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In vitro antibacterial effect of exotic plants essential oils on the honeybee pathogen *Paenibacillus larvae*, causal agent of American foulbrood

S. R. Fuselli[1,2]*, S. B. García de la Rosa[1], M. J. Eguaras[1,3] and R. Fritz[1]

[1] *Facultad de Ciencias Exactas y Naturales. Universidad Nacional de Mar del Plata (UNMdP). Funes, 3350. 7600 Mar del Plata. Argentina*

[2] *Comisión de Investigaciones Científicas (CIC). Calle 526 e/10 y 11. Rivadavia 1917. C1033AJ Buenos Aires. Argentina*

[3] *Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). Rivadavia 1917. C1033AJ Buenos Aires. Argentina*

Abstract

Chemical composition and antimicrobial activity of exotic plants essential oils to potentially control *Paenibacillus larvae,* the causal agent of American foulbrood disease (AFB) were determined. AFB represents one of the main plagues that affect the colonies of honeybees *Apis mellifera* L. with high negative impact on beekeepers worldwide. Essential oils tested were niaouli (*Melaleuca viridiflora*) and tea tree (*Melaleuca alternifolia*) from Myrtaceae, and citronella grass (*Cymbopogon nardus*) and palmarosa (*Cymbopogon martinii*) from Gramineae. The components of the essential oils were identified by SPME-GC/MS analysis. The antimicrobial activity of the oils against *P. larvae* was determined by the broth microdilution method. *In vitro* assays of *M. viridiflora* and *C. nardus* oils showed the inhibition of the bacterial strains at the lowest concentrations tested, with minimal inhibitory concentration (MIC) mean value about 320 mg L^{-1} for both oils, respectively. This property could be attributed to the kind and percentage of the components of the oils. Terpinen-4-ol (29.09%), α-pinene (21.63%) and limonene (17.4%) were predominant in *M. viridiflora,* while limonene (24.74%), citronelal (24.61%) and geraniol (15.79%) were the bulk of *C. nardus.* The use of these essential oils contributes to the screening of alternative natural compounds 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.

Additional key words: antimicrobial activity, chemical composition, *Cymbopogon martini*, *Cymbopogon nardus*, *Melaleuca alternifolia, Melaleuca viridiflora,* honeybees' pathogen.

Resumen

Actividad antibacteriana *in vitro* de los aceites esenciales de plantas exóticas frente al patógeno bacteriano *Paenibacillus larvae,* agente causal de loque americana en colonias de abejas melíferas

Se determinó la composición química y se evaluó la actividad antimicrobiana *in vitro* de cuatro aceites esenciales de plantas exóticas para el control del patógeno bacteriano *Paenibacillus larvae,* agente causal de loque americana, que afecta a los estadios de larva y pupa de la abeja melífera (*Apis mellifera* L.). Esta enfermedad produce grandes pérdidas económicas en la apicultura mundial. Los aceites esenciales analizados, pertenecientes a la familia Myrtaceae y Gramineae, fueron: niaouli (*Melaleuca viridiflora*) y árbol del té (*Melaleuca alternifolia*), citronela (*Cymbopogon nardus*) y palmarosa (*Cymbopogon martinii*), respectivamente. La composición química de los aceites esenciales se efectuó por cromatografía de gases acoplada a un espectrómetro de masas (CG-EM). La concentración inhibitoria mínima (MIC) se determinó por el método de microdilución en caldo y la concentración bactericida mínima (MBC) en agar MYPGP. Los aceites esenciales de niaoulí y citronela registraron valores promedio de MIC de 320 mg L^{-1}, atribuidos al tipo y porcentaje de sus componentes: terpinen-4-ol (29,09%), α-pineno (21,63%) y limoneno (17,4%) que predominaron en *M. viridiflora,* mientras que el limoneno (24,74%), citronelal (24,61%) y geraniol (15,79%), constituyeron los componentes mayoritarios de *C. nardus.* El excesivo uso de antibióticos para el control de esta enfermedad ha generado fenómenos de resistencia y de contaminación de todos los productos derivados de la colmena. El uso de estos aceites esenciales representa una alternativa de rotación a los productos mundialmente utilizados en la quimioterapia de loque americana, constituyendo un aporte a la búsqueda de métodos alternativos naturales para el control de esta enfermedad.

Palabras clave adicionales: actividad antimicrobiana, composición química, *Cymbopogon martini*, *Cymbopogon nardus*, *Melaleuca alternifolia, Melaleuca viridiflora,* patógenos de abejas melíferas.

* Corresponding author: sfuselli@mdp.edu.ar
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Introduction

One of the main plagues that affects the colonies of honeybees *Apis mellifera* L. is the spore forming bacterium *Paenibacillus larvae* (Genersch *et al*., 2006). This pathogen is the causal agent of American fouldbrood (AFB), with high negative impact on beekeepers worldwide. This plague is usually controlled with antibiotics which could leave toxic residues in honey and by-products (Dorman and Deans, 2000; Mc Kee, 2004) and that have already generated resistance phenomena in certain areas of Europe, USA, Canada, Argentina and Asia (Alippi and Reynaldi, 2006).

The urge to develop alternative treatment strategies follows three different directions; being one of them the treatment with natural antibacterial substances like essential oils of various plants (Genersch, 2009). Plant essential oils have been studied for their antimicrobial activity against microorganisms, including many pathogens (Dorman and Deans, 2000; Delaquis *et al*., 2002). *In vitro* antibacterial, antifungical and miticide activity of some essential oils have shown effective results in the control of bee pests (Calderone and Shimanuki, 1994; Alippi *et al*., 1996; Floris *et al*., 1996; Bazzoni and Floris, 1999; Albo *et al*., 2001, 2003; Ruffinengo *et al*., 2002, 2005, 2006; Dellacasa *et al*., 2003; Eguaras *et al*., 2005; Fuselli *et al*., 2006, 2007) offering a natural desirable alternative to antibiotics and other synthetic chemical substances.

The family Myrtaceae has at least 3,000 species distributed in 130-150 genera, with a wide distribution in tropical and warm-temperate regions of the world. Many plant essential oils belonging to Myrtaceae species, such as *Melaleuca alternifolia* and *Melaleuca viridiflora*, have been reported to have insecticidal, antifungal and nematicidal bioactivity (Lee *et al*., 2008). Tea tree oil (TTO) is a volatile essential oil derived mainly from the Australian native plant *M. alternifolia*, produced by steam distillation of the leaves and terminal branches. This oil is largely employed for its antimicrobial properties due to the compound terpinen-4-ol. It is incorporated as the active ingredient in many topical formulations used to treat infections (Carson *et al*., 2006) and as alternative natural fungicide (Terzi *et al*., 2007). Niaouli oil is extracted from *M. viridiflora* (also known as *Melaleuca quinquenervia*). This evergreen tree is native to Australia, New Caledonia and the French Pacific Islands and its oil is extracted from the young leaves and twigs by steam distillation. It is useful against enteritis, dysentery, intestinal parasites, cystitis, urinary infections and used to rheumatism and neuralgia. Niaouli oil is considered safe oil, since it is non-toxic, non-irritant and non-sensitizing (Aqil *et al*., 2007).

Some herbaceal plant species belonging to the genus *Cymbopogon* (Fam Gramineae) are of great commercial importance, between them citronella grass (*C. nardus*) and palmarosa (*C. martini*), which are used for making perfumes, soaps, cosmetics, household products and pharmaceutical products. Citronella grass is well known by its repellent effect to control plagues (Orozco Montoya *et al*., 2006) while palmarosa oil has been shown to be an effective insect repellent when applied to stored grain and beans (Kumar *et al*., 2007), and as antihelmintic and an antifungal repellent (Kumaran *et al*., 2003).

This work evaluates the *in vitro* bioactivity of tea tree (*M. alternifolia* Cheel.), niaouli (*M. viridiflora* Soland.), citronella grass [*C. nardus* (L.) Rendle] and palmarosa (*C. martinii* Stapf.) oils to control *Paenibacillus larvae* bee pest.

Material and methods

Essential oils were commercially obtained from Flora SRL, Lorenzana (Pisa), Italy. Gas chromatography combined with mass spectrometry (GC-MS)-Solid phase microextraction (SPME) analyses were carried out to characterize and identify essential oils according to Fuselli *et al*. (2008b). GC/MS analyses were made by triplicate replication and the results are reported as GC peak area percentage.

P. larvae strains were isolated from honey combs of hives exhibiting clinical symptoms of American foulbrood, belonging to different areas of Buenos Aires Province (Argentina), *i.e.* Miramar (38°15'S-57°50'W), Balcarce (37°52'S-58°15'W), Vidal (37°27'S-57°44'W), Coyunco (37°56'S-57°47'W), Chapadmalal (38°03'S-57°42'W), La Plata (34°58'S-57°54'W), Vivoratá (37°40'S-57°42'W), H. Ascasubi (39°22'S-62°39'W), Mechongué (38°09'S-59°13'W) and Mar de Cobo (37°40'S-57°19'W). Isolation and strains identification were done using previously described techniques

Abbreviations used: AFB (American fouldbrood), GC-MS (gas chromatography combined with mass spectrometry), MBC (minimal bactericide concentration), MIC (minimal inhibitory concentration), SPME (solid phase microextraction), TTO (tea tree oil).

(Gordon *et al*., 1973; Alippi, 1991, 1992) and were confirmed afterwards according to biochemical and physiological tests (Jelinski, 1985) and API CH50 kits (Api System, BioMérieux S.A., Marcy l'Étoile, Lyon, France). Reference strain of *P. larvae* from culture collections (ATCC 9545, from Istituto Nazionale di Apicoltura (INA), Italy) was also considered. The different strains of *P. larvae* were stored at –20°C on MYPGP agar (Mueller-Hinton broth-yeast extract-glucose-sodium piruvate and PO_4HK_2), with 20% v/v of glycerol until used.

The minimal inhibitory concentration (MIC) of the oils against the bacterial strains was determined by broth microdilution method. MIC of oxytetracycline was also determined in parallel experiments in order to control the sensitivity of the microorganisms tested. To perform the broth microdilution method, 100 µL of brain heart infusion broth (Difco Laboratories, 3.7% supplemented 0.1 mg mL^{-1} thiamine hydrochloride) (Shimanuki and Knox, 1991) separately autoclaved to a final concentration of 0.5% v/v was placed in each of the 96-well microtitre plates. One hundred µL from a stock solution of each exotic essential oil suspended in distilled water and emulsified with 5% v/v propylene glycol (1-2 propanediol) (The Merck Index, 1996), was added to the first well microtitre plate. Twofold serial dilution of each essential oil was carried out in the following nine microtitres plates. The eleventh microtitre plate was the growth control (brain heart infusion broth + propylene glycol) and the twelfth one the sterility control (brain heart infusion broth + propylene glycol+ test oil). One hundred microliters of microbial biomass suspension with density of 10^7-10^8 cells mL^{-1} (FDA, 1998), were added to the well microtitre plates. Microbial biomass suspension was prepared from vegetative cells of *P. larvae* cultured under microaerobic conditions at 36 ± 0.5°C on MYPGP agar for 48 h. Microtitre plates were incubated under microaerobic conditions at 36 ± 0.5°C for 72 h. The bacterial growth was indicated by the presence of a «white pellet» on the well microtitre plate bottom. From MIC negative microtitre plate, *i.e.* bacterial growth not visually observed as «white pellet», 100 µL were transferred on MYPGP solid agar (Dingman and Stahly, 1983). These media were incubated at 36 ± 0.5°C for 48 h, under microaerobic conditions, in order to determine minimal bactericide concentration (MBC) values. The antimicrobial activity analyses for four essential oils and ten *P. larvae* strains were repeated six times for each essential oil (N = 240). MIC and MBC data obtained from the essential oils were comparatively analysed using Fisher exact test, suited for small samples, and then used to estimate significant differences ($P < 0.05$) between bacterial strains. Tuckey's mean separation test for MIC and MBC of the essential oils was also estimated (StatSoft, 2000).

Results and discussion

Twenty six compounds representing between 86.9% and 97.4% of the total peak area of the headspace were identified in the exotic essential oils considered. GC/MS analysis revealed that for *C. martinii* and *C. nardus* the largely predominant specific components are the oxygenated monoterpenes, meanwhile for *M. alternifolia* and *M. viridiflora* predominant components are monoterpenes, with percentages between 76.4% and 57.0%, respectively (Table 1).

The major constituents of the oil of *C. martinii* are geraniol (37.4%), geranyl acetate (22.9%) and (E)-β-ocimene (19.6%). *C. nardus* had two largely predominant specific components, citronelal and limonene, with similar percentage values about 25%. *M. alternifolia* oil is principally comprised by p-cymene (24.2%), α-terpinene (12.8%) and α-pinene (11.3%). The main components of *M. viridiflora* are α-pinene (21.6%), limonene (17.4%) and terpinen-4-ol (29.1%) (Table 1).

The characterization of *P. larvae* strains indicated that they were Gram positive and catalase negative. They did not hydrolyze starch or produce indole. The substrates that allowed differentiate bacterial strains of *P. larvae* using API CH50, were fructose (negative), aesculin (negative) and D-tagatose (positive). The overall response from these three carbohydrates and the other techniques used allowed identifying the strains correctly.

All *P. larvae* strains were highly susceptible to citronella grass (*C. nardus*) and to niaouli (*M. viridiflora*) with MIC and MBC mean values about 320 and 590 mg L^{-1}, respectively. The other two essential oils tested, tea tree (*M. alternifolia*) and palmarosa (*C. martinii*), showed lower antibacterial activity with MIC and MBC mean values higher than 1,100 and 1,200 mg L^{-1}, respectively (Table 2).

Relationships between MIC and MBC values and the bacterial strains were found ($P < 0.05$). Tukey's means separation test for MIC and MBC values indicated that the essential oil of citronella grass (*C. nardus*) and

Table 1. Composition of the head space of exotic essential oils (Gramineae and Myrtaceae) as revealed by the SPME-GC/MS technique. The data are reported as percentage of the area of each peak respect to the total peak area

Compounds	Essential oils			
	C. martinii	***C. nardus***	***M. alternifolia***	***M. viridiflora***
Monoterpenes				
α-pinene			11.32	21.63
β-pinene			2.04	7.30
β-myrcene	2.13		1.61	3.36
α-terpinene			12.79	1.15
limonene	1.51	24.74	2.93	17.40
(Z)-β-ocimene	4.12	1.64		
(E)-β-ocimene	19.56	1.13		
γ-terpinene			14.84	3.40
p-cymene			24.18	1.40
terpinolene			6.73	1.40
Total	27.32	27.51	76.44	57.04
Oxigenated monoterpenes				
1,8-cineol			2.03	2.18
citronelal		24.61		
geranial		4.12		
linalool	4.51	1.44		
terpinen-4-ol				29.09
α-terpineol			4.28	8.07
geraniol	37.39	15.79		
nerol		6.17		
Total	41.90	52.13	6.31	39.34
Sesquiterpenes				
cariofileno	5.19	6.42		
cadineno			1.13	
(+)-ledeno			1.40	
aromadendreno			1.68	
Total	5.19	6.42	4.21	
Esters				
citronyl acetate		2.37		
geranyl acetate	22.98			
Total	22.98	2.37		
Cetones				
6-metil-5-hepten-2-ona		2.83		
4-nonanona		1.02		
Total		3.85		
Total peak area	97.39	92.08	86.96	96.38

Table 2. Mean values of the antimicrobial activity (MIC and MBC expressed as mg L^{-1}) of four exotic essential oils against ten bacterial strains of *Paenibacillus larvae,* coming from different areas of Buenos Aires Province, Argentina (N = 240)

	C. martinii	***C. nardus***	***M. alternifolia***	***M. viridiflora***
MIC	1,194.9	318.6	1,094.9	331.4
MBC	1,207.7	594.9	1,187.2	584.9

Table 3. Tuckey's mean separation test for minimal inhibitory concentration (MIC) and minimal bactericide concentration (MBC), expressed as mg L^{-1}, of four exotic essential oils against ten bacterial strains of *P. larvae,* arranged in decreasing order

MIC	*C. martinii*	*M. alternifolia*	*M. viridiflora*	*C. nardus*
C. martinii	—	+	++	++
M. alternifolia	100	—	++	++
M. viridiflora	863.5	763.5	—	ns
C. nardus	876.3	776.3	12.8	—
$W_{0.05} = 93.4$				
$W_{0.01} = 124.3$				
MBC	***C. martinii***	***M. alternifolia***	***C. nardus***	***M. viridiflora***
C. martinii	—	+	++	++
M. alternifolia	20.5	—	++	++
C. nardus	612.8	592.3	—	ns
M. viridiflora	622.8	602.3	10.0	—
$W_{0.05} = 18.4$				
$W_{0.01} = 145.8$				

Tukey's critical values (W) are given for $\alpha = 0.05$ and $\alpha = 0.01$. ++: highly significant difference. +: significant difference. ns: no significant difference.

niaouli (*M. viridiflora*) were different from the other two essential oils tested ($\alpha < 0.01$) and were no different between them in their response against the bacterial strains (Table 3).

There is not literature about the efficacy of citronella grass and niaouli essential oils to control AFB, though there are previous studies about the use of natural products against the causative agent of this disease (Eguaras *et al*., 2005; Fuselli *et al*., 2006, 2007, 2008a,b, 2009; Gende *et al*., 2008, 2009). Citronella grass and niaouli essential oils showed good antimicrobial activity, comparable to that reported by Alippi *et al*. (1996) for savory (*Satureja hortensis*) and oregano (*Origanum vulgare*) essential oils, with similar MIC values ranging from 250-450 mg L^{-1} and to *Artemisia absinthium*, *A. annua* and *Lepechinia floribunda* oils with MIC mean values of 416.7 mg L^{-1}, 401.9 mg L^{-1} and 393.6 mg L^{-1}, respectively (Fuselli *et al*., 2008b). All *P. larvae* strains showed a certain degree of sensibility to citronella grass and niaouli oils, though bacterial inhibition could be attributed to the volatile constituents of the two largely predominant specific components of *C. nardus*, limonene and citronelal and to the main components of *M. viridiflora*, which are α-pinene, limonene and terpinen-4-ol. The use of essential oils against microbial strains allows an alternative scope for the control of this serious disease, affecting honey and its by-products. These results contribute to the screening of alternative natural compounds for treating honeybee colonies suffering from AFB, and may have significant implications in the future to be incorporated in an integrated management programme.

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