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SHORT COMMUNICATION

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Antimicrobial activity of indoleacetic, gibberellic and coumaric acids against *Paenibacillus larvae* and its toxicity against *Apis mellifera*

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Abstract

Aim of study: To explore three isolated phytomolecules: indoleacetic acid (IAA), gibberellic acid (GA), and the secondary metabolite p-coumaric acid (CUM): (1) evaluating their toxicity against *Apis mellifera* larvae and adults under controlled conditions in the laboratory; (2) searching for antimicrobial activity against *Paenibacillus larvae*.

Area of study: Honey bee larvae and adults were collected from the experimental apiary of the "Centro de Investigación en Abejas Sociales (CIAS)" (-37.9348798, -57.682817), Institute of the National University of Mar del Plata (UNMdP), Argentina.

Material and methods: *Paenibacillus larvae* strains were isolated from beehives from different provinces of Argentina (Buenos Aires, Córdoba and Entre Ríos) showing clinical symptoms of the American foulbrood. All strains (S1, S2, S3, S4) were genotypically identified using PL5 and PL4 primers and characterized as genotype ERIC1. Then standard essays were performed to determined toxicity of phytomolecules in honey bees and antimicrobial activity through the broth microdilution method.

Main results: The diet with GA, IAA and CUM did not present toxic effects in larvae or adult bees, and only CUM showed antimicrobial activity against P. larvae. In this study, we obtained in vitro values of MNIC (minimum non-inhibitory concentration) of 500 µg mL^{-1} and a MIC (minimum inhibitory concentration) of 650 µg mL^{-1} for CUM.

Research highlights: The obtained results remark its potential as a natural alternative for the control of *P. larvae*, avoiding the problems generated by the use of synthetic antibiotics such as the resistance phenomena and the contamination of hive's products.

Additional key words: American foulbrood; honey bees.

Abbreviations used: AFB (American foulbrood); CUM (p-coumaric acid); GA (gibberelic acid); IAA (indoleacetic acid); MIC (minimum inhibitory concentration); OTC (oxytetracycline hydrochloride); MNIC (minimum non-inhibitory concentration).

Authors' contributions: Conceived and designed the experiments: NS, PGM, GM, PN and MM. Performed the experiments: NS, PGM, GM and MPM. Analyzed the data: NS and PGM. Contributed reagents/materials/analysis tools: SF, ME, MM and LL. Wrote the paper: NS, PGM, FMA, PN, LL and MM.

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Introduction

Apis mellifera colonies are threatened by different biotic and abiotic factors which compromise their fitness causing depopulation or entire colony losses (Steinhauer *et al*., 2018). Due to phenology and climate, there are times of the year where the bees' food resources are scarce (De Grandi-Hoffman & Chen, 2015). This phenomenon is enhanced by the beekeepers' management, who harvest almost all the colony's stored honey, leaving those bees with a nutritional challenge. The depletion of food reserves induces a stress in honey bee colonies, negatively affecting their health and increasing their susceptibility to agrochemicals and different diseases (Nazzi *et al*., 2012; De Grandi-Hoffman & Chen, 2015; Sánchez-Bayo *et al*., 2016).

One of the most important pathogens that affect bee health is the sporulated bacterium (gram positive) *Paenibacillus larvae*, the causative agent of the American foulbrood (AFB) (Hansen & Brødsgaard, 1999). For its control, the most effective treatments are based on the use of a broad spectrum of antibiotics, such as sulfathiazole and oxytetracycline hydrochloride (OTC). Those molecules are capable of inhibiting the growth of *P. larvae*, but in most cases, they have been wrongly used in its quantity and frequency of application, leading to the appearance of resistant strains and residues which contaminate the commercial products of the hive (Wilson, 1974; Hansen & Brodsgaard, 1999). Consequently, the use of antibiotics for AFB treatment and prevention is forbidden in several countries (Mutinelli, 2003), leading to an increasing need for natural alternatives for its control. In this venue, there are reports of a wide variety of natural control of *P. larvae* tested through *in vitro* assays, such as the use of essential oils, plant extracts, propolis, among others (Alonso-Salces *et al.*, 2016).

Plants contain an enormous variety of chemical compounds that are present in nectar, pollen and/or resins and seems to play an important role in honey bee health (Mao *et al*., 2013; Couvillon *et al*., 2015; Negri *et al*., 2015; Richardson *et al*., 2015; Erler & Moritz, 2016). Indeed, plant-derived compounds are involved in bees' "self-medication" a phenomenon defined as an individual responding to infection by ingesting ("pharmacophagy": *e.g.* honey, pollen, royal jelly) or to the nonedible hive products (pharmacophory: *e.g.* propolis, resins) (Erler & Moritz, 2016).

Mao *et al.* (2013) identified that p-coumaric acid (CUM), a phytochemical found in pollen and honey, up-regulates different detoxification and antimicrobial genes in *A. mellifera.* Accordingly, Liao *et al.* (2017) performed dietary trials with CUM (500 μgL^{-1}) and two pyrethroids insecticides (which are known to reduce the lifespan of bees), observing that this acid enhanced tolerance of both pyrethroids. Isidorov *et al.* (2017) carried out a study *in vitro* proving the antimicrobial activity of European propolis against *P. larvae,* where the GC-MS analysis of those extracts reveals the presence of some flavonoids and also phenolics components including p-coumaric acid. Nevertheless, there is a lack of evidence regarding the antimicrobial activity of CUM against *P. larvae.*

From a sanitary point of view, phytomolecules found in nectars or in pollen need to be continuously explored regarding its potential effects on bee health. Gibberelic acid (GA) and indoleacetic acid (IAA), are involved in the regulation of plants' nectar production and other functions (Aloni *et al*., 2006; Wiesen *et al.*, 2015). These phytomolecules are regulators of growth, development and pathogens resistances in plants, acting through transduction pathways (Richards *et al*., 2001; Denancé *et al*., 2013). In addition, these phytohormones are present in honey (Wang *et al*., 2017), but there are no reports of potential effects on bee health. Here, we aim to assess the potential bactericide effect of three isolated phytomolecules against *P. larvae*. For this purpose, we evaluated two main aspects: a) the toxicity of CUM, GA and IAA in adults and larvae of *A. mellifera*; and b) their antimicrobial activity against *P. larvae* through the broth microdilution method.

Material and methods

Biological material

Honey bee larvae and adults were collected from the experimental apiary of the "Centro de Investigación en Abejas Sociales (CIAS)" (-37.9348798, -57.682817), Institute of the National University of Mar del Plata (UNMdP), Argentina.

Paenibacillus larvae strains were isolated from beehives from different provinces of Argentina (Buenos Aires, Córdoba and Entre Ríos) showing clinical symptoms of the American foulbrood (Hansen & Brødsgaard, 1999). All strains (S1, S2, S3, S4) were genotypically identified using PL5 and PL4 primers (Piccini *et al*., 2002) and characterized as genotype ERIC1 (Giménez-Martínez *et al*., 2019).

Phytomolecules

The standards of GA, IAA and CUM were provided by Sigma Aldrich. Analytical grade alcohol (100%

purity) was used to prepare the stock. The stock solutions concentrations were 10 mM GA, 50 mM IAA and 25 mM CUM.

Toxicity of phytomolecules in honey bees

In vitro experiments were conducted in the CIAS laboratory at the UNMdP. For CUM, GA and IAA toxicity bioassays of adult honey bees, we followed the methodology described in Porrini *et al*. (2010). For this, combs-sealed brood from healthy colonies were carried to the laboratory within insulated containers and placed into an incubator (30 ± 0.79 °C, 60 ± 3.3% HR). Newly emerged bees were removed from the combs. Each treatment consisted of 30 adult bees randomly confined within acrylic boxes of 8 cm × 15 cm, using a total of three replica (N=90 individuals per treatment). The phytochemicals were administered *ad libitum* through a solution made of powdered sugar and glucose (candy), which was replaced daily. Mortality was recorded daily for 5 days (120 h). Adult bees were kept under incubator conditions during the experiment of toxicity. For honey bee larvae, the *in vitro* breeding trials were carried out according to the methodology proposed in Aupinel *et al*. (2005). We used 30 bee larvae per treatment in each of the three replica, involving a total of 90 (N=90) individuals per treatment. The bee larvae were incubated at 34 ± 0.5 °C and 90% RH. The phytochemicals were administered in individual doses diluted in the food during the whole feeding stage. Mortality was recorded daily for 8 days. The treatments for both growing stages of bees (adults and larvae) were grouped as follows: (i) Control (only candy or larvae diet respectively); (ii) control diet supplemented with the solvent (ethanol) used to do the stock solutions for the molecules tested (C Et); (iii) CUM 300/600/1200 µM; (iv) IAA 100/200/400 µM; and (v) GA 2.5/25/250 µM.

Assays of antimicrobial activity

The antimicrobial activity of the IAA, GA and CUM, were determined by the broth microdilution method on four *P. larvae* strains (S1, S2, S3, S4) within the same day (in triplicate for each antimicrobial agent and strain) and with triplicate essays (experimental replicas) (Cugnata *et al*., 2017). First, the bacterial strains were grown and maintained on Mueller-Hinton broth, yeast extract, glucose, and sodium pyruvate (MYPGP) (Dingman & Stahly, 1983) agar supplemented with 9 mg mL^{-1} of nalidixic acid to inhibit *Paenibacillus alvei* growth, and incubated under microaerobic conditions (5–10% of CO_2, 37°C, 48 hs). Afterwards, vegetative cells of *P. larvae* (previously cultivated) were suspended in sterile peptone water (peptone 0.1 % (w/v) and sodium chloride 0.85 % (w/v)) to a final optical density at 600 nm of 0.1 using a UV-VIS spectrophotometer Spectrum SP-1103 (Spectrum Instr. Co. Ltd., Shanghai, China). Brain-heart infusion (3.7 %, w/v) was used as growth media during the broth microdilution assay. *Paenibacillus larvae* growth was detected using resazurin sodium salt. We evaluated in a range of concentrations between 15.6 to 1000 µg mL^{-1} against *P. larvae* strains and determined two threshold concentration for each phytomolecule: the minimum inhibitory concentration (MIC) and the minimum non-inhibitory concentration (MNIC) of *in vitro* bacterial growth (De Graaf *et al.*, 2013). Positive and negative controls (*P. larvae* strains viability and water respectively) were used.

Statistical analyses

Kaplan-Meier survival analyses (Stalpers & Kaplan, 2018) were performed in order to compare survival curves (number of living bees *vs* time) for each treatment. The non-parametric Log-rank test was performed to determine differences between survival curves. This method builds up curves of chi-square values by comparing the observed and expected number of deaths (GraphPad Prism 5.0).

Results and discussion

In this study, we explored the beneficial properties of p-coumaric acid (CUM) and other previously unexplored phytomolecules, the phytohormones indole acetic (IAA) and gibberellic (GA) acids, on bee health. First, we determined the toxicity of these molecules in larvae and adult bees, in a range of concentrations that include those found naturally in plants (Aloni *et al.*, 2006; Wiesen *et al.*, 2015) and honey (Wang *et al*., 2017). Our results indicated that these molecules are not toxic to adult bees feed *ad libitum* for 5 days (Long-rank test, χ^2=14.14; df =10; p=0.1668) (Fig. 1A) or bee larvae survival, until 8 days *in vitro* (Long-rank test, χ^2=3.741; df=10; p=0.9583) (Fig. 1B). This is the first condition in the development of anti-parasite treatments to be used in beekeeping (*e.g*. Maggi *et al.*, 2013).

Secondly, our search of antimicrobial activity in IAA, GA and CUM by the broth microdilution method on four *P. larvae* strains (S1, S2, S3, S4) suggested that IAA and GA are not suitable antimicrobial molecules in the range from 15.6 to 1000 µg mL^{-1} to be used

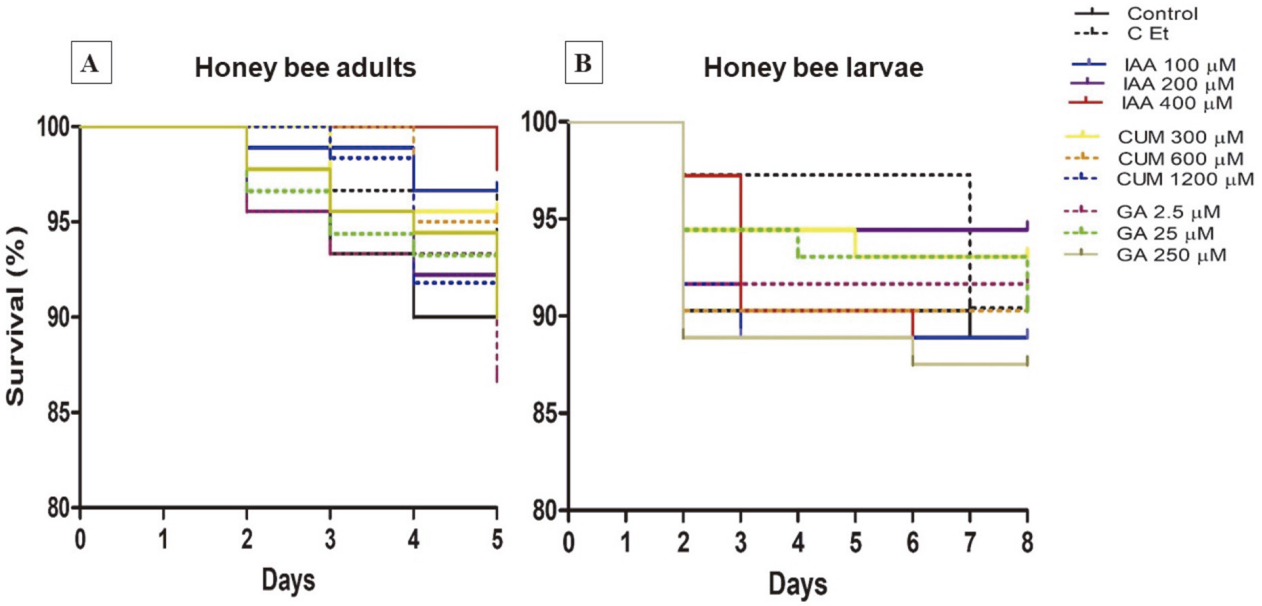

Figure 1. Kaplan-Meier plot for honey bee survival. The diet with GA (gibberelic acid), IAA (indoleacetic acid) and CUM (p-coumaric acid) did not present toxic effects in larvae and adult bees. A: Survival of adult bees (N=90 per treatment) fed *ad libitum* during 5 days (Longrank test, *p*=0.1668). B: Survival of bee larvae (N=90 per treatment) reared *in vitro* during 8 days (Longrank test, *p*=0.9583). Controls involved adult and larvae bees fed only by candy or larvae diet respectively and control diet supplemented with the solvent (ethanol) used to do the stock solutions for the molecules tested (C Et).

against *P. larvae*. Only CUM showed antimicrobial activity against *P. larvae*, obtaining a MIC equal to 650 μg mL^{-1} and MNIC to 500 μg mL^{-1} (for all *P. larvae* isolates) (Table 1).

Similar to Tunçel & Nergiz (1993) results, CUM showed antibacterial activity resembling different hydroxycinnamic acids respect their effect against gram-positive bacteria (*Bacillus cereus* and *Staphylococcus aureus*) and gram-negative bacteria (*Escherichia coli* and *Salmonella typhimurium*) showing similar MIC values (400 to 600 μg mL^{-1}). In the study of Isidorov *et al.* (2017), all propolis extracts tested inhibited the growth of *P. larvae*, with a MIC of 7.8 to 62.4 μg mL^{-1}. But this antimicrobial activity was associated with a very complex mixture of compounds present in the diethyl ether extracts of propolis (on the chromatograms of nine samples of propolis), where 278 organic components were recorded, among them, monoglycerides and diglycerides of CUM.

Table 1. Antimicrobial activity of three phytomolecules (CUM: p-coumaric acid; GA: gibberellic acid; IAA: indole acetic acid) against *Paenibacillus larvae*. The bactericidal activity of each molecule was evaluated in a range of concentrations between 15.6 to 1000 μg mL^{-1}. The results were the same for the four *P. larvae* strains used (S1, S2, S3, S4)

Phytomolecule	MIC (μg mL^{-1})	MNIC (μg mL^{-1})
CUM	650	500
GA	none	-
IAA	none	-

MIC: minimum inhibitory concentration. MNIC: minimum non-inhibitory concentration.

Similar MICs values were found between our MIC results of CUM (500 μg mL^{-1}) and other organic compounds (all assessed by broth microdilution method). For instance, MIC value for essential oils of *Artemisia absinthium* was 416 μg mL^{-1}; for *Aloysia polystachia* was 700-800 μg mL^{-1} (Fuselli *et al.*, 2008). Also, individual propolis compounds have been tested such as benzyl ferulate and pentenyl ferulate, with MIC values of 500 μg mL^{-1} (Biliková *et al.*, 2013). However, there are other organic compounds with MIC values against *P. larvae* better and closer to the synthetic antibiotic oxytetracycline hydrochloride (0.5-5 μg mL^{-1}; Gende *et al.*, 2010), such as cinnamon (*Cinnamomum zeylanicum*) essential oil (CEO): 41.67 ± 19.17 μg mL^{-1} (Gende *et al.*, 2010a), or the individual propolis compound Pinocembrin: 62.5 μg mL^{-1} (Biliková *et al.*, 2013).

In our study, we obtained *in vitro* values of MNIC for CUM (500 μg mL^{-1}) that remarks its potential as a natural alternative for the control of the American foulbrood, avoiding the problems generated by the use of synthetic antibiotics (resistance phenomena and bee product contamination). In addition to our results, previous reports also demonstrated that in the presence of pesticides, the CUM somewhat enhanced different mechanism of detoxification in honey bees (Mao *et al.*, 2013; Liao *et al.*, 2017). Thus, we found evidence suggesting that CUM is a promising molecule, which could perform either as a pharmacophagy-related compound and/or as a pharmacophory-like substance. Future studies should test CUM effects on *A. mellifera* colonies in order to improve current knowledge about their integrated management.

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