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Antimicrobial and miticide activities of *Eucalyptus globulus* essential oils obtained from different Argentine regions

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Abstract

The biological activity of *Eucalyptus globulus* essential oils derived from plant material obtained from different geographic areas was analyzed in *in vitro* experiments on *Paenibacillus larvae*, *Varroa destructor,* and *Apis mellifera*. The physicochemical properties, composition, antimicrobial, and bioactivity of these essential oils were studied. The bioactivity against *P. larvae* was analyzed by means of two *in vitro* techniques (tube dilution and bioautography). Mite and bee lethality were estimated using a complete exposure method test with oils at different concentrations. Essential oils differed in their composition, albeit their similar physicochemical properties. The minimal inhibitory concentrations range for *E. globulus* essential oil lay between 600-700 μg mL^{-1} for Mar del Plata and 900-1,200 μg mL^{-1} for Valle de Conlara essential oils against all *P. larvae* strains. The bioautography method determined that limonene accounted for the greatest antimicrobial activity with respect to the other compounds. The complete exposure method at 24, 48, and 72 h yielded lower LC_{50} values for mites exposed to *E. globulus* essential oils from Conlara. LC_{50} values for acari after 72 h of treatment with *E. globulus* from Mar del Plata and Conlara were 47.1 and 11.7 μL $capsule^{-1}$, respectively; whilst those for bees was > 20 μL $capsule^{-1}$ for both oils. The *E. globulus* essential oils tested in this study featured high efficiency against *V. destructor*, yet their antimicrobial activity against *P. larvae* proved to be lower, and innocuous to bees. The present experience promotes the use of active compounds for American fouldbrood and Varroosis management.

Additional key words: *Apis mellifera,* bioactivity, eucalyptus, *Paenibacillus larvae*, *Varroa destructor.*

Resumen

Actividad antimicrobiana y acaricida del aceites esenciales de *Eucalyptus globulus* obtenidos de diferentes regiones geográficas de Argentina

Se analizó la actividad biológica de los aceites esenciales de *Eucalyptus globulus,* derivados de material vegetal obtenido de diferentes regiones geográficas, en experimentos *in vitro* sobre *Paenibacillus larvae*, *Varroa destructor* y *Apis mellifera*. Se estudiaron las propiedades fisicoquímicas, composición, actividad antimicrobiana y bioactividad de estos aceites esenciales y se analizó la bioactividad frente a *Paenibacillus larvae* por dos técnicas *in vitro* (dilución seriada y bioautografía). Se estimó la letalidad en ácaros y abejas con los aceites esenciales a diferentes concentraciones, usando un método de exposición completa. Los aceites difirieron en su composición, aunque presentaron propiedades fisicoquímicas similares. La concentración inhibitoria mínima frente a los aislamientos de *P. larvae* para los aceites de *E. globulus* fue 600-700 μg mL^{-1} para Mar del Plata y 900-1.200 μg mL^{-1} para Valle de Conlara. La técnica de bioautografía determinó que el limoneno fue el compuesto con la mayor actividad antimicrobiana. El método de exposición completa a las 24, 48, y 72 h mostró valores de LC_{50} menores para ácaros expuestos al aceite esencial de *E. globulus* de Conlara. Los valores de LC_{50} para ácaros a las 72 h de tratamiento fueron 47,1 y11,7 μL $cápsula^{-1}$ para *E. globulus* de Mar del Plata y Conlara, respectivamente; mientras que para las abejas fue > 20 μL $cápsula^{-1}$ pa-

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ra ambos aceites. Los aceites esenciales de *E. globulus* estudiados mostraron mayor efectividad frente a *V. destructor* en relación a *P. larvae,* probando ser inocuos para las abejas. El presente trabajo promueve el uso de los componentes activos de estos aceites para un manejo de las enfermedades Loque americana y Varroosis.

Palabras claves adicionales: actividad biológica, *Apis mellifera,* eucalipto, *Paenibacillus larvae, Varroa destructor.*

Introduction

American foulbrood (AFB), an infectious disease caused by the spore-forming bacterium *Paenibacillus larvae* (Genersch *et al.*, 2006), along with the varroasis produced by *Varroa destructor* (Anderson and Trueman, 2000), an ectoparasitic mite, constitute the two main pathologies affecting brood and adult honeybee (*Apis mellifera*), thereby posing a genuine threat to apiarian producers.

A common strategy adopted to prevent and treat colonies affected with AFB is the use of antibiotics, particularly oxytetracycline hydrochloride (OTC) (Hansen and Brodsgaard, 1999). On the other hand, other investigations suggest that the early detection of this disease, the withdrawal of affected hives, and the use of hygienic behavior lines, might be sufficient to control AFB making possible to manage apiaries without antibiotics and to preserve honey quality (Basualdo *et al.*, 2008). Efforts to control *Varroa* have focused on the evaluation of synthetic acaricides such as coumaphos and fluvalinate, among others (Maggi *et al.*, 2008). In both cases, the indiscriminate use of chemicals to control said pathologies has encouraged the emergence of resistance in many countries and led to hive products contamination (Elzen *et al.*, 1999a,b; Mathieu and Faucon, 2000; Thompson *et al.*, 2002; Bogdanov, 2006; Martel *et al.*, 2006; Maggi *et al.*, 2008). Moreover the chemical control of mites and bacterial diseases may not be acceptable in pure honey production, given the contamination issues arising therefrom, as well as from other hive products consumed by the general public (Kevan, 1999).

The current tendency for chemical control is the utilization of natural products as part of an integrated control strategy. This is the case of the neem tree *Azadirachta indica* (González Gómez *et al.*, 2006) and of essential oils against *V. destructor* (Imdorf *et al.,* 1999; Eguaras *et al.*, 2005; Ruffinengo *et al.*, 2005; Maggi *et al.,* 2009). Along these lines, plant extracts and essential oils against *P. larvae* (Alippi *et al.*, 1996; Fuselli *et al.,* 2006; Gende *et al*., 2007) are now being employed to such an end. The substances contained in natural products can be considered potentially useful to control honeybee diseases (Gende *et al.*, 2008a); and their incorporation into chemical substances as a natural alternative could assist in the reduction of beehive products contamination.

Essential oils are characterized by changes in their chemical composition, which are dependent on the plant developmental state, the part used for extraction, its geographical location, and on the physical and chemical characteristics of the soil and climate in question. Several studies on *Eucalyptus spp.* cultivated in different regions worldwide have centered their attention on the variability of chemical composition. Different percentages of 1,8-cineole have been reported in *E. globulus*: 64.5% in Uruguay (Dellacasa *et al.*, 1990), 75% and 77% in Cuba (Montejo Loret de Mole *et al.*, 1985), 86.67% in USA (Nishimura and Calvin, 1979), 58% to 82% in Morocco (Ahmadouch *et al.*, 1985), and 48.7% in South Africa (Thilivahalt Ndou and Von Wandruszka, 1986). Indeed the variation in bioactivity results accounted by different researches can be attributed to the lack of constancy in the chemical composition of essential oils (Imdorf *et al.*, 1999).

In the light of the aforementioned, the aim of this study was to analyze the bioactivity of *E. globulus* essential oils extracted of plants from different geographic areas against *P. larvae* and *V. destructor,* and to ascertain the role that variation in their composition plays in the action against both diseases.

Material and methods

Plant material and oil isolation

Falciform, fully-formed leaves of *Eucalyptus globulus* Labill were collected in Mar del Plata (38° 00' 24.17" S-57° 33' 55.89" W) and in Valle de Conlara (32° 22' 18.37" S; 65° 11' 11.48" W) in July. Specimens were classified and stored in the herbarium of vascular

Abbreviations used: AFB (American foulbrood), FID (flame ionization detector), GC (gas chromatography), MIC (minimal inhibitory concentration), MS (mass spectrometry), OTC (oxytetracycline hydrochloride), TLC (thin layer chromatography).

plants located in the Faculty of Exact and Natural Sciences, Universidad Nacional de Mar del Plata (PV 89 and PV 90, respectively). Fresh plant material was dried prior to distillation (20-27°C and ~50% RH); and dried leave oils were obtained by hydrodistillation using a Clevenger-type apparatus (Richard *et al*., 1992) for 2 h. An average of 100 g of leaves was used in each experiment, and several distillations were performed until the volume required to run all trials was reached. The oils were dried over anhydrous sodium sulphate and stored in screw-capped dark glass vials at 5°-8°C until further testing.

Essential oil analyses

The oils were analyzed by GC-FID-MS, using a Perkin Elmer Clarus 500 model chromatograph equipped with a single split/splitless injector (split ratio: 1:100) connected, through a flow divisor, to two capillary columns (fused silica, 60 m × 0.25 mm i.d., 0.25 μm film thickness) coated with: a) polyethylenglycol (MW ~ 20,000) (DB-Wax, J&W Scientific) and b) 5% phenyl-95% dimethylpolysiloxane (DB-5, J&W Scientific). The polar column was connected to a FID detector, and the non-polar column to a FID detector and to an MS quadrupole detector (70 eV), using a vent system (MSVent™). Operating conditions were as follows: carrier gas was helium (constant flow: 1.87 mL min^{-1}); and oven temperature was programmed at an initial temperature of 90°C, increased at a rate of 3°C min^{-1} to 225°C, and maintained for 15 min. The injector and FID detectors temperatures were set at 255°C and 275°C, respectively; and that of the transfer line at 180°C. The ion source temperature was of 150°C, and the acquisition mass range of 40-300 m z^{-1}. Quantitative data were determined from the minor response of both FID area values, and expressed as percentages calculated from the peak-area percentage. Oil components were identified by comparing their retention indices to C_6-C_{20} alkanes on both columns, and their mass spectral data to that from electronic libraries (Willey, 2006; Adams, 2007) and to data published in the literature.

Physicochemical properties determinations

Density to 20°C, triplicate of 1.0 mL of essential oil, was weighed, and the average of the values obtained was calculated (Montes, 1981). The refractive index was determined at 20°C ± 0.05°C with an Abbe refractrometer, in compliance with AOAC official method 921.08 (AOAC, 1999).

Thin layer chromatography

The thin layer chromatography (TLC) of the essential oils was performed on silica gel plates (0.2 mm Kieselgel 60 F254, Merck). The eucalyptus oils were applied to two TLC plates using an aliquot of 5 μL (using Drummond micro-capillaries), and developed (93:7 toluene/ethyl acetate). On one plate, the separated compounds were sprayed with sulphuric acid in ethanol, and later on with vanillin in ethanol, followed by heating at 110°C (Wagner and Bladt, 1996). The other plates were used to conduct the bioautography assay.

Antimicrobial activity

Bacterial biomass preparation

Bacterial strains of *P. larvae,* collected from five locations in Buenos Aires province: La Plata, Mar de Cobo, Vivoratá, Mar del Plata and Vidal, were isolated from brood combs of beehives with clinical symptoms of American foulbrood. Isolation was achieved on MYPGP agar (Dingman and Stahly, 1983); and to inhibit *Paenibacillus alvei* growth, it was supplemented with 9 μg mL^{-1} of nalidixic acid. Plates were incubated under microaerobic conditions (5-10% of CO_2), and strains were identified using biochemical tests (Gordon *et al.,* 1973; Alippi, 1992). Pure strains were maintained on MYPGP agar with 15% v v^{-1} glycerol until used.

Vegetative cells of *P. larvae* previously cultivated on MYPGP agar for 48 h at 35 ± 0.5°C were suspended in double distilled sterile water, and the suspension was standardized according to FDA (1998) method. Concentration was adjusted to 0.5 of Mac Farland scale for measuring antimicrobial activity with serial dilution.

Determination of minimal inhibitory concentrations

The minimal inhibitory concentration (MIC) is defined as the lowest concentration of an antimicrobial agent capable of inhibiting the visible growth of a microorganism after incubation (Lennette *et al.*, 1987).

MIC individual determination was directly evaluated by turbidity observation. The oils were mixed in water and emulsified with 8% (v v^{-1}) propylene glycol (1,2-propanediol, The Merck Index, 1996). For broth microdilution, 100 µL of MYT broth (Gende *et al.*, 2008b) were placed in each of the 96-well microtitre plates and then diluted to obtain serial dilutions. Microbial biomass suspension was added to each serial dilution. Final Serial dilution concentrations ranged between 12.5-2,000 µg mL^{-1}. Positive and negative controls (with microorganisms and water, respectively) were used. Microtiter plates were incubated at 35 ± 0.5°C for 48 h so as to determine MIC values. Antimicrobial activity was tested by triplicate analyses for oil and strains. The MICs of oxytetracycline were also determined in parallel experiments as a way to control tested microorganisms sensitivity.

Bioautography

This technique was employed to define the active constituents (Iskan *et al.*, 2002). Vegetative cells of *P. larvae* previously cultivated on MYPGP agar for 48 h at 35 ± 0.5°C were suspended in double distilled sterile water and the suspension was adjusted to 2 of Mac Farland scale. Twenty milliliters of MYPGP medium were poured on TLC plates (not previously revealed) placed in Petri dishes; microbial suspension was placed on the medium and incubated at 35 ± 0.5°C for 48 h under microaerobic conditions. Microbial growth inhibition was determined by measuring the area of the inhibition zones after being revealed with a triphenyl tetrazolium chloride solution to 5% w v^{-1} in water. Bacteria reduced tetrazolium salt through dehydrogenase activity and produced intensely colored formazan, as reported by Eloff (1998). The inhibition area was observed and classified as 0, –, +, ++, in line with the length area of the inhibition zones in relation to the chromatography spot. Triplicate analyses were performed to determine oil and strains antimicrobial activity by bioautography.

Bioactivity of essential oils against *V. destructor* and *A. mellifera*

The bioactivity of *E. globulus* essential oils against *V. destructor* was established using a complete exposure method (Ruffinengo *et al*., 2005). Treatments were assayed in Petri dishes (140 × 20 mm); using new dishes for each experiment. Each essential oil was diluted in ethanol to a desirable concentration, and 1 mL of solution was then applied to the bottom of the Petri dish. Concentrations between 0 and 20 µL per cage of essential oils were used. Ethanol was evaporated from the dishes by exposing it to airflow for 3-5 min. Five newly emerged adult bees (between 0 and 3 days old) and five adhering *Varroa* mite females obtained from brood cells were placed in each Petri dish. Bees and mites were exposed to essential oils for 72 h. The bees present in Petri dishes were fed on 3 g of candy, and incubated at 30°C and 70% RH during the test. Control treatments consisted of Petri dishes with the evaporated solvent and dishes treated with fluvalinate (technical grade tau-fluvalinate). Five replicates were used for each experimental unit. The number of dead *Varroa* and dead bees was determined after visual inspection of the dish bottoms after 24, 48, and 72 h, respectively.

Statistical analysis

To determine the bioactivity of *E. globulus* essential oils (obtained from different geographic areas) against *V. destructor* and *A. mellifera,* statistical analyses were conducted in accordance with the specific software for LC_{50} values calculation, using 95% confidence interval (USEPA, 1986) and EPA software (vers. 1.5) as proposed by Lindberg *et al.* (2000). Mortality values were adjusted as a function of natural mortality in agreement with Abbott (1925).

Comparisons between LC_{50} value pairs of the different *E. globulus* oils in *V. destructor* were carried out each time (24, 48, and 72 h) by means of LC_{50} greater LC_{50} lower $^{-1}$ quotient; and statically significant differences were detected when the statistical value was higher than the corresponding critical value set forth by APHA (1992).

Results

Oils obtained by distillation with Clevenger apparatus from materials from different geographic areas yielded the same chemical composition, except for aromadendrene, trans-pinocarveol and alpha-terpineol; and featured similar physicochemical properties. Table 1 lists the essential oil composition of the *E. globulus* samples collected from Mar del Plata and Conlara. As

Table 1. Chemical composition of *E. globulus* essential oils

Compounds	Mar del Plata	Conlara
Alpha-pinene	13.71 ± 0.30	6.54 ± 0.08
Beta-pinene	0.42 ± 0.03	0.18 ± 0.00
Limonene	4.38 ± 0.04	1.68 ± 0.13
Eucalyptol (1,8-cineole)	63.49 ± 0.11	79.00 ± 0.25
p-cymene	1.05 ± 0.08	1.18 ± 0.06
Pinocarvone	0.45 ± 0.07	1.88 ± 0.15
Aromadendrene	—	1.30 ± 0.02
Trans-pinocarveol	—	4.52 ± 0.13
Alpha-terpineol	1.10 ± 0.14	—
Terpinyl-acetate	3.06 ± 0.15	1.49 ± 0.08
Viridiflor	5.35 ± 0.08	1.12 ± 0.02

Values (area %) represent media ± standard derivation of three determinations.

a whole, 11 components were fully characterized by CG/ MS analyses, eucalyptol (1,8-cineole) and alpha-pinene being the major compounds. Physicochemical properties values were as follows: density of 0.9232 g mL^{-1} at 20°C and refractive index of 1.4922 for *E. globulus* from Mar del Plata; and density of 0.9232 g mL^{-1} at 20°C and refractive index of 1.4672 for essential oil from Conlara.

Table 2 lists MIC values for the five strains of *P. larvae* in the presence of both essential oils. MIC values for *E. globulus* from Mar del Plata ranged from 600 to 700 μg mL^{-1}, while those from Conlara oil from 900 to 1,200 μg mL^{-1}. As it can be noticed, the higher percentage of 1,8-cineole is not connected to the higher antimicrobial activity values.

On silica gel thin-layer chromatograms, eucalyptus essential oils were separated into 6 and 4 spots for Mar del Plata and Conlara, respectively. Both samples were characterized by one main zone based on 1,8-cineole (Rf 0.35-0.4). In addition, only two of the bands could be identified by co-chromatography using pure standards, alpha-pinene and limonene with Rfs 0.95; 0.73, respectively.

The bioautography method was applied to strains of *P. larvae*. This bioassay allowed to evaluate the minimum concentrations of the compound responsible for the biological activity observed, at first sight, as growth inhibition areas. The clearest inhibition zones in this experiment corresponded to limonene if compared to the other compounds identified by TLC technique. Table 3 displays the results obtained for the main components in antimicrobial activity tests against *P. larvae* strains from five different geographic areas.

The percentage of mortality recorded for *V. destructor* and *A. mellifera* at 24, 48, and 72 h is shown in Table 4, being greater for mites if compared to honey bees. The essential oil of *E. globulus* from Conlara was significantly more toxic against *V. destructor* for each time

Table 2. Antimicrobial activity expressed as minimal inhibitory concentration (MIC, μg mL^{-1}) of *E. globulus* essential oils against *P. larvae* strains

	P. larvae 1	*P. larvae* 2	*P. larvae* 3	*P. larvae* 4	*P. larvae* 5
Mar del Plata	700	600	600	600-700	600
Conlara	1,000	1,200	1,000-1,200	1,000	900-1,000

Oil and strains antimicrobial activities were determined by quintuple analyses. *P. larvae* 1: La Plata. *P. larvae* 2: Cobo. *P. larvae* 3: Vivoratá. *P. larvae* 4: Mar del Plata. *P. larvae* 5: Vidal.

Table 3. Antimicrobial activities by bioautography method, expressed as area of bacterial inhibition related to chromatographic spot

Strains	Mar del Plata			Conlara		
	α pinene	Limonene	Eucalyptol	α pinene	Limonene	Eucalyptol
P. larvae 1	+	++	+	–	+	–
P. larvae 2	+	++	+	0	+	0
P. larvae 3	+	++	+	0	+	0
P. larvae 4	+	++	+	–	+	–
P. larvae 5	+	++	+	–	+	–

Oil and strains antimicrobial activities were determined by triplicate analyses. 0: no inhibitory activity. –: lesser inhibitory activity than chromatographic spot. +: inhibitory activity equal to chromatographic spot. ++: greater inhibitory activity than chromatographic spot. *P. larvae* 1: La Plata. *P. larvae* 2: Cobo. *P. larvae* 3: Vivoratá. *P. larva*e 4: Mar del Plata. *P. larvae* 5: Vidal.

Table 4. Mite and bee mean mortality (%) after 24, 48 and 72 h for different concentrations of essential oils of *E. globulus* from two different geographic locations (CL: Conlara. MdP: Mar del Plata)

Essential oils (µl capsule^{-1})	24 h		48 h		72 h	
	CL	MdP	CL	MdP	CL	MdP
Mites						
0	0	0	4	0	4	0
2.5	0	8	12	8	16	20
5	8	12	12	12	20	28
10	20	0	28	4	40	16
20	48	20	64	32	76	36
Bees						
0	0	0	0	0	0	0
2.5	0	0	0	0	0	0
5	4	0	8	0	8	0
10	0	4	4	4	4	4
20	0	0	0	0	4	4

of exposition ($p < 0.001$). Table 5 exhibits the estimated LC_{50} values for *V. destructor* obtained at each time interval for each treatment along with their comparison. EPA software version 1.5 was employed to estimate the specific values of LC_{50} based on the acari mortality percentage obtained. On the other hand, with regard to *A. mellifera*, the estimated LC_{50} values reached at each time interval for each treatment exceeded 20 µL per cage, owing to the low bee mortality (0, 0.4, and 8%) for all concentrations assayed.

For mites and bees, LC_{50} of fluvalinate decreased with time, and significant differences were identified regarding treatments in each time ($p < 0.001$).

Discussion

Among the 11 compounds identified by means of CG/MS analyses eucalyptol and alpha pinene were the main components, according with Retamar (1982). With regard to the role that environmental factors play on plants secondary metabolites, expectations lie in detecting variations in these compounds in the oils of the same species obtained from diverse geographic regions. These composition differences with respect to the geographic distribution of the plant material can explain the distinct composition of the two eucalyptus essential oils analyzed.

In general, the *E. globulus* oil shows a lesser inhibitory effect on *P. larvae* growth when compared to the essential oils of other plants, such as lemon grass (*Cymbopogon citratus*) with MIC values ranging from 50 to 100 mg L^{-1}, thyme (*Thymus vulgaris*) with values ranging from 100 to 150 mg L^{-1} (Alippi *et al.*, 2001), and cinnamon (*Cinnamomum zeylanicum*) with MIC values between 25 and 100 mg L^{-1} (Floris *et al.*, 1996; Gende *et al.*, 2008a). The antimicrobial activity data obtained in this work mirror those reported by Alippi *et al.* (1996) for the same vegetal species against *P. larvae*. The poor antimicrobial activity of *E. globulus* could be ascribed to the chemical composition rich in terpenic compounds, which is consistent with previous studies reporting that these chemical structures are the least bioactive of the essential oil components (Lattaoui and Tantaoui-Elaraki, 1994; Cosentino *et al.*, 1999).

The results achieved by bioautography suggest that limonene was the main compound responsible for the inhibitory activity against the *P. larvae* strains analyzed. These results are in line with those obtained by Fuselli *et al.* (2008), in which the antimicrobial activity of citrus oils against *P. larvae* strains is attributed to the volatile specific component, *i.e.,* limonene. The major antimicrobial activity of limonene in relation to that of the other two major components of these essential oils could be explained by the delocalized system of electrons resulting from the presence of two double bonds that allow proton exchange, thereby rendering the substances more active against microorganisms

Table 5. LC_{50} (µL Petri dish^{-1}) estimated for *V. destructo*r and *A. mellifera* for essential oils and fluvalinate from Conlara and Mar del Plata

Treatment	LC_{50} mite			LC_{50} honeybee		
	24 h	48 h	72 h	24 h	48 h	72 h
E. globulus oil-Mar del Plata	74.7^{a}	65.2^{ab}	47.1^{b}	> 20^{c}	> 20^{c}	> 20^{c}
E. globulus oil-Conlara	20.8^{c}	16.4^{c}	11.7^{c}	> 20^{c}	> 20^{c}	> 20^{c}
Fluvalinate	2.9^{d}	2.0^{d}	1.5^{d}	1.5^{d}	1.4^{d}	1.0^{d}

Different letters indicate significant differences between each other within each time ($p < 0.001$).

(Ben Arfa *et al.*, 2006). An increase in activity depends on the type of alkyl substituent incorporated into a nonphenolic ring structure, an alkenyl substituent (1-methylethenyl) results in an increased antibacterial activity (Dorman and Deans, 2000). More often than not, the inhibitory action of aroma compounds is associated with hydrophobicity, which is directly correlated to the log P (partitioning behavior of the lipophilic compounds in octanol/water) as well as to their partition in the cytoplasmic microbial membranes (Lanciotti *et al.*, 2003). The most hydrophobic compounds are generally considered to be the most toxic (Sikkema *et al.*, 1995; Weber and de Bont, 1996). In this particular case, limonene featured a log *P* value of 4.83 in comparison with 1,8-cineole with 2.74 (Onken and Berger, 1999), which would also explain why limonene is more effective against *P. larvae* than 1,8-cineole is.

If a comparison of both essential oils tested in this study is drawn, *E. globulus* from Conlara yielded high efficiency against *V. destructor*, but exhibited lesser antimicrobial activity against *P. larvae,* while they were well tolerated by worker honeybees, according to the complete exposure method. This effect may be due to the greater 1,8-cineole content. The results for *V. destructor* were congruent with the data obtained by Imdorf *et al.* (2006) and Ruffinengo *et al.* (2007), who stated that 1,8-cineole led to high mite mortality. Yet, with respect to adult bee workers mortality, results differed from those accounted by these authors. The differences noticed could be explained by the application forms adopted, being, in this study, that of complete exposure and of pulverization in the others.

Previous researches have evaluated the activity of essential oils against *V. destructor, P. larvae,* and *A. mellifera* on a separate basis; paying no attention to the composition variation associated with the geographic origin. This work centers its efforts on the relevance that knowing about oils composition has, in view of the fact that the activity against both pathogens varies depending on the geographic distribution of the plant material. With the demand for naturally occurring bioactive (both acaricid and antibacterial) agents and a never ending search for natural biologically-active products, it is hence of interest to develop a study to identify the components of essential oils with both activities.

The present experience, in which eucalyptus oil antimicrobial and miticide properties were tested *in vitro*, promotes the use of its active compounds for American foulbrood and Varroosis management in honey bee colonies.

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References

ABBOTT W., 1925. A method of computing the effectiveness of an insecticide. J Econ Entomol 18, 265-267.

ADAMCZYK S., LÁZARO R., PÉREZ-ARQUILLUÉ C., CONCHELLO P., HERRERA A., 2005. Evaluation of residues of essential oil components in honey after different anti-varroa treatments. J Agric Food Chem 53(26), 10085-10090.

ADAMS R.P., 2007. Identification of essential oil components by gas cromatography/mass spectroscopy, 4th ed. Allured Publ Co, Carol Stream, Ill, USA.

AHMADOUCH A., BELLAKDAR J., BERRADA M., DENIER C., PINEL R., 1985. Analyse chimique des huiles essentielles de cinq espèces d'*Eucalyptus* acclimatées au Maroc. Fitoterap 56, 209-220. [In French].

ALIPPI A.M., 1992. Detección de *Bacillus larvae* en poblaciones mixtas de esporas bacterianas a partir de restos larvales. Microbiol SEM 8, 115-118. [In Spanish].

ALIPPI A.M., RINGUELET J.A., CERIMELE E.L., RE M.S., HENNING C.P., 1996. Antimicrobial activity of some essential oils against *Paenibacillus larvae*, the causal agent of American Foulbrood Disease. J Herbs Spices Med Plants 4(2), 9-16.

ALIPPI A.M., RINGUELET J.A., HENNING C.P., BANDONI A., 2001. Loque americana. Actividad antimicrobiana *in vitro* de algunos aceites esenciales y mezclas de esencias sobre *Paenibacillus larvae* subsp. *larvae*. Vida Apícola 106, 41- 44. [In Spanish].

ANDERSON D., TRUEMAN J., 2000. *Varroa jacobsoni* is more than one species. Exp Appl Acarol 24, 165-189.

AOAC, 1999. Official methods of analysis, 16th ed. AOAC International, Gaithersburg.

APHA, 1992. Standard methods for the examination of water and wastewaters. 18th ed. American Public Health Association – American Water Works Association and Water Pollution Control Federation, Washington DC.

BASUALDO M.., FIGINI E., TORRES J., TABERA A., LIBONATTI C., BEDASCARRASBURE E., 2008. Control of American foulbrood disease in Argentine commercial apiaries through the use of queens selected for hygienic behaviour. Span J Agric Res 6(2), 236-240.

BEN ARFA A., COMBES S., PREZIOSI-BELLOY L., GONTARD N., CHALIER P., 2006. Antimicrobial activity of carvacrol related to its chemical structure. Lett Appl Microbiol 43(2), 149-154.

BOGDANOV S., 2006. Contaminants of bee products. Apidologie 37,1-18.

COSENTINO S., TUBEROSO C.I.G., PISANO B., SATTA M., MASCIA V., ARZEDI E., PALMAS F., 1999. *In vitro* antimicrobial activity and chemical composition of Sardinian *Thymus* essential oils. Lett Appl Microbiol 29, 130-135.

DELLACASA E., MENÉNDEZ P., MOYNA P., SOLER E., 1990. Chemical composition of *Eucalyptus* essential oils grown in Uruguay. Flav Fragr J 5, 91-98.

DINGMAN D.W., STAHLY D.P., 1983. Medium promoting sporulation of *Bacillus larvae* and metabolism of medium components. Appl Environ Microbiol 46, 860-869.

DORMAN H.J.D., DEANS S.G., 2000. Antimicrobial agents from plants: antibacterial activity of plant volatile oils. J Appl Microbiol 88, 308-316.

EGUARAS M., RUFFINENGO S., FUSELLI S., CLEMENTE G., GENDE L., GONZÁLEZ A., PONZI M., 2005. An *in vitro* evaluation of *Tagetes minuta* L. essential oil for the control of the honeybee pathogens *Paenibacillus larvae* and *Ascosphaera apis,* and the parasitic mite *Varroa destructor*. J Essent Oil Res 17, 336-340.

ELOFF J.N., 1998. A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. Planta Med 64, 711-713.

ELZEN P.J., BAXTER J.R., SPIVAK M., WILSON W.T., 1999a. Amitraz resistance in varroa: new discovery in North America. Am Bee J 139(5), 362.

ELZEN P.J., EISCHEN F.A., BAXTER J.R., ELZEN G.W., WILSON W.T., 1999b. Detection of resistance in US *Varroa jacobsoni* Oud. (Mesostigmata: Varroidae) to the acaricide fluvalinate. Apidologie 30, 13-17.

FDA, 1998. App.3.73 In: Bacteriological analytical manual. 8th ed. Food and Drug Administration, AOAC Int, Gaithersburg, MD. pp. 420-453.

FLORIS I., CARTA C., MORETTI M.D.C., 1996. Activity of various essential oils against *Bacillus larvae* White *in vitro* and in apiary trials. Apidologie 27(2), 111-119. [In French].

FUSELLI S.R., GARCÍA DE LA ROSA S.B., GENDE L.B., EGUARAS M.J., FRITZ R., 2006. Antimicrobial activity of some Argentinean wild plant essential oils against *Paenibacillus larvae larvae* causal agent of American foulbrood (AFB). J Apicult Res Bee World 45(1), 2-7.

FUSELLI S.R., GARCÍA DE LA ROSA S.B., EGUARAS M.J., FRITZ R., 2008. Chemical composition and antimicrobial activity of *Citrus* essences on honeybee bacterial pathogen *Paenibacillus larvae*, the causal agent of American foulbrood. World J Microbiol Biotechnol 24, 2067-2072.

GENDE L.B., MAGGI M.D., FORMATO G., DAMIANI N., RUFFINNEGO S., VELIS G., FRITZ R., EGUARAS M.J., 2007. Actividad antimicrobiana frente a *Paenibacillus larvae* subsp. *larvae,* propiedades fisicoquímicas, y toxicidad sobre *Apis mellifera* de los aceites esenciales de clavo de olor (*Syzygium aromaticum)* y canela (*Cinnamomum zeylanicum)*. ATTI del Congreso Malattie delle api e residui nei prodotti dell' alveare. Ed Melitense. pp. 55-61. [In Spanish].

GENDE L.B., FLORIS I., FRITZ R., EGUARAS M.J., 2008a. Antimicrobial activity of cinnamon (*Cinnamomum zeylanicum*) essential oil and its main components against *Paenibacillus larvae* from Argentine. B Insectol 61(1), 1-4.

GENDE L.B., EGUARAS M.J., FRITZ R., 2008b. Evaluation of culture media for *Paenibacillus larvae* applied to studies of antimicrobial activity. Rev Argent Microb 40, 147-150.

GENERSCH E., FORSGREN E., PENTIKA¨INEN J., ASHIRALIEVA A., RAUCH S., KILWINSKI J., FRIES I., 2006. Reclassification of *Paenibacillus larvae* subsp. *pulvifaciens* and *Paenibacillus larvae* subsp. *larvae* as *Paenibacillus larvae* without subspecies differentiation. Int J Syst Evol Microbiol 56, 501-511.

GONZÁLEZ-GÓMEZ R., OTERO-COLINA G., VILLANUEVA-JIMÉNEZ J.A., PÉREZ-AMARO J.A., SOTO-HERNÁNDEZ R.M., 2006. Toxicidad y repelencia de *Azadirachta indica* contra *Varroa destructor* (ACARI: VARROIDAE) *Azadirachta indica* toxicity and repellence of *Varroa destructor* (ACARI: VARROIDAE). Agrociencia 40, 741-751.

GORDON R.E., HAYNES W.C., PANG H.N., 1973. The genus *Bacillus*. In: Agricultural Handbook No. 427, USDA, Agricultural Research Service, Washington, DC. 283 pp.

HANSEN H., BRODSGAARD C., 1999. American Foulbrood: a review of its biology, diagnosis and bee control. Bee World 80, 5-23.

IMDORF A., BOGDANOV S., IBÁÑEZ OCHOA R., CALDERONE N., 1999. Use of essential oils for the control of *Varroa jacobsoni* in honey bee colonies. Apidologie 30, 209-229.

IMDORF A., BOGDANOV S., KILCHENMANN V., BERGER T., 2006. Toxic effects of essential oils and some of their components on *Varroa destructor* and *Apis mellifera* L. under laboratory conditions. ALP Science 495, 1-18.

ISKAN G., KIRIMER N., KÜRKCÜOOGLU M., HÜSNÜ K., DEMIRCI F., 2002. Antimicrobial screning of *Mentha piperita* essential oils. J Agric Food Chem 50(14), 3943-3946.

KEVAN P.G., 1999. Pollinators as bio indicators of the state of the environment: species, activity and diversity. Agric Ecosyst Environ 74, 373-393.

LANCIOTTI R., BELLETI N., PATRIGNANI F., GIANOTTI A., GARDINI F., GUERZONI M.E., 2003. Application of hexanal, (E)-2-hexanal, and hexyl acetate to improve the safety of fresh -sliced-apples. J Agric Food Chem 51, 2958-2963.

LATTAOUI N., TANTAOUI-ELARAKI A., 1994. Individual and combined antibacterial activity of the main components of three thyme essential oils. Riv Ital EPPOS 13, 13-19.

LENNETTE S., BALOWS R., HANSLER L., SHADONY E., 1987. Manual de Microbiología Clínica, 4th ed. Ed Panamericana, Buenos Aires. [In Spanish].

LINDBERG C., MELATHOPOULUS A., WINSTON M., 2000. Laboratory evaluation of miticides to control *Varroa jacobsoni*, a honey bee parasite. J Ecol Entom 93, 189-198.

MAGGI M.D., RUFFINENGO S.R., GENDE L.B., EGUARAS M.J., SARDELLA N.H., 2008. LC_{50} baseline levels of amitraz, coumaphos, fluvalinate and flumethrin in populations of *Varroa destructor* from Buenos Aires Province, Argentina. J Apic Res Bee World 47(4), 292-295.

MAGGI M., RUFFINENGO S., GENDE L., SARLO G., BAILAC P., PONZI M., EGUARAS M., 2010. Laboratory evaluations of *Syzygium aromaticum* (L.) Merr. et Perry essential oil against *Varroa destructor*. J Essent Oil Res 22, 119-122.

MARTEL A.C., ZEGGANE S., DRAJNUDEL P., FAUCON J.P., AUBERT M., 2006. Tetracycline residues in honey after hive treatment. Food Addit Contam 23, 265-273.

MATHIEU L., FAUCON J.P., 2000. Changes in the response time for *Varroa jacobsoni* exposed to amitraz. J Apic Res 39(3-4), 155-158.

MCLAFFERTY F.W., STAUFFER D.B., 2006. The wiley registry mass spectral library, 8th ed. John Wiley & Sons, Inc, NY.

MERCK INDEX, 1996. Encyclopedia of chemicals, drugs, and biological, 12th ed. Merck Res Lab, Division of Merck & Co, Inc Whitehouse St, NJ, USA.

MONTEJO LORET DE MOLE J., MAGRANER HERNÁNDEZ A., ROSADO PÉREZ R., BALUJA RIVERO, 1985. Estudio de la composición química del aceite esencial de *Eucalyptus globulus* Labill ssp *globulus*, obtenido en Cuba. Rev Cub Farm 14, 177-183. [In Spanish].

MONTES A.M., 1981. Bromatología. Tomos II y III. Ed Universitaria de Buenos Aires, Buenos Aires. [In Spanish].

NISHIMURA H., CALVIN M., 1979. Essential oil of *Eucalyptus globulus* in California. J Agr Food Chem 27, 432-435.

ONKEN J., BERGER R.G., 1999. Effects of *R*-(-)-limonene on submerged cultures of the terpene transforming basidiomycete *Pleurotus sapidus.* J Biotec 69, 163-168.

RETAMAR J.A. (ed), 1982. Aceites esenciales de especies vegetales diversas: sus posibilidades químicas. IPNAYS, CONICET-UNL-FIQ, Santa Fe, Argentina. [In Spanish].

RICHARD H.J., NOLEAN I., GIAMPOLI P., 1992. Techniques of analysis of the spices and aromatics. In: Epices et aromates (Richard H., ed). Tec & Lavoisier, Paris. pp. 191-211.

RUFFINENGO S., EGUARAS M., FLORIS I., FAVERIN C., BAILAC P., PONZI M., 2005. LD_{50} and repellent effect to *Varroa destructor* mite of different essential oil from Argentina wild plants species. J Ecol Entom 98, 651-655.

RUFFINENGO S., MAGGI M., FAVERIN C., GARCÍA DE LA ROSA S.B., BAILAC P., PRINCIPAL J., EGUARAS M., 2007. Essential oils toxicity related to *Varroa destructor* and *Apis mellifera* under laboratory conditions. Zootec Trop 25(1), 63-69.

SIKKEMA J., DE BONT J., POOLMAN B., 1995. Mechanisms of membrane toxicity of hydrocarbons. Microbiol Rev 59, 201-222.

THILIVAHALT NDOU T., VON WANDRUSZKA M.A., 1986. Essential oils of South African *Eucalyptus* species (Myrtaceae). S Afr J Chem 39(2), 95-100.

THOMPSON H.M., BROWN M.A., BALL R.F., BEW M.H., 2002. First report of *Varroa destructor* resistance to pyrethroids in the UK. Apidologie 33, 357-366.

USEPA, 1986. Hazard Evaluation Division. Standard Evaluation Procedure. Ecological Risk Assessment EPA 540/9-85-001. United States Environmental Protection Agency.

WAGNER H., BLADT S., 1996. Plant drug analysis: A thin layer chromatography. Atlas 2nd ed. Springer, Berlin.

WEBER F.J., DE BONT J.A.M., 1996. Adaptation mechanisms of microorganisms to the toxic effects of organic solvents on membranes. Biochim Biophys Acta 286, 225-245.