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Metabolic profiling and insecticidal activities of *Rosmarinus officinalis* L. for the management of *Aphis craccivora* Koch and *Planococcus lilacinus* Cockerell

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Abstract

Background Sucking insects are major threat to agricultural and horticultural crops. Indiscriminate application of chemical insecticides for the control of pests leads to the development of resistance, harmful to non-target organisms, consumers' health, the environment, etc. Therefore, botanical insecticides are alternate to synthetic pesticides for the control of sucking pests. In the present investigation, chemical constituents, metabolic profile, and insecticidal activities of *Rosmarinus officinalis* L. (Lamiaceae) ethanolic aqueous extract (EAE), fractions and compounds were screened against *Aphis craccivora* Koch (Hemiptera: Aphididae) and *Planococcus lilacinus* Cockerell (Hemiptera: Pseudococcidae).

Results Gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS) analysis showed that linolenic acid (24.97%), 1,8-cineole (14.26%), myrcene (10.67%), hexadecenoic acid (9.91%), and camphene (7.12%) were the major constituents in the *n*-hexane fraction. UHPLC–ESI-QTOF-IMS analysis of ethanolic aqueous extract (EAE) showed the presence of palmitoleic acid, 4-ethoxy ethyl benzoate, 7-methylrosmanol, and diosmin as major metabolites. Among extract and fractions, EAE was found more effective to *A. craccivora* (lethal dose to kill 50% of test insect *i.e.*, LD_{50} = 1.84 µL/nymph) after 96 h followed by *n*-hexane fraction (LD_{50} = 2.22 µL/insect). In *P. lilacinus*, *n*-hexane fraction displayed highest toxicity (LD_{50} = 1.46 µL/crawler) followed by ethyl acetate and *n*-butanol fraction (LD_{50} = 2.01–2.29 µL/crawler). All combinations of the EAE and fractions exhibited synergetic action. Amongst compounds, linolenic acid was found superior to *A. craccivora* (LD_{50} = 0.59 µL/nymph) and *P. lilacinus* (LD_{50} = 0.99 µL/crawler). EAE and its fractions also showed significant reproductive inhibition and deterrence to target pests. Further, EAE significantly inhibited in vivo enzyme acetylcholinesterase (AChE), glutathione-S-transferase (GST), and mixed function oxidase (MFO) in *A. craccivora* after 24 and 48 h. In *P. lilacinus*, only GST showed inhibition but AChE and carboxylesterase (CES 1) were induced after 24 h. SEM study revealed notable aberrations in the structure of the peritoneum, setae, and thoracic legs of *A. craccivora* after ingestion of EAE. Under greenhouse conditions, the higher dose of *R. officinalis* EAE (20 g/L) reported higher reduction of *A. craccivora* on leaf (82.28 to 89.36%) and twigs (70.68 to 85.72%) of cowpea after 3, 5 and 7 days of second spray.

Conclusion Based on our greenhouse study results, EAE of *R. officinalis* may be recommended for the control of *A. craccivora* in crop plants.

Keywords Rosemary, GC/GC–MS, UHPLC–ESI-QTOF-MS, Extract, Compounds, Aphid, Mealybug, Toxicity, Enzyme inhibition, Scanning electron microscopy

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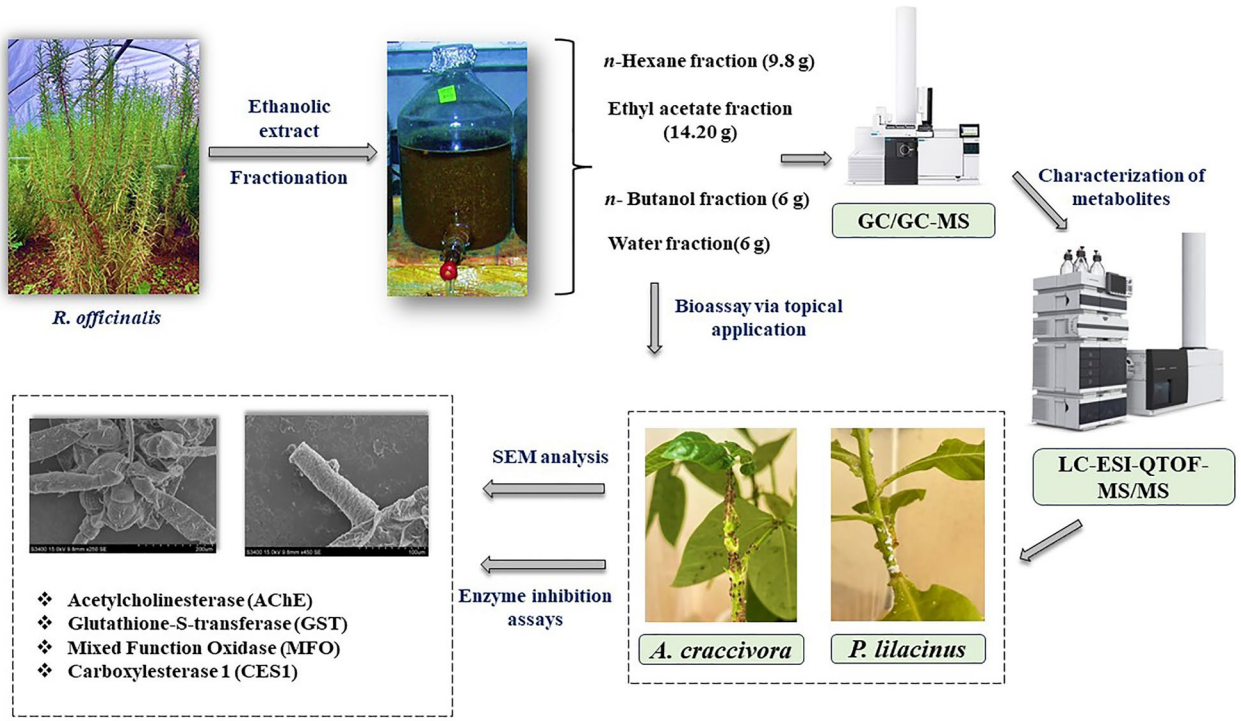
Graphical Abstract



Introduction

Aphis craccivora Koch (Hemiptera: Aphididae) and *Planococcus lilacinus* Cockerell (Hemiptera: Pseudococcidae) are major sucking pests of agricultural/horticultural crops, aromatic/medicinal plants which cause negative impact on production [1, 2]. These pests suck the sap from plants which results in reducing the vigour and growth. In severe infestation, they secrete honeydew and cause secondary damage in the form of black sooty mould, which leads to reduction in the growth of plants, photosynthesis and market value of produce [3, 4]. Application of synthetic pesticides is a primary method of pest management, which harms beneficial insects, the environment, and consumers [5–7]. Therefore, environmentally suitable, and efficient pest control from natural plant resources, against target pests is required.

Botanicals play a major role in the management of pests due to their multi-mode of action viz., contact/stomach poison, antifeedant, repellent, growth inhibitor, etc. [8–10]. The plant extracts/fractions and their isolated compounds of *Triadica sebifera*, *Trillium govanianum*, *Aconitum heterophyllum*, *Fritillaria cirrhosa* and *Cissampelos pareira* showed promising insecticidal and enzyme inhibition activities against *A. craccivora* and *P. lilacinus* [11–15]. The aromatic plant products, mainly plant extracts, essential oils (EOs) and their compounds can be utilized as alternatives to chemical insecticides because of availability, biodegradability, non-toxic to consumers, and no-resistance to insect pests [6, 16, 17].

Rosmarinus officinalis is a perennial shrub primarily utilized as a flavouring agent in cooking [18, 19]. Plant extracts and EOs are often employed in food preservation [20]. For centuries, rosemary-infused water, decoction, and ethanol extract have been associated with memory enhancement, skin rejuvenating substances, treatment of digestive problems, varicose veins, respiratory illnesses, inflammation, dizziness, and vaginitis [21]. In traditional medicine, distilled water from rosemary flowers was used as an eye ointment [22]. The plant has also been reported antibacterial, antioxidant [23], fungicidal [24], hepatoprotective, and anticancer effects [25]. *R. officinalis* powder was used to preserve seeds of French bean and wheat against stored grain pests [26]. EO of *R. officinalis* showed oviposition deterrent, and repellent against red spider mites [27]. However,

no studies were reported on the effect of rosemary extract/fraction against test insects. Therefore, current investigations were carried out, viz., (a) to study the chemical composition of *n*-hexane fraction and chemical profiling of ethanolic aqueous extract (EAE) by UHPLC–ESI-QTOF-MS; (b) insecticidal activities of EAE and its fractions, combinations, compounds against *A. craccivora* and *P. lilacinus*; (c) effect of EAE on enzyme inhibition in *A. craccivora* and *P. lilacinus*.

Materials and methods

Plant material

The vegetative parts of the rosemary plants used in this study were collected from the Agronomy field of IHBT Palampur situated at 1325 m above mean sea level (amsl) altitude (32°11′39″N latitude and 76°56′51″E longitude) dried in shade (temperature 25 ± 3 °C, humidity; 40–50%) for 15 days inside glasshouse, powdered and then used for research.

Rearing of experimental insects

A. craccivora was collected from French bean fields of Una (Himanchal Pradesh) (Latitude: 31.4697° N and Longitude: 76.2691° E) and *P. lilacinus* from the cotton field of Punjab (Latitude: 31.2000° N and Longitude: 76.3000° E). *A. craccivora* and *P. lilacinus* were constantly reared on a live French bean (*Phaseolus vulgaris* L.) and tobacco (*Nicotiana tabacum* L.) plants, respectively, in the insect rearing facility, Entomology Laboratory, CSIR-Institute of Himalayan Bioresource and Technology, Palampur (H.P.), India, under controlled conditions viz., temperature (25 ± 2 °C), relative humidity (60 ± 5%) and photoperiod (16 h:8 h light–dark) for several generations. *A. craccivora* and *P. lilacinus* were reared for more than 50 generations. Newly emerged nymphs and crawlers (3–4 days old) were used for the investigation [12].

Extraction and fractionation of EAE

Extraction and fractionation of EAE was carried out as per the standard methods reported [1, 13, 14]. The dried apical parts of *R. officinalis* were crushed into a powder using a suitable blender (Sujata SuperMix®, India). The powdered material (453 g) was placed in a clean, flat-bottomed percolator and soaked (3 L × 3 times) in 80% ethanol: DH_2O for 24 h with frequent shaking and thorough filtration. After filtration, the solvent was evaporated by rotary evaporation (Buchi, R-2010) at optimum temperature (45 °C) and pressure. About 75 g of crude EAE was dissolved in 500 mL DH_2O. Further, the dissolved part was fractionated sequentially by solvents. To prevent chemical modification of the bioactive chemicals and loss of their characteristics, the fractions were filtered before being concentrated to dryness in a rotary evaporator at a temperature (35–40 °C). The extract and fractions were stored at 4 °C until further investigation.

GC and GC–MS analysis of volatile *n*-hexane fraction

GC-FID, GC–MS and FAMES (fatty acid methyl ester) were prepared using the procedure outlined by a previous study [28]. The *n*-hexane fraction of *R. officinalis* (50 mg) was derivatized using sodium methoxide (1.55 g of sodium hydroxide in 50 mL of methanol). Using a vortex shaker (Spinix^{Tm}, Tarsons®), the mixture was rapidly agitated for 30 s. The clear solution of FAMES was then collected and evaluated by gas chromatography (GC-FID and GC–MS). The GC-FID analysis was done by using GC Shimadzu 2010 coupled with AOC-20i auto-injector, SH-Rxi-5Sil MS column (30 m × 0.25 mm i.d., 0.25 µm) and FID-detector. Nitrogen was used as a carrier gas with a flow rate of 1.24 mL/min. The initial temperature of oven was 40 °C for 4 min and programmed to 220 °C at 4 min, then held for 15 min at 220 °C. Other parameters for GC analysis were an injector temperature of 250 °C, oven temperature of 250 °C, and the split mode was used. A standard solution of *n*-alkanes (C_9–C_{23}) was used to obtain the retention indices. Individual components were identified by matching their retention indices (RI) with those reported in the literature. Further, GC–MS analysis was carried out on a Shimadzu (GC 2010) GC–MS equipped with an AOC-5000 auto-injector coupled and an SH-Rxi-5Sil MS capillary column (30 m × 0.25 mm i.d., 0.25 µm). The initial temperature of the column was 40 °C held for 4 min and was programmed to 220 °C at 4 min, then held for 44 min at 220 °C; the sample injection volume was 1 µL in the HPLC-grade dichloromethane. Helium was used as carrier gas at a flow rate of 1.28 mL/min on the split mode (1:10). After analysing the GC and GC–MS data, compounds were identified by comparing the mass spectral patterns of the fraction and their Arithmetic Index (AI) with those reported in the literature [29] and by executing a library search of the National Institute of Standards and Technology (NIST) library [30].

UHPLC–QTOF-IMS analysis of *R. officinalis* EAE

Ethanolic aqueous extract (EAE) of *R. officinalis* was dissolved in LC–MS grade methanol to get a 10 mg/mL concentration and injected into a high-resolution 6560 Ion Mobility Q-TOF LC/MS (Agilent, Santa Clara, USA). An Acquity BEH C18 column (2.1 mm × 100 mm, 1.7 µm) was utilized to separate the desired metabolite. The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The gradient program initiated at 5% B at 0 min, increased to 20% B over 4 min, and then ramped from 20 to 30% B between 4

and 6 min. It further increased to 40% B from 6 to 10 min, 50% B from 10 to 12 min, and 58% B from 12 to 18 min. After that, the concentration of B was reduced to 25% from 18 to 22 min, and then the mobile phase returned to the initial condition of 5% B by 25 min. The system was held at 5% B until 30 min to equilibrate the column. Elution was carried out with a solvent flow rate of 0.25 mL/min [31]. Mass spectrometry parameters were as follows; ionization mode, ESI positive; drying gas temperature, 300 °C; drying gas flow, 5.0 L/min; nebulizer gas pressure, 35 psi; capillary voltage, 3500 V; fragmentor voltage, 400 V; detection window, 100 ppm; MS scan range and rate, 100–1700 m/*z* at 1 spectra/s; MS threshold, 200; MS/MS threshold.

Toxicity bioassay of *R. officinalis* EAE, its fractions and compounds against test insects

Toxicity of EAE, its fractions and compounds were evaluated against target pests as per standard methods [12, 32]. Six different concentrations of EAE/fractions were prepared based on geometric progression (312.5–10,000 mg/L) by blending each EOs in 0.05% Tween 80 and distilled water solution. Briefly, 100 mg of EAE/fractions were mixed in 10 mL of 0.05% Tween 80 and distilled water then sonicated for complete dissolution for 5 to 10 min. Fresh leaf discs prepared from French bean and tea leaves (3 cm^2) for *A. craccivora* and *P. lilacinus* were used for bioassay. The leaf discs were embedded in the water-agar medium in Petri dishes (55 mm^2) for test insects to maintain the freshness. In every Petri dish, 10 nymphs and crawlers were released in each concentration (50 insects/treatment) and 1–2 µg sample was administered on the dorsal side of targeted insects by using micropipettes (Eppendorf®) in controlled environment as described above (Sect. "Rearing of experimental insects") for observation. Each treatment was replicated five times. The mortality of target insects was recorded after 72 and 96 h of treatment. For the negative control, insects were treated with distilled water containing 0.05% Tween 80. The commercially available neem-based formulation (Neem Baan®) contains azadirachtin 0.15% EC, at the recommended dose (5 mL/L) used as a positive control for comparison.

Toxicity of blends/mixtures of *R. officinalis* EAE and its fractions against test insects

Toxicity of blends/mixtures of *R. officinalis* EAE and fractions against test insects was done as per the procedure described in Sect. "Toxicity bioassay of R. officinalis EAE, its fractions and compounds against test insects". Based on the already reported study combination of extracts with fractions (EAE + HF, EAE + EAF, EAE + BF and EAE + WF) were prepared at five concentrations with different ratios (1:1, 3:1, and 1:3), the individual LD_{50} values of the EAE and fractions) for synergistic activity against target pests. To determine the co-toxicity coefficient [33–36], the following equation was followed:

CTC = [LD_{50} of compound/LD_{50} of the compound in combination] × 100.

If the mixtures provide CTC > 100 (synergistic action), CTC < 100 (individualistic action), and CTC = 100 (similar action).

Bio-efficacy of *R. officinalis* EAE against *A. craccivora* under greenhouse conditions

Efficacy of *R. officinalis* EAE at different concentrations (5, 10 and 20 mg/L) was evaluated against *A. craccivora* in greenhouse conditions. *P. vulgaris* plants were grown in plastic pots (20 × 15 cm) mixed with potting mixture (sand:soil:compost at 1:1:1 ratio). The potted plants were arranged in a completely randomized design (CRD). Four-week-old plants were infested with adults of *A. craccivora* and allowed to reproduce for seven days. After the population had crossed the economic threshold level (ETL), different concentrations/treatments were applied to plants with a hand sprayer (Kisankraft® -KK-PS3000). The number of *A. craccivora* nymphs and adults per plant was recorded by selecting three leaves one each at the top, middle, and bottom at 3, 5, and 7 days after treatment (DAT) and represented as mean no. of aphids/leaf. Similarly, one twig of 5 cm/plant was selected, and the number of aphids was recorded and represented as mean number of aphids/twigs. There were six treatments and each treatment replicated five times. The commercially available neem formulation, i.e., Neem Baan, contains azadirachtin 0.15% EC and dimethoate 30 EC (Rogar®), were used as a positive control for comparison. Tween 80™ was used as negative control.

Reproductive inhibitory activity of *R. officinalis* EAE and its fractions against *A. craccivora*

EAE and fractions were assessed for their reproductive capacity against apterous aphids after 96 h as per earlier method [37]. EAE and their fractions were tested for six different concentrations (312.5, 625, 1250, 2500, 5000 and 10,000 mg/L) based on geometric progression by blending each EAE and their fractions in 0.05% Tween 80 and distilled water solution. Briefly, 100 mg of extract/fractions were mixed in 10 mL of 0.05% Tween 80 and distilled water then sonicated for complete dissolution for 5 to 10 min. Fresh leaf discs were prepared from French bean leaves (3 cm^2) and were utilized under controlled conditions for bioassay. The leaf discs of French bean were embedded upon water-agar medium in Petri dishes (55 mm^2). In every Petri dish, 10 nymphs were released in each concentration (50 insects/treatment). From the

above concentrations prepared from stock solution, 1 µL of sample was administered on the dorsal side of the nymph of *A. craccivora* by using micropipette (Eppendorf ®) in a controlled environment. Number of newly reproduced nymphs from alive adults of *A. craccivora* was recorded after 96 h of treatment. For the negative control, insects were treated with distilled water containing 0.05% Tween 80. The commercially available neem formulation, i.e., Neem Baan, contains azadirachtin 0.15% EC, was used as a positive control for comparison.

The inhibition rate (IR) was calculated according to the formula:

$$\mathrm{IR}\,\% = [(\mathrm{C} - \mathrm{T})/\mathrm{C}] \times 100,$$

where C is the number of aphid progeny for the control and T is the number of aphid progeny in the treatment.

Deterrent activity of *R. officinalis* EAE and its fractions against *A. craccivora*

Deterrent activity of EAE and fractions against aphid was investigated [38, 39] at five concentrations (625, 1250, 2500, 5000 and 10000 mg/L). *R. officinalis* EAE and fractions were coated on the leaf disc with a brush on half of the area with respective concentrations and on the other half with DH_2O. After 15 min of drying, 10 apterous *A. craccivora* were released onto the midrib of leaf disc and inverted to hinder the insect escape. Each treatment was performed in five replicates and the number of aphids on the treated and non-treated sides were counted after 1 h interval up to 24 h. The number of live aphids on each side was counted after 24 h of treatment. Per cent deterrence index (PDI) was calculated as per the formula given below:

$$\mathrm{PDI} = (\mathrm{C} - \mathrm{T})/(\mathrm{C} + \mathrm{T}) \times 100,$$

where C is the number of aphids in the control and T is the number of aphids on the treated side.

Enzyme inhibition assays

The effect of *R. officinalis* EAE on AChE, GST, MFO and CES1 enzyme inhibition activities in *A. craccivora* and *P. lilacinus* was studied as per the standard methodology [12, 13, 40]. Four concentrations of *R. officinalis* EAE were tested in GP (1250–10,000 mg/L) upon nymphs of *A. craccivora* and crawlers of *P. lilacinus* as described in Sect. "Toxicity bioassay of R. officinalis EAE, its fractions and compounds against test insects". After 24 h treatment 20 mg of survived nymphs and crawlers were collected in triplicates for each concentration and stored in − 20 °C until further analysis. The collected samples were then homogenized in phosphate buffer (0.1 M, pH 7.4) with the mortar pestle (75 mm) under ice bath conditions. The supernatant was collected for each treatment in clean sterile microcentrifuge tubes (1.5 mL) after 30 min of centrifugation at 12,000 rpm. Before proceeding with enzyme activity, protein quantity was measured for all the treatments [41] in triplicates and average value were taken.

By adding 10 µL of homogenate, 30 µL of Milli-Q water in 160 µL of Bradford reagent (Coomassie blue G-250) developed blue colour was measured at 595 nm optical density at room temperature. The intensity of blue colour is directly proportional to the concentration of proteins in the samples. For AChE assay, dilutions were made concerning with lower concentrations of protein quantification. Diluted 25 µL homogenates in triplicates were incubated for 30 min under 4 °C with 25 µL of the reaction mixture (50 µL of DTNB, 50 µL of acetylthiocholine, and 900 µL of assay buffer). The AChE activity was spectrophotometrically measured at 410 nm and represented as milliunits per millilitre of protein (mU/mL). In GST assay, the reaction contains 100 µL of solution was added to the reaction, along with 75 µL of assay buffer, 10 µL of homogenized sample and 10 µL of glutathione. To initiate the reactions, 5 µL CDNB (1-Chloro-2,4-dinitrobenzene) was introduced into each well of 96-well microtiter plate at 37 °C. After a 60-s lag period, enzyme kinetics were measured at 340 nm absorbance for 30 min in a microplate reader. GST activity was then calculated using extinction coefficient of 0.0096 µM-1 for CDNB, and results were expressed in nanomolar per minute per millilitre (nmol/min/mL). Subsequently, to start MFO enzyme activity, all reagents were maintained at RT. To initiate biochemical reaction, 10 µL of homogenized sample, 20 µL of reaction buffer, 5 µL of coenzyme, and 15 µL of substrate were added to each well of a microtitre plate in triplicate and mixed thoroughly. After proper mixing, microtiter plate was placed in the hot air oven (Equitron®) at 37 °C for 30 min and 50 µL of stop solution was added to stop the reaction. The developed blue colour was measured at 405 nm at 10-s intervals for 10 min and expressed as U/mg. CES1 enzyme activity, 50 µL homogenate was mixed with equal volume of 1X incubation buffer in the antibody-coated microtiter plate and incubated at RT for 2 h with continuous agitation. The plate was then washed three times with 1X wash buffer, and 200 µL of 1X activity solution was immediately added, followed by 10–15 min incubation. After washing, 100 µL of detector antibody was added and incubated at RT for 30 min with continuous shaking. Subsequently, 100 µL of 1X of horseradish peroxidase (HRP) label was added and incubated at RT for 30 min with proper mixing. Following a final wash, 100 µL HRP development solution was added to each well, and the developed blue colour was optically measured at 402 nm. The CES1 activity was presented as mOD/min. GST

Table 1 Chemical composition of *n*-hexane fraction of *R. officinalis*

Sr. no	Name	AI_{Lit}	AI_{cal}	Area (%)	Mode of identification
1	α-Pinene	932	935	1.56 ± 0.03	AI, MS
2	Sabinene	969	975	0.67 ± 0.01	AI, MS
3	β-Pinene	974	982	0.61 ± 0.02	AI, MS
4	Camphene	946	961	7.12 ± 0.09	AI, MS
5	Myrcene	988	987	10.67 ± 0.09	AI, MS
6	D-Limonene	1024	1029	2.78 ± 0.09	AI, MS
7	1,8-Cineole	1026	1032	14.26 ± 0.11	AI, MS
8	*p*-Cymene	1020	1029	0.93 ± 0.01	AI, MS
9	L-Linalool	1095	1099	1.37 ± 0.03	AI, MS
10	Camphor	1141	1147	1.30 ± 0.02	AI, MS
11	Verbenone	1204	1208	0.59 ± 0.01	AI, MS
12	*Trans*-caryophyllene	1408	1422	1.05 ± 0.01	AI, MS
13	γ-Cadinene	1513	1511	2.47 ± 0.02	AI, MS
14	δ-Cadinene	1522	1521	1.46 ± 0.02	AI, MS
15	Elemol	1548	1551	0.63 ± 0.02	AI, MS
16	γ-Eudesmol	1630	1618	0.68 ± 0.01	AI, MS
17	*trans*-Phytol	1942	1947	5.75 ± 0.06	AI, MS
18	Hexadecenoic acid	1921	1926	9.91 ± 0.12	AI, MS
19	Linoleic acid	2092	2099	6.95 ± 0.14	AI, MS
20	Linolenic acid	2099	2099	24.97 ± 0.26	AI, MS
	Unidentified	–	–	4.27 ± 0.10	
Total				99.53	
Monoterpene hydrocarbons*				29.25	
Oxygenated monoterpene*				3.26	
Sesquiterpene hydrocarbons*				4.98	
Oxygenated sesquiterpene*				50.88	

*AI_{Lit}: arithmetic index (Adams, 2017), AI_{cal}: arithmetic index (calculated), MS: identified based on mass spectra

and CES1 enzyme activities were optically measured by using a microplate spectrophotometer (Biotek SYNERGY H1®).

Scanning electron microscopy (SEM) analysis of *A. craccivora*

In toxicity studies, EAE revealed promising efficacy against *A. craccivora.* Therefore, *R. officinalis* nymphs were selected to investigate their morphological characteristics for SEM studies. The *A. craccivora* samples were prepared according to standard protocols [42, 43]. Both treated and control insects were fixed in a 2.5% glutaraldehyde solution in phosphate buffer (0.1 M; pH 7.2) for 2 h after being treated with EAE (2500 and 5000 mg/L) for 24, 48 and 72 h. After fixation, the samples were washed and air-dried. Afterward, the specimens were immersed in a series of different ethanol solutions for drying, and then fixed onto aluminium stubs with the use of dual-sided sticky carbon tape. The samples were then treated to gold sputter coating (Hitachi MC1000 ion sputter, Japan) for 10 s, maintaining a vacuum pressure of 10 Pascal. The morphological characteristics of the surface were analysed using SEM images that were obtained at an appropriate resolution using a (SU 3900 Hitachi®).

Data analysis

The LD_{50} values were determined based on Probit analysis [44] by considering the mortality of test insects (number of insects responded out of number of insects used) after 72 and 96 h of treatment. The data on the per cent deterrent index, reproductive and enzyme inhibition were analysed using one-way analysis of variance (ANOVA) with SPSS software 26.0. Tukey's post hoc was used for mean comparison and levels of significance

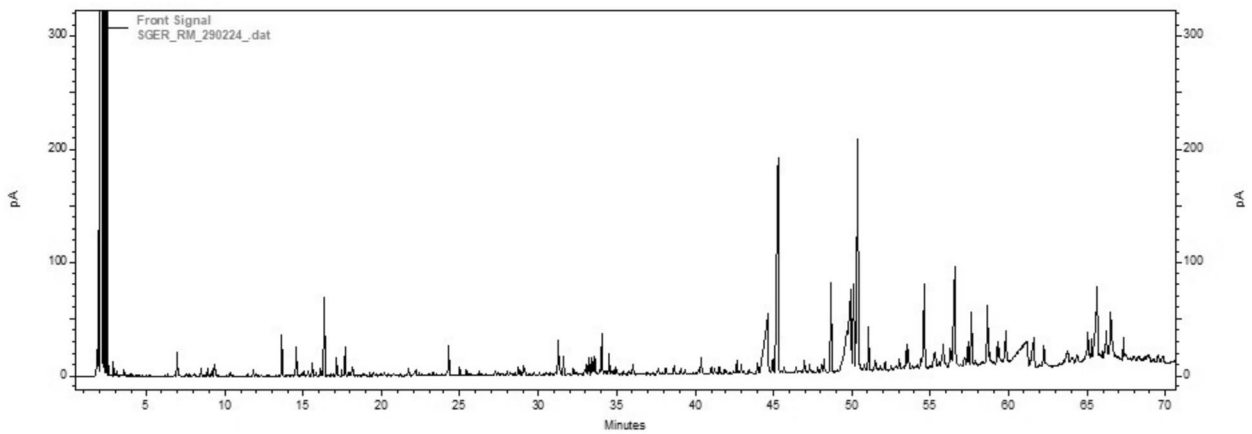


Fig. 1 Chromatogram of *n*-hexane fraction of *R. officinalis*

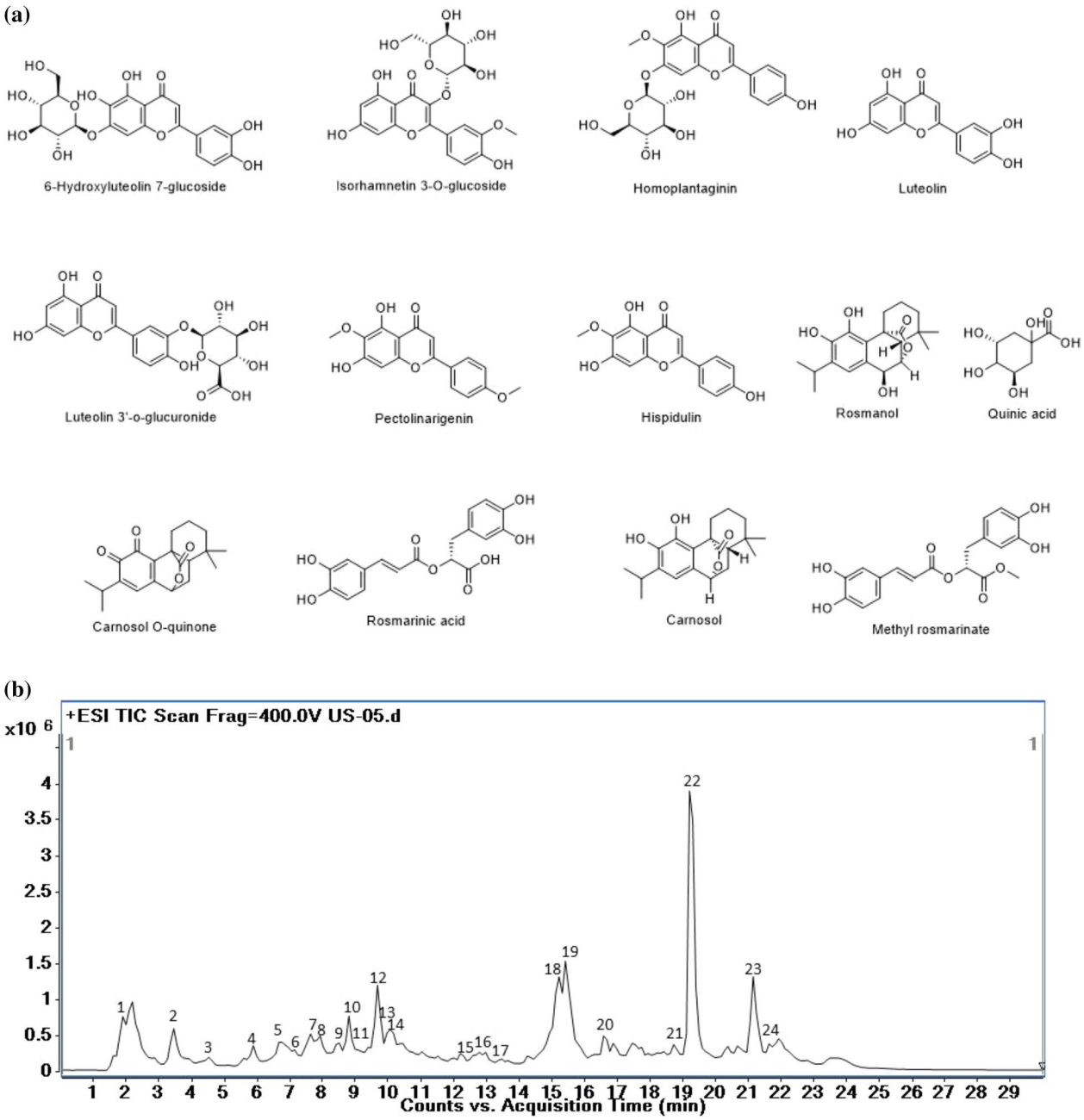


Fig. 2 **a** Major identified molecules in ethanol aqueous extract of *R. officinalis* by UHPLC–ESI-QTOF-MS. **b** TIC chromatogram of EAE of *R. officinalis*

[45]. GraphPad Prism 8.0 and ChemDraw Professional 12.0 software were used to generate graphical figures and compound structures.

Results

GC/GC–MS analysis of volatile *n*-hexane fraction

GC/GC–MS analysis of *n*-hexane fraction of *R. officinalis* is presented in Table 1 and Fig. 1. About 20 compounds accounted for 99.53% of *n*-hexane fraction, which comprises monoterpene hydrocarbons (29.25%), oxygenated monoterpenes (3.26%), and oxygenated sesquiterpenes (50.88%). The major constituents present in the *n*-hexane fraction were linolenic acid (24.97%), 1,8-cineole (14.26%), myrcene (10.67%), hexadecenoic acid (9.91%), camphene (7.71%), *trans*-phytol (5.75%), and other components includes γ-cadinene, *l*-linaloo,l α-pinene, sabinene, β-pinene, myrcene, D-limonene.

Table 2 Metabolites identified by UHPLC–ESI-QTOF-MS in EAE of *R. officinalis*

Peak no	Rt	Compound	*m/z* (actual)	*m/z* (Observed)	Fragments (MS^2)	References	Toxicity effect
1	1.898	Unidentified glycosidic derivative	542	543.14 $(M+H)^+$	381 (M + H-glucose/galactose)$^+$, 335, 231	–	–
2	4.327	Chlorogenic acid glycoside	618	619.13 $(M+H)^+$	487 (M + H-xylose/arabinose)$^+$, 355 (487-xylose/arabinose)$^+$, 221	–	*A. gossypii* (LC_{50} = 3260.84 ppm) [54]
3	5.783	Umbelliferone derivative	376	377.09 $(M+H)^+$	163 $(C_9H_7O_3)^+$	–	*Tetranychus cinnabarinus* (% Mortality = 71.20) [55]
4	6.468	Umbelliferone derivative	364	365.09 $(M+H)^+$	163 $(C_9H_7O_3)^+$	–	–
5	6.757	Quinic acid	192	193.09 $(M+H)^+$	193, 163	[48, 49]	*Myzus persicae* [56]
6	7.634	Unidentified	410	411.13 $(M+H)^+$	227, 209, 191	–	–
7	8.412	6-Hydroxyluteolin-7-*O*-glucoside	464	465.11 $(M+H)^+$	303 (M + H-glucose)$^+$	[49]	–
8	8.896	Unidentified glucosidic derivative	624	625.18 $(M+H)^+$	463 (M + H-glucose)$^+$	–	–
9	9.287	Isorhamnetin-3-*O*-glucoside	478	479.12 $(M+H)^+$	317 (M + H-glucose)$^+$	[19, 49]	–
10	9.869	Homoplantaginin	462	463.13 $(M+H)^+$	301 (M + H-glucose)$^+$	[19]	–
11	10.645	Luteolin-3′-acetyl-*O*-glucuronide	504	505.10 $(M+H)^+$	287 $(C_{15}H_{11}O_6)^+$, 207	[48, 49]	–
12	10.843	Hispidulin glucuronide	476	477.15 $(M+H)^+$	301 (M + H-glucuronic acid)$^+$, 299	[49]	–
13	11.426	Luteolin	286	287.06 $(M+H)^+$	235, 163	[49]	Antifeedant to *Acyrthosiphon pisum* [57]
14	11.618	Luteolin derivative	324	325.23 $(M+H)^+$	287 $(C_{15}H_{11}O_6)^+$	–	Toxicity to *Spodoptera frugiperda* [58]
15	12.298	Umbelliferone derivative	208	209.08 $(M+H)^+$	177, 163 $(C_9H_7O_3)^+$	–	–
16	15.217	Pectolinarigenin	314	315.09 $(M+H)^+$	301, 274, 203	[49]	Antifeedant (89.41%) to *Helicoverpa armigera* [59]
17	15.604	Pectolinarigenin isomer	314	315.09 $(M+H)^+$	301, 274, 203	[49]	Larvicidal (83.77%) to *H. armigera* [59]
18	15.797	Hispidulin	300	301.18 $(M+H)^+$	273, 231, 149	[19, 49]	Larvicidal for *S. litura* [60]
19	16.482	Rosmanol	346	347.19 $(M+H)^+$	369 $(M+Na)^+$, 347 $(M+H)^+$, 329, 301, 273	[19, 48, 49]	–
20	19.202	Carnosol-*O*-quinone	328	351.09 $(M+Na)^+$	329 $(M+H)^+$	[49]	–
21	19.883	Rosmarinic acid	360	383.19 $(M+Na)^+$	361 $(M+H)^+$, 345 $(C_{18}H_{17}O_7)^+$, 245	[19, 49]	Toxicity to *A. pisum* (LC_{50} = 0.2 ppm) [61]
22	20.660	Methoxycarnosol	360	361.21 $(M+H)^+$	273, 203	[19]	–
23	20.952	Carnosol	330	353.18 $(M+Na)^+$	331 $(M+H)^+$, 285 $(C_{19}H_{25}O_2)^+$, 183	[19, 48, 49]	–
24	21.144	Unidentified glucosidic derivative	844	845.95 $(M+H)^+$	683 (M + H-glucose/galactose)$^+$	–	–
25	21.826	Sugiol,5-dehydro	298	299.10 $(M+H)^+$	213 $(C_{16}H_{21})^+$, 169	[50]	–
26	22.020	Carnosol isomer	330	353.18 $(M+Na)^+$	331 $(M+H)^+$, 285 $(C_{19}H_{25}O_2)^+$	[19, 48, 49]	–
27	22.604	Methyl rosmarinate	374	397.20 $(M+Na)^+$	375 $(M+H)^+$, 273, 204, 127	[49]	–

Identification of metabolites in EAE of *R. officinalis* using UHPLC–QTOF-IMS

About 27 distinct peaks were identified in the EAE (Fig. 2a, b) and these assigned peaks were based on their retention time (RT), molecular weight comparison, and mass fragmentation patterns. Major phyto-constituents identified in the extract include phenolic compounds, flavonoids, and glycosidic flavonoids. The analysed data for assigned metabolites are presented in Table 2 and Figure S1-S28.

In *R. officinalis* extract, a majority of phenolics, flavonoids, and their glycosidic derivatives were

Table 3 Toxicity of EAE and its fractions of *R. officinalis* against *A. craccivora* and *P. lilacinus*

Extract/fractions	Time (h)	LD_{50} (µL/insect)	Confidence limits (µL/insect)	Slope ± SE	Chi-square	*p*-value
A. craccivora						
EAE	72	3.86	2.64–6.63	0.92 ± 0.15	4.40	0.35
	96	1.84	1.33–2.58	1.08 ± 0.15	3.05	0.55
HF	72	4.39	3.12–7.04	1.10 ± 0.16	1.90	0.75
	96	2.22	1.60–3.17	1.07 ± 0.16	2.22	0.69
EAF	72	10.01	5.59–31.30	0.79 ± 0.16	2.06	0.72
	96	4.27	2.79–8.13	0.85 ± 0.15	1.19	0.88
BF	72	11.04	7.36–21.44	1.34 ± 0.21	2.91	0.57
	96	6.79	4.74–11.67	1.21 ± 0.18	1.83	0.77
WF	72	6.59	4.23–13.70	0.93 ± 0.17	1.60	0.81
	96	4.15	2.89–6.87	1.01 ± 0.16	1.50	0.83
Azadirachtin (0.15% EC)	72	1.66	1.24–2.36	1.25 ± 0.16	1.89	0.76
	96	0.76	0.59–0.97	1.12 ± 0.89	1.48	0.89
P. lilacinus						
EAE	72	9.67	6.50–18.13	0.96 ± 0.20	0.89	0.82
	96	5.84	3.92–9.16	0.96 ± 0.19	0.41	0.94
HF	72	2.87	1.59–5.80	0.68 ± 0.19	2.24	0.52
	96	1.46	0.62–2.41	0.73 ± 0.19	1.55	0.67
EAF	72	3.57	2.45–5.82	0.99 ± 0.20	0.61	0.89
	96	2.01	1.24–3.02	0.92 ± 0.19	1.27	0.73
BF	72	3.44	2.68–4.58	1.57 ± 0.22	3.93	0.27
	96	2.29	1.76–2.98	1.51 ± 0.21	3.72	0.29
WF	72	6.46	4.19–11.09	0.87 ± 0.19	1.08	0.78
	96	4.25	2.65–6.42	0.92 ± 0.19	0.47	0.93
Azadirachtin (0.15% EC)	72	6.80	4.67–12.68	1.15 ± 0.21	0.03	0.99
	96	3.73	2.76–5.41	1.27 ± 0.20	0.47	0.93

LD_{50} values differ significantly only in case confidential intervals fail to overlap; EAE-ethanol aqueous extract; HF-hexane fraction; EAF-ethyl acetate fraction; BF-butanol fraction; WF-water fraction

acknowledged. Peak no. 1 at retention time is 1.898 min with a molecular ion peak at *m/z* 543.14 $(M+H)^+$. This was identified as a glycosidic derivative (1), based on its mass fragments at *m/z* 381 $(M+H\text{-glucose/galactose})^+$, indicating the loss of a glucose or galactose sugar moiety. Similarly, peak no. 8 (rt: 8.896 min; *m/z*: 625.18) and peak no. 24 (rt: 21.144 min; *m/z*: 845.95) were also assigned as glycosidic derivatives, based on their MS/MS fragment ion peaks which showed the loss of specific saccharide units. Peak no. 2 (rt 4.327 min; *m/z*: 619.13) was assigned as a glycosidic derivative of chlorogenic acid, displaying two major fragment ion peaks at *m/z* 487 $(M+H\text{-xylose/arabinose})^+$ and 355 $(487\text{-xylose/arabinose})^+$, respectively. Additionally, peak no. 7 (rt 8.412 min; *m/z*: 465.11), 9 (rt 9.287 min; *m/z*: 479.12), 10 (rt 9.869 min; *m/z*: 463.13), 11 (rt 10.645 min; *m/z*: 505.10), and 12 (rt 10.843 min; *m/z*: 477.15) were identified as flavonoid glycosides. These assignments were based on their observed fragment units and comparison with literature data, as presented in the Table. Peak no. 3, 4, and 15 were assigned as umbelliferone derivatives from the observed fragment at 163 $(C_9H_7O_3)^+$. Peak no. 7 was assigned as 6-hydroxyluteolin-7-*O*-glucoside, based on its mass fragment ion peak at *m/z* 303 $(M+H\text{-glucose})^+$. Similarly, peaks no. 9, 10, 11, and 12 were identified as isorhamnetin-3-O-glucoside (9), homoplantaginin (10), luteolin-3'-acetyl-O-glucuronide (11), and hispidulin glucuronide (12), respectively, based on fragment ion peaks observed after the loss of glucose and glucuronic acid moieties. Peaks no. 13, 20, 21, 23, 24, 26, and 27 were identified as luteolin, carnosol-*o*-quinone, rosmarinic acid, carnosol, carnosol isomer, and methyl rosmarinate, respectively, and were quantitatively dominant in the rosemary extract.

Toxicity of EAE, its fractions of *R. officinalis* and compounds against test insects

The experimental results on efficacy of EAE and its fractions against test insects are presented in Tables 2, 3, 4,

Table 4 Toxicity of compounds identified in volatile fraction of *R. officinalis* against *A. craccivora* and *P. lilacinus*

Compounds	Time (h)	LD_{50} (µL/insect)	Confidence limits (µL/insect)	Slope ± SE	Chi-square	*p*-value
A. craccivora						
Linolenic acid	72	1.93	1.49–2.63	1.53 ± 0.22	3.88	0.27
	96	0.99	0.79–1.24	1.83 ± 0.23	4.27	0.23
1,8-Cineole	72	2.35	1.71–3.68	1.22 ± 0.21	2.82	0.96
	96	1.20	0.93–1.54	1.62 ± 0.22	0.72	0.87
Myrcene	72	1.52	1.17–2.02	1.47 ± 0.21	1.74	0.63
	96	1.01	0.79–1.28	1.70 ± 0.22	0.64	0.89
Camphene	72	2.38	1.73–3.68	1.25 ± 0.21	0.33	0.95
	96	1.76	1.29–2.57	1.22 ± 0.20	1.51	0.68
Hexadecenoic acid	72	3.79	2.50–7.96	1.08 ± 0.21	0.26	0.97
	96	2.34	1.68–3.74	1.17 ± 0.21	1.62	0.65
P. lilacinus						
Linolenic acid	72	1.24	0.88–1.77	1.14 ± 0.20	0.33	0.95
	96	0.59	0.44–0.74	1.89 ± 0.25	5.11	0.16
1,8-Cineole	72	1.53	1.10–2.23	1.15 ± 0.20	2.43	0.49
	96	0.63	0.46–0.79	1.77 ± 0.24	2.39	0.51
Myrcene	72	1.45	1.12–1.89	1.54 ± 0.21	0.62	0.89
	96	0.92	0.73–1.14	1.90 ± 0.23	3.16	0.36
Camphene	72	3.82	2.72–6.53	1.38 ± 0.27	0.42	0.94
	96	1.57	1.19–2.15	1.38 ± 0.21	1.26	0.74
Hexadecenoic acid	72	2.29	1.54–4.25	0.95 ± 0.19	0.64	0.89
	96	1.37	0.94–2.05	1.04 ± 0.20	2.13	0.54

*LD_{50} values differ significantly only in case confidential intervals fail to overlap

5. Among extract/fractions, EAE was found more effective against *A. craccivora* after 72 and 96 h of treatment (LD_{50} = 3.86 and 1.84 µL/nymph, respectively) followed by *n*-hexane (LD_{50} = 4.39 and 2.22 µL/nymph,) and water fraction (LD_{50} = 6.59 and 4.15 µL/ nymph) as compared to *n*-butanol fraction. However, the positive control was superior to the extract and fractions. In *P. lilacinus,* *n*-hexane fraction revealed high efficacy (LD_{50} = 2.87 and 1.46 µL/crawler, respectively) after 72 and 96 h of treatment followed by *n*-butanol (LD_{50} = 3.44 and 2.29 µL/crawler) and ethyl acetate fraction (LD_{50} = 3.57 and 2.01 µL/crawler) as compared to water fraction and EAE. However, *n*-hexane, ethyl acetate and *n*-butanol fractions were superior to the positive control (Neem Baan) after 96 h of treatment.

Among the compounds, linolenic acid (LD_{50} = 1.93 µL/nymph) was found more superior against *A. craccivora* after 72 h of treatment (Table 4) preceded by myrcene (LD_{50} = 1.52 µL/nymph), 1,8-cineole, camphene (LD_{50} = 2.35–2.38 µL/nymph) than hexadecenoic acid. Similarly, 96 h after treatment, linolenic acid and myrcene (LD_{50} = 0.99 and 1.01 µL/nymph) were highly effective preceded by 1,8-cineole (LD_{50} = 1.20 µL/nymph) in contrast to camphene and hexadecenoic acid. All the tested compounds are superior against *A. craccivora* as compared to the positive control. In *P. lilacinus,* linolenic acid (LD_{50} = 1.24 µL/crawler) was found more superior within 72 h of exposure and was preceded by myrcene and 1,8-cineole (LD_{50} = 1.45–1.53 µL/ crawler) than hexadecenoic acid and camphene. Similarly, 96 h after treatment, linolenic acid and 1,8-cineole (LD_{50} = 0.59–0.63 µL/crawler) was found highly effective preceded by myrcene (LD_{50} = 0.92 µL/crawler) in contrast to hexadecenoic acid and camphene. All the tested compounds are superior to the positive control.

Bio-efficacy of *R. officinalis* EAE against *A. craccivora* under greenhouse conditions

The efficacy of *R. officinalis* EAE against *A. craccivora* in greenhouse conditions is presented in Tables 5 and 6. In leaf, among different concentrations of *R. officinalis* EAE evaluated against *A. craccivora*, after first spray, higher dose (20 g/L) was significantly more effective (10.13 aphids/leaf) ($F_{5,29}$ = 52.10; $p < 0.0001$) in controlling *A. craccivora* after 3 days of treatment and was at par with next concentration at 10 g/L (12.80 aphids/leaf) as compared to the lower dose (5 g/L) as control (32.70 aphids/leaf). However, the higher dose of *R.*

Table 5 Bio-efficacy of *R. officinalis* EAE against *A. craccivora* on cowpea plants in greenhouse conditions

Treatments	Pre-count	No. of aphids/leaf (days after I spray)			No. of aphids/leaf (days after II spray)		
		3 DAT	5 DAT	7 DAT	3 DAT	5 DAT	7 DAT
R. officinalis (20 g/L)	85.07 ± 5.72 (9.20 ± 0.32)	10.13 ± 0.75 (3.25 ± 0.11)^{bc}	11.77 ± 0.56 (3.49 ± 0.07)^{bc}	24.27 ± 3.22 (4.93 ± 0.32)^{abc}	10.63 ± 1.30 (3.32 ± 0.18)^{ab}	11.07 ± 1.25 (3.38 ± 0.19)^{ab}	15.07 ± 1.16 (3.93 ± 0.15)^{b}
R. officinalis (10 g/L)	84.80 ± 2.41 (9.20 ± 0.12)	12.80 ± 0.87 (3.64 ± 0.12)^{bc}	19.83 ± 1.67 (4.49 ± 0.27)^{bc}	32.20 ± 5.28 (5.65 ± 0.45)^{bc}	19.17 ± 3.05 (4.38 ± 0.35)^{ab}	18.01 ± 1.55 (4.29 ± 0.18)^{ab}	20.23 ± 1.70 (4.54 ± 0.18)^{bc}
R. officinalis (5 g/L)	80.97 ± 5.21 (9.10 ± 0.22)	18.13 ± 2.80 (4.27 ± 0.30)^{c}	29.33 ± 2.99 (5.43 ± 0.37)^{c}	40.03 ± 4.22 (6.33 ± 0.32)^{cd}	24.80 ± 3.59 (4.96 ± 0.41)^{b}	25.80 ± 1.29 (5.12 ± 0.12)^{b}	27.27 ± 1.98 (5.25 ± 0.19)^{c}
Neem Baan (5 mL/L)	50.37 ± 5.44 (7.31 ± 0.37)	6.17 ± 0.92 (2.56 ± 0.41)^{ab}	5.20 ± 1.56 (2.24 ± 0.74)^{a}	10.33 ± 0.40 (3.29 ± 0.06)^{ab}	9.63 ± 0.87 (3.17 ± 0.14)^{ab}	8.37 ± 0.62 (2.97 ± 0.11)^{ab}	14.30 ± 0.98 (3.84 ± 0.13)^{b}
Dimethoate (1.5 mL/L)	36.47 ± 7.63 (6.11 ± 0.61)	0.00 ± 0.00 (0.71 ± 0.00)^{a}	0.00 ± 0.00 (0.71 ± 0.00)^{a}	4.10 ± 0.23 (2.14 ± 0.05)^{a}	0.93 ± 0.52 (1.12 ± 0.21)^{a}	0.16 ± 0.10 (0.79 ± 0.08)^{a}	1.53 ± 0.53 (1.14 ± 0.43)^{a}
Control	78.80 ± 8.45 (8.98 ± 0.40)	32.70 ± 4.56 (5.70 ± 0.41)^{d}	88.63 ± 5.89 (9.42 ± 0.31)^{d}	88.60 ± 13.30 (7.61 ± 0.89)^{d}	55.37 ± 9.26 (7.36 ± 0.65)^{c}	70.10 ± 11.21 (8.29 ± 0.69)^{c}	86.74 ± 4.28 (9.33 ± 0.23)^{d}
F-value	$F_{5,29}$ = 11.49; p > 0.067	$F_{5,29}$ = 24.87; $p < 0.0001$	$F_{5,29}$ = 71.53; $p < 0.0001$	$F_{5,29}$ = 10.92; $p < 0.0001$	$F_{5,29}$ = 19.88; $p < 0.0001$	$F_{5,29}$ = 28.48; $p < 0.0001$	$F_{5,29}$ = 184.42; $p < 0.0001$

*Figures in parentheses indicate √x + 0.5 transformations; Means followed by the same letters within a column are not statistically different (p > 0.05)

Treatments	% ROC in leaf (days after I spray)			% ROC in leaf (days after II spray)		
	3 DAT	5 DAT	7 DAT	3 DAT	5 DAT	7 DAT
R. officinalis (20 g/L)	65.08 ± 7.31 (54.17 ± 4.42)^{bc}	86.56 ± 0.42 (68.50 ± 0.35)^{ab}	65.42 ± 2.09 (54.01 ± 1.25)^{c}	89.36 ± 1.31 (71.08 ± 1.15)^{bc}	82.88 ± 3.13 (65.84 ± 2.22)^{bc}	82.28 ± 1.98 (65.24 ± 1.51)^{b}
R. officinalis (10 g/L)	57.00 ± 7.36 (49.18 ± 4.33)^{bc}	78.50 ± 3.10 (62.59 ± 2.15)^{bc}	54.94 ± 2.04 (47.40 ± 1.17)^{c}	80.84 ± 3.04 (64.31 ± 2.23)^{cd}	70.30 ± 7.00 (57.49 ± 4.35)^{bc}	76.02 ± 3.45 (60.87 ± 2.23)^{bc}
R. officinalis (5 g/L)	41.30 ± 8.91 (39.74 ± 5.27)^{c}	68.60 ± 4.49 (56.13 ± 2.77)^{c}	42.08 ± 3.31 (40.39 ± 1.94)^{d}	75.22 ± 3.59 (60.48 ± 2.64)^{d}	58.36 ± 8.29 (49.96 ± 4.89)^{d}	67.98 ± 3.45 (55.66 ± 2.15)^{c}
Neem Baan (5 mL/L)	78.54 ± 5.29 (63.11 ± 3.78)^{ab}	89.06 ± 6.24 (76.03 ± 7.43)^{ab}	84.04 ± 2.61 (66.72 ± 2.04)^{b}	90.36 ± 0.85 (71.98 ± 0.84)^{ab}	86.64 ± 2.75 (68.89 ± 2.15)^{bc}	83.46 ± 1.04 (66.04 ± 0.79)^{b}
Dimethoate (1.5 mL/L)	100.00 ± 0.00 (100.00 ± 0.00)^{a}	96.48 ± 3.52 (93.04 ± 6.96)^{a}	93.66 ± 1.08 (75.61 ± 1.26)^{a}	99.08 ± 0.52 (90.01 ± 4.19)^{a}	99.78 ± 0.22 (96.80 ± 3.20)^{a}	98.34 ± 1.66 (94.65 ± 5.35)^{a}
F-value	$F_{5,29}$ = 11.49; $p < 0.0001$	$F_{5,29}$ = 6.99; $p < 0.001$	$F_{5,29}$ = 80.29; $p < 0.0001$	$F_{5,29}$ = 17.12; $p < 0.0001$	$F_{5,29}$ = 9.29; $p < 0.0001$	$F_{5,29}$ = 19.76; $p < 0.0001$

*Figures in parentheses indicate angular/arcsine transformations; Means followed by the same letters within a column are not statistically different ($p > 0.05$)

officinalis EAE was at par with the positive control, i.e., Neem Baan (6.17 aphids/leaf). All the concentrations and Neem Baan are not superior to dimethoate. After 5 and 7 days after treatment, a similar trend was observed as reported at three days after treatment. After second spray, higher dose (20 g/L) was significantly more effective (7.64 aphids/leaf) ($F_{5,29} = 19.88$; $p < 0.0001$) in controlling *A. craccivora* after 3 days of treatment and was at par with next concentration at 10 g/L (13.17 aphids/leaf) followed by lower dose (5 g/L) as compared to control (55.37 aphids/leaf). However, two higher doses of *R. officinalis* EAE were at par with Neem Baan. All the concentrations of *R. officinalis* and Neem Baan are not superior than dimethoate. However, a similar trend in controlling the *A. craccivora* after 5 and 7 days after treatment (II spray) was also reported. For per cent reduction over control, all the concentrations showed significant reduction in the *A. craccivora* population. Among them, higher concentration showed higher reduction of aphid (65.08 to 86.56%) after 3, 5 and 7 days after treatment and was at par with next concentration at 10 g/L (49.18 to 62.59%) and Neem Baan (78.54 to 89.06%) as compared to lower dose (41.3 to 68.6%). However, a similar trend was observed in the second spray, but the per cent reduction of aphid was higher in the higher concentration (82.88 to 89.63%).

In twig, among different concentrations of *R. officinalis* EAE evaluated against *A. craccivora*, after first spray, higher dose (20 g/L) was significantly more effective (11.20 aphids/leaf) ($F_{5,29} = 97.93$; $p < 0.0001$) in controlling *A. craccivora* after 3 days of treatment and was at par with 10 g/L (14.70 aphids/leaf) and was followed by lower dose (5 g/L) as control (4.88 aphids/leaf). All the concentrations of *R. officinalis* and Neem Baan are not superior than dimethoate. At 5 and 7 days after treatment, higher dose of *R. officinalis* (20 g/L) was more effective against

Table 6 Bio-efficacy of *R. officinalis* EAE against *A. craccivora* on cowpea plants in greenhouse conditions

Treatments	Pre-count	No. of aphids/twig (days after I spray)			No. of aphids/twig (days after II spray)		
		3 DAT	5 DAT	7 DAT	3 DAT	5 DAT	7 DAT
R. officinalis (20 g/L)	88.80 ± 1.98 (9.45 ± 0.11)	11.20 ± 1.17 (3.40 ± 0.18)^{b}	13.20 ± 0.66 (3.70 ± 0.09)^{b}	18.40 ± 1.40 (4.34 ± 0.16)^{bc}	7.00 ± 0.76 (2.73 ± 0.14)^{b}	13.80 ± 0.75 (3.78 ± 0.10)^{b}	17.40 ± 1.61 (4.21 ± 0.19)^{b}
R. officinalis (10 g/L)	83.90 ± 1.35 (9.18 ± 0.07)	14.70 ± 1.76 (3.88 ± 0.22)^{b}	23.90 ± 2.10 (4.92 ± 0.23)^{c}	24.60 ± 1.87 (4.99 ± 0.19)^{c}	14.70 ± 1.11 (3.89 ± 0.14)^{c}	23.40 ± 1.07 (4.88 ± 0.11)^{c}	27.30 ± 2.40 (5.25 ± 0.23)^{c}
R. officinalis (5 g/L)	73.50 ± 7.44 (8.56 ± 0.44)	23.60 ± 2.82 (4.88 ± 0.28)^{c}	32.60 ± 1.86 (5.74 ± 0.16)^{d}	34.50 ± 3.07 (5.89 ± 0.25)^{d}	19.80 ± 1.08 (4.50 ± 0.12)^{c}	29.80 ± 1.98 (5.49 ± 0.18)^{c}	35.20 ± 2.49 (5.96 ± 0.22)^{d}
Neem Baan (5 mL/L)	45.00 ± 0.22 (6.75 ± 0.01)	12.40 ± 1.32 (3.57 ± 0.18)^{b}	4.80 ± 0.52 (2.29 ± 0.11)^{a}	11.10 ± 1.03 (3.39 ± 0.16)^{a}	0.00 ± 0.00 (0.71 ± 0.00)^{a}	7.30 ± 1.06 (2.77 ± 0.19)^{ab}	11.00 ± 0.91 (3.38 ± 0.14)^{b}
Dimethoate (1.5 mL/L)	37.80 ± 7.25 (6.05 ± 0.64)	0.00 ± 0.00 (0.71 ± 0.00)^{a}	0.00 ± 0.00 (0.71 ± 0.00)^{a}	0.00 ± 0.00 (0.71 ± 0.00)^{a}	0.00 ± 0.00 (0.71 ± 0.00)^{a}	0.00 ± 0.00 (0.71 ± 0.00)^{a}	0.00 ± 0.00 (0.71 ± 0.00)^{a}
Control	59.10 ± 12.63 (7.55 ± 0.80)	45.70 ± 0.83 (6.80 ± 0.06)^{d}	49.20 ± 3.06 (6.67 ± 0.23)^{e}	57.30 ± 3.11 (6.13 ± 0.25)^{d}	33.80 ± 3.04 (5.83 ± 0.26)^{d}	48.90 ± 3.77 (7.00 ± 0.28)^{d}	51.40 ± 1.86 (7.20 ± 0.13)^{e}
F-value	$F_{5,29}$ = 9.53; p > 0.085	$F_{5,29}$ = 97.93; $p < 0.0001$	$F_{5,29}$ = 96.23; $p < 0.0001$	$F_{5,29}$ = 47.15; $p < 0.0001$	$F_{5,29}$ = 83.92; $p < 0.0001$	$F_{5,29}$ = 88.19; $p < 0.0001$	$F_{5,29}$ = 106.78; $p < 0.0001$

*Figures in parentheses indicate √x + 0.5 transformations; Means followed by the same letters within a column are not statistically different ($p > 0.05$)

Treatments	% ROC in twig (days after I spray)			% ROC in twig (days after II spray)		
	3 DAT	5 DAT	7 DAT	3 DAT	5 DAT	7 DAT
R. officinalis (20 g/L)	75.40 ± 2.72 (60.47 ± 1.85)^{b}	69.26 ± 3.52 (56.38 ± 2.16)^{b}	47.96 ± 8.42 (43.61 ± 5.08)^{bc}	78.40 ± 3.42 (62.58 ± 2.40)^{b}	70.68 ± 3.83 (57.38 ± 2.38)^{b}	85.72 ± 4.13 (54.28 ± 2.46)^{b}
R. officinalis (10 g/L)	67.60 ± 4.44 (55.46 ± 2.69)^{b}	43.84 ± 7.67 (41.39 ± 4.52)^{c}	33.74 ± 2.49 (35.45 ± 1.52)^{c}	54.74 ± 6.52 (47.70 ± 3.83)^{c}	51.36 ± 3.16 (45.79 ± 1.82)^{c}	66.36 ± 5.75 (42.83 ± 3.35)^{c}
R. officinalis (5 g/L)	48.26 ± 6.26 (43.93 ± 3.68)^{c}	24.78 ± 6.58 (29.02 ± 4.48)^{c}	29.06 ± 7.09 (31.91 ± 4.63)^{c}	39.06 ± 7.13 (38.37 ± 4.31)^{c}	37.70 ± 5.89 (37.67 ± 3.51)^{c}	31.76 ± 3.35 (34.18 ± 2.07)^{c}
Neem Baan (5 mL/L)	72.66 ± 3.31 (58.63 ± 2.12)^{b}	88.82 ± 1.54 (70.65 ± 1.45)^{ab}	69.20 ± 4.13 (56.49 ± 2.62)^{b}	100.00 ± 0.00 (100.00 ± 0.00)^{a}	84.80 ± 2.49 (67.31 ± 1.96)^{bc}	78.58 ± 1.62 (62.50 ± 1.18)^{b}
Dimethoate (1.5 mL/L)	100.00 ± 0.00 (100.00 ± 0.00)^{a}	100.00 ± 0.00 (100.00 ± 0.00)^{a}	100.00 ± 0.00 (100.00 ± 0.00)^{a}	100.00 ± 0.00 (100.00 ± 0.00)^{a}	100.00 ± 0.00 (100.00 ± 0.00)^{a}	100.00 ± 0.00 (100.00 ± 0.00)^{a}
F-value	$F_{5,29}$ = 22.28; $p < 0.0001$	$F_{5,29}$ = 41.45; $p < 0.0001$	$F_{5,29}$ = 29.38; $p < 0.0001$	$F_{5,29}$ = 320.51; $p < 0.0001$	$F_{5,29}$ = 47.81; $p < 0.0001$	$F_{5,29}$ = 55.95; $p < 0.0001$

*Figures in parentheses indicate angular/arcsine transformations; means followed by the same letters within a column are not statistically different ($p > 0.05$)

A. craccivora than other concentrations. However, Neem Baan and dimethoate are superior to *R. officinalis.* In second spray, among different concentrations of *R. officinalis* EAE evaluated, higher dose (20 g/L) was significantly more effective (7.0 aphids/leaf) ($F_{5,29}$ = 83.92; $p < 0.0001$) in controlling *A. craccivora* after 3 days of treatment and was followed by 10 and 5 g/L (3.89 and 4.50 aphids/leaf, respectively) as compared to control (33.80 aphids/leaf). However, a similar trend was observed after 5 and 7 days after treatment. The positive control was superior to all the concentrations of *R. officinalis*. For per cent reduction over control, all the concentrations showed significant reduction in the *A. craccivora* population. Among them, higher concentration showed higher reduction of aphid (47.96 to 75.40%) after 3, 5 and 7 days after treatment and was at par with next concentration at 10 g/L (33.74 to 67.60%) and Neem Baan (69.20 to 88.82%) as compared to lower dose (24.78to 48.26%). However, a similar trend was observed in the second spray, but the per cent reduction of aphids was higher in the higher concentration (70.68 to 85.72%).

Toxicity of blends/mixtures of EAE and its fractions of *R. officinalis* against test insects

To examine the interaction between *R. officinalis* EAE and its fractions, toxicity of different mixtures (1:1, 3:1, and 1:3 ratio, w/w) against aphid and mealy bug is presented in Table 7. Among blends/mixtures, EAE + HF (1:1) showed superior efficacy (LD_{50} = 1.19 µL/nymph against nymphs of aphid at 72 h and was followed by EAE + WF (LD_{50} = 1.84 µL/nymph) as compared to EAE + BF. Similarly, EAE + WF and EAE + HF were more effective (LD_{50} = 0.69–0.75 µL/insect) after 96 h as compared to EAE + BF and EAE + EAF. Similarly (3:1 ratio), EAE + HF and EAE + WF showed more promising toxicity (LD_{50} = 0.46–0.53 µL/nymph) to *A. craccivora* after

Table 7 Toxicity and synergistic effect of blends/mixtures of EAE and fractions of *R. officinalis* against *A. craccivora* and *P. lilacinus*

Blends/mixtures	Time (h)	LD_{50} (µL/insect)	Confidence limits (µL/insect)	Slope ± SE	Chi-square	*p*-value	Co-toxicity coefficient	Interaction type
A. craccivora								
EAE + HF (1:1)	72	1.19	0.77–2.08	0.84 ± 0.19	0.37	0.94	319.36	Synergistic
	96	0.75	0.47–1.11	0.96 ± 0.19	1.37	0.71	259.52	Synergistic
EAE + EAF	72	2.37	1.62–4.30	1.01 ± 0.20	1.34	0.72	161.42	Synergistic
	96	1.13	0.80–1.59	1.16 ± 0.20	2.07	0.55	171.77	Synergistic
EAE + BF	72	2.34	1.42–5.45	0.72 ± 0.19	1.63	0.65	163.46	Synergistic
	96	1.01	0.57–1.54	0.87 ± 0.19	0.50	0.92	193.07	Synergistic
EAE + WF	72	1.84	1.07–5.42	0.67 ± 0.19	0.77	0.85	208.23	Synergistic
	96	0.69	0.37–1.09	0.81 ± 0.19	1.41	0.70	280.44	Synergistic
EAE + HF (3:1)	72	0.46	0.31–0.66	1.07 ± 0.20	0.03	0.99	834.67	Synergistic
	96	0.27	0.18–0.36	1.30 ± 0.21	0.34	0.95	727.96	Synergistic
EAE + EAF	72	0.66	0.47–1.01	1.21 ± 0.21	1.67	0.65	583.19	Synergistic
	96	0.43	0.32–0.60	1.31 ± 0.20	0.60	0.89	447.57	Synergistic
EAE + BF	72	1.25	0.76–3.54	0.82 ± 0.20	0.48	0.92	305.30	Synergistic
	96	0.51	0.34–0.82	0.93 ± 0.19	0.33	0.95	380.00	Synergistic
EAE + WF	72	0.53	0.33–1.21	0.77 ± 0.19	0.22	0.97	716.88	Synergistic
	96	0.25	0.16–0.36	0.98 ± 0.20	0.35	0.95	795.20	Synergistic
EAE + HF (1:3)	72	0.98	0.52–5.22	0.73 ± 0.19	0.13	0.98	389.59	Synergistic
	96	0.50	0.31–1.16	0.81 ± 0.19	0.47	0.92	389.01	Synergistic
EAE + EAF	72	1.04	0.68–2.16	0.97 ± 0.20	0.68	0.88	368.99	Synergistic
	96	0.49	0.34–0.71	1.09 ± 0.19	0.11	0.99	400.51	Synergistic
EAE + BF	72	2.43	1.42–7.63	0.94 ± 0.21	0.18	0.98	157.82	Synergistic
	96	1.43	0.90–3.43	0.88 ± 0.19	1.84	0.60	136.64	Synergistic
EAE + WF	72	2.22	1.41–5.31	0.96 ± 0.20	0.31	0.96	172.18	Synergistic
	96	1.04	0.73–1.64	1.05 ± 0.19	0.23	0.97	187.38	Synergistic
Full mixture	72	3.16	2.32–4.50	1.23 ± 0.20	0.24	0.97	–	–
	96	1.96	1.47–2.53	1.50 ± 0.21	1.14	0.78	–	–
P. lilacinus								
EAE + HF (1:1)	72	1.45	1.01–2.39	1.03 ± 0.19	4.04	0.26	666.23	Synergistic
	96	0.73	0.47–1.05	1.02 ± 0.19	1.32	0.72	802.02	Synergistic
EAE + EAF	72	1.19	0.83–1.83	1.02 ± 0.19	0.39	0.94	808.04	Synergistic
	96	0.79	0.45–1.19	0.91 ± 0.19	0.93	0.82	740.41	Synergistic
EAE + BF	72	3.28	1.97–9.56	0.86 ± 0.20	1.55	0.67	294.33	Synergistic
	96	0.91	0.53–1.45	0.83 ± 0.19	2.30	0.51	644.01	Synergistic
EAE + WF	72	2.44	1.64–4.57	0.96 ± 0.19	0.89	0.83	396.54	Synergistic
	96	1.47	0.99–2.31	0.96 ± 0.19	0.42	0.94	396.65	Synergistic
EAE + HF (1:3)	72	1.37	1.02–2.01	1.28 ± 0.21	0.18	0.98	704.23	Synergistic
	96	0.99	0.74–1.36	1.32 ± 0.20	0.69	0.87	586.61	Synergistic
EAE + EAF	72	0.63	0.44–0.84	1.29 ± 0.21	1.51	0.68	1537.87	Synergistic
	96	0.59	0.43–0.78	1.46 ± 0.21	4.07	0.25	977.34	Synergistic
EAE + BF	72	2.46	1.51–6.43	0.83 ± 0.19	0.98	0.80	393.68	Synergistic
	96	0.76	0.45–1.16	0.88 ± 0.19	2.45	0.42	769.79	Synergistic
EAE + WF	72	2.49	1.67–4.83	0.99 ± 0.20	3.98	0.26	387.74	Synergistic
	96	1.22	0.79–1.95	0.90 ± 0.19	0.48	0.92	477.74	Synergistic
EAE + HF (3:1)	72	0.97	0.67–1.45	1.05 ± 0.20	4.37	0.23	999.86	Synergistic
	96	0.51	0.36–0.67	1.43 ± 0.21	0.98	0.81	1135.87	Synergistic
EAE + EAF	72	0.76	0.51–1.07	1.11 ± 0.20	3.08	0.38	1270.89	Synergistic
	96	0.52	0.37–0.67	1.59 ± 0.23	4.74	0.19	1130.57	Synergistic

Table 7 (continued)

Blends/mixtures	Time (h)	LD_{50} (µL/insect)	Confidence limits (µL/insect)	Slope ± SE	Chi-square	*p*-value	Co-toxicity coefficient	Interaction type
EAE + BF	72	3.70	2.07–14.70	0.790.20	0.89	0.83	294.33	Synergistic
	96	1.29	0.84–2.25	0.87 ± 0.19	0.01	1.00	450.04	Synergistic
EAE + WF	72	2.91	1.84–7.07	0.94 ± 0.20	1.19	0.75	331.97	Synergistic
	96	1.76	1.15–3.08	0.95 ± 0.19	0.24	0.97	331.72	Synergistic
Full mixture	72	4.09	3.06–5.96	1.32 ± 0.21	0.61	0.89	–	–
	96	3.34	2.48–4.74	1.27 ± 0.20	0.12	0.98	–	–

**EAE* ethanol aqueous extract, *HF* hexane fraction, *EAF* ethyl acetate fraction, *BF* butanol fraction, *WF* water fraction

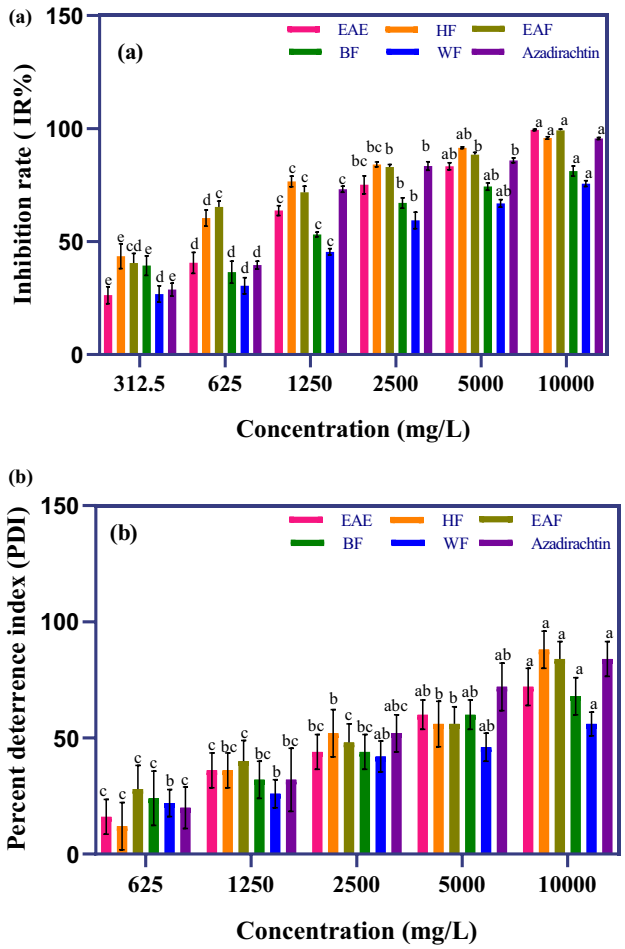


Fig. 3 **a** Reproduction inhibition rate (IR%) and **b** percent deterrent activity (PDI) of EAE and fractions of *R. officinalis* against *A. craccivora*. Bars represent the standard deviation (± SD) of five replications. Means ± SD in the error bars differs significantly by Tukey's HSD ($p < 0.0001$)

72 h of application and was followed by EAE + EAF (LD_{50} = 0.66 µL/nymph) than EAE + BF. Likewise, for 96 h, EAE + WF and EAE + HF exhibited more promising toxicity (LD_{50} = 0.25–0.27 µL/nymph) than EAE + EAF and EAE + BF (LD_{50} = 0.43–0.51 µL/nymph). At 1:3 ratio, EAE + HF and EAE + EAF (LD_{50} = 0.98–1.04 µL/insect) were found most active after 72 h in contrast to EAE + WF and EAE + BF (LD_{50} = 2.22–2.43 µL/insect). In addition, after 96 h, EAE + EAF and EAE + HF displayed higher efficacy (LD_{50} = 0.49–0.50 µL/nymph) preceded by EAE + WF (LD_{50} = 1.04 µL/nymph) than EAE + BF (LD_{50} = 1.43 µL/insect). Additionally, full mixture of EAE and its fractions showed comparatively low toxicity (LD_{50} = 3.16 and 1.96 µL/insect, respectively) after 72 and 96 h exposure aligned to blends.

In *P. lilacinus*, EAE + EAF and EAE + HF (1:1 ratio) was found more toxic (LD_{50} = 1.19 and 1.45 µL/crawler) after 72 h of treatment followed by EAE + WF (LD_{50} = 2.44 µL/ crawler) than EAE + BF. Correspondingly, 96 h later, EAE + HF and EAE + EAF was found more promising (LD_{50} = 0.73 and 0.79 µL/crawler) followed by EAE + BF (LD_{50} = 0.91 µL/crawler) than EAE + WF. For (1:3, ratio), EAE + EAF was found more promising after 72 h (LD_{50} = 0.63 µL/crawler) preceded by EAE + HF (LD_{50} = 1.37 µL/insect) than EAE + BF and EAE + WF. After 96 h of exposure, EAE + EAF and EAE + BF (LD_{50} = 0.59 and 0.76 µL/crawler) were found more effective and were followed by EAE + HF (LD_{50} = 0.99 µL/crawler) than EAE + WF (LD_{50} = 1.22 µL/crawler). In a 3:1 ratio, EAE + EAF and EAE + HF (LD_{50} = 0.76 and 0.97 µL/ crawler) was found promising after 72 h preceded by EAE + WF (LD_{50} = 2.91 µL/crawler) in contrast to EAE + BF (LD_{50} = 3.70 µL/insect). Likewise, after 96 h EAE + HF and EAE + EAF was more effective (LD_{50} = 0.51 and 0.52 µL/ crawler) followed by EAE + BF and EAE + WF (LD_{50} = 1.29 and 1.76 µL/insect, respectively). Moreover, the full mixture of EAE and its fractions showed lower toxicity than blends.

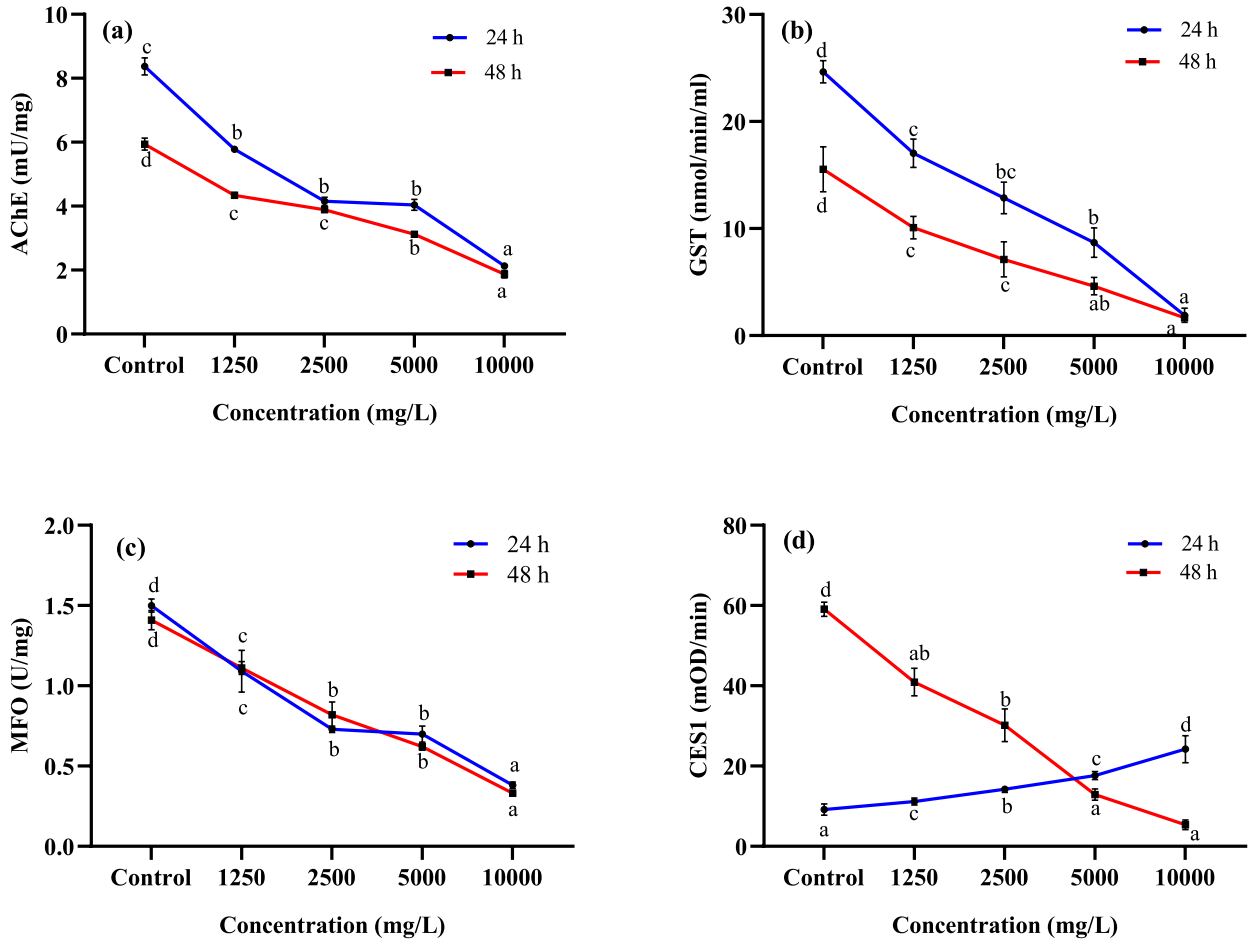


Fig. 4 Enzyme inhibition of *A. craccivora* treated with EAE of *R. officinalis*. **a** AChE, **b** GST, **c** MFO, **d** CES 1. Bars represent the standard error (± SE) of three replications. Means followed by the same letters within a figure do not differ significantly by Tukey's HSD test ($p > 0.05$)

Reproductive inhibitory activity of *R. officinalis* EAE/ fractions against test insects

Effect of EAE and its fractions on reproductive capacity of aphid after 96 h of exposure is displayed in Fig. 3a and Table S1. Results showed that EAE and fractions at higher concentration (10,000 mg/L) exhibited substantially significant reproductive inhibitory capacity (75.65–99.57%) except EAF and is at par with next concentration (5000 mg/L) in contrast to other lower doses (312.5–1250 mg/L).

Deterrent activity of EAE and its fractions of *R. officinalis* against *A. craccivora* adults

Deterrent activity of EAE and fractions of *R. officinalis* against adults of *A. craccivora* is presented in Fig. 3b and Table S2. All the concentrations differed significantly ($F_{4,24} = 3.68–10.17$; $p < 0.0001$) to deter the aphid. The percent deterrence index (PDI) was higher in the higher concentration at 10,000 mg/L (between 56 and 88%) and was at par with the next higher dose (5000 mg/L) than lower doses. All the concentrations of EAE and its fractions were at par or comparable with Neem Baan®.

Effect of EAE of *R. officinalis* on enzymatic inhibition in test insects

The effect of AChE, GST, MFO, and CES1 enzymes on aphid and mealy bug after 24 and 48 h of exposure is displayed in Figs. 4, 5 and Table S3. All the tested concentrations of the extract (EAE) were significantly inhibited AChE ($F_{4,14} = 86.30$ and 166.35), GST ($F_{4,14} = 49.84$ and 15.82), MFO ($F_{4,14} = 40.62$ and 73.65) CES 1 ($F_{4,14} = 66.54$) against *A. craccivora* at the level of $p < 0.0001$, after 24 and 48 h in contrast to the control. In *P. lilacinus* also, EAE inhibited AChE ($F_{4,14} = 40.31$), GST ($F_{4,14} = 10.75$ and 15.69), and CES 1 ($F_{4,14} = 100.31$ and 58.48) at the level of $p < 0.0001$, after 24 and 48 h exposure. Among the concentrations, higher dose of EAE (10,000 mg/L) showed significant inhibition

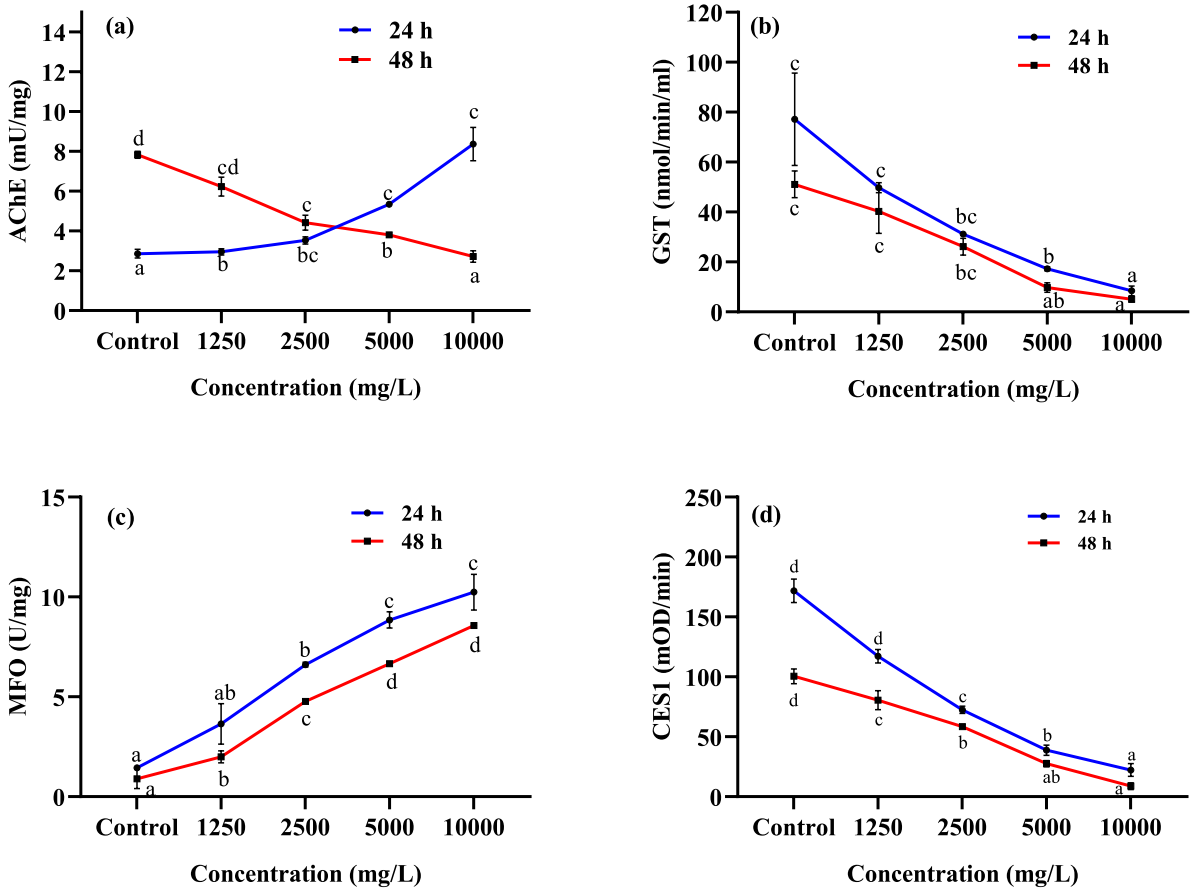


Fig. 5 Enzyme inhibition activities of *P. lilacinus* treated with *R. officinalis* EAE. **a** AChE, **b** GST, **c** MFO, **d** CES 1. Bars represent the standard error (± SE) of three replications. Means followed by the same letters within a figure do not differ significantly by Tukey's HSD test ($p > 0.05$)

of AChE (2.13 ± 0.02 and 1.87 ± 0.12 mU/mg), GST (1.90 ± 0.66 and 1.66 ± 0.24 nmol/mL/min.) and MFO (0.38 ± 0.02 and 0.33 ± 0.01 U/mg, respectively) in aphid after 24 and 48 h of treatment in contrast to control. For CES1 activity, none of the concentrations of *R. officinalis* inhibited enzyme after 24 h but slight inhibition (5.37 ± 1.21 mOD/min) was detected after 48 h of exposure. In *P. lilacinus*, induction of AChE and CES 1 was found after 24 h of exposure but inhibition was seen in all the concentrations of the extract in AChE (2.72–6.23 mU/mg) and CES1 (8.78–80 mOD/min after 48 h of treatment. GST inhibition was observed in *P. lilacinus* after 24 h of treatment (8.40–49.77 nmol/mL/min) and then decreased gradually after 48 h (5.09–40.26 nmol/mL/min) compared to control. MFO was significantly induced by EAE in mealybug after exposure.

Scanning electron microscopy (SEM) study of *A. craccivora* treated with EAE

A. craccivora nymphs were topically treated with EAE of *R. officinalis*, and the impact on the cuticle, antenna, and antennal sensilla was examined by SEM. The peritoneum, leg setae, and thoracic limb of the treated *A. craccivora* showed many abnormalities after 24, 48, and 72 h treatment as shown in Figs. 6, and 7. At 24 and 48 h after treatment, the colour of *A. craccivora* was changed from pale yellow to deep brown, and thick encrustations appeared on its outermost layer (Fig. 6a–f). After 48 and 72 h of treatment, *A. craccivora* nymphs led to repeated swellings on the outer layer, significant leg abnormalities, damaged setae, and peritoneal degradation were shown. In the untreated control, distinct cuticle layer, legs, and setae were observed without deformities. Consequently, within 48 h, the abdomen shortened, the setae separated from the cuticle, and the body turned black and filled with fluids, which resulted in the oozing of body fluid as compared to the control (Fig. 7a, b). After 72 h (Fig. 6g–i), there was full disintegration of the external habitus,

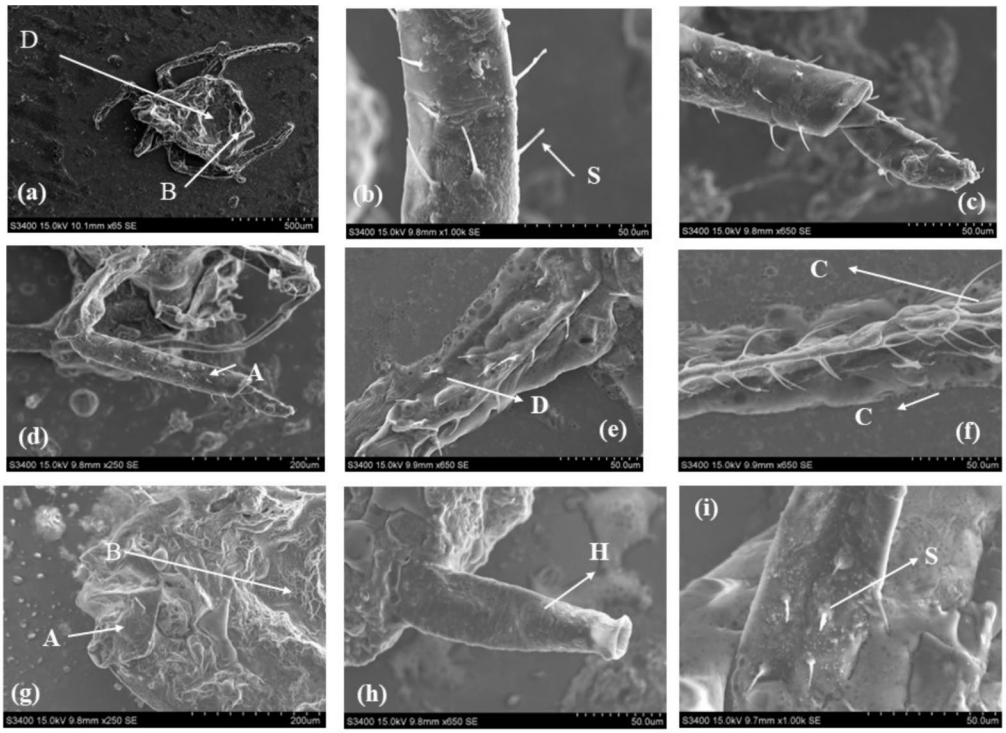


Fig. 6 Scanning electron microscopy examination of the disintegration encountered in *A. craccivora* after treatment with ethanol aqueous extract of *R. officinalis*. **a**–**d** 24 h after treatment; **e**–**f** 48 h after treatment; **g**–**i** 72 h after treatment. A-damaged antenna; B-abdominal cuticle; S-swollen setae; H-swollen habitus; C-thoracic leg; D-deformity/disintegration

complete shrinkage in the abdomen region, and encrustations in the cuticle with a division of setae and antenna from the main body observed.

Discussion

GC and GC–MS examination of *n*-hexane fraction of aerial parts of *R. officinalis*, chemical profiling of EAE by UHPLC–ESI–QTOF–MS; insecticidal activities (toxicity, repellent, deterrent, synergistic, reproductive/enzyme inhibition, and SEM of EAE and its fractions/compounds and combinations in test insects is discussed. *n*-Hexane fraction of aerial parts of *R. officinalis* comprises linolenic acid, 1,8-cineole, and myrcene, which were major constituents. Present results align with earlier findings in which EAE of *R. officinalis* leaves reported camphor, phytol, borneol, caryophyllene oxide, thymol and others [46]. In the present study, 1,8-cineole and phytol are higher than previous reports [46], whereas camphor (4.28%) was higher in earlier reports. In a similar study, phthalazine derivatives (30.8%) and verbenone (5.7%) were the major compounds in the ethanolic extract of rosemary [47], but no report in the current study.

UHPLC–ESI-QTOF-MS analysis of EAE showed that major phyto-constituents are phenolic compounds, flavonoids, and glycosidic flavonoids. EAE exhibited a total of 27 peaks in which luteolin, carnosol-*O*-quinone, rosmarinic acid, carnosol, carnosol isomer, and methyl rosmarinate are qualitatively dominant. Among the different identified phenolic acids, quinic acid ([M − H] + m/z 193.09), rosmanol [$(M - H)^+$ m/z 347.19, $(M+Na)^+$, m/z 369] and rosmarinic acid [$(M+Na)^+$ m/z 383.19, $(M+H)^+$ m/z 361] also reported in previous studies with similar molecular weight but different MS/MS ion fragments [19, 48–50]. Similarly, 6-hydroxyluteolin-7-O-glucoside was also observed in the previous study with the same (m/z) and MS^2 (m/z) fragments [49]. Pectolinarigenin ($[M-H]^+$ m/z 315.09) flavone was found in the present study with different fragments and different species of *Salvia hypoleuca* and *Salvia pedicellate* [51, 52]. The main flavonoids identified in the EAE of *R. officinalis* were rosmanol, rosmadial, and their isomers, carnosol isomer, methyl rosmarinate, and methoxycarnosol, which is consistent with a similar study [49, 53] reported the same compounds with similar molecular mass but at different elution times because of the presence of isomers. However, the current research luteolin derivative, umbelliferone derivative, pectolinarigenin isomer, and chlorogenic acid glycoside were not present in earlier investigations. Among different metabolites,

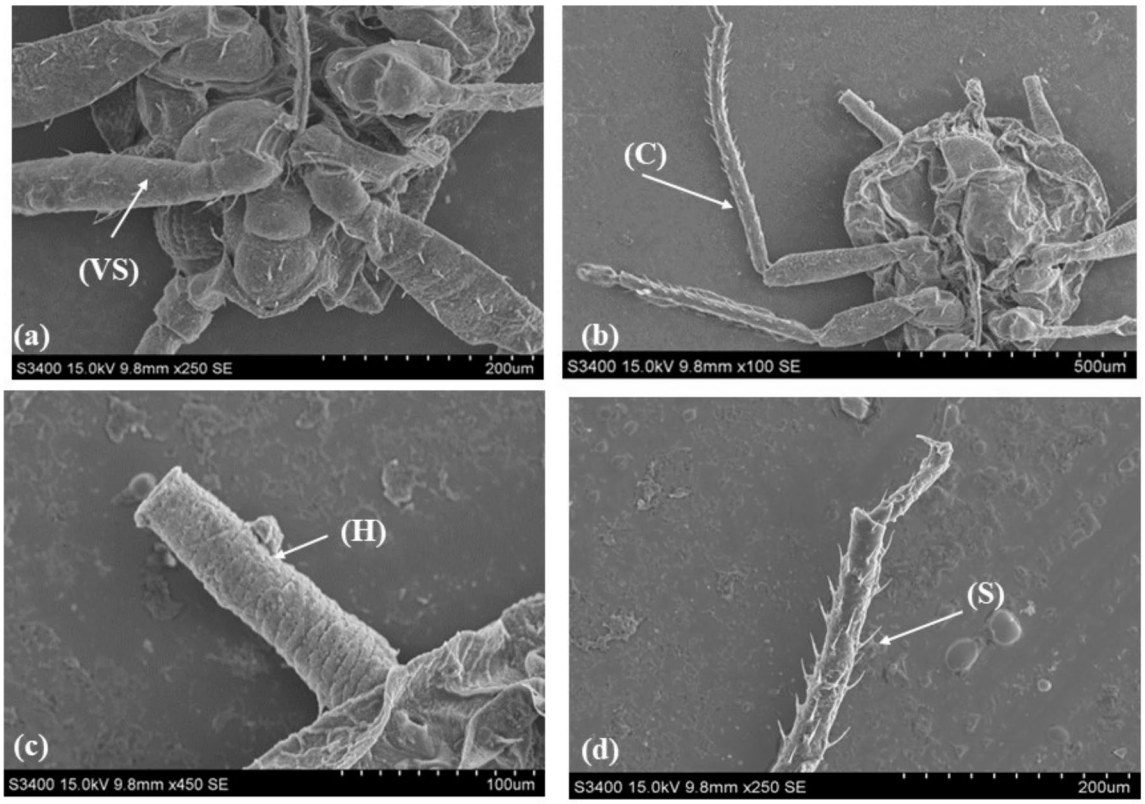


Fig. 7 Scanning electron microscopy examination of *A. craccivora* in untreated control. **a**–**d** A&B-untreated ventral side of the *A. craccivora*. C-thoracic leg; VS-ventral side; H-habitus; S-setae

few were reported only for their individual toxicity effect against different pests [54–61].

Insecticidal activities of extract/fractions/oils vary depending on the type of extraction, choice of solvents used during the extraction process, the duration of exposure of solvent and concentration [62]. The activity also depends on type of insect (chewing/sucking pests), stage of the insect, type of assay/evaluation. In the current study, EAE was highly effective to *A. craccivora* (LD_{50} = 1.84 µL/nymph,) as compared to previous studies in which ethanolic extract of *Citrullus colocynthis* (800 µg/mL) against cabbage aphid [9] and *Ricinus communis* against *Myzus persicae* (LC_{50} = 553 mg/L) [63] were less effective at higher concentration. The efficacy of EAE in the current research may be attributable to the occurrence of luteolin, carnosol-*O*-quinone, rosmarinic acid, carnosol, carnosol isomer, and methyl rosmarinate, etc. In a similar study, ethanolic extract of *Aconitum heterophyllum* (LC_{50} = 2837.2 mg/L) [13] and *Triadica sebifera* (LC_{50} = 6756.4 mg/L) [11] was less effective against *A. craccivora* than the current investigation. Likewise, *n*-hexane fraction of *R. officinalis* in the present study was more effective against *A. craccivora* in contrast to *n*-hexane fraction of goat and Crofton weeds (LC_{50} = 2881–2590 mg/L) [28, 64]. Similarly, *n*-hexane fraction of EAE of *Trillium govanianum* (LC_{50} = 2234.60 mg/L) was also comparatively less effective [1] than the present investigation. In the current investigation, *n*-hexane fraction exhibited higher toxicity to *P. lilacinus* preceded by ethyl acetate and *n*-butanol fraction. Effect of *n*-hexane fraction on aphid might be the presence of major compounds such as linolenic acid, 1,8-cineole, and myrcene. Earlier reports showed aqueous leaf extracts of *Eucalyptus globulus*, *Piper betle* and *Ocimum sanctum* showed less effective against *Maconellicoccus hirsutus* [65] than the present study. Similarly, plant extracts of *Cymbopogon citratus*, *Plectranthus amboinicus*, *P. madagascariensis* and *Mentha piperita* were found less promising against *P. citri* [66] than the current study.

In the present study under greenhouse conditions, the higher dose of *R. officinalis* EAE (20 g/L) after second spray on cowpea at 3–7 days reported higher reduction of aphids on leaf and twigs of cowpea as compared to control. However, Neem Baan was comparable with the *R. officinalis* in reducing the aphid population. Present results are superior to previous findings in which leaf ethanol extract of *T. sebifera* at 2% showed reduction

in the population of *A. craccivora* in cowpea leaves (83.3%) and twigs (68.2%) after 7 days of treatment under greenhouse conditions [67]. In a similar study, neem oil (7.5 mL/L) also showed reduction in *M. persicae* population (73%) on sweet pepper plants in the greenhouse after seven days of treatment [68]. In another study, ethanolic extract of *Azadirachta indica* and *Withania coagulans* (10%) showed reduction in *Myzus persicae* population (50.3 to 54.2%) on cucumber in the greenhouse after 7 days [69], but these extracts are inferior at higher doses than the present study.

No reports on combinations of *R. officinalis* EAE and its fractions against *A. craccivora*. However, the current study aimed to study combined effects of the extract and fractions [70]. Instead of individual extract/fraction/EOs, combinations were advantageous because of multi-action may prevent pests from developing resistance [35, 36]. Plant extracts comprise many bioactive compounds that have the most potent biological /distinct impact on insects [71]. In the current study, among blends, EAE + WF and EAE + HF (3:1 ratio) exhibited a significant effect on aphid and mealybug. Current findings are comparable with earlier results where the mixture of leaf/bark EAE of *T. sebifera* (1:3) was found less (LC_{50} = 179.31 mg/L) promising [11] than present findings.

Among compounds, linolenic acid, myrcene, and 1,8-cineole were more toxic towards aphid, whereas linolenic acid and 1,8-cineole to mealybug. Current findings are in line with previous studies, in which 1,8-cineole and myrcene from EO of *R. officinalis* showed acute toxicity against larvae of *Trichoplusia ni* and *Spodoptera littoralis* [72, 73]. Similarly, carvacrol, alpha-bisabolol and chamazulene exhibited toxicity to *A. craccivora* and *Tribolium castenum* [74].

EAE and its fractions at higher concentration in this study showed higher reproductive inhibition followed by the next concentration (5000 mg/L). The current results align with those of prior studies, where EO and methanolic extract of *Cyperus rotundus* at higher concentration (10,000 mg/L) showed higher reproductive inhibition (90–97.58%) [12]. In the same investigation, the EO of *Murraya koenigii* also showed promising inhibition in *A. craccivora* [75]. Similarly, as the concentration increases, the deterrent activity of the EAE against *A. craccivora* also increased. Higher concentration of extract/fractions showed promising deterrent activity (56–88%). Present results are in agreement with earlier study, where saponins isolated from *Quillaja saponaria* showed low deterrent activity to pea aphid [38].

Several natural plant compounds that are used to combat insect infestations substantially alter enzymatic activities [76]. Current investigation revealed that the EAE of *R. officinalis* significantly reduced inhibitory activity of AChE, GST, MFO, and CES1 in aphid. The inhibition of these detoxification enzymes in the aphid and mealy bug may be due to the presence of linolenic acid, 1,8-cineole and myrcene in the *n*-hexane fraction; phenolic compounds, flavonoids, and glycosidic flavonoids in the EAE. Present findings confirmed that EAE significantly decreased the inhibition of AChE in aphids and mealy bugs than the untreated control. AChE controls the propagation of nerve impulses between cholinergic synapses. When AChE is inhibited, acetylcholine is concentrated in the synaptic cleft, causing insect paralysis [77]. Therefore, our findings imply that the ability of the extract to inhibit AChE may be related to the insecticidal activity of *R. officinalis*. Therefore, it is confirmed that the prime mode of action of several plant extracts in insects is the suppression of AChE [78]. However, in a similar study leaf/bark ethanolic extract of *T. sebifera* and methanolic extract of *Thalictrum foliolosum* significantly inhibited AChE in *A. craccivora* [11, 79]. In the current study, all concentrations of EAE significantly inhibited GST and MFO in aphid, but MFO showed induction in mealy bug. Detoxification of pesticide groups involves GST in insects [80]. GSTs can transform both endogenous and exogenous toxins into inactive substances and render them water-soluble [81]. Moreover, plant extracts/EOs with their metabolites and chemical compounds, disrupt GST [82, 83]. Current findings agree with previous report in which ethanolic extract of *Aconitum heterophyllum* and *Fritillaria cirrhosa* suppressed the GST in *A. craccivora* [13, 15]. Additionally, owing to increased MFO activity, numerous studies have demonstrated that MFO is crucial for the detox process in the body of insects and emergence of pesticide resistance in several insect genera [84]. *R. officinalis* extract inhibited MFO in aphid and induced in mealy bug in all concentrations than *A. heterophyllum* extract which induced MFO in *A. craccivora* [13]. A similar study found that the secondary metabolite of *Ginkgo biloba* exhibited a more rapid MFO activity response to ginkgo flavonoids and ginkgolide [85] when tested against *Hyphantria cunea* larvae. However, P450 monooxygenases, GST, and carboxyl/cholinesterases play a crucial role in sustaining metabolic processes among phytophagous insects by eliminating xenobiotic substances [86, 87] and changing substances from ester components into acid and alcohol [88]. Our in vivo enzyme studies indicated that EAE of rosemary significantly inhibited all the enzymes after 24–48 h of exposure in the aphid. Although a slight induction of the enzyme was recorded in CES 1 after 24 h, but displayed inhibition after 48 h. The findings of our

study are consistent with similar reports, where EO of *Artemisia absinthium* containing carvacrol, α-bisabolol, and chamazulene decreased CES1 and GST in citrus psyllid, *Diaphorina citri* than control [87]. An additional method for assessing the toxicological efficacy of extract/EOs against insects based on morphological, histological, and sensory examinations, with a focus on the epicuticle layers of the head, thorax, and abdomen [89]. In the current investigation, EAE on aphid showed crumbling of habitus in the posterior abdominal segments and multiple swellings in the sensilla. Earlier reports also showed that application of *Tagetes minuta* EO against *A. craccivora* and *P. xylostella* [43, 90] caused significant disintegration in the peritoneal membrane, head, and thorax.

Conclusion

GC and GC–MS analysis showed that linolenic acid (24.97%) and 1,8-cineole (14.26%), were the major constituents in the *n*-hexane fraction of *R. officinalis*. EAE contains the presence of palmitoleic acid, 4-ethoxy ethyl benzoate, 7-methylrosmanol, and diosmin are major metabolites. EAE and its *n*-hexane fraction showed the promising effect on aphid (LD_{50} = 1.84–2.22 µL/nymph). In mealybug, *n*-hexane fraction exhibited promising toxicity (LD_{50} = 1.46 µL/crawler) than ethyl acetate and *n*-butanol fraction (LD_{50} = 2.01–2.29 µL/crawler). Among compounds, linolenic acid was found more superior to both insects (LD_{50} = 0.59–0.99 µL/insect). *R. officinalis* extract/fractions also showed significant reproductive inhibition and deterrent activity against target pests. Further, EAE significantly inhibited AChE, GST and MFO in the aphid. In mealybug, only GST showed inhibitory activity. The identified lead (s) will be evaluated against the target pests in the field for validation and recommendation.

Abbreviations

EAE	Ethanolic aqueous extract
HF	*n*-Hexane fraction
EAF	Ethyl acetate fraction
BF	*n*-Butanol fraction
WF	Water fraction
GC	Gas chromatography
GC–MS	Gas chromatography and mass spectrometry
UHPLC–ESI-QTOF-IMS	Ultra-high performance liquid chromatography–quadrupole time-of-flight-ion mobility mass spectrometry
RT	Retention time
LD_{50}	Lethal dose to kill 50% of test population
LC_{50}	Lethal concentrations to kill 50% of test population
AChE	Acetylcholinesterase
GST	Glutathione-S-transferase
MFO	Mixed function oxidase
CES 1	Carboxylesterase 1
SEM	Scanning electron microscopy
FAMES	Fatty acid methyl esters
NIST	National Institute of Standards and Technology
CRD	Completely randomized design
DAT	Days after treatment
PDI	Percent deterrence index

Supplementary Information

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Additional file 1.

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Author contributions

U.K. done Data curation, Methodology, Investigation, Formal analysis, Software, Conceptualization, Writing—original draft, S.G.E.R done Conceptualization; Methodology, Resources; Supervision; Funding acquisition, Project Administration; Writing—review & editing.

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Availability of data and materials

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Declarations

Ethics approval and consent to participate

Not applicable.

Competing interests

The authors declare no competing interests.

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