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ORIGINAL ARTICLE

Antibacterial activity of some plant-derived essential oils against plant pathogenic bacteria

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Abstract

Essential oils (EOs) are natural products being considered as an alternative to chemicals to control plant pathogenic bacteria (PPB). In the present study, the EOs from *Thymus kotschyanus* Boiss. & Hohen., *Thymus daenensis* Celak, *Ferulago angulata* (Schlecht.) Boiss., *Ziziphora clinopodioides* Lam., *Eucalyptus camaldulensis* Dehnh., *Echinophora cinerea* (Boiss.) Hedge *et* Lamond., and *Trachyspermum ammi* L. were obtained and their major constituents were identified by gas chromatography/mass spectrometry (GC–MS). The effect of the EOs against nine species of PPB was evaluated using a disk diffusion assay. *T. ammi* EO showed the highest antibacterial activity with an inhibition zone of 52.25 and 46.25 mm against *Xanthomonas citri* pv. *citri* and *Pectobacterium carotovorum* subsp. *carotovorum*, respectively. All EOs exhibited an anti-biofilm formation effect on *Erwinia amylovora*. Minimum inhibitory concentration and minimum bactericidal concentration of the EOs were determined using the macro-dilution method. Synergistic effects of the EOs were determined using the serial dilution checkerboard method. Transmission electron microscopy revealed that exposure of the PPB to the EOs caused unified cell structure including bacterial aggregation due to the lysis of the cell wall, shrinkage, and deformation. In conclusion, the present results suggest that the EOs might be a promising source of antibacterial activity against PPB.

Keywords Anti-biofilm · Synergistic effects · Electron microscopy · Shrinkage

Introduction

Plant pathogenic bacteria (PPB) are a main category of phytopathogens causing remarkable damage to a variety of cultivated and uncultivated plants globally (Singh 2017). Several control measures have been applied commonly to reduce the damage caused by PPB including the plantation of resistant/tolerant cultivars and chemical treatment (Chaube and Singh 2018). The application of copper-based fungi-bactericides and antibiotics is the easiest and the most accessible chemical practice to control PPB (Sharma et al. 2022). The extensive applications of antibiotics such as streptomycin and erythromycin are relatively expensive and antibiotic-resistant strains would probably emerge within PPB populations (Corona and Martinez 2013; Sandoval-Motta and Aldana 2016). The copper-based compounds used against PPB have low efficiency and often cause phytotoxicity (Lalancette and McFarland 2007). Also, the residue of these compounds persists in the environment affecting living organisms adversely (Maag et al. 2000; Bakshi and Kumar 2021). Since copper-based compounds have fungicidal and bactericidal effects, they cause devastating effects on the soil microbiome and thus interfere with the process of organic matter decomposition in the soil (Bakshi and Kumar 2021). The low efficiency of antibiotics is mainly due to the increased resistance rate of PPB to several antibiotics (Sundin and Wang 2018). This has been restricting the use of antibiotics all around the world (Stockwell and Duffy 2012). Taken together, novel approaches are required as an alternative to conventional control measures against bacterial diseases. There is a need for compounds that, in addition

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to the appropriate bactericidal effect, overcome resistance development among PPB and have fewer side effects than commonly applied chemicals. Plant-derived essential oils (EOs) are a major group of environment-friendly compounds with antibacterial properties that have been used during the last decade (Ootani et al. 2013).

EOs are aromatic, hydrophobic, concentrated, and volatile compounds that are found in individual or complex secretory cells and trichomes, secretory glands, secretory ducts, and in the surface or internal parts of leaf, flower, fruit, bud, and branch (Swamy 2020; de Oliveria and de Aguiar Andrade 2022). EOs as antimicrobial agents have two main characteristics: 1) they are safe for humans and the environment due to their natural origin, and 2) they harbor less risk for microbial resistance as they contain various compounds that might have different mechanisms against microbes (Ganesan et al. 2015; Ghorbanpour and Varma 2017). It is difficult to assay the sensitivity of a microorganism against potential antimicrobial compounds and why the sensitivity varies from one race to another. Predicting the action of EOs requires a comprehensive investigation of the target site, mode of action, and possible interactions of the EO with the surrounding environment (Hyldgaard et al. 2012).

The antimicrobial activity of the EOs from several plant species, including eucalyptus (*Eucalyptus* spp.) (Hendry et al. 2009; Cock 2009; Damjanović-Vratnica et al. 2011; Ishnava et al. 2013; Sliti et al. 2015), *Ferulago angulata* (Schlecht.) Boiss. (Taran et al. 2010), *Thymus daenensis* Celak (Moghimi et al. 2016), *Thymus kotschyanus* Boiss. & Hohen. (Mehdizadeh et al. 2012), *Echinophora cinerea* (Boiss.) Hedge *et* Lamond. (Ghasemi Pirbalouti and Gholipour 2016), *Ziziphora clinopodioides* Lam. (Shahbazi 2015) and *Trachyspermum ammi* L. (Moein et al. 2015) have been demonstrated against different bacterial species. Although there are numerous studies about the antiviral effects of the EOs, little information exists on the inhibitory properties of these EOs against PPB.

This study aimed to evaluate the antimicrobial activity of EOs from *Eucalyptus camaldulensis*, *F. angulata*, *T. daenensis*, *T. kotschyanus*, *E. cinerea*, *Z. clinopodioides* and *T. ammi* against some economically important PPB including *Pectobacterium carotovorum* subsp. *carotovorum* (Jones 1901) Waldee 1945, *Erwinia amylovora* (Burrill 1882) Winslow et al. 1920, *Ralstonia solanacearum* (Smith 1896) Yabuuchi et al. 1996, *Xanthomonas citri* subsp. *citri* (Gabriel et al. 1989) Schaad et al. 2007, *Xanthomonas oryzae* pv. *oryzae* (ex Ishiyama 1922) Swings et al. 1990, *Pseudomonas syringae* pv. *syringae* (van Hall 1902) Janse 1982, *P. syringae* pv. *phaseolicola* (Burkholder) Gardan, Bollet, Abu Ghorrah, Grimont & Grimont, *P. syringae* pv. *tomato* (Okabe 1933) Young, Dye & Wilkie 1978, and *P. syringae pv. morsprunorum* (Wormald 1931) Young, Dye & Wilkie 1978. Furthermore, the EOs composition and their effect on biofilm formation by the PPB were assayed. The possible effects of EOs on the physical structure of the PPB were also visualized.

Materials and methods

Plant materials

Table 1 shows the details of the plant species which were collected in 2019. The plants were sampled during three growth stages: flowering, pre, and post-flowering. Healthy and succulent leaves were collected from March through June 2019. Treatments were prepared from leaves that were dried, ground to pass a 5-mm mesh Wiley mill screen, and stored in jars at room temperature until use.

EO preparation

EO extraction was conducted by steam distillation method using a Clevenger apparatus (Heidolph, laborota 4003, Germany) (Rezaei and Jaymand 2006). Before analysis, 100 g of tissue powder was placed within the apparatus bulb and 1,200 ml of deionized distilled water (DDW) was added. The mixture was heated for 4 h, and the upper phase containing the EO was isolated. The EO was immediately transferred into clean vials. The vials were sealed with parafilm, wrapped with aluminum foil, and stored at 4 °C.

Table 1 Details of the plant species used in this study

Common name	Scientific name	Location (city: province)	Geographical specification
Thyme	*Thymus kotschyanus* Boiss. & Hohen	Taleghan: Isfahan	32.6577°N 51.6692°E
Thyme	*Thymus daenensis* Celak	Yasouj: Kohgilouyeh & Boyerahmad	32.6577°N 51.6692°E
Chavir	*Ferulago angulata* (Schlecht.) Boiss	Taleghan: Isfahan	32.6577°N 51.6692°E
Kakuti-e-kuhi	*Ziziphora clinopodioides* Lam	Yasouj: Kohgilouyeh & Boyerahmad	32.6577°N 51.6692°E
River red gum	*Eucalyptus camaldulensis* Dehneh	Khorramabad: Lorestan	33°29′16″N 48°21′21″E
Fialeh	*Echinophora cinerea* (Boiss.) Hedge *et* Lamond	Khuzestan, Dezful	32°22′57″N 48°24′07″E
Ajwain	*Trachyspermum ammi* L	Khuzestan, Izeh	32°00′N 49°55′E

Gas chromatography-mass spectrometry (GC–MS)

EOs of *T. kotschyanus, T. daenensis, Z. clinopodioides, T. ammi, F. angulata, E. cinerea* and *E. camaldulensis* were determined from GC–MS analysis. Aliquots (1 µl) of extracts were introduced with an automatic sample injector (Model 7683, Agilent Technologies, CA) into Agilent 6850 series GC system with quadruple MS detector (model 5973) coupled through HP-5MS column (30-mm long, 0.25 mm internal diameter, 0.25 µm film thickness, Agilent Technologies). The starting temperature was 60 °C for 1 min, then increased to 4 every min until reaching 90 °C. After 3 min at 90 °C, the temperature was increased by 2 °C per min up to 121 °C. The temperature was held for 2 min at 121 °C, followed by the third increase of 6 °C until 182 °C. The program was completed after 1 min at the final temperature. Qualitative identification of the different constituents was performed by comparison of their relative retention times and mass spectra with those of authentic reference compounds, or by retention indices (RI) and mass spectra (Davies 1990; Adams 2004). This experiment was repeated twice.

Preparation of PPB

Gram-negative PPB including *X. oryzae* pv. *oryzae* (*Xoo*), *X. citri* pv. *citri* (*Xcc*), *P. syringae* pv. *phaseolicola* (*Psp*), *P. syringae* pv. *syringae* (*Pss*), *P. syringae* pv. *tomato* (*Pst*), *P. syringae* pv. *morsprunorum* (*Psmo*), *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*), *E. amylovora*, and *R. solanacearum* were obtained from the microbial collection of the Department of Plant Pathology at Ferdowsi University of Mashhad (Mashhad, Iran) and Shahid Chamran University of Ahvaz (Ahvaz, Iran). The strains were stored at 4 °C in sterile distilled water throughout the study and used as stock cultures.

Disk diffusion method

To investigate the antibacterial effect of EOs, a bacterial suspension with a concentration of 10^8 colony-forming units (CFU) per ml (using a spectrophotometer (WPAS2000) ($OD_{600\ nm} = 0.1$)) was prepared from a 24 h bacterial culture and 100 µl of the suspension was cultured on a Petri dish containing nutrient agar (NA, Merck, Germany). Then, 10 µl of EOs were placed on 6 mm standard paper disks. After absorbing the EOs with the paper disks, the disks were placed on the culture medium. Then, two disks containing the EOs were placed inside each 12-cm Petri dish and the Petri dishes were incubated at 28 °C for 48 h (Burt 2004). A disk was soaked in DDW and a standard antibiotic disk of ampicillin (30 mg/disk) was used as negative and positive controls, respectively. All Petri dishes were sealed with sterile parafilm. The antibacterial effect of the EOs was determined by measuring the inhibitory halo with a caliper. Three biological replicates and three technical replicates were considered per treatment.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

MIC and MBC of the PPB that had been sensitive to the EOs in disk diffusion assay were determined using the macrodilution method. The inoculum was obtained from a 24 h bacterial culture. The standard 0.5 McFarland suspension was prepared. A mixture of each bacterium and EO was incubated at 25 °C for 24 h in Mueller Hinton agar medium (Hi-Media, India). The first concentration in which bacteria did not grow was considered as MIC. Then, the contents of non-growing tubes were re-cultured in the plate containing EOs and incubated at 25 °C for 24 h. The first plate in which the bacterial growth had been inhibited was considered MBC (Nasr et al. 2005; Parvin et al. 2010; Jafari et al. 2011; Salehi et al. 2011). Three biological replicates and three technical replicates were considered per treatment.

Effect of the EOs on biofilm formation

The method described by O'Toole (2011) with some modifications was used to test the effect of EOs on biofilm formation by the PPB. The bacteria were grown overnight in Luria–Bertani broth (LB, Sigma-Aldrich, USA) medium at 28 °C, diluted to obtain optical density at 600 nm wavelength ($OD_{600\ nm}$) = 0.4. Then, 10 µl of the bacterial suspension was placed in 96-well microtiter plates and filled with 150 µl of LB broth. The plate was incubated at 28 °C for 24 h. The cultures were dried and stained overnight with a 0.1% crystal violet dye solution. The excess stain was removed by washing DDW. The stained biofilms were quantified by measuring their $OD_{600\ nm}$. A 30% acetic acid was used as a blank. The experiments were performed in quadruplicate wells. Three biological replicates and three technical replicates were considered per treatment.

Determination of fractional inhibitory concentration (FIC)

The interaction among the EOs was calculated according to the FIC index using the Modified Dilution Checkboard method (Pillai et al. 2005).

$$FICI = FICA + FICB = \frac{MICAcombined}{MICAalone} + \frac{MICBcombined}{MICBalone}$$

The 96-well polypropylene microtiter plate was used to determine the FIC. To test the synergistic effect, the Serial Dilution Checkboard method was used. To this end, 75 µl

of each compound (MIC, 1/2, 1/4, 1/8 and inhibitory concentration of each compound) was added to each well in a row. Then, 75 µl of another compound was added to wells in the reverse row. Sixteen wells were used to determine the synergistic effect of each two EOs. Finally, 11 µl of bacterial suspension was added to each well and the plate was placed in a shaker at 25 °C for 48 h. The 5 µl of each well was evenly poured on Petri dishes containing NA medium which had been divided into 16 sections. The Petri dishes were incubated at 25 °C for 24 h and the FIC value was calculated based on the growth of bacteria in each section. If the FIC index (FICi) value was more than 0.5, the combined effect of EOs would be synergistic. If $0.5^{<}$ FICi $^{<}1$, the combination of EOs had an additive effect. If the FICi was more than 1 and less than 4, the combination of EOs against the pathogen would be neutral. FICi $^{>}4$ indicates the antagonistic effect of the combination of EOs. Three biological replicates and three technical replicates were considered per treatment.

Scanning electron microscopy (SEM)

For this experiment, PPB were cultured in LB medium at the MIC in a shaking incubator at 28 °C for 24 h. The microorganism was collected and washed twice with 0.1 M phosphate-buffered solutions (PBS, pH 7.0). The suspension was filtered by a polycarbonate filter and fixed in a 2.5% glutaraldehyde solution and kept at 4 °C for 2 h. After several washing with DDW, the sample was dehydrated successively with 6 ethanol solutions (30%, 50%, 70%, 80%, 90%, and 100%) for 10 min. The fixed bacterial cells were coated with gold nanostructure using Desk sputter coater-DSR1 (NSC, Iran). The cells were photographed using SEM (Tescan, FE SEM/Mira3 Lmu, HV = 20 kV, Czechia).

Statistical analysis

The data from EOs-PPB interaction, biofilm formation, and synergistic effect of EOs were analyzed by one-way ANOVA in SPSS 19.0 statistical software. Duncan's multiple range test was used to measure the differences between data means at 99% significance level ($p < 0.01$). The graphs were drawn using Microsoft Office Excel 2013.

Results

GC–MS analysis

EOs of *T. kotschyanus, T. daenensis, T. ammi*, *Z. clinipodioides, E. camaldulensis*, *F. angulata*, and *E. cinerea* were obtained from the leaves. The seven EOs were analyzed by GC–MS and their chemical compositions were identified (Table S1). A total of 24, 34, 10, 45, 27, 36, and 47 EO constituents of *T. kotschyanus, T. daenensis, T. ammi*, *Z. clinipodioides, E. camaldulensis, E. camaldulensis*, *F. angulata*, *E. cinerea* were identified, representing 95.86%, 95.16%, 98.103%, 85.19%, 75.79%, 67.58%, and 48.14% of their total compounds, respectively. The most frequent EO component of *T. kotschyanus* is carvacrol with 57.94% area. Secondly, thymol methyl ether (20.06%), gamma terpinene (3.61%), 1,8-cineole (2.64%), and linalool (2.12%) had the highest amount among the EO components. In *T. daenensis,* the compounds including paracimen (28.70%), orthocimen (16.64%), carvacrol (14.43%), linalool (9.11%), borneol (4.2%), and aromadendren (2.70%) were the most important compounds covering the 79.97% of the EO compounds. The main constituents of the *T. ammi* EO were carvacrol, paracymene, and gamma terpinen with area percentages of 76.6%, 13.40% and 6.78%, respectively. The *Z. clinipodioides* EO contained pulegone (20.38%), alpha-terpineol (14.24%), terpin-4-ol (7.20%), isomenthol (5.68%), thymol (4.38%), bornyl acetate (3.93%), 1,8-cineole (6.57%) and carvacrol (2.27%) showing a total of 64.11% of the EO components. The 1,8 cineol with 20% area had the highest amount within *E. camaldulensis* EO. Then, paracymen (9.13%), 4-ol terpene (6.346%), alpha-pinene (4.73%), gamma-terpinene (2.19%) and other compounds composed the *E. camaldulensis* EO. The most important chemical compounds of *F. angulata* EO included isobornyl acetate (15.31%), cis-beta-ocimene (9.22%), methyl eugenol (6.46%), camphene (5.16%), cis-verbenol (3.97%), spathulenol (3.76%), linalool (3.48%), myrcene (3.17%), alpha-muurolene (2.21%), α-terpineol (2.06%). These compounds included 54.8% of the total compounds identified in *F. angulata* EO. The compounds including linalool (6.38%), e-ocimene (5.76%), carvacrol (5.16%), citronellol (5.08%), myrcene (4.4%), thymol (4.06%), spathulenol (3.5%), myrtenyl acetate (2.85%), alpha-terpineol (2.64%), limonene (2.22%) were the most important compounds within the EO of *E. cinerea*.

Antibacterial activity assay

Disk diffusion method

P. carotovorum* subsp. *carotovorum The antibacterial activities of EOs against eight PPB were summarized in Fig. 1. All EOs had a significant effect on *Pcc*, except for *F. angulata* EO. The highest average diameter of the inhibitory area (46.25 ± 2.39 mm) against the bacterial growth was related to *T. ammi* EO, while the lowest inhibition was found using eucalyptus EO with an average diameter of 8.91 ± 0.9 mm.

E. amylovora *F. angulata* EO did not form any inhibitory zone on the culture plate of *E. amylovora.* The highest effect of the EO was found using *T. ammi* EO with an average diameter of inhibition growth of 34 ± 1.41 mm and the low-

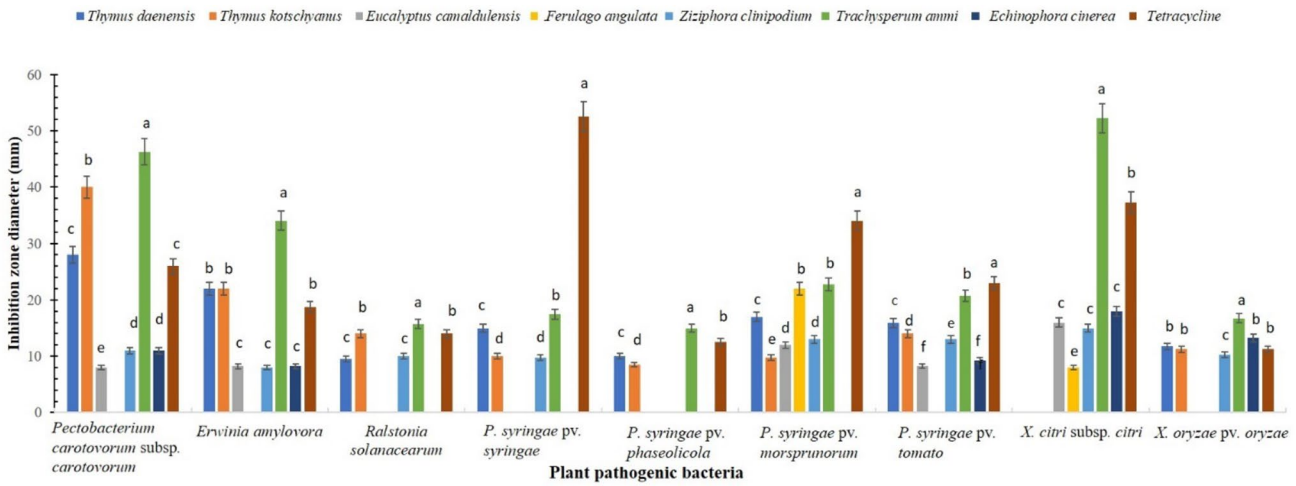

Fig. 1 Summary of the EOs antimicrobial activity. Means were compared based on the Duncan multiple range test at 1% of probability level. Values within a column followed by the same letter do not differ at the 1% significance level. All data represent means ± standard error of the mean (SEM) from three independent experiments. Tetracycline was used as positive control. The missing bars for some PPB show the lack of inhibition zone

est effect related to the EOs of *E. camaldulensis* and *E. cinerea* with the average diameter of the halo 25 ± 1.50 and 8.25 ± 1.71 mm, respectively (Fig. 1).

R. solanacearum *E. camaldulensis*, *F. angulata*, and *E. cinerea* EOs did not have any inhibitory effect on *R. solanacearum*. The most halos of growth inhibition with an average diameter of 15.75 ± 0.48 mm were found using *T. ammi* EO and the least effect in *T. daenensis* EO with an average halo diameter of 9.50 ± 0.65 mm was observed. In the positive control, the inhibition of bacterial growth was 13 ± 0.91 mm (Fig. 1).

X. citri* pv. *citri *T. daenensis* and *T. kotschyanus* EOs had no effect on *Xcc*. The highest and lowest inhibitory effect on the growth of this bacterium was observed by *T. ammi* EO with an average of 52.25 ± 1.11 and *F. angulata* EO with an average of 8 ± 0.41 mm, respectively. In the positive control, this amount was 37.25 ± 0.63 mm (Fig. 1).

X. oryzae* pv. *oryzae *E. camaldulensis* and *F. angulata* EOs did not form any inhibition zone on *Xoo* culture. The results of the variance analysis of the inhibition halo of EOs on *Xoo* bacteria at the 1% level showed that *T. ammi* EO has the largest diameter of the inhibition halo with a size of 16.75 ± 0.25 mm and the smallest one (10.25 ± 0.47 mm) was found using EO of *Z. clinipodioides* (Fig. 1).

MIC and MBC

The minimum inhibitory and bactericidal concentrations of the seven EOs against the PPB were shown in Table 2. *T. ammi* EO with MIC and MBC values of 0.2 and 1 µl/ml, respectively, has the most effect on *Pcc*, followed by the most effect on the EOs from *T. kotschyanus* and *T. daenensis* with MIC and MBC values of 0.4, 1 and 0.6, 1.1 µl/ml, respectively. The lowest effect was found using the EO of *E. camaldulensis* with the MIC and MBC values of 25 and 27.5 µl/ml, respectively. *E. amylovora* showed the highest sensitivity to *T. ammi* and *T. daenensis* EOs, with the MIC value of 0.2 and 0.8 µl/ml, respectively. Then, *T. kotschyanus* exhibited a significantly high effect with inhibition of 1.25 µl/ml. The lowest effect of EO on this bacterium was observed using *E. cinerea* EO with MIC and MBC values of 22.5 and 25 µl/ml, respectively. As presented in Table 2, the highest and lowest effect on *R. solanacearum* was obtained when *T. kotschyanus* and *Z. clinipodioidas* EOs were used resulting in MIC values of 1.25 and 22.5 µl/ml, respectively. The MBC of *T. kotschyanus* and *Z. clinipodioidas* EOs were measured as 2.5 and 25 µl/ml, respectively. Secondly, *T. ammi* EO had a significantly high effect on this bacterium with MIC and MBC values of 1.5 and 2.5 µl/ml, respectively. The lowest effect on *Xcc* was found by *Z. clinipodioidas* EO with MIC and MBC values of 20 and 22.5 µl/ml, respectively, and the highest effect was obtained using *T. ammi* EO with MIC and MBC values of 0.2 and 1 µl/ml, respectively. *T. ammi* EO had the most effect on *Xoo* with MIC and MBC values of 1.25 and 2.5 µl/ml, respectively. In contrast, *Z. clinipodioidas* EO had the least effect with MIC and MBC values of 22.5 and 25 µl/ml, respectively. Also, *E. cinerea* EO showed a slight effect on this bacterium with MIC value of 17.5 µl/ml. The antibacterial effect of *T. kotschyanus* EO against *Pss* was found higher than other EOs with MIC and MBC values of 1.5, 2.5 µl/ml, respectively, while *Z. clinipodioidas* EO

Table 2 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) value of the plant-derived EOs against some plant pathogenic bacteria

PPB	EO	MIC (µl/ml)	MBC (µl/ml)
P. carotovorum subsp. *carotovorum*	*E. camaldulensis*	25	27.5
	F. angulata	–	–
	T. daenensis	0.6	1
	T. kotschyanus	0.4	1
	E. cinerea	6	7.5
	Z. clinipodioides	8.5	10
	T. ammi	0.2	1
E. amylovora	*E. camaldulensis*	8.5	10
	F. angulata	–	–
	T. daenensis	0.8	1
	T. kotschyanus	1.25	2.5
	E. cinerea	22.5	25
	Z. clinipodioides	20	22.5
	T. ammi	0.2	1
R. solanacearum	*E. camaldulensis*	–	–
	F. angulata	–	–
	T. daenensis	4.5	5
	T. kotschyanus	1.25	2.5
	E. cinerea	–	–
	Z. clinipodioides	22.5	25
	T. ammi	1.5	2.5
X. citri subsp. *citri*	*E. camaldulensis*	4	5
	F. angulata	4.5	5
	T. daenensis	–	–
	T. kotschyanus	–	–
	E. cinerea	3.5	5
	Z. clinipodioides	20	22.5
	Trachyspermum ammi	0.2	1
X. oryzae pv. *oryzae*	*E. camaldulensis*	–	–
	F. angulata	–	–
	T. daenensis	3.5	5
	T. kotschyanus	2	2.5
	E. cinerea	17.5	20
	Z. clinipodioides	22.5	25
	T. ammi	1.25	2.5
P. syringae pv. *syringae*	*E. camaldulensis*	–	–
	F. angulata	–	–
	T. daenensis	9	10
	T. kotschyanus	1.5	2.5
	E. cinerea	–	–
	Z. clinipodioides	20	22.5
	T. ammi	2	2.5
P. syringae pv. *phaseolicola*	*E. camaldulensis*	–	–
	F. angulata	–	–
	T. daenensis	9	10
	T. kotschyanus	4.5	5
	E. cinerea	–	–
	Z. clinipodioides	–	–
	Trachyspermum ammi	7.5	10

Table 2 (continued)

PPB	EO	MIC (µl/ml)	MBC (µl/ml)
P. syringae pv. *tomato*	*E. camaldulensis*	6.5	7
	F. angulata	–	–
	T. daenensis	1.75	2.5
	T. kotschyanus	0.8	1
	E. cinerea	6.5	7.5
	Z. clinipodioides	25	27.5
	T. ammi	0.8	1
P. syringae pv. *morsprunorum*	*E. camaldulensis*	25	27.5
	F. angulata	7	7.5
	T. daenensis	1.5	2.5
	T. kotschyanus	0.8	1
	E. cinerea	–	–
	Z. clinipodioides	22.5	25
	T. ammi	0.6	1

PPB plant pathogenic bacteria, *EO* essential oil, *MIC* minimum inhibitory concentration, *MBC*: minimum bactericidal concentration

had the least antibacterial effect with MIC and MBC values of 20 and 22.5 µl/ml, respectively. The MIC of *T. kotschyanus*, *T. ammi* and *T. daenensis* EOs against *Psp* was 4.5, 7.5 and 9 µl/ml, respectively. Also, the MBC for these EOs was 5, 10 and 10 µl/ml, respectively. The MIC of *T. ammi* and *T. kotschyanus* EOs on *Pst*, was 0.8 µl/ml and their MBC was determined to be 1 µl/ml. This bacterium showed the least sensitivity to *Z. clinipodioidas* EO with MIC and MBC values of 25 and 27.5 µl/ml, respectively. In the case of *Psmo* EOs of *E. camaldulensis* and *Z. clinipodioidas* with the MIC value of 25 and 22.5 µl/ml, respectively, had the least effect. Also, the maximum effect on this bacterium was related to *T. ammi* EO with MIC and MBC values of 0.6 and 1 µl/ml, respectively.

Determination of fractional inhibitory concentration (FIC)

As shown in Table 3, the combination of *E. cinerea-Z. clinipodioides* EO against *Pcc* showed a neutral effect. However, the combination of other EOs against this bacterium had a synergistic effect. The combination of EOs from *T. daenensis-T. kotschyanus, T. daenensis-T. ammi* and *T. kotschyanus-T. ammi* against *E. amylovora* had a synergistic effect while the combination of EOs of *T. daenensis-E. camaldulensis*, *T. kotschyanus-E. camaldulensis* and *T. ammi-E. camaldulensis* was neutral against the bacterium. The combination of EOs from *T. daenensis-T. ammi* and *T. ammi-T. kotschyanus* against *R. solanacearum* was of the neutral type and the combination of EOs of *T. daenensis-T. kotschyanus* was an additive type. The combination of EOs from *T. ammi-E. camaldulensis*, *T. ammi-E. cinerea* and *T. ammi-F. angulata* had a synergistic effect against the bacterium *Xcc*. Also, the combination of EOs of *F. angulata-E. cinerea* had an additive effect against *Xcc*. While the combination of EOs from *F. angulata-E. camaldulensis* and *E. cinerea-E. camaldulensis* resulted in neutral effect against *Xcc*. The multiple application of EOs of *T. daenensis-T. kotschyanu* and *T. daenensis-T. ammi* was found to be synergistic against *Xoo*, and the mixture of EOs from *T. ammi-T. kotschyanus* was neutral against *Xoo*. The combination of EOs from *T. daenensis-T. kotschyanus, T. daenensis-T. ammi* and *T. kotschyanus-T. ammi* against *Pss* had a synergistic effect. The combination of EOs of *T. daenensis-T. kotschyanu* and *T. daenensis-T. amm* against *Psp* had a synergistic effect. Also, the reaction of EOs from *T. ammi-T. kotschyanus* against the bacterium was found to be additive. The combination of all the EOs applied against the *Pst* was of neutral type. All EOs used against *Psmo* had a synergistic effect.

Effect of the EOs on the formation of bacterial biofilm

To identify anti-biofilm agents, the EOs were screened. It was found that the studied EOs did not affect the biofilm formation of *Pcc*, *Xcc*, *Pss,* and *Pst.* (Fig. 2). The results showed that all EOs had an inhibitory effect on *E. amylovora* biofilm. The highest inhibition of the biofilm formation of this bacterium was observed when the EO of *E. cinerea* or *E. camaldulensis* were used, and the lowest inhibition was found by the EO of *Z. clinipodioides*. According to the results, the EOs of *Z. clinipodioides*, *T. daenensis*, and *T. kotschyanus* did not show any significant inhibitory effect against the formation of *Xoo* biofilm. In the investigation

Table 3 FIC index of the EOs from different plant species against some PPB

PPB	EO	FIC_i	Reaction
P. carotovorum subsp. *carotovorum*	*T. kotschyanus-T. ammi*	0.5	S
	T. kotschyanus- Z. clinipodioides	0.5	S
	T. kotschyanus- E. cinerea	0.5	S
	T. kotschyanus- T. daenensis	0.5	S
	T. daenensis- T. ammi	0.5	S
	T. daenensis- Z. clinipodioides	0.5	S
	T. daenensis- E. cinerea	0.5	S
	T. ammi- Z. clinipodioides	0.5	S
	T. ammi- E. cinerea	0.5	S
	E. cinerea- Z. clinipodioides	1.5	I
E. amylovora	*T. daenensis- T. kotschyanus*	0.5	S
	T. daenensis- T. ammi	0.5	S
	T.daenensis- E. camaldulensis	2.25	I
	T. kotschyanus- E. camaldulensis	1.625	I
	T. kotschyanus- T. ammi	0.5	S
	T. ammi- E. camaldulensis	1.625	I
R. solanacearum	*T. daenensis- T. kotschyanus*	1	A
	T. daenensis- T. ammi	2.25	I
	T. ammi- T. kotschyanus	2.25	I
X. citri subsp. *citri*	*T. ammi- E. camaldulensis*	0.5	S
	T. ammi- E. cinerea	0.5	S
	T. ammi- F. angulata	0.5	S
	F. angulata- E. cinerea	0.875	A
	F. angulata- E. camaldulensis	1.625	I
	E. cinerea- E. camaldulensis	2.25	I
X. oryzae pv. *oryzae*	T. *daenensis- T. kotschyanus*	0.5	S
	T. daenensis- T.ammi	0.5	S
	T. ammi- T. kotschyanus	1.125	I
P. syringae pv. *syringae*	*T.daenensis- T. kotschyanus*	0.5	S
	T.daenensis- T.ammi	0.5	S
	T. kotschyanus- T.ammi	0.5	S
P. syringae pv. *phaseolicola*	*T.daenensis- T. kotschyanu*	0.5	S
	T.daenensis- T.ammi	0.5	S
	T.ammi- T. kotschyanus	0.75	A
P. syringae pv. *tomato*	*T.daenensis-. E. camaldulensis*	2.25	I
	T.daenensis- T. kotschyanus	2.25	I
	T.daenensis- T.ammi	2.25	I
	T.daenensis- E. cinerea	2.25	I
	T. kotschyanus- E. camaldulensis	2.25	I
	T. kotschyanus- T.ammi	2.25	I
	T. kotschyanus- E. cinerea	2.25	I
	T.ammi- E. camaldulensis	2.25	I
	T.ammi- E. cinerea	2.25	I
	E. cinerea- E. camaldulensis	2.25	I
P. syringae pv. *morsprunorum*	*F.angulata- T.daenensis*	0.5	S
	F.angulata- T. kotschyanus	0.5	S
	F.angulata- T.ammi	0.5	S
	T.daenensis- T. kotschyanus	0.5	S
	T.daenensis- T.ammi	0.5	S
	T. kotschyanus- T.ammi	0.5	S

The three divisions of interactions of EOs are summarized with the following signs: *S* synergistic, *I* indifferent, *A* additive, *PPB*: plant pathogenic bacteria, *EO* essential oil, *FICi*: fractional inhibitory concentration index

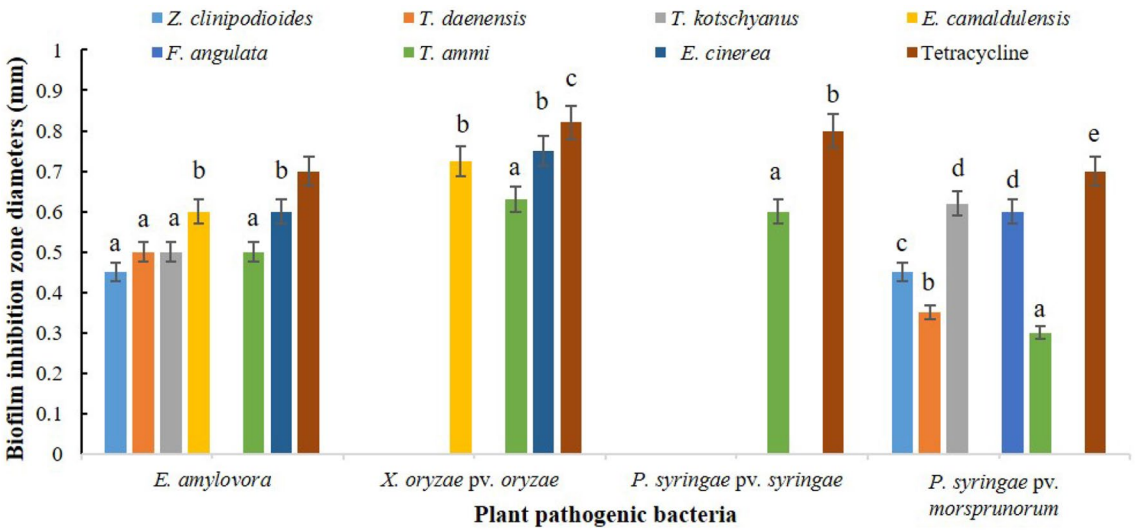

Fig. 2 The effect of the EOs from different plants on biofilm formation of the PPB. All data represent means ± standard error of the mean (SEM) from three independent experiments. Tetracycline was used as positive control. The missing bars for some PPB show the lack of inhibition zone

of the effect of EO on *Pss*, it was found that the only EO of *T. ammi* prevents the formation of biofilm and the other two EOs were ineffective. Also, among EOs affecting *Psmo*, eucalyptus EO had no inhibition on biofilm formation. The highest inhibitory effect against *Psmo* biofilm formation was obtained by *E. cinerea* and *T. kotschyanus* EOs. In contrast, the lowest inhibitory effect was found by *T.ammi* EO.

Ultrastructure changes of PPB

The results of SEM of the EOs on PPB showed that the population of bacteria decreased drastically in MIC concentrations. The cells indicated an obvious unified cell structure including bacterial aggregation due to the lysis of the cell wall, shrinkage, and deformation (Fig. 3). The bacteria changed from a normal (rod-shaped) shape to an oval, round, and irregular. Also, the cell wall was wrinkled and plastinated. Other conditions were also evident such as keeping the overall shape of the bacteria, and cell wall shriveling and flattening. According to the results, the EO from *T. ammi* was able to affect *Xcc, Pst,* and *E. amylovora* cells adversely. Also, *T. kotschyanus* EO had a negative effect on *Pcc* cells (Fig. 3).

Discussion

The resistance of bacteria to antibiotics has been increasing since their usage. Research on the discovery of new substances with relatively stronger antimicrobial properties is expanding and due to the effectiveness of plant-derived EOs against PPB, they are considered an interesting issue in the research area (Mangalagiri et al. 2021). Plant-derived EOs have antimicrobial effects on a wide range of organisms, and their side effects are less than those of common antibiotics (Semeniuc et al. 2017).

In recent years, some researchers have tried to find anti-biofilm compounds (Mishra et al. 2020). The complex structure of the biofilm promotes the development of antibiotic resistance and becomes extremely difficult to eradicate. One of the advantages of using medicinal plants as anti-biofilm is that the majority of them have balanced biological toxicity and fewer side effects. Also, easy access, reasonable price, and lower risk of bacterial resistance are other advantages of plant-derived compounds (Zhang et al. 2022). Pourkhosravani et al. (2021) showed the antibacterial and anti-biofilm profiles of cinnamon and cardamom EOs alone and in combination together against infectious bacterial strains. They found that cinnamon EO and its combination with cardamom EO had the highest anti-biofilm activity at the lowest MIC value. In this research, *T. ammi* EO had stronger antibacterial and anti-biofilm properties than other EOs. This EO can be used as a natural antibacterial compound due to its monoterpene compounds. The present findings seem to be consistent with other research in which *T. ammi* EO affected food-pathogenic bacteria (Jebelli Javan et al. 2019).

Recently, many researchers have tested different plant EOs on *Pcc* bacteria. For instance, Cai et al (2022) found that *Polygonum orientale* L. EO had effective inhibitory activity against *Pcc*, thus this plant could have potential application in controlling the bacterium. The amount of halo in *T. daenensis* and *T. kotschyanus* EOs in this study was 28.5 ± 0.65 and 40.25 ± 1.10 mm, respectively, and these differences could be due to the type of plant species. In a

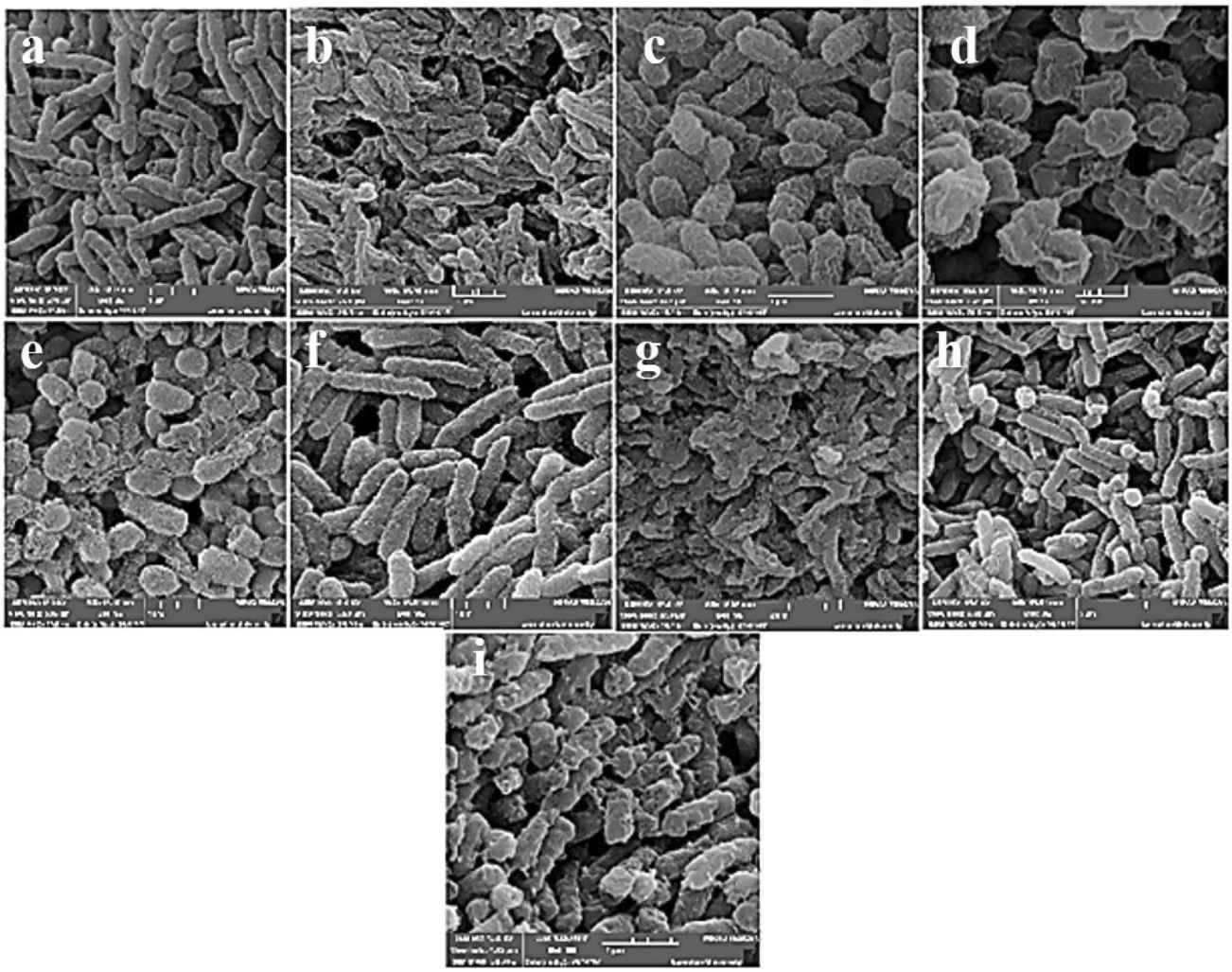

Fig. 3 SEM photographs of the interaction of plant-derived EOs and plant pathogenic bacteria including **a** *Xcc*, **b** the effect of *T. ammi* EO on *Xcc*, **c** *E. amylovora* and **d** the effect of *T. ammi* EO on *E. amylovora*, **e** the effect on tetracycline on *E. amylovora*, **f** *Pst* and **g** the effect of *T. ammi* EO. **h** *Pcc* and **i** the effect of *T. kotschyanus* EO on *Pcc*

study on the antibacterial activity of *T. ammi* and thyme (*T. vulgaris*) EOs, it was found that *T. ammi* EO had a greater effect on *Pcc* by creating a growth inhibition zone diameter of 46.67 mm (Jafarpour and Golparvar 2013).

The use of combinations of EOs and their isolated components is thus new approaches to increasing the efficacy of EOs to control PPB and take advantage of their synergistic and additive effects (Bassolé and Juliani 2012). Based on our results, the EOs combination used against *Psmo* caused synergistic interactions. The combination of different EOs produced synergism suggesting that several chemical components act and the target bacterium cannot develop resistance to multiple components of two or more EOs. Thus, doses of the combined agents are required to improve their antibacterial activities (Basavegowda and Baek 2022).

In a study, the antibacterial effect of *T. vulgaris* EO on three strains of *E. amylovora* was investigated and the average inhibition was reported as 28.11 ± 10.71 mm. Karami-Osboo et al. (2010) stated the amount of growth inhibitory halo of this bacterium under the influence of *T. vulgaris* EO as 25 mm. In our study, the inhibition zone for *T. daenensis* and *T. kotschyanus* EOs was measured as 20.75 ± 1.71 and 21 ± 3.65 mm, respectively, and the reason for this difference could be the difference in the type of plant species, bacterial strain, and the difference in EO compositions in different regions (Kokoskova et al. 2011).

The results of Hosseini-Nejad et al. (2012) about the antibacterial property of *T. vulgaris* EO on *R. solanacearum* by disk diffusion method showed the development of a halo with a diameter of 34.8 mm. Also, the growth inhibitory halo of eucalyptus EO (*E. globolus*) was stated to be 6.5 mm. Moghaddam et al. (2014) reported that the diameter of the inhibitory halo caused by the EO of *Ocimum ciliatum* on this bacterium was 9 mm. The diameter of the halo inhibiting the

growth of *R. solanacearum* in the treatment with the EO of *Macleaya cordata* R. Br. was determined to be 8.5 ± 0.6 and 18.6 ± 1.9 mm (Li and Yu 2015).

Iacobellis et al. (2005) stated that *T. ammi* has a relatively high inhibitory effect on *Xanthomonas* bacteria. Also, Mahmoudi et al. (2010) studied the antibacterial activity of *T. ammi* EO against leaf spot bacteria (*Xanthomonas arboricola* pv. *pruni*), and stated the mean halo diameter as 40.77 ± 0.25 mm. In the present study, this EO had a high inhibitory effect on *Xcc* bacteria by creating halos with an average diameter of 52.25 ± 1.11 mm. Jadhav and Deobhankar (2013), Inhibitory halo diameter on the growth of *Xanthomonas citri* bacteria. reported 21.2, 15.6, 2.7 and 24.4 mm using EOs of *Eucalyptus globules*, *Tridax procumbens, Emblica officinalis* and *Calotropis procera* respectively. In a research, *Spiraea alpina* EO against *X. oryzae* pv. *oryzae* and *X. campestris* pv. *citri* was evaluated. This EO created halos with an average of 15.3 and 13.7 mm, respectively (Teng et al. 2010). The average inhibitory halo for the growth of *Xanthomonas oryzae* pv. *oryzae* and *Xanthomonas citri* due *to Ocimum ciliatum* EO were reported to be 6 ± 0.5 and 10 ± 1 mm, respectively (Moghaddam et al. 2014). The results obtained from the study of Pawar and Pandit (2014) showed that the diameter of the inhibitory halo of *Ocimum sanctum* extract against strains of *Xanthomonas campestris* pv. *mangiferaeindicae* is 20.36, 20.11 and 16.27 mm. Therefore, it can be concluded that the inhibitory power of different EOs, in addition to the type of plant species, will be different depending on the type of bacteria and even the bacterial strain.

In a study, Mahmoudi et al. (2010) investigated the antibacterial activity of EO against *Pss* were investigated. In this study, the diameter of the halo inhibiting the growth of this bacterium was equal to 20.22 ± 0.16 mm. In the present study, the inhibitory halo value was 17.5 ± 0.26 mm. In a study, the average diameter of the halo inhibiting the growth of *Pss* bacteria due to the use of EO of *Ocimum ciliatum* was stated to be 2 ± 1 mm (Moghaddam et al. 2014). In the research of Balestra et al. (2009), the effect of *Allium sativum* and *Ficus carica* extracts on *Pst* was investigated and it was determined that the average diameter of the aura of non-growth of bacteria was 14 ± 1.2 and 21 ± 1.8 mm, respectively.

The high MIC of eucalyptus EO in the research shows that this EO has a weak performance in preventing the growth of bacteria. In a study by Mehrsorosh et al. (2014), the minimum inhibitory concentration of *T. vulgaris* EO on *Pectobacterium carotovorum* was 145 µg/ml. Alamshahi et al. (2010) investigated the effect of several plant EOs on *Pectobacterium carotovorum* and showed that thyme EO (*Thymus vulgaris*) has the highest growth inhibition rate among EOs, and this amount is 5 µl/ml. This difference in the amount of inhibition in our research compared to Alamshahi et al. (2010) can be due to the difference in the plant species used. Alamshahi et al. (2010) studied the effect of *Eucalyptus camaldulensis* EO on *Pectobacterium* and showed that the minimum inhibitory concentration of this EO is 5–150 µl/ml which is consistent with our result.

In a study, the average value of the minimum concentration of inhibition and lethality against *E. amylovora* bacteria was expressed from 0.09 to 0.18 µl/ml (Mihajilov-Krstev et al. 2010). Hosseini-Nejad et al. (2012) investigated the antibacterial properties of thyme (*Thymus vulgaris*) EO against *R. solanacearum* bacteria and showed that the minimum inhibitory concentration of this EO is equal to 1 µl/ml. In the present study, the minimum inhibitory concentration for *T. daenensis* and *T. kotschyanus* EOs was measured as 4.5 and 1.25 µl/ml, respectively. The minimum inhibitory concentration of *R. solanacearum* bacteria with the application of *Macleaya cordata* EO was 125 to 500 µg/ml (Li and Yu 2015).

Antibacterial activity of orange, fennel, and pine EOs on *Xcc* has been investigated by Sauer et al. (2015) and the MIC and MBC levels for these EOs have been reported as 0.238, 1.81, 7.81, and 7.81, 14.99, 0.477 µl/ml, respectively. In the study by Gormez et al. (2015), the minimum inhibitory concentration for *Pss*, *Psp,* and *Pst* under the influence of *Satureja hortensis* EOs, has been measured as 31.25, 15.63 and 7.81 µg/ml, respectively. This value for *Calamintha nepeta* EO against three bacteria was found to be 7.81 µg/ml.

The antibacterial effect of tea tree, clove, lemon grass, and Indian hyacinth EO on *X. vesicatoria* has been investigated using electron microscopy and it has been found that these EOs directly affected the bacterial cell wall (Lucas et al. 2012). Bacterial cells of *R. solanacearum* treated with *Macleaya cordata* EO have been severely damaged and, consequently, lost their rod structure. Also, a large number of deformed and incomplete bacterial cells has been observed. It has been concluded that the EO caused a change in the permeability of the bacterial cell so that the substances leaked from the bacterial cytoplasm (Li and Yu 2015). These results were consistent with our results according to which the EO from *T. ammi* and *T. kotschyanus* were able to affect the bacterial cells adversely. Taken together, the EOs used in this assay can inhibit the growth of the bacteria and negatively affect their cell structure demonstrating that the plant-derived EOs are a promising source of antibacterial compounds. Further experiments are required to identify and validate the antibacterial components within these EOs.

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Declarations

Conflict of interest The authors do not express any competing interest in the work done and the manuscript written.

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