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SHORT COMMUNICATION

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A potential role of tannins in the control of American foulbrood

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Abstract

Aim of study: To evaluate the use of tannins extracts in the control of the American foulbrood pathology and to investigate if these extracts present levels of toxicity on *Apis mellifera.*

Area of study: Paenibacillus larvae strains C1 and C2 were from Balcarce, province of Buenos Aires, strain C6 from Rio Cuarto, in Cordoba province and strain C9 from Concordia in Entre Rios province. Bees larvae used for toxicological assays were collected in Santa Paula experimental apiary, Mar del Plata (belonging to the Centro de Investigación en Abejas Sociales (CIAS-IIPROSAM) from UNMdP.

Material and methods: The minimal inhibitory concentration (MIC) of five different tannin extracts were obtained by agar diffusion method on four *P. larvae* strains; using the MIC value, the toxicity test on *A. mellifera* larvae was performed afterwards.

Main results: The MIC value was in the range of 6.9 to 898.6 µg/mL. Three tannin extracts did not show toxicity against bee larvae; however, those that were fed with the latter showed a significant increase in weight.

Research highlights: Three tannins extracts showed a good antimicrobial activity against *P. larvae* and they did not show toxicity against bee larvae.

Additional key words: *Paenibacillus larvae*; antimicrobial activity; honeybee.

Abbreviations used: MIC (minimal inhibitory concentration); MYPG (mannitol egg yolk polymyxin agar)

Authors' contributions: The three authors participated in all stages of the work, including the conception and design of the research, the revision of the intellectual content and the drafting of the paper. All authors read and approved the final manuscript.

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Introduction

Antimicrobial resistance is a phenomenon of adaptation of bacteria to resist antimicrobial agents. The overuse of antibiotics has led to the evolution of new strains of bacteria that are resistant or even more lethal than the susceptible strain. This fact leads to many health-related problems (Iwu *et al.*, 1999).

In order to avoid the resistance process generated by antibiotics, plant compounds with known antimicrobial activity have been widely used, *i.e.* quinones, phenols, alkaloids, flavonoids, terpenoids, essential oil, tannins, lignans, glucosinolates and others secondary metabolites (Chandra *et al.*, 2017).

Tannins have been studied to have a defence role against predation by animals (Isaza, 2007). These water-soluble compounds with molecular weight ranging between 500 and 5000 Da can precipitate proteins and alkaloids (Sieniawska & Baj, 2017). Some studies suggested that the microbial cell membrane is the main site of the biocidal action of tannins (McAllister *et al.*, 2005; Liu *et al.*, 2013) through cell aggregation and alteration of cell membranes and functions. In general, the antimicrobial activity of tannins has been reported to be higher against

gram-positive than against gram-negative bacteria (Smith & Mackie, 2004).

American foulbrood disease is the most serious disease of bee larvae caused by the gram-positive bacteria *Paenibacillus larvae*, which can form spores (Genersch *et al.*, 2006). Beekeepers use synthetic antibiotics such as oxytetracycline hydrochloride (Genersch, 2010), as well as the common practice of burning infected hives (Hansen & Brødsgaard, 1999); although this practice is not accepted by beekeepers due to economic loss. However, the use of these compounds causes serious problems such as the presence of chemical residues in the commercial products of the hive and the appearance of resistant strains of *P. larvae* (Miyagi *et al.*, 2000; Evans & Lopez, 2004). In this context, the development of alternative natural methods to control the disease is an important long-term strategy.

The aim of this study was to evaluate the antimicrobial activity of five commercial tannin extracts against *P. larvae* strains and their toxicity against *Apis mellifera* adults and larvae.

Material and methods

Chemicals

Tannins extracts (TAN´ACTIV C (from *Castanea sativa*), TAN´ACTIV R (from *Quercus robur*), TAN´ACTIV GTC/E (from *Rhus semialata*), TAN´ACTIV QS SOL (from *Schinopsis lorentzii*) and TAN´ACTIV T80 (from *Caesalpinia spinosa*)) were obtained from SilvatTeam S.p.a. (San Michele Mondovi, Italy). For the five commercial tannin extracts, 0.01 g of each were weighed, then they were dissolved in distilled water, and finally they were filtered through a 0.45 micron filter; and four serial dilutions were made, obtaining the following concentrations: 10000, 5000, 2500, 1250 and 625 µg/mL.

Biological material

Paenibacillus larvae strains were isolated from honey combs of beehives exhibiting clinical symptoms of American foulbrood, located in the provinces of Buenos Aires, Córdoba and Entre Ríos in Argentina. Strains C1 and C2 were obtained from Balcarce, province of Buenos Aires (37°52′S-58°15′W), strain C6 from Río Cuarto, in Cordoba province (33°08′00″S 64°21′00″O) and strain C9 from Concordia in Entre Ríos province (31°23′32″S 58°01′01″O). Bacterial strains were grown and maintained according to Nordström and Fries (1995). All strains used are genetically the same; all of them are ERIC 1 (Giménez.Martínez *et al.*, 2019).

Bees larvae used for toxicological assays were collected in Santa Paula experimental apiary, Mar del Plata (National Route 226, Km 10, Argentina) (37°55′48″S 57°40′59″O), belonging to the Centro de Investigación en Abejas Sociales (CIAS-IIPROSAM) from UNMdP.

Determination of minimal inhibition concentration (MIC)

To obtain the minimum inhibitory concentration (MIC), the agar diffusion analysis was performed on mannitol egg yolk polymyxin agar (MYPG) plates. The diameter of the inhibition zone reflects the susceptibility of the strain, and its size depends on the nature of the diffusion of the extract through the agar layer. This is characterized by two diffusion concepts proposed by Bonev *et al.* (2008): (i) free diffusion model, based on the assumption that the antimicrobial drug diffuses freely in the solid agar; and (ii) dissipative diffusion model, which takes account of extract loss through interactions with the agar matrix.

For the antimicrobial assay for each extract, five concentrations were prepared: 10000, 5000, 2500, 1250 and 625 µg/mL, it was made for triplicate. When obtaining the MIC value, the average value of the measurement of the inhibition halos was taken.

Distilled water was used for the negative control, because it was used to make the dilutions of the commercial tannin extracts.

Toxicity analysis on honeybee larvae

For the toxicity test on *A. mellifera* larvae, these were fed with a diet containing 50% of royal jelly, obtained from a commercial supplier, and 50% of sugar solution composed by yeast extract, D-glucose (Sigma-Aldrich, St. Louis, MO, USA), and D-fructose (Fluka, St. Gallen, Switzerland). Each larvae was fed daily with the volumes and compositions according to Aupinel *et al.* (2005). All larvae were standardized in the larval stage L1, and received a total of 160 µL diet during the six-day rearing period. On day four (stage L4) the MIC value obtained for each extract was administered on the feed, and then continued feeding with a normal diet until the end of the trial. In each independent experiment, six groups of 32 larvae were feeding; one with the standard diet (control), the other five was fed with the MIC obtained for each commercial tannin extract (OECD, 2013). The plates were kept dark at a temperature between 34 and 35 °C, in an incubator. Once the trial was finished, all the larvae that survived were weighed in analytical balance to make a comparison.

Statistics

To analyse the antimicrobial activity of the extracts, the measurements of the inhibition halos were recorded and the MIC value was obtained with their average value. For the toxicity assay of honeybee larvae, a dose-response analysis was performed. Once the assay was finished, the larvae that survived were weighed in analytical balance to obtain an average weight and their respective standard deviation, and a t test was performed to compare the means obtained. The entire assay was performed using GraphPadPrism v 7.00 for Windows, GraphPad Software, La Jolla, CA, USA (www.graphpad.com).

Results and discussion

The MYPG plates loaded with tannin extract ranging in concentrations from 625 to 10000 µg/mL, showed significant antimicrobial activity compared to the MYPG plate with distillate water (negative control), which did not display any inhibitory effect (Table 1). The inhibition halo increased with the tannin extract concentration. The corresponding R^2 values of linear regression were higher for the free diffusion model (Table 1). Thus, diffusion of tannin extract through the solid agar overlay can be considered as a free diffusion process that yields the best suit model for the determination of MICs.

According to Michielin *et al.* (2009), the extracts can be classified as antimicrobial agents based on the MIC values. Duarte *et al.* (2007) and Wang *et al.* (2008) classified the extracts and natural compounds as: strong inhibitors (MIC<500 µg/mL); moderate inhibitors (MIC between 600 and 1500 µg/mL); weak inhibitors (MIC>1600 µg/mL). This classification is useful to detect the potential biological activity of various plant materials. Based on this the extracts ACTIV C, ACTIV R, ACTIV GTC/E and ACTIV QS SOL would be strong inhibitors, but the extract TAN´ACTIV T80 would be a moderate inhibitor.

In the case of larval toxicity tests, the ACTIV C, ACTIV R and T80 extracts did not show mortality greater than 20%, in the other case the GTC/E and QS-SOL extracts were very toxic for honeybee larvae (Fig. 1).

Tannins have traditionally been considered antinutritional compounds; however, current studies have shown

Table 1. MIC values obtained after agar diffusion analysis for commercial tannin extracts.

Tannin extracts	Free diffusion model		Dissipative model	
	MIC (µg/mL)	R^2	MIC (µg/mL)	R^2
TAN´ACTIV QS SOL	6.90	0.94	119.48	0.94
TAN´ACTIV C	30.36	0.86	253.94	0.84
TAN´ACTIV GTC/E	89.74	0.97	423.58	0.94
TAN´ACTIV R	232.82	0.89	876.45	0.87
TAN´ACTIV T80	898.60	0.85	2068.74	0.82

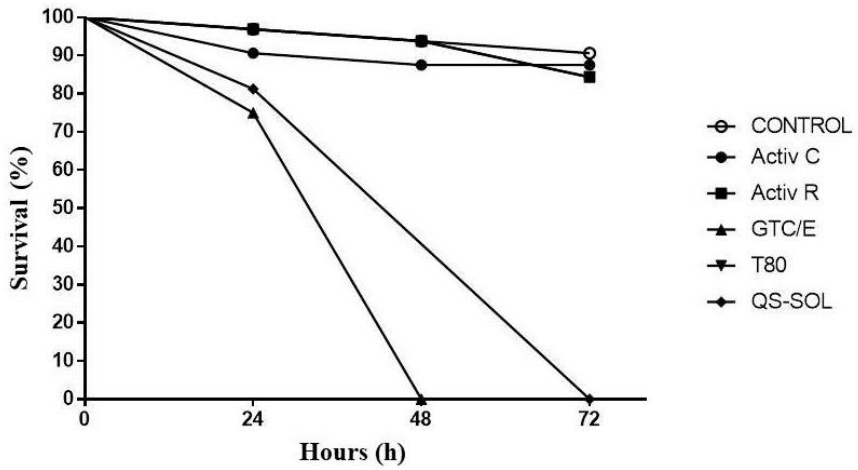

Figure 1. Survival analysis of *A. mellifera* larvae against commercial tannin extracts. The x-axis indicates the post-exposure hours (L4). The Activ T80 and Activ R extracts showed a similar mortality and are overlapped in the graph

that their properties can be both beneficial or negative depending on their chemical structure and the amounts in which they are naturally found (Yuan *et al.*, 2020). Tang *et al.* (2014) suggested that the negative effect of tannic acid (a commercial preparation of plant tannins) on the activity of glutathione s-transferase depends on the concentration of this compound. In turn, Yuan *et al.* (2020) observed that diets containing 3% of tannic acid significantly reduced the development index in *Hyphantria cunea* larvae. These results matched with the high mortality presented by ACTIV GTC/E and ACTIV QS-SOL extracts in our study.

On the other hand, the ACTIV C, ACTIV R and T80 extracts did not show toxic effect on *A. mellifera* larvae, and after analyzing the weight of the larvae fed with these extracts it was observed that they presented higher weights than those of the control group: 0.1021; 0.0982 and 0.0951 g respectively, and it was statistically different (p<0.05) (Fig. 2). Bernklau *et al.* (2019) found that low concentrations of gallic acid (a component present in tannins) were effective in both promoting tolerance to pathogens and increasing the longevity of bees.

Mao *et al.* (2015) analysed the effect of diets supplemented with p-coumaric acid (a secondary metabolite component of pollen and honey) on the Hippo signalling pathway, which is responsible for organ and tissue development in larvae (Schneck, 2004; Li *et al.*, 2019). Therefore, it could be inferred that a supplemented diet with secondary metabolites such as tannins could be positively regulating the expression of the signalling pathways responsible for larval development, adding this to its bactericidal activity.

After analysing our results, some assertions can be made on commercial tannin extracts: 1) they showed antimicrobial activity against *P. larvae*; 2) they influenced larval development by activating some metabolic pathways. Therefore, including these compounds in a control strategy would not only avoid the problems caused by synthetic antibiotics, but also provide beneficial effects in several aspects on the health of bees.

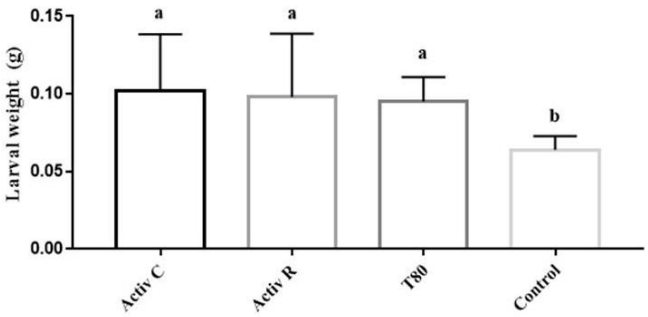

Figure 2. Average values of the larval weights of *A. mellifera* fed with commercial tannin extracts. Different letters indicate significant differences between treatments (p<0.05).

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