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Larvicidal and antibiofilm potential of three mountain plants: *Centaurea ensiformis*, *Origanum hypericifolium*, *Paeonia turcica*

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Author contributions
Fatma Bursali designed the study and conducted the larvicidal activity experiments. Rukiye Yavaser Boncooğlu extracted the plant material and performed antioxidant activity assays. Mustapha Touray carried out larvicidal activity experiments, curated the data, and edited the manuscript. Mehmet Aytar conducted the antibiofilm assay. Ali Celik provided the plant material, wrote and edited the manuscript. All authors contributed significantly to the preparation of the manuscript.

Competing interests
The authors declare no conflicts of interest.

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Abbreviations
LC, lethal concentration; LC-MS/MS, liquid chromatography-tandem mass spectrometry; DPPH, 2,2-diphenyl-1-picrylhydrazyl; TPC, total phenolic content; GAE, gallic acid equivalent; ESI, electrospray ion source.

Citation
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Abstract
Background: Plants are known to produce a diverse group of natural metabolites with different biological activities. *Centaurea ensiformis* P.H. Davis, *Origanum hypericifolium* O. Schwartz & P.H. Davis, and *Paeonia turcica* Davis & Cullen are endemic plant species that grow on mountains in select regions in Türkiye and have been used in traditional Turkish medicine for various ailments. **Methods:** As first, we evaluated the larvicidal and antibiofilm activities of ethanol, ethyl acetate, acetone, and water extracts obtained from these plants. Antioxidant activities of the extracts were also investigated. **Results:** All tested extracts were effective at concentrations > 25 ppm on *Aedes aegypti* larval mortality with the lethal concentration 50 (LC_{50}) values ranging between 32.82–48.35 ppm and LC_{90} between 46.26–63.2 ppm. *O. hypericifolium* was the most effective plant, ethanol extracts presented LC_{50} values of 32.82 ppm. Extracts demonstrated varying degrees of antibiofilm activity depending on the dose and bacterial species. *Origanum hypericifolium* extracts notably inhibited biofilms of *Staphylococcus aureus* (up to 98% inhibition), while *P. turcica* showed moderate efficacy against the same bacterial species. *Pseudomonas aeruginosa* biofilms displayed high resistance to all extracts. **Conclusion:** The results indicated that these endemic Turkish plants possess promising larvicidal and antibiofilm potential, particularly *Origanum hypericifolium*. Extracts analyzed by liquid chromatography-tandem mass spectrometry contained caffeic acid, myricetin, cinnamic acid, quercetin, gallic acid, epicatechin, and ascorbic acid. Further research should explore their potential applications in mosquito control and biofilm-related infections.

Keywords: antioxidant; antibiofilm; larvicidal; *Centaurea ensiformis*; *Origanum hypericifolium*; *Paeonia turcica*; LC-MS/MS

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Highlights

Phenolic compounds from plants exhibit antimicrobial and insecticidal properties.

Endemic plants growing on Turkish mountains are widely used in folk medicine.

We evaluated the larvicidal and antibiofilm activities of extracts obtained from endemic plants.

Medical history of objective

Medicinal herbs have been widely used in Anatolia for centuries. *Centaurea ensiformis* P.H. Davis, *Origanum hypericifolium* O. Schwartz & P.H. Davis and *Paeonia turcica* Davis & Cullen are endemic plant species that grow on mountains in select regions in Türkiye and have been used in traditional Turkish medicine for various ailments. The use of these plants as medicine is based on oral tradition, with no specific ancient texts documenting their usage. They are composed of compounds with sedative, antispasmodic, analgesic, anti-inflammatory effects. Modern research has shown that these plants have important biological properties of importance in disease treatment.

Background

The species *Aedes aegypti* (Diptera: Culicidae) is a hematophagous black and white striped mosquito that feeds from humans and other animals for oogenesis [1–3]. This daytime-feeding behavior is a public health nuisance. Also, *Ae. aegypti* vectors deadly viral pathogens like yellow fever, dengue, chikungunya and Zika virus, and several other arboviruses characterized with high fever, rash, nausea, joint swelling, headache, fatigue, severe joint, and retro-orbital pain [1–3]. Annually, up to 700,000 deaths are attributed to such arboviral diseases [4]. *Ae. aegypti* is well adapted to human habitats and can be found breeding in almost any kind of container like flowerpots, discarded car-tire casings, tree-holes, and bamboo stumps [5]. Originally endemic to areas with tropical climate in Africa, *Ae. aegypti* mosquito is an invasive species spreading worldwide on ships during international travel and trade. Also, climate change has impacted the geographical and spatial range of this mosquito to higher latitudes [6–8]. Control of container breeding species like *Ae. aegypti* species is largely aimed at reducing the numbers of larval habitats with standing water, repelling host seeking females through the use of mosquito repellent or reducing mosquito populations by application of larvicides (*Bacillus thuringiensis var. israelensis,* Spinosad, and insect growth regulators) and spraying of insecticides (pyrethroids), and/or by releasing transgenic insects (e.g. self-limiting OX513A adult males) [5, 9]. Few larvicide products are available for mosquito control and *Ae. aegypti* has developed resistance to commonly used insecticides [10].

Biofilms are complex structures composed of microorganisms in extracellular polymeric mucilage, often attached to surfaces. Although biofilms can have beneficial roles in wastewater treatment, biodegradation, numerous food production systems, these structures can have negative implications on global health [11, 12]. In medical settings, groups of pathogenic bacteria can form biofilms on surfaces of medical implants such as catheters, sutures, and dental implants. This enhances the ability of these microbes to tolerate antibiotics, evade host defense systems and other external stresses such as pH changes, osmolarity, etc. [11, 12]. There is a need for the development of treatment options such as novel anti-biofilm agents, nanoparticles-based antibiotics formulation, CRISPR gene editing technologies, and photodynamic therapy to combat formation and eradication of biofilm infections [13, 14].

Over the years, there has been added pressure to develop new and safer alternative antimicrobial and insecticidal compounds, and it is well established that secondary metabolites from plants have a wide range of biological effects including antioxidants, antimicrobials, and insecticidal compounds that can be used in the treatment and prevention of a wide range of diseases as well as control of harmful pests [15–17]. Plants are known to produce a diverse group of natural plant metabolites which can be phenolics, alkaloids, glycosides, or terpenes, and have different antibacterial, antiparasitic, insecticidal, repellency, antifungal effects, etc. Among these important secondary metabolites phenolic compounds are quite important. They are produced via mainly shikimic acid and malonic acid pathways bearing one or more hydroxyl groups which can be methylated or glycosylated [18]. Phenolic compounds may be classified into five subgroups including phenolic acids, flavonoids, coumarins, lignin, and tannins [19]. Quantification and determination of these compounds are important since several bioactivities such as antioxidant, antimicrobial, anti-inflammatory, cytotoxic, larvicidal are related to phenolic compounds [20].

Centaurea ensiformis (Ece sarıbaşı local name) has a woody rootstock with sterile rosettes, stems can reach 35 cm and has simple 1–2 short branches with thinly-adpressed-tomentose leaves. *Paenoia turcica*, locally known as Ayıgülü, Eşek Gülü, or Bocur, can be found in the mountains of Eastern Anatolia Region and the Mediterranean region of Türkiye [21–24]. It produces deep maroon, red colored flowers on 65 cm stems in late spring. The blooms are large, up to 12 cm in diameter, and bear a boss of rich orange stamens. *Centaurea ensiformis* (locally known as peygamber cicegi) can grow up to 35 cm, has leaves covered with spear-shaped thin, flattened coarse hairs and its flowers are yellow in color. *C. ensiformis* has only been described from the Sandras Mountains in SW Anatolia [21–25]. All three plants are known for their medicinal properties and are used in traditional medicine for various ailments. They have antispasmodic, antimicrobial, expectorant, antioxidant, anticholinesterase, and carminative properties [26–28].

As a first, we evaluated the larvicidal and antibiofilm activities of different extracts (ethanol, ethyl acetate, acetone, and water) obtained from *C. ensiformis, O. hypericifolium*, and *P. turcica*. We also assessed the antioxidant activities of these extracts of plants and performed LC-MS analysis.

Materials and methods

Plant materials

The aerial parts of *Centaurea ensiformis* and *O. hypericifolium* were collected from Mount Sandras, Denizli, and Türkiye, during the flowering period between June and August 2023. These plants were found on this mountain at 1,860–1,970 m above sea level. The rhizomes of *P. turcica* were collected in June 2023, on Mount Samsun (elevation 1,130 m), Kusadası-Aydın (Figure 1).

Dr. Ali Celik further identified all collected plants. The voucher specimens are deposited at the Herbarium of the Biology Department, Faculty of Science, Pamukkale University (Herbarium No. AÇE 2546, Herbarium No. AÇE 2545 and Herbarium No. AÇE 2551 respectively). The samples were air-dried and stored in a polyethylene bag until use.

Chemicals

1,1-diphenyl-2-picryl-hydrazyl (DPPH•), methanol, acetone, ethyl acetate, Folin-Ciocalteu reagent, Na_2CO_3, myricetin, quercetin, gallic acid, caffeic acid, cinnamic acid, kaempferol, epicatechin, flavone and chlorogenic acid were obtained from Sigma-Aldrich (Steinheim, Germany). Rutin trihydrate was purchased from Riedel-de Haën (Seelze, Germany), ascorbic acid was from Fluka, adipic acid and succinic acid were from Cambridge Isotope Laboratories Inc. (Andover, MA, USA).

Preparation of plant extracts

Aerial parts of *C. ensiformis* and *O. hypercifolium* and roots of *P. turcica* were washed with tap water, followed by distilled water, and then dried at room temperature in the shade. *C. ensiformis* and *O. hypercifolium* were ground using a blender and *P. turcica* was cut into

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Figure 1 Plants used in the study. A, *Centaurea ensiformis.* B, *Paeonia turcica.* C, *Origanum hypericifolium*. Photos were taken by Ali Celik.

small pieces with pruning shears. Each plant sample was extracted with four different solvents including water, ethanol, acetone, and ethyl acetate. For this, 15 g of plant was mixed with 200 mL of solvent at 60 °C for 2 h and filtered through Whatman No. 1 filter paper. Extraction process was repeated with 200 mL of fresh solvent for 20 h and the filtrates were pooled. Ethanol, acetone, and ethyl acetate were removed by a rotary evaporator (IKA RV 05 Basic 1B, Staufen, Germany) whereas water was removed using a lyophilizator (Labconco, Freezone, Tokyo, Japan) up to dryness. Extracts were stored at −20 °C in dark glass bottles until they were used.

Quantification of total phenolics

The amount of phenolic compounds was estimated using the Folin-Ciocalteu method as described in Singleton et al. [29]. The assay was conducted by diluting 0.5 mL of extract solution (5.0 mg/mL) with 11.0 mL of distilled water in a 50 mL Erlenmeyer. Then, 0.5 mL of Folin-Ciocalteu reagent and after 3 min 0.75 mL of Na_2CO_3 solution (2%) was added. The containers were sealed and shaken at room temperature in darkness for 2 h at 120 rpm on a benchtop shaker (Promax 2020, Heidolph Instruments GmbH & Co KG, Kelheim, Germany). Absorbances of the solutions at 760 nm were recorded against blank using a Shimadzu, UV 1900i UV-Vis spectrophotometer (Kyoto, Japan). The total phenolic contents (TPC) of extracts were calculated using the linear regression equation of the standard curve of gallic acid. Results of triplicate experiments were expressed as gallic acid equivalents per gram extract (mg GAE/g extract).

Antioxidant activity determination by DPPH assay

The free radical scavenging activities of extracts were determined by the DPPH method according to Brand-Williams et al. with minor modifications [30]. For this, 1.5 mL of extract solutions of different concentrations (10–500 µg/mL) were mixed with 0.5 mL of DPPH solution (0.1 mM in methanol). The solutions were vortexed and incubated at 25 °C in darkness for 30 min. Absorbance was recorded at 517 nm against sample blank (extract solution + methanol) for each concentration. A control was also prepared and BHT was used as the standard antioxidant. DPPH scavenging activity was calculated from Equation (1).

$$\text{DPPH Scavenging \%} = [(A_{control} - A_{sample})/A_{control}] \times 100 \quad (1)$$

Where A control was the absorbance of the initial DPPH solution, A sample was the absorbance of extract + DPPH solution. Results of triplicate experiments were expressed as half-maximal inhibitory concentration (IC_{50}) values, the extract concentration able to scavenge 50% of DPPH radicals, obtained from plots of scavenging percent versus extract concentration.

Larvicidal bioassay

Late 3rd stage larvae of *Ae. aegypti* were used in the study. These mosquitoes were maintained at ± 2 °C, and 60 ± 10% relative humidity, and under a light/dark (14:10) phase. Adults were fed on 10% sugary water; females were fed on sheep blood through a membrane for oogenesis [3]. Larvae were fed with ground fish food (Tetramin®). The larvicidal bioassay was performed in 24 well plates according to Yang et al. and Li et al. with small modifications [31, 32]. Obtained extracts were dissolved in dimethyl sulfoxide (DMSO) and different concentrations (100, 75, 50, and 25 ppm (µg/mL) were prepared in distilled water. Ten *Ae. aegypti* larvae were introduced into the wells of the plate and were treated with 2 mL of the plant extracts. Experiments were done under laboratory conditions. Larval mortality was assessed after 48 h; unresponsive larvae were recorded as dead when touched with a brush. Negative control with dimethyl sulfoxide–water was also included. The treatments each had 6 replicates, and the experiments were repeated thrice. Mortality was calculated using the following Equation (2):

$$\text{Percentage of Mortality} = (\text{Number of Dead Larvae})/(\text{Total Number of Larvae}) \times 100 \quad (2)$$

Data from tests with mortality < 5% in the control group was used.

Antibiofilm bioassay

The antibiofilm activity of the extracts was tested against biofilm-forming bacteria *Escherichia coli* (3055), *Klebsiella pneumoniae* (5108), *Pseudomonas aeruginosa* (PAO1) and *Staphylococcus aureus* (RN4220). For this purpose, overnight cultures were diluted 1:100 with 1% glucose LB broth, then 200 µL of these diluted cultures were added to wells of a 96-well microplate. Plant extracts were added to the wells to obtain final concentrations of 0.1–0.5–1 mg/mL. Bacterial culture without added extract was used as a positive control, and fresh medium was used as a negative control. The plates were incubated at 37 °C for 24 h without stirring for biofilm production. Bacteria that did not adhere after incubation were removed by washing three times with distilled water. Adhering bacteria were fixed by drying at 60 °C for 1 h [33]. Bacteria attached to the plate were stained and measured spectrophotometrically [34]. The decrease in biofilm formation for each bacterial strain was expressed as antibiofilm activity and calculated by the Equation (3) below. The experiment was performed as three replicates.

$$\text{Percentage of Inhibition of Biofilm Formation} = (1 - OD_{sample}/OD_{control}) \times 100 \quad (3)$$

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis

Stock solutions of standards were prepared in methanol at 1,000 ppm concentration. Dilutions were prepared using methanol to obtain 0.5 and 2 ppm concentrations. Ten mg of the plant extracts were dissolved in 10 mL of water in a vial. Then, 50 µL of internal standards, adipic acid, and succinic acid, were added into a capped autosampler vial and 10 µL of sample was injected to LC-MS/MS. The samples in autosampler were kept at room temperature during the experiment. Experiments were performed by a Zivak® High-Performance Liquid Chromatography and Zivak® Tandem Gold Triple quadrupole (Aydin, Türkiye) mass spectrometry equipped with a Zivak C18 column (250 × 2 mm i.d., 5 µm particle size). The mobile phase was 0.1% formic

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acid in acetonitrile and flow rate of the mobile phase was 0.25 mL/min, and the column temperature was 37 ˚C. The injection volume was 10 µL.

The LC-MS/MS method was conducted using the method proposed by Tohma et al. with slight modifications [35]. A gradient of acidified formic acid and acetonitrile system was determined to be the best mobile phase for appropriate abundance of ionization and compound separation by the electrospray ion source (ESI). A triple quadrupole mass spectrometry system was used due to its fragmented ion stability [36]. Optimum ESI parameters were determined as 2.40 mTorr Collision-Induced Dissociation (argon gas) gas pressure, 5,000 V ESI needle voltage, 600 V ESI shield voltage, 350 ˚C drying gas temperature, 55 ˚C API housing temperature, 55 psi nebulizer gas pressure and 35.00 psi drying gas pressure. Detailed information on experiment parameters is given in Supplementary Table S1.

Validation of experiments and uncertainty evaluation

Adipic acid and succinic acid were used as an internal standard for validation experiments. Linearity, repeatability, limit of detection, and limit of quantification were determined. The linearity for each compound was determined by analyzing standard. Calibration curves were used to calculate the concentration of each analyte. Linear regression equations of the reported compounds are also presented in Supplementary Table S2. Limit of detection and limit of quantification of the LC-MS/MS methods for the compounds were calculated [35].

Data analysis

Obtained data were presented as percentage mean ± standard error. Differences in larval susceptibility and antibiofilm activity after treatment with plant extracts was determined using analysis of variance and Tukey's test with the plant species, extract type, concentration and their interactions as main factors taken into consideration. Probit analysis for the lethal concentration 50 (LC_{50}) and LC_{90} values was also included. Graphpad (v9) and SPSS software (v23) were used for statistical analysis and graph generation.

Results

Total phenolic and antioxidant activity

Results on the TPC of extracts of *C. ensiformis, O. hypericifolium*, and *P. turcica* are presented in Table 1. It was determined that the phenolic content of *O. hypericifolium* water extract was 193.8 ± 1.60 mg GAE/g. TPCs of *P. turcica* and *C. ensiformis* ethanol extracts were calculated as 77.6 ± 1.00 mg GAE/g and 27.8 ± 2.44 mg GAE/g, respectively. DPPH radical scavenging activities of extracts of *C. ensiformis*, *P. turcica, O. hypericifolium* were displayed in Figure 2. Acetone extract of *C. ensiformis*, ethanol and water extracts of *O. hypericifolium* and ethyl acetate extract of *P. turcica* had the lowest IC_{50} values. All extracts of *P. turcica* had low IC_{50} in a range of 5.490–26.48 µg/mL. DPPH radical scavenging percent of *C. ensiformis* was lower compared to other plants' extracts. The IC_{50} values are mostly related to TPC (Table 1).

Table 1 TPCs and inhibitory concentrations (IC_{50}) on DPPH radical of *C. ensiformis, O. hypericifolium* and *P. turcica* extracts

	TPC (mg GAE/g extract)			IC_{50} (µg/mL)		
Extract	***C. ensiformis***	***O. hypericifolium***	***P. turcica***	***C. ensiformis***	***O. hypericifolium***	***P. turcica***
Ethyl acetate	7.20 ± 1.30	21.1 ± 2.58	91.8 ± 0.49	838.5 ± 19.03	110.5 ± 2.143	5.490 ± 0.027
Acetone	18.5 ± 1.18	74.6 ± 2.03	30.5 ± 0.68	141.0 ± 7.889	26.71 ± 0.512	26.48 ± 0.347
Ethanol	27.8 ± 2.44	136.9 ± 2.85	77.6 ± 1.00	143.5 ± 2.119	8.523 ± 0.296	7.031 ± 0.396
Water	21.9 ± 1.21	193.8 ± 1.60	49.1 ± 1.56	433.9 ± 16.73	5.285 ± 0.354	26.13 ± 0.201

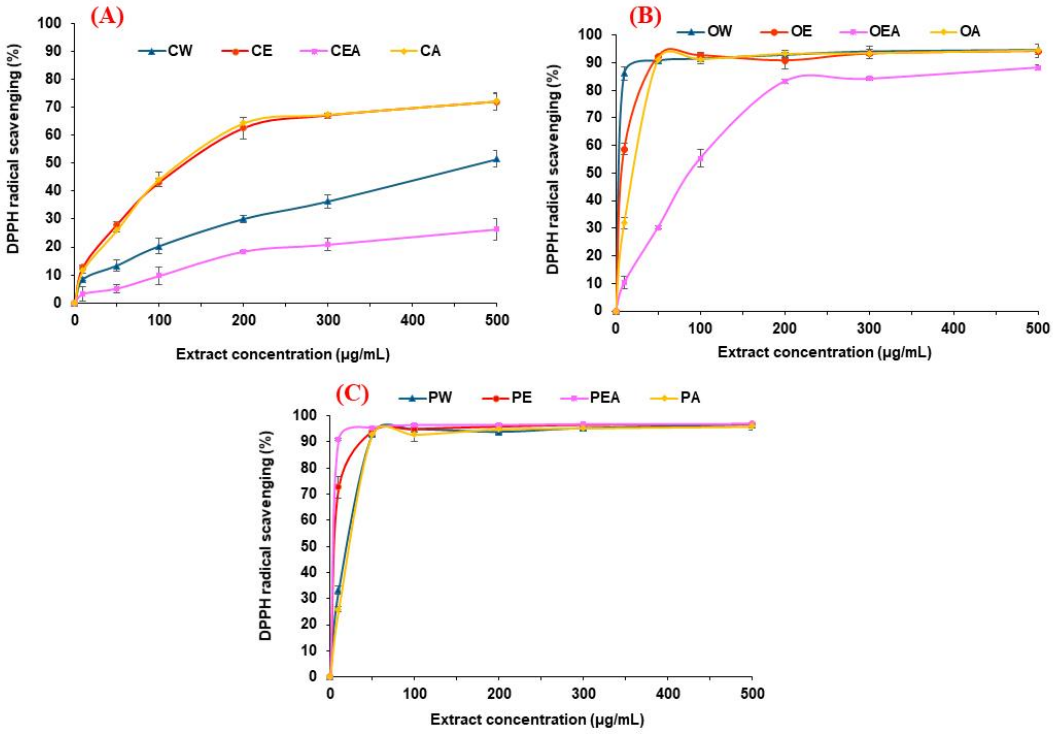

Figure 2 DPPH radical scavenging activities of water (W), ethanol (E), ethyl acetate (EA) and acetone (A) extracts of *Centaurea ensiformis* (C), *Origanum hypericifolium* (O) and *Paeonia turcica* (P)

Larvicidal activity

Comparison of different extracts from *C. ensiformis*, *O. hypericifolium, P. turcica* plants showed that *Ae. aegypti* larval mortality was concentration dependent with the highest mortality being observed at the 100 and 75 µg/mL for all tested plant extracts (Figure 3). A downward trend was observed in *Ae. aegypti* mortality rate. According to three-way ANOVA, there were significant interactions between the effects of the plant, tested concentration, and extract type on larval mortality ($P < 0.05$, see Table 2). Simple main effects analysis showed that extract type (F = 1.64; df = 2,647; $P = 0.195$) had no effects on the larvicidal properties of the plants. The LC_{50} values obtained ranged between 32.82 and 48.35 ppm and LC_{90} was between 46.26 and 63.2 ppm. Negative control had no effects on mosquito larvae. All tested extracts were effective at concentrations > 25 ppm with the LC_{50} values ranging between 32.82–48.35 ppm and LC_{90} between 46.26–63.2 ppm. *Origanum* was the most effective plant, ethanol extracts presented LC_{50} values of 32.82 ppm (Table 3). Water extract from all plants had no larvicidal effects (data not included).

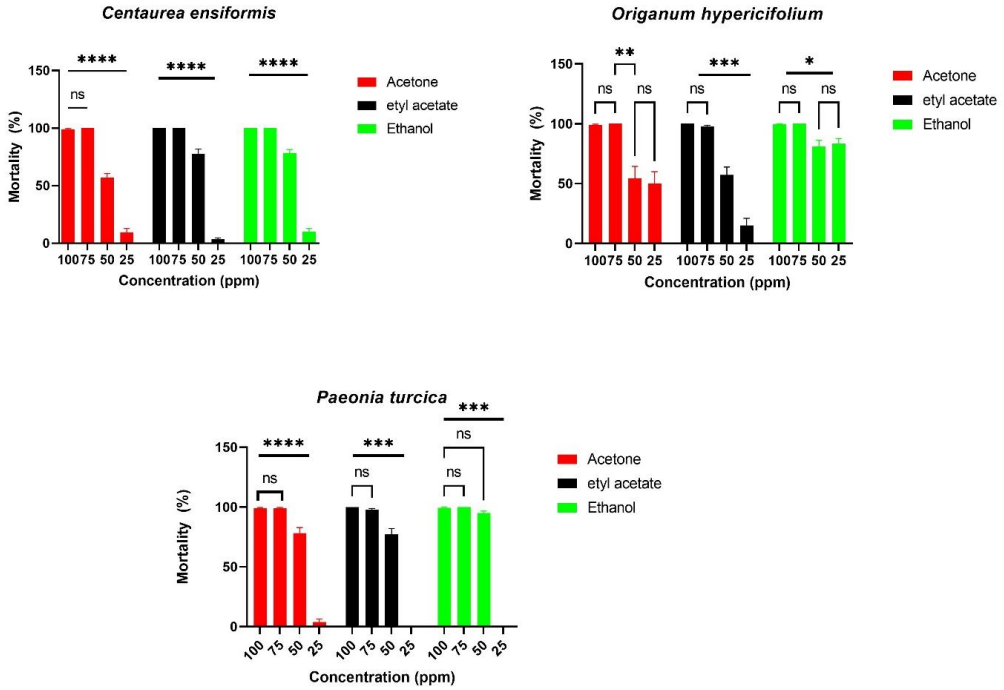

Figure 3 Larvicidal effects of *Centaurea ensiformis, Paeonia turcica* and *Origanum hypericifolium* against *Aedes aegypti*. Difference in larvicidal effects determined using ANOVA and Tukey's test. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. ns, no significance.

Table 2 Statistical data on the larvicidal effects and on the antibiofilm activity presented by plant extracts

Source	Larvicidal effects			Antimicrobial activity		
	df	F	*P*	df	F	*P*
Plant	2	140.59	< 0.001	2	199.778	< 0.01
Extract	2	1.64	0.195	3	317.026	< 0.01
Concentration	3	1213.18	< 0.001	2	553.259	< 0.01
Plant × Extract	4	5.745	< 0.001	6	225.164	< 0.01
Plant × Concentration	6	101.02	< 0.001	4	56.669	< 0.01
Extract × Concentration	6	8.671	< 0.001	6	12.569	< 0.01
Plant × Extract × Concentration	12	3.356	< 0.001	12	106.503	< 0.01

* level of significance $P \leq 0.05$.

Table 3 LC_{50} and LC_{90} values presented by *Centaurea ensiformis, Origanum hypericifolium, Paeonia turcica* plant extracts

Plant	Sample	LC_{50} (µg/mL) (LCL-UCL)	LC_{90} (µg/mL) (LCL-UCL)	X^2
Centaurea ensiformis	Etyl acetate	40.52 (38.64–42.35)	56.01 (53.25–59.43)	1.99
	Acetone	48.35 (46.28–50.03)	61.65 (58.96–65.84)	0.07
	Ethanol	37.75 (35.92–39.59)	55.87 (52.73–59.80)	3.91
Origanum hypericifolium	Etyl acetate	46.52 (18.46–60.29)	63.20 (51.05–361.18)	2.34
	Acetone	37.19 (23.03–51.16)	59.56 (44.67–147.05)	2.71
	Ethanol	32.82 (31.01–34.62)	52.20 (48.86–56.49)	1.21
Paeonia turcica	Etyl acetate	42.12 (39.87–44.0)	54.36 (52.10–57.29)	0.43
	Acetone	44.88 (42.65–46.71)	58.40 (55.88–61.93)	2.30
	Ethanol	37.63 (35.39–39.62)	46.26 (44.07–48.73)	0.002

LCL-lower limit (95% confidence limit); UCL-upper limit (95% confidence limit). $^{*}P \leq 0.05$, level of significance of chi-square values.

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Antibiofilm activity

The extracts of the plants tested in our study presented varying degrees of antibiofilm activity depending on the dose and the bacteria tested (Table 2, 4). Biofilms produced by *S. aureus* RN4220 were the most affected by all tested extracts. Ethanol, ethyl acetate, and acetone extracts of *O. hypericifolium* caused 96–98% inhibition at 0.5 mg/mL. *P. aeruginosa* biofilms were highly resistant. Negligible effects were exhibited by the extracts against *P. aeruginosa*. Ethanol, ethyl acetate, and acetone extracts of *Paeonia turcica* had activity against *S. aureus* as 88 ± 3% at 1 mg/mL, 89 ± 2% at 0.1 mg/mL, and 87 ± 2% at 1 mg/mL respectively. Moreover, ethanol and acetone extracts of *Centaurea ensiformis* had inhibitory effects of biofilm against *S. aureus* as 77 ± 1% at 1 mg/mL and 82 ± 0% at 0.5 mg/mL respectively. Statistical analysis of plant extracts was given in Table 4.

LC-MS/MS analysis

Plant extracts were analyzed by LC-MS/MS using 11 standard antioxidant compounds (Table 5). Standard chromatograms of secondary metabolites were presented in Supplementary Figure S1. Among the extracts of *C. ensiformis*, caffeic acid (14.65 mg/kg) and cinnamic acid (11.27 mg/kg) were the major phenolic compounds in ethanol and water extracts, respectively. Ethanol extract of *C. ensiformis* also contains chlorogenic acid, epicatechin, ascorbic acid, and rutin which contribute to the high TPC, remarkable DPPH radical scavenging activity and larvicidal activity compared to other extracts. *O. hypericifolium* extracts were rich in cinnamic acid, quercetin, gallic acid, epicatechin, and ascorbic acid which are known compounds with high biological activities. *P. turcica* mainly contained myricetin, gallic acid, quercetin, kaempferol, and epicatechin. Myricetin was detected in all extracts of *P. turcica* at considerable amounts.

Table 4 Antibiofilm activity percentages of plant extract

Centaurea ensiformis	**Concentration (mg/mL)**	***E. coli***	***K. pneumoniae***	***P. aeruginosa***	***S. aureus***
Water extract	0.1	56 ± 4	23 ± 7	0 ± 0	0 ± 0
	0.5	25 ± 3	0 ± 0	15 ± 3	0 ± 0
	1	65 ± 6	0 ± 0	19 ± 2	38 ± 10
Ethanol extract	0.1	33 ± 2	2 ± 3	0 ± 0	50 ± 4
	0.5	49 ± 9	17 ± 7	0 ± 0	74 ± 3
	1	53 ± 0	27 ± 3	0 ± 0	77 ± 1
Ethyl acetate extract	0.1	0 ± 0	0 ± 0	0 ± 0	68 ± 8
	0.5	57 ± 6	0 ± 0	0 ± 0	46 ± 8
	1	68 ± 9	0 ± 0	0 ± 0	29 ± 2
Acetone extract	0.1	38 ± 3	0 ± 0	0 ± 0	77 ± 4
	0.5	34 ± 0	33 ± 2	0 ± 0	82 ± 0
	1	29 ± 5	67 ± 2	0 ± 0	81 ± 1
Origanum hypericifolium	**Concentration (mg/mL)**	***E. coli***	***K. pneumoniae***	***P. aeruginosa***	***S. aureus***
Water extract	0.1	27 ± 1	0 ± 0	0 ± 0	62 ± 2
	0.5	48 ± 6	0 ± 0	41 ± 5	77 ± 2
	1	35 ± 6	0 ± 0	22 ± 6	66 ± 11
Ethanol extract	0.1	36 ± 7	8 ± 0	0 ± 0	66 ± 2
	0.5	54 ± 3	12 ± 2	13 ± 3	98 ± 1
	1	0 ± 0	76 ± 3	0 ± 0	97 ± 2
Ethyl acetate extract	0.1	1 ± 1	15 ± 1	0 ± 0	65 ± 2
	0.5	51 ± 4	18 ± 4	6 ± 2	94 ± 4
	1	64 ± 4	91 ± 1	0 ± 0	96 ± 0
Acetone extract	0.1	28 ± 6	10 ± 4	0 ± 0	67 ± 3
	0.5	0 ± 0	3 ± 1	0 ± 0	97 ± 0
	1	0 ± 0	28 ± 2	0 ± 0	92 ± 1
Paeonia turcica	**Concentration (mg/mL)**	***E. coli***	***K. pneumoniae***	***P. aeruginosa***	***S. aureus***
Water extract	0.1	1 ± 1	0 ± 0	0 ± 0	2 ± 2
	0.5	5 ± 1	25 ± 3	0 ± 0	18 ± 6
	1	4 ± 1	51 ± 3	0 ± 0	65 ± 5
Ethanol extract	0.1	31 ± 1	20 ± 2	0 ± 0	40 ± 6
	0.5	0 ± 0	2 ± 1	0 ± 0	87 ± 2
	1	0 ± 0	20 ± 2	0 ± 0	88 ± 3
Ethyl acetate extract	0.1	59 ± 8	31 ± 1	0 ± 0	89 ± 2
	0.5	1 ± 1	45 ± 6	0 ± 0	87 ± 1
	1	2 ± 2	49 ± 7	0 ± 0	87 ± 1
Acetone extract	0.1	34 ± 2	50 ± 3	0 ± 0	50 ± 6
	0.5	1 ± 1	60 ± 5	0 ± 0	85 ± 1
	1	17 ± 3	64 ± 4	0 ± 0	87 ± 2

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Table 5 Amount of secondary metabolites in Ethyl acetate, acetone, ethanol and water extracts of *Centaurea ensiformis, Origanum hypericifolium, Paeonia turcica* in mg/kg concentration obtained by LC-MS/MS analysis

	Centaurea ensiformis				*Origanum hypericifolium*				*Paeonia turcica*			
Standards	Ethyl acetate	Acetone	Ethanol	Water	Ethyl acetate	Acetone	Ethanol	Water	Ethyl acetate	Acetone	Ethanol	Water
Myricetin	1.095	0.623	0.459	1.177	0.373	0.256	0.425	0.283	4.702	6.563	4.339	4.116
Quercetin	0.707	0.722	1.253	0.909	1.447	1.662	1.880	1.242	2.238	0.423	1.039	1.518
Cinnamic acid	0.625	0.905	2.247	11.27	0.458	0.484	0.786	10.51	0.460	0.711	1.632	2.074
Rutin	0.112	0.136	2.011	0.491	0.076	0.092	0.385	0.421	0.149	0.137	0.094	0.081
Gallic acid	1.231	2.751	0.954	0.779	0.914	0.821	1.462	0.946	2.603	1.492	0.528	3.233
Caffeic acid	0.375	0.509	14.65	1.670	0.569	0.377	0.468	1.561	0.211	0.414	0.960	4.529
Flavone	0.019	0.002	0.041	0.032	0.015	0.010	0.009	0.012	0.008	0.016	0.090	0.160
Kaempferol	0.932	0.427	0.87	1.453	0.490	0.739	0.993	0.938	1.770	2.703	1.368	1.329
Chlorogenic acid	0.151	0.757	3.118	0.211	0.296	0.056	0.230	0.605	0.040	0.082	0.040	0.067
Epicatechin	1.613	1.281	2.782	3.537	1.022	0.993	1.123	5.590	1.801	0.953	1.308	2.821
Ascorbic acid	0.474	0.921	2.600	2.389	0.803	1.111	2.076	13.57	0.432	0.245	0.780	1.335

Discussion

The primary aim of this study was to evaluate the larvicidal and antibiofilm activities of different extracts (ethanol, ethyl acetate, acetone, and water) obtained from *C. ensiformis, O. hypericifolium* and *P. turcica.* These plants are known for their medicinal properties and are used in traditional medicine for various ailments. We also assessed the antioxidant activities of these extracts of plants and performed LC-MS analysis.

DPPH assay is a simple, economic, rapid, and efficient method to assess the antioxidant activity of samples via radical scavenging [37–39]. The results showed that *C. ensiformis* had a lower radical scavenging activity compared to the other two plants. In a previous study, the amounts of phenolic compounds in ethyl alcohol and ethyl acetate extracts of *Centaurea ensiformis* were reported to be 64.61 µg/mL and 54.89 µg/mL equivalent pyrocatechol, respectively [26]. Additionally, TPC of essential oil of *O. hypericifolium* was calculated to be 1.2480 ± 0.03 mmol GAE/L by Çelik et al. [40]. Herein, TPC of *P. turcica* ethanol extract was calculated as 77.6 ± 1.00 mg GAE/g extract, similar to the findings of Orhan *et al.* where it was 7.6 mg GA/100 mg extract [41]. On the other hand, methanol extract of *P. turcica* was reported to contain 729.32 ± 9.74 mg GAE/100 g root phenolic compounds [42]. The observed differences in DPPH radical scavenging activity may be attributed to the polarities of the phytochemicals and the extraction solvent used. Ugur et al. reported similar findings, demonstrating that the ethyl alcohol and ethyl acetate extracts of *C. ensiformis* scavenged DPPH radicals at 94.97% and 91.75% respectively, in a concentration-dependent manner [26]. Additionally, Karamenderes *et al.* observed an 86.19 ± 2.94% DPPH radical scavenging activity for the methanol extract of *C. ensiformis*, with results consistent with their TPC analysis [43]. In our study, the IC_{50} of the acetone extract of *O. hypericifolium* was determined to be 26.71 ± 0.512 µg/mL. This differs slightly from Özer et al. who reported an IC_{50} of 38.29 ± 2.03 µg/mL for the acetone extract of the same plant [44]. Similarly, previous studies reported IC_{50} values of 234.50 ± 1.92 µg/mL for *P. turcica* methanol extract and 23.69 ± 0.338% DPPH scavenging activity for *P. turcica* ethanol extract, indicating lower activity compared to our findings [41, 42].

Regarding the larvicidal effects, larval mortality rate increased with higher extract concentration (100 µg/mL and 75 µg/mL being the most effective) for all the tested plant extracts (*C. ensiformis*, *O. hypericifolium*, *P. turcica*). LC_{50} and LC_{90} of these plants ranged between 32.82–48.35 ppm and 46.26–63.2 ppm, respectively, for all extracts except water extracts, which had no effect. *Origanum* extract (specifically the ethanol extract) exhibited the strongest larvicidal effect with an LC_F value of 32.82 ppm. Numerous studies can be found in the literature exploring the insecticidal effects of different plant species against important mosquito species as such plants are potential sources of compounds whose properties could lead to the development of new biopesticides [45–49]. Insecticidal effects can vary according to extract type. Lim et al. evaluated the larvicidal properties of different extracts (hexane, chloroform, and ethyl acetate) from *Ocimum americanum* (hoary basil), *Curcuma longa* (turmeric), and *Petroselinum crispum* (parsley) against *Aedes albopictus* [48]. They reported that hexane extracts of *P. crispum* and *O. americanum* had the greatest larvicidal activity with LC_{50} values of 14.35 and 26.60 ppm. Prabhu et al. demonstrated that the ethanolic extract of *Piper betle* killed 100% of *Culex quinquefasciatus* (LC_{50} values = 143.91 µg/mL) and contained Caryophyllene (4.97%), Alpha-caryophyllene (3.46%), 1 H-Cycloprop(e)azulene (3.75%), Benzene (3.95%), Cyclohexane (1.81%), alpha cubebene (1.00%), 2,4a-Methanonaphthalene (0.46%) based on GC-MS analysis [45]. In another study, Nachammai et al. reported that aqueous extract of *Cladophora* sp. presented dose-dependent larvicidal activity against *Cx. quinquefasciatus* causing 100% mortality at 50 mg/mL concentration after 45 mins [49]. They showed that the contents of the aqueous extract of *Cladophora* sp. comprised of N1,2,4-Oxadiazole, 3-(1,3-benzodioxol-5-yl)-5-[(4-iodo-1H-pyrazol-1-yl)methyl]- (20.874), -Methyl-1-adamantaneacetamide (21.918), Cyclo barbital (20.685), trans- (19.896), Benzene pentanoic acid, 3,4-dimethoxy-, methyl ester (11.542), 4-Dehydroxy-N-(4,5-ethylenedioxy-2-nitrobenzylidene) tyramine (19.785), Quinoline, 1,2,3,4-tetrahydro-1-((2-phenylcyclopropyl)sulfonyl)-, Bicyclo[3.1.1]heptane, 2,6,6-trimethyl, 2,3-bis (methyl thio) (14.952), 1H-Pyrrolo[3,4-c]pyridine-1,3,4(2H,5H)-trione, 6-methyl- (14.475), Benzoic acid, 3-(4-morpholylazo)- (12.053) compounds in the extract.

The plant extracts showed different levels of effectiveness against biofilms formed by two bacteria, *S. aureus* and *P. aeruginosa*. This activity depended on both the dose of the extract and the type of bacteria. All extracts from *O. hypericifolium* were highly effective, inhibiting biofilm formation by 96–98% at a concentration of 0.5 mg/mL. Similarly, *P. turcica* extracts also showed good activity at higher concentrations (0.1 mg/mL to 1 mg/mL), inhibiting biofilm formation by 87–89%. *C. ensiformis* extracts (ethanol and acetone) had a moderate effect on *S. aureus* biofilms with inhibition around 77–82%

at 0.5 mg/mL to 1 mg/mL concentration. All tested extracts showed negligible effects against *P. aeruginosa* biofilms. Few studies assessed the antibiofilm activities of plants in the genera *Centaurea, Origanum*, and *Paeonia* [50–53]. *P. aeruginosa*, *S. aureus*, and *E. coli* are serious disease-causing bacteria that have developed resistance to current antibiotics. Biofilm formation is one of the mechanisms to virulence activity as well as hiding from antibiotics [54, 55]. Semiz et al. reported that essential oil of *O. hypericifolium* exhibited > 60% antibiofilm activity at 50 mg/mL concentration against *S. aureus* ATCC 29213, *M. luteus* NRRL-B 1013, *E. faecalis* ATCC 19433, and *P. fluorescens* ATCC 55241 [53]. Essential oil of *C. furfuracea* had no inhibitory effect on *S. aureus* ATCC 6538-P biofilm formation but the highest antibiofilm activity of the methanol extract was 87.90% on *S. aureus* ATCC 6538-P at 50 mg/mL [51].

The study's focus on ethanol and acetone extracts is a limitation. While these solvents are commonly used for plant extraction, other solvents like aqueous or hexane could potentially yield different bioactive compounds with distinct properties. Aqueous and hexane extracts have different polarities compared to ethanol and acetone. This means they can extract different types of compounds from the plant material. Additionally, compounds extracted with different solvents might exhibit unique biological activities, such as antimicrobial, antioxidant, or anti-inflammatory properties. Certain compounds might be more active or stable when extracted with specific solvents.

Biological activities of plant extracts and phenolic compounds are generally stated to be proportional [56]. Therefore, it is important to determine the phenolic constituents as well as phenolic amount. We identified caffeic acid, cinnamic acid, chlorogenic acid, epicatechin, ascorbic acid, rutin, quercetin, gallic acid, and ascorbic acid, myricetin, kaempferol and epicatechin in the plant extracts. Kaempferol, luteolin, rutin, caffeic acid, gallic acid, and chlorogenic acid were also determined by Özer et al. in methanol and acetone extracts of *O. hypericifolium* [44]. Carvacrol has been reported as the main essential oil in *C. ensiformis* [57]. Baykan-Erel et al. identified four phenolic compounds, one aminoacid, two acetophenone glycosides, three phenylpropanoide glycosides, one coumarin glucoside, four flavon glycosides, two neolignan glycosides, two megastigmane glycosides and schikimic acid methyl ester as the secondary metabolites of *C. ensiformis* [58]. Caffeic acid and cinnamic acid are phenolic acids with known antioxidant, larvicidal, antimicrobial, and anticancer activities [59]. Their derivatives have been studied for larvicidal activities against *Ae. aegypti* by França et al. and Thanasoponkul et al. [60, 61]. Myricetin, which was detected in all extracts of *P. turcica*, is a flavonol and its biological activities have been attributed to the more hydroxyl-bearing molecular structure compared to other flavonols [62]. Quercetin and kaempferol may be involved in larvicidal activity like the results of Pontual et al. where *Moringa oleifera* flower extract was investigated for its larvicidal activity on *Aedes aegypti* [63].

Conclusion

This study explored the efficacy of plant-extracts as a natural alternative to prevent biofilm formation and to manage *Aedes* larvae. These endemic plants exhibited promising larvicidal activity and antibiofilm activity. Future studies should attempt to determine the bioactive component, including its bioactivity, stereochemistry, optimal dosage, and toxicity, and explore potential applications in mosquito control and biofilm-related infections.

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