Fractionation of hexane extracts from *Achyrocline satureioides* and their biological activities against *Paenibacillus larvae*

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Abstract

Previous studies carried out in our laboratory described the antimicrobial activity of the whole hexanic extract (HE) of *Achyrocline satureioides* (Lam.) DC against *Paenibacillus larvae*, the causal agent of American Foulbrood (AFB) a disease of the honey bee larvae. In this study, the HE was partitioned into five main fractions by chromatographic techniques leading to the isolation of four known compounds: two prenylated phloroglucinol α-pyrones (**1** and **3**), 5,7-dihydroxy-3,8-dimethoxyflavone (gnaphaliin A) (**2**), and 23-methyl-6-*O*-demethylauricepyrone (**4**). Isolated compounds were further analyzed towards structural elucidation using ^{1}H RMN and ^{13}C RMN spectroscopic techniques. For the first time, the antimicrobial activity of the isolated compounds was evaluated against *P. larvae* strains by broth microdilution method and compared with that of the whole HE. Compounds **1–4** displayed minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values ranging between 0.07 and 62.5 µg/mL and 0.26 and 12.5 µg/mL, respectively. The lowest MIC and MBC values were obtained with compounds **3** and **4**, respectively. The antimicrobial activity of each single compound and the combination of them showed that the presence of all compounds is needed for the antimicrobial efficacy of whole HE.

Introduction

Honeybees play a vital role in the pollination of crops, fruit, and wild flowers. Thus, factors affecting bee health also affect a sustainable and profitable agriculture as well as many non-agricultural ecosystems.

Honeybees are affected by numerous pathogens like viruses, bacteria, fungi, and parasites, which represent a significant threat to bee health. The Gram-positive, spore-forming bacterium, *Paenibacillus larvae* (*P. larvae*), is the causative agent of American foulbrood (AFB), the most severe bacterial disease that affects larvae of the honeybee *Apis mellifera*. AFB presents worldwide distribution, causing a significant decrease in honeybee populations

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and production of honey, pollen, propolis, royal jelly, and beeswax [1, 2].

AFB is a notifiable disease in many countries, and its treatment is often regulated by law. AFB control measures include the burning destruction of infected hives or the use of antibiotics for the treatment of the disease, although it is banned in most European countries, limiting the international marketing. In Argentina, since 2016, the Servicio Nacional de Sanidad y Calidad Agroalimentaria (SENASA) has forbidden veterinary products used in beekeeping made on the base of the active principle oxytetracycline, used for the treatment of AFB [3]. The fact that antibiotics can mitigate, but not eliminate the disease provokes infected hives to be treated constantly to prevent a foulbrood outbreak [4–6]. However, antibiotics are only effective on the rod cell but not on the spore, which can persist in the hives for years [7]. In addition, chemical residues can persist in honey affecting its quality and safety for human consumption. All these factors contribute to increase the antimicrobial resistance to antibiotics.

As a consequence, the requirement for a reduction in the use of antibiotics in food prompts a need to develop safer active compounds. In recent years, many researchers have been investigating new strategies that can be alternatives to the use of antibiotics. The use of natural substances (i.e., plant extracts and essential oils or their compounds) in apiculture offers an interesting alternative against synthetic antibiotics. Plant extracts are of particular interest because they are relatively safe, widely accepted by consumers, and can be exploited for multiple uses. Taking into consideration the large potential of plants as sources for antimicrobial compounds, many systematic investigations were carried out and screened in different parts of the world [8–12].

South America has historically been a notable reservoir of plant resources. Many of them have been evaluated for their biological activities, including for the control of diseases in honeybees. In this way, essential oils [13–16] and propolis [17] have been tested for the control of *P. larvae*, and several plant extracts showed antibacterial activity against this pathogen also [10, 18, 19].

It has been shown that extracts from *Achyrocline satureioides* (Lam.) DC have antimicrobial activity against Gram-positive and Gram-negative bacteria [20]. This plant belongs to the *Asteraceae* family, and it is known as "Marcela" in folk medicine; it is a native plant from the southeastern region of South America and grows in sandy or stony soils, on hilly or plain terrain. It is common in Brazil, Uruguay, Venezuela, Colombia, Bolivia, Paraguay, Peru, and also central and northeastern of Argentina [21–23]. In this last country, it mostly grows in humid areas and sandy soils, such as the mountains of Córdoba, San Luis, and Tandil and on coastal dunes of the Province of Buenos Aires [24].

The extracts of *A. satureioides* are characterized by their high content of polyphenols and flavonoids [25, 26]. Numerous authors found that the yield of the dry extract and the content of flavonoids vary according to the extraction method used. On the other hand, pharmacological activities of *A. satureioides* have been the subject of numerous studies in the past few years [25]. The large volume of related literature and the positive results reported by most authors reflect the undoubted potential of this plant as a natural medicine. Also, toxicological studies of *A. satureioides* aqueous extracts, performed in mice and rats, demonstrated low acute toxicity when it was administered intraperitoneally and no toxicity was observed after oral administration [27].

In a previous study, we determined that extracts from *A. satureioides* presented an antagonistic effect on the growth of *P. larvae* strains. Four different extracts were obtained by liquid–liquid extraction from an aqueous-ethyl alcohol macerate of the aerial parts of the plant. The crude hexanic extract (HE) showed the highest anti-*P. larvae* activity [28, 29]. The biological activity of the HE against the growth of *P. larvae* strains tested in our work was comparable with those informed by other authors for different natural products and for the antibiotic oxytetracycline [30–32].

The aim of the present study was to isolate and identify by ^{1}H NMR and ^{13}C RMN spectroscopic techniques the compounds present in the HE of *A. satureioides* and test, for the first time, their antibacterial activity against *P. larvae*, comparing them with the inhibitory activity of the whole HE.

Material and methods

All reagents and solvents were of analytical grade and used without further purification. All experiments were carried out with ultrapure water from LABCONCO equipment model 90,901–01 (HPLC grade water).

Cleaning and drying of plant material

The aerial parts of *A. satureioides* were collected from Santa Mónica, Córdoba, Argentina (32° 5′ 26.5″ S, 64° 37′ 1.9″ 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. Leaves, fine stems, and flowers were collected, cleaned, and dried. Dried specimens were stored until they were used for extraction.

Extract preparation

The air-dried powdered plant material (200 g) was extracted by maceration with an ethanol–water (1:1) mixture at room

temperature. The extracting mixture was renewed until the material was exhausted (until it came out colorless). This macerate was filtered with Whatman N° 2 paper obtaining the hydro-alcoholic extract (HAE). The HAE was concentrated under reduced pressure in a rotary evaporator, and the aqueous solution obtained (approximately 2 L) was submitted to liquid–liquid extraction with hexane. Then, the HE was concentrated to dryness in a rotary evaporator under reduced pressure and was kept in dark flasks at 4 °C until they were used.

Phytochemical screening of HE

Preliminary phytochemical screening tests of the dry HE were carried out to identify useful constituents by standard methods [33], testing for alkaloids, tannins, flavonoids, anthraquinones, steroids, and triterpenoids.

Test for alkaloids

A small amount (0.09 g) of the HE was dissolved in 15 mL of 5% HCl and kept in a water bath at 60 °C for about 2 min. The extract was then filtered off and allowed to cool, and then 1 mL of the extract was separated in two test tubes. Few drops of Dragendorff's reagent (potassium iodide-bismuth nitrate) were added in the tube and appearance of orange-red precipitate was taken as positive. Few drops of Mayer's reagent (composed of mercuric chloride and potassium iodide dissolved in distilled water) were added to the second tube and appearance of buff-colored precipitate designates the existence of alkaloids.

Test for steroids and triterpenoids

The HE (0.09 g) was extracted with two 15 mL portions of petroleum ether. The extract was filtered, and evaporated in a water-bath, up to 5 mL. Then, 10 mL of water–methanol (10:90) was added and stirred. The mixture was allowed to completely separate into two layers. The ether layer was separated by two-dimensional TLC on silica gel G eluting first with cyclohexane-ethyl acetate (95:5) and then, with petroleum ether—ethyl ether—acetic acid (80:20:1) and sprayed with Liebermann-Burchard reagent. Then, it was heated at 105 °C for 10–15 min. The residue was extracted with two 10 mL portions of ethanol–water (1:7) at 60 °C, and it was filtered. The filtrate (called *solution 1* hereafter) was used for the determination of flavonoids, anthraquinones, and tannins.

Test for flavonoids

One milliliter of *solution 1* was put into two clean test tubes. One test tube was reserved as a control. Concentrated HCl (0.5 mL) was added to the other test tube followed by addition of magnesium turnings. The turning to a red color confirmed the presence of flavonoids.

Test for tannins

One milliliter of the *solution 1* was added to a 1 mL of solution of gelatin containing 10% NaCl. The mixture was centrifuged at 2000 rpm, the residue was dissolved in 2 mL of urea solution 10 M, and few drops of 5% ferric chloride were added. The occurrence of a blackish blue color showed the presence of gallic tannins and a green-blackish color indicated presence of catechol tannins [34].

Test for anthraquinones

Five milliliters of *solution 1* was placed in a dry test tube. One milliliter of hydrogen peroxide (20 volumes) and 1 mL of H_2SO_4 (5%) were then added. This mixture was heated in a steam bath for 15 min. It was then cooled and extracted with 3 mL of benzene and stirred with 1 mL of 5% NaOH containing 2% NH_4OH. A pink-red color in the ammoniacal (lower) layer showed the presence of anthracene derivatives.

Hexanic extract fractioning

Column chromatography (CC)

The HE was subjected to silica gel CC for the isolation of the active principles. An appropriate column of 5-cm diameter and 50-cm length was used. A small plug of fiberglass was placed at the bottom of column helped with a glass rod. The column was packed with 30 gr of Silica gel (60 Å pore size, 35–75-μm diameters, Sigma, USA). It was washed with water, rinsed with acetone, and then completely dried. A mixture of n-hexane–acetone (8:2, v/v) was used as mobile phase. A 3-mm layer of sand was carefully placed on the flat top of the dry silica gel bed, and the column was clamped for pressure packing and elution. Then, the HE was seeded on top of the silica bed, the mobile phase poured carefully in, and N_2 gas pressure of 50 psi was applied. The knob at the bottom was slowly opened to release the solvent. One hundred fractions of 2 mL each were separately collected and subjected to thin-layer chromatography (TLC) using a mixture of n-hexane:acetone (8:2 v/v) as mobile phase. The content of those flasks showing a TLC band with the same retardation factor (Rf) value were gathered in a single flask and the solvent evaporated by using a rotary evaporator. The number of flasks was reduced from one hundred to five. These purified fractions were kept in a dark flask at 4 °C until they were used for further analysis on thin layer or radial centrifugal chromatography.

TLC

TLC analysis of the HE of *A. satureioides* was performed with silica gel on PET foils (20×20 cm of 0.25-mm thick) with fluorescent indicator at 254 nm (Fluka, Germany). Different mixtures of n-hexane and ethyl acetate were used as mobile phase (see below). The chromatographic plates were revealed with UV light at 365 nm and 254 nm with anisaldehyde solutions sprayed onto them, heating the plates at 120 °C.

Centrifugal thin-layer chromatography

A Harrison Research Chromatotron (model 7924 T) was used in this experiment. The rotor stationary phase was made of silica gel 60 F256 with calcium sulfate hemihydrate (Merck) with an average particle size of 15 μm, a thickness of 2 mm, and a diameter of 24 cm. The rotation rate was 800 rpm. Solvent was delivered from a reservoir at a rate of 1 mL/min to 10 mL/min.

Identification of compound via *spectroscopic techniques*

The ^{1}H RMN and ^{13}C RMN spectra of isolated compound were recorded in Bruker Avance 400 at 400 and 100 MHz, respectively, in CDCl$_3$, with TMS as the internal reference. The compounds were identified by comparison with published spectroscopic data.

Microbial strains

Two strains of *P. larvae* were used in this study: *P larvae Tipica* (Pl T) and *P. larvae* strain 9 (Pl 9). The first one belonged to the Centro de Investigación en Abejas Sociales de la Universidad Nacional de Mar del Plata (CIAS-UNMDP), and Pl 9 was isolated from infected colonies from Rio Cuarto region. It was biochemically and molecularly identified using previously described techniques [35]. There were grown on MYPGP agar (Mueller–Hinton broth, yeast extract, potassium phosphate, glucose, and sodium pyruvate), where *P. larvae* colonies were small, regular, mostly rough, flat or rose, and whitish to beige colored. They were Gram-positive rod, catalase negative; they could hydrolyze gelatin and casein, but not starch. The identification and molecular confirmation of *Pl* 9 strain were carried out using the rRNA 16S gene. The data was incorporated to Gen-Bank's database, which confirmed the identity of the microorganism [29]. The *P. larvae* strains were kept at − 20 °C on MYPGP agar with 20% v/v of glycerol until used.

Minimum inhibitory concentration (MIC) determination

The antimicrobial activity of the HE and the isolated compounds against *P. larvae* strains was determined by a broth microdilution method [36–38]. Colonies grown for 48 h in a MYPGP agar plate were suspended in sterile peptone water until reaching a final optical density at 600 nm of 0.1.

Preparation of HE/compounds dilutions: 6 mg of HE/compounds was dissolved in 15% dimethyl sulfoxide (DMSO), which was previously determined as the non-toxic concentration of solvent for the growth of *P. larvae*. HE dilutions were prepared at a concentration range of 0.048 to 600 μg/mL. In a microplate, 100 μL of brain heart infusion was placed in each well, then 100 μL of HE was added to well 1, and serial dilutions were performed until well 11. Finally, 50 μL of the previously prepared inoculum were added to each well, except for the negative control. The microplate was incubated in microaerophilia at 37 °C for 48 h, and then 10 μL of resazurin (0.01%) was added to each well. It was incubated again during 1:30 h at the same conditions. MIC was defined as the lowest concentration of compounds that inhibited visible growth indicated by a blue color and bacterial growth was shown by a pink color; DMSO at 15% was used as the negative control (blue color). The assays were repeated three times.

Minimum bactericidal concentration (MBC) determination

For the minimum bactericidal concentration (MBC) determination, 100 μL of the MIC dilution and the previous ones were inoculated in MYPGP agar and incubated in microaerophilia at 37 °C for 48 h. MBC was considered as the last dilution without cell growth [39, 40].

Determination of associations between compounds

Briefly, 100 μl of dilutions of compound "A" was distributed in the rows of a microplate and 100 μl of compound "B" was placed in the columns. Then, different combinations were made between isolated compounds so as to reach different combinations of them [41]. Then, 50 μl of the inoculum was added to each well (optical density at 600 nm: 0.8–0.12); microplates were incubated at 37 °C for 24 h and revealed with rezasurin. The interactions were stablished using the fractional inhibitory concentration index (FICI) with the formula: FICI = (MIC Ab/MIC A) + (MIC Ba/MIC B), where MIC A and MIC B were the minimum inhibitory concentrations of compounds "A" and "B" and MIC Ab and MIC Ba were the minimum inhibitory concentration of A or B at a defined concentration of B or A, respectively. Synergistic effect was observed when FICI value < 1; an additive effect

when FICI value = 1; an indifferent effect when $1 <$ FICI value ≤ 2 and an antagonistic effect when FICI value > 2.

Results and discussion

HE yields and chemical compositions

The yields of HE from *A. satureioides* were expressed as percentage (w/w) of original dry vegetal and varied between 0.5 and 0.75%. Phytochemical tests on samples of HE for the identification of bioactive constituents were carried out using the standard phytochemical procedures described in experimental section. The results confirmed the presence of tannins and flavonoids in the HE, which was in agreement with preliminary studies carried out in our laboratory [42]. The presence of anthraquinones and terpenoids were also observed, while alkaloids were not found. The results are shown in Table 1.

For the testing of the biological activity of natural products and for the structure elucidation of potential bioactive compounds, very small quantities of the substances in extreme pure stage are necessary. Therefore, isolation of pure compounds from their extracts is one of the most important requirements in natural product research.

The compounds present in the HE were investigated in order to establish the chemical profile potentially responsible for the antimicrobial activity. Firstly, the HE was fractionated by CC on silica gel and the collected fractions were analyzed by TLC as previously described in the experimental section. Eluted samples showing the same *Rf* values were put together, resulting in five main fractions (F1 to F5) (Table 2). The obtained fractions were dried under vacuum using a rotary evaporator preparing them for a subsequent purification. The F1 and F5 fractions were purified by preparative TLC, F2 by radial centrifugal chromatography and F3 and F4 by successive column chromatography (Table 2).

Several mobile phases with varying solvent ratios were tested for F1 and F5 (data not shown here). The TLC plates were developed using a mixture 15% n-hexane:ethyl acetate as the mobile phase for F1. Separation of F5 components was achieved by two successive TLCs using the same solvent system but with different proportions: 25% and 30%, respectively. In the semi-preparative TLC plates, two spots were collected from F1 and F5. Each of the desired bands was scraped off the backing material. The isolated compounds were placed in tubes with acetone, left standing for 1 h, filtered and stored in dry and previously weighed dark glass containers until further characterization by spectroscopic techniques.

Two main compounds were separated from F1 and F5, hereinafter referred to as compound **1** and **2**, respectively (Fig. 1). Comparing the ^{1}H NMR and ^{13}C NMR data (see Table 3) with Refs. [20, 43], the obtained products were identified as (*S*)-3-[{5,7-dihydroxy-2,2-dimethyl-8-(2-methylbutanoyl)-2*H*-chromen-6-yl}methyl]-6-ethyl-4-hydroxy-5-methyl-2*H*-pyran-2-one, (**1**) and 5,7-dihydroxy-3,8-dimethoxyflavone (gnaphaliin A) (**2**).

F2 was further purified by radial centrifugal chromatography. The chromatotron was first fitted with a 1-mm silica gel plate, and 50 mL of n-hexane was placed in the solvent reservoir. Once the plate was equilibrated, the F2 was loaded

Table 1 Phytochemical constituents of HE of *A. satureioides*

Test	*Observation*	Inference
Tests with Dragendorff's and Mayer's alkaloidal reagents for alkaloids	No colored ppt with any reagent	Absence of alkaloids
Liebermann-Burchard's reagent for Steroids and Triterpenoids test	Appearance dark green color	Steroids and triterpenoids presents
Test for flavonoids	Appearance of red color	Flavonoids present
FeCl$_3$ solution test for tannins	Appearance dark green color	Tannins present
Test for anthraquinones	Appearance pink-red color in the alkaline layer	Anthraquinones present

Table 2 *Rf* values of fractionated compounds of HE and separation technique used

Fraction	*Rf* values	mg	Yield (%)	Separation technique
F1	0.47	16	1.21	Preparative TLC
F2	0.47 / 0.38	40	3.02	Radial centrifugal chromatography
F3	0.47 / 0.38 / 0.25	178	13.43	Successive column chromatography
F4	0.38 / 0.25	116.2	8.76	Successive column chromatography
F5	0.14	115.4	8.71	Preparative TLC
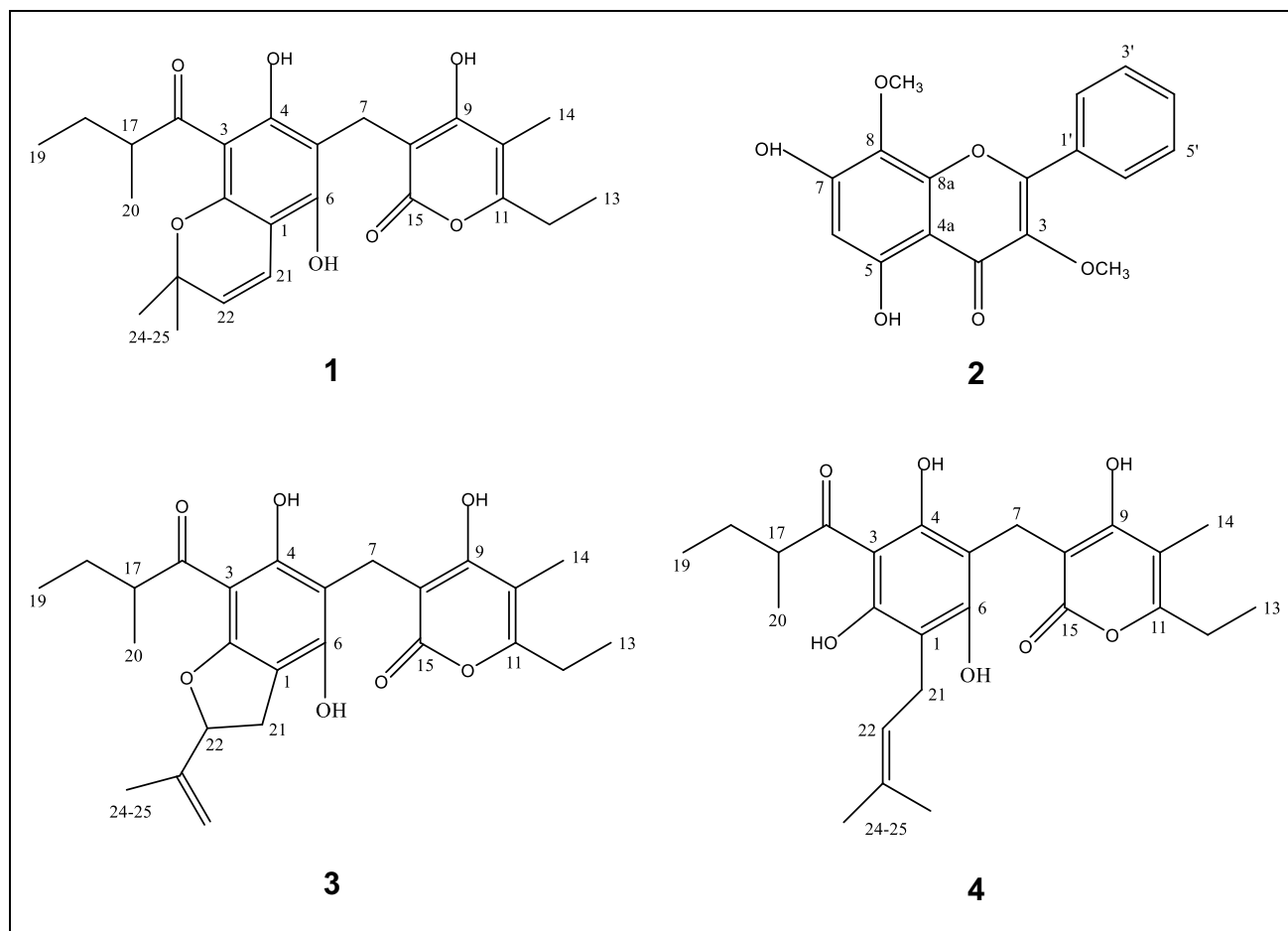
Fig. 1 Molecular structure of compounds 1 to 4

onto the plate via the reservoir inlet. The purification step was carried out using a mixture of n-hexane and ethyl acetate in a gradient as mobile phase. The eluents were collected in series of one hundred forty fractions for each solvent system. After TLC analysis (using a mixture of n-hexane and ethyl acetate (8:2 v/v) as the mobile phase), fractions of each solvent system with similar *Rf* value were combined as it was assumed that the same compound was present in each collection. Therefore, the number of fractions was reduced to eleven and all were analyzed by spectroscopic techniques. The results showed that the main compound of these fractions was 1, described above.

Reverse phase column chromatography was applied for the isolation of compounds from 100 mg of a mixture of F3 and F4. The purification step was carried out using a gradient mixture of n-hexane and ethyl acetate as the eluent. Collected fractions (two hundred fractions) were monitored by TLC using a mixture of n-hexane and ethyl acetate (4:1, v/v) as mobile phase. Fractions with similar *Rf* value were combined as it was assumed that the same compound was present in each collection. Therefore, the number of

fractions was reduced to ten and all were analyzed by spectroscopic techniques. The results showed that four compounds were isolated. Two of them were the compounds **1** and **2**, described above. Comparing the ^{1}H NMR and ^{13}C NMR data (see Table 3) with Refs. [12, 20], the third compound was identified as another prenylated phloroglucinol α-pyrones, 3-[{4,6-dihydroxy-7-(2-(*S*)-methylbutanoyl)-2-(prop-1-en-2-yl)-2,3-dihydrobenzofuran-5-yl}methyl]-6-ethyl-4-hydroxy-5-methyl-2*H*-pyran-2-one and the fourth was the compound known as 23-methyl-6-*O*-desmethylauricepyrone hereinafter referred to as compound **3** and **4**, respectively (Fig. 1).

At the end of the purification process, the amount of the isolated compounds was: **1** (4.1% yield based on whole HE), **2** (3.0%), **3** (0.3%) and **4** (2.5%).

It is important to clear out that different composition and concentrations would be obtained if the plants were collected from a different place. Nowadays, it is known that variability in both parameters could be observed due to the recollection place, the weather conditions, the part of the plant, or the presence of a plague. In addition, the harvesting

Table 3 NMR data of compounds **1 – 4** in CDCl$_3$

Position	1 δH, J (Hz)[a]	1 δC[b]	3 δH, J(Hz)[a]	3 δC[b]	4 δH, J(Hz)[a]	4 δC[b]	position	2 δH, J (Hz)[a]	2 δC[b]
1	-	104.1	-	99.9	-	109.8	2		159.9
2	-	155.45	-	160.6	-	161.5	3		139.4
3	-	104.5	-	103.4	-	106.6	4		179.5
4		162.0	-	160.0	-	160	5		154.4
OH-4	16.25 s		14.0 s		15.01 s		OH-5	12.16 s	
5	-	106.1	-	107.1		107.1	6	6.45 s	94.4
OH-5							7		151.5
6		158.96	-	162.0	-	158.25	OH-7	5.18 s	
OH-6	10.61 s		10.37 s		10.57 s		8		125.8
7a	3.68brs	17.6	3.59brs	18.2	3.58brs	18.9	8a		142.7
7b	3.61brs		3.51brs		3.52brs		4a		105.7
OH-7							1'		130.7
8	-	102.2		102.4	-	100.3	2'	8.14 m	128.6
9		167.54		166.5	-	170.26	3'	7.52 m	128.4
OH-9	9.96 s		8.52brs		-		4'	7.52 m	131.0
10	-	108.3		108.1	-	110.1	5'	7.52 m	128.5
11	-	161.4	-	161.5	-	161.84	6'	8.14 m	128.6
12	2.55 c (7.5)	24.4	2.56 c (7.5)	24.3	2,60 c (7.5)	25.1	3-OMe	3.87 s	60.6
13	1.18 t (7.5)	11.7	1.02 (7.5)	11.9	1.10 t (7.6)	11.8	8-OMe	3.98 s	56.6
14	1.94 s	9.5	1.95 s	9.41	1.97 s	9.7			
15	-	169.4	-	169.2	-	170			
16	-	211.1	-	211.9	-	211.6			
17	3.78 sext (6.7)	45.8	3.93 sext (6.6)	45.87	3.97 sext (6.6)	46			
18a	1.86 m	26.8	1.82 m	27.0	1.83 m	28.2			
18b	1.42 m		1.40 m		1.38 m				
19	0.92 t (7.2)	12.1	0.91 t (7.3)	11.9	0.91 t (7.4)	12.3			
20	1.16 d (6.6)	16.8	1.16 d (6.8)	16.64	1.13 d (6.7)	17.2			
21	6.72 d (10.0)	117.5							
21a			3.0 dd (15.0;8.0)	31.6	3.27 sa	22.7			
21b			3.32 dd (15.0;9.6)		3.29 sa				
22	5.43 d (10.0)	124.9	5.40 t (8.6)	89.7	5.11 m	124			
23	-	78.3	-	142.27	-	129.86			
24	1.47 s	27.8			1.76 s	18.0			
24a			5.14 s	114.2					
24b			5.02 s						
25	1.47 s	27.8	1.77 s	16.75	1.65 s	25.9			

[a]Spectra recorded at 400 MHz. [b]Spectra recorded at 100 MHz

time, how to keep it dry, the age of the plant, and the extraction method also influence the quality and quantity of the compounds. However, in this study, it was observed that the chemical composition of *A. satureioides* did not show significant differences with the composition of hexane extracts of the same plant collected from other geographical area or collection period reported by other authors. This could be relevant if a different hexane extraction method is applied [20, 29]. These results would indicate that *A. satureioides* has a relatively stable chemical composition which is essential from the point of view of its application as an antibacterial agent or for any other use.

Evaluation of antibacterial activity of isolated compounds and HE

The antimicrobial activity of extracts obtained from *A. satureioides* with solvents of different polarities against *P.*

larvae growth was previously studied, HE being the most active [29]. In order to identify the metabolites responsible for the antibacterial activity against *P. larvae* described for HE, the antibacterial activity of compounds **1–4** was assayed. The results are summarized in Table 4, and the antibacterial activity of HE was included for comparative purposes.

It was observed that all isolated compounds and the whole HE were active against the growth of *P. larvae* strains except for compound **2** that did not show activity against Pl T, within the range of concentrations studied. The evaluation of the antimicrobial activity of the compounds, **1** to **4**, displayed MIC and MBC values ranging from 0.07 to 62.5 µg/mL and 0.26 to 12.5 µg/mL, respectively. The best MIC was obtained with compound **3** followed by **4** and the HE. The lowest anti-*P larvae* activity was demonstrated for compound **2**, chemically recognized as a flavonoid. This result was consistent with other authors who observed that flavonoids isolated from different plant extracts presented lower antimicrobial activity against *P. larvae* compared to other chemical compounds [19]. Compounds **3** and **4** also showed the strongest bactericidal effect on PI 9, with MBCs of 0.50 and 0.26 µg/mL, respectively. The whole HE had previously showed a MIC of 0.4 µg/mL and 2.34 µg/mL for Pl 9 and Pl T, respectively, which was in the range obtained for the isolated compounds, but it was only observed bactericidal effect of it on Pl T.

The antimicrobial activity of a phytochemical has been defined as significant when MIC is below 10 µg/mL, moderate when 10 µg/mL < MIC < 100 µg/mL or low when MIC > 100 µg/mL [44]. Therefore, the results showed that both the isolated compounds and HE significantly inhibited the growth of *P. larvae* strains, with MIC values below 10 µg/mL, in most cases. All values were comparable or less than those informed by other authors for different natural products against *P. larvae* [18, 19].

It is important to emphasize that compounds **1**, **3**, and **4** isolated in this work match with compounds numbered

1, 2, and 5 previously reported by Casero et al. [20], which also demonstrated antimicrobial activity against clinically significant bacteria, reporting better activity on Gram-positive bacteria. Similar results were obtained in our work considering that *P. larvae* is Gram-positive bacteria.

The results presented here showed that all the isolated compounds and the whole HE had very effective bioactivity and could be considered to replace the use of traditional antibiotics for the treatment against AFB in bees. This is more promising taking into consideration that more recent studies by our research group have shown that the HE from this plant is not toxic to larvae and bees at the MIC concentrations reported in this work [45].

Associations between compounds

Above was described the antimicrobial activity of the EH and four isolated compounds of *A. satureioides* against two *P. larvae* strains. The main interest in the isolation and identification of single compounds of a HE is based on the need to identify the degree of biological activity each one has in the whole HE so that this one can be replaced by an individual agent or a mix. This implies that interactions between isolated compounds should be done in order to asses if these compounds have better or worse activity than the whole extract. Many times, it has been reported that the antimicrobial activity of the whole vegetal fraction was more effective and had a wider range than the individual activity of isolated compounds [46, 47].

Therefore, different combinations between isolated compounds were performed in order to evaluate the antimicrobial interactions: synergisms, additivity, antagonisms, and indifference. In all combinations, between compounds **1**, **2**, and **3**, a synergistic effect was observed for both strains (FICI values < 1), except for the **1/3** mixture for Pl 9, which showed an additive effect (FICI value = 1). No antagonism was seen in any of the experiments. The remaining combinations of the other interactions showed inconclusive results.

The results obtained with the combinations **1/3** were used to build isobolograms. Plotting the MIC value of one compound of a combination on the x-axis and the other on the y-axis resulted in the isobolograms shown in Fig. 2. The line that connects the two points on the graph shows all the combinations that would theoretically result in additivity, those that are below the line would correspond to a synergistic effect and those that are above it to an antagonistic effect [48].

The MIC values of **1/3** combinations on Pl 9 strains were located on the straight line showing the additive effect of

Table 4 Antibacterial activity of the isolated compounds from the HE of *A. satureioides* on *P. larvae* strains

Compounds	MIC (µg mL^{-1})		MBC (µg mL^{-1})	
	Pl 9	Pl T	Pl 9	Pl T
1	1.05	62.5	6.5	12.5
2	2.5	NI	6.1	ND
3	0.07	62.5	0.5	12.5
4	0.26	ND	0.26	ND
HE	0.4	2.34	–-	4.68

NI no inhibition, *ND* not determined
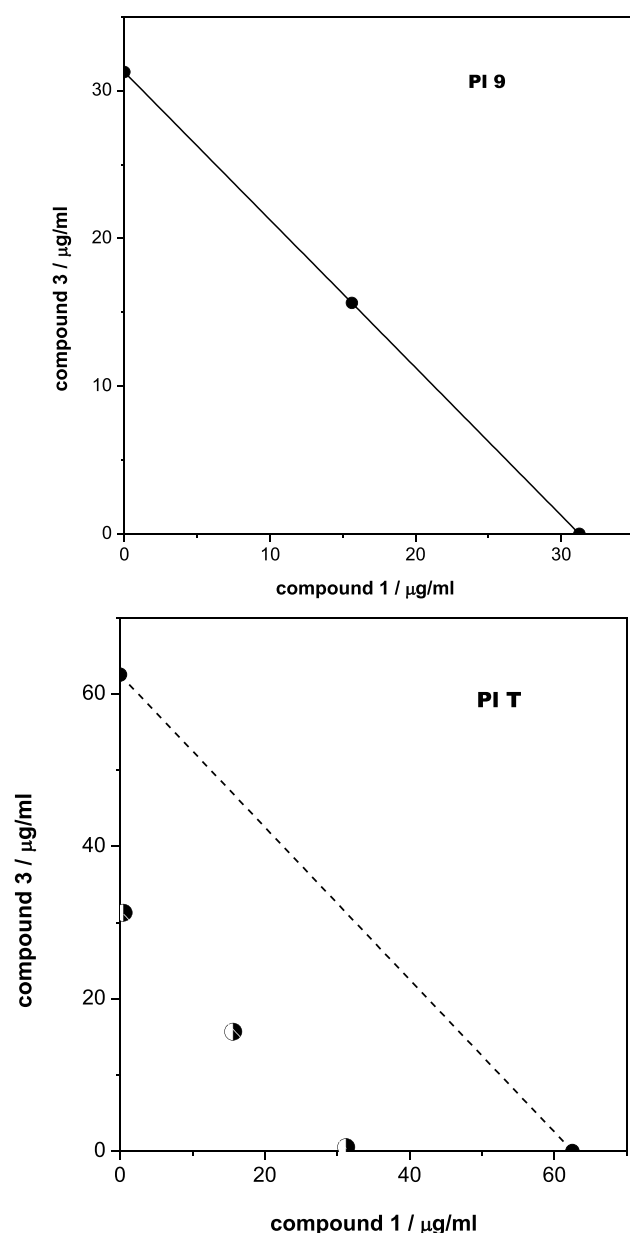
Fig. 2 MIC isobolograms for combinations of compounds 1 and 3 against Pl 9 and Pl T.

both compounds while for Pl T the three combinations were below the lines, indicating a synergistic effect (Fig. 2).

It could be noticed in this research work that the concentrations needed to reach inhibition with each single compound was higher than the effective concentration of the whole HE, supporting the idea of a better activity of a complex mixture of compounds. Similar statements were given by many authors who studied the antimicrobial interactions between different essential oils, or by the terpene constituents of them describing the whole extract as a more effective inhibitor than single compounds [46–49]

Conclusions

The HE of A. *satureioides* constitutes an alternative as a new source of antibacterial compounds for the treatment of AFB. This research helped in identifying, for the first time, the active principles of the HE responsible for the antibacterial activity against *P. larvae*, the causal agent of AFB disease in honey bee colonies. Though (*S*)-3-[{5,7-dihydroxy-2,2-dimethyl-8-(2-methylbutanoyl)-2*H*-chromen-6-yl}methyl]-6-ethyl-4-hydroxy-5-methyl-2*H*-pyran-2-one (**1**), gnaphaliin A (**2**), 3-[{4,6-Dihydroxy-7-(2-(*S*)-methylbutanoyl)-2-(prop-1-en-2-yl)-2,3-dihydrobenzofuran-5-yl}methyl]-6-ethyl-4-hydroxy-5-methyl-2*H*-pyran-2-one (**3**) and 23-methyl-6-*O*-desmethylauricepyrone (**4**) were identified as the major chemical compounds.

The results showed that both the isolated compounds and whole HE significantly inhibited the growth of *P. larvae* strains, with MIC values below 10 µg/mL, in most cases.

In addition, different combinations between compounds 1 and 4 were tested, showing, in most cases, a synergistic effect for both *P. larvae* strains (FICI values < 1). However, the complete HE resulted more effective than the combinations studied and the individual compounds, except for compounds 3 and 4 towards Pl 9.

To conclude, these products represent a potential alternative to replace traditional antibiotics and reduce residues in honey. However, the efficacies of these agents need to be further evaluated under field conditions in beehives.

Declarations

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