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Efficacy of natural propolis extract in the control of American Foulbrood

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Abstract

Paenibacillus larvae is the causative agent of American Foulbrood (AFB), a severe disease that affects larvae of the honeybees. Due to the serious effects associated with AFB and the problems related to the use of antibiotics, it is necessary to develop alternative strategies for the control of the disease. The aim of the present work was to evaluate the effect of a propolis ethanolic extract (PEE) against *P. larvae* and its potential for the control of AFB. *In vitro* activity of PEE against *P. larvae* isolates was evaluated by the disk diffusion method and the minimum inhibitory concentration (MIC) was determined. Toxicity for honeybees was evaluated by oral administration of PEE and its lethal concentration was assessed. Lastly, colonies from an apiary with episodes of AFB on previous years were divided into different groups and treated with sugar syrup supplemented with PEE by aspersion (group one), sugar syrup by aspersion (group two), fed with sugar syrup supplemented with PEE (group three) and fed with sugar syrup only (group four).

All isolates were sensitive to PEE and the MIC median was 0.52% (range 0.32–0.64). PEE was not toxic for bees at least at 50%. Field assays showed that 21 and 42 days after the application of the treatments, the number of *P. larvae* spores/g of honey was significantly lower in colonies treated with PEE compared to the colonies that were not treated with PEE. To our knowledge, this is the first report about the use of propolis for the treatment of beehives affected with *P. larvae* spores.

Keywords: American Foulbrood; *Paenibacillus larvae*; Propolis ethanolic extract; Honeybee disease; Natural treatments

1. Introduction

American Foulbrood (AFB) is one of the most severe bacterial diseases that affects larvae of

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honeybee *Apis mellifera*, causing a decrease of bee population and honey production. The causative agent is *Paenibacillus larvae*, a gram positive and spore-forming bacterium that is distributed worldwide (Genersch et al., 2006).

A common strategy for the prevention and treatment of affected colonies is the use of antibiotics, particularly oxytetracycline hydrochloride (OTC) (Hansen and Brodsgaard, 1999). However, several problems may be associated with its extended use. Chemical residues can persist in honey affecting its quality for human consumption while application of antibiotics may reduce the lifetime of bees and raise the risk of resistant strains emergency (Thompson et al., 1960; Martel et al., 2006). The presence of *P. larvae* OTC-resistant strains has been reported so far in Argentina, the United States, Italy, New Zealand and United Kingdom (Alippi, 1996; Miyagi et al., 2000; Evans, 2003). In Uruguay we did not find OTC-resistant *P. larvae* isolates among a collection of local isolates although 22% of them resulted resistant to sulfisoxazole, another antimicrobial drug frequently used for the control of AFB (Piccini and Zunino, 2000). Due to the serious effects associated with AFB and the problems related to the use of antibiotics, it is necessary to develop new strategies for the control of the disease.

Propolis is a natural product derived from plant resins and produced by honeybees to seal the walls and entrance of the hive and contributes to protect the colony against different pathogens (Ghisalberti, 1979). It has several biological properties such as antibiotic, antifungal, antiviral, anti-inflammatory activity (Manolova et al., 1985; Marcucci, 1995; Drago et al., 2000; Tichy and Novak, 2000; Santos et al., 2003).

Propolis has empirically been used in apiculture for the prevention of AFB and other honeybee diseases for years. As it is present in the hives in a solid state that cannot be consumed directly by the honeybees, beekeepers usually use ethanolic extracts. However, systematic studies about their potential effects were lacking.

The aim of the present work was to evaluate the use of a propolis ethanolic extract (PEE) as a natural alternative for the control of AFB.

2. Materials and methods

2.1. *P. larvae* isolates

Fifty *P. larvae* isolates were randomly selected from the collection of the Department of Microbiology, IIBCE. These isolates were obtained from worker bees, larvae and honey from different provinces of Uruguay between 1999 and 2002. Four isolates of *P. larvae* from Argentina were also used (Table 1). *P. larvae* isolates corresponded to the different genotypes observed in previous works (Antúnez et al., 2007). Isolates were routinely grown on J medium (Hornitzky and Nicholls, 1993).

2.2. Preparation of PEE

A concentrated propolis solution was prepared homogenizing 400 g of propolis in 1 l of ethanol 96%, and incubated for 10 days at 22–25 °C. Then, it was

Table 1
P. larvae isolates collection and propolis MIC

Number of isolates	Origins	Genotype	MIC (%)
32	Lavalleja, Colonia, Durazno, Flores, Florida, Maldonado, Paysandú, Río Negro, Soriano, Salto, San José, Treinta y Tres, Tacuarembó (Uruguay) and Buenos Aires (Argentina)	ERIC I BOX A	0.56
21	Canelones, Colonia, Durazno, Flores, Florida, Maldonado Río Negro, Salto, San José, Tacuarembó (Uruguay) and Río Negro (Argentina)	ERIC I BOX C	0.64
1	Buenos Aires (Argentina)	ERIC II BOX B	0.32
1	Buenos Aires (Argentina)	ERIC III BOX PLP	0.62

filtered through paper filter and incubated until ethanol evaporated and the product obtained a honey-like consistence. This extract was diluted in ethanol 96% to a final concentration of 10% of the extract to form the PEE (starting solution).

2.3. Antimicrobial susceptibility test

Susceptibility patterns of *P. larvae* isolates to PEE were assessed by the disk diffusion method following the general guidelines of National Committee for Clinical Laboratory standards (Bauer et al., 1966; NCCLS, 1986). Disks containing different PEE concentrations (100%, 10%, 1%) were used. Stock solutions were prepared in ethanol and disks used as negative controls contained ethanol only. All assays were carried out by duplicate.

2.4. Determination of the minimum inhibitory concentration of propolis

The minimal inhibitory concentration (MIC) was directly assessed by the observation of turbidity. One millilitre of the PEE starting solution was added to MYT broth, Mueller–Hinton broth supplemented with yeast extract 1.5% and 0.1 ml/l of thiamine (Gende et al., in press). It was serially diluted and 1 ml of a *P. larvae* bacterial suspension (equivalent to 0.5 McFarland) was added to each serial dilution tube with agitation. All sample tubes (as well as positive and negative controls) were incubated at $35 \pm 0.5\ ^{\circ}C$ for 48 h in order to determine MIC values. The lowest concentration of propolis that prevented bacterial growth, determined by the absence of turbidity, was defined as the MIC.

2.5. Determination of the lethal concentration of PEE on bees

Toxicity of PEE on bees was evaluated using a technique developed by (Maggi et al., in press). Bees were collected from healthy colonies from the experimental Apiary of the UNMdP, JJ. Nagera station located in Mar del Plata, placed on route 11 km 32 (S38°10′06″, W57°38′10″), Buenos Aires province, Argentina. Between 300 and 400 adult worker bees were located in special cages (16 cm × 12 cm × 6 cm) and were stabilized with queen pheromone. Ten ml of sugar syrup 2:1 (2 kg sugar in 1 l water) supplemented with PEE were placed into each box. Different PEE concentrations, between 50% and 3%, were evaluated. A negative control was performed using sugar syrup without PEE. The assay was carried out by quintuplicate. Boxes were incubated at 22 °C and 65% RH during 24 h, 48 h and 72 h. Each day dead bees were counted and discarded. At the end of the experiment, bees were sacrificed and mortality percentages were calculated.

2.6. Field experiment

The efficiency of PEE for the control of AFB on *P. larvae* naturally infected colonies was evaluated on *A. mellifera* colonies located in Apiary Scarzella, placed in Paso Severino (S34°05′58″, W56°12′53″), Florida province, Uruguay.

Thirty apparently healthy colonies (without clinical symptoms of AFB) were used. Colonies consisted of four to six brood combs and two honey combs in the brood area divided by a horizontal separation, eight to ten combs of adult bees, and 10–12 kg of honey reserves at the beginning of the assay (total honey reserves weighed approximately 15 kg). Colonies were divided into two groups of ten each (groups one and two) and two groups of five each (groups three and four), in a randomized design.

The number of *P. larvae* spores in honey samples was used as a parameter to evaluate the effect of PEE on *P. larvae*. Previous reports demonstrated the significant relationship between the number of *P. larvae* spores in honey samples and clinical symptoms of AFB, supporting the value of honey analysis for sanitary control of bee colonies (Graff et al., 2001; Antúnez et al., 2004).

Colonies were inspected and honey samples were extracted from each one, from the brood area, to ensure that it was in direct and permanent contact with nurses' bees and used to feed larvae. Honey samples were sent to the laboratory for analysis.

PEE stock was diluted in sugar syrup 1:1 (1 kg of sugar in 1 l of water) at a final concentration of 6% (concentration commonly used by beekeepers), and 50 ml were administered by aspersion over the

brood combs of each colony of the group one. As a control, 50 ml of sugar syrup 1:1 were aspersed over the brood combs of colonies of the group two. Both treatments were performed once a week, during 3 consecutive weeks after the last summer crop (April, 2006).

PEE was also diluted in sugar syrup 2:1 (2 kg of sugar in 1 l of water) at a final concentration of 6% and 2 l were used to feed each colony of the group three, and as a control, 2 l of sugar syrup 2:1 was used to feed each colony of the group four. In this case, both treatments were performed only once, after the last summer crop (April, 2006).

Twenty one, 42 and 85 days after the first application of the treatments, colonies were inspected and honey samples were extracted and sent to the laboratory for analysis.

Beehive 21, which belonged to group one (treated with PEE by aspersion) did not produce any honey, so it was eliminated from the study.

2.7. Quantification of *P. larvae* spores

For enumeration of *P. larvae* spores, 20 ml of honey (approximately 27 g) were diluted with 20 ml of sterile distilled water (SDW) and processed as described before (Antúnez et al., 2004). Spore suspensions were spread onto J plates and incubated under microaerofilic conditions (5–10% of CO_2) for 96 h and the number of *P. larvae* colony forming units (CFU)/g of honey was determined. Three to five colonies from each sample were selected for initial identification assessing colony shape and margins, microscopic characterization and catalase production (Alippi, 1992). *P. larvae* specific confirmation was carried out by PCR, using the primers PL 5 (5′-CGAGCGGACCTTGTGTTTCC-3′) and PL 4 (5′-TCAGTTATAGGCCAGAAAGC-3), which amplify a fragment of the *P. larvae* 16S rRNA gene (Piccini et al., 2002).

2.8. Statistical analyses

Mann–Whitney two-sample test was performed in order to compare the number of *P. larvae* spores of colonies treated with PEE and colonies treated with sugar syrup only. p values under 0.05 were considered significant.

3. Results

3.1. Antimicrobial susceptibility test and determination of MIC

All *P. larvae* isolates were highly susceptible to the assessed propolis concentrations while ethanol did not inhibit bacterial growth. Inhibition diameters around the disks obtained when the minimum concentration of propolis was used (1%) ranged between 20 mm and 30 mm. The CIM median was estimated in 0.55% ranging between 0.32% and 0.64 % (Table 1).

3.2. Lethal concentration on bees

Toxicity analysis for honeybees, evaluated by oral administration of PEE, demonstrated that propolis is not toxic at least at 50%, the maximum concentration used in the assay (Fig. 1).

3.3. Field experiment

The effect of PEE on the counts of *P. larvae* in honey was assessed by aspersion and by feeding, both on production colonies naturally contaminated with *P. larvae* spores that did not exhibit clinical symptoms of AFB. In order to monitor this effect, the number of *P. larvae* spores/g of honey was measured at different times after the treatments.

In the first case (aspersion) colonies were initially divided into two groups with similar *P. larvae*

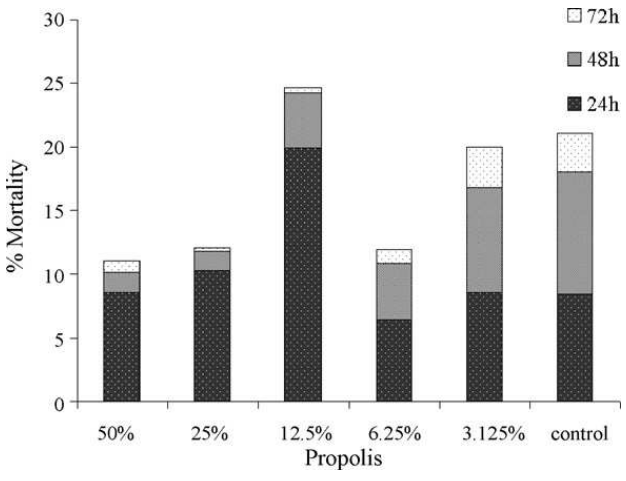

Fig. 1. Lethal concentration of propolis on *A. mellifera*.

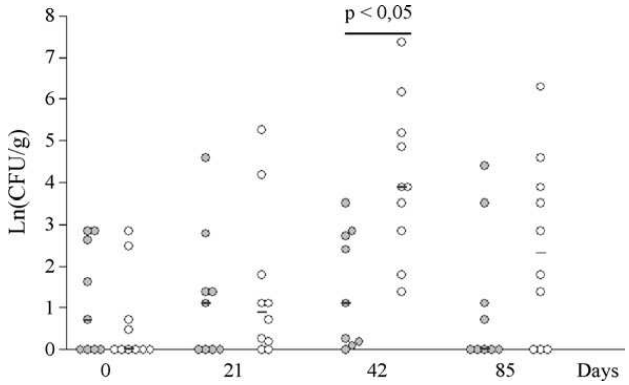

Fig. 2. Evaluation of the effect of PEE administered by aspersion on the number of *P. larvae* spores/g of honey. Each gray dot represents the lnCFU/g of honey from each colony of the group treated with sugar syrup supplemented with PEE, while each white dot represents the lnCFU/g of honey from each colony of the group treated with sugar syrup only.

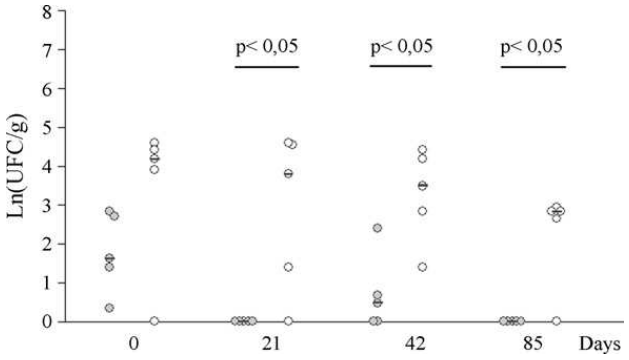

Fig. 3. Evaluation of the effect of PEE administered by feeding on the number of *P. larvae* spores/g of honey. Each gray dot represents the lnCFU/g of honey from each colony of the group fed with sugar syrup supplemented with PEE, while each white dot represents the lnCFU/g of honey from each colony of the group fed with sugar syrup only.

infection rates, 55% and 40% of *P. larvae* positive colonies, and no significant differences were observed between the numbers of *P. larvae* spores/g of honey ($p > 0.05$) (Fig. 2).

After the aspersion-based application, the PEE-treated group of hives exhibited a significant decrease in the number of spores/g of honey compared to the control group, treated with sugar syrup only ($p = 0.004$, 42 days after application). After 85 days the number of spores/g of honey of the treated hives was clearly lower compared to the control group although the difference between them was not significant ($p = 0.165$). However, the percentage of infected hives in the PEE-treated group decreased from 55% to 44% while the control group exhibited an increase of 40–70% (Fig. 3).

In the second case (feeding), colonies were also initially divided into two groups that showed similar infection rates (100% and 80%). At the beginning of the experiment, no significant differences were observed between the number of *P. larvae* spores/g of honey in both groups ($p > 0.05$) (Fig. 2).

At the end of the assay, the hives fed with PEE exhibited a significant decrease in the number of *P. larvae* spores/g of honey compared to the control group ($p = 0.036$, 0.016 and 0.036 at 21, 42 and 85 days after application, respectively). Infection percentage diminished from 100% to 0% in the PEE-treated group but remained constant in the control group, fed with sugar syrup only.

Clinical symptoms of AFB were not observed in any colony during the course of the experiment.

4. Discussion

The present work reports the first systematic study about the use of the propolis ethanolic extract for the treatment of *P. larvae*-affected beehives.

These results indicate that PEE has a direct *in vitro* antibacterial activity against *P. larvae* vegetative cells and that very low concentrations of propolis are required to inhibit its growth. This effect was seen in all *P. larvae* isolates included in the study obtained from different geographic areas of Uruguay and Argentina. These results are in accordance with previous works that reported the antibacterial activity of PEE against diverse pathogens like *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Bacillus subtilis*, *Corynebacterium diphtheriae*, *Streptococcus pneumoniae*, *Moraxella catarrhalis*, *Haemophilus influenza* and *Pseudomonas aeruginosa* between others (Drago et al., 2000; Garedew et al., 2004).

The antibacterial activity of PEE could be related to the chemical composition of propolis, which includes phenolic compounds (flavonoids and aromatic acids), terpenes and essential oils among others (Sforcin, 2007). Eighteen flavonoids, eleven phenolic acid

esters and four aromatic carboxylic acids were isolated and identified from Uruguayan propolis (Kumazawa et al., 2002). Although chemical composition of propolis may vary since it is related to the local flora, it always exhibits significant biological activities (Markham et al., 1996; Kujumgiev et al., 1999; Garedew et al., 2004). It has been proposed that the antimicrobial activity could be due to the synergism between its different components. It has been observed that not even a single component has shown an activity higher than the total extract (Kujumgiev et al., 1993; Serra Bonvehi et al., 1994). In samples from different geographic locations, different substance combinations could be essential for the biological activity (Kujumgiev et al., 1999). For example, the antibacterial and antifungal activities of European and Uruguayan propolis are mainly due to flavonones, flavones, phenolic acids and their esters while in the case of Brazilian propolis such activities are due to prenylated *p*-coumaric acids and diterpenes (Ghisalberti, 1979; Kujumgiev et al., 1993; Marcucci, 1995; Kumazawa et al., 2002; Bankova, 2005). The mixture and combined effects of its different components decrease the chance of propolis-resistant bacterial strains emergency, due to the several target sites probably present in a bacterial cell (Rios et al., 1988; Denyer and Stewart, 1998).

Propolis extraction methods may also influence its activity, due to the solubility properties of the different compounds (Sforcin, 2007). The most common solvents used in biological assays are ethanol and water, although it has been reported that the ethanolic extract shows higher antimicrobial activity than water extracts or volatile compounds, since it possess all water and ethanol extractable and biologically active components. In addition, the ethanolic extract contains several bioactive components that are not found when other solvents are used (Garedew et al., 2004).

In vivo antibacterial effect of propolis was also demonstrated, since a significant decrease in the number of *P. larvae* spores/g of honey was found in naturally infected beehives treated with PEE. The proposed mechanism of action, includes the oral ingestion of PEE by adult honeybees and its delivery to larvae with feeding, facilitating the interaction and direct antibacterial effect on *P. larvae* vegetative cells. The addition of honey to the larval diet is around the third day of the larval stadium, coinciding with germination and multiplication of vegetative cells of *P. larvae* (Shuel and Dixon, 1960; Hansen and Brodsgaard, 1999). We propose that this mechanism cannot prevent the infection of new larvae with *P. larvae* spores, but can inhibit the replication of vegetative cells in the larval gut. Moreover, we cannot rule out a possible indirect effect of the propolis due to the stimulation of the bee immune system. Several authors have reported the stimulating effect of propolis in the innate and adaptive immune response of mouse, bovines and humans. *In vitro* and *in vivo* assays demonstrated that propolis activates macrophages, increasing their microbicidal activity, enhances the lytic activity of natural killer cells and stimulates antibody production (Sforcin, 2007). Insect's immunity shares important features with the innate immune response of vertebrates so it would be interesting to elucidate the mechanism of action of propolis on the honeybee immunity. Enhancement of the defense response of honeybees by propolis could also be important for the control of other honeybee diseases (Evans et al., 2006).

5. Conclusions

We can conclude that a propolis-based therapy against AFB is effective, safe for bees and can be easily performed by beekeepers.

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