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CHEMICAL COMPOSITION AND ANTIMICROBIAL ACTIVITY OF CINNAMON, THYME, OREGANO AND CLOVE ESSENTIAL OILS AGAINST PLANT PATHOGENIC BACTERIA

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Abstract

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Essential oils are volatile substances from plants and many of them have antimicrobial activity. For that reason, they have become known as a useful alternative to chemical preservatives and pesticides. In this study, we tested essential oils of four aromatic plants. Cinnamon (*Cinnamomum zeylanicum*), thyme (*Thymus vulgaris*), oregano (*Origanum vulgare*) and clove (*Syzygium aromaticum*) essential oils were investigated for their composition and antimicrobial effect against plant pathogenic bacteria (*Pectobacterium* spp. and *Pseudomonas* spp.). Both are commonly associated with diseased fruit trees in orchards and gardens. The chemical composition of the tested essential oils was identified by gas chromatography coupled with mass spectrometry. The cinnamon essential oil was most effective form tested oil. The experimental results indicated that the wild strains of tested bacteria are more resistant to essential oils than commonly used laboratory strains. In conclusion, certain essential oils could be used for the control of postharvest bacterial pathogens. The findings of the present study suggest that the essential oils have a potential to be used as antimicrobial agents.

Keywords: essential oils; plant pathogens; antibacterial; Pectobacterium; Pseudomonas

INTRODUCTION

Different bacterial plant pathogens with several genera of fungi and viruses cause many economically important diseases of plants around the world (Anderson *et al.*, 2004; Narayanasamy, 2011; Strange and Scott, 2005). Genus *Pectobacterium* is characterized as gram-negative, rod-shaped and fermentative bacterium. These bacteria cause soft rot and other diseases in a wide range of plant species, including several economically important ones (Alamshahi *et al.*, 2010). *Pseudomonas syringae* is gram-negative polyphagous plant pathogenic bacterium. This bacterium usually survives as an epiphyte on host plants and under appropriate environmental conditions to become pathogenic. Bacteria form genus *Pseudomonas* cause significant losses to stone fruits. Variety of symptoms such as blossom blast, spur dieback, leaf necrosis, bark cankers, gummosis of woody tissues and bacterial spot are associated with this bacterium (Huang and Lakshman, 2010; Kokoskova *et al.*, 2011). Most plants have developed tolerance to several of plant pathogens. Waxy cuticular layers and natural antimicrobial compounds provide passive protection against pathogens that are not specialized to attack a specific host (Dangl and Jones, 2001). Gram-negative bacteria from genus *Pectobacterium* and *Pseudomonas* are ones of the most occurring plant pathogenic bacteria in fields (Holtsmark *et al.*, 2008; Mansfield *et al.*, 2012). It is difficult to protect agricultural product from bacterial rot. Some products based on copper hydroxide are commercially available, but they are primary used as fungicides. Various plants which are known for their antimicrobial and antifungal activity are for centuries used to improve taste and aroma of

food. Essential oils represent a complex mixture of natural substances. Essential oils are known for their antibacterial and antifungal activity and have been empirically used as antimicrobial agents (Bakkali *et al.*, 2008; Burt, 2004). The spectrum of activity and mechanisms of action is still unknown for most of them. Some authors reported better efficiency of essential oils in vapour phase and in this case they could be used in lower concentrations with lower impact to treated product (Frankova *et al.*, 2016; Goñi *et al.*, 2009; Laird and Phillips, 2012; Nedorostova *et al.*, 2009). Chemical composition of essential oils is commonly studied by gas chromatography coupled with mass spectrometry (Kloucek *et al.*, 2012; Nedorostova *et al.*, 2011; Velázquez-Nuñez *et al.*, 2013). This work evaluates the antibacterial activity of selected essential oils against important plant pathogens.

MATERIAL AND METHODS

Bacterial strains

Nine clinical isolates of plant pathogenic bacteria from Collection of Phytopathogenic Bacteria (Crop Research Institute, Czech Republic) namely *Pectobacterium carotovorum* sub. *carotovorum* (CPPB-54), *Pectobacterium carotovorum* subs. *antroseptica* (CPPB-83), *Pectobacterium carotovorum* subs. *carotovorum* (CPPB-143), *Pseudomomas fluorescens* (CPPB-195), *Pseudomomas fluorescens* (CPPB-89), *Pseudomonas fluorescens* (CPPB-171), *Pseudomonas putida* (CPPB-74), *Pseudomonas putida* (CPPB-99), *Pseudomonas syringae* pv. *lachrymans* (CPPB-152), and two strains from Czech collection of microorganism (Brno) *Pectobacterium carotovorum* subsp. *carotovorum* (CCM 1008), *Pseudomonas syringae* (CCM 7018) were used in this study. Used strains are not indicated as resistant. Bacteria were cultivated at 25 °C in Tryptone Soya Broth or Pseudomonas Agar Base both from Oxoid (UK). Before tests strains was inoculated to liquid Tryptone Soya Broth (Oxoid, UK) and cultivated overnight, 72 h in the case of *Pseudomonas* strains.

Essential oils

Cinnamon leaf (*Cinnamomum zeylanicum*), thyme (*Thymus vulgaris*), oregano (*Origanum vulgare*), and clove (*Syzygium aromaticum*) essential oils from Biomedica (CZ) and Sigma-Aldrich (CZ) were stored in air-tight sealed glass bottles at 4 °C till further use. The relative composition of essential oils was determined and the compounds were identified by GC-MS.

Chemical composition

Identification of essential oil constituents was processed by GC-MS method. Samples of tested essential oils were prepared in hexane to final concentration l µL/mL. Analyses were carried out using Agilent 7890A GC coupled to an Agilent MSD5975C MS detector (Agilent Technologies, Palo Alto, CA, USA) with a HP-5MS 5% Phenyl Methyl Siloxan column (30 m×0.25 mm, 0.25 µm film thickness). One µL of the sample was injected in split mode 1:12, the injector temperature was 250 °C, the electron ionization energy set at 70 eV. The oven temperature started at 60 °C and was programmed to 231 °C at a rate of 3 °C/min, and then kept constant for 10 min. (Kloucek *et al.*, 2012). The identification of constituents was based on comparison of their mass spectra and relative retention indices (RI) with the National Institute of Standards and Technology Library (NIST, USA), as well as authentic standards and literature (Adams, 2007). Used standards from Sigma-Aldrich (CZ) and their RI are in Tab. I.

Antimicrobial assays

Bacterial cultures were grown in Tryptone Soya Broth at 25 °C for 24 h respectively 72 h in the case of *Pseudomonas* strains before the tests. An inoculum was then created by dilution in the same medium to a final cell concentration of 10^6 CFU/mL, which was confirmed by density measurement in McFarland

I: *Retention indexes of used standards*

RI	standard	RI	standard	RI	standard
921	anisole	**1089**	(+)-fenchone	**1223**	(-)-menthone
937	α-pinene	**1102**	linalool	**1234**	α-citronellol
952	camphene	**1112**	rose oxide	**1244**	citral
963	benzaldehyde	**1146**	camphor	**1247**	(-)-carvone
979	β-pinene	**1149**	isopulegol	**1259**	geraniol
993	myrcene	**1158**	(+/-)-citronellal	**1271**	cinnamaldehyd
999	butylisothiocyanate	**1169**	borneol	**1287**	(-)-bornyl acetate
1019	α-terpinene	**1176**	(+)-menthol	**1296**	thymol
1028	p-cymene	**1181**	4-terpineol	**1306**	carvacrol
1032	limonene	**1198**	d-dihydrocarvone	**1361**	eugenol
1034	eucalyptol	**1199**	estragole	**1387**	geranyl acetate
1063	γ-terpinene	**1202**	2-decanol	**1420**	α-caryophyllene
				1448	citronellyl propionate

II: *Essential oils composition by GC-MS*

RI[b]	coumpound	Cinnamon[c]	Clove	Oregano	Thyme
931	α-thujene				0.32
937	[a] α-pinene	1.04		0.41	1.31
952	[a] camphene	0.33		0.17	1.52
963	[a] benzaldehyde	0.18			
979	[a] β-pinene	0.21		0.80	0.12
981	1-octen-3-ol			0.22	0.73
988	3-octanone			0.14	
992	β-myrcene			0.52	1.36
998	3-octanol			0.19	
1005	α-phellandrene	0.80		0.13	0.14
1019	α-terpinene	0.12		0.42	1.12
1027	[a] p-cymene	1.22		10.32	17.88
1031	[a] d-limonene	0.71		0.67	0.36
1034	eucalyptol	0.12		0.99	1.47
1062	γ-terpinene			2.56	5.27
1089	terpinolene			0.16	0.13
1100	[a] linalool	1.87		1.45	4.81
1146	[a] camphor			0.69	1.98
1167	[a] borneol			1.03	1.85
1178	[a] 4-terpineol	0.10		0.96	1.93
1190	α-terpineol	0.24		0.56	0.18
1194	methyl salicylate		0.12		
1238	thymol methyl ether				0.70
1240	2-methyl-3-phenyl-propanal			0.18	
1247	[a] (-)-carvone				1.01
1269	[a] cinnamaldehyde	1.35			
1285	bornyl acetate	1.33		0.14	
1296	[a] thymol			5.14	44.34
1297	menth-1-en-9-ol				2.88
1305	[a] carvacrol			67.14	
1350	cubebene		0.12		
1364	[a] eugenol	76.85	81.74		0.22
1376	copaene	0.68	0.42		0.16
1419	[a] caryophyllene	2.97	12.25	1.09	6.94
1446	cinnamyl acetate	1.38			
1453	α-caryophyllene	0.53	1.49	0.35	
1509	β-bisabolene			0.11	
1523	δ-cadinene	0.14	0.31	0.27	0.32
1529	eugenol acetate	2.21			
1573	caryophyllene oxide	0.48	2.54	1.35	0.19
1763	benzyl benzoate	3.87			
Total:		98.72	98.99	98.17	99.22

[a] Identification confirmed by co-injection of authentic standard.
[b] RI: identification based on Kovat's retention indices (HP-5MS capillary column) and mass spectra.
[c] Relative proportions were calculated in % by dividing individual peak area by total area of all peaks.

units (densitometer McFarland type DEN-1B, Biosan, LV). A modification of EUCAST microdilution method (2003) was used for antimicrobial testing. A two-fold serial dilution ranging from 1024 to 64 mg/L was prepared from a stock solution in tryptone soy broth with 1% of Tween 80 in 96-wells microtitration plates, 10 µl of inoculum was added to 190 µl Tryptone Soya Broth with 1% of Tween 80. The microtitration plates were incubated at 25 °C for 72 h. After incubation, the minimum inhibitory concentrations (MICs) were recorded. MICs is defined as the lowest concentration of tested substance which absolutely inhibit by naked eye visible growth of the microorganism. Each plate contained two negative control without inocula and two positive controls without tested substances. All

tests for each compound, were replicate three times, and the results were calculated as median MICs value from these receptions. All tests were done in microbiological safety cabinet to protect samples form contamination.

RESULTS

Essential oils composition

The chemical compositions of tested essential oils were analysed by GC-MS method from Kloucek *et al.* (2012). The percentage of identified compounds and modes of identification are listed in Tab. II. The chromatographic analyses resulted in the identification of 43 components, representing more than 98% of the oils, the others being only in trace amounts (<0.1%). The major identified component of cinnamon and clove oil was eugenol (77%; 82%). In oregano oil, we detected as major compounds carvacrol (67%) and p-cymene (10%). Thymol (44%) was main compound in thyme oil.

Antibacterial assay

Completed antibacterial test shows high (Tab. III) variability between tested strains. In general, we can say that cinnamon essential oil was most effective, but in the other hand in case of clove was the less effective in tested concentrations. From tested strain *Pectobacterium carotovorum* subs. *antroseptica* (CPPB-83) were most sensitive and *Pseudomonas fluorescens* (CPPB-171), which was most sensitive strain from all tested bacteria. *Pectobacterium carotovorum* subs. *carotovorum* and *Pseudomonas syringae* pv. *lachrymans* were not inhibited in tested concentration of essential oils.

DISCUSSION

Plant extracts and essential oils are used for centuries as natural preservatives in food. For their antimicrobial activity are used as pharmaceuticals in alternative medicine and natural therapies (Badawy and Abdelgaleil, 2014; Castillo *et al.*, 2014).

Cimanga *et al.* (2002) reported the high antibacterial efficacy of thyme essential oil and its major components, which are α-pinene, p-cymene, carvacrol and thymol against post-harvest pathogens. In comparison to the previously reported results (de Azeredo *et al.*, 2011; Hosseini Nezhad, 2012) the isolates tested in this study are less sensitive to used essential oils. Usually is problematic to compare outcomes achieved in different studies, because of authors used the various testing methods, numerous bacterial strains and several sources of tested antimicrobial substances. Essential oils are biological products with high variability (Kaya *et al.*, 2013). For example Karami-Osboo *et al.* (2010) registered antibacterial effect of thyme oil, carvacrol and thymol on *Pectrobacterium* by measuring of inhibition zones of agar diffusing method, but this process say very small data about MIC of tested compounds (Jorgensen and Turnidge, 2015). Disc diffusion method is useful method for qualitative preliminary screening of antimicrobial activity (Jorgensen and Turnidge, 2015). In our test, quantitative microdilution method was used. Badawy and Abdelgaleil (2014) described components of some essential oils and their minimum inhibitory concentrations obtained by the agar dilution method. In their study the oil of *Thymus occidentalis* effectively inhibited grow of *Pectobacterium*, MIC values of 350 mg/L. Kokoskova *et al.* (2011) related high antimicrobial activity to three essential oils from *Thymus vulgaris, Origanum compactum* and *Origanum vulgare.* They detected p-cymene (16%), geraniol (8%), thymol (6%) and carvacrol (8%) as main compounds in thyme oil. For *Origanum compactum* carvacrol (36%), p-cymene (22%) and thymol (18%), for *Origanum vulgare* thymol (28%) and carvacrol (19%) were identified as major compounds. All three oils were tested against plant pathogens *Pseudomonas syringae* and *Pectobacterium carotovorum* by evaluating of the inhibition zones. Oussalah *et al.* (2006) reported **s**trong antimicrobial

III: *Minimum inhibitory concentrations (MICs) of essential oils against plant pathogens*

Bacterial strain	Cinnamon	Thyme	Oregano	Clove
	[mg/L]			
***Pectobacterium carotovorum* subs. *carotovorum* (CPPB-54)**	>1024	>1024	>1024	>1024
***Pectobacterium carotovorum* subs. *antroseptica* (CPPB-83)**	128	128	256	512
***Pectobacterium carotovorum* subs. *carotovorum* (CPPB-143)**	>1024	>1024	>1024	>1024
***Pseudomomas fluorescens* (CPPB-195)**	1024	1024	128	>1024
***Pseudomomas fluorescens* (CPPB-89)**	1024	>1024	>1024	>1024
***Pseudomonas fluorescens* (CPPB-171)**	128	128	512	1024
***Pseudomonas putida* (CPPB-74)**	512	>1024	>1024	>1024
***Pseudomonas putida* (CPPB-99)**	128	>1024	>1024	>1024
***Pseudomonas syringae* pv. *lachrymans* (CPPB-152)**	>1024	>1024	>1024	>1024
***Pectobacterium carotovorum* subs. *carotovorum* (CCM 1008)**	128	256	256	1024
***Pseudomonas syringae* (CCM 7018)**	256	512	254	1024

action of *Origanum compactum*, *Origanum heracleoticum* and *Thymus vulgaris* against *Pseoudomonas putida*. They reached MIC values of 0.05%. Some studies (Zheng *et al.*, 2013) reported inhibitory effects of essential oil compounds against numerous bacteria and determined MIC for *Pseoudomonas fluorescens* were 400–700 µg/mL for carvacrol; 800–1200 µg/mL for thymol; 1600–4000 µg/mL for eugenol, respectively. Therefore, they observed similar MIC values like us in this study. In our study, cinnamon oil has strong antibacterial effect, which could be caused by eugenol in combination with benzyl benzoate, both from cinnamon leaf essential oil (Ali *et al.*, 2002). Joshi *et al.* (2016) proved that plant phenolic volatiles eugenol and carvacrol inhibit quorum sensing in pectobacteria and reduce their virulence. Essential oils could be safely used as effective pesticides with low risks for human health and environments. They could develop good alternative for conventionally used chemical constituents (Karami-Osboo *et al.*, 2010; Zarubova *et al.*, 2015).

CONCLUSION

In the present study, we evaluated and compared the antimicrobial activities of four plant essential oils against a range of postharvest plant pathogens. Oregano and cinnamon showed a high effect against the test microorganisms. In conclusion, essential oil can act effectively against postharvest plant pathogens at low concentrations. Further studies are needed to evaluate the efficacy of model products of these essential oils. Result show that laboratory strains could be more sensitive to natural antimicrobials than wild strains.

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