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Essentials oils of some *Mentha* spp. and their relation with antimicrobial activity against *Paenibacillus larvae*, the causative agent of American foulbrood in honey bees, by using the bioautography technique

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Abstract

Essential oils of three mint species (*Mentha* aff. *arvensis*, *Mentha* aff. *rotundifolia* and a probably feral hybrid of *Mentha* spp.) were investigated for their antimicrobial properties against *Paenibacillus larvae* (White). The bioactivity of the oils was compared using the combination of *in vitro* techniques such as microdilution, agar dilution and bioautography. The chemical composition of the oils was analyzed by GC/MS. Using the bioautography assay, menthol, menthone, menthofuran and piperitone oxide were found to be responsible for the antimicrobial activity of these oils. A quantitative structure-activity relationship (QSAR) model was developed for four terpenoids with significant antimicrobial activity using Hyperchem 8.0 and Gaussian 03 software. The QSAR approach leads to a better understanding of the structural properties of these terpenoids which are responsible for bioactivity. The present work reports the first systematic study about the use of QSAR properties to correlate antimicrobial activity of natural substances against *P. larvae*.

Key words: natural control, bacterial disease, honey bees, QSAR.

Introduction

Healthy honey bee colonies are essential for good honey production and effective pollination (Gillard *et al*., 2008). The occurrence of infectious diseases is an important factor that affects the colony development. Among these diseases, the highly contagious is American foulbrood (AFB), which is caused by the spore-forming bacterium *Paenibacillus larvae* (White). The spores germinate in the larval midgut of young larvae, penetrate the midgut epithelium, and finally enter the body cavity. Infection results in death of the larva and degradation of the larval tissues to a brownish sticky mass, that later become dry scales. Detection of AFB in apiary is based on these observational clinical symptoms. Since it is the most severe diseases of honey bees, often it is necessary to destroy honey bee colonies once that clinical symptoms have emerged (Genersch, 2010).

The no-legal or no-rational use of antibiotics for these infections has been cause of a series of problems for the environment and for the human health, among which stands out the appearance of resistance (Moellering-Jr, 2000) and the accumulation of residues of antibiotics in beehive products (especially in honey), thereby decreasing their quality and hindering marketing opportunities (Fuselli *et al*., 2005). For this reason the employment of essential oils has been proposed as a valid alternative from many years and diverse studies have demonstrated the efficacy, *in vitro* and *in vivo*, of several essential oils against *P. larvae* (Floris *et al*., 1996; Gende *et al*., 2010a), implying that these substances and/or their main compounds (Gende *et al*., 2008b, Gende *et al*., 2010b) could also be promising sources of new drugs and could lead to the improvement of natural therapeutic options for the management of the AFB.

The composition of the essential oils can vary with the climate, geographical area, seasons, soil conditions, crop period and extraction technique (Bertini *et al*., 2005; Carvalho-Filho *et al*., 2006). Essential oils present antimicrobial activity against a variety of bacteria and yeasts, including resistant species to antibiotics and antifungal agents (Bertini *et al*., 2005; Lima *et al*., 2006).

Several species of *Mentha*, belonging to the Lamiaceae family, have been investigated for the essential oils produced by their leaves (Sartoratto *et al*., 2004; Bertini *et al*., 2005).

The quantitative structure-activity relationship (QSAR) investigation was applied to find a correlation between the different physicochemical parameters of the compounds studied and their biological activity (Chang *et. al*., 2007). The transport process in QSAR analysis is most frequently modeled by the logarithm of the octanol-water partition coefficient (Log P = log Kow). Various biological effects depend on the reactivity. Reactivity is related to the electronic structure and can be modeled with quantum descriptors (Netzeva *et al*., 2005). We have taken into account the premise that by devising better descriptors it may be possible to achieve sufficient flexibility to model a range of properties (Burden *et al*., 2009). Besides, the data structuring

proved that exist a relationship between the molecular structures of the essential oil compounds and their antimicrobial activity (Voda *et al*., 2004).

This investigation concerns the antimicrobial screening of three *Mentha* spp. oils using different bioassay techniques in combination. The antimicrobial activities of the oils were determined and compared by using techniques such as microdilution and bioautography agar overlay methods. The aim of this study was to determine the antibacterial activity of the principal compounds of *Mentha* spp. essential oils against *P. larvae* and also to develop a set of physicochemical properties in order to characterize these compounds. In addition, the characterization with QSAR properties was developed for the first time in a systematic study about the use of QSAR properties to correlate antimicrobial activity of natural substances against *P. larvae*.

Materials and methods

Plant material and essential oil extraction

Samples of leaves from *Mentha* spp. were collected in Colinas Verdes (37°54'S; 57°49'W), Buenos Aires Province, Argentina, in summer. Specimens were classified with the collaboration of the chair of Vascular plants, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata. The vegetal material was determined and called as *Mentha* A (*Mentha* aff. *arvensis*), *Mentha* B (*Mentha* sp. probably a feral hybrid) and *Mentha* C (*Mentha* aff. *rotundifolia*). The fresh plant material has been dried prior to distillation (20-27 °C and ~ 20% RH); and the essential oils from dried leaves were obtained by hydro-distillation for 2 h using a Clevenger-type apparatus (Richard *et al*., 1992). 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 CG-FID-MS, using a Perkin Elmer Clarus 500 model chromatograph equipped with an Autosampler installed on 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 aprox. 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: the carrier gas was helium (constant flow: 1.87 ml/min); and oven temperature was programmed at an initial temperature of 90 °C, increased at a rate of 3 °C/min to 225 °C, and maintained for 15 min. The injector and FID detectors temperatures were set at 255 and 275 °C, respectively; transfer line at 180 °C. The ion source temperature was of 150 °C, and the acquisition mass range of 40-300 m/z. Quantitative data were determined from the respective 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 C6-C20 alkanes on both columns, and their mass spectral data to that from electronic libraries (Adams, 2007; Wiley, 2006), in house library, co-chromatography with standards and to data published in the literature.

Essential oils were subjected to ultraviolet-visible (UV-vis) spectroscopy at a concentration of 200 ppm for *Mentha* A and B and 20 ppm for *Mentha* C in ethanol (Montes, 1981) using a Shimadzu UV-2101PC scanning spectrophotometer.

The infrared spectroscopy of the samples was recorded as a thin liquid film on NaCl windows with FTIR Mattson, model Genesis II spectrophotometer. The spectra were accumulated from eight scans measured with a resolution of 2 cm^{-1} in the range of 500-4,500 cm^{-1}.

The thin layer chromatography (TLC) of the essential oils was performed on silica gel plates (0.2 mm Kieselgel 60 F254, Merck). A plate was used for the bioautography as described below, the mint oils were applied to two TLC plates using an aliquot of 5 µL (using Drummond micro-capillaries), and developed (93:7 toluene/ethyl acetate), and the other was revealed by spraying with sulphuric acid in ethanol, and later with vanillin in ethanol, followed by heating at 110 °C (Wagner and Bladt, 1996). The Rf measures were determined by triplicate analyses for each oil.

The yields of the essential oils were calculated. Density to 20 °C and the refractive index were also determined.

Antimicrobial activity

Bacterial strains of *P. larvae* were isolated from brood combs of beehives with clinical symptoms of AFB in six Argentinian localities: Cobo, Necochea, Entre Rios, Vidal, Mar del Plata and Coyunco. Isolations were achieved on MYPGP agar (with 9 µg/mL of nalidixic acid; 5-10% of CO_2), strains were identified using biochemical tests (Alippi, 1992) and tests based on PCR and restriction fragment analysis of the 16S rRNA genes (rDNA) (D'Alessandro *et al*., 2006).

Pure strains were maintained on MYPGP agar with 15% v/v 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 method (1998). Concentration was adjusted to 0.5 of Mac Farland scale for measuring antimicrobial activity with serial dilution.

The minimal inhibitory concentration (MIC) (Lennette *et al*., 1987), was directly evaluated by turbidity observation. The oils were emulsified in water adding 8% (v/v) propylene glycol (1, 2-propanediol, The Merck Index, 1996). For broth microdilution, 100 µl of MYT broth (Gende *et al*., 2008a) and microbial biomass suspension was added to each serial dilution. Final serial dilution concentrations ranged between 2,000-12.5 µg/mL. Posi-

tive 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.

Minimal bactericide concentration (MBC) was specified. To that end, 100 µL were transferred from MIC negative tubes to MYPGP solid agar (Dingman and Stahly, 1983), and incubated at 35 ± 0.5 °C under microaerobic conditions for 48 h in order to delimit MBC values. The antimicrobial activities were determined by quintupled analyses for oil and strains.

Bioautography technique was employed to define the active constituents (Iskan *et al*., 2002). Bacterial suspension was adjusted to 2 of Mac Farland scale. Twenty millilitres 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 in water. Bacteria reduced tetrazolium salt through dehydrogenase activity and produced intensely coloured formazan, as reported by Eloff (1998). The inhibition area was observed and classified as 0, −, + in line with the length area of the chromatographical spot. Triplicate analyses were performed to determine oil and strains antimicrobial activity by bioautography.

Identified compounds by TLC that showed antibacterial activity in the bioautographic method were chosen for QSAR analysis.

Computational chemistry was used to study the chemical structures selected. Geometry optimizations were performed in all the cases (Foresman, 1996).

The semi-empirical method AM1 and the Polak Ribiere optimizer, implemented in the program Hyperchem (Hyperchem 8.0 software, Hypercube Inc.) were used. The QSAR properties (Log P, Refractivity, Polarizability, Volume and the Surface area) were obtained. Also, other descriptors are shown: binding energy, heat of formation, dipolar moment, ionization energy and electron affinity.

Gaussian (Frisch *et al*., 2004) offers an entire range of electronic structure methods, the DFT (density functional methods) include the effect of electron correlation and are more accurate. The B3LYP/6-31G (d) level was applied. The following items were extracted: (n,n), which is the distance between non polar groups; (n,O) and (O,n), which are the distances between non polar groups and oxygen atom (in the case of piperitone oxide, the oxygen in the keto group was considered); µ, dipolar moment (Debye); IE, ionization energy (eV, electron volt); EA, electron affinity (eV, electron volt); Atomic Charge O, atomic charge in oxygen atom (with hydrogens summed into heavy atoms).

Molden (Molecular Structure Editor) a visualization program was used (Schaftenaar, 1991). With Molden we can build the designs of the respective molecules from previously optimized geometries.

Table 1. Relative % peak area of *Mentha* spp. essential oils. The oils were analyzed on DB-Wax column and DB-5 column, J & W Scientific.

Compounds	*Mentha* A	*Mentha* B	*Mentha* C
α pinene	0.3		1.0
α pinene + thujeno		1.3	
β pinene	0.3	1.4	1.7
sabinene	0.1	0.8	0.7
myrcene		0.4	4.7
limonene	0.4	3.6	2.7
1,8-cineol	0.1	5.3	9.8
cis β ocimene		0.2	0.3
p-cymene		0.1	0.3
3-octanol	0.3	0.3	0.2
menthone	10.5	8.6	
isomenthone	4.3	2.1	
neomenthol	1.4	0.9	
terpinen-4-ol		0.2	0.7
menthol	73.6	34.5	0.5
menthyl acetate	1.7	1.3	
β caryophyllene		1.2	2.3
linalool		2.0	0.2
linalyl acetate		2.2	
piperitenone		1.0	0.3
piperitone epoxide			0.7
β farnesene			0.3
pulegone	1.2	4.4	
caryophyllene oxide	0.4		0.8
piperitone	1.6	1.0	0.3
piperitone oxide		0.4	63.6
menthofuran		19.0	
carvone		2.6	
borneol		0.5	
α terpineol		0.4	

Statistical analysis

A cluster analysis, as statistical study, was carried out using the BioEstat 5.0 software package in order to determine the relationship between the different samples of *Mentha* spp. using the minimal inhibitory concentration (MIC) in µg/mL. Euclidean distance was selected as a measure of similarity, and the unweighted pair-group method with arithmetic average (UPGMA) was used for cluster definition.

Results and discussion

Characteristic of mint essential oils

The main compounds of the essential oils extracted from the different plant species studied are given in table 1. *Mentha* A and *Mentha* B showed menthol as main compound in their composition; while piperitone oxide was present as a predominant component in *Mentha* C essential oil.

The yield (Y) and physicochemical properties as density (D) and refraction indexes (RI) at 20 °C for *Mentha* A and *Mentha* B essential oils data were Y = 2.5-2.7% and 0.5-1.5% v/w, density = 0.89 g/mL (20 °C, the same value for both samples), RI = 1.4600 and 1.4690, respec-

tively. While for *Mentha* C these values were as follows: Yield 1.9-2.5% v/w, a density of 1.06 g/mL (20 °C), a refraction index of 1.4920.

UV spectra were displayed, a difference in the UV absorption curves of *Mentha* A and *Mentha* B in relation to *Mentha* C essential oils was observed. *Mentha* C showed a peak absorbance at 260 nm, absent in the other two oils spectra (data not shown).

On infrared spectra, *Mentha* A and *Mentha* B showed the same bands pattern for both oils (data not shown), varying the intensity of the picks. It could be observed that *Mentha* C essential oil did not present the characteristic band of the OH group between 3,200 and 3,600 cm^{-1}, indicating the absence of this functional group in this essential oil.

Antimicrobial activity of mint essential oils and their components

Table 2 shows the MIC and MBC range values for the six strains of *P. larvae* in the presence of *Mentha* A, *Mentha* B and *Mentha* C essential oils.

Statistical analysis, through the Euclidean distance, revealed the similarity in the antimicrobial activity between *Mentha* B and *Mentha* C essential oils in relation with *Mentha* A (data not shown).

Until now only Alippi *et al*. (1996) studied *Mentha piperita* against *P. larvae*, while there were no reported trials of other *Mentha* ssp. essential oils against this honeybee pathogen. In the present study, *Mentha* B essential oil had the greatest inhibitory effect on the growth of *P. larvae* in relation with other *Mentha* species studied.

On silica gel thin-layer chromatograms, essential oils were separated into bands according with their composition. Results of Rf distances for the three oils are shown in table 3.

The bioautography aided in the identification of the antimicrobial active component. This assay was applied to all essential oils using *P. larvae* strains. This experiment showed inhibition zones corresponding to menthol (on 83.3% of the bacterial isolates) and menthone (on 33.3%) for *Mentha* A essential oil; menthol, menthofuran (in all of the isolates) and menthone (66.6%) for *Mentha* B and piperitone oxide (in all of the isolates, 4 with inhibitory activity equal to chromatographic spot) for *Mentha* C, in comparison with the other compounds identified by TLC technique. Table 3 shows the results obtained for the main components in the tests of antimicrobial activity against *P. larvae* strains from six different geographical origins (table 3).

The predominant component in *Mentha* B essential oil was menthol. The antibacterial effect of this substance was demonstrated by several authors (Bassolé *et al*., 2010; McKay and Blumberg, 2006). Menthol along with carvacrol and thymol showed the best antibacterial activity in the microdilution method against the three major pathogens of the cultivated button mushroom, *Agaricus bisporus* (Sokovic and van Griensven, 2006).

Table 2. Antimicrobial activity (expressed as MIC and MBC) of *Mentha* spp. essential oils against different *P. larvae* strains.

Essential oils	Antimicrobial activity (µg/mL)	*Paenibacillus larvae* isolations					
		P.l Cb	*P.l* R88	*P.l* ER	*P.l* V	*P.l* Tp	*P.l* Cy
Mentha A	MIC	1800	1400-1600	1000	1400-1600	1200	1200-1400
	MBC	2000	1800-2000	1600	1800-2000	1800	1600-1800
Mentha B	MIC	600	600	600-700	700	700	600-700
	MBC	1000	1000-1200	1000	1200	1200	1000-1200
Mentha C	MIC	600-1000	600	900-1000	600	600-900	1000
	MBC	1600-1800	1800-2000	>2000	1800-2000	>2000	>2000

P.l Cb = Cobo; *P.l* R88 = Necochea; *P.l* ER = Entre Rios; *P.l* V = Vidal; *P.l* Tp = Mar del Plata; *P.l* Cy = Coyunco.

Table 3. TLC spots data by bioautography method expressed as area of bacterial inhibition.

Essential oil	Principal compounds	Rf-value	Colour band	*Paenibacillus larvae* isolations					
				P.l Cb	*P.l* R88	*P.l* ER	*P.l* V	*P.l* Tp	*P.l* Cy
Mentha A	Menthol	0.45	Blue	0	−	−	−	−	−
	Menthone	0.70	Blue-green	0	0	−	0	−	0
	Iso-menthone	0.55	Blue-green	0	0	0	0	0	0
Mentha B	Menthol	0.40	Blue	−	−	−	−	−	−
	Menthofuran	0.90	Red-violet	−	−	−	−	−	−
	1,8 cineol	0.40	Blue	0	0	0	0	0	0
	Menthone	0.75	Blue-green	−	−	−	0	0	−
Mentha C	Piperitone oxide	0.60	Yellow-orange	+	+	−	+	+	−
	1,8-cineol	0.40	Blue	0	0	0	0	0	0
	Myrcene	0.95	Violet-blue	0	0	0	0	0	0

(0): No inhibitory activity; (−): Lesser inhibitory activity than chromatographic spot; (+): Inhibitory activity equal to chromatographic spot; *P.l* Cb = Cobo; *P.l* R88 = Necochea; *P.l* ER = Entre Rios; *P.l* V = Vidal; *P.l* Tp = Mar del Plata; *P.l* Cy = Coyunco.

On the other hand, Mahboubi and Haghi (2008) described that piperitone was one of the main components of essential of *Mentha pulegium* from plants of south Iran responsible for the effect on *Staphylococcus aureus*. Other study showed that piperitone completely inhibited *Aspergillus flavus* at low concentrations (Cárdenas-Ortega *et al*., 2005).

Data published by Maggiore *et al*. (2012) suggest that essential oils of *Mentha* spp., especially *M. pulegium* essential oil, could be a promising source of potential protoscolicidal agents.

Essential oils are complex mixtures of compounds with low molecular weights, but the antimicrobial effect of total oil is related to one or a few active ingredients (Maggiore *et al*., 2012). To try to find a relationship between the chemical structure and bioactivity we used quantitative structure-activity relationship (QSAR) properties as tool allowing to link this potential between the chemical structure and the bioactivity of a compound. We studied QSAR properties in menthol, menthone, piperitone oxide and menthofuran.

Investigations into the effects of terpenoids upon isolated bacterial membranes suggest that their activity is a function of the lipophilic properties of the constituent terpenes (Knobloch *et al*., 1986), the potency of their functional groups and their aqueous solubility (Knobloch *et al*., 1988). The lipophilicity of terpenoids indicates the ease with which they can pass across lipid membranes (Mazzatorta *et al*., 2005). The inhibitory action of these compounds is, in most cases, related to the hydrophobicity, which is expressed with the log P.

Quantitative structure-activity relationship (QSAR)

The following QSAR properties were obtained: Log P, 1-octanol-water partition coefficient; R, refractivity; P, polarizability; V, volume and the surface area (table 4).

In table 5, other descriptors are shown: E, binding energy (kcal/mol); H, heat of formation (kcal/mol); μ, dipolar moment (Debye); IE, ionization energy (electron volt); EA, electron affinity (electron volt).

The following items were extracted, as showed in table 6: n, non polar group; O, oxygen; (n,n) = distance between non polar groups; (n,O) and (O,n) = distance between non polar groups and oxygen (in the case of piperitone oxide, the oxygen atom in the keto group was considered); μ, dipolar moment (Debye); IE, ionization energy (electron volt); EA, electron affinity (electron volt); atomic charge O, atomic charge in oxygen (tables 4, 5 and 6).

We proposed the (n,n) descriptors as more suitable though one may appreciate that they are related to the volume and surface area. The information seems to be similar but these descriptors represent the non-polar part of the molecule at highlight its importance. In addition, it can be seen how they correlate with the data shown in table 3.

Data from the table 6 are best suited, because with Gaussian, B3LYP to get more accurate results, given

Table 4. Descriptors from Hyperchem 8.0 software, QSAR properties.

Essential oil	Principal compounds(*)	Descriptors (**)					
		Log P	R, Å^3	P, Å^3	Mass, amu	V, Å^3	Surface area grid, Å^2
Mentha A	Menthol (=1)	2.78	47.44	18.99	156.27	580.22	362.23
	Menthone (=2)	3.15	46.52	18.44	154.25	569.34	360.39
Mentha B	Menthol (=1)	2.78	47.44	18.99	156.27	580.22	362.23
	Menthofuran	0.80	46.76	17.83	150.22	539.49	345.53
	Menthone (=2)	3.15	46.52	18.44	154.25	569.34	360.39
Mentha C	Piperitone oxide	2.02	41.31	16.46	154.21	517.46	334.63

(*) Principal compounds that showed antimicrobial activity by bioautography;(**) Log P, 1-octanol-water partition coefficient; R, refractivity; P, polarizability; V, volume. (=) because they correspond to the same compound, the values of the parameters are repeated.

Table 5. Computational Descriptors from Hyperchem 8.0.

Essential oil	Principal compounds(*)	Computational descriptors (**)				
		E kcal/mol	H kcal/mol	μ D	IE eV	EA eV
Mentha A	Menthol (=1)	−2911.19	−100.70	1.538	−10.47	3.28
	Menthone (=2)	−2788.50	−82.20	2.798	−10.18	0.96
Mentha B	Menthol (=1)	−2911.19	−100.70	1.538	−10.47	3.28
	Menthofuran	−2527.32	−29.43	0.957	−8.72	0.77
	Menthone (=2)	−2788.50	−82.20	2.798	−10.18	0.96
Mentha C	Piperitone oxide	−2449.72	−63.16	3.298	−10.36	0.52

(*) Principal compounds that showed antimicrobial activity by bioautography. (**) E, binding energy (kcal/mol); H, heat of formation (kcal/mol); μ, dipolar moment, (Debye); IE, ionization energy (electron volt); EA, electron affinity (electron volt). For geometry optimization, the semiempirical AM1 method was used and the Polak Ribiere optimizer. (=) because they correspond to the same compound, the values of the parameters are repeated.

Table 6. Computational descriptors from Gaussian 03.

Essential oil	Principal compounds(*)	Computational descriptors (**) (n,n) Å	(n,O) Å	(O,n) Å	μ D	IE eV	EA eV	Atomic charge O
Mentha A	Menthol (=1)	8.56	3.28	5.15	1.487	−6.963	2.048	−0.2513
	Menthone (=2)	8.40	3.89	4.88	2.786	−6.365	−0.322	−0.4538
Mentha B	Menthol (=1)	8.56	3.28	5.15	1.487	−6.963	2.048	−0.2513
	Menthofuran	7.88	6.24	4.31	1.015	−5.483	0.845	−0.4590
	Menthone (=2)	8.40	3.89	4.88	2.786	−6.365	−0.322	−0.4538
Mentha C	Piperitone oxide	6.08	5.07	5.13	3.288	−6.656	1.004	−0.4595

(*) Principal compounds that showed antimicrobial activity by bioautography. (**) n, non polar group; O, oxygen; (n,n), distance between non polar groups; (n,O) and (O,n), distance between a non polar group and oxygen (in the case of piperitone oxide, the oxygen atom in the keto group is considered); μ, dipolar moment (Debye); IE, ionization energy (electron volt); EA, electron affinity (electron volt); Atomic charge O, atomic charge in oxygen. For geometry optimization, density functional calculations at the B3LYP/6-31G (d) level were used. (=) because they correspond to the same compound, the values of the parameters are repeated.

that this method works with electronic correlation.

In our case, menthone was the substance that presented the highest log P value (with similar concentrations on both essential oils of *Mentha* A and *Mentha* B), followed by menthol, but using that reasoning *Mentha* A should have had greater antimicrobial activity (due to its higher concentration in these substances). Vermuë *et al*. (1993) showed that a high logP did not always result in the greater toxicity of the compounds. Ben Arfa *et al*. (2006) explains that carvacryl acetate has a logP of 3.59 and thus hydrophobicity equivalent to carvacrol, but exhibited no effect on micro-organism growth. These results indicate that another factor other than hydrophobicity may be involved.

Antimicrobial activity of menthol can be explained because the alcohols are known to possess bactericidal rather than bacteriostatic activity against vegetative cells. The highest antimicrobial activity of the *Mentha* B in relation with *Mentha* A could be explained by different chemical composition. The stereochemistry of the major constituents had an influence on bioactivity (Hinou *et al.*, 1989). It can also be seen in table 4 that the volume and surface area varies between the constituents, as well as electronic-energetic characteristics and the distance between groups (tables 5 and 6).

Menthone was the major component (50.9%) of *Satureja odora* essential oil, showing MIC values of 800-1000 μg/mL against *P. larvae* and marked antimicrobial activity by bioatography method (Gende, 2009).

As described on table 1, the chromatographic analysis of *Mentha* B essential oil presented menthofuran in its composition, compound that was absent in the rest of the analyzed samples. Piperitone oxide on *Mentha* C seems to be the major constituent responsible for bioactivity within the tested major pure compounds supported by the bioautography assay.

The piperitone oxide and menthofuran had lower n, n values than menthol, that is a non-polar descriptor and it could be related to a critical part of the active site. There is, precisely, a very good correlation with the corresponding antimicrobial response (table 6). Similar values on the distances n, n between menthol and menthone were observed, 8.5 Å (table 6). The most noticeable difference between the chemical composition of *Mentha* A and *Mentha* B is the presence of menthofuran in the last one. Menthofuran showed more inhibitory activity than menthol and menthone and a non polar distance of 7.88 Å. Piperitone oxide showed the most inhibitory activity and a distance of 6.08 Å.

Statistically, the antimicrobial activity of *Mentha* B and *Mentha* C was similar, this result could be attributed to the extension of the non-polar area.

Another observation deriving from these results is that other terpenes in the essential oils may show some synergistic effects since the activity of the oils would be related to the respective composition, the structural configuration of the constituents and their functional groups and possible synergistic interactions between components (Dorman and Deans, 2000).

Conclusions

Based on our results, we may conclude that the bioautography is a good method to identify the substances responsible for the antimicrobial activity. But, in the evaluation of the application of this technique it should be take care to consider the concentration of the compounds in the essential oil, because if a compound is in small quantities it can give a false negative, while when is in equal concentrations (i.e. evaluated pure) may be as or more active than others. This methodology gives an idea of what are the most active compounds of the essence in the ratio analysis.

In the future would be useful gather more biological data and analyze individual components and, in addition, search other molecular descriptors in order to perform a multiple regression analysis.

The present work reports the first systematic study about the use of QSAR properties to correlate antimicrobial activity of natural substances against *P. larvae*. We are currently working in a study of individual substances for providing a control measure system for AFB and other honeybee diseases.

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