Antimicrobial Activity of *Pimpinella anisum* and *Foeniculum vulgare* Essential Oils Against *Paenibacillus larvae*

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Abstract

The essential oil obtained by hydrodistillation from the fruits of *Pimpinella anisum* L. (green anise) and *Foeniculum vulgare* Miller (fennel) were analyzed by GC and GC/MS and physicochemical properties. The oils of *P. anisum* and *F. vulgare* were found to be especially rich in (E)-anethole, 96.3% and 92.7%, respectively. The MICs were determined by the tube dilution method against *Paenibacillus larvae*. The oils showed MICs values were 300 µg/mL and 250 µg/mL for *P. anisum* and *F. vulgare*, respectively. Both oils presented great similarity in physiochemical properties values and antimicrobial activity.

Key Word Index

Pimpinella anisum, *Foeniculum vulgare*, Apiaceae, essential oil composition, (E)-anethole, antimicrobial activity, *Paenibacillus larvae*.

Introduction

American foulbrood (AFB) is a severe bacterial disease affecting larvae of the honeybee *Apis mellifera*, caused by *Paenibacillus larvae* (1). The disease is usually not recognized until signs of infection are detected in hives during routine hive management procedures. In some cases, the disease may not be recognized until considerable damage has been done (2). It occurs throughout the world and is found in many beekeeping areas of Argentina (3).

Compounds isolated from aromatic plants have been demonstrated through in vitro tests to possess antimicrobial activity against *Bacillus larvae*, *Ascosphaera apis*, and *Bacillus alvei* (4). Essential oils have been used in bee colonies for the control of chalkbrood, a fungal disease caused by the pathogen *Ascosphaera apis* (5,6), and varroasis, an affection caused by the parasitic mite *Varroa destructor* (7). Also it has been reported that cinnamon oil was effective on colonies of honeybees infected with American Foulbrood disease (8).

Pimpinella anisum L. (green anise) and *Foeniculum vulgare* Miller (fennel) belong to the Apiaceae family. *Pimpinella anisum* is an aromatic plant attaining a height of 75 cm, native of Egypt; it is extensively cultivated in Greece, India and Turkey. Spain and Egypt are the principal producers of the essential oils (9). The fruit is used as a flavoring agent in confections, candies, chewing gums, tobacco, pickles, animal feeds, liquors and pharmaceutical preparations. It is also used in perfumes and soaps. The fruit is chewed to sweeten the breath and help digestion. Green anise oil is a pale yellowish liquid; it is stimulant, eupeptic, carminative, mildly expectorant and diuretic. It is found to be useful in flatulence and spasms (10). *Foeniculum vulgare* is an erect growing perennial herb, native to southern Europe and the Mediterranean area. Reaching a height of 1.5 m, the plant has yellow flowers on a compound umbel. The oil content varies strongly from 0.6% to 6%; fruits in the center of a umbel are generally larger, more green and possess a stronger fragrance. Fennel seed is used in the food and flavor industry for addition to meats, vegetable products, fish sauces, soups, salad dressings, stews, breads, pastries, teas and alcoholic beverages. The oil and the oleoresin of fennel are used in condiments, soaps, creams, perfumes and liqueurs. Several types of fennel differing in morphology and leaf color are available for ornamental use and as a fresh vegetable. As a medicinal plant, fennel seed has been used as an antispasmodic, carminative, diuretic, expectorant, laxative, stimulant and stomachic (9).

The objectives of this work were to compare the chemical composition, physicochemical properties and antimicrobial

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Table I. Percentage composition of the oils of *Pimpinella anisum* and *Foeniculum vulgare*

Compound	RI*	*P. anisum*	*F. vulgare*
limonene	1022	< 0.1	0.7
fenchone	1084	-	2.1
menthol[1]	1184	0.1	0.2
methyl chavicol	1196	1.0	3.1
(Z)-anethole	1240	0.5	0.4
p-anisaldehyde, dimethyl acetal[†]	1248	0.8	0.1
(E)-anethole	1281	96.3	92.7

*retention indices, relative to n-alkanes series on DB-1 column; [1]correct isomer not identified; [†]artifact

Table II. Minimal inhibitory concentration (μg/mL) of the oils of *Pimpinella anisum* and *Foeniculum vulgare* against three bacterial strains of *Paenibacillus larvae*

Oils	Strains		
	La Plata	Cobo	Sierra de los Padres
Pimipinella anisum	300	300	300
Foeniculum vulgare	250	250	250

activity against *Paenibacillus larvae* of the oils of *P. anisum* and *F. vulgare*.

Experimental

Plant material and oil isolation: *Pimpinella anisum* and *F. vulgare* fruits were purchased from a herborist in Mar del Plata, Buenos Aires, Argentina, in August 2004. The oils of fruits were obtained by hydrodistillation using a Clevenger-type European Pharmacopoeias apparatus (11) for 2 h; an average of 100 g of fruits was used for each experiment. The oils were dried over anhydrous sodium sulphate and stored in screw-capped dark glass vials at 5–8°C until further tests were made. The yields were 0.5–2% and 0.5–1.2% (v/w) for green anise and fennel, respectively.

Essential oil analysis: The oils were analyzed by GC and GC/MS, using a Shimadzu GC-17A chromatograph equipped with a flame ionization detector (FID). The separations were performed using a DB-1 fused silica column (60 m x 0.248 mm, film thickness 0.25 μm). Oven temperature was programmed from 60–240°C at 3°C/min and the final temperature was held for 10 min. Injector and detector temperatures were set at 230°C and 250°C, respectively; carrier gas N_2 at a flow of 0.9 mL/min. The GC/MS analysis was performed on a Perkin-Elmer, QMass 910 GC operating at 70 eV; equipped with a DB-5 fused silica column (30 m x 0.25 mm, film thickness 1.0 μm). The injector and detector temperatures were 250°C; oven temperature programmed from 60°C (5 min), 60–220°C at 3°C/min and 220°C (8 min); carrier gas He at a flow of 1mL/min. The identification of components was based on comparison of their mass spectra with those reported in literature (12) and by computer search of their 70 eV mass spectra with those stored in the library of the GC/MS data system, as well as by retention indexes. Quantitative data was obtained by electronic integration of FID area percents without the use of collection factors.

Thin layer chromatography: Thin layer chromatography (TLC) analysis of oils was performed on Sílicagel (Kieselgel 60H Merck) with mobile phase toluene/ethyl acetate (93:7). An aliquot of 10 μL (using Drummond micro-capillaries) of the sample was applied onto TLC plates, in duplicate. The separated compounds were sprayed with solution A: 5% sulphuric acid in ethanol, and later with solution B: 1% vanillin in ethanol, followed by heating at 110°C (13).

Physicochemical properties determinations: Density to 20°C, triplicate of 1 mL of essential oil was weighed and the average of the obtained values was calculated (14). Refraction index was determined according to AOAC official method (921.08, 2003) at 20°C ± 0.05°C, with Abbé refractrometer of total reflection. Acid index was obtained by titration with an aqueous solution of NaOH 0.1. For this 1 g of oil was dissolved in alcohol 96°, previously neutralized with NaOH using blue of thymol as indicator. Spectroscopy studies were subjected to ultraviolet-visible (UV-Vis) spectroscopy at a concentration of 12.5 ppm in ethanol (15), using Shimadzu UV-2101PC scanning spectrophotometer. The IR spectrum of the sample was recorded as a thin liquid film on NaCl windows, 8 scans, 2 cm^{-1} resolution were obtained with FTIR Mattson, model Genesis II spectrophotometer.

Paenibacillus larvae bioassay: Bacterial strains of *P. larvae* were isolated from brood combs of bee hives with clinical symptoms of American Foulbrood corresponding to three localities of Buenos Aires province: La Plata, Cobo and Sierra de los Padres. Isolation was made on MYPGP agar (Mueller-Hinton-yeast extract-glucose-sodium pyruvate) and in order to inhibit *Paenibacillus alvei* growth; it was supplemented with 9 μg/mL of nalidixic acid. Plates were incubated under microaerobic conditions (5–10% of CO_2), and the strains were identified using biochemical tests (16–18). The pure strains were maintained on MYPGP agar with 15% v/v glycerol until used.

Vegetative cells of *P. larvae* previously cultivated on MYPGP agar during 48 h at 35°C ± 0.5°C were suspended in double distilled sterile water and the suspension was standardized according to FDA method (19). The concentration was adjusted to 0.5 of Mac Farland scale for measuring antimicrobial activity with serial dilution method.

Determination of minimal inhibitory concentrations: The minimal inhibitory concentration (MIC) is the lowest concentration of an antimicrobial agent that is capable to inhibit the bacterial growth, being the last dilution in which there is not observed growth of microorganisms (20). MIC individual determination was evaluated directly by turbidity observation. The oils were mixed in water and were emulsified with 8% (v/v) propylene glycol (1,2-propanediol, The Merck Index, 1996). One mL of each stock solution was added to MYT (Mueller-Hinton, yeast extract and thiamine) broth the composition of which was the following: 2.0 g/L extract meat, 17.5 g/L hydrolyzed casein, 1.5 g/L starch, 1.5% yeast extract and 0.1 mg/L thiamine (autoclaved separately) and then they were diluted to obtain serial dilutions. Serial dilution concentrations ranged

between 2000–12.5 µg/mL. Microbial biomass suspension was added to each serial dilution tube, at room temperature with agitation, using a Vortex dispersing tool for tubes (Fbr® by Decalab SRL). Positive and negative (without microorganisms) controls were used. All samples tubes and controls were incubated at 35°C ± 0.5°C for 48 h in order to determine the MIC values. The antimicrobial activities were determined by triplicate analyses for oil and strains.

Statistical analysis: MICs data obtained from anise and fennel oils were analysed by Fisher exact test, specially suited for small samples, which was used to estimate significant differences ($p < 0.05$) between bacterial strains.

Results and Discussion

The oil of *P. anisum* and *F. vulgare* were characterized by higher amount to contain (E)-anethole 96.3% and 92.7%, respectively. Table I shows the principal compounds of the oils screened.

TLC analysis showed a single spot at Rf 0.91 and Rf 0.93 for anise and fennel, respectively, which corresponded with that of standard anethole.

Physicochemical properties of the oils are related with the chemical composition, for that they can be used as approach of purity, identification and verification. Density and refraction indexes values are high for oils with majority composition with bencenic nucleus (14). For these oils the following values were: density of 0.9484 g/mL and 0.9609 g/mL to 20°C, refraction index of 1.544 and 1.491 for anise and fennel, respectively. While the acid index was 1.04 mg KOH/g oil and 1.77 mg KOH/g for the green anise and fennel, respectively, being this valuation a quantitative determination of the total acidity of an oil. This value is related with its conservation state (freshness) and aging being higher when the acidity increases (21).

The different strains analyzed are shown in Table II, the green anise and fennel presented good antimicrobial activity, with MIC mean values of 300 µg/mL and 250 µg/mL, respectively. No differences between MIC values and bacterial strains from La Plata, Cobo and Sierra de los Padres ($p < 0.05$) were found for both oils. These oils yielded good antimicrobial activity compared with other oils such rosemary and laurel whose MIC values were of 700 µg/mL, and camomile oils whose MIC values ranged from 500 to 650 µg/mL (23). De et al. (10) reported the antimicrobial activity of isolated anethole, compared with standard anethole, indicated that it is effective against different microorganisms including bacteria, yeast and fungal strains.

According to the obtained results, the oils of green anise and fennel showed similar physical and microbial properties; this was due to the great (E)-anethole concentration that presented both oils.

The oils of green anise and fennel tested could be efficient for to control AFB in the apiaries; toxicological risks and other undesirable effects would be avoided as resistance factors, developed by the indiscriminate use of antibiotics.

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