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Purification of Substances from *Achyrocline satureioides* with Inhibitory Activity Against *Paenibacillus larvae*, the Causal Agent of American Foulbrood in Honeybees' Larvae

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Abstract *Achyrocline satureioides* extracts were tested in vitro against the growth of *Paenibacillus larvae*. Four different extracts were obtained by liquid-liquid extraction from an aqueous-ethyl alcohol macerate of the aerial parts of the plant. The biological activity was tested by the broth microdilution technique. Hexane extract showed the highest activity (minimum inhibitory concentration=0.060±0.037 mg/mL). Transmission electron microscopy experiments showed that the main effect exerted by the hexane extract on the cell was at the cellular membrane level. The hexane extract was analyzed by thin-layer chromatography, and the activity of its components was tested by bioautography. Four growth inhibition zones were observed in the bioautographic experiments (using hexane-acetone (7:3) as mobile phase), with that at *Rf*=0.57 showing the largest zone of inhibition. High-performance liquid chromatographic experiments, using ultraviolet and electrospray ionization coupled to tandem mass spectrometric detection, showed the presence of one compound with a *m*/*z* ratio of 442, which may be related to phloroglucinols α-pyrone compounds recently discovered. The high antibacterial activity of the hexane extract and of the isolated compound determined in this work may be useful for the development of future new alternatives for the treatment of American foulbrood.

Keywords *Achyrocline satureioides* · *Paenibacillus larvae* · Antibacterial activity · Bioautography

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Introduction

American foulbrood (AFB) is the most serious bacterial disease of the honeybee (*Apis mellifera*) brood. AFB is produced by the Gram-positive, spore-forming bacterium, *Paenibacillus larvae*. AFB is difficult to handle for the beekeepers because the spores produced by the pathogen are environmentally stable. Larvae are most susceptible to infection during the early larval stages, i.e., 12–36 h after egg hatching. During this time window, the oral uptake of a dose of about ten spores or fewer via contaminated larval food is sufficient to successfully initiate a fatal infection [1]. Once clinical disease symptoms are visible, infected colonies are likely to succumb to the disease if left untreated.

One of the main control measures in many countries, e.g., New Zealand and the UK, is burning the bees, brood combs, and all movable parts; the hive box and other components such as queen excluders are often sterilized by scorching, by gamma irradiation or by immersion in hot paraffin. However, in some other countries, like Argentina, USA, and Canada, one of the most frequent control measures is the use of antibiotics, especially oxytetracycline (OTC), which only controls vegetative cells leaving spores accumulating in the beehive and contaminating honey, remaining infective for many years. This may lead to recurrence of AFB later in the life of the colony and to the development of resistant strains [2–5]. Also, chemical residues can persist in honey affecting its quality and safety for human consumption.

Nontoxic, natural compounds can be an alternative to the use of antibiotics to control AFB and considerably diminish the resistance of pathogens as well as the amount of antibiotic residues in beehive products [1]. Several studies have reported on natural compounds with antagonistic properties against *P. larvae* such as propolis [6], propolis ethanol extract [7], essential oils [1, 8–14], biosurfactants synthesized by strains of *Bacillus subtilis* isolated from honey and honeybee-gut [15], different bacteria isolated from bees and bee-gut [16–18] or from apiarian sources [19]. Thus, in this work, we decided to study the antibacterial activity of some plant extracts against the growth of *P. larvae*.

For a long period, plants have been a valuable source of natural products for maintaining humans' and animals' health [20]. However, the use of plant-derived compounds as antimicrobials has been virtually nonexistent since the advent of antibiotics in the 1950s. Nevertheless, during the last years, the public is becoming increasingly aware of problems related with the overprescription and misuse of antibiotics, and the pace in the search for new antimicrobial compounds is quickening as scientists realize that the effective life span of an antibiotic is limited. Nowadays, almost 25 % of the drugs going to the market come from a vegetal compound. Approximately 300,000 species are known in the plant kingdom, although the total number of species is much higher. Considering that only 1 % of these species has been studied extensively in terms of chemical composition and therapeutic use, there is still enormous potential of bioactive molecules to be discovered. In this regard, essential oils and other derivate compounds from different plant species have proven active against the growth of *P. larvae* [21–23].

Achyrocline satureioides (Lam.) DC. is native to southeastern South America and grows from the sea level to 2000 m high [24]. It belongs to the Asteraceae family, and it is known as Marcela in folk medicine. This subshrub is very well known because of its medicinal properties and is commonly used in Brazil, Paraguay, Uruguay, and Argentina. In a previous study, we determined that extracts from *A. satureioides* presented an antagonistic effect against the growth of *P. larvae* strains [23]. It was demonstrated in that work that either the decoction or the water remaining after steam distillation was more active than the essential oil in inhibiting the growth of *P. larvae*.

In the present work, we performed a bioassay-guided fractionation of a water extract of *A. satureioides* and determined the most active fractions. Hexane, benzene, ethyl ether, and ethyl acetate were used as extractants, and the antibacterial activity of each extract was tested against *P. larvae* strains. The extract with the highest antibacterial activity was further analyzed by a bio-guided fractionation using bioautography. The fraction with the highest antibacterial activity was analyzed by high-performance liquid chromatography with diode-array and tandem mass spectrometry detection (HPLC-PAD-MS/MS).

Material and Methods

Plant Specimens

The aerial parts of *A. satureioides* were collected at Villa Jorcoricó, Córdoba, Argentina (32° 41′ 38″ S, 64° 42′ 16″ W) during the flowering season. A voucher specimen was stored at the Herbarium of the Faculty of Agronomy and Veterinary, Universidad Nacional de Río Cuarto, as file herbarium 4658. The air-dried plant material was kept in plastic bags at 4 °C until its use.

Plant Extracts

A water-ethanol (1:1, *v*/*v*) macerate was obtained with 300 g of the powdered dried plant material. The solvent mixture was changed frequently until it came out colorless. Then, the aqueous-ethanol extract was concentrated under reduced pressure in a rotary evaporator, and the aqueous solution thus obtained (approximately 2 L) was submitted to liquid-liquid extraction with solvents of increasing polarity. Hexane, benzene, ethyl ether, and ethyl acetate were used successively, rendering the hexane, benzene, ethyl ether, and ethyl acetate extracts, respectively. Then, each extract was concentrated to dryness in a rotary evaporator under reduced pressure, and the extracts were kept in dark flasks at 4 °C until they were used.

Bacterial Strains

Eleven *P. larvae* bacterial strains were isolated from infected colonies or provided by Instituto Nacional de Tecnología Agropecuaria, Estación Experimental Agropecuaria, Balcarce, Argentina. Isolation and biochemical identification were done using previously described techniques [25]. On Mueller-Hinton broth, yeast extract, potassium phosphate, glucose, and sodium pyruvate (MYPGP) agar, *P. larvae* colonies were small, regular, mostly rough, flat or rose, and whitish to beige colored. They were Gram positive and catalase negative. These microorganisms hydrolyzed gelatin and casein, but they neither hydrolyzed starch nor withstood serial transfers in nutrient broth. The identification and molecular confirmation of *P. larvae* strains was carried out by using the rRNA 16S gene. The data was incorporated to GenBank's database, which confirmed the identity of each microorganism. The strains of *P. larvae* were kept at −20 °C on MYPGP agar with 20 % *v*/*v* of glycerol until used.

Antibacterial Activity Assessment

The broth microdilution technique according to Mann and Markham [26] was used to determine the inhibitory effect of hexane, benzene, ethyl ether, and ethyl acetate extracts, and hexane extract fractions on all the *P. larvae* strains included in this study. A cell suspension

of each *P. larvae* strain was prepared at a cell concentration of 10^6–10^7 CFU/mL, equivalent to 0.5 on the MacFarland scale. Microplates were poured with 170 μL of the bacterial inoculum and supplemented with 20 μL of each of the different samples to be assayed for antimicrobial activity. The same inoculum (170 μL) supplemented with 20 μL of the solvent without any substance was used as positive control, and 170 μL of the culture medium without inoculum supplemented with 20 μL of the different dilutions was used as negative control. The microplates were incubated microaerophilically at 37 °C for 14–15 h, and afterward, 10 μL of 0.01 % resazurin were added, and the plates were incubated again at 37 °C for 2 h. Positive controls presented a pink color (bacterial growth) and negative controls a blue color (bacterial inhibition).

TLC and Bioautography

Thin-layer chromatographic analysis of the hexane extract of *A. satureioides* was performed with silica gel on PET foils with fluorescent indicator at 254 nm (Fluka, Germany). A mixture of hexane and acetone (7:3 *v*/*v*) was used as mobile phase. The chromatographic plates were revealed with UV light of 254 nm and with I_2 and $FeCl_3$ solutions sprayed onto them.

Thin-layer chromatography (TLC) plates 3 cm wide by 7 cm long were cut for the bioautographic analysis [27]. The components of the hexane extract were separated by using the same solvent system as for the TLC analysis, and the plates were sterilized under UV light for 1 h previous to the bioautographic analysis. A bacterial inoculum of one of the most sensitive strains determined in our study (*P. larvae* strain number 7, GenBank access code AY530295) was activated in MYPGP agar at 37 °C for 72 h under microaerophilic conditions. A dilution equivalent to the number 1 of the McFarland scale was made with physiological solution. One milliliter of this solution was added to 20 mL of MYPGP 0.6 % agar at 40 °C and poured into a Petri dish containing the TLC plate with the separated hexane extract components. The Petri dishes were incubated 24 h at 37 °C under microaerophilic conditions, once the growth medium solidified. Finally, 1 mL of a 5 % 2,3,5-triphenyl tetrazolium (TTC) in water was sprayed on the Petri dish, and the dishes were incubated again during 24–48 h under microaerophilic conditions to reveal growth inhibition zones. Visualization of growth inhibition zones is based upon the capacity of living cells to reduce TTC to 1,3,5-triphenylformazan, which has a reddish color. The assays were repeated three times.

Flash Chromatography

Twenty-five grams of silica (60-Å pore size, 35–75-μm diameters, Sigma, USA) were packed in a glass column prepared for flash chromatography. A mixture of hexane-acetone (7:3, *v*/*v*) was used as mobile phase. The hexane extract was seeded on top of the silica bed, the mobile phase poured carefully in and a N_2 gas pressure of approximately 50 psi was applied. The eluent of the column was collected in a series of 2-mL flasks, and a TLC analysis was performed for each flask. The content of those flasks showing a TLC band with the same *Rf* value was gathered in a single flask and the solvent distilled in a rotary evaporator. Thus, approximately 4 mg of the band with Rf=0.57 were obtained. This purified fraction was kept in a dark flask at 4 °C until its use.

Electron Microscopy of Hexane Extract-Treated Bacteria

A cell suspension of one of the most sensitive *P. larvae* strains in our study (strain number 7, GenBank access code number AY530295) was prepared at a cell concentration equivalent to 3

on the MacFarland scale. A volume of 0.80 mL of this suspension was poured into Eppendorf tubes containing 0.20 mL of the hexane extract at a concentration of 8 mg/mL in dimethyl sulfoxide (DMSO) the first tube, and serial dilutions factor 2 of that solution with DMSO the remaining tubes. The positive control was prepared with 0.80 mL of the cell suspension and 0.20 mL DMSO. The Eppendorf tubes were incubated at 37 °C under microareophilic conditions for 72 h.

After the incubation period, the bacteria was fixed for 3 h at 4 °C by using 2.5 % glutaraldehyde in 0.20 M pH 7.4 S-collidine buffer and washed twice with the S-collidine buffer. The cells were fixed again for 1 h at room temperature with 1 % osmium tetroxide, rinsed two times with S-collidine buffer, and dehydrated in crescent concentrations of acetone (successive washings in 30, 50, 70, and 90 % acetone for 5 min each, three washings in 100 % acetone for 5 min each).

Preinclusion of cells was performed with EMbed 812 epoxy resin-acetone (1:1 *v*/*v*) overnight at room temperature. Inclusion of cells was performed with EMbed 812 epoxy resin at 56 °C for 48 h. In between these steps, the cell suspension was centrifuged at 5500 rpm for 10 min, the supernatant was discarded, and the steps continued with the pellet. Ultrathin sections were stained with uranyl acetate and lead citrate and were examined with an Elmiskop 101 (Siemens) transmission electron microscope at an accelerating voltage of 80 kV.

HPLC-PAD-MS/MS Analysis of the Most Active Band

An Alliance 2695 quaternary HPLC pump (Waters, USA) coupled with a 2998 Photodiode Array Detector (PDA) (Waters, USA) and a Micromass Quattro Ultima™ Pt (Waters, USA) tandem mass spectrometric detector was used. Peak purity was assessed with the PDA specific functions. Mass spectrometric detector working conditions were as follows: 3.25 kV of capillary voltage, 35 V of cone voltage, and desolvation temperature 250 °C. Nitrogen was used as nebulizer and desolvation gas with a flow of 75 and 500 L/h, respectively. The MS/MS analysis was performed in the daughter ion spectrum (DIS) mode which allowed for the determination of the approximate molecular mass.

A Luna C18(2) (150×3.0 mm) (Phenomenex, USA) column was used for the chromatographic separation. The mobile phase was composed of acetonitrile-0.01 M oxalic acid (20:80) and ran in isocratic mode at a flow rate of 0.20 mL/min.

Results and Discussion

Liquid-Liquid Extraction Yields

The yields of each extract obtained by the liquid-liquid extraction made on the aqueous concentrate of *A. satureioides* were as follows: hexane extract=0.52 %, benzene extract= 0.50 %, ethyl ether extract=0.17 %, and ethyl acetate extract=0.06 %. Yields are expressed as percentage weight by weight of original dry vegetal.

Antibacterial Activity

Table 1 summarizes the results of the antibacterial activity tests of different *A. satureioides* extracts against the growth of various *P. larvae* strains. The average MIC value obtained when using the hexane extract was 0.060 mg/mL (SD=0.037 mg/mL), and strains 1 and 7 were the most sensitive with a MIC value of 0.016 mg/mL while strains 4 and 8 were the most resistant

Table 1 Minimum inhibitory concentration values (mg/mL) determined by the growth inhibition tests with *A. satureioides* extracts obtained with hexane (HE), benzene (BE), ethyl ether (EE), and ethyl acetate (EAE) against different *P. larvae* strains

Strain number	*P. larvae* strain provenance	GenBank access code	HE	BE	EE	EAE
1	INTA Balcarce, Buenos Aires	AY530295	0.016	0.063	1.000	8.000
2	INTA Balcarce, Buenos Aires	AY530295	0.063	0.063	0.500	8.000
3	INTA Balcarce, Buenos Aires	DQ079623	0.032	0.063	0.500	8.000
4	INTA Balcarce, Buenos Aires	DQ079623	0.125	0.125	1.000	8.000
5	INTA Balcarce, Buenos Aires	DQ079623	0.063	0.063	0.500	4.000
6	INTA Balcarce, Buenos Aires	AY530295	0.063	0.063	1.000	4.000
7	Río Cuarto, Córdoba	AY530295	0.016	0.125	1.000	8.000
8	Río Cuarto, Córdoba	AY530295	0.125	0.250	1.000	4.000
9	Río Cuarto, Córdoba	AY530295	0.032	0.250	1.000	8.000
10	Río Cuarto, Córdoba	DQ079623	0.063	0.250	0.500	8.000
11	Río Cuarto, Córdoba	AY530295	0.063	0.125	0.500	4.000
Average±SD			0.060±0.037	0.131±0.081	0.773±0.261	6.545±2.018

with a MIC value of 0.125 mg/mL. Also, the growth of all the *P. larvae* strains tested in this work was inhibited by the benzene (MIC value of 0.131±0.081 mg/mL), the ethyl ether (MIC value of 0.773±0.261 mg/mL), and the ethyl acetate (MIC value of 6.545±2.018 mg/mL) extracts, showing that *A. satureioides* has active compounds against the growth of *P. larvae*.

The observation of growth inhibition zones in all 11 bacterial strains is indicative of the antibacterial activity of the extracts. However, the hexane extract was the most active extract against the growth of *P. larvae* strains giving that its MIC value was the lowest observed, i.e., 0.060±0.037 versus 0.131±0.081, 0.773±0.261, and 6.545±2.018 mg/mL for benzene, ethyl ether, and ethyl acetate extracts, respectively. The biological activity of the hexane extract against the growth of the 11 *P. larvae* strains tested in our work is comparable with those informed by other authors for different natural products [13, 28]. In the first work, MIC values between 38 and 50 μg/mL were informed for the essential oil of *Cinnamomum zeylanicum*, while MIC values between 350 and 400 μg/mL, and between 650 and 700 μg/mL, were informed for the essential oils of *Lavandula officinalis* and *Mentha piperita*, respectively. Similar MIC values for *C. zeylanicum* were informed by the same laboratory when the essential oil was tested against other strains of *P. larvae*.

The MIC values obtained in our work with the hexane extract of *A. satureioides* against the growth of *P. larvae* were significantly lower than those informed for the essential oils of *Citrus paradise* (average MIC values of 385 μg/mL), *Citrus sinensis* (average MIC values of 840 μg/mL), *Citrus nobilis* (average MIC values of 815 μg/mL), and *Citrus limon* (average MIC values of 764 μg/mL) [11]. Also, significantly higher MIC values were informed [10] for the essential oils of *Lippia turbinata* Griseb, *Tagetes minuta* L., *Mintosthachys mollis* H. B. K. Gris, *Schinus molle* var. areira, and *Acantholippia seriphioides* A. Gray (average MIC values

of 867, 805, 769, 667, and 236 μg/mL, respectively). Also, the MIC value of the most active extract (the hexane extract) of *A. satureioides* against the growth of *P. larvae* compares favorably with that informed [13, 29] for the known antimicrobial oxytetracycline (MIC values between 3 and 6 μg/mL, and between 0.125 and 128 μg/mL, respectively).

Transmission Electron Microscopy Analysis

Figure 1 shows the results of the transmission electron microscopy study of the effect of the hexane extract on the growth of *P. larvae* strain number 7, GenBank access code number AY530295 (one of the most sensitive strains determined in our study). Increasing concentrations of the hexane extract were used to analyze its effect on the bacterial growth. Hexane extract-treated *P. larvae* cells contained multilamellar mesosome-like structures (Fig. 1b, c) that were not seen in untreated cells (Fig. 1a). In addition, the contents of some treated cells appeared depleted and amorphous (Fig. 1c, d).

The preliminary studies conducted in our laboratory about the mechanism of action of the hexane extract on *P. larvae* cells, using transmission electron microscopy, showed that the main effect is exerted at the cellular membrane level. Increasing the concentration of the hexane extract in the growth medium induced a higher level of cellular membrane disruption

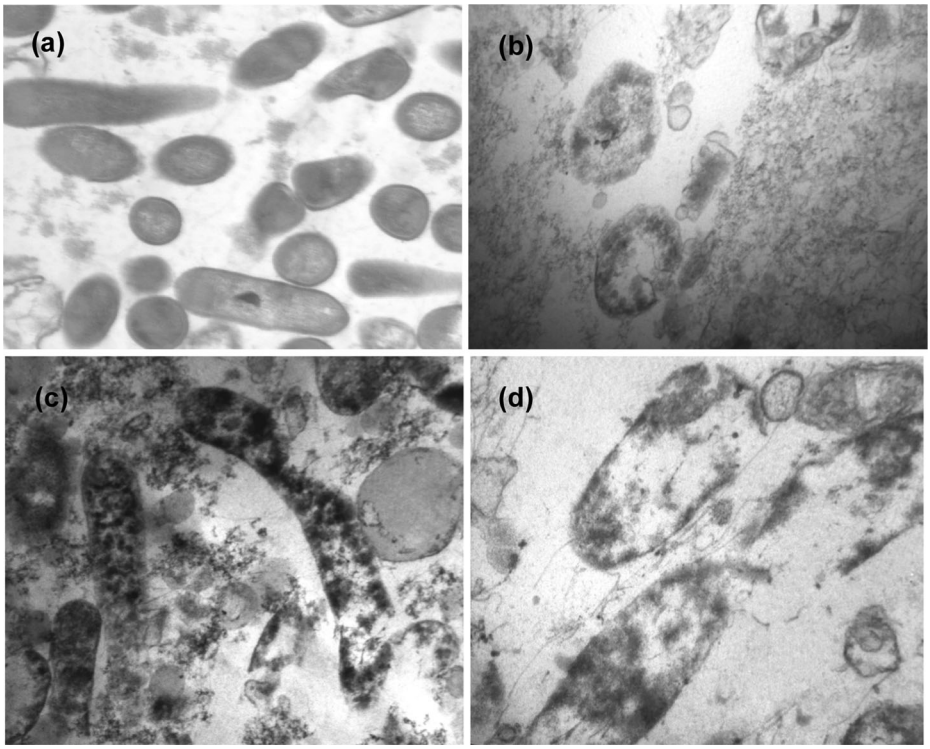

Fig. 1 Antibacterial effect of the hexane extract against the growth of *Paenibacillus larvae* (strain number 7, GenBank access code number AY530295) observed with transmission electron microscopy. **a** Control bacterial cells observed at ×21,560; **b** bacterial cells treated with 2 mg/mL of hexane extract, observed at ×50,000; **c** bacterial cells treated with 4 mg/mL hexane extract, observed at ×30,000; **d** bacterial cells treated with 8 mg/mL of hexane extract, observed at ×50,000

(Fig. 1). Cytotoxicity of low polarity compounds may include cellular membrane damage [30, 31]. It is already known that surface-active compounds are able to disrupt phospholipid membranes by disrupting the hydrophobic interactions between lipids and proteins [31]. The permeabilization of the membranes is associated with loss of ions and reduction of membrane potential, collapse of the proton pump, and depletion of the ATP pool. Consequently, assumptions regarding the mechanism of action of the hexane extract used in our work may be based on the nature of its components. In this context, low polarity compounds are expected to be present in this extract as it was obtained by using a low-polarity solvent such as hexane. In the present study, *P. larvae* cells in the stationary phase of growth were inhibited by the hexane extract.

Thin-Layer Chromatography

The composition of the hexane extract was examined by TLC experiments. A total of nine thin-layer chromatographic bands were observed (results not shown) when a mixture of hexane/acetone (7:3, *v*/*v*) was used as the mobile phase. Seven of these thin-layer chromatographic bands were revealed with ultraviolet light (254 nm), and the other two bands were revealed with $FeCl_3$. Similar results were obtained in other laboratories using extraction techniques based on maceration [32] instead of the liquid-liquid extraction protocol used in our study.

Bioautography

A bioautographic analysis was performed with the *A. satureioides* hexane extract to determine which bands carried the biological activity. A bacterial inoculum of one of the most sensitive strains determined in our study (*P. larvae* strain number 7, GenBank access code AY530295) was used for this study. Four growth inhibition zones were observed, which corresponded with two thin-layer chromatographic bands revealed with $FeCl_3$ (*Rf* values of 0.15 and 0.23), and with two thin-layer chromatographic bands revealed with UV light of 254 nm (*Rf* values of 0.49 and 0.57). The inhibition zone observed over the band with *Rf*=0.57 was the largest. This last result means either that the components in the chromatographic band are present in small quantities but they are very active against *P. larvae* or that the components are present in large amounts but their activity is low.

Flash Chromatography

Column chromatography on silica gel was used to obtain an additional quantity of the compounds present in the thin-layer chromatographic band with *Rf* 0.57. Approximately 4 mg of compounds were obtained. This quantity was dissolved in 1 mL of DMSO, rendering a solution of concentration 4 mg/mL. This solution was used later in the biological activity tests performed against the growth of all of the *P. larvae* strains and in the HPLC-PAD-MS/MS analysis.

Antibacterial Activity of Compounds in TLC Band with *Rf*=0.57

The antibacterial activity of the compounds present in the band with *Rf*=0.57 obtained in the thin-layer chromatographic experiments was assessed against the two strains of *P. larvae* which showed the highest sensitivity to the hexane extract (*P. larvae* strains number 1 and 7, GenBank access code numbers AY530295 and AY530295). The average MIC value obtained was 0.065±0.030 mg/mL. This MIC value was close to that obtained when the entire hexane extract was used, i.e., 0.060±0.037 versus 0.065±0.030 mg/mL, respectively. The closeness of

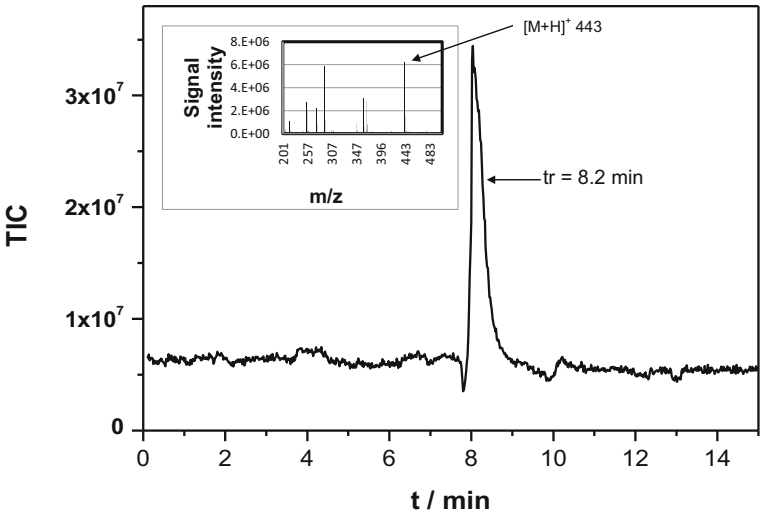

Fig. 2 HPLC-MS/MS chromatogram of the TLC band with *Rf*=0.57. Mobile phase: 0.01 M oxalic acid (A), acetonitrile (B). Isocratic mode: 80 % A, 20 % B. Flow rate=0.20 mL/min. Column: Luna C18(2), 150×3.0 mm

the observed MIC values could be explained by proposing that the antibacterial activity of the entire hexane extract is mainly due to the compound present in the TLC band with *Rf*=0.57.

HPLC-ESI-MS/MS Analysis of the Band with *Rf*=0.57

The HPLC-ESI-MS/MS analysis of the TLC band with *Rf*=0.57 is shown in Fig. 2. A major peak was observed at approximately 8.20 min under the chromatographic conditions used. This result, altogether with the peak purity analysis, indicated that only one compound was present under the chromatographic peak. The mass spectra of the compound in the chromatographic peak at t_r=8.20 min showed a molecular ion with a *m/z* ratio of 443 (inset in Fig. 2), which is consistent with a molecular formula $C_{25}H_{30}O_7$ (calculated mass 442.20). In a recent study performed by Casero [32] on secondary metabolites of *A. satureioides* and their biological activity against the growth of various microorganisms, it was reported that the hexane macerate of the aerial parts of the plant showed notable antibacterial activity. Seven biological active compounds were isolated from this macerate. Compounds named As-1 and As-2, which molecular weights determined by high-resolution mass spectrometry were 442, showed remarkable antibacterial activity against the growth of *Staphylococcus aureus* and were identified as phloroglucinol α-pyrone derivates [32]. Thus, we propose in this work that a phloroglucinol α-pyrone derivative is the active compound in the fraction that showed the highest antibacterial activity. Moreover, phloroglucinols are compounds of known antibacterial activity [33, 34] and have shown minimum inhibitory concentrations as low as 0.002 mg/mL against the growth of *Enterococcus faecalis* and *S. aureus* [34].

Conclusions

Extracts of *A. satureioides* showed high antibacterial activity against 11 *P. larvae* strains. This bacterium is the causative agent of American foulbrood in the honeybee larvae. An aqueous-

ethanol macerate of the aerial parts of the plant was submitted to a bioassay-guided fractionation to determine the most bioactive fractions. Four different extracts were obtained by liquid-liquid extraction using successively hexane, benzene, ethyl ether, and ethyl acetate as extracting solvents. The hexane extract showed the highest activity against the growth of all *P. larvae* strains tested in our study (average MIC value of 0.060; standard deviation 0.037 mg/mL), and the bioautographic analysis of this extract showed a large inhibition zone coincident with that of the thin-layer chromatographic band at Rf=0.57 (using a mixture 7:3, *v*/*v* of hexane and acetone). Transmission electron microscopy revealed that the extract produced cellular membrane disruption. The high-performance liquid chromatographic analysis (ultraviolet and electro-spray tandem mass spectrometric detection) of the TLC band showed the presence of one component, with an estimated m/z ratio of 442. This mass matches that of recently discovered phoroglucinol α-pyrone derivates obtained by extraction from hexane macerates of *A. satureioides*, as described in the bibliography. Thus, it is proposed that the active component found in the hexane extract used in our work belongs to the family of phloroglucinols. The antibacterial activity of the hexane extract and of the isolated compound could be of interest for the development of new alternatives for the treatment of American foulbrood and in vivo assays are being done in order to determine the right dose for its application to the beehive.

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