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STUDIES OF THE BIOLOGICAL AND THERAPEUTIC EFFECTS OF ARGENTINE STINGLESS BEE PROPOLIS

Yanina Brodkiewicz[a], Karenina Marcinkevicius[b], Marcos Reynoso[a], Virginia Salomon[c], Luis Maldonado[c], Nancy Vera[a]*

[a] *Cátedra de Farmacoquímica, Inst. de Estudios Farmacológicos Dr. A. Sampietro, Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Ayacucho 471, 4000, Tucumán, Argentina.*

[b] *Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Ayacucho 471, 4000, Tucumán, Argentina.*

[c] *Laboratorio de Agroindustrias, Estación experimental agropecuaria. Instituto Nacional de Tecnología Agropecuaria (INTA). Famaillá, 4132, Tucumán. Argentina.*

ABSTRACT

Meliponids are native bees of the Americas, characterized by having no sting (ANSA). Some live in the Yunga forests of northern Argentina, a place rich in a diversity of this type of bees of which at least thirty-three species may be found. Propolis is a resinous substance that bees collect from plants exudates. Chemical composition and functional properties vary according to the flora of the place where the hives are. They have been valued by humans for their biological properties for centuries. This study is aimed at investigating the antinociceptive, antioxidant and anti-biofilm activities of propolis from the stingless bees *Tetragonisca fiebrigi* Schwarz and *Scaptotrigona jujuyensis* Schrottky found in Estación Experimental Agropecuaria Famaillá of INTA, Tucumán, Argentina. Analgesic activity of the extracts was estimated against acetic acid induced writhing, tail immersion method and formalin test. Antioxidant capacity was evaluated using DPPH free radical scavenging and β-carotene bleaching assays. Propolis anti biofilm activity was tested on *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATTC 6538P bacteria. Phytochemical constituents were obtained using standard chemical methods. The propolis alcoholic extracts of the studied species possess antinociceptive activity at both central and peripheral levels as demonstrated by the three algesia tests used. Both propolis extracts were effective antioxidants in DPPH and β-carotene linoleic acid model systems. *S. jujuyensis* propolis tested at all doses against *S. aureus* and *P. aeruginosa* presented a selective biofilm inhibition unrelated to bacterial growth inhibition, thus achieving a reduction in pathogenicity. The chemical studies revealed the presence of sterols, triterpenes, catechins, coumarins, flavonoids, phenols, tannins and anthocyanidins. Chemical composition observed in the *T.fiebrigi* and *S. jujuyensis* propolis, suggest that those responsible for the activity would be chemical compounds of a non-phenolic nature. Our data indicate that geopropolis is a natural source of bioactive substances with promising beneficial properties for human health. Isolation and identification of compounds responsible for the pharmacological activities displayed by propolis has started.

Keywords: Geopropolis; *Scaptotrigona jujuyensis*; *Tetragonisca fiebrigi*; Antinociceptive activity; Antioxidants; antimicrobial activity.

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***Address for Correspondence:**

Dr. Nancy Roxana Vera, Cátedra de Farmacoquímica, Inst. de Estudios Farmacológicos Dr. A. Sampietro, Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Ayacucho 471, 4000, Tucumán, Argentina.

Conflict of interest

The authors declare that there are no conflicts of interest and they have no actual or potential competing financial interests

1. INTRODUCTION

In recent decades, several researches have shown that analgesics represent one of the most studied therapeutic classes in the world. This fact is understandable due to the high consumption of these drugs worldwide, although they may have some adverse effects and low therapeutic efficacy. Thus, the effort to develop new drugs has been the focus in the screenings of extracts from natural sources, which historically have led to the discovery of many clinically important drugs in the current therapy [1,2]. Natural products from bees have been extensively employed since ancient times because of their broad pharmacological activity [3,4].

Propolis has been widely utilized as a medicine and dietary supplement for its broad biological, antimicrobial, anti-inflammatory, immunomodulatory, and antioxidant activities [5-7]. Additionally, a large number of studies have indicated that the reactive oxygen scavenging and antimicrobial activity may be attributed to the various natural phenolic components and flavonoids with antioxidant effects and reducing activity present in propolis [8,9].

Previous phytochemical studies demonstrated that *Apis mellifera* propolis predominantly contains complex phenolic compounds which are responsible for its activities [10]. However, chemical constituents and related bioactivities of each type of propolis depend on bee species, preference for resin and food plants, geographical regions, variation in plant resin compositions and accessible plant species [11,12]. In general, the composition of *Apis mellifera* propolis primarily consists of resin (50%), wax (30%), essential and aromatics oils (10%), bee pollen (5%), and other substances (5%) Stingless bees are another bee species, a eusocial group which plays an important role in pollination they produce a variety of propolis popularly known as geopropolis. It consists of a mixture of resin, wax and soil with distinctive physicochemical characteristics [13]. In the Yunga forests of northern Argentina, a rich place in a diversity of this type of bees, at least thirty-three species may be found [14].

Although many studies about propolis have been published, most of them are from *Apis mellifera*. In contrast, very little is known about the chemical composition and biological activity of stingless bees propolis although it is frequently used in folk medicine.

Other investigators have studied geopropolis collected by *Melipona scutellaris* a native Brazilian stingless bee1 showed that *Melipona scutellaris* geopropolis has antinociceptive and anti-inflammatory properties [15,16]. Ferreira Campos et al., (2015) reported phenolic compounds, aromatic acids, alcohols, terpenes and sugars in ethanol extracts of *Tetragonisca fiebrigi* propolis. These compounds have been identified in other studies of stingless bees propolis found in Brazil [18,19]. *T. fiebrigi* ethanol extract showed antimicrobial activity against gram positive, gram negative and fungal bacteria, that cause respiratory pneumonia and common nosocomial infections in the urinary tract, and in post-surgical, gastrointestinal and skin wounds [20]. Brodkiewicz et al., 2017 reported that oral administration of *Scaptotrigona jujuyensis* and *T. fiebrigi* propolis in rats showed antiinflamatory activity and had no toxic effects.

The aim of this work was to evaluate the antinociceptive, antioxidant and antibiofilm activities of ethanolic extracts of *Tetragonisca fiebrigi* Schwarz and *Scaptotrigona jujuyensis* Schrottky, (Apoidea) propolis to validate their traditional use. In addition, we assessed the phytochemical composition and measured the content of total phenolic compounds and flavonoids in the extracts in order to correlate them with the assayed activities. This work constitutes the first in vivo validation study of the antioxidant, antibiofilm and antinociceptive effect of stinglees bee propolis from Northwestern Argentina.

2. MATERIALS AND METHODS

2.1 Propolis sample

In this work we used propolis of *S. jujuyensis* and *T. fiebrigi*. The hives are located in the Famaillá Agricultural Experiment Station of INTA, in the province of Tucumán, Argentina. The bees were identified and deposited in the Museo Argentino de Ciencias Naturales Benardino Rivadavia, Buenos Aires, Argentina. Samples of *S. jujuyensis* and *T. fiebrigi* propolis were collected and kept in a dry place and stored at 4 °C until its processing. Dry propolis was subjected to exhaustive maceration with 70% ethanol (1:7 w/v) in a shaker (300 rpm) at room temperature for 72 h. The ethanol extract solution was then filtered and concentrated using a rotary evaporator to obtain the ethanol extracts of *S. jujuyensis* propolis (**ESP**) and *T. fiebrigi* propolis (**ETP**).

2.2 Physical and chemical determinations

Humidity: 4 g of powdered samples were heated in an oven at 105°C for 6 hours and cooled to room temperature in a desiccator until constant weight [22].

Ash: 4 g of powdered samples were ashed at 550 ± 25 ° C for 4 hours, and cooled to room temperature in a desiccator until constant weight [22].

Waxes: 2 g of powdered samples were treated with n-hexane in a soxhlet for 6 hours. The extracts were concentrated to dryness in a water bath at 70°C, and then cooled to room temperature in a desiccator until constant weight [23].

Resins: soxhlet cartridge after extracting wax was extracted with ethanol 96% in a soxhlet until negative reaction to % ferric chloride (about 3 hours). The extracts were diluted to 100 ml with ethanol in a volumetric flask at 20°C. 50 ml were concentrated to dryness in a water bath at 100°C, then cooled in a desiccator until constant weight. The other 50 ml was kept in an amber flask to determine the content of total phenolic compounds and total flavonoids [23].

Mechanical mixtures: the residues, that stays in the soxhlet cartridge after extracting wax with hexane and resins with ethanol, is dryed in an oven at 105°C for 4 hours, and cooled to room temperature in a desiccator until constant weight [23].

2.3 Phytochemical screening

For the identification of the different groups of secondary metabolites present in the extracts of propolis, the techniques and procedure described by Miranda and Cuéllar, (2002) were used.

2.4 Determination of total phenol content

Total phenolic content was estimated by the Folin–Ciocalteu method [25]. Absorbance at 765 nm was measured after 30 min of incubation at room temperature. Gallic acid (0-10 mg/l) was used for the standard calibration curve. The results were expressed as mg gallic acid equivalent (GAE)/g dry weight, and calculated as mean value ± SD (n = 3).

2.5 Total flavonoid content

Total flavonoid content was determined by the colorimetric method of Christel et al., (2000). Absorbance was measured at 430 nm. Total flavonoid contents were calculated from a calibration curve using quercetin equilibrant (g QE /100 g dry matter) (Sigma Chem. Co., USA).

2.6 Antioxidant activity

2.6.1 DPPH scavenging activity

The free radical scavenging activity of the extracts and positive controls (Quercetin and butylated hydroxytoluene) were investigated using 1,1 biphenyl - 2-picrylhydrazyl (DPPH) radical scavenging method [27]. Absorbance at 517 nm was measured versus ethanol as a blank. All experiments were carried out in triplicate. The degradation of DPPH was evaluated against a control (0.25 ml of DPPH solution and 0.75 ml ethanol 96 %). Antioxidant activity was expressed as:

Scavenging activity % = [(Abs control – Abs sample)/ Abs control] x 100

2.6.2 β-carotene bleaching method

The antioxidant activity of ethanolic extracts from propolis was evaluated using β–carotene–linoleate model system, as described by Sun and Ho, (2005). The absorbance at 470 nm was measured. Quercetin or BHT was used as positive control and distilled water or solvent were the negative control. All samples were assayed in triplicate. The antioxidant activity (AA) was measured in terms of successful bleaching of β–carotene by using the equation:

$$AA= [1- (A_0 – A_t / A_0^0 – A_t^0)] \times 100$$

Where A_0 and A_0^0 were the absorbance values, before incubation for test sample and control respectively. A_t and A_t^0 were the respective absorbance of the test sample and the control after incubation for 120 min. The results were expressed as % of the prevention of bleaching of β–carotene [29].

2.7 Antimicrobial activity

2.7.1 Bacterial growth

Overnight cultures of *P. aeruginosa* ATCC 27853 and *S. aureus* ATTC 6538P were diluted to reach 2.5×10^6 CFU/mL in Luria–Bertani (LB) and Mueller-Hinton (MH) media was used for *P. aeruginosa* and *S. aureus*, respectively. The diluted culture (190 µL) was placed in each of the 96 wells of a micro titer polystyrene plate. Solutions of extracts in DMSO–distilled water (1:1) were prepared separately and 10 µL of each was pipetted to the plastic micro titer plate wells individually (eight replicates) in order to reach final concentrations of 200, 100, 50, and 5 µg/mL. Control wells (eight replicates) the final concentration of DMSO is 2.5 %. Medium control was prepared using sterile LB and contained the diluted culture (190 µL) and 10 µL of a solution of DMSO–water (1:1) in which MH for to each microorganism. After 24 h incubation at 37 °C, bacterial growth was detected as turbidity (600 nm) using a micro titer plate reader (Power Wave XS2, Biotek, VT, USA).

2.7.2 Biofilm formation assay

For biofilm quantification, a micro method based on a protocol previously reported was employed [30]. Absorbance (540 nm) of ethanol solutions of crystal violet was determined using a micro titer plate reader (Power Wave XS2. Biotek, Vermont, USA). Ciprofloxacin, a known biofilm inhibitor, was incorporated in the same bioassay as a positive control at 5 µg/mL. At this concentration, ciprofloxacin inhibited the biofilm formation but did not significantly modify the bacterial growth.

2.8 Studies in vivo

2.8.1 Animals

Wistar male rats (weighing 220–240 g) were used for this study and were obtained from the Bioterio of the Facultad de Bioquímica, Química y Farmacia, Instituto de Biología (INSIBIO), Universidad Nacional de Tucumán. All animals were kept under normal laboratory conditions of humidity, temperature (25±1 ºC) and light (12 h dark/light cycle), and allowed free access to food and water ad libitum. The studies were conducted in accordance with the internationally accepted principles for laboratory animal use and care (EEC Directive of 1986; 86/609/EEC). Prior to initiation of dosing, all rats were acclimated for 7 days and evaluated for weight gain and any gross signs of disease or injury.

2.8.2 Antinociceptive assays

2.8.2.1 Formalin-induced nociception

The formalin test was carried similar to that described by Gorzalczany et al., (2011). Rats were injected with 20 µl of 2.5% formalin solution, into the sub-plantar region of the right hind paw 30 min after treatment with sterile water (control, p.o.), extracts of *S. jujuyensis* propolis (**ESP**) and *T. fiebrigi* propolis (**ETP**) (250, 500 and 1000 mg/kg b.w.) and reference drugs ibuprofen syrup (100 mg/kg b.w.) and morphine syrup (1 mg/kg b.w.). Licking time of the injected paw, was recorded as nociceptive response at 0–5 min (neurogenic phase) and 15–30 min (inflammatory phase) after formalin injection

2.8.2.2 Acetic acid-induced writhing method

The acetic acid method was carried out as described by Reynoso et al. (2016). Thirty minutes before to acetic

acid injection, rats (n = 6 per group) were treated with extracts of *S. jujuyensis* propolis (**ESP**) and *T. fiebrigi* propolis (**ETP**) (250, 500 and 1000 mg/kg b.w., p.o.), sterile water (control, p.o.), morphine syrup (1 mg/kg b.w., p.o.) and ibuprofen syrup (100 mg/kg b.w., p.o.). Each group was administered 10 ml/kg b.w., i.p., of an aqueous solution of acetic acid (1.0%). After five minutes the rats were observed and the number of writhing was counted for 30 min.

2.8.2.3 Tail immersion test

To evaluate the central analgesic property the tail immersion test was performed [33]. One to two cm of tail of the rats pretreated with extracts of *S. jujuyensis* propolis (**ESP**) and *T. fiebrigi* propolis (**ETP**) (250, 500 and 1000 mg/kg b.w., p.o.), morphine syrup (1 mg/kg b.w., p.o.), ibuprofen syrup (100 mg/kg b.w., p.o.) and sterile water (p.o.) were immersed in warm water kept constant at 54 ± 0.5°C. The latency between tail immersion and deflection of tail was recorded. A latency period of 20 s was maintained to avoid tail tissue damage in mice. The latency period of the tail with draw al response was taken as the index of antinociception and was determined at 30, 60, 90, 120 and 150 min after the administration of the drug and extracts.

2.9 Statistical analysis

All experimental values are expressed as the mean ± the standard deviation of at least two independent experiments. Statistically significant differences from the vehicle group were identified by Student's test or ANOVA followed by Tukey test for paired data. The level of $p \leq 0.05$ was used to determine statistical significance.

3. RESULTS

3.1 Physical and chemical parameters

The results of the characterization of geopropolis samples of *T. fiebrigi* and *S. jujuyensis* are shown in Table 1. Humidity values are in the range 1.58 - 2.57 g/100g, wax in the range 76.43-67.30 g/100g, resins between 7.66 and 6.36, ash in the range 2.55–3.51 g/100g, and mechanical mixtures between 14.23 and 23.77 g/100g**.**

3.2 Phytochemical screening

The phytochemical screening of the main groups of chemical constituents of the propolis under study was qualitatively determined by simple reactions of coloration and precipitation. Table 2 shows the results of the phytochemical screening of ethanolic extracts of *T. fiebrigi* (**ETP**) and *S. jujuyensis* (**ESP**) propolis.

Table 1: Physical and chemical parameters

Parameter	Geopropolis	
	ETP	**ESP**
Humidity [g/100g]	1,58 ± 0,53	2,57 ± 0,43
Ash [g/100g]	2,55 ± 0,70*	3,51 ± 0,23*
Wax [g/100g]	76,43 ± 2,78*	67,30 ± 4,92*
Resins [g/100g]	7,66 ± 0,98	6,36 ± 1,55
Mechanical sludges [g/100g]	14,33 ± 1,73*	23,77 ± 3,74*
Total phenolic substances [(1)g/100g]	0,12 ± 0,02	0,23 ± 0,05
Total flavonoid substances [(2)g/100g]	0,08 ± 0,04	0,08 ± 0,02

1= gallic acid equivalent
2= quercetin hydrate equivalent
Values represent the mean ± SEM (n=6). * The asterisks denote the significance levels between the values in the same row, $p < 0.001$ (one-way ANOVA, followed by Tukey's test).

Table 2: Phytochemical screening of the ethanol extracts *T. fiebrigi* (ETP) and *S. jujuyensis* (ESP) propolis.

Phytoconstituents	**Test**	**ETP**	**ESP**
Catechins	Catechine	**+**	**+**
Lactones	Baljet	+	+
saponins	foam	-	-
coumarins	UV fluorescence	+	+
flavonoids	Shinoda	+	+
sterols and Triterpenes	Liebermann - Burchard	+	+
phenols and tannins	Ferric chloride	+	+
quinones	Bornträger	-	+
cardenolics	Kedde		
anthocyanidins	Anthocyanidins	+	+
alkaloids	Dragendorf	-	-
	Mayer		
	Wagner		

(+) Presence (-) Absence

The polyphenol and flavonoid contents of ethanolic extracts of *T. fiebrigi* (**ETP**) and *S. jujuyensis* (**ESP**) propolis are shown in Table 1.

Total phenolic concentration in the extracts was expressed as g/GAE 100 g dry weight. The amount of total phenolic compounds in the ETP and ESP was 0.12 and 0.23 g GAE/100 g respectively.

Flavonoid concentration in the extracts was expressed as quercetin equivalents (QE)/100 g dry weight. The flavonoid content of the ethanol extract of *T. fiebriyi* (**ETP**) and *S. jujuyensis* (**ESP**) propolis was 0.08 ± 0.04 and 0.08 ± 0.02 g QE/100 g dry weights respectively.

3.3 DPPH radical scavenging activity

The DPPH antioxidant assay is based on the ability of DPPH, a stable free radical, to decolorize in the presence of antioxidants. Figure 1A shows that the scavenging effects of samples on DPPH radical and were in the following order: **QUER** = **BHT** >**ESP** > **ETP**. The effective concentration 50 (IC 50), defined as the concentration at which the DPPH radicals were scavenged by 50 %, was 2.27 ± 0.12 mg/ml for the ETP and 1.72 ± 0.28 mg/ml for the ESP. Though the antioxidant potential of extracts was found to be low ($P < 0.05$) in comparison with BHT and quercetin (0.002 ± 0.001 mg/ml and 0.080 ± 0.010 mg/ml respectively), the study revealed that ETP and ESP have a prominent antioxidant activity, 88.89 and 93.29 % at a concentration of 7 mg/ml respectively.

3.4 Antioxidant activity determined by β-carotene bleaching method

The antioxidant potential of the propolis extract was also evaluated by the β-carotene bleaching method. Figure 1B shows the decrease in absorbance of the β-carotene emulsion in presence of 10 mg/ml of the extracts and 1 mg/ml of the reference antioxidants (BHT and Quercetin). The addition of 10 mg/ml of ETP and ESP extracts was effective in inhibiting the oxidation of linoleic acid and subsequent bleaching of β- carotene, in comparison with the control ($p < 0.05$), which contained no antioxidant component. The percentages of activity were ETP (67.03 %), ESP (55.02 %), BHT (95.00 %) and Quercetin (93.00 %). The results indicated that the ETP and ESP extracts were effective antioxidants in a β-carotene linoleic acid model system.

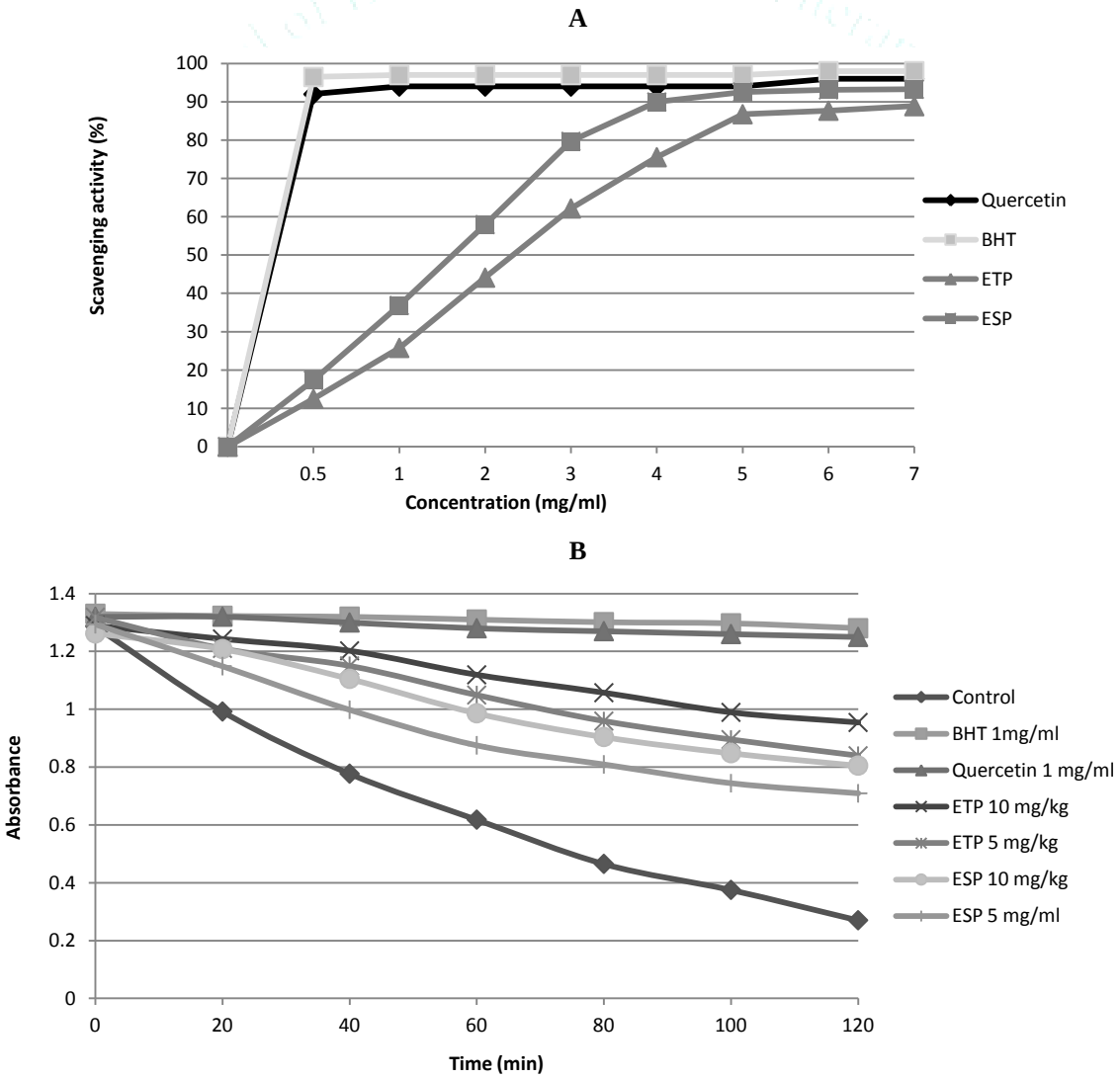

Figure 1: Antioxidant activity of *T. fiebrigi* (**ETP**) and *S. jujuyensis* (**ESP**) propolis. (A) DPPH radical scavenging activity. (B) Inhibition of lipid peroxidation. Quercetin and BHT were used as reference anti-oxidant. Values represent the mean ± S.E.M. (n=6).

3.5 Bacterial growth

The effects of *T. fiebrigi* (**ETP**) and *S. jujuyensis* (**ESP**) propolis on (a) *S. aureus* ATTC 6538P and (b) *P. aeruginosa* ATCC 27853 are shown in Figure 2 in comparison with the control experiment.

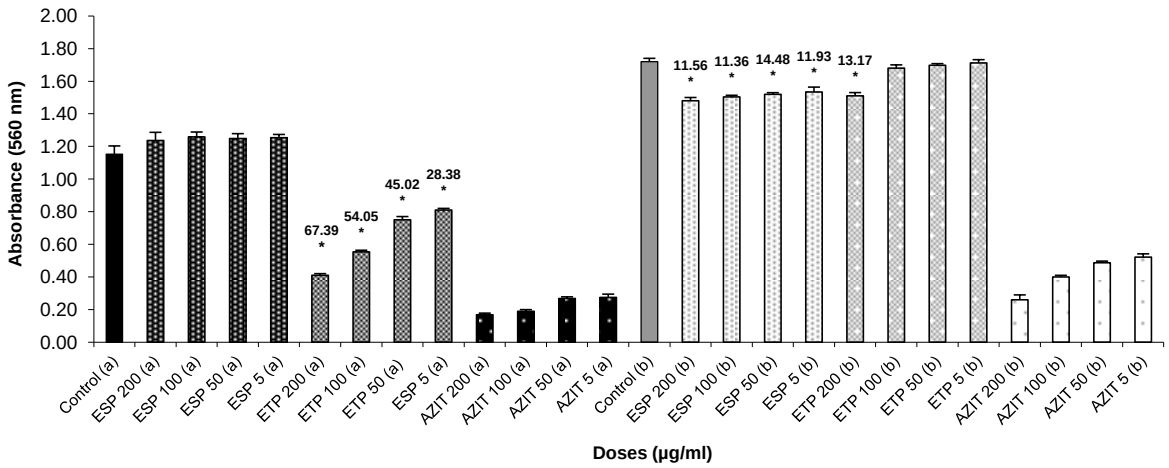

Figure 2: Effect of *T. fiebrigi* (**ETP**) and *S. jujuyensis* (**ESP**) propolis at 200 µg/ml, 100 µg/ml, 50 µg/ml and 5 µg/ml on *S. aureus* ATTC 6538P growth (**a**) and *P. aeruginosa* ATCC 27853 growth (**b**). Bacterial growth was assessed by reading the absorbance at 560 nm. Vertical bars represent the standard deviation (n = 8). ***** The asterisks denote the significant difference compared with the control group, by means of ANOVA, followed by the Tukey test ($p < 0.05$). Values in parentheses are percentage of inhibition.

T. fiebrigi (**ETP**) propolis moderately inhibited *S. aureus* bacterial growth (67.39, 54.05, 45.02 and 28.38%) at all the concentrations assayed (200, 100, 50, 5 µg/ml). However, none of them inhibited *P. aeruginosa* bacterial growth.

3.6 Biofilm formation

The absorbance of biofilm (Figure 3), formed after 24 h incubation in the control media, stained with crystal violet, was 2.75.

The decrease in biofilm production observed in *S. aureus* by *T. fiebrigi* (**ETP**) propolis may be due to the growth inhibition at all doses tested. However, *S. aureus* and *P. aeruginosa* biofilm production was inhibited by *S. jujuyensis* (**ESP**) propolis at all doses tested (Fig. 3). Since this inhibition was not related to that of bacterial growth (Figure 2), there was a reduction in pathogenicity.

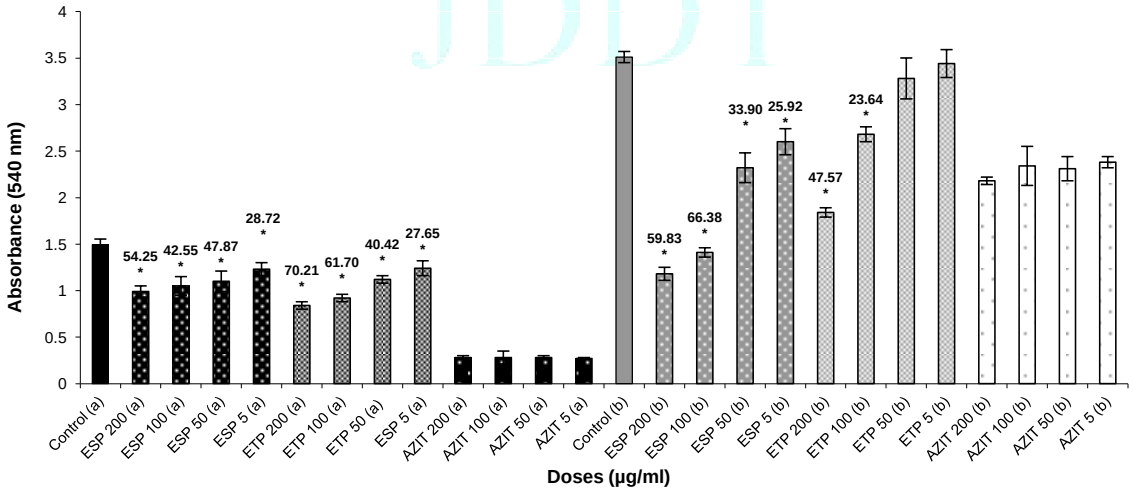

Figure 3: Effect of *T. fiebrigi* (**ETP**) and *S. jujuyensis* (**ESP**) propolis at 200 µg/ml, 100 µg/ml, 50 µg/ml and 5 µg/ml on *S. aureus* ATTC 6538P biofilm production (**a**) and *P. aeruginosa* ATCC 27853 biofilm production (**b**). Vertical bars represent the standard deviation (n = 8). ***** The asterisks denote the significant difference compared with the control group, by means of ANOVA, followed by the Tukey test ($p < 0.05$). Values in parentheses are percentage of inhibition.

T. fiebrigi (**ETP**) propolis only selectively inhibited the production of biofilm of *P. aeruginosa* (47.57 and 23.64%) at doses of 200 and 100 µg / ml respectively.

3.7 Antinociceptive study

3.7.1 Formalin-induced pain

Overall, the ethanol extracts of *T. fiebrigi* (ETP) and *S. jujuyensis* (ESP) propolis showed a significant ($P < 0.05$) antinociceptive activity in both phases of the formalin-induced paw licking test (Figures 4A and 4B). Morphine was used as positive control (1 mg/kg b.w., p.o.) and the response time of the animals decreased significantly when compared to the negative control in both phases, while the other positive control, ibuprofen (100 mg/kg b.w., p.o.), was effective only in the second phase (Figure 4B).

In the first phase (Figure 4A), *T. fiebrigi* (ETP) and *S. jujuyensis* (ESP) propolis produced a significant ($p<0.05$) dose dependent inhibition of nociceptive reaction with a peak effect inhibitory effect (71.13 and 75.28 %) at the highest dose (1000 mg/kg). This effect was statistically significant ($p<0.05$), but lower than that produced by morphine (86.54 % inhibition). In the second phase, the duration of the nociceptive reaction in the control group was 264.01±10.51 seconds. The ethanolic extract of *T. fiebrigi* (ETP) propolis significantly ($p<0.05$) inhibited the biting and licking response with a higher inhibitory effect (85.56 %) produced at the same dose.

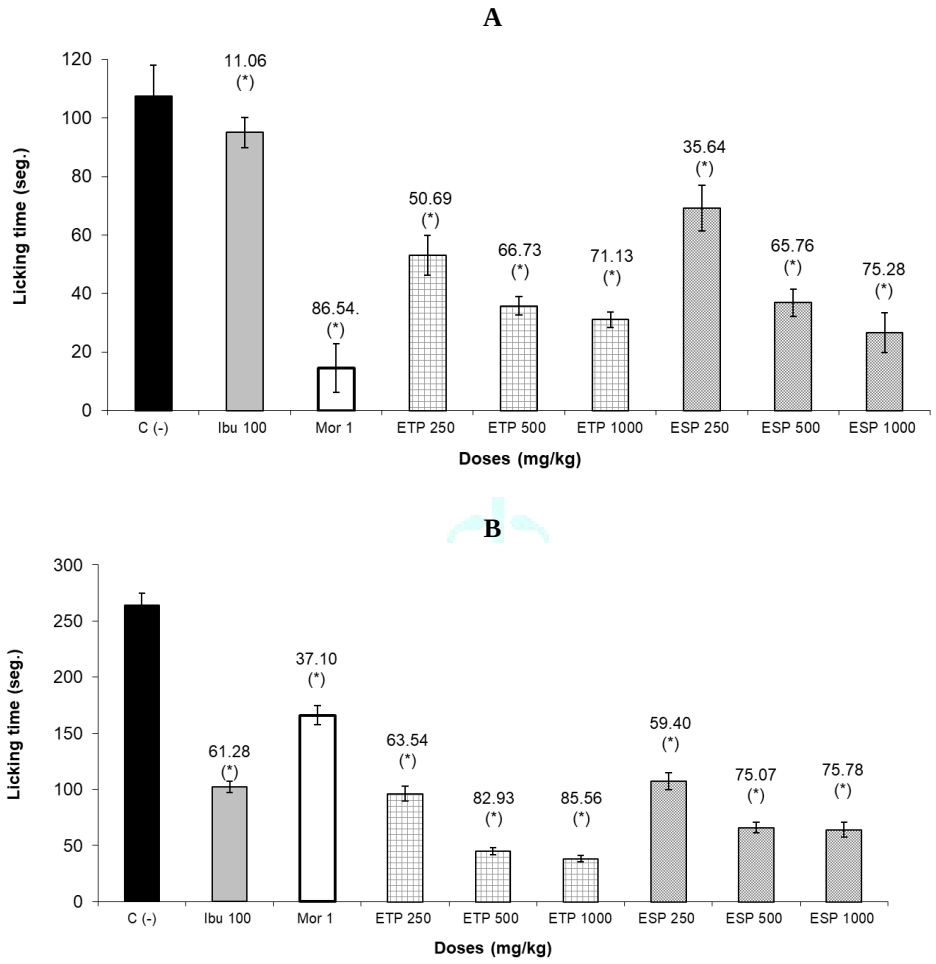

Figure 4: Effect of ethanolic extracts of *T. fiebrigi* (**ETP)** and *S. jujuyensis* (**ESP**) propolis on the nociceptive response of the formalin test in first phase (A) and second phase (B). Control, ibuprofen (Ibu 100 mg/kg b.w.), morphine (Mor 1 mg/kg b.w.), *T. fiebrigi* propolis (250-500-1000 mg/kg b.w.) and *S. jujuyensis* propolis (250-500-1000 mg/kg b.w.). Values in parentheses are percentage of inhibition. * The asterisks denote the significance levels compared with the control group, $p<0.05$ (one-way ANOVA, followed by Tukey's test). Values represent the mean ± SEM (n=6)

3.7.2 Acetic acid-induced writhing method

The oral antinociceptive doses of ethanolic extracts of *T. fiebrigi* (**ETP)** and *S. jujuyensis* (**ESP**) propolis (1000 mg/kg b.w.) produced a significant inhibition of acetic acid i.p. induced abdominal constriction in rats (Figure 5).

The calculated inhibition for the ETP and ESP were 77.97 % and 58.71 % respectively, significantly lower compared with dose morphine (89.99 %) and ibuprofen (94.49 %).

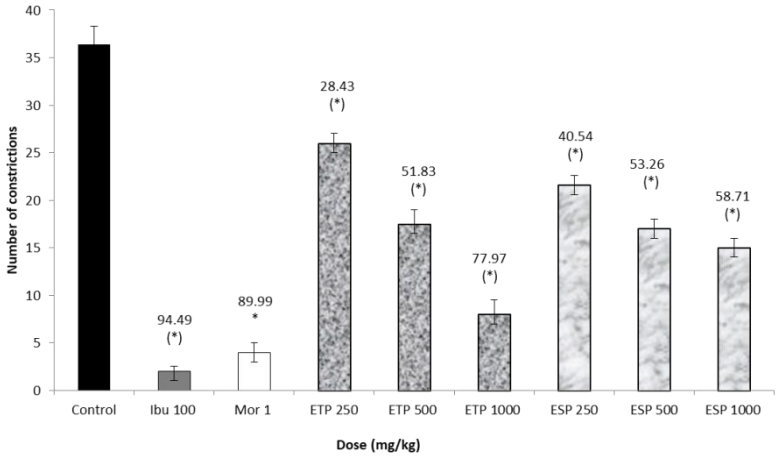

Figure 5: Effect of oral administration on acetic acid induced writing in rats. The intensity of nociception behavior was cuantified by counting the total number or writhes occurring 20 min following the stimulus injection. Rats were orally treated with control, ibuprofen (Ibu 100 mg/kg b.w.), morphine (Mor 1 mg/kg b.w.), ethanolic extracts of *T. fiebrigi* (**ETP)** and *S. jujuyensis* (**ESP**) propolis (EE 250-500-1000 mg/kg b.w.) Values in parentheses are percentage of inhibition. * The asterisks denote the significance levels compared with the control group, $p<0.05$ (one-way ANOVA, followed by Tukey's test). Values represent the mean ± SEM (n=6)

3.7.3 Tail immersion test

A significant reduction of the painful sensation due to tail immersion in warm water was observed following oral administration of *T. fiebrigi* (**ETP**) and *S. jujuyensis* (**ESP**) propolis at doses of 500 and 1000 mg/kg b.w. (Table 1). The inhibitory effects of **ETP** and **ESP** became pronounced at 60 min, 65.36 % and 67.54% respectively, post dosing 1000 mg/kg b.w. The inhibitory effect of the ethanol extracts of both propolis was lower than that produced by morphine and statistically significant ($p<0.05$). Ibuprofen had no effect in this test.

4. DISCUSSION

The aim of this work was to evaluate the antinociceptive, antioxidant and antibiofilm activities of ethanolic extracts of *T. fiebrigi* and *S. jujuyensis* propolis to validate the traditional usage of this stingless bee's propolis.

Our results showed that the ethanol extract of *T. fiebrigi* (ETP) and *S. jujuyensis* (ESP) propolis had a significantly antinociceptive effect on three classical nociception models in rats: the formalin, the acetic acid induced writhing and the tail immersion tests, all of which are useful methods for screening prospective antinociceptive compounds.

The intraperitoneal injection of acetic acid elicited writhing, a syndrome characterized by a wave of abdominal musculature contractions followed by extension of the hind limbs. This response is a sensitive procedure to establish peripherally acting analgesia that involves local peritoneal receptors on the surface of the cells lining the peritoneal cavity [34]. A significantly effective protection was observed in the groups of animals treated with ethanolic extracts of *T. fiebrigi* and *S. jujuyensis* propolis as compared with the standard drug (Ibuprofen) (Figure 5). Inhibition percentages were dose-dependent for the *T. fiebrigi* extract. The analgesic effect produced by the ethanolic extract of *S. jujuyensis* propolis was high in the three doses studied (250, 500 and 1000 mg / kg). The agent that reduced writhing renders an analgesic effect preferably by inhibition of prostaglandin synthesis, a peripheral mechanism of pain inhibition [35]. However, the test of abdominal constrictions has a low specificity, since several compounds, such as antihistamines, neuroleptics and adrenergic blockers may also inhibit constrictions [36]. Hence, we used the formalin test, a chemical model of nociception, which provides a more specific response compared with the model of abdominal constrictions induced by acetic acid [37].

A subcutaneous injection of formalin produces a distinct biphasic nociception. The first phase starts immediately after the formalin injection and continues for 5 min, after which nociception appears to diminish. The second phase starts as a return to high levels of nociception beginning 15~30 min after the formalin injection and continues for 60 min [38]. These phases have obvious and different properties that are very useful tools, not only for assessing the potency of analgesics, but also for elucidating the mechanisms of pain and analgesia. The action of analgesics is different in the first and second phases. Drugs such as narcotics (e.g. morphine, codeine, meperidine) which primarily act centrally, inhibit both phases equally, but peripherally acting drugs such as ibuprofen, aspirin, oxyphenbutazone, dexamethasone, and hydrocortisone only inhibit the second phase of formalin-induced nociception [39,40]. The results of the present study showed that the ethanol extract of *T. fiebrigi* and *S. jujuyensis* propolis inhibited both the early and the late phases of formalin-induced pain (Figure 4), thus suggesting its central and peripheral antinociceptive actions. These results are similar to those obtained by Lima Cavendish (2015) in red propolis of *Apis mellifera* where the alcohol extract (30

mg / kg) decreased the response in the first and second phases, while acetylsalicylic acid, used as a positive control (300 mg / kg) inhibited only the second phase. Additionally, the extracts of *T. fiebrigi* and *S. jujuyensis* propolis produced a greater inhibition in the second phase. This effect was comparable to that produced by the standard drug (Morphine)(Figure 4B).

Findings in the formalin test suggest that the ethanolic extracts of geopropolis act through a peripheral mechanism, as established in the mouse writhing test, demonstrating possible effectiveness in the treatment of chronic inflammatory pain by inhibition of associated inflammatory processes, basically associated to the release and/or action of inflammatory mediators. This assertion is supported by the report of Brodkiewicz et al., 2017 where ethanolic extracts of *T. fiebrigi* and *S. jujuyensis* propolis showed an anti-inflammatory activity via inhibition of histamine, serotonin, substance P and prostaglandin synthesis.

In the tail immersion test, oral pre-treatment with the *T. fiebrigi* and *S. jujuyensis* propolis caused a profound and dose dependent analgesia in the treated animals although the analgesic effect was lower than that produced by morphine (Table 3). Animal response in this test is usually integrated at the lower level in the central nervous system, thus giving information about the pain threshold. Therefore, it is used to detect narcotic and non-narcotic analgesics. The ethanolic extracts of *T. fiebrigi* and *S. jujuyensis* propolis reached their maximum analgesic level 60 min after administration, similar to morphine. Ibuprofen did not show any activity in this test.

Table 3: Effect of *T. fiebrigi* (**ETP**) and *S. jujuyensis* (**ESP**) propolis on pain with the tail immersion test

		Interval following treatment (h)					
		0.0	**0.5**	**1.0**	**1.5**	**2.0**	**2.5**
Treatment	**Dose (mg/Kg, p.o.)**	**Reaction time (seg)**					
Control	SW	2.05±0.15	2.25±0.10	2.40±0.10	2.40±0.11	2.05±0.05	2.05±0.04
Ibuprofen	100	2.10±0.13	2.35±0.06	2.35±0.18	2.35±0.09	2.29±0.05	2.25±0.05
Morphine	1	2.10±0.10	3.85±0.15 *	4.40±0.22 *	4.00±0.20 *	3.40±0.17 *	3.10±0.15 *
S. jujuyensis propolis	250	2.10± 0.09	2.74±0.20 *	3.06±0.33 *	2.81±0.08 *	3.36±1.57 *	3.12±0.43 *
	500	2.15± 0.09	4.13±0.62 *	3.85±1.53 *	4.57±1.64 *	3.15±0.26 *	3.39±0.23 *
	1000	2.10± 0.05	3.87±0.96 *	5.06±0.27 *	3.42±0.60 *	2.88±0.11 *	2.48±0.15 *
T. fiebrigi propolis	250	2.20± 0.05	2.65±0.13 *	2.31±0.01	2.83±0.15 *	2.21±0.11	2.41±0.12 *
	500	2.20± 0.09	3.29±0.45 *	4.68±1.70 *	3.52±1.14 *	2.89±1.14 *	3.27±0.57 *
	1000	2.15± 0.15	4.05±0.08 *	4.35±3.18 *	3.18±0.88 *	3.35±0.04 *	2.84±0.06 *

Values represent the mean ± SEM and are in seconds (n=6). * The asterisks denote the significance levels compared with the control group, $p < 0.05$ (one-way ANOVA, followed by Tukey's test). SW (sterile water).

Our results have shown that the alcoholic extracts of propolis of the species *T. fibrigi* and *S. jujuyensis*, have a significant antinociceptive effect in laboratory animals at high doses. Similar results were found for another meliponid species, *Melipona scutellaria* [15].

In recent years, various investigations have undertaken the study of propolis as an antioxidant of natural origin for the prevention and treatment of various diseases of oxidative origin [42].

The extracts were able to scavenge DPPH radicals, but especially that of *T. fiebrigi* propolis (Figure 1A), with IC50 of 2.27 ± 0.12 mg/ml. promoted a stronger DPPH radical scavenging activity than the others.

In the β-carotene bleaching assay, linoleic acid produces hydroperoxides as free radicals during incubation at 50 1C. The presence of antioxidants in the extracts minimizes the oxidation of β-carotene by hydroperoxides. There was a correlation between the degradation rate and the bleaching of b-carotene since the extract with the lowest β-carotene degradation rate exhibited the highest antioxidant activity. The results indicated that the ETP and ESP extracts were effective antioxidants in a β-carotene linoleic acid model system at the doses tested (Figure 1B). However, all extracts assayed had a lower antioxidant activity than BHT and Quercetin, in agreement with the results from Ozsoy et al. (2008).

Biofilm production of *S. aureus* and *P. aeruginosa* was inhibited by *S. jujuyensis* (**ESP**) propolis (Figure 3), but it was not related to the bacterial growth inhibition (Figure 2). The percentage of biofilm inhibition by propolis extracts was higher than the percentage of growth inhibition by the same compounds. The relation between biofilm production (measured at DO540 nm) and bacterial growth (measured at DO560 nm) was defined as specific biofilm produced, i.e., biofilm that each bacterium forms [43]. The specific biofilm production for the control media was 0.53 and 0,60 for *P. aeruginosa and S. aureus*. If DO relation of the

treatments is lower than that of the control, it indicates that the specific production of biofilm was inhibited. Under the conditions studied, the *S. jujuyensis* propolis extract tended to reduce biofilm specific production of *S. aureus* and *P. aeruginosa* at all doses tested. The specific biofilm production was on average 0.33 in *S. aureus* and 0.46 in *P. aeruginosa*. *T. fiebrigi* ethanolic extracts reduced the specific production of biofilm in *P. aeruginosa* only at the highest doses tested (specific production of biofilm 0,43 and 0,40 at 200 and 100 µg / ml respectively).

Chemical constituents and related bioactivities of each type of propolis depend on bee species, preference for resin and food plants, geographical regions, variation in plant resin compositions and accessible plant species [11]. Phenolic compounds are reported by many authors as being responsible for the biological activity observed in *Apis mellifera* propolis [5,44], but there are very few data on the propolis of ANSA.

The chemical studies revealed the presence of sterols, triterpenes, catechins, coumarins, flavonoids, phenols, tannins and anthocyanidins (Table 2). The propolis evaluated had a low content of phenolic compounds, flavonoids and resins and a high content of waxes (Table1). Similar results were obtained by Franchin et al., 2013. The differences in chemical composition observed in the ANSA propolis analyzed in this paper suggest that those responsible for the observed activity would be chemical compounds of a non-phenolic nature.

5. CONCLUSIONS

Although meliponid hive products have many advantages, meliponiculture has not become popular in Argentina yet, as it has in Mexico and Brazil. The studies published on these species are very scarce and have not been carried out at the same chemical and pharmacological level. These results signify that these geopropolis alcoholic extracts are an important source of natural analgesics, antioxidants and antipathogenics which might play a vital role as novel potential therapeutic agents for the alleviation of infection and inflammatory pain. Chemical compounds of a non-phenolic nature would be responsible for the observed activity. Isolation of pharmacologically active molecules through directed bioassay is now in progress.

Conflict of interest

The authors declare that there are no conflicts of interest and they have no actual or potential competing financial interests

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