

ANTIBACTERIAL ACTIVITY OF ESSENTIAL OILS
AGAINST THE ETIOLOGICAL AGENT OF AMERICAN
FOULBROOD DISEASE (*PAENIBACILLUS LARVAE*)

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Summary

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Antibacterial activities of eleven essential oils against *Paenibacillus larvae* (15 field strains and the reference BCCM / LMG 9820 strain) were studied by the disk diffusion method and the method of serial dilutions in agar. The minimal inhibitory concentration (MIC) of essential oils was determined within 1%–0.015% v/v. Highest activity: MIC \leq 0.06–0.015% v/v was shown by essential oils of cinnamon, thyme, clove, peppermint, lemongrass, sage and oregano. Variable activity exhibited marjoram and tee tree oils. Citrus essential oils showed the lowest inhibitory effect with MIC \geq 0.12–1.0% v/v for mandarin oil and \geq 0.25–0.5% v/v for grapefruit oil. Established antibacterial activity against *Paenibacillus larvae* encourages further research to include essential oils as an alternative means in the measures for prevention and control of American foulbrood without the use of antibiotics.

Key words: American foulbrood, essential oils, minimal inhibitory concentration, *Paenibacillus larvae*

INTRODUCTION

American foulbrood is a highly contagious disease affecting the larval stage in the development of honey bees (*Apis mellifera* L.). The etiological agent is the spore forming bacterium *Paenibacillus larvae* (Genersch *et al.*, 2006). This is the most serious bee disease (Ratnieks, 1992; Shimanuki & Knox, 1997), leading to death not only of affected colonies, but often to the perishment of entire apiaries (Matheson & Reid, 1992). It spreads rapidly within the apiary during the routine beekeeping practice, as well as by bee thieves, flying and roaming of bees (Delaplane, 1991).

Methods for American foulbrood control vary in different countries. In EC member states, including the Republic of Bulgaria, the use of antibiotics and sulfonamides in beekeeping is prohibited, while in others – USA, Canada, Australia, their application for preventive purposes is a routine practice. It is therefore not surprising that *P. larvae* strains resistant to antibiotics have been isolated (Miyagi *et al.*, 2000). Another problem accompanying this practice is the accumulation of antibiotic residues in bee products (Bogdanov, 2006). This requires the development of alternative means for American

foulbrood control without the use of antibiotics.

In recent years the interest of researchers has been focused on identifying natural substances with antimicrobial properties (including essential oils) which, in optimal concentrations, are readily accepted by the bees, do not accumulate in bee products and provide a stimulating effect on the development of colonies (Bogdanov, 2006; Zhelyazkova *et al.*, 2009).

Essential oils are concentrated volatile compounds from the metabolism of plants with multiple pharmacological effects. According to a number of authors, they exhibit antimicrobial, antiparasitic and insecticide activity (Ghannoum, 1988; Romerio *et al.*, 1989; Karpouhtsis *et al.*, 1998; Pessoa *et al.*, 2002). The essential oils of some wild plants are reported to have antifungal and acaricide activity on *Ascosphaera apis* and *Varroa destructor*, causing ascospherosis and varroasis of bee brood and adults (Eguaras *et al.*, 2005). The antibacterial activity of essential oils against *Paenibacillus larvae* has been studied (Albo *et al.*, 2003; Eguaras *et al.*, 2005; Fuselli *et al.*, 2008). In our country, the activity of certain oils to a limited number of strains of *P. larvae* (n=5) was tested by Gurgulova *et al.* (2006).

The aim of the present work was to determine the *in vitro* activity of various essential oils to field strains (isolated from apiaries in Bulgaria) and a reference *Paenibacillus larvae* strain with respect to their utilization as alternative means for prevention and control of American foulbrood without antibiotics.

MATERIALS AND METHODS

The antibacterial activity of 11 essential oils against 15 field *P. larvae* strains and

the reference *P. larvae* BCCM/LMG 9820 strain was studied by the disk diffusion method (Bauer *et al.*, 1966) and by determining the minimum inhibitory concentrations (MIC) according to the serial dilution method in agar by Eguaras *et al.* (2005) with some modifications (utilization of TSA instead of MYPGP agar, of Tween 20 instead of propylene glycol and 15 µL bacterial suspension spread on agar instead of 20 µL). The strains were isolated from bee brood with signs of American foulbrood and identified by conventional microbiological test methods (Anonymous, 2008).

Essential oils

Essential oils from thyme (*Thymus vulgaris*), cloves (*Syzygium aromaticum*), cinnamon (*Cinnamomum aromaticum*), marjoram (*Origanum majorana*), tea tree (*Melaleuca alternifolia*), sage (*Salvia sclarea*), peppermint (*Mentha x piperita*), oregano (*Origanum vulgare*), grapefruit (*Citrus paradisi*), lemon grass (*Cymbopogon citratus*) and mandarin (*Citrus reticulata* var. *madurensis*) were obtained from ArtMedics Ltd., Sofia and Lavena AD, Shoumen, Bulgaria.

Disc diffusion method

Suspensions with 0.5 McFarland standard density were prepared from the tested strains and were spread in amount of 100 µL on Columbia agar (BD) with 5% sheep red blood cells with a sterile Drigalski spatula. Sterile 6-mm filter paper discs (NCIPD, Sofia) were placed on the agar surfaces after absorption of inocula and 10 µL aliquots of essential oils were added. A disc impregnated with saline was used as control. The Petri dishes were left for 30 min at room temperature for diffusion of the oils in agar and incubated at 35 °C for 48 h. The

zone of growth inhibition was measured in millimetres and determined in triplicate. Activity of essential oils was interpreted as strong (with growth inhibition zone of ≥ 20 mm), medium (20–12 mm) and no activity (<12 mm) (Rota *et al.*, 2008).

Determination of minimum inhibitory concentration (MIC) of essential oils by serial dilution in agar

Serial twofold dilutions of essential oils ranging from 1 % v/v to 0.015 % v/v were prepared in trypticase soy agar incorporated with 1 % Tween 20 (Sigma) at 50 °C. After solidification of agar and evaporation of condensation water at 35 °C, plates were inoculated with of 15 μ L of bacterial suspensions containing approximately 10^7 cfu/mL. Trypticase soy agar with 1% Tween 20, but with no oil was used as positive growth control. After absorption of the bacterial suspensions in agar, plates were incubated inverted at 35 °C for 48–72 h. The MICs were determined as the lowest concentrations of any oil inhibiting the visible growth of test strains on the agar plate. The presence of 1–2 colonies was ignored. The MICs by the serial dilution method in agar were determined twice.

Statistical analysis

The data from the disc diffusion method are presented in millimetres as mean \pm standard deviation of three parallel measurements (StatMost, version 2.5).

RESULTS

The results from the effect of essential oils on *P. larvae* by the disc diffusion method are presented in Table 1.

According to accepted criteria, the data show that the essential oils of

cinnamon, thyme, clove, peppermint, lemon grass, sage and oregano exhibited a strong inhibitory effect against all tested *P. larvae* strains. Tea tree oil showed a strong activity – for three strains, the growth inhibition zones were in the middle range. Variable values showed marjoram oil. It exhibited a strong activity against the reference *P. larvae* LMG 9820 strain and four of field strains, and a medium one against the others. Mandarin and grapefruit oils showed either medium or lack of activity in some strains ($d < 12$ mm).

Table 2 summarizes the minimum inhibitory concentrations of essential oils by the serial dilution method in agar. The lowest concentrations inhibiting the growth of colonies on agar (0.015% v/v) were those of cinnamon, clove and lemon grass oils. MICs of the essential oils of thyme, peppermint, sage, oregano and tea tree were within the range of 0.03–0.06% v/v, the latter one inhibited three strains at 0.12% v/v. Marjoram oil showed MIC in the 0.06–0.12% v/v range. Oils of mandarin and grapefruit inhibited the growth of *P. larvae* at MIC ≥ 0.12 –1.0% v/v.

DISCUSSION

The application of natural plant products for control of bacterial, fungal and parasitic bee and brood diseases has several advantages over conventional means. In the specialized literature resistance of bacteria to essential oils has not yet been documented (Hitokoto *et al.*, 1980). In addition, natural substances in bee products decompose rapidly, their quantity in honey is low and they do not have an adverse effect on the health of consumers (Nozal *et al.*, 2002). Although in small amounts, essential oils and organic acids are normally contained in various types of honey. According to some authors their use as alternative means for

Table 1. Antibacterial activity (inhibition zone, mm) of essential oils against *P. larvae* (15 field isolates and a reference strain BCCM/LMG 9820) by the disc diffusion method. Data are presented as mean±SD

	Essential oils										
	cinnamon	thyme	cloves	marjoram	mandarin	grapefruit	tea tree	peppermint	lemon grass	sage	oregano
Field <i>P. larvae</i> strains											
1	44.3±2.0	59.0±1.0	40.3±0.6	17.3±0.6	10.7±0.6	14.3±1.5	21.0±1.0	26.0±1.0	56.3±1.5	36.0±1.0	61.0±2.6
2	43.3±1.5	60.0±1.0	41.3±1.5	19.7±1.2	14.3±0.6	16.0±1.0	29.0±1.0	35.0±1.0	57.7±1.5	37.7±2.0	55.3±4.2
3	46.0±1.0	50.7±1.5	38.3±1.5	18.7±0.6	11.3±0.6	13.3±1.5	26.7±2.0	29.0±2.6	61.3±3.2	35.0±2.6	51.7±2.5
4	47.7±1.5	49.7±2.1	36.7±1.5	19.0±2.0	14.7±0.6	15.3±1.5	25.3±1.5	31.3±1.5	63.0±2.6	40.0±1.0	53.0±2.6
5	49.7±1.5	51.7±1.5	35.3±1.5	17.3±1.5	14.3±0.6	16.0±1.0	27.0±3.0	33.0±2.0	64.3±2.1	47.3±2.5	55.7±2.0
6	50.7±3.0	57.7±2.5	39.7±2.5	24.0±1.7	11.0±1.0	12.0±1.0	28.7±1.5	37.0±2.6	66.0±2.6	54.0±3.6	60.7±3.0
7	45.7±5.1	60.3±1.5	50.3±2.5	16.3±1.5	12.0±2.6	16.3±1.5	18.3±0.6	39.0±2.0	63.0±2.0	59.3±2.0	63.7±3.5
8	39.9±1.5	49.7±2.0	58.7±2.3	19.0±1.0	10.0±1.0	16.7±2.0	23.7±1.5	41.0±2.0	65.3±1.5	38.7±1.5	65.3±3.8
9	40.3±2.5	49.0±1.0	59.0±3.6	32.0±2.0	16.3±1.5	17.0±1.0	24.0±1.0	36.7±2.0	66.7±2.5	35.7±1.5	62.0±4.6
10	42.7±2.5	52.7±2.5	62.7±2.5	24.0±2.0	10.7±1.5	11.3±2.0	18.0±1.0	34.3±1.5	66.3±3.0	57.3±2.5	60.7±2.5
11	45.0±2.0	60.0±2.0	41.3±1.5	19.3±2.0	13.0±1.0	14.3±1.5	23.3±2.0	36.3±2.5	65.0±2.0	34.0±2.6	57.7±3.5
12	50.7±2.0	48.0±2.0	42.7±2.5	21.0±1.0	12.3±1.5	14.0±2.0	25.0±1.0	39.7±3.0	62.7±1.5	33.0±3.6	63.7±3.2
13	52.7±2.5	45.6±1.5	48.0±2.6	20.6±1.2	10.7±1.5	15.3±0.6	19.7±1.5	40.7±2.0	58.7±2.5	43.0±3.0	60.0±2.0
14	54.7±1.5	60.7±2.1	50.3±1.5	17.0±1.0	10.0±1.0	12.3±1.5	27.3±1.5	31.3±2.5	56.3±2.5	46.3±3.5	60.7±3.0
15	52.7±3.5	62.7±2.5	49.3±2.0	17.7±0.6	11.3±1.5	14.3±2.5	26.3±3.0	35.0±1.0	58.3±2.0	31.7±2.0	57.7±2.5
Reference strain BCCM/LMG 9820											
	50.7±2.0	55.0±1.0	54.7±4.2	35.0±1.0	15.0±1.0	18.3±0.6	25.7±1.5	26.0±1.0	59.0±2.0	34.7±1.5	58.7±1.5

Table 2. Minimal inhibitory concentrations in of essential oils against *P. larvae* (15 field isolates and a reference strain BCCM/LMG 9820) by the serial dilution method in agar; n – number of field strains

Essential oils											
cinnamon	thyme	clove	marjoram	mandarin	grapefruit	tea tree	peppermint	lemon grass	sage	oregano	
MIC of field strains, % v/v											
0.015 n=15	0.03 n=9	0.015 n=15	0.12 n=11	1.0 n=6	0.5 n=11	0.12 n=3	0.03 n=4	0.015 n=15	0.06 n=15	0.06 n=15	
	0.06 n=6		0.06 n=4	0.5 n=7	0.25 n=4	0.06 n=5	0.06 n=11				
				0.25 n=1		0.03 n=5					
				0.12 n=1		0.015 n=2					
MIC of the reference strain BCCM/LMG 9820, % v/v											
0.015	0.06	0.015	0.06	0.12	0.25	0.03	0.06	0.015	0.06	0.06	

prevention and control of bee diseases guarantees ecologically clean bee products (Zhelyazkova *et al.*, 2009).

This study established a relationship between the measured zones of *P. larvae* growth inhibition and MIC values at dilution of the oils in agar. Most of the tested essential oils showed high activity against *P. larvae*, which is reported by other authors (Alippi *et al.*, 1996; Albo *et al.*, 2003; Fuselli *et al.*, 2006; Gende *et al.*, 2008). Marjoram and tee tree oils exhibited variable activity. We found out a low activity of mandarin and grapefruit oils which is in agreement with other publications (Smith-Palmer *et al.*, 1998) and previous studies of ours on pathogens of veterinary medical importance (Rusenova & Parvanov, 2009). Fuselli *et al.* (2008) have reported a high activity of *Citrus paradisi* (grapefruit) oil to *P. larvae* with MIC 385.0 mg/L. In a study of antibacterial activity of essential oils against *P. larvae*, Gurgulova *et al.* (2006) established a high activity of oils from savory, thyme and white marjoram with MIC = 0.012–0.025% v/v. Differences in MIC values and the interpretation of the activity of essential oils against *P. larvae* can be explained as a lack of internationally accepted criteria for reporting results, and with varying composition of the oils, the structural configuration of components and their possible synergistic interactions. Some environmental and natural climatic factors affecting the growth of producing plants can also have an impact (Chang *et al.*, 2001). These reasons require a differentiated approach to the selection and use of essential oils for prevention and control.

The antibacterial activity of essential oils is explained by the presence of different active compounds. Gende *et al.* (2008) attribute the high activity of

cinnamon oil against *P. larvae* to its constituents cinnamaldehyde and eugenol. Thyme oil is rich in thymol and carvacrol, which determine its antibacterial activity (Rota *et al.*, 2008). The activity of grapefruit oil is due to the presence of volatile compounds, mainly limonene and myrcene (Fuseli *et al.*, 2008). They are responsible for the different mechanism of action of essential oils on the microbial agent. Investigations in this regard have shown abnormalities in permeability of the cytoplasmic membrane, leakage of K⁺ ions from the cell and clumping of intracellular substances (Rasooli *et al.*, 2006; Shapira & Mimran, 2007).

The high *in vitro* activity against *P. larvae* of most of the essential oils tested in this study and the evidence for their stimulating effect on development and productivity of bee colonies (Zhelyazkova *et al.*, 2009) shows that essential oils can be a reliable alternative means for prevention and control of American foulbrood without the use of antibiotics. However, further studies are needed on the efficacy of their implementation *in vivo*.

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