Antifungal Activity in Propolis Extracts Obtained in Jujuy, Argentina

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Abstract

Propolis is a substance collected and produced by Apis mellifera from resins and buds vegetal. It has antifungal, antibacterial, antiviral and antioxidative activity. The objective of our work was evaluating in vitro activity of propolis extracts on the mycelium and spores of Aspergillus section Nigri and Penicillium chrysogenum series isolated from the cuticle of apiary-related ants. Propolis extracts from three localities in this province were used in this study. Spore germination inhibition was studied by means of immersion in propolis dilutions, whereas mycelium growth inhibition was observed by comparing fungal explants with those dilutions. Total flavonoid and phenolic contents in different extracts were evaluated. Significant differences were seen among propolis dilutions, being 0.3 and 0.03 g/mL concentrations the most effective ones. Propolis controls fungi coming from hive-related ants that may affect honey production.

Keywords — *propolis, antifungal, Aspergillus Niger group, Penicillim chrysogenum group*

I. INTRODUCTION

In 2005 and 2006, Argentina became the second largest honey producer worldwide, as 80% of crops are pollinated by Apis mellifera [1]. Beekeeping is currently threatened by environmental and sanitary factors that cause colony mortality and affect production. However, there are fungi and bacteria that cohabit with bees and are harmful to them [2].

Propolis is made by bees from resinous exudates, buds and sprouts they collect and transport to the hive. The compound gets a resinous appearance, its taste is bitter, and it features a variety of colors from yellow to green, reddish and brown. It is credited for its antibacterial, antifungal, antiparasitic and antiviral activity [3, 4, 5, 6, 7], in relation to its phenolic compound content, especially flavonoids [8, 9]. Its chemical composition is really complex, and it comprises more than 160 active components, including phenols, flavonoids, benzoic acid, caffeic acid and their by-products [10, 11]. Biological activity, which is attributed to the synergic action of several substances [12, 13, 14], depends on vegetation, geographical area, season of the year, collection method and bee species.

In the hives, bees use propolis to seal openings, cover cells, and provide asepsis and protection. However, some ant species violate this hermetic environment and enter the hive, and they may develop a dominant behavior allowing them to colonize degraded areas and cause a significant environmental impact and economic loss. Beekeeping is one of the productive sectors most severely affected by these insects. For this reason, ants are considered one of the main natural enemies of *A. mellifera* [15].

In the area of Valles Templados (for its meaning in Spanish, temperate valleys) in Jujuy province, the presence of six species of ants was registered inside beehives: *Camponotus substitutus*, *Camponotus mus*, *Acromyrmex hispidus*, *Solenopsis* sp., *Linepithema humile* and *Crematogaster* sp., which did not cause beekeepers an economic damage [16]. On the ground, these ants are extremely exposed to the spread of disease and they tend to carry fungi and bacteria on their cuticles [17].

The presence of *Penicillium*, *Fusarium* and *Aspergillus* has been shown in the bees' digestive tract, as well as in honey and other products made by these insects. Some species tend to produce mycotoxins that are harmful both for humans and animals [18, 19]. The purpose of our work was to evaluate in vitro the antifungal activity of propolis extracts on mycelium and spores from isolated fungi on the cuticle of ants associated with Jujuy apiaries.

II. MATERIALS AND METHODS

A. Fungi

Ant specimens of Camponotus mus and Linephitema humile species, which were inoculated in Sabouraud dextrose agar, were obtained from the inside of A. mellifera hives. Colonies identified as Penicillium chrysogenum series and Aspergillus section Nigri [19, 20] were isolated.

B. Propolis

It was obtained through special grids during March, April and May. These grids were placed at random in hives in Los Nogales, Severino and

Tilquiza localities in Jujuy province. Samples were kept in glass jars away from light. A stock solution was prepared with 30 g of propolis in 100 mL of alcohol at 96%, and 1/10 and 1/20 dilutions based on said stock solution.

C. Antifungal activity

To determine propolis action on spore germination, a 106 spore suspension was immersed in different propolis concentrations for 24, 48 and 72 hours, using water as control. Later, malt extract17 was inoculated in agar in triplicates and the plates were incubated at 30 ºC for 5 days.

Activity on mycelium was carried out by comparing an explant of each fungus to paper disks with 10 µL of each propolis dilution, which were stored on plates with the same medium. The inhibition halo was measured at 5 days.

D. Total phenolic and flavonoid content analysis

Total phenolic compound content was determined by spectrophotometry based on the oxidation-reduction reaction with Folin-Ciocalteu reagent [21]; 0.5 mL of propolis ethanolic solution, 10 mL of water and 1 mL of Folic-Ciocalteu reagent were introduced in test tubes, shaken, and left to stand for 8 minutes. Later, 4 mL of Na2CO3 at 20% were added, test tubes were left in the dark for 1 hour, and the absorption factor of each sample was registered at 760 nm. The calibration curve was created using gallic acid solutions.

Total flavonoids analysis was performed by means of Liu's test [22], using a 0.5 mL portion of propolis ethanolic solution, adding 0.5 mL of AlCl3 at 2%, and then incubating it at room temperature for 1 hour. The absorption factor was measured at 540 nm. Quercetin solutions were used to create the calibration curve.

E. Statistical analysis

Results were analyzed statistically using generalized linear models with Poisson distribution, for forming colony evaluation. To compare the inhibition halo, a general linear model was presented with a mean comparison test. Tukey and Kruskal Wallis mean tests were used to analyze phenolic and flavonoid results respectively, in R software with an interphase in RStudio and an interphase in Infostat [23, 24].

III. RESULTS

Administered tests showed no colony formation in the plates inoculated with spores exposed to 0.3 g propolis/mL solution, whereas those treated with 0.03 and 0.003 dilutions presented between 2 and 5 colonies. Control plates had over 50 colonies in the same time period.

In mycelium tests, solutions of 0.3 and 0.003 g propolis/mL revealed inhibition halos between 4.4 and 15.3 mm depending on concentration and propolis sample (table 1).

Table 2 portrays mean values of total phenolic and flavonoid content with the corresponding standard deviation, and, for each sampling site, it shows in letters the results of the statistical tests applied. In the extracts analyzed, total phenolics ranged between 33.4 mg GAE/g of EEP and 53.5 mg GAE/g of EEP. As regards flavonoids, the lowest registered value obtained was 12.8 mg QE/g of EEP corresponding to Severino extracts, and the highest was 15.4 mg QE/g of EEP in Los Nogales extracts.

Statistical analysis revealed significant differences among propolis dilutions ($p>0.05$), where 0.3 g propolis/mL solutions showed larger inhibition halos as compared to Penicillium chrysogenum series and Aspergillus section Nigri. Inhibitory activity in apiary propolis differed among the different localities under study (figure 1); similarly, results (figure 2) showed a difference in inhibitory activity in the two fungi species. Total phenolic content quantification in the different sites showed significant differences ($p<0.05$; $p<0.0001$; g.1.2), and so did total flavonoid quantification ($p<0.05$; $p<0.0036$; g.1.2).

TABLE I

Inhibition halos in millimeters (mm) of the three propolis dilutions from three localities in Jujuy.

Concentration g/mL	Village	Fungus	24 hs	48 hs	72 hs	96 hs	120 hs
0.3	Severino	*Penicillium chrysogenum* series	5.4	12.2	14.0	14.0	14.0
0.03			8.3	13.5	15.4	15.4	15.4
0.003			4.7	6.1	6.8	6.8	6.8
0.3	Tilquiza		5.0	8.0	13.5	13.5	13.5
0.03			5.3	7.1	7.1	12.3	12.3
0.003			4.4	4.8	6.9	6.9	6.9
0.3	Nogales		7.3	14.8	15.3	15.3	15.3
0.03			5.7	5.7	13.5	13.5	13.5
0.003			4.8	4.8	7.7	7.7	7.7
0.3	Severino	*Aspergillus* section *Nigri*	9.3	9.9	9.9	12.3	12.3
0.03			9.8	9.8	11.2	12.8	12.8
0.003			6.5	6.6	7.3	7.3	7.3
0.3	Tilquiza		8.8	9.5	11.3	11.3	11.3
0.03			9.2	10.1	11.7	11.7	11.7
0.003			7.1	6.2	6.4	6.4	6.4
0.3	Nogales		7.8	9.6	12.4	12.4	12.4
0.03			7.7	8.6	11.9	11.9	12.0
0.003			6.0	6.9	7.5	7.5	7.5

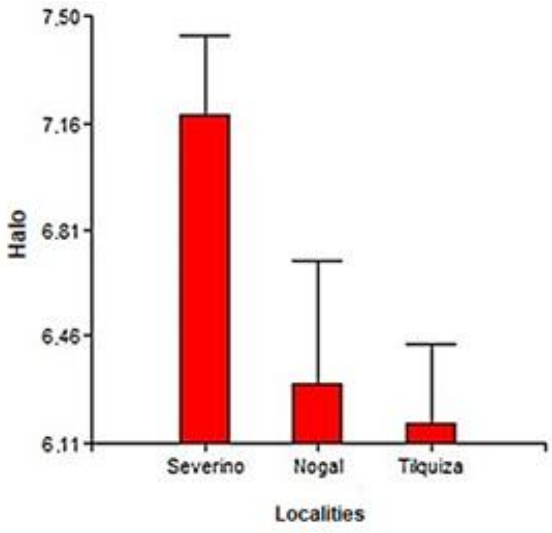

Figure 1. Average inhibition halo of propolis (mm) from 3 localities (Severino, Los Nogales and Tilquiza) in Valles Templados in Jujuy.

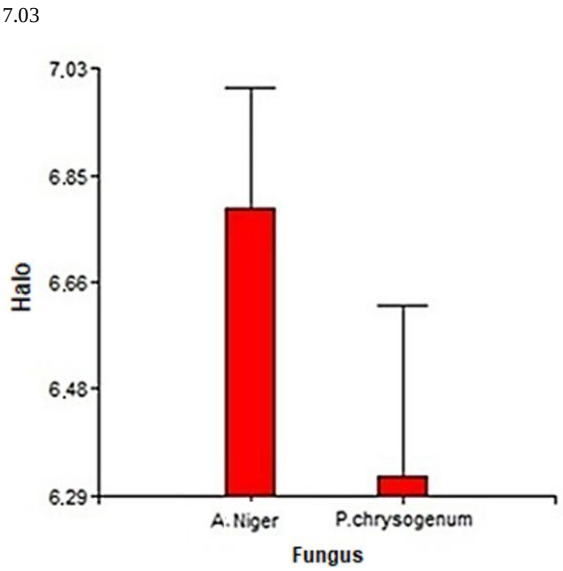

Figure 2. Average inhibition halo of propolis (mm) against *Aspergillus* section *Niger* and *Penicillum chrysogenum* series.

TABLE III

Total phenolic and flavonoid contents of propolis samples from three localities in Jujuy.

Sampling sites	Total Phenolic Content (% p/p) ± SD	Kruskal Wallis Test	Total Flavonoid Content (%P/P) ± SD	Tukey Test
Severino	33.48 ± 0.24	B	12.83 ± 0.13	A
Nogales	43.22 ± 1.17	AB	15.42 ± 0.03	B
Tilquiza	53.6 ± 1.76	A	21.14 ± 0.04	C

***Values represent the mean of three repetitions ± the standard deviation (SD).**

IV. DISCUSSION

Propolis extracts affect the growth of Alternaria alternata, Ascosphaera apis, Aspergillus flavus, Aspergillus Niger, Aspergillus versicolor, Botrytis cinérea, Candida albicans, Penicillium digitatum, Penicillium chrysogenum, Plasmopara vitícola, Saccharomyces cerevisiae, Trichoderma viridae, and others [2, 4, 25, 26, 27]. Likewise, this study revealed inhibition of Penicillium chrysogenum series and Aspergillus section Nigri spores, which is consistent with the results obtained for spore inhibition by other authors [28], who used propolis from a very different origin, with a large inhibition variability per country due to flora diversity.

Mycelium inhibition results evidenced antifungal action by means of halo presence. Similar data were registered by García et al. [29] with ethanolic extracts of propolis in Candida albicans inhibition.

The propolis solutions used showed effectiveness to inhibit the fungi under study, where spore and mycelium development were lower in the more concentrated solutions. This bioactivity is associated with phenolic content, such as flavonoids and phenolic acids [10, 11]. The chemical composition in propolis extracts varies according to location and the existing botanical species in each region, which influences propolis effectiveness [12, 13, 14].

In Argentina and Brazil, phenols and flavonoids are considered quality indicators for propolis production [30, 31]. When determining total phenolics, the extracts under study showed high values of phenolic content with an average 43.41 mg GAE/g of EEP, consistent with data reported by Palomino and Penadillos [32, 33]. As regards flavonoid quantification, the results are similar to those registered in ethanolic extracts from Peru [34]. The results obtained show a high level of biologically active compounds, such as phenols and flavonoids, in the extracts under study.

V. CONCLUSIONS

Propolis extracts from Jujuy apiaries present antifungal activity against fungi spores and mycelium isolated on the surface of hive-related ants. This is an indication that it is possible to use propolis to control fungi that may be harmful for apiculture.

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