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Evaluation of some essential oils as alternative antibiotics against American foulbrood agent *Paenibacillus larvae* on honey bees *Apis mellifera* L

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In this study, antibacterial activities of some essential oils against *Paenibacillus larvae* were investigated as an alternative to synthetic antibiotics used against American foulbrood (AFB), which causes serious damage to the beekeeping industry. Ten essential oils belonging to various species and carvacrol, the major component of *Origanum onites* oil, were analyzed. Besides examining the antibacterial activities of the essential oils in the experiments, composition of the oils was investigated by gas chromatography (GC) and GC–mass spectroscopy (GC–MS) analysis. The minimum inhibition concentrations (MICs) of the effective essential oils were also determined. The conclusions resulting from these observations were that *Origanum onites* oil showed the strongest antibacterial activity. *Rosmarinus officinalis*, *Seseli andronakii*, *Pimpinella anisum*, *Heracleum platytaenium*, *Anethum graveolens*, *Bifora radians*, and *Seseli tortuosum* were respectively found to be effective coming after *Origanum onites*. *Seseli petraeum* and *Seseli resinosum*, two different species within the same genus showed no antibacterial activity against AFB. The results show that composition of the essential oils is very important for antibacterial effect; in fact antibacterial effect is determined by the major component in the essential oil composition. However, intercomponent synergetic effect is as important as the major component itself according to the results of carvacrol experiments.

Keywords: *Apis mellifera*; *Paenibacillus larvae*; antibiotic; American; foulbrood; essential oils; treatment; colony losses

Introduction

American foulbrood (AFB) caused by the spore forming bacterium *Paenibacillus larvae* is the most serious disease of bacterial origin affecting the larval and pupal stages of honey bees (1–3). In areas where disease incidence is high, antibiotics have been applied as an alternative to burning infected hives. *In vitro* tests have demonstrated that a lot of synthetic antibiotics possess antibacterial activity against *P. larvae* such as oxytetracycline, tylosine, democlocycline, doxycycline, minocycline, paromomicin, tobramycine, etc. (4–11). However, both *P. larvae* is a highly pathogenic disease and in recent years has become resistant to conventional antibiotics worldwide (6, 12, 13). The use of new, commercial antibiotics can create strains that are unaffected by a suite of compounds (6) and can further affect the beekeeping industry since many antibiotics leave residues in hive products (14). For this reason, a ban has been introduced in the European Union (EU) member states and Turkey on the use of synthetic antibiotics against AFB (15, 16). In untreated colonies, spores in the combs can remain viable for a long time through extreme temperature changes. Beekeeping equipment and products from infected hives, including honey from colonies affected by AFB, can become contaminated and can promote the spread of the disease within and among colonies (17–19). There is no alternative but to burn infected colonies to eliminate the source of infection, however, beekeepers often prefer not to do this which leads to the spread of disease routinely. As a consequence of the fact that chemicals cannot be used against AFB, and with AFB being a serious cause of severe losses in the beekeeping industry, research activities have focused on the use of natural compounds like essential oils extracted from different plants. Essential oils have no residue problem in bee products. They have a synergetic effect among their components and because their composition is complex and their mode of action is not yet well understood, it is very difficult for resistance to build up. Thus, a small number of extracted essential oils were investigated in terms of their antibacterial activity, antioxidant characteristics and composition. Various essential oils like savory, thyme, lemon-grass, oregano and their mixture have been used in some studies on AFB. (20–24). Turkey is one of the largest reserves across the world for essential oils and floral diversity. Its bridging position between Asia and Europe, and multi-seasoned climate contribute

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to this plant diversity. Turkey is located on the crossroads of three different floristic regions (namely Europe-Siberia, Iran-Turan, Mediterranean). The flora of Turkey is made up of 10000 species from 173 families and 1225 genera, 3000 endemic species, and sixteen endemic genera (25–33). In this study, essential oils extracted from ten plant species were examined for the control and prevention of AFB disease antibacterial activities.

Experimental

Plant material

A: Seseli petraeum M. Bieb. was collected in October 2002, from Trabzon, Maçka, around Sümela Monastery (ED 1654). B: *Seseli resinosum* Freyn et Sint. was collected on 12 September 2001, in Bartin-Amasra (ED 1641). C: *Seseli andronakii* Woron. was collected in October 2002, from Gümüshane to Köse (ED 1653). D: *Seseli tortuosum* L. was collected on 12 September 2002 in Ankara-Beynam Forest (ED 1655). E: *Bifora radians* Bieb. was collected on 7 June 1996 in Eskisehir: Eskisehir to Seyitgazi (ESSE 12054). F: *Anethum graveolens* L. was obtained from a commercial firm on 19 April 1995. G: *Heracleum platytaenium* Boiss. was collected on 10 June 1994 in Manisa, Spil Mountain, Atalanı High Plato. H: *Pimpinella anisum* L. was obtained from a commercial firm on 21 December 1995. I: *Rosmarinus officinalis* L. was collected on April 2000 in Tarsus, Aladag. J: *Origanum onites* L. was obtained from a commercial firm on 24 May 2000. Voucher specimens are deposited in the Herbarium of the Faculty of Pharmacy, Gazi University in Ankara, Turkey (ED) and Anadolu University in Eskisehir, Turkey (ESSE).

Essential oil distillation

Crushed fruits of *Seseli petraeum, S. andronakii, S. resinosum, S. tortuosum, Anethum graveolens, Heracleum platytaenium* and whole fruits of *Pimpinella anisum* and aerial parts of *Bifora radians*, *Rosmarinus officinalis* were subjected to separate water distillation for 3 hours using a Clevenger-type apparatus. The yields of essential oils were 3.4%, 2.1%, 2.1%, 2.2%, 3.6%, 6.5%, 2.6%, 0.7%, and 1.9% (v/w), respectively on a dry weight basis. *Origanum onites* L. yielded 2.0% oil by steam distillation.

Gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS) conditions for Bifora radians, Anethum graveolens, Heracleum platytaenium, Pimpinella anisum, Origanum onites *and* Rosmarinus officinalis

The gas chromatography (GC) analysis was carried out using an Agilent 6890N GC system. In order to obtain the same elution order with GC–mass spectrometry (GC–MS), simultaneous injection was achieved using the same column and appropriate operational conditions. Flame ionization detection (FID) temperature was 300°C.

GC–MS

The GC–MS analysis was carried out with an Agilent 5975 GC MSD system. Innowax FSC column (60 m × 0.25 mm, 0.25 m film thickness) was used with helium as carrier gas (0.8 mL/minute). GC oven temperature was kept at 60°C for 10 minutes and programmed to 220°C at a rate of 4°C/minute, and kept constant at 220°C for 10 minutes and then programmed to 240°C at a rate of 1°C/minute. Split ratio was adjusted 40:1. The injector temperature was at 250°C. MS were taken at 70 eV. Mass range was from *m*/*z* 35 to 450.

GC and GC–MS conditions for Seseli petraeum, S. andronakii, S. resinosum and S. *tortuosum*

GC analysis using a Hewlett–Packard (HP) 6890 system. An HP-Innowax FSC column (60 m × 0.25 mm ∅, with 0.25 mm film thickness) was used with nitrogen as carrier gas. The oven temperature was kept at 60°C for 10 minutes and programmed to 220°C at a rate of 4°C/minute, then kept constant at 220°C for 10 minutes and then programmed to 240°C at a rate of 1°C/minute. The injector temperature was at 250°C. The percentage compositions were obtained from electronic integration measurements using FID (250°C).

GC–MS

A HP GCD system. Innowax FSC column (60 m × 0.25 mm ∅) was used with helium as carrier gas. GC oven temperature was kept at 60°C for 10 minutes and programmed to 220°C at a rate of 4°C/minute, and then kept constant at 220°C for 10 minutes and programmed to 240°C at a rate of 1°C/minute. Split flow was adjusted at 50 mL/minute. The injector temperature was at 250°C. MS were taken at 70 eV. Mass range was from *m/z* 35 to 425.

The components of essential oils were identified by comparison of their mass spectra with those in the Baser Library of Essential Oil Constituents, Wiley GC–MS Library, Adams Library, Mass Finder Library and confirmed by comparison of their retention indices. Alkanes were used as reference points in the calculation of relative retention index (RRI) values. Relative percentage amounts of the separated compounds were calculated from FID chromatograms.

Isolation of Paenibacillus larvae *strains*

The suspected combs were collected from eighty-one colonies in forty-five apiaries in different regions of

Turkey. This sample area included all regions of Turkey which attracts a large number of migratory beekeepers in the summer. The suspected combs were analyzed by diagnosis methods using heating method, then brain heart infusion (BHI) agar (Acumedia) with 3 g/mL nalidixic acid and biochemical tests (12, 15, 34). All isolated strains were stored at –20°C in microorganism kits.

Agar-diffusion method

Agar-diffusion method was used for screening the antibacterial activity of the essential oils and pure compound carvacrol. A stock solution of each oil was prepared in dimethylsulfoxide (DMSO, Carlo-Erba, France). Bacterial suspensions were prepared as 2×10^7 bacteria/mL. A Thoma counting chamber (Hawskley, UK) was used to determine the number of bacteria. An aliquot of 25 mL of BHI broth was poured into the plates. The bacterial suspension (1 mL) was transferred and spread with a Drigalsky spatula on the surface of BHI plates. Using a sterile cork-borer, 9 mm diameter wells were punched on the medium. Each well was filled with 10 L of the pure oil. Plates were incubated at 37°C for 24 hours. Oxytetracycline (Sigma, Germany) was used as standard antibacterial agent.

Micro-dilution method

Micro-dilution broth susceptibility assay was used for the antibacterial evaluation of the ten oils and the main compound of *Origanum onites*; carvacrol. Stock solutions of oils and pure compound were prepared in DMSO. The dilution series were prepared from 5 mg/mL to 0.078 mg/mL in sterile distilled water in micro test tubes from where they were transferred to 98-well microtite plates. Bacterial suspensions grown overnight in BHI broth were standardized to approximately 2×10^7 bacteria/mL. A Thoma counting chamber was used to determine the number of bacteria. Each bacterial suspension (100 L) was then added to each well. The last row containing only the serial dilutions of antimicrobial agent without microorganism was used as negative control. Sterile distilled water and medium served as a positive growth control. After incubation at 37°C for 24 hours, the first well free from turbidity was determined as the minimal inhibitory concentration (MIC) oxytetracycline (Sigma, Germany) was used as standard antibacterial agent.

Results and discussion

The suspected combs were analyzed by diagnosis methods of AFB and fourteen of eighty-one combs were identified as *Paenibacillus larvae* (Table 1).

Table 1. Strains which are isolated from different location in Turkey.

Location	Code	Sample number	Code in experiment	Identification
Ankara	06	01	06/01	*Paenibacillus larvae*
Antalya	07	09	07/09	*P. larvae*
Antalya	07	19	07/19	*P. larvae*
Antalya	07	20	07/20	*P. larvae*
Muğla	48	19	48/19	*P. larvae*
Muğla	48	52	48/52	*P. larvae*
Muğla	48	62	48/62	*P. larvae*
Muğla	48	63	48/63	*P. larvae*
Muğla	48	64	48/64	*P. larvae*
Ordu	52	01	52/01	*P. larvae*
Ordu	52	04	52/04	*P. larvae*
Trabzon	61	01	61/01	*P. larvae*
Erzincan	24	02	24/02	*P. larvae*
Erzincan	24	03	24/03	*P. larvae*

Ten essential oils obtained from various sources were analyzed by GC and GC–MS to determine their main constituents and evaluated for their antibacterial properties against *Paenibacillus larvae* which causes AFB.

The composition and relative percentages of the essential oils (A–J) were elucidated using GC and GC–MS analyses. As a result, ten major compounds of the oils were identified (Table 2).

The antibacterial activity of the essential oils and carvacrol were analyzed by statistical methods, one-way analysis of variance (ANOVA) and Duncan tests (Tables 3 and 4). According to the test results, antibacterial properties of *Seseli petraeum* and *S. resinosum* were found to have negative antibacterial activity. *Seseli tortuosum*, *Bifora radians*, *Anethum graveolens*, *Heracleum platytaenium*, *Pimpinella anisum* were classified in the same class for antibacterial effectivity. *Seseli andronakii* and *Rosmarinus officinalis* had more antibacterial activity respectively. However, *Origanum onites* showed the strongest antibacterial activity against *Paenibacillus larvae* (32.5238 mm). Carvacrol, which is a major component of the oil of *O. onites*, was also investigated for antibacterial affect. Antibacterial properties of carvacrol were in strength between *R. officinalis* and *O*. onites.

The microdilution broth susceptibility assay was examined to determine MIC values and the results are summarized in Table 5.

In recent years, a large number of investigations have been performed on the antimicrobial activities of essential oils (20–33). Antimicrobial evaluations of essential oils are difficult because of their volatility, insolubility in water, and because of their complex chemical compounds. Some factors are important when testing oils such as the assay technique, growth

Table 2. Major components of ten essential oils (A–J) identified by GC and GC–MS.

RRI	Compound	A (%)	B (%)	C (%)	D (%)	E (%)	F (%)	G (%)	H (%)	I (%)	J (%)
1032	α-pinene	1.8	13.7	4.7	13.5	—	—	—	—	10.1	1.0
1118	β-pinene	tr	37.5	0.5	1.3	—	—	—	—	2.6	—
1132	sabinene	9.5	4.3	0.2	19.7	—	—	—	—	—	—
1203	limonene	0.4	2.0	0.5	2.3	—	38.2	—	—	2.2	—
1213	1,8-cineole	—	—	—	—	—	—	—	—	46.9	—
1255	γ-terpinene	11.3	tr	1.1	2.6	—	—	—	—	1.3	3.3
1280	*p*-cymene	6.5	tr	0.3	1.1	—	—	—	—	1.9	8.3
1483	octyl acetate	—	—	—	—	—	—	87.9	—	—	—
1504	daucene	6.1	—	1.6	—	—	—	—	—	—	—
1532	camphor	—	—	—	—	—	—	—	—	7.1	—
1553	linalool	—	—	—	—	—	—	—	—		9.3
1612	β-caryophyllene	—	—	—	—	—	—	—	—	3.5	—
1726	germacrene D	7.8	6.2	8.7	2.1	—	—	—	—	—	—
1751	carvone	—	—	—	—	—	56.4	—	—	—	—
1845	(*E*)-anethole	—	—	—	—	—	—	—	94.6	—	—
2000	(*E*)-2-tridecenal	—	—	—	—	52.9	—	—	—	—	—
2045	carotol	20.7	—	52.7	tr	—	—	—	—	—	—
2100	(*E*)-2-tetradecenal	—	—	—	—	24.6	—	—	—	—	—
2183	(*E*)-sesquilavandulol	—	—	—	37.0	—	—	—	—	—	—
2202	4α-hydroxygermacra-1(10)-5-diene	2.8	21.7	—	—	—	—	—	—	—	—
2239	carvacrol	0.5	—	—	—	—	—	—	—	—	66.4

Notes: A, *Seseli petraeum*; B, *Seseli resinosum*; C, *Seseli andronakii*; D, *Seseli tortuosum*; E, *Bifora radians*; F, *Anethum graveolens*; G, *Heracleum platytaenium*; H, *Pimpinella anisum*; I, *Rosmarinus officinalis*; J, *Origanum onites*; tr, trace (< 0.1).

Table 3. The results of the ANOVA test.

	SS (KT)	DF	MS (KO)	*F*	*P*
Intergroup	37495.727	10	3749.573	342.551	0.000*
Intragroup	4936.657	451	10.946		

Notes: SS, sum of squares; DF, differential factor; MS, median of squares. $^{*}P \leq 0.05$ important.

Table 4. The summary of Duncan test results.

	Essential oils	Medium inhibition diameter (mm)
Duncan	*Seseli petraeum*	0.0000 (0.0000)[a]
	S. resinosum	0.0000 (0.0000)[a]
	S. tortuosum	6.8452 (0.7902)[b]
	Bifora radians	7.2738 (0.7907)[b]
	Anethum graveolens	7.3512 (1.6313)[b]
	Heracleum platytaenium	8.0940 (0.6939)[b]
	Pimpinella anisum	8.2262 (1.9575)[b]
	S. andronakii	9.7738 (1.2601)[c]
	Rosmarinus officinalis	11.5417 (5.3840)[d]
	Carvacrol (Sigma)	22.3810 (1.5094)[e]
	Origanum onites	32.5238 (8.9067)[f]

Notes: The standard deviations are given in parentheses. The oils which have the same superscript letter are in the same category for the antibacterial activity.

medium, the microorganism and the oil itself (34–39).

In previous investigations, savory, thyme, lemongrass, oregano and mixed solutions of these oils have been examined (20–24). Following on from these research activities, the essential oil, thymol has been made as a preparation and used against Varroa and tracheal mites. But still essential oils have not been used

Table 5. Antimicrobial activity of MIC values (mg/mL).

AFB	Essential oils				Standard negative control
Paenibacillus larvae	f:6 *Origanum onites* 0.078	e:5 *Carvacrol (sigma) 0.156	d:4 *Rosmarinus officinalis* 0.625	b:2 *Anethum graveolens* 2.5	MIC (mg/mL) Oxytetracycline (terramycine) 2

against the foulbrood disease. This study includes the first extensive data on the antibacterial activity of some essential oils against *Paenibacillus larvae* strains in Turkey.

In this study, *Origanum onites* oil was determined to be the most effective essential oil against the AFB agent. Even though carvacrol is the major compound of this oil, carvacrol showed less activity than *O onites* oil. This result suggests that essential oils might have some synergistic affects within their components (29–31). It is not just the major compound itself which is important for antibacterial property several compounds working in synergy are important in essential oils.

Although it is reported that Seseli genus have antimicrobial activities against some bacterial genus (25, 26) *S. petraeum* and *S. resinosum* had no antimicrobial activity on *Paenibacillus larvae*, while *S. tortuosum* showed very weak antibacterial activity. The main difference between the species is their geographical locations in Turkey. *Seseli petraeum* and *S. resinosum* grow in the north of Turkey, while *S. tortuosum* is endemic to both sides: north and south of Turkey (32). The different locations probably cause the different compositions of the oils. The essential oils which were determined as antibacterial agents on *P. larvae* were *Bifora radians*, *Anethum graveolens*, *Heracleum platytaenium*, *Pimpinella anisum*, *S. andronakii* and *Rosmarinus officinalis*, similar to other bacterial groups before (27, 28).

Compared to antibiotics, essential oils have antibacterial activity as much as synthetic antibiotics but do not leave residues in honey bee products as they are an organic composition. Furthermore, essential oils could be an alternative product to prevent honey bee colonies from bacterial diseases; even the vegetative forms of *Paenibacillus larvae* causes several losses in honey bee colonies. In the first place, we should prefer the use of the essential oils showing very strong antibacterial activity such as *Origanum onites*, *Rosmarinus officinalis* or *Seseli andronakii*. Further studies are required to verify activities in *in vivo* experimental models on honey bees.

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