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ORIGINAL RESEARCH ARTICLE

Total phenolic and flavonoid contents of oakmoss lichen *Evernia prunastri* extracts and their insecticidal activities against larvae of two vector mosquitoes, *Aedes aegypti* and *Culex pipiens*

Ozge Tufan-Cetin[1] · Aysegul Cengiz[2] · Zeynep Nur Gultekin[2] · Sevval Kahraman[2] · Burak Polat[2] · Samed Koc[2,3] · Huseyin Cetin[2]

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Abstract

Aedes aegypti L. and *Culex pipiens* L. are among the world's most common and medically important mosquitoes (Diptera: Culicidae). Each year, millions of people are infected with pathogens transmitted by these mosquitoes and thousands of people die from mosquito-borne diseases. Although chemical insecticides are widely used for vector control, most of them are highly toxic to the environment and human health. The harmful effects of chemical pesticides have led scientists to search for the less toxic, natural, and eco-friendly products. In this regard, lichens, a symbiotic association, are one of the organisms studied in the search for new insecticidal active substances. In this research, the toxicity of acetone, ethanol and methanol extracts from oakmoss lichen, *Evernia prunastri* (L.) Ach., a widely available epiphytic macro-lichen species, was investigated in of *Ae. aegypti* and *Cx. pipiens*. The extracts showed varying levels of toxic effect on the two mosquito species depending on the concentration and it is clear that methanol and ethanol extracts of *E. prunastri* were more toxic than the acetone extract according to the LC_{50} values. The LC_{50} values of methanol, ethanol and acetone extracts for *Cx. pipiens* older instar (third–fourth) larvae at 48 h were 104.3, 153.9 and 490.0 ppm, for young instars (first–second), they were 203.4, 326.6 and 606.9 ppm, respectively. The LC_{50} values of methanol, ethanol and acetone extracts for 48 h on *Ae. aegypti* young instar larvae were 390.9, 440.0 and 2934.8 ppm, and 537.1, 515.3 and 1837.1 ppm for older instar larvae, respectively. The highest contents of total flavonoids and phenolic compounds were determined in acetone extract of *E. prunastri*. The phenolic compounds in extracts range from 7.48 to 10.28 mg gallic acid/g and the flavonoid content of extracts range from 37.68 to 91.76 mg catechin/g. This is the first study on the toxic effect of *E. prunastri* extracts on mosquitoes and indicates that lichen extracts can be a source for developing environmentally friendly larvicides.

Keywords *Aedes aegypti* · *Culex pipiens* · Extract · Larvicidal · Mosquito · Oakmoss Lichen

Introduction

Mosquitoes transmit many diseases caused by microorganisms (viruses, bacteria and protozoans). Each year, more than 700,000 people die from mosquito-borne diseases, such as dengue, malaria, West Nile fever, yellow fever and Zika virus. Although many important mosquito (Diptera: Culicidae) species from the genera *Aedes* and *Culex* are well known worldwide, *Aedes aegypti* L. and *Culex pipiens* L. are among the world's most common vector mosquito species (Silver 2008). Because mosquitoes develop in stagnant water habitats, they can survive even in small puddles around human settlement areas. Therefore, water resources used by people for drinking and domestic purposes are

✉ Huseyin Cetin
hcetin@akdeniz.edu.tr

[1] Department of Environmental Protection Technology, Vocational School of Technical Sciences, Akdeniz University, Antalya, Turkey

[2] Department of Biology, Faculty of Science, Akdeniz University, Antalya, Turkey

[3] Laboratory Animals Application and Research Centre, Akdeniz University, Antalya, Turkey

suitable habitats for mosquito breeding (Silver 2008; Suman et al. 2018). Drought in many parts of the world as a result of climate change has begun to pose problems for people's access to safe and usable water. Drought may eliminate stagnant water, but it can also cause flowing water to stagnate. Due to global warming lakes become shallower and river flows decrease as well as the release of wastewater from industrial, agricultural, and human activities into these habitats, are being also created new breeding areas for mosquitoes (Jeppesen et al. 2015; Ma et al. 2022).

Although adult control practices are preferred worldwide, larval control should be prioritized for successful control of mosquitoes. Most of the larvicides recommended by the World Health Organization (WHO) for the control of mosquitoes are of chemical origin, and many of them (e.g. Chlorpyrifos, Fenthion, Pirimiphos-methyl and Temephos) are banned in European countries and Türkiye. The toxic effects of these chemicals on the environment, non-target species and human health, as well as emerging resistance to pesticides, increase the importance of biological products and alternative methods (Kumar and Sahgal 2022). Biologically based microbial insecticides recommended by WHO as alternatives to chemical larvicides, such as *Bacillus thuringiensis israelensis* (Bti), *B. sphaericus* (Bs- recently *Lysinibacillus sphaericus*) or spinosad, are widely used worldwide for larval mosquito control (WHO, 2013). However, researchers show that mosquitoes may have also developed resistance to these biological products in many parts of the world and that some bacterial based larvicides are not effective enough in highly polluted breeding sites (Hongyu et al. 2004; Dawson et al. 2019; Su et al. 2019).

We need new, environmentally friendly and effective larvicidal products. Many studies are carried out today to fulfill this important need. Biological activity studies of extracts and secondary metabolites obtained from natural materials of some organisms such as flowering plants, macro fungus and lichens on mosquito larvae are increasing day by day (Pavela et al. 2019; Ramzi et al. 2022). Especially the results of biological activity studies of symbiotic organisms such as lichens containing a wide variety of unique substances are remarkable (Cetin et al. 2008; Gandhi et al. 2019). Lichens are organisms formed by the association of fungi with green algae and/or cyanobacteria. In addition to this living partnership, it has been discovered that yeasts, which are members of the Basidiomycota, can also be found in lichen content (Spribille et al. 2016). The free-living species that make up the lichen association do not have as wide ecological tolerance as lichens (Ranković and Kosanić 2021). This tolerance is due to the fact that lichens produce over one thousand secondary metabolites that protect them from various adverse environmental effects (Goga et al. 2018). Lichens have proven a wide range of biological effects, including analgesic, antibiotic, antipyretic, antioxidant, anticancer and antiviral (Do et al. 2022).

Evernia prunastri (L.) Ach. is a widespread Holarctic lichen (Fig. 1) with distribution in North America, Europe, Northern Africa, Türkiye, Russia, Kazakhstan, and Japan (Nimis and Martellos 2023). This lichen is known as perfume lichen and oakmoss lichen. Studies are available about investigating the antimicrobial, antioxidant, cytotoxic and anti-acetylcholinesterase effects of various solvent (ethanol, methanol and acetone) extracts and secondary components (e.g. atranorin, evernic acid and usnic acid) of *E. prunastri* (Aoussar et al. 2020; Salah et al. 2022). However, no study has been identified to determine its toxic effect on insects. Therefore, the aim of this research was to investigate whether acetone, ethanol, and methanol extracts from *E. prunastri*, can be used as an alternative larvicide source for the control of mosquitoes.

Materials and methods

Lichen samples and preparation of extracts

Evernia prunastri samples collected from oak trees (*Quercus* sp.) on Gulluk Mountain in Antalya, Türkiye in October 2022 were identified from current keys according to their morphological and biochemical characteristics and some specimens are preserved as number 309 in the personal lichen fungarium (herbarium) of Dr. Ozge Tufan-Cetin at Akdeniz University (Fig. 1).

After identification, the lichen samples were weighed at 100 g each, cut into small pieces of about 5–10 mm length and treated with 500 ml of solvent (acetone, ethanol or methanol) in dark conditions at room temperature for three days, and filtered through filter paper at the end of this period. Filtrates were removed from solvents with the rotary evaporator at 30 °C and stored in refrigerator at 4 °C for more processing.

The concentrations tested were based on preliminary studies. Stock solutions of each solvent extract (1000 ppm) was prepared by dissolving 1 g lichen extract in 1 l of dechlorinated tap water. Test concentrations: 100, 250, 500, 750 and 1000 ppm were prepared by serial dilution of stock solution.

Mosquitoes

Eggs of two mosquito species (*Aedes aegypti* Bora Bora Lab strain and *Culex pipiens* Dosemealti field strain collected from pool in May 2022) were obtained from the Vector Ecology and Control Laboratory at Akdeniz University and used in the toxicity studies. After the eggs were hatched, the

Fig. 1 *Evernia prunastri* in its natural habitat on a tree trunk

larvae were fed with Tetramin fish food. The young (first–second) and the older (third–fourth) instar larvae were used for toxicity test.

Larvicidal bioassays

The larval toxicity assays were conducted at 24 ± 2 °C temperature, 40 ± 10% relative humidity with a photoperiod of 12:12 h light and dark conditions according to the method of Koc et al. (2021) Ten young or older instar larvae of *Ae. aegypti* or *Cx. pipiens* were released in a hard plastic clear cup (250 ml) containing 100 ml of the test solution, using a Pasteur pipette. Larval mortality was recorded after 24 and 48 h exposure and the mean percent mortalities were obtained from the three replicates. Dead larvae were identified when they could not move after probing with a needle in the cervical or siphon region. Dechlorinated tap water was used as a negative control group.

Estimation of total phenolic and flavonoid contents

In order to determine the total phenolic content, 900 µl of distilled water, 5 ml of 0.1 N Folin-Ciocalteau solution and finally 4 ml of 7.5% sodium bicarbonate solution were added to 100 µl sample extract. The mixture was kept at room temperature in a dark environment for 90 min and the absorbance was measured at 765 nm wavelength on a Shimadzu UV-Vis 160 A model spectrophotometer. The total phenolic content of the samples was calculated as mg gallic acid equivalent/g (Spanos and Wrolstad 1990).

Total flavonoid content was determined as colorimetrically according to the method proposed by Zhishen et al. (1999). To 1 ml of sample extract, 4 ml of distilled water was added followed by 300 µl of 5% sodium nitrite solution. After 6 min, 600 µl of 10% aluminum chloride solution was added and the mixture was kept for 6 min. Then 2 ml of 4% sodium hydroxide solution and 2.1 ml of distilled water were added and the absorbance value of the mixture was read in a spectrophotometer at 510 nm wavelength and the results were expressed as mg catechin equivalent/g.

Statistical analysis

All data were statistically analyzed with the Statistical Package of Social Sciences (SPSS) 20.0 for Windows. A probit analysis was used to determine lethal concentration values (LC_{50} and LC_{90}) and upper (max) and lower (min) confidence limits, chi-square (χ^2) and p values. The statistical significance of the percent mortality averages was determined using one-way ANOVA analysis, and then Duncan's

Table 1 Total phenolic (mg gallic acid/g extract) and flavonoid (mg catechin/g extract) contents

Extract	Total phenolic content (mg gallic acid/g) ± SE	Total flavonoid content (mg catechin/g) ± SE
Acetone	10.28 ± 0.25	91.76 ± 1.49
Ethanol	7.48 ± 1.18	80.53 ± 2.32
Methanol	8.67 ± 1.02	37.68 ± 1.09

multiple range test ($p < 0.05$) was applied to determine which averages differed from each other. Two-way ANOVA analysis was used to determine whether or not an effect of variables (mosquito species, solvents, larval instars, exposure times, and concentrations) on the percent mortality averages.

Results

The total phenolic and flavonoid contents of the *E. prunastri* extracts are presented in Table 1. According to the ratios of total flavonoids and phenolic compounds, the highest values were determined in acetone extract of *E. prunastri*. Total flavonoid ratios in acetone, ethanol and methanol extracts were 91.76, 80.53 and 37.68 mg catechin/g and total phenolic ratios were 10.28, 7.48, and 8.67 mg gallic acid/g, respectively.

Calculated lethal concentration values by Probit analysis of *E. prunastri* extracts on two mosquito species larvae are given in Table 2. Furthermore, the larvicidal effect of various concentrations of *E. prunastri* on both *Cx. pipiens* (Fig. 2) and *Ae. aegypti* (Fig. 3) was graphed. The distribution of the mean percentages of death according to 5 different extract concentrations, 3 types of solvents, two exposure times (24–48 h) and two larval instars (young and older) were used in our study are shown on the graphs. In addition, statistical differences between the mean mortality percentages of the applied concentrations are shown in the graph separately according to each exposure time of extract.

According to our results, it was determined that solvent type, exposure times and tested concentrations were significantly effective on mortality rates of both mosquito species. However, there was no effect of *Ae. aegypti* larval instar type (larval age) on mortality ($F = 3.170$, $p = 0.077$), while larval age had a significant effect on the mortality of *Cx. pipiens* mosquitoes ($F = 35.094$, $p = 0.001$). Moreover, according to Probit analysis results, when young and older instars of *Cx. pipiens* mosquito larvae were compared in terms of LC_{50} and LC_{90} values, it was found that older instars were more sensitive to all tested extracts than young instars. The LC_{50} values of methanol, ethanol and acetone extracts for *Cx. pipiens* older instar larvae at 48 h were 104.3, 153.9 and 490.0 ppm, for young instars, they were 203.4, 326.6 and 606.9 ppm, respectively (Table 2). According to the

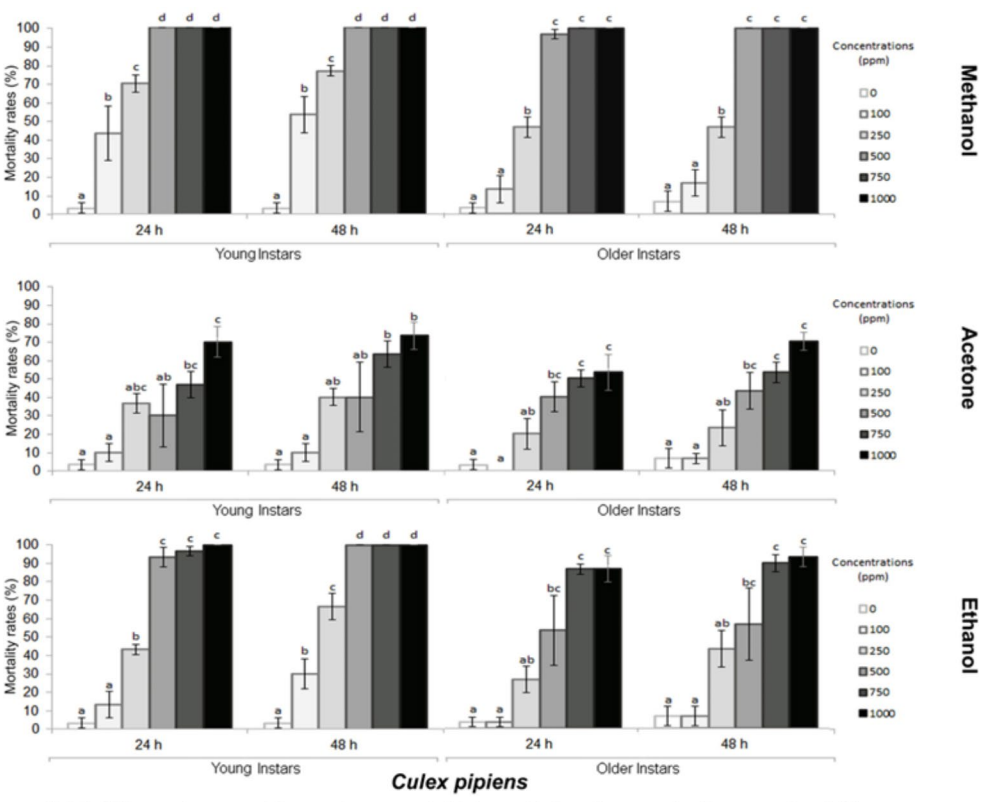

Fig. 2 Mean mortality percentages of *Cx. pipiens* larvae treated with *E. prunastri* extracts at various concentrations

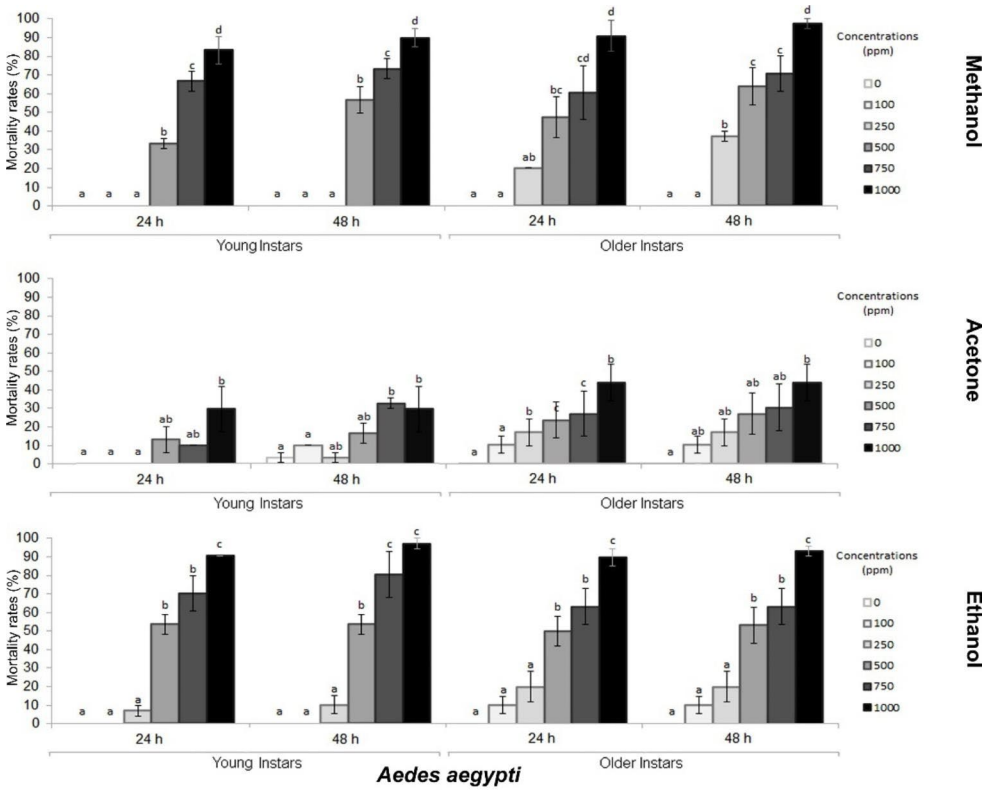

Fig. 3 Mean mortality percentages of *Ae. aegypti* larvae treated with *E. prunastri* extracts at various concentrations

LC_{50} values, it is clear that ethanol and methanol extracts of *E. prunastri* were more toxic than the acetone extract. The assessment of ethanol and methanol extracts' toxicity on both instars, in terms of LC_{90} values, revealed that the older instars exhibited greater sensitivity in comparison to the young instars. Conversely, the acetone extract displayed similar toxicity levels on both instars.

When both instars of *Ae. aegypti* mosquito larvae were compared in terms of LC_{50} and LC_{90} values, it was found that the young instars were more sensitive to methanol and ethanol extracts. The LC_{50} values of methanol, ethanol and acetone extracts, in the term of 48 h on *Ae. aegypti* young instar larvae were 390.9, 440.0 and 2934.8 ppm, and 537.1, 515.3 and 1837.1 ppm for older instar larvae, respectively. The assessment of ethanol and methanol extracts' toxicity on both instars, in terms of LC_{90} values, revealed that the younger instars exhibited greater sensitivity in comparison to the older instars. Conversely, the acetone extract displayed higher levels of toxicity in older instars.

In general, *Cx. pipiens* larvae were found to be more susceptible to extracts than *Ae. aegypti* mosquito larvae. In all extracts, there was no statistical difference ($F = 0.556$, $p = 0.456$) in terms of the 24 and 48 h mortalities of both mosquitoes in both larval instars (Figs. 2 and 3). In methanol and ethanol extracts, the mortality rates for *Cx. pipiens* mosquitoes generally increased ($F = 213.858$, $p = 0.001$) with increasing concentrations and for *Ae. aegypti* mosquitoes, although there were small variations in general at some concentrations, mortality rates increased ($F = 180.851$, $p = 0.001$) as the concentration increased. The maximum average percent mortality in the control groups was 4.7% (Fig. 3). The methanol extract caused 96–100% mortality in both instars of *Cx. pipiens* at concentrations of 500 ppm and 750 ppm, while mortality in *Ae. aegypti* larvae at the same concentrations ranged between 33.3 and 73.3%. The ethanol extract caused 93.3–100% mortality in the young and older instars of *Cx. pipiens* at concentrations of 500 ppm and 750 ppm, while mortality in *Ae. aegypti* larvae at the same concentrations ranged between 53.3 and 96.7%. While acetone extract caused ranged between 3.3 and 43.3% mortality in the young and older instars of *Ae. aegypti*, the mortality rate in *Cx. pipiens* mosquito at concentrations of 500 ppm and above varied between 30.0 and 73.3%.

Discussion

The development of environmentally friendly products for pest control has become even more important due to the mutagenic, teratogenic, and carcinogenic effects, as well as the toxicity, posed by chemical pesticides on non-target organisms. In addition to the search for new insecticidal active ingredients by investigating the effects of extracts and essential oils of flowering plants on harmful insects, the

Table 2 Lethal concentration (LC_{50} and LC_{90}) values and confidence limits of test extracts on young and older instars larvae of *Culex pipiens and Aedes aegypti* mosquitoes

Lethal concentrations (ppm*)	*Culex pipiens*											
	Methanol				Acetone				Ethanol			
	Young instars		Older instars		Young instars		Older instars		Young instars		Older instars	
	24 h	**48 h**	**24 h**	**48 h**	**24 h**	**48 h**	**24 h**	**48 h**	**24 h**	**48 h**	**24 h**	**48 h**
LC_{50}	247.0	203.4	128.0	104.3	768.5	606.9	674.3	490.0	407.9	326.6	234.2	153.9
Min	ND*	92.6	40.4	26.6	535.7	524.8	277.9	294.0	309.9	202.6	158.8	86.9
Max	ND	350.7	202.3	163.6	1575.0	716.6	> 2000	927.2	517.4	465.9	316.4	221.5
LC_{90}	947.1	417.3	331.0	292.0	3178.1	2802.9	5214.0	2794.1	1047.3	924.2	524.3	348.0
Min	ND	262.8	209.0	186.9	1558.7	2021.3	1368.1	1274.7	775.3	615.2	380.1	239.1
Max	ND	2283.5	1390.9	1013.2	36286.8	4527.4	> 50,000	49782.9	1815.2	2349.1	948.1	862.4
$\chi 2$	114.7	25.0	15.2	11.6	7.7	1.3	18.2	9.1	6.0	11.9	10.3	12.1
p	0.0001	0.0001	0.002	0.009	0.051	0.717	0.0001	0.028	0.09	0.007	0.016	0.007
Lethal concentrations (ppm)	***Aedes aegypti***											
	Methanol				Acetone				Ethanol			
	Young instars		Older instars		Young instars		Older instars		Young instars		Older instars	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
LC_{50}	515.3	390.9	564.9	537.1	1638.5	2934.8	2162.9	1837.1	457.0	440.0	521.8	515.3
Min	356.9	188.3	ND	373.4	ND	ND	1340.3	1202.7	263.5	224.4	480.9	356.9
Max	727.0	627.5	ND	684.3	ND	ND	5660.2	4119.1	800.3	832.8	563.2	727.0
LC_{90}	1265.6	977.4	907.4	942.0	4718.3	42964.3	38900.8	29025.5	1547.1	1427.4	1030.1	1265.6
Min	853.5	613.1	ND	729.3	ND	ND	11532.4	9701.8	858.9	777.2	922.8	853.5
Max	3813.9	5619.0	ND	1921.2	ND	ND	> 50,000	> 50,000	12659.4	19333.1	1188.3	3813.9
$\chi 2$	11.7	21.8	43.9	13.5	9.9	15.1	3.8	1.7	15.5	19.8	3.4	11.7
p	0.008	0.0001	0.0001	0.004	0.019	0.002	0.284	0.621	0.001	0.0001	0.326	0.008

* ppm: parts per million; ND: Not determined

number of studies on lichen extracts and secondary metabolites has increased in recent years (Prabha et al. 2016; Emsen and Aslan 2018; Sachin et al. 2018).

Lichens can produce a large number of secondary metabolites and lichen acids to withstand extreme environmental conditions and some of them have toxic effects on stored product pests such as weevils (Coleoptera: Curculionidae) and public health pests such as mosquitoes (Diptera: Culicidae) (Emsen et al. 2015; Yildirim et al. 2012; Khader et al. 2018; Moreira et al. 2016).

Acetone extracts of *Lecanora muralis* (Schreb.) Rabenh., *Letharia vulpina* (L.) Hue, *Peltigera rufescens* (Weiss) Humb lichens were tested on the Wheat Weevil *Sitophilus granarius* L. adults and 96 h after application, the concentration 20 mg/ml of extracts resulted in more than 86.86% mortality (Emsen et al. 2015). In another study, lichen extracts of *L. vulpina* and *P. rufescens* and two main lichen components (usnic acid and diffractaic acid) were tested at various concentrations against the adults of the maize weevil *Sitophilus zeamais* Motschulsky. The mortality rates at 96 h were found as 96.9, 95.9, 96.9 and 76.7% for extracts of *L. vulpina*, *P. rufescens* and the compounds of usnic acid and diffractaic acid repectively (Yildirim et al. 2012). Although the acetone extracts of lichens had significant toxic effects on the stored food pests, *S. granarius and S. zeamais*, at low concentrations, in our research, the acetone extract exhibited very low toxic effects on *Ae. aegypti*.

Khader et al. (2018) reported the toxicity of methanol extracts of six lichen species (*Leptogium papilosum*, *Parmelia erumpens*, *Parmotrema reticulatum*, *Parmot. kamatti*, *Parmot. tinctorum* and *Roccella montagnei*) on *Ae. aegypti*, *Anopheles stephensi* and *Cx. quinquefasciatus* mosquitoes. They found all the lichen extracts showed complete mortality against *Cx. quinquefasciatus* and this mosquito was found more susceptible to lichen extracts than *Ae. aegypti* and *An. stephensi*. The results of our research are consistent with this study and it was found that *Ae. aegypti* mosquito larvae found more resistant to three extracts of *E. prunastri* than *Cx. pipiens*.

Toxicity of the methanol extract of *Ramalina usnea* (L.) R. Howe and three fractions on third-instar larvae of *Ae. aegypti* reported by Moreira et al. (2016) They found that the extract and fractions showed high activity, killing 100% and 96.6% of the larvae at a concentration of 150 ppm after 24 h.

Cetin et al. (2008) investigated the insecticidal effects of usnic acid enantiomers ((−)- and (+)-usnic acids) isolated from *Cladonia foliacea* (Huds.) Wield and *Ramalina farinacea* (L.) Ach lichens against *Cx. pipiens* larvae. Some lichen compounds: cabraleadiol monoacetate, 3,6-dimethyl-2-hydroxy-4-methoxybenzoic acid, lichexanthone and 4-O-methylcryptochlorophaeic acid were found toxic on the second instar larvae of *Ae. aegypti* (Kathirgamanathar et al. 2006). The major secondary metabolites of most lichen extracts, atranorin, gyrophoric acid, (+)-usnic acid and 3-hydroxyphysodic acid were showed high larvicidal activities on mosquito *Culiseta longiareolata* Macquart (Cetin et al. 2012).

Three solvents with various polarity were used in this study. The relative polarity ratios of methanol, ethanol and acetone were 0.762, 0.654 and 0.355, respectively (Reichardt and Welton 2010). According to our results, methanol extracts of *E. prunastri* were found to be more toxic than ethanol and acetone extracts. This result may be due to the polarity of the solvent type and solvent polarity may affect the quality and toxicity of the extract. In previous research, the impact of various solvents on extraction yields, phytochemical constituents, antioxidant and in vitro anti-inflammatory activities of *Severinia buxifolia* (Poir.) Ten. (Rutaceae) were studied by Truong et al. (2019) They reported that methanol was the most effective solvent for the extraction, resulting in the highest extraction yield as well as the highest content of alkaloid, flavonoid, phenolic and terpenoids. In another study that used four solvents (acetone, ethanol, ethyl acetate and methanol), the richest extract of ginger (*Zingiber officinale* Roscoe) in terms of phenolic content was found in methanol and the lowest phenolic content was found in acetone (Ezez and Tefera 2021). In our research when the contribution of phenolic and flavonoid compound content to larvicidal activity was evaluated, it was observed that lower phenolic and flavonoid content resulted in better insecticidal activity. Polarity of solvents changes the solubility of substances and we supposed that the insecticidal substances in the lichen could not show sufficient effect due to the high proportion of total flavonoids and phenolic compounds in the acetone extract than methanol and ethanol extracts.

In addition, in previous studies the main secondary metabolites of *E. prunastri* were found to be atranorin, chloroatranorin, evernic acid and usnic acid (Yoshimura et al. 1994). The toxic effects of these metabolites on the mosquitoes and some other insects have been reported by some investigators (Cetin et al. 2008, 2012). We believe that the larvicidal activity of *E. prunastri* may be attributed to the biological activities of these components and the synergistic relationships between these substances. In future studies, we will examine the potential of these components to be used individually or in specific proportions as potential biopesticides in both laboratory and field testing.

Conclusions

The data obtained using lichens are promising for the discovery of new pesticide active ingredients that are safe for the environment and can control the development of resistance in insects. In our study, the toxicity of various extracts of *E. prunastri* lichen was investigated for the first time and found to be effective against *Ae. aegypti* and *Cx. pipiens* larvae, which are common vector species that threaten public health. The findings of this study indicate that further studies should be carried out on this subject with lichens.

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Declarations

Conflict of interest The authors have no competing interests to declare that are relevant to the content of this article.

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

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