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In vitro inhibition of *Paenibacillus larvae* by different extracts and pure compounds from *Flourensia* spp

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ABSTRACT

Paenibacillus larvae, a sporulating Gram-positive bacterium, is the etiological agent of American foulbrood disease in *Apis mellifera* L. Plant extracts could be a natural alternative to control this pathology. The current study assessed the anti-*P. larvae* effect of extracts and pure principal products from the *Flourensia* genus: *F. riparia*, *F. fiebrigii* and *F. tortuosa*. Their inhibitory effect was assayed against different *P. larvae* strains according to the disk diffusion technique and subsequently, the minimal inhibitory concentrations (MIC) of extracts by the agar dilution method was determined. Furthermore, toxicity of the most effective extracts against *P. larvae* was tested in bees. All extracts inhibited growth of the different *P. larvae* strains assayed. However, the magnitude of the antagonistic effect depended on the chemical nature of the extract and the *P. larvae* strain. Chloroform extracts (CE) and ethyl ether extracts (ETE) from *F. riparia* and ETE from *F. fiebrigii* were most active against *P. larvae* Azul, the most sensitive indicator strain with MIC values of 250 ppm (CE) and 2000 ppm (ETE) for *F. riparia*, and 2500 ppm (ETE) for *F. fiebrigii*. Hexane extracts from the three species did not present any significant inhibitory effect. These results would indicate that one or some of the more polar compounds would cause inhibition of this pathogen. Toxicity assays demonstrated that even the highest concentrations assayed (125,000 ppm) did not show lethal effects on exposed bees during *in vitro* conditions.

1. Introduction

Honey bees (*Apis mellifera*) are not only important for the honey they produce, but are also vital as pollinators of agricultural and horticultural crops. In recent years, serious losses of bee colonies and a reduction in bee populations have been reported all over the world (Bacandritsos et al., 2010; Watanabe, 2008; vanEngelsdorp et al., 2007, 2010). Different causes can be attributed to this phenomenon: bacterial beehive infections, virus and parasite attacks, and pesticides used to control plant or crop diseases are among the most important causes.

In particular, honeybee open brood is susceptible to *Paenibacillus larvae*, the ethiological agent of American foulbrood (AFB), potentially a lethal disease for the colony (Genersch, 2010). Young larvae are infected by ingesting spores within the larval food provided by adult worker bees (nurses), and diseased larvae will die from infection when sporulation occurs. They will then transmit spores throughout the hive. This disease can kill the colony as spores become widespread unless colonies demonstrate a resistance either physiological or behavioral (Bastos et al., 2008). However, while some honeybees possess inherent mechanisms of resistance, high levels of spores (around 3000 *P. larvae* spores per adult) (Gende et al., 2011) can produce clinical infections that inevitably leads to the demise of colonies. AFB is considered to be the most serious illness plaguing apiculture today and has a nearly cosmopolitan distribution. Also, it is difficult for beekeepers to manage or control because the pathogen produces environmentally stable spores which are virulent as well as resistant to heat, to desiccation, and to chemical disinfectants (Williams, 2000; Thompson and Maus, 2007).

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Burning the AFB affected combs and/or hive is the most widely used solution in the European Community countries. However, a common strategy employed in some other countries for the prevention and treatment of AFB affected colonies is the use of antibiotics (Antúnez et al., 2008). Sulfathiazole was used in the 1940s; but the persistence of residues in harvested honey resulted in its discontinuation of its use in beekeeping. Oxytetracycline hydrochloride has been used for decades to control AFB. There is no Maximum Residue Limit of tetracyclines established for honey according to the European Community regulations (Mutinelli, 2003; Reybroeck et al., 2012). Nevertheless, tetracycline resistant strains were identified in USA, Canada and Argentina (Reynaldi et al., 2008) raising many concerns among the scientific community because of the possibility of their worldwide spreading. Other antibiotics, such as tylosin and lincomycin, have also been successfully used to control AFB, but concerns still remain regarding the emergence of resistant strains or the residues that they may leave in hive products.

Plants, herbs, spices and their derived extracts, essential oils and/or pure compounds can reduce or inhibit the growth of bacteria, yeast and molds (Ríos and Recio, 2005; Bakkali et al., 2008; Mahady et al., 2008). Some natural alternatives to control *P. larvae* have been evaluated such as essential oils (Fuselli et al., 2006; Gende et al., 2008), plant extracts (González and Marioli, 2010) as well as biocontrol agents from antagonistic aerobic spore-forming bacteria isolated from honey and other apiarian sources (Alippi and Reynaldi, 2006; Sabaté et al., 2009, 2012). Also, different natural alternatives to treat AFB in apiary trails such as essential oils (Albo et al., 2003), propolis extracts (Antúnez et al., 2008), cinnamon essential oil (Gende et al., 2009a) were assessed.

As our study is based on the phytochemical analysis of plants belonging to the *Flourensia* genus, chloroform and ethyl ether extracts from *Flourensia riparia*, and ethyl ether extract from *Fluorensia fiebrigii* (Uriburu et al., 2004, 2005, 2007) were analyzed in order to obtain data related to the chemical composition of this genus that would support the classification of *Flourensia* in the subtribe Ecliptinae. *Flourensia* DC. (Asteraceae) genus exists exclusively in America and involves 32 different species; it grows in a semiarid weather and its importance is related to its narrow distribution (Delbón et al., 2012). Chemical composition and biological activity studies of species growing in México, Chile and Argentina, have reported that they can be used as potential bactericidal, fungistatic and insecticide agents (Delbón et al., 2012, Castillo et al., 2010, Mendez et al., 2012; Silva et al., 2012). The aim of the present work was to investigate the antimicrobial activity of extracts and some pure compounds from three species, *F. riparia*, *F. fiebrigii* and *F. tortuosa* against *P. larvae*. No information about the antimicrobial properties of these species has been published yet.

2. Materials and methods

2.1. P. larvae strains and growth conditions

P. larvae strains (Azul, 1 and III) were kindly provided by Dr. Terzolo and Eng. Borracci from INTA Balcarce, Argentina, and strain 35A (Río Negro) was purchased from CIDEFI. All strains were activated on MYPGP (1.5% yeast extract, 1% Mueller–Hinton broth (Britania), 0.2% glucose, 0.3% K_2HPO_4 and 0.1% sodium pyruvate; pH 7.0; 1.5% agar; (Dingman and Stahly, 1983) at 37 °C for 72 h without any special atmosphere.

2.2. Plant species

Three different species of *Fluorensia* spp. were analyzed. *F. riparia* was collected in December 1995, in El Maray (Salta, Argentina) and identified by Eng. Novara. A voucher specimen (N° 10765) was deposited in the Museum of the Facultad de Ciencias Naturales, Universidad Nacional de Salta (Salta, Argentina). *F. fiebrigii* was collected in March 1999, Parque Nacional Los Cardones (Salta, Argentina). A voucher specimen (N° 11244) was deposited at the Museum of the Facultad de Ciencias Naturales, Universidad Nacional de Salta (Salta, Argentina). *F. tortuosa was* collected in March 2009, Camping La Aguadita, Andalgalá (Catamarca, Argentina) and identified by Dr. Ariza Espinar. A voucher specimen (Delbón 2) is on deposit at the Museo Botánico (Córdoba, Argentina).

Leaves, fine stems, flowers and/or fruits of the different plant species were collected, cleaned and dried. Dried specimens were stored until they were used for extraction.

2.2.1. Flourensia spp. samples

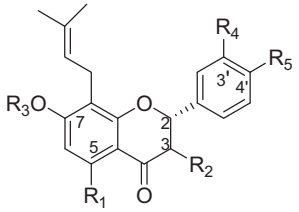
2.2.1.1. Extracts. Dried and powdered aerial parts of *F. riparia* (1.2 kg) were treated with methanol (MeOH) according Uriburu et al. (2004). Then, the solvent was evaporated, and the residue was partitioned between hexane–MeOH–H_2O 10:3:1. The organic phase was separated and the polar phase was extracted three times with hexane, which was joined with the first organic phase to afford the hexane extract (6 g). After removal of the MeOH under reduced pressure, the aqueous layer was extracted with chloroform to obtain the chloroform extract (4 g).

On the other hand, aerial parts of *F. riparia* (876 g) were treated with ethanol (EtOH). The residue, after evaporation of the solvent was suspended in MeOH–H_2O 9:1, and extracted with hexane. The aqueous-alcoholic phase was concentrated and extracted with ethyl ether, to obtain the ethyl ether extract (3.2 g), according to Uriburu et al. (2005).

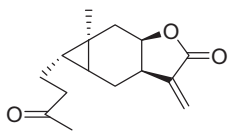
The dried aerial parts of *F. fiebrigii* (1.6 kg) were extracted with EtOH–H_2O (98:2). The resulting extract was concentrated under reduced pressure and the residue was suspended in MeOH–H_2O 9:1. After 24 h, the resulting suspension was filtered. The MeOH–H_2O solution was washed with hexane to obtain the hexane extract (7 g), and the MeOH was evaporated *in vacuo*. The remaining aq. solution was extracted with ethyl ether to obtain the ethyl ether extract (3 g) (see Uriburu et al., 2007).

Finally, aerial parts of *F. tortuosa* (632 g) were treated with EtOH at room temperature. The residue, after evaporation of the solvent was suspended in MeOH:H_2O 9:1, and extracted with hexane, to afford the hexane extract (7 g). The aqueous-alcoholic phase was concentrated and extracted with chloroform, to obtain the chloroform extract (4 g).

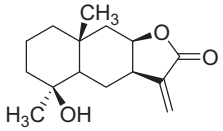
2.2.1.2. Pure compounds. Pure compounds were isolated from *F. riparia* and *F. fiebrigii* and identified by spectra dates according to Uriburu et al. (2004, 2007). The structures of the pure compounds assayed are given in Fig. 1. It can be observed that compounds of different biosynthetic origin have been found like sesquiterpene lactones (carabrone and isoalantolactone), benzofurans (6-methoxytremetone), coumarins (scopoletin), flavanones (8-prenylnaringenin, 8-prenyleryodictiol, 5,3′-dihydroxyisobavachin-7-*O*-methyl ether, exiguaflavanone K and (2S)-8-(3″-methylbut-2″-enyl)-7,3′,4′-trihydroxyflavanone), and dihydroflavonols (glepidotin B, 8-prenyldihydroisorhamnetin and scariosin). Most of them were isolated from the chloroform extract from *F. riparia* (Uriburu et al., 2004), whereas isolantolactone and 6-methoxytremetone were also observed in the hexane extract. The *F. fiebrigii* ethyl ether extract revealed 6-methoxy-tremetone, 8-prenyleryodictiol, 5,3′-dihydroxyisobavachin-7-*O*-methyl ether and (2S)-8-(3″-methylbut-2″-enyl)-7,3′,4′-trihydroxyflavanone (Uriburu et al., 2007). The latter compound was only observed in the ethyl ether extract of this species.



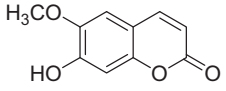
	R_1	R_2	R_3	R_4	R_5
1. 8-prenylnaringenin	OH	H	H	H	OH
2. 8-prenyleryodictiol	OH	H	H	OH	OH
3. 5,3´-dihydroxyisobavachin -7-*O*-methyl eter	OH	H	CH_3	OH	OH
4. exiguaflavanone K	OH	H	H	OCH_3	OH
5. (2S)-8-(3"-methylbut-2"-enyl)-7,3',4'-trihydroxyflavanone	H	H	H	OH	OH
6. glepidotin B	OH	OH	H	H	H
7. 8-prenyldihydroisorhamnetin	OH	OH	H	OCH_3	OH
8. scariosin	OH	OH	CH_3	OCH_3	OH



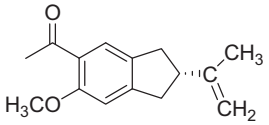
9. carabrone



10. isoalantolactone



11. scopoletin



12. 6-methoxytremetone

Fig. 1. Structures of compounds isolated from *Flourensia* spp.

2.3. Anti- P. larvae activity of the different extracts and/or pure compounds

Vegetative cells of each *P. larvae* strain grown in MYPGP agar for 72 h of incubation at 37 °C were suspended in MYPGP broth. An aliquot of 0.30 mL of this solution was poured into a Petri dish and 10 mL of melting MYPGP agar were added in order to obtain a lawn of the bacterium. Chloroform, ethyl ether extracts and pure compounds were dissolved in methanol while hexane extracts were dissolved in chloroform. The final concentration of the studied samples was known (extract concentration range from 50,000 to 100 ppm, pure compounds concentration range from 2500 to 100 ppm), and were assayed by the disk diffusion technique. All plates were incubated at 37 °C during 24–48 h. After the incubation period, the zones of growth inhibition of the bacterium were measured with a caliper. The experiments were carried out in duplicate. Disks used as negative controls contained methanol or chloroform.

2.4. Minimal inhibitory concentrations (MICs) of the different extracts obtained from Flourensia spp. against P. larvae

The minimal inhibitory concentration of each extract or pure compound was determined according to the agar dilution technique (Sabaté et al., 2012). Briefly, different dilutions were made in MYPGP agar, which were left to solidify in Petri dishes. *P. larvae* cell suspensions were prepared as mentioned before, and 10 μl of each suspension were seeded onto MYPGP agar, supplemented with the different extracts and compound concentrations (concentration range from 2500 to 100 ppm). Inoculated plates were examined for growth after 24–48 h of incubation at 37 °C. MYPGP agar without any compounds was used as the *P. larvae* growth control. The antimicrobial effect of the different solvents was also analyzed against each selected *P. larvae* strain. All experiments were carried out in duplicate.

2.5. Insecticidal activity bioassays

The complete exposure method proposed by Ruffinengo et al. (2005) was implemented to analyze the effect of the extracts obtained from *F. riparia* and *F. fiebrigii* ether ethylic extracts on *A. mellifera* L. The treatments were assayed in unmodified Petri dishes (90 × 20 mm). Each viscous substance was dissolved in 96% ethanol in order to obtain the solutions of the botanical extracts. The concentrations used in the assay were 2000; 4000; 8000; 16,000; 31,250; 62,500 and 125,000 ppm. A milliliter of each solution was applied to the bottom of the Petri dish with the aim to cover all the inner surface of the capsule and by this way ensure the contact of the extracts with the bees. One hour later, 5 (five) newly-emerged adult worker bees (between 0 and 3 days) were placed inside each dish. Before this action, bees remained with no food for 4 h. A candy (mixture of sugar and water) was located inside as resource food for bees. Complete exposure was tested for each extract concentration, and solvent (alcohol 96%) was included as control. Before and during experiments, bees were placed in an incubator at 33–34 °C and 60% RH. Five replicates were done for each treatment. Bee percentage mortality was controlled at 24, 48 and 72 h.

A chi square test was carried out to evaluate treatment toxic effects on bees.

3. Results

3.1. Antimicrobial effects of the different extracts

The analysis of antimicrobial activity by halo inhibition of the extracts from the different *Flourensia* tested against *P. larvae* strains, revealed that all of them, to a different degree, showed an inhibitory effect (Table 1). The hexane and chloroform extracts from *F. tortuosa* mainly inhibited *P. larvae* 1, but only at elevated concentrations (48,000 ppm). *F. riparia* extracts were more active, especially chloroform and ethyl ether extracts. *F. fiebrigii* had a remarkable effect on *P. larvae* Azul. No antagonistic effects were detected with the pure solvents used as negative controls.

3.2. MIC values of selected plant extracts

F. riparia chloroform and ethyl ether extracts, and also *F. fiebrigii* ethyl ether extract, were chosen to determine their MIC against *P. larvae* because they showed the highest anti-*P. larvae* effect. The *F. riparia* chloroform extract demonstrated the lowest MIC value against *P. larvae* Azul and *P. larvae* 1 (for both 250 ppm), contrary to the MIC values of the ethyl ether extract from *F. fiebrigii*: 1250 ppm (*P. larvae* Azul), 2500 ppm (*P. larvae* 1) and 5000 (*P. larvae* III) (Table 2).

Table 1
Anti-*P. larvae* effects of different extracts (concentration range 50,000–40,000 ppm) recovered from *Flourensia* species.

Fluorensia	Extract	*P. larvae* 35A	*P. larvae* Azul	*P. larvae* 1	*P. larvae* III
F. tortuosa	Hexane	–	+	++	++
	Chloroform	–	+	++	++
F. riparia	Hexane	–	+	–	++
	Ethyl ether	–	++	++	++
	Chloroform	++	++	++	++
F. fiebrigii	Hexane	–	++	+	++
	Ethyl ether	–	++	+	+

++: Inhibition zone 1.0–2.6 mm.
+: Inhibition zone 0.5–0.9 mm.
–: No inhibition.

Table 2
Minimal inhibitory concentration (MIC, in ppm) of selected extracts of *Fluorensia* spp. against *P. larvae* strains.

Fluorensia	Extract	*P. larvae* 35A	*P. larvae* Azul	*P. larvae* 1	*P. larvae* III
riparia	Ethyl ether	2000	250	500	2000
	Chloroform	2500	250	250	2000
fiebrigii	Ethyl ether	NT	1250	2500	5000

NT: Not tested.

3.3. Pure compounds

The pure compounds isolated from the chloroform extract of *F. riparia* showed variable results in their anti-*P. larvae* ability. Exiguaflavanone K and 8-prenyldihydroisorhamnetin were active against *P. larvae* Azul with a very low MIC (625 ppm and 500 ppm, respectively) in regards to the pure compounds and extracts studied. The same result was determined for *P. larvae* 35A. However, carabrone (2000 ppm), isoalantolactone (1250 ppm), scariosin (2000 ppm), glepidotin B (2500 ppm) 8-prenylnaringenin (1250 ppm) and scopoletin (2500 ppm) were not active at all.

On the other hand, (2S)-8-(3″-methylbut-2″-enyl)-7,3′,4′-trihydroxyflavanone from *F. fiebrigii* ethyl ether extract, was the most active pure compound with a MIC equal to 500 ppm against the two *P. larvae* strains studied. Results showed 8-prenyleryodictiol had an important effect on *P. larvae* Azul (MIC 500 ppm) but moderate on *P. larvae* 35A (1000 ppm).

Finally, 5,3′-dihydroxyisobavachin-7-*O*-methyl eter and 6-methoxytremetone, isolate from both species *Fluorensia*, *F. riparia* and *F. fiebrigii* showed the weakest *P. larvae* inhibition (Table 3).

Table 3
Minimal inhibitory concentrations (MIC) of different pure compounds, from *Fluorensia* spp. extracts, against *P. larvae*.

Fluorensia	Pure compound	MIC (ppm)	
		P. larvae 35A	*P. larvae Azul*
F. riparia	Carabrone	NI	NI
	Isoalantolactone	NI	NI
	Scopoletin	NI	NI
	8-Prenylnaringenin	NI	NI
	Exiguaflavanone K	625	625
	Glepidotin B	NI	NI
	8-Prenyldihydroisorhamnetin	500	500
	Scariosin	NI	NI
F. fiebrigii	8-Prenyleryodictiol	1000	500
	(2S)-8-(3″-Methylbut-2″-enyl)-7,3′,4′-trihydroxyflavanone	500	500
F. riparia and *F. fiebrigii*	6-Methoxytremetone	NI	NI
	5,3′-Dihydroxyisobavachin-7-*O*-methyl eter	NI	NI

NI: No inhibition with values < 1000 ppm.

3.4. Toxicity tests on *Apis mellifera* L. of two selected extracts

For both vegetal extracts, no mortality of bees was observed during treatment even at the highest concentrations and after 72 h of treatment. Table 4 depicts bee mortality (expressed in percentage) for *F. riparia* and *F. fiebrigii* ether ethylic extracts. No statistical differences were detected among treatments and time for bee toxicity (Chi Square test, $p > 0.05$).

4. Discussion

In the field of honeybee health, there is an interesting area for future research involving alternative natural substances to control the AFB or another bee illness. Ideally, the reduction or elimination of *P. larvae* in *Apis mellifera* colonies would involve treatments with acceptable antimicrobial activity with no side effects on honeybees and minimize residues in honey and wax constituting a viable alternative to reduce antimicrobial resistance. In this way, an alternative for new drug discovery or development can be natural products, extracts, essential oils or isolated from new plants or from known medicinal plants (Gende et al., 2009a; Santos et al., 2012).

Many researchers have evaluated the biological activity of essential oils against bee pathologies, and have obtained and reported variable results. For example, Fuselli et al. (2006) evaluated the anti-*P. larvae* spectrum of five essential oils from Argentinian wild plants and they informed high MIC, from 200 to 1100 mg/L, according to the essential oil tested. Conversely, Gende et al. (2009b) proved the anti-*P. larvae* effects of *Pimpinella anisum* and *Foeniculum vulgare* essential oils with positive results and relatively high MICs (300 μg/mL and 250 μg/mL, respectively). But, when they analyzed the essential oils from *Cinnamomum zeylanicum*, a higher inhibitory effect was achieved against *P. larvae* (MIC between 25 and 100 μg/mL) (Gende et al., 2008).

It should be mentioned that essential oils are unique, complex mixtures of different phytochemicals (plant secondary metabolites), generally comprising two quite different groups with diverse biosynthetic origins. The main group comprises terpenes and terpenoids, whereas the other group consists of aromatic and aliphatic compounds of low molecular weight. They contain two or three main compounds (20–70%) compared to others that are scarcer, and the principal compounds generally determine the biological properties (Bakkali et al., 2008).

Propolis is another natural source that has been assayed for active anti-*P. larvae* metabolites (Bastos et al., 2008; Antúnez et al., 2008). The chemical composition of propolis is highly complex, composed of: 30% of wax, 50% of plant resins and balms, 10% of essential and aromatic oils and 5% of pollen and other substances (the percentages correspond to mass fractions); the exact composition depends on the plant and the local flora, which is highly variable. A comparative study of the chemical composition of propolis-producing plants that grow in Brazil (*Baccharis dracunculifolia* DC., *Eucalyptus citriodora* Hook and *Araucaria angustifolia*

Table 4
Bee means mortality (%) after 24, 48 and 72 h for different concentrations expressed in ppm of *F. riparia* and *F. fiebrigii* ethyl ether extracts.

Extract	Time (h)	Concentrations (ppm)							
		0	2000	4000	8000	16,000	31,250	62,500	125,000
F. riparia	24	0	0	0	0	0	4	4	12
	48	0	0	4	0	0	4	12	12
	72	4	0	4	8	0	4	12	24
F. fiebrigii	24	0	4	0	0	0	4	4	12
	48	0	4	0	0	0	4	0	12
	72	4	4	4	8	0	4	12	12

Bert.) with propolis samples isolated from each of the three plants assayed revealed that the chemical composition and the concentration of secondary metabolites in leaf extracts and propolis samples was similar (Sforcin, 2007).

In this work, the antimicrobial activity of different extracts from three scarcely studied species: *Flourensia riparia*, *F. fiebrigii* and *F. tortuosa*, were evaluated against *P. larvae*. The phytochemical study of these species revealed secondary metabolites such as prenylated flavonoids, sesquiterpene lactones and *p*-hydroxyacetophenone derivatives (Uriburu et al., 2004, 2007). Consequently, from a biosynthetic origin of secondary metabolites point of view, it could be said that, the chemical composition of these extracts is more similar to propolis extracts than to essential oils.

Among the pure compounds assayed against *P. larvae*, it can be seen that the flavonoids (**1**–**8**, Fig. 1) had inhibitory effect when compared with the remaining compounds that were structurally different. Flavanones have been reported to be strong inhibitors of certain Gram-positive bacteria, but only when they contained 5,7-dihydroxy, 5,7,4′-trihydroxy or 2′-hydroxy and 2′,4′- dihydroxy groups (Tsuchiya et al., 1996; Alcaráz et al., 2000). However, the current study determined that the most active flavonoid assayed was (2S)-8-(3″-methylbut-2″-enyl)-7,3′,4′-trihydroxyflavanone (**5**), which is a 5-deoxyflavonoid, whereas the other compounds with inhibitory effect (**2, 3, 4, 7**) contained one or more of the required groups in their structures.

Flavanone seems to have low toxicity because the related flavonoids are widely distributed in edible plants and beverages, and have been used in medicine (Tsuchiya et al., 1996). However, *in vivo* confirmation of their toxicity or side-effects on bee health is a pre-requisite in evaluating the practical usefulness of these compounds. For example, Maggi et al. (2010), working with antagonistic compounds to fight against the ectoparasitic mite *Varroa destructor*, reported interesting acaricide activity of *Sysygium aromaticum*. This oil also produced high bee mortality, even at low concentrations. Thus, in this research, the ethyl ether extracts of both, *F. riparia* and *F. fiebrigii* were assessed on *A. mellifera* to verify the possible toxic action. They showed similar results to the control group, causing no toxic effects or the bee death. These results are remarkable because others compounds, such as Amazonian oils have shown relevant anti-*P. larvae* effects; but, when their bee-toxicity was assayed, it was observed that the Andiroba oil was dangerous for bee health (Santos et al., 2012).

The extracts and pure compounds from *Flourensia* assayed in the current study could be potential anti-*P. larvae* agents.

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