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Antibacterial and Biochemical Activities of Phenylpropenes and Monoterpenes on Phytopathogenic Bacteria

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ABSTRACT

In the present study, six monoterpenes and two phenylpropenes were evaluated for their antibacterial effect against three phytopathogenic bacteria, *Agrobacterium tumefaciens*, *Ralstonia solanacearum* and *Erwinia carotovora* var. *carotovora*. The inhibitory effects of these compounds on polygalacturonase and dehydrogenases activities were also tested. The results revealed that *trans*-cinnamaldehyde, (-)-citronellal, (-)-terpinen-4-ol had the highest antibacterial activity against *A. tumefaciens*. Their minimum inhibitory concentration (MIC) values were 1000, 1500 and 1500 mg/l, respectively. Similarly, *trans*- cinnamaldehyde (MIC = 2000 mg/l), and (-)-citronellal (MIC = 2000 mg/l) were the highest activity compounds against *E. carotovora var. carotovora*. Moreover, (-)-citronellal caused the greatest antibacterial effect against *R. solanacearum* with MIC value of 1000 mg/l. Further, *trans*- cinnamaldehyde showed the highest inhibitory effects on polyglacturonase and dehydrogenases activities of *A. tumefaciens*, while (-)-citronellal represented the most potent effect of inhibition on polyglacturonase and dehydrogenases activities of *E. carotovora* var. *carotovora* and *R. solanacearum*.

Keywords: Phytopathogenic bacteria; minimum inhibitory concentration; monoterpenes; phenylpropenes; polygalacturonase; dehydrogenases

INTRODUCTION

Plant pathogenic bacteria are responsible for huge economic losses in agriculture by decreasing the yields and marketing values of crops (Obradovic *et al*., 2008). *Agrobacterium tumefaciens*, *Erwinia carotovora* subsp. *carotovora* and *Ralstonia solanacearum* are soil borne bacteria that cause crown gall, soft rot and lethal wilt, respectively. These three bacteria are among the most common plant pathogenic bacteria worldwide. They attack numerous plant families including fruits, vegetables and flowers, and cause devastating loss in the production of infected crops (Hayward, 1991; Wright 1998; Wang *et al.,* 2000).

The control of plant diseases caused by phytopathgenic bacteria is mainly focusing in the continuous use of synthetic chemicals. However, the use of synthetic chemicals emerges several environmental and health problems such as pollution of environment components, food toxic residues and development of resistance (Vidaver 2002; Montesinos and Bardaji 2008; Yuliar *et al.,* 2015). Resistance of many bacterial strains to commonly used antibiotics becomes evidence and decreases their efficacy in the management of plant diseases (Sundin and Wang 2018).

The increasing awareness of the drawbacks of synthetic chemicals and the increasing demand on safe products for controlling plant diseases encourage the search for new alternatives for plant disease control. Plant materials, such as plant extracts, essential oils and plant secondary metabolites are among the most promising alternatives for controlling plant diseases (Isman, 2008).

Monoterpenes and phenylpropenes are two classes of plant secondary metabolites with low molecular weights and boiling points. These compounds are commonly present as the major constituents of plant essential oils. Monoterpenes and phenylpropenes have been described to display wide spectrum of biological effects against agricultural pests, such as fungicidal, insecticidal and herbicidal activities (Grodnitzky and Coats, 2002; Singh *et al*., 2002; Wuryatmo *et al*. 2003; Cheng *et al.,* 2008; Ahuja *et al*. 2015; Saad *et al.,* 2018). However, the antibacterial activity of monoterpenes and phenylpropenes against plant pathogenic bacteria are poorly studied. Monoterpenes and phenylpropenes have been reported to possess inhibitory effect on the growth of *A. tumefaciens* and *E. carotovora* var. *carotovora* (El-Zemity *et al.,* 2008; Abdel Rasoul *et al.,* 2012), *E. amylovora* (Sato *et al.,* 2007; Scortichini and Rossi 2008) and *Xanthomonas campestris* pv. *phaseoli* var. *fuscans* (Cantore *et al.,* 2009).

Therefore, the aim of this study was to evaluate the antibacterial efficacy of six monoterpenes and two phenylpropenes against three plants pathogenic bacteria *A. tumefaciens*, *E. carotovora* var. *carotovora* and *R. solanacearum*. Also, the inhibitory effects of these compounds on the activity of two exocellular enzymes polygalacturonase and dehydrogenases were studied in order to understand their possible mechanism of action.

MATERIALS AND METHODS

Chemicals

Two phenylpropenes and six monoterpenes were selected to study their antibacterial and biochemical effects. Tested compounds were (–)-citronellal (95%), *p*-cymene (99%), (–)-menthone (90%), α-pinene (98%), α-terpinene (85%), (–)-terpinen-4-ol (95%), *trans*-cinnamaldehyde (99%) and eugenol (99%). These compounds were bought from Sigma Aldrich Chemical Co. (Steinheim, Germany). Figure 1. shows the chemical structures of tested monoterpenes and phenylpropenes.

Test bacteria

Bacterial strains of *Agrobacterium tumefaciens* (Erwin Frink Smith & Town.) (Family: Rhizobiaceae; Class: *Alpha Proteobacteria*), *Erwinia carotovora* var. *carotovora* (Erwin Frink Smith) (Family: Enterobacteriaceae; Class: Gamma Proteobacteria) and *Ralstonia solanacerium* (Erwin Frink Smith) (Family: Burkholderiaceae; Class: Betaproteobacteria), were obtained from Laboratory of Microbiology, Department of Plant Pathology, Alexandria University. Nutrient agar medium (NA) which prepared by mixing peptone (10 g), meat extract (5 g), sodium chloride (2.5 g) and agar (10 g) in one liter of distilled water was used for maintaining bacterial strains.

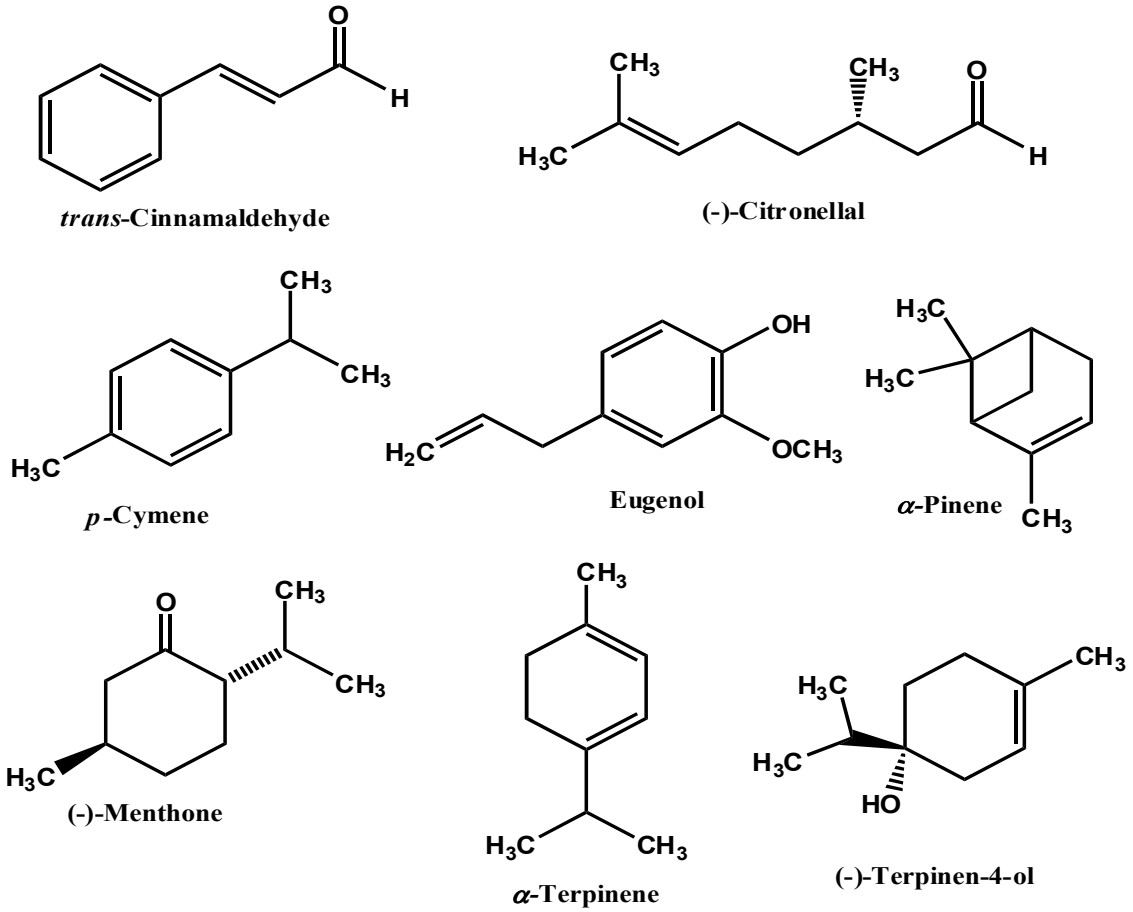

Figure 1. Chemical structure of monoterpenes and phenylpropenes.

Minimum inhibitory concentration (MIC) assay

The minimum inhibitory concentrations (MICs) of the tested phenylpropenes and monoterpenes on the three bacterial strains were determined by using agar dilution method (ESCMID 2000). Stock solutions of tested compounds were first prepared in acetone. A series of concentrations of each compound were prepared by adding different volumes of the prepared solutions to molten NA to give a series of concentrations ranged between 10 and 10000 mg/l. Then the media were poured into Petri dishes.

The petri dishes were left for solidifications and 2 µl of bacterial cultures (approximately 10^8 CFU/ml) was spotted on the surface of agar using 2 µl standard loops. Three spots were made per each plate. The inoculum spots were left to dry. The plates were incubated at 37°C for 24 h. The lowest concentration of each compound in which no visible growth of bacterial strain in the plate was observed and taken as MIC value.

Dehydrogenases activity assay

These compounds have been estimated on dehydrogenases activity of E. carotovora var. carotovora, A. tumefaciens, and R. solanacerium by using a methylene blue technique (Schoenhard, 1962). The phenylpropenes and monoterpenes were evaluated at 10, 50, 100, 500 and 1000 mg/l. Inhibition percentage (I %) of dehydrogenases was calculated form the following equation:

$$\mathbf{I\,(\%) = ((T - C)/\ T_{max} - C) \times 100}$$

T: Is known as the time of reduction (min) for 90% of methylene blue in the treatment, T_{max} : Is known as the maximum time of reduction (min) for the 90% methylene blue recorded in treatments, C: Is known as the time of reduction (min) for 90% of methylene blue in control treatment. Values of IC_{50}, concentration causing 50% of enzyme inhibition, of tested compounds were calculated using Probit analysis (Finney, 1971).

Polygalacturonase activity assay

The phenylpropenes and monoterpenes were evaluated at 10, 50, 100, 500 and 1000 mg/l. Inhibition percentage (I %) of polyglacturonase activity was calculated according to (Ayers *et al.,* 1966; Nasuno and Starr,1966)

The increase in the absorbance at 550 nm (OD_{550}) was used to determine the enzyme activity. Three replicates were used in each treatment. Absorbance of reaction mixture containing enzyme at zero time was used as control.

The equation as follows:

$$\mathbf{I\,\% = ((A\ control - A\ treatment)/A\ control) \times 100}$$

(I %): Is known as the inhibition of polygalacturonase activity

A: Is known as the absorbance

Statistical analysis

Probit analysis was carried out to determine IC_{50} values, 95% confidence limits and other statistical parameters of tested compounds on both enzymes (Finney, 1971) using SPSS v21.0 software program (Chicago, USA).

RESULTS AND DISCUSSION

Results

Antibacterial effect of monoterpenes and phenylpropenes

The eight compounds were assayed for *in vitro* antibacterial activity against three plants pathogenic bacteria *A. tumefaciens*, *E. carotovora* var. *carotovora* and *R. solanacerium.* Table 1 shows the values of MIC for the tested compounds on the three bacterial strains. It was clear that phenylpropenes and monoterpenes possessed variable levels of antibacterial activity.

Table 1. Minimum inhibitory concentration (MIC) of monoterpenes and phenylpropenes on plant pathogenic bacteria

Compound	MIC (mg/l)		
	Agrobacterium tumefaciens	*Erwinia carotovora* var. *carotovora*	*Ralstonia solanacearum*
trans-Cinnamaldehyde	1000	2000	1500
(-)-Citronellal	1500	2000	1000
p-Cymene	5000	>6000	>5000
Eugenol	2000	2500	2000
(-)-Menthone	2000	2500	2000
α-Pinene	4000	6000	4000
α-Terpinene	2000	3000	3000
(-)-Terpinen-4-ol	1500	2500	2000

Generally, *A. tumefaciens* was more susceptible than *E. carotovora* var. *carotovora* and *R. solanacearum* to the monoterpenes and phenylpropenes. *trans*-Cinnamaldehyde was the most active compound against *A. tumefaciens* and *E. carotovora* var. *carotovora* with MIC values of 1000 and 2000 mg/l, respectively. α-Pinene and *p*-cymene were the less active compounds against *A. tumefaciens* and *E. carotovora* var. *carotovora*. In addition, (-)-citronellal showed the highest antibacterial activity against *R. solanacearum* (MIC = 1000 mg/l) followed *trans*- cinnamaldehyde (MIC = 1500 mg/l), while *p*-cymene showed the lowest antibacterial activity (MIC >5000 mg/l). Eugenol, (-)-menthone, α-terpinene and (-)-terpinen-4-ol displayed moderate antibacterial activity against the three plant pathogenic bacteria.

Effect of monoterpenes and phenylpropenes on dehydrogenases activity

These results cleared that (-)-citronellal caused the highest effect of inhibition on dehydrogenases activity of E. carotovora var. carotovora and R. solanacearum with IC50 values of 125.10 and 83.41 mg/l, respectively (Tables 3 and 4). In contrary, p-cymene exhibited the lowest inhibitory effect on the dehydrogenases activity of *A. tumefaciens* and *E. carotovora* var. *carotovora.* Moreover, *trans*-cinnamaldehyde caused the highest inhibitory effects on dehydrogenases of *A. tumefaciens* (IC_{50} = 75.18 mg/l).

Eugenol, (-)- menthone, α-terpinene and (-)-terpinen-4-ol had a moderate effect of inhibition on the dehydrogenases activity of the three plant pathogenic bacteria.

Effect of monoterpenes and phenylpropenes on polyglacturonase activity

The results of the inhibitory effect of the tested compounds on polyglacturonase of the three bacteria detected that all of the tested compounds caused pronounced inhibition of the enzyme(Tables 2, 3 and 4). (-)- Citronellal revealed the highest inhibition on polyglacturonase activity of *E. carotovora* var. *carotovora* (IC_{50} =13.99 mg/l) and *R. solanacearum* (IC_{50} = 15.69 mg/l) respectively, while *trans*-cinnamaldehyde was the most potent inhibitor on polyglacturonase from *A. tumefaciens* (IC_{50} = 11.49 mg/l). p-Cymene showed the lowest inhibition of polyglacturonase from *A. tumefaciens* and *R. solanacearum*, while (-)-terpinen-4-ol was the less active on the enzyme from *E. carotovora* var. *carotovora*.

Table 2. The effect of Inhibition monoterpenes and phenylpropenes on polyglacturonase and dehydrogenases activities of *Agrobacterium tumefaciens*

Compound	Polyglacturonase			Dehydrogenases		
	IC_{50}[a] (mg/l) (95% Confidence limit)	Slope[b] ± SE	Intercept[c] ±SE	IC_{50} (mg/l) (95% Confidence limit)	Slope ± SE	Intercept ±SE
trans-Cinnamaldehyde	11.49 (6.29-17.67)	0.94±0.11	-1.00±0.20	75.18 (52.75-103.66)	0.84±0.09	-1.58±0.19
(-)-Citronellal	13.36 (8.10-19.40)	0.66±0.11	-1.19±0.21	93.28 (65.63-129.82)	0.82±0.09	-1.61±0.19
p-Cymene	61.79 (12.15-167.11)	0.92±0.09	-1.65±0.19	224.49 (153.61-348.76)	0.70±0.09	-1.65±0.19
Eugenol	19.40 (11.46-28.78)	0.88±0.10	-1.31±0.19	108.50 (76.10-153.13)	0.79±0.09	-1.60±0.19
(-)-Menthone	20.55 (11.79-30.99)	0.82±0.09	-1.07±0.19	119.78 (37.44-375.19)	0.67±0.09	-1.40±0.19
α-Pinene	27.83 (14.67-44.15)	0.66±0.09	-0.95±0.18	219.44 (153.69-329.16)	0.75±0.09	-1.76±0.20
α-Terpinene	53.62 (17.01-115.82)	0.97±0.09	-1.67±0.19	172.75 (113.77-273.85)	0.63±0.08	-1.41±0.19
(-)-Terpinen-4-ol	52.01 (36.46-70.66)	0.93±0.09	-1.59±0.19	146.08 (96.25-226.41)	0.64±0.08	-1.38±0.19

[a] **The Inhibitory concentration of 50 % from enzyme.**
[b] **Slope of the concentration inhibition regression line ±standard error (SE).**
[c] **Intercept of the regression line ±SE**

Table 3. The effect of inhibition monoterpenes and phenylpropenes on polyglacturonase and dehydrogenases activities of *Erwinia carotovora* var. *carotovora*

Compound	Polyglacturonase			Dehydrogenases		
	IC_{50}[a] (mg/l) (95% Confidence limit)	Slope[b] ± SE	Intercept[c] ±SE	IC_{50} (mg/l) (95% Confidence limit)	Slope ± SE	Intercept ±SE
trans- Cinnamaldehyde	25.80 (16.80-36.21)	0.96±0.10	-1.35±0.20	141.33 (99.80-202.45)	0.78±0.09	-1.67±0.19
(-)-Citronellal	13.99 (2.87-30.33)	0.98±0.10	-1.13±0.20	125.10 (90.66-172.73)	0.87±0.09	-1.79±0.19
p-Cymene	84.40 (19.66-246.42)	0.96±0.09	-1.84±0.20	369.41 (247.87-617.15)	0.71±0.09	-1.82±0.20
Eugenol	73.95 (24.56-170.59)	0.88±0.09	-1.64±0.19	176.14 (123.47-259.09)	0.75±0.09	-1.69±0.19
(-)-Menthone	47.86 (18.57-91.62)	1.02±0.10	-1.71±0.10	255.37 (88.55-1795.9)	0.68±0.09	-1.64±0.19
α-Pinene	35.97 (19.13-57.21)	0.61±0.09	-0.09±0.18	317.05 (214.41-516.83)	0.71±0.09	-1.77±0.20
α-Terpinene	102.15 (75.15-137.31)	0.93±0.09	-1.86±0.20	324.16 (124.93-2183.4)	0.62±0.09	-1.56±0.19
(-)-Terpinen-4-ol	126.72 (95.76-167.60)	1.00±0.09	-2.11±0.20	231.70 (152.05-381.07)	0.63±0.09	-1.50±0.19

[a] The Inhibitory concentration of 50 % from enzyme.
[b] Slope of the concentration inhibition regression line ±standard error (SE).
[c] Intercept of the regression line ±SE.

Table 4. The effect of Inhibition monoterpenes and phenylpropenes on polyglacturonase and dehydrogenases activities of *Ralstonia solanacearum*

Compound	Polyglacturonase			Dehydrogenases		
	IC_{50}[a] (mg/l) (95% Confidence limit)	Slope[b] ± SE	Intercept[c] ±SE	IC_{50} (mg/l) (95% Confidence limit)	Slope ± SE	Intercept ±SE
trans- Cinnamaldehyde	50.19 (36.14-66.80)	1.01±0.09	-1.72±0.20	122.49 (83.25-151.12)	0.94±0.09	-1.92±0.20
(-)-Citronellal	15.69 (8.45-24.46)	0.81±0.09	-0.97±0.19	83.41 (32.88-182.33)	0.98±0.09	-1.88±0.20
p-Cymene	129.67 (61.64-272.27)	0.98±0.09	-2.02±0.20	158.34 (106.45-242.28)	0.67±0.09	-1.49±0.19
Eugenol	47.77 (9.86-116.61)	0.78±0.09	-1.30±0.19	109.62 (81.05-147.36)	0.93±0.09	-1.90±0.20
(-)-Menthone	45.09 (11.59-100.70)	0.74±0.09	-1.22±0.18	190.62 (125.66-306.46)	0.63±0.08	-1.44±0.19
α-Pinene	86.90 (60.20-122.05)	0.79±0.09	-1.53±0.19	258.03 (180.97-391.51)	0.77±0.09	-1.85±0.20
α-Terpinene	132.22 (98.81-177.15)	0.96±0.09	-2.03±0.20	233.33 (152.73-387.77)	0.62±0.09	-1.48±0.19
(-)-Terpinen-4-ol	95.43 (68.24-131.22)	0.85±0.09	-1.69±0.19	143.18 (99.67-208.20)	0.74±0.09	-1.60±0.19

[a] The Inhibitory concentration of 50 % from enzyme.
[b] Slope of the concentration inhibition regression line ±standard error (SE).
[c] Intercept of the regression line ±SE.

Discussion

The current research summarizes the antibacterial inhibitory effect of six monoterpenes and two phenylpropenes against the phytopathogenic bacteria *A. tumefaciens*, *E. carotovora* var. *carotovora* and *R. solanacearum*. *trans*-Cinnamaldehyde exhibited potent antibacterial activity against *E. carotovora* var. *carotovora* and *A. tumefaciens*. Meanwhile, eugenol displayed moderate antibacterial activity against *A. tumefaciens*, *E. carotovora* var. *carotovora* and *R. solanacearum*. These results are comparable with those reported by (El-Zemity *et al.,* 2008). It has been observed that the presence of phenolic components in the oils increased their antibacterial potential against different microorganisms (Penalver *et al*., 2005). Similarly, (El-Zemity and Ahmed, 2005) noticed that the essential oils with high content of phenolic monoterpenes, such as carvacrol, eugenol and thymol showed strong antifungal activity. Also, (Abdel Rasoul *et al.,*2012) reported the effect of inhibiton 12 monoterpenes on the growth of *E. carotovora var. carotovora* and *A. tumefaciens*. They found that myrcene and thymol were the most potent antibacterial compounds, and these two compounds and (*R*) linalool inhibited polyglacturonase and dehydrogenases. The results of the current study also indicated that the tested phenylpropenes and monoterpenes were more effective against *A. tumefaciens* and *R. solanacearum* than *E. carotovora* var. *carotovora*. These findings pointed out that the activity of monoterpenes and phenylpropenes may differ with the bacterial species under investigation.

It is known that, the essential oils and their major constituents, monoterpenes and phenylpropenes, may cause their antimicrobial activity by elevating leaking and permeability of cell membranes (Lambert *et al.,* 2001; Oussalah *et al*., 2006). This may lead to loss of ions, reducing in membrane potential, and interruption of the proton pump (Di Pasqua *et al.,* 2006; Turina *et al*., 2006). Essential oils and their major constituents, monoterpenes and phenylpropenes, may also inhibit protective enzymes and consecutively inhibit various vital biochemical pathways (Xing *et al.,* 2012). They may cross the cell wall and the cytoplasmic membrane and damage the structure of different fatty acids, polysaccharides and phospholipids layers (Longbottom *et al.,* 2004). They may also coalesce in the cytoplasm and damage proteins and lipids (Burt, 2004). The results of the biochemical studies of the current study revealed that the tested monoterpenes and phenylpropenes caused significant inhibition of polyglacturonase and dehydrogenases of the three tested bacterial strains. Strong inhibition was observed on polygalacturonase activity.

In conclusion, the tested monoterpenes and phenylpropenes showed variable levels of antibacterial activity against the three plant pathogenic bacteria with *trans*-cinnamaldehyde, (–)-citronellal, (–)-menthone, eugenol and (–)-terpinen-4-ol being the most active compounds. These compounds also caused potent inhibition of polyglacturonase. These results indicated that monoterpenes and phenylpropenes could be possible candidates for biocontrol of plant pathogenic bacteria.

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التاثير الابادى البكتيرى و البيوكيميائي لمركبات التربينات الاحادية و الفينيل بروبين ضد البكتيريا النباتية

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في هذه الدراسة ، تم تقييم ستة من monoterpenes واثنين من phenylpropenes ضد ثلاثة انواع من البكتيريا المسببة للامراض النباتية ، *Agrobacterium tumefaciens* ، *Ralstonia solanacearum* و *Erwinia carotovora var. carotovora* ، وذلك باستخدام طريقة تخفيف أجار. تم اختبار التأثيرات المثبطة لهذه المركبات على انزيمى polygalacturonase و dehydrogenases أيضا لفهم طريقة ميكانكية العمل المحتملة من مركبات monoterpenes واثنين من phenylpropenes. كشفت نتائج الفحص المضاد للبكتيريا أن trans-cinnamaldehyde، (-) - citronellal، (-) - terpinen-4-ol كان أعلى نشاط مضاد للجراثيم ضد *A. tumefaciens*. كانت قيم تركيز المثبط الأدنى (MIC) 1000و 1500 و 1500 ملغم / لتر على التوالي. وبالمثل ، كان trans-cinnamaldehyde (MIC = 2000 mg / l) و citronellal (MIC = 2000 mg /l) -أكثر المركبات فعالية ضد *E. carotovora var. carotovora*. علاوة على ذلك ، كان السترونيال أكبر تأثير مضاد للبكتيريا ضد *R. solanacearum* وبقيمة MIC 1000 ملجم / لتر. في المقابل ، أظهر p-cymene أدنى نشاط مضاد للبكتيريا ضد السلالات البكتيرية الثلاثة التي تم اختبارها. بشكل عام ، كان *A. tumefaciens* و *R. solanacearum* أكثر حساسية من *E. carotovora var. carotovora* إلى المركبات التي تم اختبارها. علاوة على ذلك ، أظهرت trans-cinnamaldehyde أعلى آثار مثبطة على أنشطة polyglacturonase و dehydrogenases من *A. tumefaciens* ، في حين عرض citronellal أقوى تأثير كابح على polyglacturonase و dehydrogenases وأنشطة *E. carotovora var. carotovora* و *R. solanacearum*. استنادا إلى قيمها IC50 ، تسببت جميع المركبات التي تم اختبارها تثبيط أعلى على polyglacturonase من dehydrogenase.