

Larvicidal and Antibiofilm Potential of Three Mountain Plants: *Centaurea ensiformis*, *Origanum hypericifolium*, *Paeonia turcica*

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Abstract

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 *Paenonia 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. As a 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. All tested extracts were effective at concentrations > 25ppm on *Aedes aegypti* larval mortality with the LC₅₀ values ranging between of 32.82–48.35 ppm and LC₉₀ between 46.26–63.2 ppm. *O. hypericifolium* was the most effective plant, ethanol extracts presented LC₅₀ 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. The results indicate that these endemic Turkish plants possess promising larvicidal and antibiofilm potential, particularly *Origanum hypericifolium*. Further research should elucidate the bioactive compounds responsible for these activities, optimize extraction methods, and explore potential applications in mosquito control and biofilm-related infections.

Introduction

The species *Aedes aegypti* (Diptera: Culicidae) is a hematophagous black and white striped mosquito that feeds from human and other animals for oogenesis (Rueda, 2004). 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 (Madewell 2020; Wint et al. 2022; Bursali and Simsek 2023). Annually, up to 700,000 deaths are attributed to such arboviral diseases (Ketkar et al. 2019). *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, bamboo stumps (Levin et al. 2014). 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 (Kraemer et al. 2019; Iwamura et al. 2020; Touray et al. 2023). 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) (Levin et al. 2014; Patil et al. 2018). Few larvicide products are available for mosquito control and *Ae. aegypti* has developed resistance to commonly used insecticides (Zulfa et al. 2022).

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 (Sharma et al. 2019; Amankwah et al. 2021). 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 within to tolerate antibiotics, evade host defense systems and other external stresses such as pH changes, osmolarity, etc. (Sharma et al. 2019; Amankwah et al. 2021). 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 (Zuberi et al. 2017a; Zuberi et al. 2017b).

Over the years, there has been an 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 (Calixto 2019; Silvério et al. 2020; Ayaz et al. 2021). 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 (Silva et al. 2022). Phenolic compounds may be classified into five subgroups including phenolic acids, flavonoids, coumarins, lignin and tannins (Mahajan et al. 2020). Quantification and determination of these compounds are important since several bioactivities such as antioxidant, antimicrobial, anti-inflammatory, cytotoxic, larvicidal are related to phenolic compounds (Phuyal et al. 2020).

Centaurea ensiformis (Ece sarbaşı 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. *Paenonia turcica*, locally known as Ayıgülü, Eşek Gülü or Bocur, can be found on mountains of Eastern Anatolia Region and Mediterranean region of Türkiye (Baytop 1994; Baytop 1999; Tuzlacı 2006; Tanker et al. 2007). 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 Sandras Mountains in SW Anatolia (Baytop 1994;1999; Tuzlacı 2006; Tanker et al. 2007; Davis 1956). 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 (Ugur et al. 2009; Yılmaz et al. 2017; Demirboğa et al. 2021).

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.

Methods

Plant materials

The aerial parts of *Centaurea ensiformis* and *O. hypericifolium* were collected from Mount Sandras, Denizli, Türkiye, during flowering period between June and August 2023. These plants were found on this mountain at 1860–1970 m above sea level. The rhizomes of *P. turcica* were collected in June 2023, on Mount Samsun (elevation 1130 m), Kusadası-Aydın (Fig. 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 (FCR), Na₂CO₃, 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. hypericifolium* 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. hypericifolium* were ground using a blender and *P. turcica* was cut into 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 2h and filtered through Whatman No:1 filter paper. Extraction process was repeated with 200 mL of fresh solvent for 20h 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 lyophilizator (Labconco, Freezone, 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 were estimated using the Folin-Ciocalteu method as described in Singleton et al. (1999). 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 Na₂CO₃ solution (2%) was added. The containers were sealed and shaken at room temperature in darkness for 2h 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 (Japan). 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. (1995) with minor modifications. 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 Eq. 1.

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

where, A_{control} was the absorbance of initial DPPH solution, A_{sample} was the absorbance of extract + DPPH solution. Results of triplicate experiments were expressed as IC₅₀ 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 in insectary at 28 ± 2°C, and 60 ± 10% RH, and under a light/dark (14:10) phase. Adults were fed on 10% sugary water; females were fed on sheep's blood through a membrane for oogenesis (Bursali and Simsek 2023). Larvae were fed with ground fish food (Tetramin®). The larvicidal bioassay was performed in 24 well plates according to Yang et al. (2021) and Li et al. (2022) with small modifications. Obtained extracts were dissolved in 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 (Sigma, Corning Costar Multiple Well Plates, CLS3524) and were treated with two 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 formula:

$$\text{Percentage of mortality} = (\text{Number of dead larvae}) / (\text{Total number of larvae}) \times 100$$

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 was 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 hours 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 hour (Stepanović et al. 2007). Bacteria attached to the plate were stained and measured spectrophotometrically (Li et al. 2014). The decrease in biofilm formation for each bacterial strain was expressed as antibiofilm activity and calculated by the formula below. The experiment was performed as three replicates.

Percentage of inhibition of biofilm formation = $(1 - OD_{\text{sample}} / OD_{\text{control}}) \times 100$

LC-MS/MS analysis

Stock solutions of standards were prepared in methanol at 1000 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, was added into 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® HPLC 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 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. (2019) with slight modifications. 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). Triple quadrupole mass spectrometry system was used due to its fragmented ion stability (Gören et al. 2009). Optimum ESI parameters were determined as 2.40 mTorr CID (argon gas) gas pressure, 5000 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 1.

Validation of experiments and uncertainty evaluation

Adipic acid and succinic acid was used as an internal standard (IS) for validation experiments. Linearity, repeatability, limits of detection (LOD) and quantification (LOQ) were determined. The linearity was for each compound was determined by analyzing standard solution. Calibration curves were used to calculate the concentration of each analyte. Linear regression equations of the reported compounds are also presented in Supplementary Table 2. LOD and LOQ of the LC-MS/MS methods for the compounds were calculated (Tohma et al. 2019).

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 LC₅₀ and LC₉₀ 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 total phenolic content 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* was displayed in Fig. 2. Acetone extract of *C. ensiformis*, ethanol and water extracts of *O. hypericifolium* and ethyl acetate extract of *P. turcica* had the lowest IC₅₀ values. All extracts of *P. turcica* had low IC₅₀ 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 IC₅₀ values are mostly related to total phenolic content (Table 1).

Table 1
Total phenolic contents and inhibitory concentrations (IC₅₀) on DPPH radical of *C. ensiformis*, *O. hypericifolium* and *P. turcica* extracts

Extract	Total phenolic content (mg GAE/g extract)			IC ₅₀ (µg/ml)		
	<i>C. ensiformis</i>	<i>O. hypericifolium</i>	<i>P. turcica</i>	<i>C. ensiformis</i>	<i>O. hypericifolium</i>	<i>P. turcica</i>
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

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 extract (Fig. 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 LC₅₀ values obtained ranged between of 32.82 and 48.35 ppm and LC₉₀ was between 46.26 and 63.2 ppm. Negative control had no effects on mosquito larvae. All tested extracts were effective at concentrations > 25ppm with the LC₅₀ values ranging between of 32.82–48.35 ppm and LC₉₀ between 46.26–63.2 ppm. *Origanum* was the most effective plant, ethanol extracts presented LC₅₀ values of 32.82 ppm (Table 3). Water extract from all plants had no larvicidal effects (data not included).

Table 2
Statistical data on the larvicidal effects and on the antibiofilm activity presented by plant extracts; * level of significance $p \leq 0.05$.

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

Table 3
LC₅₀ and LC₉₀ values presented by *Centaurea ensiformis*, *Origanum hypericifolium*, *Paeonia turcica* plant extracts; LCL-lower limit (95% confidence limit); UCL-upper limit (95% confidence limit). *p ≤ 0.05, level of significance of chi-square values.

Plant	Sample	LC ₅₀ (µg/mL) (LCL-UCL)	LC ₉₀ (µg/mL) (LCL-UCL)	χ ²
<i>Centaurea ensiformis</i>	Ethyl 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
<i>Origanum hypericifolium</i>	Ethyl 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
<i>Paeonia turcica</i>	Ethyl 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

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 (Tables 2 and 4). Biofilms produced by *S. aureus* RN4220 were the most affected by all tested extracts. Ethanol, ethyl acetate, 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, acetone extracts of *Paeonia turcica* had activity against *S. aureus* as 88 ± 3% at 1 mg/mL, 89 ± 2% at 0.1 mg/mL, 87 ± 2% at 1 mg/mL respectively. Moreover, ethanol and acetone extracts of *Centaurea ensiformis* had inhibitory effect 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.

Table 4
Antibiofilm activity percentages of plant extract

<i>Centaurea ensiformis</i>	Concentration (mg/mL)	<i>E. coli</i>	<i>K. pneumoniae</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>
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
<i>Origanum hypericifolium</i>	Concentration (mg/mL)	<i>E. coli</i>	<i>K. pneumoniae</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>
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
<i>Paeonia turcica</i>	Concentration (mg/mL)	<i>E. coli</i>	<i>K. pneumoniae</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>
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

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 Material. 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 contained chlorogenic acid, epicatechin, ascorbic acid and rutin which contribute to the high total phenolic content, 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 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

	<i>Centaurea ensiformis</i>				<i>Origanum hypericifolium</i>				<i>Paeonia turcica</i>			
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 (Kedare and Singh 2011; Gulcin 2020). 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⁻¹ and 54.89 µg mL⁻¹ equivalent pyrocatechol, respectively (Ugur et al, 2009). Additionally, total phenolic content of essential oil of *O. hypericifolium* was calculated to be 1.2480 ± 0.03 mmol GAE L⁻¹ by Celik et al. (2010). 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. (2010) where it was 7.6 mg GA/100 mg extract. On the other hand, methanol extract of *P. turcica* was reported to contain 729.32 ± 9.74 mg GAE/100 g root phenolic compounds (Cetiz et al. 2023). 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. (2009) 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. Additionally, Karamenderes et al. (2007) observed an 86.19 ± 2.94% DPPH radical scavenging activity for the methanol extract of *C. ensiformis*, with results consistent with their total phenolic content analysis. In our study, the IC₅₀ of the acetone extract of *O. hypericifolium* was determined to be 26.71 ± 0.512 µg/ml. This differs slightly from Özer et al. (2020) who reported an IC₅₀ of 38.29 ± 2.03 µg/ml for the acetone extract of the same plant. Similarly, previous studies reported IC₅₀ values of 234.50 ± 1.92 µg/mL for *P. turcica* methanol extract (Cetiz et al, 2023) and 23.69 ± 0.338% DPPH scavenging activity for *P. turcica* ethanol extract (Orhan et al, 2010), indicating lower activity compared to our findings.

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*). LC₅₀ and LC₉₀ 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 LC₅₀ 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 (Prabhu et al. 2022; Amutha et al. 2023; Sari et al. 2023; Lim et al. 2023; Nachammai et al. 2023). Insecticidal effects can vary

according to extract type. Lim et al. (2023) 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*. They reported that hexane extracts of *P. crispum* and *O. americanum* had the greatest larvicidal activity with LC₅₀ values 14.35 and 26.60 ppm. Prabhu et al. (2022) demonstrated that the ethanolic extract of *Piper betle* killed 100% of *Culex quinquefasciatus* (LC₅₀ 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. In another study, Nachammai et al. (2023) 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. They showed that the contents of 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-morpholyazo)—(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* (Ayaz et al. 2021; Chemsal et al. 2018; Jin et al. 2023; Semiz et al. 2018). *P. aeruginosa*, *S. aureus*, *E. coli* are serious disease-causing bacteria that have developed resistance to current antibiotics. Biofilm formation is one of the mechanisms employed to increase virulence activity as well as hide from antibiotics (Schillaci et al. 2013; Frassinetti et al. 2020). Semiz et al. (2018) are 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 (Semiz et al. 2018). 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 (Chemsal et al. 2018).

Biological activities of plant extracts and phenolic compounds are generally stated to be proportional (Ranilla et al. 2010). 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. Caffeic acid and cinnamic acid are phenolic acids with known antioxidant, larvicidal, antimicrobial, and anticancer activities (Ruwizhi and Aderibigbe 2020). Their derivatives have been studied for larvicidal activities against *Ae. aegypti* by França et al (2021) and Thanasoponkul et al (2023). 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 (Park et al. 2016). Quercetin and kaempferol may be involved in larvicidal activity like the results of Pontual et al. (2012) where *Moringa oleifera* flower extract was investigated for its larvicidal activity on *Aedes aegypti*.

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.

Declarations

Author contributions

Methodology, FB, RYB, MT, MA; formal analysis, FB, RYB, MT, MA; plant materials, AC; investigation, FB, RYB, MT, MA; data curation, FB, RYB, MT, MA; writing—original draft preparation, FB, RYB, MT, MA. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures



Figure 1

Plants used in the study. A: *Centaurea ensiformis* B: *Paeonia turcica* C: *Origanum hypericifolium*

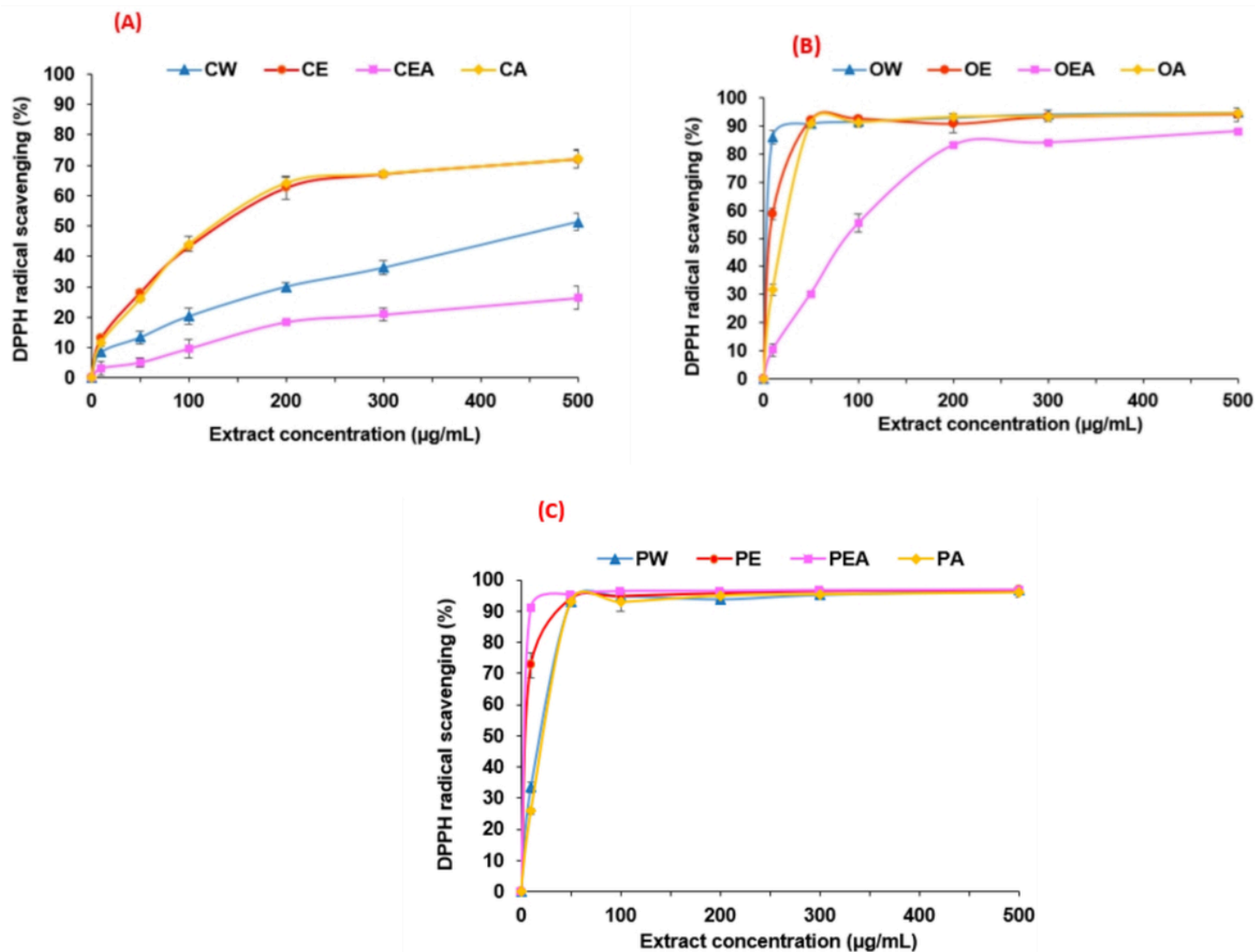


Figure 2

DPPH radical scavenging activities of extracts of *Centaurea ensiformis*, *Paeonia turcica*, *Origanum hypericifolium*

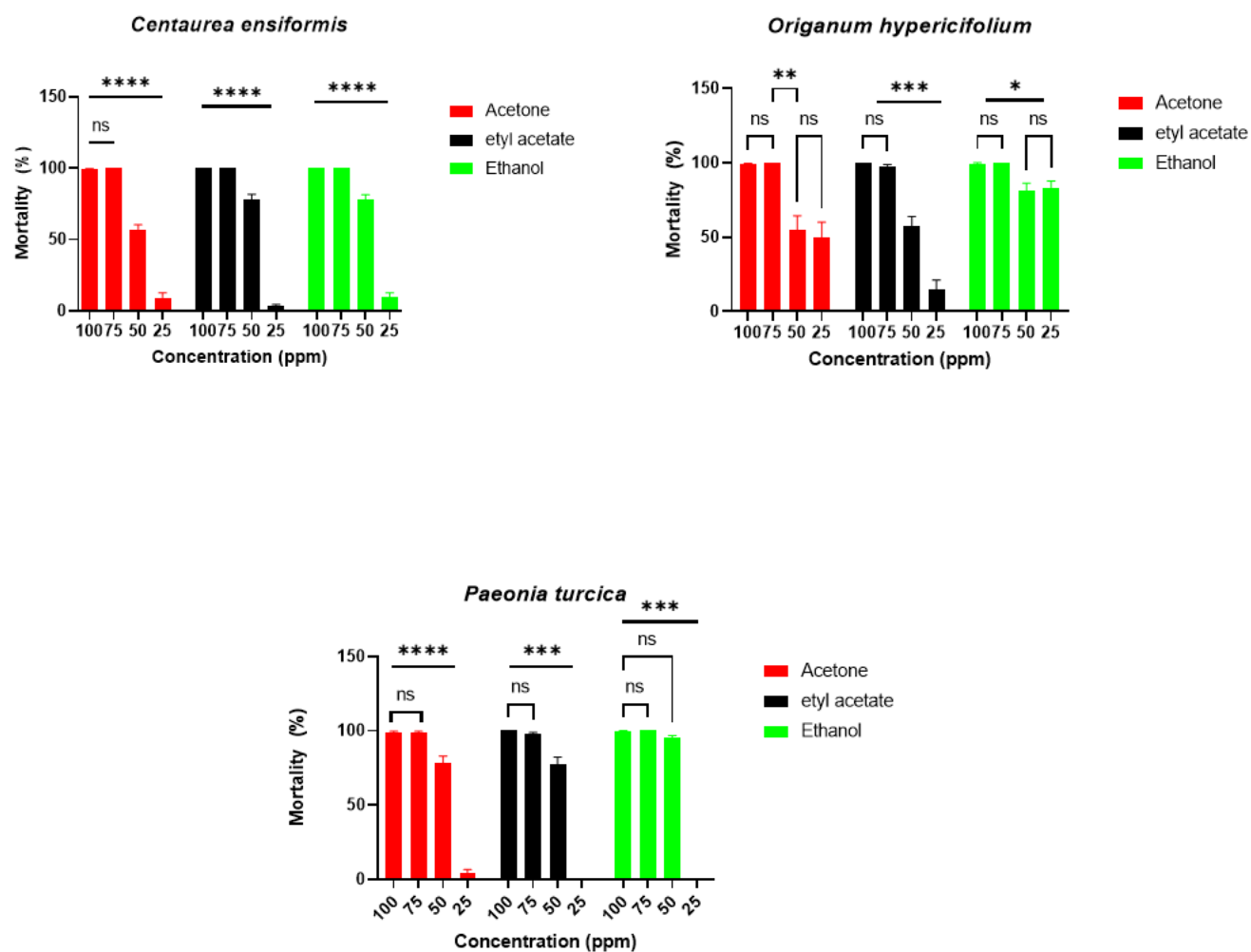


Figure 3

Larvicidal effects of *Centaurea ensiformis*, *Paeonia turcica* and *Origanum hypericifolium* against *Aedes aegypti* (difference in larvicidal effects determined using ANOVA and Tukey's test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

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