Isolation of compounds from Brazilian propolis active against the honey bee pathogen *Paenibacillus larvae*

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ABSTRACT

Propolis, a plant resin used by honeybees, is collected from specific botanical sources in different regions worldwide yielding different types with distinct color, aroma, and chemical composition. Among other biological properties, propolis has exhibited antimicrobial activity against several organisms including *Paenibacillus larvae*, the causative organism of the honey bee disease American Foulbrood (AFB). The longer term goals of this study are to isolate the chemical components of propolis which could be used as an alternative treatment for AFB and other diseases. Brown propolis from Mariana, Minas Gerais (Brazil) was extracted in 65% isopropanol and filtered. This propolis solution was adjusted to pH 2, 7, and 8.5 and partitioned against ethyl acetate. A spectrophotometric assay of bacterial growth was conducted to determine if any of the fractions inhibited *P. larvae*. It was postulated that only pH 2, organic acid fraction would likely inhibit growth. Using solid phase extraction (SPE), two aromatic acid reference compounds with different pKa's were used to standardize the system with elution step gradients of KCl (10 mM, 15 mM, 25 mM, 35mM, 50 mM, 60 mM, 75 mM, 1 M). Acids were detected by UV280; they were shown to separate as expected by pKa. This SPE method was employed on the propolis extracts and fractions collected. SPE fractions were assayed for inhibition of *P. Larvae* growth using the spectrophotmetric bacterial assay. We found that the 10 mM KCl fraction from the pH 2 ethyl acetate extraction inhibited the *P. Larvae*. Future experiments will be directed towards analyzing active fractions with LC/MS and LC/MS/MS.

INTRODUCTION

Propolis is a resinous substance collected from various plant sources by honey bees. Geographic variables influence the overall appearance and composition of propolis. For years it has been used by humans universally for various purposes such as varnish for string instruments, as a constituent of 'bio-cosmetics', and 'health foods' (Matsuda, 1994; Wollenweber and Buchmann, 1997; Kujumgiev, 1999). Beyond this, propolis has exhibited extensive antibacterial, antifungal, and antiviral activity in many studies (Kujumgiev et al., 1999).

Paenibacillus larvae is the causative organism of the honey bee disease American Foulbrood (AFB). The rapid transmission of *P. larvae* spores through honey, contaminated bee keeping equipment, and larval food (Bastos et al., 2008) make this disease hard to manage. Currently, antibiotics such as tylosin and oxytetracycline are being used to treat AFB. However, the threat of these chemicals being left in bee products and emerging antibiotic resistance has forced bee keepers to think of new strategies to control AFB. For these reasons, their interest has peaked by the prospect of using propolis, which contains natural antimicrobial compounds, as a means to control disease. Antimicrobial compounds, however, have other uses as well, including uses as antiseptics and even potentially as antibiotics for control of animal and human bacterial diseases.

MATERIALS & METHODS

Organic extraction with ethyl acetate: 10 mL of brown propolis extract was adjusted to pH 2, and then extracted three times with ethyl acetate. Sodium chloride was added to facilitate separation of organic and aqueous phases. Ethyl acetate (organic phase) was collected using a separatory funnel. Two more 10 mL aliquots of propolis extracts were adjusted to pH 7 & 8.5 respectively. They were both extracted with ethyl acetate as described yielding three fractions.

Spectrophotometric propolis assay: To test for *P. larvae* inhibition, a high-thorough spectrophotometric assay previously developed was utilized (Wilson, unpublished). Assay was conducted by transferring samples that were dried with a nitrogen stream and resolubilized in 65% isopropanol into a 96-well plate. 100 µL of each fraction was transferred by pipet into wells of the plate. The samples in each of the wells were dried completely with nitrogen. 100 µL of brain/heart infusion broth was transferred into the plates and incubated at 34°C with shaking at 450 RPM for fifteen minutes to resolubilize the sample residues. Each sample well contained 200 µL of a 1:100 dilution of liquid *P. larvae* bacteria culture; while reference wells contained a corresponding amount of sample with brain/heart infusion broth. Control wells only contained 1:100 dilution of liquid culture. Plates were incubated at 34°C, and optical density was measured at 600 nm every hour for six hours.

Solid Phase Extraction: To develop a method for the solid phase extraction of propolis, benzoic and salicylic acid samples were eluded with trial gradients of potassium chloride on the 'Baker - 10 Extraction System' using an amino (NH2) column. The method employed was adapted from Barkawi et al. (2008). The samples were prepared by evaporating the ethyl acetate, resolubilzing the residue in 65% isopropranol and adjusting to pH 7. The column was pretreated with 1 mL of ethyl acetate, 1 mL of acetonitrile, and 1 mL of 0.2 M of imadizole buffer, pH 7. The column was then rinsed three times with 1 mL of deionized water. 0.6 mL of 65% isopropanoyl (solvent in which sample was dissolved) was added to condition the column. 0.6 mL of each sample was added to their individual columns respectively. Each sample was vacuum filtrated and flow-through fractions collected. Three elution's of 0.6 mL of 10 mM, 15 mM, 25 mM, 35 mM, 50 mM, 60 mM, 75 mM, and 1 M of KCl were taken and sixteen fractions collected. The same method was employed using organic extraction propolis fractions.

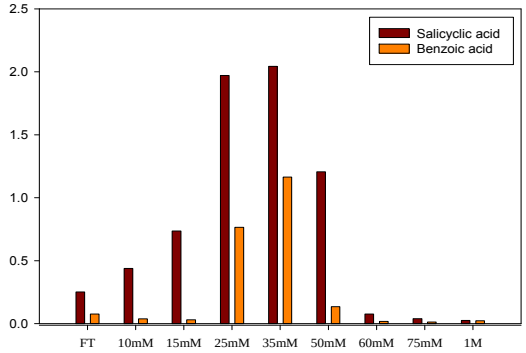

Fig 1: Salicylic acid (pK_a = 3.0) and Benzoic acid (pK_a = 4.2) detection in trial gradients of KCl. Absorbance of salicylic acid measured at 280 nm

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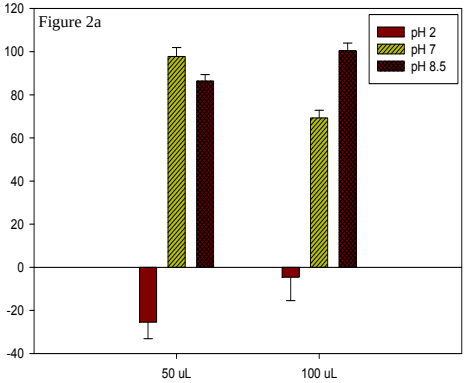

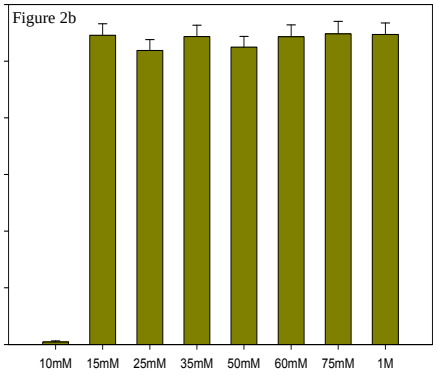

Fig 2: pH 2, 7, 8.5 ethyl acetate propolis extracts (fig. 1a) and pH 2 10 mM KCl SPE fraction inhibition of *P. larvae* growth.

CONCLUSIONS

- The developed reversed phase SPE method can separate acids based on their pK_a
- The most active component(s) of "brown" propolis from Mariana, Minas Gerias, Brazil are acidic since only the pH 2 ethyl acetate extraction of propolis strongly inhibited *P. larvae* growth
- The active component(s) are weak acids since only the 10 mM reversed phase SPE fraction of at pH 2 exhibited inhibition of *P. larvae* growth
- Propolis inhibition of bacterial growth is not an artifact created by low pH
- Active compounds found in the pH 2 10 mM SPE propolis fractions have a pK_a higher than 4.2