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In vitro study of the antimicrobial activity of Brazilian propolis against *Paenibacillus larvae*

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Abstract

The honey bee disease American foulbrood (AFB) is a serious problem since its causative agent (*Paenibacillus larvae*) has become increasingly resistant to conventional antibiotics. The objective of this study was to investigate the *in vitro* activity of propolis collected from various states of Brazil against *P. larvae*. Propolis is derived from plant resins collected by honey bees (*Apis mellifera*) and is globally known for its antimicrobial properties and particularly valued in tropical regions. Tests on the activity of propolis against *P. larvae* were conducted both in Brazil and Minnesota, USA using two resistance assay methods that measured zones of growth inhibition due to treatment exposure. The propolis extracts from the various states of Brazil showed significant inhibition of *P. larvae*. Clear dose responses were found for individual propolis extracts, particularly between the concentrations of 1.7 and 0.12 mg propolis/treatment disk, but the source of the propolis, rather than the concentration, may be more influential in determining overall activity. Two of the three tested antibiotics (tylosin and terramycin) exhibited a greater level of inhibition compared to most of the Brazilian samples, which could be due to the low concentrations of active compounds present in the propolis extracts. Additionally, the majority of the Brazilian propolis samples were more effective than the few collected in MN, USA. Due to the evolution of resistance of *P. larvae* to conventional antibiotic treatments, this research is an important first step in identifying possible new active compounds to treat AFB in honey bee colonies.

Keywords: American foulbrood; *Apis mellifera*; Antibiotic

1. Introduction

The honey bee disease American foulbrood (AFB), caused by the bacterium *Paenibacillus larvae* (formerly *Bacillus larvae,* Genersch et al., 2006), is a serious problem in beekeeping worldwide. AFB is highly infectious and can be fatal to colonies. At a minimum it decreases honey productivity of colonies and increases costs to beekeepers for labor and treatments to control disease transmission (Message and De Jong, 1999; Hansen and Brødsgaard, 1999; Eischen et al., 2005).

The bacteria in the group *Bacillus* and *Paenibacillus* are spore forming, and the vegetative cells of *P. larvae* have become increasingly resistant to tetracycline treatments (Kochansky et al., 2001; Cox et al., 2005). Interestingly, the resistance of *P. larvae* to currently applied antibiotics appears to have developed independently across North America in the past decade (Miyagi et al., 2000; Evans,

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2003), which has implications for its ability to become resistant to antibiotics worldwide. The use of new, commercial antibiotics can create strains that are unaffected by a suite of compounds (Kochansky et al., 2001) and can further affect the beekeeping industry since many antibiotics leave residues in hive products (i.e. Feldlaufer et al., 2004). In untreated colonies, spores in the combs can remain viable for a long time through extreme temperature changes. Beekeeping equipment and products from infected hives, including honey from colonies affected by AFB, can become contaminated and can promote the spread of the disease within and among colonies (Hansen and Brødsgaard, 1999; Schuch et al., 2003). As a result, beekeepers often burn infected colonies to eliminate the source of infection.

Larvae are infected by ingesting spores within the larval food provided by adult worker bees. Diseased larvae will ultimately die from infection when sporulation occurs and then will transmit spores throughout the hive. The disease can kill the colony as spores become widespread unless colonies demonstrate mechanisms of resistance. Young larvae from some colonies demonstrate physiological resistance to infection (Rothenbuhler and Thompson, 1956; Crailsheim and Riessberger-Gallé, 2001; Evans, 2004). Another form of resistance is behavioral: adult bees in some colonies exhibit hygienic behavior and remove diseased larvae before they become contagious (Rothenbuhler, 1964; Spivak and Reuter, 2001).

Larval food and honey are the principal means of dispersal of this disease. Various studies have demonstrated that commercial honey, internationally, contains live spores of this bacterium, which generally constitutes a risk of contamination (i.e. Lauro et al., 2003; Iurlina and Fritz, 2005). While AFB is fairly common across the US, Europe and Australia, it has only recently affected South American countries. AFB was not documented in Argentina until 1992 (Alippi, 1992) and has only just been detected in a few honey, pollen and adult bee samples in southern Brazil. However no clinical symptoms of the disease have been detected in live bee colonies in Brazil (Schuch et al., 2003) or in the bordering regions of Uruguay (Antúnez et al., 2004). There is also some evidence that the more recently introduced strains of AFB show resistance to some conventional antibiotics (i.e. oxytetracycline), but not to others (i.e. tylosin; Schuch et al., 2003; Alippi et al., 2005). If the disease does spread through Brazil, beekeepers may try to control it with antibiotics with limited effectiveness, and possibly lose the emerging and very promising honey export market.

As an additional possible natural mechanism of disease resistance, bees collect resin from trees and shrubs and typically use it as a form of cement, called propolis, to seal any cracks or openings in the nest. Propolis is known for its anti-microbial properties and is commonly used by people especially outside of the US to treat a number of bacterial and viral infections (see Ghisalberti, 1979). Brazilian propolis has been widely documented for its properties as an antimicrobial, antifungal, antitumor and antiviral agent (Grange and Davey, 1990; Dobrowolsky et al., 1991; Fernandes et al., 1995; Park et al., 1995, 1997, 2002; Ota et al., 2001; Bastos, 2003). Because of the range of its antimicrobial properties, propolis may be essential to honey bees, particularly those in the wild (Haydak, 1953; Hoyt, 1965; Ghisalberti, 1979; Seeley, 1985). Propolis from temperate regions has been tested against AFB both in cultures and in field studies, although not in recent years (Lindenfelser, 1967, 1968; Mlagan and Sulimanovic, 1982). Until now, no one has tested the antimicrobial properties of Brazilian propolis against *P. larvae*, even though it is well known for its strong inhibitory effects against other Gram-positive bacteria in laboratory cultures (Aga et al., 1994; Sforcin et al., 2000; Bastos, 2001).

The objective of this study was to investigate the *in vitro* activity of propolis collected from various states of Brazil against *P. larvae* and to compare the activity of propolis with conventional antibiotics and, as a reference, two propolis samples from Minnesota, USA.

2. Materials and methods

2.1. Propolis extracts

2.1.1. Brazilian propolis

Two sets of propolis samples were collected from colonies from a variety of states within Brazil. In the first set, 16 samples of crude propolis of different colors were collected from colonies located in the states of Minas Gerais, Goiás, Paraná and Bahia, Brazil. A second set of 16 samples of crude propolis was collected from different colonies only in the states of Minas Gerais and Paraná.

The green propolis in both sets that originated from Minas Gerais was derived from *Baccharis dracunculifolia* (Bastos, 1998, 2001, 2003; Bastos and Oliveira, 1998; Bastos et al., 2000; Santos et al., 2003). The green propolis from Paraná had a distinct botanical origin from that of green propolis in Minas Gerais, although the specific plant source remains unknown. Black propolis produced in Minas Gerais has been identified as a mixture of resins from *B. dracunculifolia* and *Vernonia polyanthes*. The plant sources of the other colors of propolis were not known.

Ethanol extractions of the two sets of Brazilian propolis samples were done in Brazil as follows: 50 g of crude propolis from each sample was added to 100 mL of 70% ethyl alcohol. This solution was kept in the dark at ambient temperature for 14 days and agitated once per day. After this time, the solution was filtered through filter paper.

The first set of samples was tested in Minas Gerais, Brazil and the second set was shipped to MN where they were tested at the University of Minnesota.

2.1.2. Minnesota propolis

As a reference for the results of tests done with Brazilian propolis, propolis was collected from colonies located in two locations within MN: the first near the University of

Minnesota, St. Paul campus, and the second in the southeastern part of the state, near the town of Houston, MN. These were the same propolis samples used by Gekker et al. (2005). The botanical origin of the MN propolis has not been identified, but is probably comprised to some degree from poplar trees, *Populus* spp. (see Ghisalberti, 1979).

Ethanol extractions of MN propolis were done at the University of Minnesota. 50 g of crude propolis was ground and placed in 100 mL of 95% ethyl alcohol. The solution was covered from light and kept under constant, moderate agitation for 8 days after which point the extracts were filtered following Gekker et al. (2005). After extraction, all propolis extracts (from Brazil and MN) were stored in amber flasks and analyzed for the amount of dissolved solids to obtain the real concentration (Instructional Norms No. 3, 2001). A portion of these extracts was tested in MN and another was shipped to Brazil for testing there.

Dose–response tests were done on four samples (two Brazilian extracts—2 and 19b—and the two MN extracts). Serial dilutions were made from the initial extracts so that the concentrations ranged from approximately 260 to 2 mg/mL (see Gekker et al., 2005). The dilutions made were 3-fold and a total of four dilutions were made for each extract. The concentrations of the propolis extracts determined by dissolved solids was 16.9%, 26.6%, 24.6%, and 26.0%, respectively, which correspond to 169, 266, 246, and 260 mg/mL of propolis in ethanol. The 3-fold serial dilutions were made from these initial concentrations. While the exact concentrations tested across extracts were slightly different (refer to Fig. 1), the magnitude of change for each dilution was equivalent.

2.2. In vitro assay in Brazil

2.2.1. Obtaining microorganisms in Brazil

An ampule containing lyophilized cells of *P. larvae* CCT 4443, originating from a collection of Argentinean spore cultures from the Tropical Foundation of Research and Technology Andre Tosello, Campinas, São Paulo, Brazil, was opened according to technical specifications, adding 0.2mL sterile distilled water. The cellular suspension obtained in this manner was transferred to two test tubes containing 5 mL of Brain Heart Infusion (BHI), and incubated at 37 °C for 24 h.

Once activated, the *P. larvae* culture was transferred and incubated for 48 h at 37 °C in tubes with agar J (5.0 g triptone, 15.0 g yeast extract, 3.0 g K_2HPO_4, 20.0 g agar, 1 L of water, and 2.0 g glucose at a pH of 7.5) following Gordon et al. (1973). The tubes were stored at 5 °C.

With the objective of maintaining the cultures of *P. larvae* for future research, the *P. larvae* were removed from colonies grown in agar J, and these were transferred to glass flasks containing 4 mL of semi-solid agar J and incubated at 37 °C for 48 h. After this time, the flasks were sealed and stored at 5 °C.

2.2.2. Sporulation technique

The inoculum that was previously stored was transferred into a tube containing 0.5 mL of BHI. It was incubated at 37 °C for 72 h. After the incubation time, it was streaked in four tubes of agar J, inclined and incubated at 37 °C for 72 h. This procedure was repeated with the same lines two more times. The growth obtained was rinsed with 0.5 mL sterile distilled water and transferred into an inclined Roux flask containing Bailey and Lee agar specifically for sporulation (Bailey and Lee, 1962). It was incubated at 37 °C for 21 days.

At the end of the incubation time, the superficial growth was rinsed using a pipette and sterile distilled water. All of the rinsed growth was transferred into centrifuge tubes. This material was centrifuged for 20 min at 3000 rpm at 4 °C five times, re-suspended and exposed to thermic shock at 80 °C for 10 min. This spore suspension was stored at 5 °C and used as tests of pure antimicrobial activity or to prepare serial dilutions.

2.2.3. Test of antimicrobial activity of propolis extracts against P. larvae

This resistance assay was adapted from the technique of radial diffusion on two layers of perforated agar following Grove and Randall (1990). For the first layer, 15 mL of BHI-T agar (commercial BHI agar from Merck with 0.1% thiamine added) was used in Petri dishes. For the second layer, a flask with 3 mL BHI-T semi-solid medium (commercial BHI from Merck with 0.75% agar and 0.1% thiamine added) was added to 10 μL spore suspension (2×10^9 CFU/mL). This medium was poured over the first layer of solidified BHI-T agar. The agar was perforated using a metal cylinder of 7 mm diameter, and 50 μL of propolis extract was inoculated into the perforated well. The amount of actual propolis in the wells varied according to the concentration of the extracts. For example, Bahia Sample 24 and Minas Gerais Sample 9a (see Table 2) had concentrations of 15.5% and 24.4%, which resulted in 7.75 and 12.2 mg of propolis in the well, respectively. After the wells were full, the plates were incubated at 35 °C for 72 h. For the controls, commercial disks of the antibiotics, vancomycin (30 μg, Sigma) and tetracycline (30 μg, Sigma), were used and sterile filter paper disks saturated with 70% EtOH were used as a control for the propolis extracts. Vancomycin was used because it is a broad spectrum antibiotic effective against Gram-positive bacteria.

After the incubation period, the zones of growth inhibition of the bacterium were measured with a digital micrometer. Assays on each propolis extract from both Brazil and MN, USA were replicated five times.

2.3. In vitro assay in Minnesota

2.3.1. Obtaining microorganisms in MN

As a way to compare activity of the propolis extracts on various strains of *P. larvae*, an *in vitro* assay was also con-

ducted in MN, USA. *P. larvae* spores were collected from several diseased colonies in MN by collecting scales of dried larvae that contain bacterial spores from combs. In this way, the spores may have included multiple strains of *P. larvae* (Evans, 2003). Five scales were macerated with 10 mL sterilized water in screw-cap tubes. This suspension was heat-shocked at 80 °C for 10 min to kill non-spore forming bacteria.

2.3.2. Test of antimicrobial activity of propolis extracts against P. larvae

The resistance assay used in MN followed the protocol for the disk diffusion method as described by Shimanuki and Knox (2000). To prepare the bacterial cultures, 0.2 mL of the spore suspension was evenly spread over brain–heart infusion (BHI) agar plates (Difco Laboratories) fortified with 0.1 mg/L thiamine hydrochloride and pH adjusted to 6.6 using hydrochloric acid. A 6.35 mm filter paper disk (Whatman 7) was saturated with the antibiotic (propolis extract, tylosin, or methanol/ethanol control) and dried. Each disk absorbed approximately 20 μL of solution (see Kochansky et al., 2001). A treated disk was then placed on the center of the plates with the spore suspension. The plates were then incubated at 34 °C for 72 h. The diameter of the zone of growth inhibition around the disk was then measured without correcting for disk diameter. If there was no visible zone of inhibition, the diameter was scored as 0.

For the controls, the antibiotic tylosin (Sigma) was chosen because preliminary tests provided evidence that *P. larvae* from the MN colonies was resistant to terramyacin (Miyagi et al., 2000). A solution of 400 mg/L was prepared for the antibiotic treatment. Both methanol and ethanol were used as negative controls because tylosin was in a methanol solution while the propolis extracts were prepared in ethanol (see above).

For the dose response tests, the disks were treated in the same manner. A disk was saturated with one of the diluted propolis extracts (ranging from approximately 260 to 2 mg/mL). This resulted in disks containing approximately 5, 1.7, 0.5, 0.17, and 0.06 mg of each extract/disk (for exact amounts refer to Fig. 1).

In summary, the assays presented here provide a robust analysis of the activity of propolis extracts collected from various areas of Brazil against multiple strains of *P. larvae*. Table 1 provides a summary of the sources of propolis

Table 1
Summary of methods

	Minas Gerais, Brazil		St. Paul, Minnesota	
Origin of propolis samples	Various states in Brazil	Two locations in MN	Two states in Brazil	Two locations in MN
Extraction Procedure	70% EtOH, 14 d	95% EtOH, 8 d	70% EtOH, 14 d	95% EtOH, 8 d
P. larvae spore origin	Culture bank, Argentina		Scales from diseased colonies in MN	
Resistance assay methods	Grove and Randall (1990)		Shimanuki and Knox (2000)	

Table 2
Average zones of inhibition of two antibiotics and the Brazilian and Minnesota propolis extracts tested against *Paenibacillus larvae* cultures in Brazil

State of origin (Sample#)	Botanic origin	Color	Dose (mg of propolis)	Avg. zone of inhibition in mm ± SD (*n*)
Brazil				
Bahia (24)	Unknown	Red	7.75	20.5 ± 2.1 (5)
Minas Gerais (9a)	*B. dracunculifolia*	Green	12.20	20.2 ± 1.0 (5)
Bahia (23)	Unkown	Red	12.40	20.1 ± 2.1 (5)
Minas Gerais (19a)	*B. dracunculifolia*	Green	12.60	19.8 ± 0.7 (5)
Minas Gerais (13)	*B. dracunculifolia*	Green	11.95	19.3 ± 1.5 (5)
Goiás (5)	Unknown	Brown	2.70	18.3 ± 1.4 (5)
Minas Gerais (11)	*B. dracunculifolia* and *V. polyanthes*	Black	8.60	18.1 ± 3.6 (5)
Minas Gerais (3)	*B. dracunculifolia*	Green	9.10	17.7 ± 1.6 (5)
Minas Gerais (35)	*B. dracunculifolia* and *V. polyanthes*	Black	7.50	17.6 ± 2.2 (5)
Minas Gerais (10a)	*B. dracunculifolia*	Green	10.40	17.5 ± 4.5 (5)
Paraná (6)	Unknown	Green	3.75	17.3 ± 2.6 (5)
Minas Gerais (4a)	Unknown	Brown	5.20	16.6 ± 3.0 (5)
Minas Gerais (3T)	*B. dracunculifolia*	Green	7.30	16.3 ± 1.4 (5)
Minas Gerais (34)	*B. dracunculifolia* and *V. polyanthes*	Black	7.05	15.5 ± 2.2 (5)
United States				
University of Minnesota	Unknown	Red	15.70	14.1 ± 0.5 (5)
Southeastern Minnesota	Unknown	Red	14.30	12.9 ± 1.0 (5)
Antibiotic				
Vancomycin	Antibiotic		30.0 μg	19.1 (1)
Tetracycline	Antibiotic		30.0 μg	31.3 (1)

samples and methods used in each location. By applying two different conventional methods to test for antibacterial activity, the consistency of any effects would be apparent.

2.4. Statistical analysis

Differences among the activity of the propolis extracts as measured by the zones of inhibition were analyzed using one-way ANOVA, followed by post-hoc Tukey's HSD tests. A Pearson correlation was calculated to determine if the concentration of propolis was related to the zones of inhibition.

3. Results

3.1. In vitro assay in Brazil

The results obtained on the susceptibility of *P. larvae* to the propolis extracts and the controls are shown in Table 2. The zones of inhibition of all the Brazilian samples varied between 20.5 ± 2.1 and 15.5 ± 2.2 mm demonstrating some antimicrobial activity of all the Brazilian propolis samples against *P. larvae*. An ANOVA of the Brazilian-derived samples revealed significant differences in their inhibitory activity ($F = 12.29$; df = 13, 56, $p = 0.02$). However, post-hoc pairwise comparisons of means indicated the differences were only between the two samples that showed the highest average zones of inhibition (samples 9a from Minas Gerais and 24 from Bahia) and the two that showed the lowest (samples 34 and 3T, both from Minas Gerais).

The negative control (ethanol) did not inhibit bacterial growth, and vancomycin and tetracycline had zones of inhibition of 19.1 and 31.3 mm, respectively. An ANOVA comparing the two extracts with the highest average inhibition, the two with the lowest average inhibition and the two antibiotics was significant ($F = 17.57$; df = 5, 16; $p < 0.001$). The Tukey's test showed that tetracycline had a significantly larger zone of inhibition than vancomycin and all four propolis extracts ($p < 0.01$ for each comparison).

An ANOVA comparing the two high and two low Brazilian samples with the two MN propolis samples revealed significant differences in inhibitory activity ($F = 22.38$; df = 5,24; $p < 0.001$) (Table 2). Tukey's comparisons showed that both MN samples had significantly less inhibitory activity than the two Brazilian extracts with highest average inhibition (samples 9a and 24, $p < 0.001$ for each comparison). The MN propolis collected from the University had the same inhibitory activity as the two Brazilian samples with lowest average zones of inhibition (samples 34 and 3T, $p > 0.238$). The MN sample from southeastern MN was significantly less effective than the Brazilian sample 3T ($p = 0.015$) but not different from the lowest, Brazilian Sample 34 ($p = 0.089$).

Among the Brazilian propolis extracts, there was a significant correlation ($r = 0.575$, Bonferroni $p = 0.031$) between the concentration of soluble solids and zones of inhibition; that is, the higher the propolis concentration, the greater the antimicrobial activity (refer to Table 2).

3.2. In vitro assay in Minnesota

The results of the tests conducted in MN are shown in Table 3. The range of the zones of inhibition for the Brazilian propolis extracts was from 34.7 ± 1.0 to 29.3 ± 5.9 mm. There were no statistical differences in the zones of inhibition among the Brazilian propolis extracts ($F = 1.58$; df = 15, 40, $p = 0.124$), and so they were pooled for subsequent analysis. There was a highly significant difference among the zones of inhibition for tylosin, the two MN propolis extracts, and the pooled Brazilian extracts ($F = 93.89$; df = 3, 62, $p < 0.001$). The Tukey's post hoc comparison of means indicated that tylosin had a significantly larger zone of inhibition compared to all the Brazilian extracts ($p < 0.001$) and each of the MN propolis extracts had significantly smaller zones compared to both the antibiotic ($p < 0.001$) and the Brazilian extracts ($p < 0.001$). The solvents (ethanol and methanol) showed no growth inhibition with zones of inhibition of 0.

To study the correlation between the concentration of the propolis extracts and their antimicrobial activity we used dose response tests. No significant differences in the zone of inhibition were observed for any propolis samples between the highest two concentrations (5 mg propolis/disk and 1.7 mg propolis/disk; Fig. 1). In all cases, significant differences were observed between the concentrations of 1.7 and 0.12 mg propolis/disk. The middle concentration, 0.5 mg propolis/disk, showed intermediate zones of inhibition between the highest and lowest concentrations for each propolis extract indicative of a clear dose response.

4. Discussion

The propolis extracts derived from the various states of Brazil significantly inhibited the growth of multiple strains of *P. larvae* in cultures. The antibiotics terramycin and tylosin had significantly more activity against *P. larvae* compared to the most active propolis samples from Brazil and MN, respectively. However vancomycin had similar effects as the Brazilian propolis samples. It is important to note that the doses of antibiotic versus propolis were significantly different, especially in regards to the active components. For the Brazilian study, 30 μg of antibiotic was used, whereas the concentration of propolis in the wells ranged from 2.7 to 12.6 mg. In the MN study, 8 μg of antibiotic and doses ranging from 1.92 to 5.32 mg of propolis were used. With the antibiotics, 100% of the material is active against the bacteria. However, the concentration of active components in propolis has not been determined. In fact, it is not known what the active components acting against *P. larvae* are, and in general active components of propolis extracts are understudied.

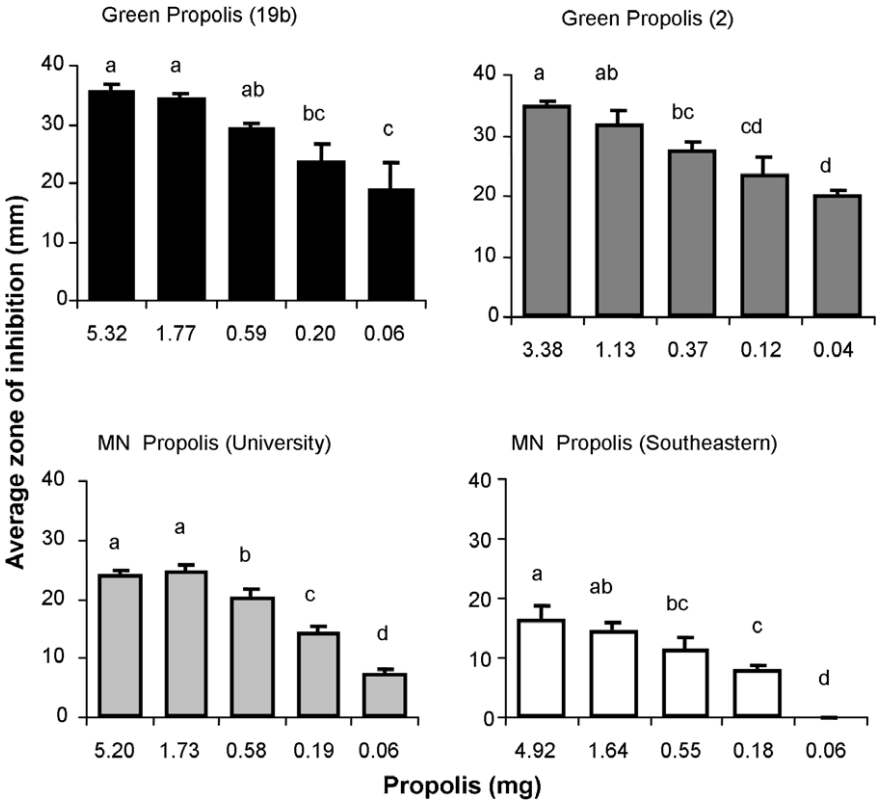

Fig. 1. Antimicrobial activity of four different propolis extracts at different dilutions. Three replicates for each extract were done for every concentration. Dose responses to Brazilian- and MN-derived propolis extracts listed in Table 3. Significant differences ($p < 0.05$) are indicated by the letters above the bar, determined by ANOVA and Tukey's tests. (a) Brazilian extract 19b ($F = 17.43$, df = 4, 10, $p < 0.001$). (b) Brazilian extract 2 ($F = 23.20$, df = 4, 10, $p < 0.001$). (c) MN, University extract ($F = 126.84$, df = 4,10, $p < 0.001$). (d) MN, Southeastern extract ($F = 46.29$, df = 4,10, $p < 0.001$). Standard deviations are indicated on graphs.

Table 3
Average zones of inhibition of the antibiotic tylosin and the Brazilian and Minnesota propolis extracts tested against *Paenibacillus larvae* cultures in Minnesota

State of origin (Sample#)	Botanic origin	Color	Dose (mg of propolis)	Avg. zone of inhibition in mm ± SD (*n*)
Brazil				
Minas Gerais (19b)	*B. dracunculifolia*	Green	5.32	34.7 ± 1.0 (3)
Minas Gerais (17)	*B. dracunculifolia*	Green	4.22	34.0 ± 1.2 (3)
Minas Gerais (2)	*B. dracunculifolia*	Green	3.38	34.0 ± 2.8 (4)
Minas Gerais (4b)	*B. dracunculifolia*	Green	3.48	33.5 ± 3.8 (4)
Minas Gerais (5)	*B. dracunculifolia*	Green	3.18	33.5 ± 3.0 (4)
Paraná (3)	Unknown	Green	3.34	33.3 ± 1.2 (4)
Minas Gerais (10b)	*B. dracunculifolia*	Green	4.38	33.0 ± 1.4 (4)
Paraná (11)	Unknown	Green	4.32	32.8 ± 1.0 (4)
Minas Gerais (8)	*B. dracunculifolia*	Green	4.52	32.8 ± 1.3 (4)
Minas Gerais (20)	*B. dracunculifolia*	Green	3.42	32.3 ± 1.7 (3)
Minas Gerais (18)	*B. dracunculifolia*	Green	3.60	32.0 ± 0.6 (3)
Minas Gerais (1)	*B. dracunculifolia* and *V. polyanthes*	Black	3.34	30.8 ± 2.5 (4)
Minas Gerais (14)	*B. dracunculifolia* and *V. polyanthes*	Black	3.92	30.3 ± 2.1 (3)
Minas Gerais (9b)	*B. dracunculifolia*	Green	4.18	30.0 ± 1.7 (4)
Minas Gerais (12)	*B. dracunculifolia*	Green	4.34	29.3 ± 5.9 (3)
Minas Gerais (21)	*B. dracunculifolia*	Green	1.92	29.3 ± 0.6 (3)
United States				
University of Minnesota	Unknown	Red	5.20	19.0 ± 1.0 (3)
Southeastern Minnesota	Unknown	Red	4.92	15.0 ± 1.0 (3)
Antibiotic				
Tylosin	Antibiotic		8.0 μg	43.3 ± 1.0 (4)

Propolis samples consist of many (80–300) different chemical compounds in complex and varied mixtures (Bankova et al., 2000, 2002; Salatino et al., 2005). The active portion of propolis extracts is often in quantities less than 3% of the total mixture (da Silva et al., 2006). Additionally it is clear that in different samples, different combinations of compounds, which widely range in activity levels and could act synergistically, are required for significant biological activity (Kujumgiev et al., 1999). So despite the obvious difference in the dose of antibiotic versus total propolis extract used in this study, it is possible that the amount of active compounds tested was less than the dose of antibiotic. Therefore, the finding of strong activity of various propolis samples against *P. larvae*, even at such low doses of active compounds, is quite promising.

Identification of the chemical profiles of propolis and the resins from which they are derived is currently a popular area of research. Propolis from temperate regions throughout the world is often identified as poplar-type propolis, and the compounds of propolis derived from various poplar species are quite similar across the globe (Greenaway et al., 1990; Bankova et al., 2000; Bankova, 2005). In general, propolis from temperate regions (poplar-type propolis) is typically characterized by the presence of flavonones, flavones, phenolic acids and their esters. The green Brazilian propolis from Minas Gerais is derived from *Baccharis dracunculifolia* (Bastos, 1998, 2001, 2003; Bastos and Oliveira, 1998; Bastos et al., 2000; Santos et al., 2003), and its chemical properties are well documented (i.e. Salatino et al., 2005). Brazilian green propolis is identified as mainly consisting of diterpenes and a specific group of phenolic acids, prenylated *p*-coumaric acids (for general review see Bankova et al., 2000; Bankova, 2005; Salatino et al., 2005).

With respect to the two MN, USA propolis extracts used in this study, the majority of the Brazilian extracts exhibited a higher level of antibacterial activity. The only exceptions were two samples of Brazilian propolis with the lowest average zones of inhibition that did not display significantly different activity compared to the MN propolis extracts when tested in Brazil. General differences in chemistry of temperate propolis (flavonoids) and tropical propolis (terpenes) may account for the differences in activity against *P. larvae*; however, more temperate samples should be tested to confirm this idea. Additionally the results of this study indicate that: (1) Brazilian propolis has similar antimicrobial properties against *P. larvae* independent of geographic origin and (2) plant source may determine level of activity, as green propolis derived from *B. dracunculifolia* appeared to be more effective in general.

Significant dose responses were found for individual propolis extracts, particularly between the concentrations of 1.7 and 0.12 mg propolis/disk. However, it is clear that the propolis source was more important in determining its overall activity against *P. larvae* than the concentration. For example, Sample 5 from the state of Goiás, Brazil (Table 2) had the lowest concentration (5.4%) but showed just slightly less activity against the bacterium compared to Sample 13 from Minas Gerais with a concentration of 23.9%. Similarly, propolis extracts from MN with higher concentrations than any extracts from Brazilian propolis (31.4% and 28.6%, Table 2) were only as active as two Brazilian samples with the lowest activity and relatively low concentrations (14.6% and 14.1%).

These findings appear to be rather robust considering that the difference between the activity between the Brazilian and MN propolis samples was observed using two different sets of methods. The first difference in methods was that the propolis samples from MN were extracted in 95% EtOH for 8 d whereas the samples from Brazil were extracted in 70% EtOH for 14 d; this may have led to slight differences in the presence or amounts of some of the chemical components (Krell, 1996). However, in a subsequent trial, the extraction procedure used in Brazil was replicated on the same propolis sample from the University of Minnesota and found that the zone of inhibition increased from 19.0 ± 1.0 mm ($n = 3$) in 95% EtOH 8 d extraction to 25.8 ± 3.3 mm ($n = 4$) in the 70% EtOh 14 d extraction. However, the increased activity was still lower than the Brazilian samples tested in MN, confirming our initial findings. Sawaya et al. (2002) examined the effectiveness of propolis extracted in different concentrations of ethanol and water against strains of the fungus *Candida* and found that extracts with 50% EtOH or higher performed similarly, which also supports our results.

The other differences in methods concerned the origin of the *P. larvae* spores (cell culture from Argentina vs. scales collected from several AFB diseased colonies in MN), and the resistance assay methods [based on Grove and Randall (1990) or Shimanuki and Knox (2000)]. These differences may account for the overall greater zones of inhibition observed in all samples tested in MN compared to samples in Brazil. Small differences in the results from these techniques were also noted in Sawaya et al. (2002).

Lindenfelser (1967) examined the activity of 15 propolis samples collected from across the US against *P. larvae* using the tube dilution method. He found that all 15 samples showed a similar level of bactericidal action at concentrations less than 100 μg/mL; this is the same order of magnitude at which the dose response data presented for this study were most clear. Additionally, Mlagan and Sulimanovic (1982) tested two temperate propolis samples from Yugoslavia against eight strains of *P. larvae* using the disk diffusion method (the same that was used for the MN assays). They found that 5% and 10% propolis extracts all inhibited the growth of the bacteria and had an average zone of inhibition of 15.1 and 15.6 mm, respectively, which is similar to what we have shown for the two MN propolis samples.

Although these results are promising, more studies are needed to confirm the findings. For example, it may be important to test propolis samples against a variety of strains of *P. larvae*. In the present study, it is possible that

multiple *P. larvae* strains were tested in MN since the scales originated from several colonies from different sites (Evans, 2003), but this was not confirmed. While Mlagan and Sulimanovic (1982) tested propolis against eight *P. larvae* strains in laboratory cultures and found similar results, strain differences may be more important when considering the effect of treatments in the field where different strains can have vastly different virulence properties (Ashiralieva and Genersch, 2006).

Further studies should also be conducted to isolate the active compounds of propolis and then to use appropriate concentrations of just the active substances to determine its effectiveness in treatments. Furthermore, the mode of action for propolis against Gram-positive bacteria needs to be understood in order to determine the potential value of propolis as a natural treatment. Field studies have been conducted by Lindenfelser (1968) and Mlagan and Sulimanovic (1982) who fed bees a low dose of temperate propolis extract in solution and found that the treatments only temporarily prevented the spread of the disease in infected colonies. However, as has been shown here, it appears that Brazilian propolis and its active compounds may be a more effective treatment option. It is also possible that feeding the bees a propolis solution may not be the optimal form of treatment, but this needs to be investigated further (Simone, Soares, & Spivak, unpublished data).

Due to the evolution of resistance of *P. larvae* to conventional antibiotic treatment, this research is an important first step in identifying possible new active compounds within propolis to treat AFB in honey bee colonies. This study succeeded in making the initial step to understand the general bioactivity of various propolis extracts in order to determine that further research should be conducted concerning its active compounds and its potential as an antibacterial agent against American foulbrood. Field bioassays are underway to test the effectiveness of Brazilian propolis extracts on AFB infected colonies to determine if the difference in effectiveness between conventional antibiotics and propolis is biologically important.

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