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RESEARCH ARTICLE

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Oral and contact toxicity of the extract obtained with hexane from *Achyrocline satureioides* on larvae and adult honey bees

Diana C. Pimentel-Betancurt[1], Natalia V. Tonello[2], Francisco Padilla-Alvarez[3], María F. Paletti-Rovey[1], María M. Oliva and Juan M. Marioli[2]

[1] *National University of Río Cuarto, Faculty of Exact, Physico-Chemical and Natural Sciences, Dept. Microbiology and Immunology. X5804BYA Río Cuarto, Argentina.* [2] *National University of Río Cuarto, Faculty of Exact, Physico-Chemical and Natural Sciences, Dept. Chemistry. X5804BYA Río Cuarto, Argentina.* [3] *University of Córdoba, Campus of Rabanales, Dept. Zoology. 14071 Córdoba, Spain.*

Abstract

Aim of study: To evaluate the antimicrobial activity of the hexanic extract (HE) of *Achyrocline satureioides* on *Paenibacillus larvae* - a gram-positive spore-forming bacillus that affects the larval stage and causes American Foulbrood (AFB) - and its oral and contact toxicity on larvae and adult honey bees.

Area of study: A. satureioides plants were collected in Santa Monica (32° 05' 29" S, 64° 36' 54" W, Córdoba. Argentina). The larvae and adults of *Apis mellifera* were obtained from the experimental apiary of the University of Córdoba, Spain.

Material and methods: P. larvae 9 was previously isolated and identified in the Laboratory of General Microbiology (Dept. of Microbiology, National University of Río Cuarto, Argentina). The HE was obtained by liquid-liquid extraction. The minimum inhibitory concentration (MIC) of HE was determined by a microdilution method. This concentration and 2 ½ MIC were used for *in vitro* toxicity tests. Oral toxicity was tested on larvae, feeding them with both concentrations of the HE, while on adult bees the HE was spread to determine contact toxicity.

Main results: The HE showed antimicrobial activity, the MIC obtained was 0.4 µg/mL. The HE presented very low toxicity at the MIC and 2 ½ MIC, with survival percentages to be around 95% for adult bees and larvae.

Research highlights: The results show that this extract could be used for the development of an alternative product for a safe and effective treatment of AFB.

Additional key words: *Apis mellifera*; antibacterial activity; hexanic extract; *Paenibacillus larvae*

Abbreviations used: AFB (American Foulbrood); HE (hexanic extract); MBC (minimal bactericidal concentration); MIC (minimal inhibitory concentration).

Authors' contributions: Conceived and designed the experiments, analyzed the data, contributed reagents/materials/analysis tools: DCPB, FPA, MMO, JMM. Performed the experiments: DCPB, NVT, MFPR. Wrote the paper: DCPB, MMO, JMM.

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Correspondence should be addressed to Diana C. Pimentel-Betancurt: dpimentelb@exa.unrc.edu.ar

Introduction

The honeybee, *Apis mellifera*, is a social insect living in a colony or a hive comprising between 40 to 80 thousands individuals. Honeybees are recognized to have an essential role in nature for their efficient abilities as pollinators, considering that they pollinate more than one third of human crops, contributing to the cross-pollination of many fruits and forages (Watson & Stallins, 2016). In addition, they produce honey and other bee

products such as propolis, royal jelly and bee wax, which are used by humans for several purposes. Thus, they are very important for mankind survival. Nevertheless, like all living organisms, honeybees can be altered with diseases and pests.

One of the most important pathogen of honeybees is *Paenibacillus larvae,* a gram positive and spore-forming bacterium, responsible for American Foulbrood (AFB), a highly virulent, contagious and fatal disease of the honeybee larvae (Genersch, 2010). The control of AFB in many countries, like UK and New Zealand, consists in burning the hives suspected to have AFB-affected colonies, leading to several economical losses. In these countries the use of antibiotics is banned or regulated by law; however in other countries the use of antibiotics, such as oxytetracycline hydrochloride or sulfathiazole, is a common strategy for the prevention and treatment of affected colonies, although they are not effective against the infectious spores, hence they only suppress clinical symptoms and mask the disease. As a result of the continuous use of antibiotics, serious problems have been generated, like the appearance of resistant strains of bacteria and the chemical residues that can persist in honey affecting its quality and safety for human consumption (Lodesani & Costa, 2005). Consequently, there is a need to develop new and effective AFB treatments, less prone to generate bacterial resistance, leave residues in honey and that are safe for honeybees.

Non-toxic natural compounds are being studied as an alternative to the use of antibiotics for the control of AFB and to considerably diminish the appearance of resistance in *P. larvae*, as well as the amount of antibiotic residues in beehive products. Several studies have reported antagonistic properties of natural compounds against *P. larvae* such as propolis (Antúnez *et al.*, 2008; Bastos *et al.*, 2008), essential oils and other derivated compounds from different plant species (Albo *et al.*, 2003; Gende *et al.*, 2009; Flesar *et al.*, 2010; Sabaté *et al.*, 2012).

Achyrocline satureioides (Lam.) DC, popularly known as "Marcela del campo", belongs to the Asteraceae family and has been used extensively in popular medicine. In recent years, extracts from *A. satureioides* have been the target of numerous *in vitro* and *in vivo* experimental testings confirming the ethnopharmacological uses described for them (Barioni *et al.*, 2013). González & Marioli (2010) tested *A. satureioides* extracts against the growth of *P. larvae*. They found that the water remaining after hydro-distillation and the decoctions were good inhibitors of the bacterial growth. In a more recent study (González *et al.*, 2015), they showed that the compounds carrying the biological activity of *A. satureioides* against the growth of *P. larvae* were mainly contained in the hexanic extract obtained by liquid-liquid extraction from an aqueous-ethyl alcohol macerate of the aerial parts of the plant. However, the toxicity of the whole hexanic extract (HE), or its individual components, to the honeybee larvae or adult bees has not yet been tested.

Thus, the aims of this study were to evaluate the antimicrobial activity of the HE of *A. satureioides* on *P. larvae* and its oral and contact toxicity on larvae and adult honey bees, with a view to develop products based on this extract or its compounds for the control of AFB, in the future.

Material and methods

Plant material

Plant samples of *A. satureioides* were collected in Santa Monica, in the southern region of Córdoba Province (32° 05' 29" S, 64° 36' 54" W, Argentina) in the summer of 2016. A voucher specimen was conditioned and deposited at the Herbarium of the Faculty of Agronomy and Veterinary, National University of Río Cuarto, for verification of taxonomic identity as file herbarium 4658.

The aerial parts were dried at room temperature for 3 days; the hydro-alcoholic extracts were obtained by crushing the dried material and macerating it until depletion in a water: ethanol (1:1) mixture. The alcohol was then evaporated under low pressure. The HE was obtained by liquid-liquid extraction. Finally, the hexane was evaporated under low pressure (Gonzalez *et al.*, 2015).

Phytochemical tests

The preliminary phytochemical evaluation of HE was carried out following Sanabria´s (1983) methodology. For this, the presence of alkaloids, flavonoids, tannins, naphthoquinones and terpenoids were determined.

Bacterial strain

P. larvae 9 was previously isolated and identified in the Laboratory of General Microbiology (Dept. of Microbiology, National University of Río Cuarto, Argentina) from a hive located in the southern region of Río Cuarto (González & Marioli, 2010). The identification was phenotypically corroborated by biochemical characterization and confirmed by mass spectrometry (MALDI-TOF). The maintenance and storage of the strain was performed on MYPGP agar (Mueller-Hinton broth, yeast extract, potassium phosphate, glucose and sodium pyruvate) (González & Marioli, 2010).

Determination of minimum inhibitory concentration (MIC) of HE of *A. satureioides*

The antimicrobial activity of the HE against *P. larvae* was determined by the broth microdilution method

(Cugnata *et al.*, 2017). Colonies grown for 48 h in a MYPGP agar plate were suspended in sterile peptone water (peptone 0.1% (w/v) and sodium chloride 0.85 % (w/v)) until reaching a final optical density at 600 nm of 0.1, measured with a UV-VIS spectrophotometer Spectrum.

— Preparation of HE dilutions: 6 mg (0.006 g) of the HE was dissolved in 15% dimethylsulfoxide (DMSO), which was previously determined as the nontoxic solvent concentration for the growth of *P. larvae*. HE dilutions were prepared in a concentration range from 0.048 to 600 µg/mL.

— Minimum inhibitory concentration (MIC): 100 µL of brain-heart infusion was placed in a 96 well microplate, then 100 µL of HE was added to well-1 and serially dilutions were performed until well-11. Finally, 50 µL of the previously prepared inoculum was added to each well. The microplate was incubated in microaerophilia at 37 °C for 48 h, then 10 µL of 0.01% resazurin were added to each well. It was incubated again during 1½ h under the same conditions and MIC was visually determined by the changing in color from blue (oxidized) to pink (reduced by microbial metabolism). The MIC was established as the last dilution where resazurin was not reduced, that is, it remained blue (Mann & Markham, 1998). DMSO at 15% was used as a negative control.

Determination of the minimum bactericidal concentration (MBC)

To determine the MBC, 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. The MBC was considered as the last dilution without cell growth (Finelgold *et al.*, 1992).

Apis mellifera material

The larvae and adults of *A. mellifera* were obtained from the experimental apiary managed by the beekeeping research group of the University of Córdoba, Spain. The collection was carried out from February to June 2018 from hives free of signs or symptoms of disease. To obtain the sample of bees, insects were chosen from the outer and middle frames of the hive, in order to have a representative sample of the population of worker bees. The larvae were obtained from a central breeding pattern; larvae were free of symptoms of disease and with an age of 1 to 2 days of development.

Toxicity test of hexanic extract (HE) on adult bees

The toxicity of HE was assessed at the MIC concentration (0.4 µg/mL) and at 2 ½ times MIC (1 µg/mL). The toxicity of higher concentrations of the HE (1000 and 2000 µg/mL) was also assessed. Petri dishes (150 × 15 mm) were padded with filter paper on the inner bottom. Immediately after arriving from the field, the bees were slept with carbon dioxide gas for no more than 3 minutes, and then transferred to Petri dishes. Fifteen adult worker bees were placed in each Petri dish and 1 mL of each concentration was sprayed using a manual sprayer. They were fed with candy and water using a device placed inside each unit; fifteen bees in a Petri dish were sprayed with DMSO at 15% as the negative control and 0.07% deltamethrin (Deltavex Pour-On, Spain) was used as the positive death control. In addition, a survival check was performed where the bees were sprayed with distilled water. Petri plates were placed in incubators at 28 ºC ± 1°C and 60% relative humidity. The mortality of bees was assessed daily by visual inspection for 72 h (De Almeida *et al.*, 2015; Piana *et al.*, 2015). Four replicates for each experimental group were run.

Oral toxicity test of hexanic extract (HE) on larvae

First stage larvae were transferred from the hive combs to sterile 48 well culture plates. Twenty larvae were placed per plate, which were fed with a nutritious preparation containing 50% royal jelly, 12% D-glucose, 12% D-fructose, 2% yeast extract and 24% distilled water. The larvae were placed on 5 µL of food previously placed at the bottom of the wells, and viability was checked by movement. All process was carried out in a laminar flow cabin, and the tools and materials used were previously sterilized under UV light for 15 minutes (Schmehl *et al.*, 2016).

First, the toxicity of the solvent (DMSO) on the larvae was determined in order to know its non-toxic concentration. Therefore, five concentrations of DMSO were evaluated: 50%, 25%, 15%, 1.2% and 0.008%. The HE was used at the concentrations of 0.4 µg/mL and 1 µg/mL, equivalent to the MIC and 2½ times the MIC, respectively. In addition, it was decided to test higher concentrations of HE to determine at which concentration toxic effects on larvae were first observed; therefore the toxicity of HE at 48, 120, 480 and 960 µg/mL was assessed. Honeybee larvae were fed daily with the different diets (controls and HE treatments) for a period of 3 days.

DMSO (1.2%) and distilled water were used as negative control and 0.07% deltamethrin was used as a positive control of death. Each plate was incubated in dark at 34°C and 95% humidity throughout the experiment. Larval mortality was recorded by visual inspection during the period of 24, 48 and 72 h. The dead larvae were removed daily and the diet was replaced every day (Zhu *et al.*, 2014; De Almeida *et al.*, 2015). The test was performed four times.

Analysis of results

The data obtained were analyzed under a completely randomized experimental design with four replications per treatment using the IBM SPSS Statistics statistical program. To observe the variation in the survival rate of individuals in each treatment throughout the test, survival graphs were performed using the GraphPad Prism 6 program; in addition, the data were analyzed by the variance analysis method (ANOVA) of a factor.

Results

Phytochemical tests

The presence of several types of secondary metabolites was found in the HE of *A. satureioides* when qualitative identification tests were performed. Flavonoids, tannins, naphthoquinones and steroids were detected (Table 1).

Determination of the minimum inhibitory concentration (MIC) of HE of *A. satureioides*

The MIC of the HE against *P. larvae* using the broth microdilution technique was 0.4 µg/mL. No bactericidal effects were observed. The MIC and 2 ½ times the MIC were used for the toxicity tests on larvae and bees (0.4 and 1 µg/mL).

Toxicity test of the HE of *A. satureioides* by contact on adult bees

The toxicity of different concentrations of HE on bees was assessed. In Fig. 1, the survival rates of bees in the different treatments were observed at 24, 48 and 72 h of exposure. During the test, the percentages remained around 95% for all the treatments: negative control (99.6% ± 0.8%), DMSO (99.0% ± 1.0%) and treatment with EH at 0.4 (99.6% ± 0.8) and 1 µg/mL (97.9% ± 2.7), meaning that there was no acute toxicity in bees when they were in contact with HE at MIC and 2½ times MIC concentrations. In the positive control (deltamethrin), it was observed a lethal effect on bee survival at 24 h of exposure.

The HE was also tested at higher concentrations than MIC (1000 and 2000 µg/mL) to analyze what would happen with an HE overdose. At 1000 µg/mL the survival rate slightly decreased to 91.0% ± 3.8% at the end of the test, and at the concentration of 2000 µg/mL a greater toxic effect by contact in bees was observed since the survival rate decreased to 66.6% ± 6.6. The negative control showed statistically significant differences (p<0.05) with the positive control, EH at 1000 µg/mL and EH at 2000 µg/mL. Also, the treatment with EH at 2000 µg/mL showed statistically significant difference with the negative control, DMSO, positive control, EH at 0.4 µg/mL and 1 µg/mL. The NOAEL (No Observed Adverse Effect Level) in bees was 1 µg/mL (p=0.05).

Table 1. Preliminary phytochemical studies of hexanic extracts of *A. satureioides*

Phytochemical tests	Results
Börntrager´s test (naphthoquinones)	+++
Liberman Burchard´s test (phytoesteroles)	++
Mayer´s test (alkaloids)	-
Dragendorff´s test (alkaloids)	-
Ferric chloride (phenolic compounds)	+++
Shinoda´s test (flavonoids)	+++
Alcaline reactions test (flavonoids)	++

The results are shown as strong presence (+++), moderate presence (++), weak presence (+) and absence of compound (-).

Oral toxicity test of the HE of *A. satureioides* on larvae

In order to perform the larval oral toxicity test, it was necessary to determine the previous concentration of DMSO without any toxic effect on it. The maximum non-toxic concentration found was 1.2% in water, so the HE dilutions were prepared at this DMSO concentration, thus avoiding toxic effects produced by the solvent.

First, the toxicity of HE at concentrations of MIC and 2 ½ times MIC was assessed. The results showed that the survival rate of larvae remained close to 95% until 72 h (Fig. 2); therefore, HE at these concentrations had no toxic effect on larvae compared to positive control. The statistical analysis of covariance showed that the negative control only presented a statistically significant difference with the positive control (p<0.05). This indicated that the HE of *A. satureioides* did not cause toxic effects on larvae at concentrations of 0.4 and 1 µg/mL.

When higher HE concentrations were used, the results indicated that the survival rate of the larvae was similar for all the treatments: control, DMSO 1.2%, DMSO 0.6%, and HE (480 µg/mL, 120 µg/mL and 48 µg/mL) (Fig. 3). Therefore, the larvae were not affected by HE at these concentrations. On the other hand, the survival rate of the larvae began to decrease in the positive control and in the treatment with HE at 960 µg/mL, after 24 h of exposure till 72 h.

The statistical analysis showed significant differences in the negative control with respect to positive control and the treatment with HE at 960 µg/mL, indicating that HE of *A. satureioides* caused toxic effects on larvae at the

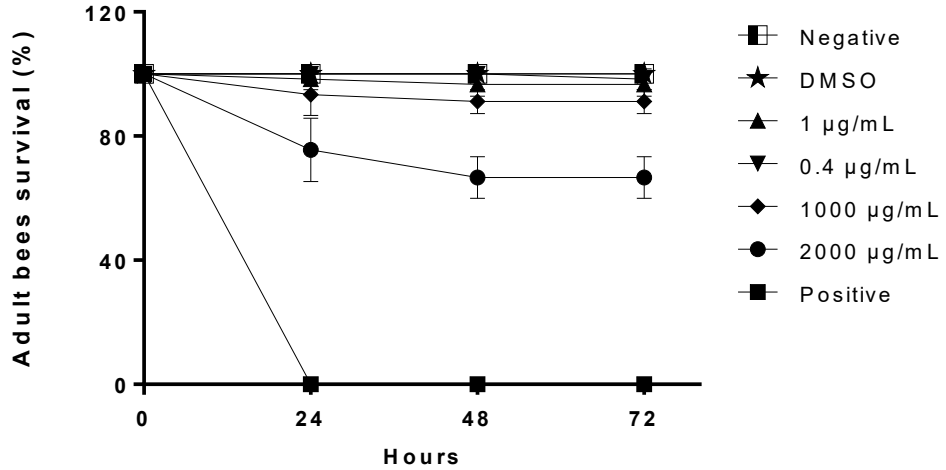

Figure 1. Toxicity tests of hexanic extract (HE) of *A. satureioides* on bee survival. Honey bees were sprayed with distilled water as negative control, deltamethrin diet (0.07%) as a positive control, DMSO as a solvent control, and different concentrations of HE (0.4, 1, 1000 and 2000 µg/mL).

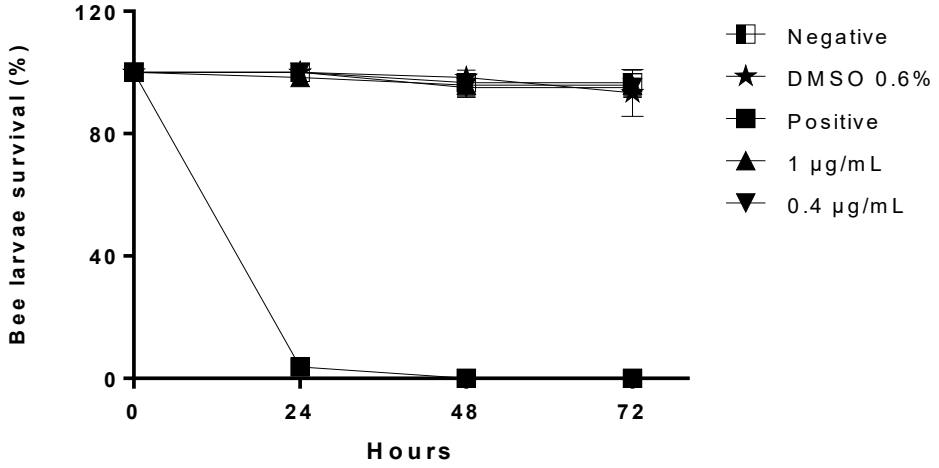

Figure 2. Toxicity tests of HE of *A. satureioides* on larval survival. Larvae were fed with a normal diet as a negative control; deltamethrin diet (0.07%) as a positive control; DMSO (0.6%) as a solvent control, and different concentrations of HE (0.4 and 1 µg/mL).

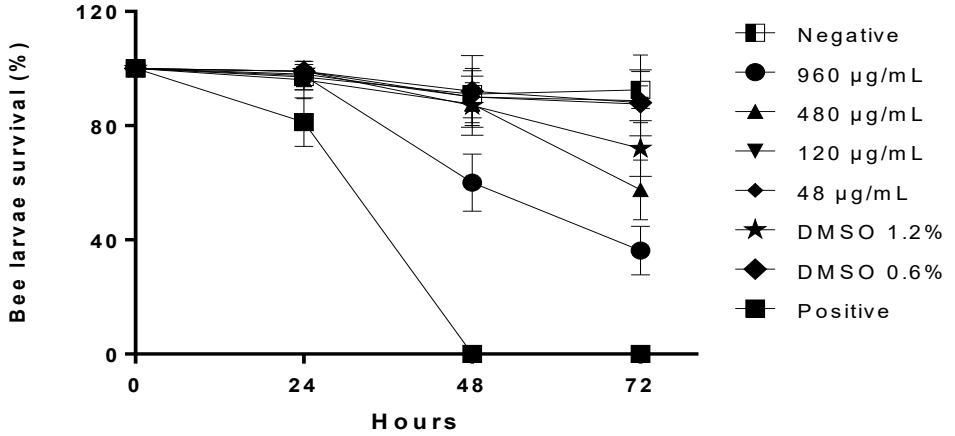

Figure 3. Toxicity tests of HE of *A. satureioides* on larval survival. Larvae were fed with a normal diet as a negative control deltamethrin diet (0.07%) as a positive control DMSO (1.2 and 0.6%) as a solvent control, and different concentrations of HE (48, 120, 480 and 960 µg/mL).

concentration of 960 µg/mL, since the survival rate at the end of the test was 37%.

Discussion

The Asteraceae family is particularly rich in phenolic compounds (Emerenciano *et al.*, 2001; Heiden *et al.*, 2007; Katinas *et al.*, 2007) and *A. satureioides*, a member of this family, has been reported as one of the species with higher flavonoid content like others such as *Ginkgo biloba* and *Epilobium parviflorum* (Arredondo *et al.*, 2004). In this work, a qualitative assay of the HE of this plant was analyzed and the results showed that flavonoids were present as it was reported by other authors who studied the chemical composition of *A. satureioides* extracts and considered them as an interesting source of flavonoids, regardless of whether the extraction was performed with polar or nonpolar solvents (De Souza *et al.*, 2007; Fachinetto *et al.*, 2007; Santin *et al.*, 2010). In addition, the presence of other phenolic compounds such as tannins was observed in the HE of this plant species, which coincided with a study carried out by Casero *et al.* (2015) where they isolated and identified from the HE of *A. satureioides* four new compounds of phenolic nature derived from phloroglucinol which also presented antimicrobial activity against gram positive and gram negative bacteria.

The test for naphthoquinones and/or anthraquinones indicated the possible presence of these compounds in the HE of *A. satureioides*, which has been reported in other species belonging to the Asteraceae family, such as *Senecio subpanduratus* (Gratti *et al.*, 2014). The steroid and/or triterpenoid test (CCF Libermann-Burchard) indicated the presence of these metabolites in the HE of *A. satureioides*, which is consistent with a study conducted by Gillij *et al.* (2008) where they found monoterpenoids and sesquiterpenes.

The antibacterial activity of the HE of *A. satureioides* was evaluated against the strain *P. larvae* 9 and the effective action of this extract was probed. The HE inhibited the growth of this pathogenic bacteria at concentrations lower than or equal to conventional antibiotics such as oxytetracycline, whose reported value was 0.5 to 1 µg/mL (Sabaté *et al.*, 2012). According to Alippi *et al.* (2007), *P. larvae* can be considered susceptible to antibiotics when MIC < 4 µg/mL, intermediate for MIC values between 4 and 8 µg/mL and resistant when MIC >16 µg/mL. Therefore, the low concentration of HE necessary to inhibit the growth of this microorganism indicated the high sensitivity of strain 9 to HE.

The biological activity of HE was compared with other investigations carried out with *A. satureioides*; *e.g.* González *et al.* (2015) reported mean MIC values for the HE of 60 µg/mL, with the lowest value of 16 µg/mL. Likewise, Sabaté *et al.* (2012) found a MIC range from 16 to 125 µg/mL for the HE of the same plant, resembling more to Gonzalez *et al.*´s (2015) study than to this study, where a better effect was observed with a minor MIC. These differences could be attributed to the place of collection of the plant material and/or the date of collection, since it is well known that the qualitative and quantitative composition and, therefore, the biological activities of a plant extract, could vary according to the different geographic, climatic factors, altitude, harvest time and growth stage. Consequently, the chemical composition is not homogeneous and different chemotypes could be originated (Ferraro *et al.*, 2008). This was evidenced in a research conducted by Cezarotto *et al.* (2011) with the essential oil of the aerial parts of *A. satureioides* where they found a significant variation in chemical composition and antibacterial activity when the plant was collected during the same year but at different times.

Comparing the results obtained in this study with other research works of the antibacterial activity against *P. larvae* of different extracts of other plant species, it could be observed that the HE of *A. satureioides* presented better activity than the methanolic-dichloromethane extracts of *Humulus lupulus* L (MIC=2 µg/mL), *Myrtus communnis* L. (MIC=2-8 µg/mL) and *Eucalyptus gunnii* Hook.f. (MIC=16-32 µg/mL) (Flesar *et al.*, 2010). The crude extracts of Brazilian species exhibited MIC values of 12.76 mg/mL for *Nasturtium officinale* and 30.51 mg/mL for *Copaifera officinalis* (Piana *et al.*, 2015); the species *Chromolaena odorata,* also belonging to the Asteraceae family, presented a MIC range between 16 and 64 µg/mL (Chaimanee *et al.*, 2017) and the ethyl acetate fraction of the species *Buddleja thyrsoides* Lam. showed MIC results of 1.68-3.36 mg/mL (Boligon *et al.*, 2013).

The antimicrobial activity of essential oils of different plant species against *P. larvae* was reported by other authors; *Cinnamomum zeylanicum* oil showed MIC values between 38 and 50 µg/mL, *Lavandula officinalis* between 350 and 400 µg/mL, *Mentha piperita* between 650 and 700 µg/mL, *Citrus paradise* MIC of 385 µg/mL, *Citrus sinensis* MIC of 840 µg/mL, *Citrus nobilis* MIC of 815 µg/mL, *Citrus limon* MIC of 764 µg/mL (Fuselli *et al.*, 2008), *Mentha* spp. MIC >600 µg/mL (Gende *et al.*, 2014) and *Pimenta dioica* (L.) Merr. MIC of 78 µg/mL (Ansari *et al.*, 2015). A MIC value of 142 µg/mL has also been reported when glycerol monolaurate nanocapsules were evaluated (Lopes *et al.*, 2016). Therefore, it could be observed that the HE of *A. satureioides* in this study demonstrated significantly lower MIC values than essential oils, which are usually described as the most biologically active plants compounds.

The better effect of HE on *P. larvae* could be due to a combination of the activities of the different compounds that constitute it, since it has been observed that single compounds did not show better activity than the whole extract (Flesar *et al.*, 2010; Boligon *et al.*, 2013; Piana

et al., 2015). These observations could be indicating the existence of synergistic interactions between the metabolites present in the HE of this plant that enhance the effect for the control of pathogenic bacteria. Joray *et al.* (2010), evaluating the interactions between the metabolites of *A. satureioides* in relation to their antibacterial activity, found the existence of a combined action between the metabolites of the plant.

The results obtained in this study in relation to antimicrobial activity demonstrate that *A. satureioides* HE had good antimicrobial properties, and taking into consideration that when performing the preliminary phytochemical analysis, metabolites such as flavonoids, tannins, steroids, triterpenoids and naphthoquinones were identified in abundance, it could be said that they were directly responsible for the activity on *P. larvae*, since several investigations frequently reported the antibacterial activity of these secondary metabolites (Flesar *et al.*, 2010; Piana *et al.*, 2015; Chaimanee *et al.*, 2017). It was described that for flavonoids, the bacteriostatic effect was attributed to the presence of phenolic hydroxyls in their structure, which easily penetrate through the bacterial cell membrane, combine and precipitate protoplasmic proteins, denaturing them and acting as protoplasmic poisons (Coloma, 2009).

In vitro confirmation of toxicity or side effects on the larvae and bees health is a prerequisite for assessing the practical usefulness of extracts with biological activity. For example, an interesting "biological" activity of *Sysygium aromaticum* against *Varroa destructor* has been reported, but the oil produced high bee mortality, even at low concentrations (Maggi *et al.*, 2010). As regards essential oils with good effects for the control of *P. larvae*, it has been reported that andiroba oil is toxic for bees (Santos *et al.*, 2012); similarly, nanoemulsions of the same oil presented a significant toxic effect for the larvae reporting a mortality of 26%. On the other hand, nanoemulsion with Copaiba oil did not present toxic effects on larvae or bees (De Almeida *et al.*, 2015). A research work conducted with glycerolmonolaurate showed high toxic effect on bees with a survival rate of 55%, but the nanoencapsulation of the same decreased the toxic effect, with a survival rate of 95% at the end of the test (Joray *et al.*, 2010). Chaimanee *et al.* (2017) found that the extract of *Piper ribesioides* and *Piper sarmentosum* were highly toxic to bees at 48 h; on the contrary, the crude extracts of *Allium sativum, Cymbopogon citratus, Senna alata* and *Thunbergia laurifolia* did not cause toxic effects in adult bees, even at high concentrations. A study of ethyl acetate, butanolic and dichloromethane fractions and the crude extract of the species *Buddleja thyrsoides* Lam. showed a clear mortality of bees only in the treatment with the dichloromethane fraction (Boligon *et al.*, 2013). The investigation of the toxic effect of the crude extract of *Calendula officinalis* and the ethyl acetate fraction of *Cariniana domestica* showed, respectively, 66.68% and 26.64% survival in bees at the end of the experiment. On the other hand, no toxic effects were found to be caused by the crude extract of *Nasturtium officinale* and the *n*-butanol fraction of *C. domestica* (Piana *et al.*, 2015), as well as the crude extract and fractions of *Scutia buxifolia* (Boligon *et al.*, 2013). Our results showed that the HE of *A. satureioides* did not cause the death of larvae and bees at the same concentration that exhibited antimicrobial activity against *P. larvae*; even at higher concentrations of MIC no bee/larvae mortality was observed until 72 h. Considering that HE did not have toxic effects on larvae or bees at concentrations of MIC and 2½ times MIC, the HE could be used at a concentration of 0.4 µg/mL for the control of the pathogen in hives. Furthermore, it could be used up to a concentration of 480 µg/mL, since such concentrations would not affect the health of *Apis mellifera.*

In summary, the results presented in this research demonstrated the antibacterial activity of the HE of *A. satureioides* on *P. larvae* at relatively low concentrations. The toxicity analysis of the HE of *A. satureioides* on larvae and adult bee of *Apis mellifera* showed no toxicity at concentrations of MIC (0.4 µg/mL) and 2 ½ times MIC (1 µg/mL) until 72 h of the test. Therefore, since HE is safe for larvae and bees at the concentration that inhibits the growth of the pathogen, it could be used in the future as a biopesticide for the control of AFB in hives.

References

Albo G, Henning C, Ringuelet J, Reynaldi F, De Giusti M, Alippi A, 2003. Evaluation of some essential oils for the control and prevention of American Foulbrood disease in honey bees. Apidologie 34: 417-437. https://doi.org/10.1051/apido:2003040

Alippi A, López A, Reynaldi F, Grasso D, Aguilar O, 2007. Evidence for plasmid-mediated tetracycline resistance in *Paenibacillus larvae*, the causal agent of American Foulbrood (AFB) disease in honeybees. Vet Microbiol 125: 290-303. https://doi.org/10.1016/j.vetmic.2007.05.018

Ansari M, Al-Ghamdi A, Usmani S, Al-Waili N, Nuru A, Sharma D, *et al.*, 2015. *In vitro* evaluation of the effects of some plant essential oils on *Paenibacillus larvae*, the causative agent of American foulbrood. Biotechnol Biotechnol Equip 30 (1): 49-55. https://doi.org/10.1080/13102818.2015.1086690

Antúnez K, Harriet J, Gende L, Maggi M, Eguaras M, Zunino P, 2008. Efficacy of natural propolis extract in the control of American Foulbrood. Vet Microbiol 131: 324-331. https://doi.org/10.1016/j.vetmic.2008.04.011

Arredondo MF, Blasina F, Echeverry C, Morquio A, Ferreira M, Abin-Carriquirv JA, *et al.*, 2004. Cytoprotection by *Achyrocline satureioides* (Lam) D.C. and

some of its main flavonoids against oxidative stress. J Ethnopharmacol 91: 13-20. https://doi.org/10.1016/j.jep.2003.11.012

Barioni E, Santin J, Daufenback I, Rodrigues S, Ferraz De Paula V, Wagner T, *et al.*, 2013. *Achyrocline satureioides* (Lam.) D.C. hydroalcoholic extract inhibits neutrophil functions related to innate host defense. Evid-Based Compl Altern Med 2013: art 787916. https://doi.org/10.1155/2013/787916

Bastos E, Simone M, Jorge D, Soares A, Spivak M, 2008. *In vitro* study of the antimicrobial activity of Brazilian propolis against *Paenibacillus larvae*. J Invertebr Pathol 97: 273-281. https://doi.org/10.1016/j.jip.2007.10.007

Boligon A, Faccim T, Zadra M, Piana M, Filipi C, Pedroso V, *et al.*, 2013. Antimicrobial activity of Scutia buxifolia against the honeybee pathogen *Paenibacillus larvae*. J Invertebr Pathol 112: 105-107. https://doi.org/10.1016/j.jip.2012.11.009

Casero C, Machín F, Méndez Álvarez S, Demo M, Ravelo A, Pérez Hernández N, *et al.*, 2015. Structure and antimicrobial activity of phloroglucinol derivatives from *Achyrocline satureioides*. J Nat Prod 78: 93-102. https://doi.org/10.1021/np500735f

Cezarotto V, Giacomelli S, Mack J, Barin J, Silva U, Linares C, 2011. Seasonal variation, chemical composition and antimicrobial activity of essential oil of *Achyrocline satureioides* (Lam.) D.C. Lat Am J Pharm 30: 1536-1541.

Chaimanee V, Thongtue U, Sornmai N, Songsri S, Pettis J, 2017. Antimicrobial activity of plant extracts against the honeybee pathogens, *Paenibacillus larvae* and Ascosphaera apis and their topical toxicity to *Apis mellifera* adults. J Appl Microbiol 123 (5): 1160-1167. https://doi.org/10.1111/jam.13579

Coloma J, 2009. Evaluación "*in vitro*" de la actividad antifúngica de los alcaloides del agua de cocción del proceso de desamargado del Chocho (Lupinus mutabilis Sweet). Tesis de grado bioquímico farmacéutico. Esc Sup Politecn Chimborazo, Fac Ciencias, Riobamba, Ecuador. 92 pp.

Cugnata N, Guaspari E, Pellegrini M, Fuselli S, Alonso Salces R, 2017. Optimal concentration of organic solvents to be used in the broth microdilution method to determine the antimicrobial activity of natural products against *Paenibacillus larvae*. J Apic Sci 61 (1): 37-53. https://doi.org/10.1515/jas-2017-0004

De Almeida Vaucher R, Giongo JL, Perger Bolzan L, Saldanha Correa M, Pedroso Fausto V, Filippi Dos Santos Alves C, *et al.*, 2015. Antimicrobial activity of nanostructured Amazonian oils against Paenibacillus species and their toxicity on larvae and adult worker bees. J As Pac Entomol 18: 205-210. https://doi.org/10.1016/j.aspen.2015.01.004

De Souza K, Bassani V, Schapoval E, 2007. Influence of excipients and technological process on anti-inflammatory activity of quercetin and *Achyrocline satureioides* (Lam.) D.C. extracts by oral route. Phytomedicine 14: 102-108. https://doi.org/10.1016/j.phymed.2005.10.007

Emerenciano VP, Militao JSL, Camposa CC, Romoffc P, Kapland MAC, Zambond M, *et al.*, 2001. Flavonoids as chemotaxonomic markers for Asteraceae. Biochem Syst Ecol 29: 947-957. https://doi.org/10.1016/S0305-1978(01)00033-3

Fachinetto J, Bagatini M, Durigon J, Da Silva A, Tedesco S, 2007. Efeito anti proliferativo das infusões de *Achyrocline satureioides* DC (Asteraceae) sobre o ciclo celular de Allium cepa. Rev Bras Farmacogn 17 (1): 49-54. https://doi.org/10.1590/S0102-695X2007000100011

Ferraro G, Anesini C, Ouviña A, Retta D, Filip R, Gattuso M, 2008. Total phenolic content and antioxidant activity of extracts of *Achyrocline satureioides* flowers from different zones in Argentina. Lat Am J Pharm 27 (4): 626-628.

Finelgold S, Baron E, Braily S, 1992. Diagnóstico microbiológico, aislamiento e identificación de microorganismos patógenos. Ed Médica Panamericana Bs As 36: 514-533.

Flesar J, Havlik J, Kloucek P, Rada V, Titera D, Vendar M, *et al.*, 2010. *In vitro* growth-inhibitory effect of plant-derived extracts and compounds against *Paenibacillus larvae* and their acute oral toxicity to adult honey bees. Vet Microbiol 145: 129-133. https://doi.org/10.1016/j.vetmic.2010.03.018

Fuselli S, García De La Rosa S, Eguaras M, Fritz R, 2008. Chemical composition and antimicrobial activity of Citrus essences on honeybee bacterial pathogen *Paenibacillus larvae*, the causal agent of American foulbrood. World J Microbiol Biotechnol 24: 2067-2072. https://doi.org/10.1007/s11274-008-9711-9

Gende L, Maggi M, Fritz R, Eguaras M, Bailac P, Ponzi M, 2009. Antimicrobial activity of Pimpinella anisum and Foeniculum vulgare essential oils against *Paenibacillus larvae*. J Essent Oil Res 21: 91-93. https://doi.org/10.1080/10412905.2009.9700120

Gende L, Mendiara S, Fernández N, Van Baren C, Di Leo Lira A, Bandoni A, *et al.*, 2014. 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. Bull Insectol 67 (1): 13-20.

Genersch E, 2010. American Foulbrood in honeybees and its causative agent, *Paenibacillus larvae*. J Invertebr Pathol 103: 10-19. https://doi.org/10.1016/j.jip.2009.06.015

Gillij YG, Gleiser RM, Zygadlo, JA, 2008. Mosquito repellent activity of essential oils of aromatic plants growing in Argentina. Bioresour Technol 99: 2507-2515. https://doi.org/10.1016/j.biortech.2007.04.066

González M, Marioli J, 2010. Antibacterial activity of water extracts and essential oils of various aromatic plants

against *Paenibacillus larvae*, the causative agent of American Foulbrood. J Invertebr Pathol 104: 209-213. https://doi.org/10.1016/j.jip.2010.04.005

González MJ, Beoletto VG, Agnese AM, Audisio MC, Marioli M, 2015. Purification of substances from *Achyrocline satureioides* with inhibitory activity against *Paenibacillus larvae*, the causal agent of American Foulbrood in honeybees larvae. Appl Biochem Biotechnol 175: 3349-3359. https://doi.org/10.1007/s12010-015-1506-5

Gratti A, Feijóo M, Barrientos E, Peneff R, Laztra E, 2014. Caracteres anatómicos, morfofuncionales y químicos foliares de Senecio subpanduratus (Asteraceae). Dominguezia 30 (1): 41-46. http://www.dominguezia.org/volumen/articulos/3015.pdf

Heiden G, Barbieri RL, Wasun RA, Scur L, Sartori M, 2007. A familia Asteraceae em Sao Mateus do Sul, Parana. Rev Bras Biocienc 5: 249-251.

Joray M, Rollán M, Ruiz G, Palacios S, Carpinella M, 2010. Antibacterial activity of extracts from plants of central Argentina-isolation of an active principle from *Achyrocline satureioides*. J Med Plant Nat Prod Res 77 (1): 95-100. https://doi.org/10.1055/s-0030-1250133

Katinas L, Gutiérrez DG, Grossi MA, Crisci JV, 2007. Panorama de la familia Asteaceae (= Compositae) en la República Argentina. Bol Soc Arg Bot 42 (1-2): 113-129.

Lodesani M, Costa M, 2005. Limits of chemotherapy in beekeeping development of resistance and the problem of residues. Bee World 86 (4): 102-109. https://doi.org/10.1080/0005772X.2005.11417324

Lopes L, Santos C, De Almeida Vaucher R, Gende L, Raffin R, 2016. Evaluation of antimicrobial activity of glycerol monolaurate nanocapsules against American foulbrood disease agent and toxicity on bees. Microb Pathog 97: 183-188. https://doi.org/10.1016/j.micpath.2016.05.014

Maggi M, Ruffinengo S, Gende L, Sarlo G, Bailac P, Ponzi M, *et al.*, 2010. Laboratory evaluations of Syzygium aromaticum (L.) Merr: et Perry essential oil against Varroa destructor. J Essent Oil Res 22: 119-122. https://doi.org/10.1080/10412905.2010.9700278

Mann CM, Markham JL, 1998. A new method for determining the minimum inhibitory concentration of essential oils. J Appl Microbiol 84: 538-544. https://doi.org/10.1046/j.1365-2672.1998.00379.x

Piana M, Brum T, Boligon A, Alves C, Freitas R, Nunes L, *et al.*, 2015. *In vitro* growth-inhibitory effect of Brazilian plants extracts against *Paenibacillus larvae* and toxicity in bees. An Acad Bras Cienc 87 (2): 1041-1047. https://doi.org/10.1590/0001-3765201520140282

Sabaté D, González M, Porrini M, Eguaras M, Audisio M, Marioli J, 2012. Synergistic effect of surfactin from Bacillus subtilis C4 and *Achyrocline satureioides* extracts on the viability of *Paenibacillus larvae*. World J Microbiol Biotechnol 28: 1415-1422. https://doi.org/10.1007/s11274-011-0941-x

Sanabria A, 1983. Preliminary phytochemical analysis. Methodology and its application in the evaluation of 40 plants of the Compositae family. Thesis, Nat Univ of Colombia, Fac Pharmaceut Chem, Santa Fe de Bogotá.

Santin J, Lemos M, Klein L, Niero R, De Andrade S, 2010. Antiulcer effects of Achyrocline satureoides (Lam.) DC (Asteraceae) (Marcela), a folk medicine plant, in different experimental models. J Ethnopharmacol 130: 334-339. https://doi.org/10.1016/j.jep.2010.05.014

Santos R, Filippi Dos Santos Alves C, Schneider T, Quintana Soares Lopes L, Aurich C, Giongo JL, *et al.*, 2012. Antimicrobial activity of Amazonian oils against Paenibacillus species. J Invertebr Pathol 109: 265-268. https://doi.org/10.1016/j.jip.2011.12.002

Schmehl D, Tomé H, Mortensen A, Ferreira G, Ellis J, 2016. Protocol for the *in vitro* rearing of honey bee (*Apis mellifera* L.) workers. J Apic Res 55 (2): 113-129. https://doi.org/10.1080/00218839.2016.1203530

Watson K, Stallins JA, 2016. Honey bees and colony collapse disorder: a pluralistic reframing. Geogr Comp 10: 222-236. https://doi.org/10.1111/gec3.12266

Zhu W, Schmehl D, Mullin C, Frazier J, 2014. Four common pesticides, their mixtures and a formulation solvent in the hive environment have high oral toxicity to honey bee larvae. PloS one 9 (1): e77547. https://doi.org/10.1371/journal.pone.0077547