºC (±2), 60% (±5) relative humidity and 14/10 h light/dark photoperiod in laboratory conditions. The fresh *N. oleander* leaves were washed with distilled water and were dried for a week in shady room. Air dried *N. oleander* leaves were grounded using a mill and blender. The 20 g shade-dried and powdered samples were extracted with 200 ml water in a shaking water bath (Memmert, WNB 14) at 50 ºC for 6 hours. The mixture was extracted after being heated in a shaking water bath at 55 ºC. Extract was filtered with (Whatman No: 1) filter paper. After filtration of the extract, the solvents were evaporated in a Rotary Evaporator (IKA, RV 10D, Germany) at 50 ºC and the water in the extract was frozen in the Freeze-Drying machine (Labconco, Free Zone, USA) and then drawn out.

Larvicidal Assays

The larval mortality bioassay was carried out according to the test method described by Cetin *et al*. (2007) with minor modifications. For the assays, 2 g of crude extract was dissolved in 100 ml distilled water containing 0.3% Tween 80 to make 1% stock solutions. A series of concentrations (62.5–8000 ppm) were then prepared in 250-ml capacity glass jars. Field collected larvae nests were opened under fume hood and then larvae were transfered into the petri dish with 20 larvae (late third to early fourth instar) per dish. The larvae were fed on fresh leaves. Each dose of extract (1 ml per larvae) was topically applied to larvae. Control experiments consisted of 250-ml of distilled water containing 0.3% Tween 80 only for each treatment. Three replicates were carried out for each dilution and for the control. Mortality was recorded after an exposure of 24, 48, 72 and 96 hours. Mortality test values were determined by observing individuals that were neither moving when prodded with a fine brush nor feeding in the test area [18].

Data analysis

Percent mortality data were subjected to ANOVA and the means were compared to determine significant differences using Duncan's multiple range tests ($P < 0.05$). Probit analysis of dose-mortality data were conducted to estimate the LD50 and LD90 values and associated 95% confidence limits for each treatment [19].

Results and Discussion

The larvicidal effect of aqueous extracts of leaves of *N. oleander* were investigated against the *T. wilkinsoni*. Table 1 shows mean percent larval mortality to varying concentrations and exposure times of aqueous extract, and Table 2 provides the results of LD50 and LD90 values of *Nerium oleander* leaf extract at 24, 48, 72 and 96 hours against to *T. wilkinsoni*. All the extracts tested (from 62.5 to 8000 ppm) demonstrated significant larvicidal activity on *T. wilkinsoni*, with LD50 values ranging from 28.96 to 165.02 ppm. Among the eight doses tested, the dosage of 4000 and 8000 ppm caused 100% larval mortality after 24 h. Larval mortality was not observed throughout the study in the control group. After 24 h, the leaf extract of *N. oleander* exhibited significant larvicidal activity; the LD50 and LD90 values are 322.5 ppm (Limits; 165.02-551.86 ppm) and 2573.49 ppm (Limits; 1309.17-9664.74 ppm) respectively. Larval mortality was observed throughout the study with all doses in different levels. As expected, larval mortality rates increased with increasing doses of the extract used. Some researchers have determined the larvicidal activity of various plant extracts against PPM [20, 21]. Unal and Akkuzu [22] demostrated that *Azadirachta indica* A. Juss extracts showed high larvicidal activity against *T. wilkinsoni*. Up to now, there were no reports on the larvicidal effect of *N. oleander* leaf extracts against *T. wilkinsoni*. Additionally, there are some reports show that the extracts of *N. oleander* can be used as a control agent against some pest and vector species such as mosquitoes [23-25]. PPM is one of the most harmful pest in the Mediterranean Basin and it causes serious morphological and physiological losses in *Pinus* forests. After heavily damage by PPM, resistance ability of the damaged tree to drought, pathogens and herbivores may be reduced. Until now, although different methods were used to control this pest, the problem is still unsolved in many parts of the Mediterranean Basin where the pest occurs. In this point, *N. oleander* extracts have significant larvicidal activity against PPM larvae and it might be used for potential larvicides.

Table 1: Concentrations LD50 and LD90 lethal larvae of *Thaumetopoea wilkinsoni* against *Nerium oleander* leaf extracts at 24, 48, 72 and 96 hours of exposure.

Time (h)	LD50 (ppm)	Limits (ppm)	LD90 (ppm)	Limits (ppm)
24	322.50	165.02-551.86	2573.49	1309.17-9664.74
48	190.00	93.16-314.10	1154.67	638.77-3737.74
72	94.24	28.96-181.70	737.15	392.71-2934.22
96	78.17	38.29-109.70	169.67	119.08-572.31

Table 2: Toxicity of aqueous leaf extract from *Nerium oleander* on *Thaumetopoea wilkinsoni* (Mortality (%) ± SE)

Time (hour)	Test doses (ppm)								
	Control	62.5	125	250	500	1000	2000	4000	8000
24	0.0±0.0 $a^{\alpha}A^{\beta}$	23.3±6.7 aB	33.3±3.3 aB	36.7±3.3 aB	60.0±1.0 aC	70.0±5.7 aC	76.7±.6.7 aC	100.0±0.0 aD	100.0±0.0 aD
48	0.0±0.0 aA	33.3±3.3 aB	36.7±3.3 aB	43.3±3.3 aB	66.7±3.3 bC	90.0±5.8 bD	96.7±3.3 bE	100.0±0.0 aE	100.0±0.0 aE
72	0.0±0.0 aA	43.3±8.8 aB	63.3±3.3 bBC	73.4±3.3 bC	76.7±3.3 cC	90.0±5.8 bD	100.0±0.0 bE	100.0±0.0 aE	100.0±0.0 aE
96	0.0±0.0 aA	43.3±8.8 aB	73.3±3.3 cC	96.6±3.3 cD	100.0±0.0 dE	100.0±0.0 bE	100.0±0.0 bE	100.0±0.0 aE	100.0±0.0 aE

$^{\alpha}$: Means within a column followed by the same lowercase letter are not significantly different (P< 0.05).
$^{\beta}$: Means within a row followed by the same uppercase letter are not significantly different (P< 0.05).

Conclusion

The results of this study indicate that *N. oleander* can be used as a commercial insecticide against *T. wilkinsoni*. However, future research needs to be performed to test its effects against non-target organisms in the environment. Recently, many studies have been conducted to find plant-derived compounds as a natural and eco-friendly larvicides. In this regard, it is necessary to conduct more detailed research to find active components of this plant that are more effective larvicidal activities and less side effect. Using of plant-derived larvicides instead of chemical larvicides reduces the damage would have occurred in the environment. Additionally, field studies are needed to understand the effectiveness of current extracts in field circumstances.

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