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Antimicrobial activity of nanostructured Amazonian oils against *Paenibacillus* species and their toxicity on larvae and adult worker bees

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ABSTRACT

Antimicrobial activity of Amazonian oils Andiroba and Copaiba against *Paenibacillus larvae* has been recently determined, indicating their potential use for the control of American Foulbrood Disease (AFB), but the use of essential oils in the environment still represents a challenge. The oils present several volatile elements in its composition, such high volatility being the cause of a sharp decline in antimicrobial activity. In this context the nanostructuration of these amazon oils may decrease the volatile characteristic of such products. The following research aimed to evaluate the activity of nanoemulsions prepared with Andiroba and Copaiba oils against *Paenibacillus* species. The toxicity of nanoemulsions has also been investigated with larvae and adult worker bees. Nanoemulsions (NE1, 10% Andiroba oil; NE2, 10% Copaiba oil; and NE3, 10% medium-chain triglyceride as negative control) were prepared in a high pressure homogenizer. The particle sizes were determined as 192, 211, and 178 nm for NE1, NE2, and NE3, respectively. The z potential values were −56.4, −47.1, and −27.2, respectively. NE1 and NE2 showed minimal inhibitory concentration (MIC) values lower than 0.39% for most *Paenibacillus* species tested. None of the strains were inhibited by negative control NE3. The time-response effect of the nanoemulsions has been tested on *P. larvae* ATCC9545, resulting in a decrease in the number of viable cells to less than 1 log CFU/ml for NE1. The nanoemulsion NE1 showed a significant toxic effect for the larvae (26% mortality) when compared with NE2 (13%) and NE3 (7%). The toxic effect of nanoemulsions has also been evaluated for 72 h in adult worker bees and low mortality rate was only observed for the NE1 treatment (8.3%). This study shows for the first time that nanoemulsions of Copaiba oil can be a potential candidate for the treatment or prevention of AFB.

Introduction

American foulbrood (AFB) is among the most economically important honeybee disease. The etiological agent of AFB is the Gram-positive, spore-forming bacterium *Paenibacillus larvae* (Genersch, 2010a). The extremely tenacious spores are the infectious form. Such spores drive disease transmission within colonies (Lindström et al., 2008) as well as between colonies as soon as they end up in the honey stores of an infected colony. Infected bees generally die from the disease late in larval development, forming a dry 'scale' with approximately 2×10^9 bacterial spores/bee (Genersch, 2010b). Clinical symptoms of AFB are typical, with the brown, viscous larval remains forming a ropy thread when drawn out with a matchstick. The decaying larvae desiccate into hard scales, consisting of millions of bacterial spores (Genersch et al., 2005).

Currently, the treatment of AFB is carried out by the use of oxytetracycline, which has been continuously applied, so that strains may develop resistance. Besides, the use of antibiotics may generate residues in the honey, affecting quality and commercialization. Throughout the past few years an increased attention has been devoted to the use of natural substances, such as essential oils and antimicrobial peptides, to treat infected beehives (Benitez et al., 2012; Sabaté et al., 2012). Recently, our research group described the potential of Copaiba oil for treatment or

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prevention of AFB, since it showed high antimicrobial activity against *Paenibacillus* species (Santos et al., 2012).

Copaiba oils are produced by exudation of the trunks of trees belonging to the genus *Copaifera*. The medicinal properties of copaiba oils were known among American Indians, who probably observed that animals rubbed themselves on copaiba tree trunks to heal their wounds (Veiga-Júnior et al., 2006). Pharmacological studies have demonstrated that it presents anti-inflammatory, gastro-protective, analgesic, wound-healing (Paiva et al., 2004; Brito et al., 2005; Carvalho et al., 2005), anti-nociceptive (Gomes et al., 2007), anti-tumor (Lima et al., 2003), and antimicrobial (Costa-Lotufo et al., 2002; Tincusi et al., 2002) properties. Previous studies have demonstrated that the oleoresin did not show toxicity in mammalian cells, neither caused lesions nor bleeding in the stomach of treated mice (Gomes et al., 2007; Veiga-Júnior et al., 2007).

Carapa guaianensis is a tall tree that grows wild throughout South America, West India, and South Africa. In Brazil, it can be found prevalently in areas of the Amazon rainforest. From the nuts of this plant, an oil called Andiroba oil is extracted, which has a long history of traditional use in South America (Gilbert et al., 1999; Moura et al., 2002). Among its properties, the anti-inflammatory (Penido et al., 2006a), anti-allergic and analgesic actions (Penido et al., 2006b) are noticeable. Insecticide and repellent activity against *Aedes aegypti* and *Aedes albopictus* (Miot et al., 2004; Silva et al., 2004) and larvicidal activities (Mendonça et al., 2005; Silva et al., 2006) have been reported. Because of this, Andiroba oil has aroused great interest from pharmaceutical and cosmetic industries.

Nanoemulsions are a group of usually spherical dispersed particles used for pharmaceutical and biomedical aids and vehicles that show great promise for the future of drug therapy and biotechnology. Such particles are generally in the size range of 100 nm or less in diameter. They may exist as water-in-oil and oil-in-water forms (where the core of the particle is either water or oil, respectively) (Sarker, 2005). Nanoemulsions are often produced by high-energy emulsification using high shear stirring, high-pressure homogenizers, or ultrasound. This approach is widely used due to its ease for large scale production and low cost (Solans et al., 2005), having resulting systems with high stability, transparency, and low viscosity that are attractive for many applications (Wu et al., 2001; Sonneville-Aubrun et al., 2004; Yuan et al., 2008).

Despite the many bioactivities associated to Copaiba and Andiroba oils, there is limited information on their formulation as emulsions or nanoemulsions (Dias et al., 2012). This research aimed to evaluate the physicochemical properties of nanoemulsions of Copaiba and Andiroba oils, and to investigate their antimicrobial activity against *Paenibacillus* species and their toxicity on larvae and adult worker bees *Apis meliffera*.

Materials and methods

Essential oils

Andiroba oil (*C. guaianensis*) code RF3150 and Copaíba oil (*Copaifera officinalis*) code RF3350 were purchased from *Beraca Sabará Químicos e Ingredientes S/A* (São Paulo, Brazil).

Oils characterization

Oils compositions and yield were analyzed applying gas chromatography (GC). Analyses were performed with Agilent Technologies 6890N GC-FID system, equipped with DB-5 capillary column (30 m × 0.32 mm; 0.50 mm) and connected to an FID detector. The thermal programmer was 60 °C (1 min) to 180 °C at 3 °C/min; injector temperature 220 °C; detector temperature 220 °C; split ratio 1:10; carrier gas Helium; flow rate: 1.0 ml/min. The volume injected 1 μl diluted in chloroform (1:10). Two replicates of samples were processed in the same way. Component relative concentrations were calculated based on GC peak areas without using correction factors (Boligon et al., 2013). GC–MS analyses were performed on an Agilent Technologies AutoSystem XL GC–MS system operating in the EI mode at 70 eV, equipped with a split/splitless injector (220 °C). The transfer line temperature was 220 °C. Helium was used as gas carrier (1.0 ml/min) and the capillary columns used were an HP 5MS (30 m × 0.35 mm; film thickness 0.50 mm) and an HP Innowax (30 m × 0.32 mm i.d., film thickness 0.50 mm). The temperature programmer was the same as the one used for the GC analyses. The injected volume was 1 μl of the essential oil diluted in chloroform (1:10). Identification of the constituents was performed on the basis of retention index (RI), determined with reference of the homologous series of *n*-alkanes, C_7–C_{30}, under identical experimental conditions, comparing with the mass spectra library search (NIST and Wiley), and with the mass spectra literature date Adams (1995). The relative amounts of individual components were calculated based on the CG peak area (FID response).

Microorganisms

Six isolates of *Paenibacillus* species from the collection of the Ministry of Agriculture (LANAGRO/RS) Brazil were used in this study. The test organisms included isolates of *Paenibacillus alginolyticus*, *Paenibacillus azotofixans*, *Paenibacillus borealis*, *Paenibacillus gluconolyticus*, *Paenibacillus validus* and *P. larvae* (ATCC9545). The microorganisms were grown in Mueller-Hinton broth (Difco, Sparks, MD, USA) at 37 °C for 24 h and maintained in nutrient agar slopes (Difco).

Nanoemulsions

Nanoemulsions were prepared using the oil phase containing a lipophilic surfactant (Span 80®) dispersed in Andiroba oil, Copaiba oil or medium-chain triglyceride (MCT; blank) and an aqueous phase containing hydrophilic surfactant (Tween 20®) dispersed in water (Table 1). Both phases were mixed and homogenized under magnetic stirring for 15 min at room temperature, resulting in a coarse emulsion. Afterwards, the coarse emulsions were individually subjected to high pressure homogenization (Emulsiflex-C3®, Avestin, Canada) at 1000 MPa to get the final emulsions. The particle size and polydispersity indices (PDI) were measured by photon correlation spectroscopy (Malvern Zetasizer/Nanosizer®) and z potential by electrophoretic mobility, after dilution of 20 μl of the samples in 20 ml of 1 mM NaCl. The pH of the nanoemulsions has been analyzed by Digimed direct readings potentiometer (São Paulo, Brazil) previously calibrated with pH buffers 4.0 and 7.0. All determinations were performed for three independent preparations.

Antimicrobial activity

The minimum inhibitory concentrations (MIC) of NE1, NE2 and NE3 were determined for *Paenibacillus* species in Mueller-Hinton broth by microdilution techniques (CLSI, 2008). The nanoemulsions were diluted in sterile saline (NaCl 0.85%). The assay was carried out in 96-well microtitre plates. Each nanoemulsion was mixed with an inoculum prepared in the same medium at a density adjusted to 0.5 of the McFarland scale (1.5×10^8 CFU/ml) and diluted 1:10 for the microdilution broth procedure. Microtiter trays were incubated at 37 °C and the MICs were recorded after 24 h of incubation. The MIC was defined as the lowest concentration of compounds that inhibits bacterial growth. This test was performed in triplicate on separate occasions. The compound 2,3,5-triphenyltetrazolium chloride was used as an indicator of bacterial growth.

Toxicity studies

In order to evaluate the toxicity of nanoemulsions, larvae and adult worker bees of *A. mellifera* were kindly provided by the association of