



Antimicrobial activity of tea tree oil nanoparticles against American and European foulbrood diseases agents



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ABSTRACT

Paenibacillus larvae and *Melissococcus plutonius* are the primary bacterial pathogens of honeybees and the causative agents of American and European foulbrood disease (AFB and EFB) respectively. Such diseases have been gaining importance since there are few therapeutic options beyond the reporting of microorganisms resistant to conventional antibiotics. Due to the inefficiency and/or low efficacy of some antibiotics, researches with nanotechnology represent, possibly, new therapeutic strategies. Nanostructured drugs have presented some advantages over the conventional medicines, such as slow, gradual and controlled release, increased bioavailability, and reduced side-effects, among others. In this study, *in vitro* antimicrobial activity of tea tree oil (TTO) nanoparticles against *Paenibacillus* species, including *P. larvae* and *M. plutonius* strains was evaluated. Minimal inhibitory concentration (MIC) in Mueller–Hinton or KSBHI broth by the microdilution method was assessed. TTO registered MIC values of 0.18–6.25%, while the MIC values obtained for the TTO nanoparticle were of 0.01–0.93%. The possible toxic effect of TTO and TTO nanoparticle has been assessed by the spraying application method in the concentrations higher than the MICs. Bee mortality was evident only in treatment with TTO and the TTO nanoparticles show no toxic effects after 7 days of observation. Our results showed for the first time that TTO nanoencapsulation presented a high activity against *Paenibacillus* species and *M. plutonius* strains showing that the use of nanotechnology may represent one alternative way for the treatment or prevention of AFB and EFB.

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Introduction

American foulbrood (AFB) and European foulbrood (EFB) are the two major bacterial diseases affecting honeybees, leading to a decrease in viability of the hive, decreasing honey production, and resulting in significant economic losses to beekeepers.

The etiologic agent of AFB is the *Paenibacillus larvae* (a spore-forming, Gram-positive bacterium) (Shimanuki, 1997) and the *Apis mellifera* larvae are more vulnerable during the early larval stage—where arguably an undeveloped immune system and/or a lack of energy

storage results in death (Brødsgaard et al., 1998). The death occurs due to a systemic infection after the germinated bacterial spores proliferate in the midgut and then breach the midgut epithelium via a paracellular route (Yue et al., 2008).

The EFB is closely related to AFB in symptomatology. The causative agent of EFB is a Gram-positive lanceolate coccus, *Melissococcus plutonius*. This bacterium was originally described in 1912 by White and was first cultured and characterized by Bailey (White, 1912; Bailey, 1957). EFB affects mainly unsealed larvae and kills them at the age of 4 to 5 days. The dead larvae turn yellowish, then brown, decompose, and become watery. The larval remains often give off a foul or sour smell due to secondary invaders, such as *Enterococcus faecalis* and *Paenibacillus* sp. (Arai et al., 2012).

P. larvae are the most devastating bacterial pathogen of honey bees. AFB is a non-rare, globally occurring brood disease which is classified as

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a noticeable disease in most countries (Genersch et al., 2006; Genersch, 2010). EFB occurs in most areas in the world where apiculture is practiced, and is recognized as an economically important disease for apiculture (Arai et al., 2012).

The treatment of AFB and EFB is difficult since antibiotics can only mitigate but will not eliminate these diseases and therefore infected hives must be treated constantly to prevent a foulbrood outbreak. When untreated, AFB and EFB destroy the hive's bee population and can annihilate an apiary. Some antimicrobials have been demonstrated to be an effective treatment of AFB and EFB (Waite et al., 2003); however, the use of these antibiotics must be discontinued with sufficient time prior to honey flow in order to prevent residues in the honey.

Some authors have suggested the use of essential oils to control the diseases (Albo et al., 2003). Recently, our research group evaluated the *in vitro* activity of two Amazonian oils (Andiroba and Copaiba) against *Paenibacillus* species, including *P. larvae*. The minimal inhibitory concentration (MIC) of Andiroba oil demonstrated values of 1.56–25%, while the MIC values obtained for Copaiba oil were of 1.56–12.5%. Bee mortality was evident only in treatment with Andiroba oil while Copaiba oil did not show toxic effects after 10 days of observation (Santos et al., 2012).

Tea tree oil (TTO), derived from *Melaleuca alternifolia*, has widely been used as an antimicrobial and anti-inflammatory agent. The broad-spectrum antimicrobial activity of TTO is mainly attributed to terpinen-4-ol and 1,8-cineole, major components of the oil. These substances indicate some activities, such as antibacterial, antifungal, antiviral, and antiprotozoal activities, all promoting TTO as a therapeutic agent (Furneri et al., 2006). There are no records of the use of TTO in AFB and EFB.

The field of nanotechnology is one of the most active areas of research in modern materials science. Nanoparticles present completely new or improved properties based on specific characteristics such as size, distribution and morphology. The emergence of this technology in the last decade presents opportunities for exploring the bactericidal effect of nanoparticles. Several nanoparticles have found applications in diverse products such as medical instruments and devices and industrial processes, including water treatment and food processing as antimicrobial agents (Kora and Arunachalam, 2011).

This research evaluates for the first time the antimicrobial activity of tea-tree oil nanoparticles against AFB and EFB etiologic agents. Toxicity against honeybees *A. mellifera* was also investigated.

Materials and methods

TTO and TTO nanoparticles

TTO was purchased from Importadora Química Delaware Ltda, Brazil. Dimethyl sulfoxide (DMSO) was used to dilute TTO. Tea tree nanoparticles were obtained from Inventiva® (Porto Alegre, Brazil). Briefly, solid lipid nanoparticles were prepared with 7.5% of tea tree oil using a proprietary method from Inventiva®, based on high pressure homogenization. Cetyl palmitate was used as solid lipid and polysorbate 80 as surfactant. Total solid content was 18.6%. Particle size and zeta potential were evaluated in diluted samples (500×) using Zeta Sizer Nanoseries, Malvern. The pH was assessed by direct use of Digimed potentiometer.

TTO characterization

Oil composition and yield were analyzed using the gas chromatography (GC) carried out using an Agilent Technologies 6890N GC-FID system, equipped with DB-5 capillary column (30 m × 0.25 mm × 2.5 μm film thickness) and connected to a flame ionization detector (FID). The injector and detector temperatures were set to 250 °C. The carrier gas was helium, at a flow rate of 1.3 mL/min. The thermal programmer was 100–280 °C at a rate of 10 °C/min. Two replicates of samples were

processed in the same way. Component relative concentrations were calculated based on GC peak areas without using correction factors. The injection volume of the TTO was 1 μL (Boligon et al., 2013a,b). GC-mass spectroscopy (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 (250 °C). The transfer line temperature was 280 °C. Helium was used as carrier gas (1.5 mL/min) and the capillary columns used were an HP 5MS (30 m × 0.25 mm × 2.5 μm film thickness) and an HP Innowax (30 m × 0.32 mm i.d., film thickness 0.50 μm). The temperature programmed was the same as that used for the GC analyses. The injected volume was 1 μL of the essential oil.

Identification of the constituents of TTO was performed on the basis of retention index (RI), determined with reference of the homologous series of *n*-alkanes, C₇–C₃₀, under identical experimental conditions, comparing with the mass spectra library search (NIST and Wiley), and with the mass spectra literature data Adams (1995). The relative amounts of individual components were calculated based on the CG peak area (FID response).

Microorganisms

In this study, eight isolates of *Paenibacillus* species from the collection of the National Agricultural Laboratory (LANAGRO/RS-Brazil) and two strains of *M. plutonius* (National Institute of Animal Health, Japan) were used. The test organisms included environmental isolates such *Paenibacillus alginolyticus*, *Paenibacillus pabuli*, *Paenibacillus azotofixans*, *Paenibacillus borealis*, *Paenibacillus gluconolyticus*, *Paenibacillus validus*, *Paenibacillus thiaminolyticus* and *P. larvae* (ATCC 9545). The *Paenibacillus* were grown in Mueller–Hinton broth (Difco Becton Dickinson Co., Sparks, MD, USA) at 37 °C for 24 h and maintained on slopes of nutrient agar (Difco). The strains of *M. plutonius* used in this work were DAT561 (atypical strain) and DAT606 (typical strain). Cultural and biochemical characteristics of the strains are described in Arai et al. (2012).

Antibacterial assay

The MIC of TTO was determined by microdilution techniques in Mueller–Hinton broth (Difco) for *Paenibacillus* species (CLSI, 2008) and KSBHI broth (BHI broth [Becton Dickinson] supplemented with 0.15 M KH₂PO₄ and 1% starch) for *M. plutonius* strains. A negative control was performed with DMSO.

Effect of TTO and TTO nanoparticles on *A. mellifera*

On the basis of the highest MIC for each microorganism (Table 1), we assessed toxicity of 6.25% TTO and 12.5% TTO nanoparticles in honeybees (*A. mellifera*). In the 12.5% TTO nanoparticle solution, 0.93% TTO

Table 1
MICs of TTO and TTO nanoparticles.

Microorganism	MIC (%) ^a	
	TTO	TTO nanoparticles
<i>P. larvae</i> ATCC9545	1.5	0.11
<i>P. borealis</i>	1.5	0.11
<i>P. validus</i>	0.18	0.11
<i>P. azotofixans</i>	0.18	0.01
<i>P. gluconolyticus</i>	1.5	0.46
<i>P. alginolyticus</i>	1.5	0.46
<i>P. apiarius</i>	1.5	0.23
<i>P. thiaminolyticus</i>	1.5	0.46
<i>M. plutonius</i> DAT561 ^b	6.25	0.93
<i>M. plutonius</i> DAT606 ^c	3.12	0.93

^a MIC of TTO and TTO nanoparticles was expressed as a percentage of TTO contained in the broth and TTO/TTO nanoparticles mixtures.

^b Atypical strain.

^c Typical strain.

was contained. The spraying application method according to Damiani et al. (2009) was used. A device with candy and water was placed inside each unit as food for the bees. Six bees in a modified Petri dish sprayed with DMSO were included as negative death control, and six bees in a modified Petri dish sprayed with deltamethrin (DTT) 0.07% (Pirisa-Piretro Industrial Ltda, Brazil) were included as positive death control. Four replicates for each experimental group were run. Bioassay dishes were placed in incubators at $28 \pm 1^\circ\text{C}$ and 60% RH. Mortality of bees was evaluated daily by visual inspection for up to seven days.

Statistical analysis

Differences in survival after 7 days of observation were assessed by Kaplan–Meier analysis followed by the Logrank test. A *P* value of <0.05 was considered statistically significant. All statistical analyses were performed with the software package GraphPad Prism 4.00 for Windows (GraphPad Software, San Diego, CA, USA).

Results

Characterization of TTO

Fifteen components, representing 95.86% of the total composition, were identified in TTO oil, sample used in this research. The results indicated that terpinen-4-ol (41.98%) was the most abundant compound, followed by γ -terpinene (20.15%), α -terpinene (9.85%), 1,8-cineole (6.03%) and terpinolene (4.15%) as seen in Fig. 1.

Peak	Compounds	RI ^a	RI ^b	Amount (%)	ISO 4730 range (%)	Mol. Formula
1	α -Pinene	937	939	3.51	1-6	C ₁₀ H ₁₆
2	Sabinene	976	976	0.46	Tr-3.5	C ₁₀ H ₁₆
3	α -Terpinene	1016	1018	9.85	5-13	C ₁₀ H ₁₆
4	p -Cymene	1025	1026	2.27	0.5-8.0	C ₁₀ H ₁₄
5	Limonene	1032	1031	1.39	0.5-1.5	C ₁₀ H ₁₆
6	1,8-Cineole	1037	1038	6.03	Tr-15	C ₁₀ H ₁₈ O
7	γ -Terpinene	1062	1062	20.15	10-28	C ₁₀ H ₁₆
8	Terpinen-4-ol	1178	1177	41.98	30-48	C ₁₀ H ₁₈ O
9	Terpinolene	1089	1088	4.17	1.5-5	C ₁₀ H ₁₆
10	α -Terpineol	1190	1189	2.43	1.5-8	C ₁₀ H ₁₈ O
11	Aromadendrene	1440	1439	1.04	Tr-3	C ₁₅ H ₂₄
12	Ledene	1521	1525	0.80	Tr-3	C ₁₅ H ₂₄
13	δ -Cadinene	1539	1538	0.63	Tr-3	C ₁₅ H ₂₄
14	Globulol	1583	1583	0.97	Tr-1	C ₁₅ H ₂₆ O
15	Viridiflorol	1591	1590	0.18	Tr-1	C ₁₅ H ₂₆ O
Total identified (%)				95.86		

Relative proportions of the essential oil constituents were expressed as percentages. Tr = Trace amounts. ^aRetention indices experimental (based on homologous series of *n*-alkane C₇–C₃₀). ^bRetention indices from literature (Adams, 1995). International Organization for Standardization (ISO) standard no. 4730.

Relative proportions of the essential oil constituents were expressed as percentages. ^aRetention indexes experimental (based on homologous series of *n*-alkane C₇–C₃₀). ^bRetention indexes from literature (Adams, 1995).

Fig. 1. Qualitative and quantitative analyses of TTO essential oil. Relative proportions of the essential oil constituents were expressed as percentages. Tr = Trace amounts. ^aRetention indices experimental (based on homologous series of *n*-alkane C₇–C₃₀). ^bRetention indices from literature (Adams, 1995). International Organization for Standardization (ISO) standard no. 4730.

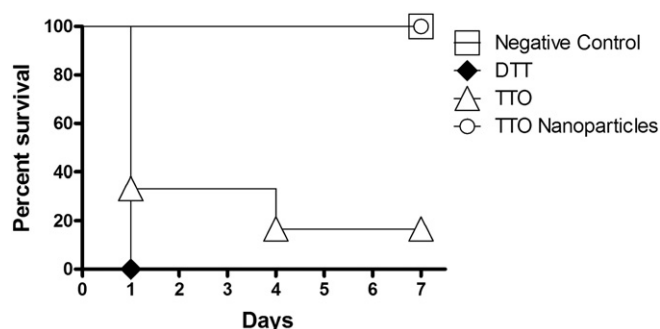


Fig. 2. Effects of TTO and TTO nanostructures spraying applications on bees. For further details, see Materials and methods.

Characterization of TTO nanoparticles

The formulation prepared was evaluated regarding their physical-chemical properties. The particle size was 287 ± 2 nm and the polydispersion index was 0.203 ± 0.022 . The formulation presented a zeta potential of -14.2 ± 1.7 mV.

Antimicrobial susceptibility test and determination of MIC

All *Paenibacillus* species and the *M. plutonius* strains were susceptible to TTO, and the MIC ranged 0.18 to 6.25 when it was used as a crude

essential oil (TTO in Table 1). The bactericidal activity increased when the oil was added to bacterial cultures in the nanoparticle form (TTO nanoparticles), and the MIC ranged 0.01 to 0.93% in the form (Table 1).

Lethal concentration on bees

Toxicity analyses for honeybees, evaluated by the spraying application method, demonstrated that bee mortality was evident in treatment with DTT (positive death control group) and TTO after 7 days of treatment (Fig. 2). The TTO nanoencapsulation significantly reduced this toxic effect since 100% of the honeybees remained alive.

Discussion

Bee diseases AFB and EFB can be treated with anti-infectious agents such as Streptomycin, Tetracyclines, Sulfonamides, Erythromycin, Tylosin, Chloramphenicol, and Nitrofurans. However, in the EU and the USA the use of these agents in beekeeping is strictly regulated due to the lack of tolerance for residues of these drugs in honey (Murray et al., 2007). Another problem associated to antibiotic use is the antimicrobial resistance. *P. larvae* resistance to antimicrobial drugs has become widespread in the past few years (Miyagi et al., 2000; Mussen, 2000; Evans, 2003; Cox et al., 2005). Therefore, the development of strategies of natural origin for the control of AFB and EFB is of great importance.

The present work reports the first study about the use of TTO and TTO nanoparticles for the treatment of *P. larvae* and *M. plutonius*. Results indicate that TTO and TTO nanoparticles showed *in vitro* antibacterial activity against the etiologic agents of AFB and EFB and that low concentrations (especially TTO nanoparticles) are required to inhibit its growth. In this research, TTO nanoparticles showed better results for *Paenibacillus* species and *Melisococcus* species with MIC of 0.11 to 0.01% and 0.93%, respectively (Table 1).

Interactions between components present in the essential oils and the structures of bacteria play a key role in antimicrobial actions. Therefore, antibacterial activity described in TTO could be related to their chemical composition that is rich in terpinen-4-ol, but other components may be important such as γ -terpinene, α -terpinene, 1,8-cineole, terpinolene, ρ -cymene, α -pinene, α -terpineol, aromadendrene, δ -cadinene, limonene, sabinene, globulol, and viridiflorol (Carson et al., 2006). In the present study, the major compound found in TTO was terpinen-4-ol, a widely known substance that present intense antimicrobial activity, both in bacteria and in fungi. Despite the antimicrobial activity of TTO being attributed mainly to terpinen-4-ol, it has been proposed that the antimicrobial activity could be due to the synergism between its different components (Cox et al., 2001). The presence of such compounds in the essential oil from TTO was previously reported by James and Callander (2012) and other species of the genus *Melaleuca* also showed these components in their chemical composition (Amri et al., 2012).

TTO presents physical characteristics that jeopardize its use in pharmaceutical formulations. Its lipophilicity leads to miscibility problems in water-based products. The composition of TTO may change during storage; light, heat, exposure to air, and moisture can affect its stability. Moreover, TTO presents high rate of volatilization (Carson et al., 2006). Then, nanoencapsulation of TTO can offer significant advantages. It is possible to be seen in our results that there is an antimicrobial action potentiation of TTO, where small concentrations are sufficient to inhibit the growth of etiologic agents of AFB and EFB.

In recent years, the interest of researchers has been focused on identifying natural substances with antimicrobial properties which are readily accepted by the bees, do not accumulate in bee products and provide a stimulating effect on the development of colonies (Bogdanov, 2006; Rusenova and Parvanov, 2009). Recently, our group evaluated the effect of crude extract and fractions of *Scutia buxifolia* against six *Paenibacillus* species, including *P. larvae*. All *Paenibacillus* species were sensitive to crude extract and fractions of *S. buxifolia*. The MICs ranged from 50 to

1.56 mg/mL. The *S. buxifolia* showed no toxic effects for the bees after 15 days of observation (Bologon et al., 2013a,b).

In this research, TTO and TTO nanoparticles were sprayed on *A. mellifera* adults to verify the possible toxic effects during 7 days. TTO demonstrates high toxicity to bees (Fig. 2). There are several papers showing the toxic effects of TTO on insect life cycle. Callander and James (2012) showed that TTO was capable of eradicating the Australian sheep blowfly (*Lucilia cuprina*) and sheep body lice (*Bovicola ovis* Schrank). In this same study, *Sarcoptes scabiei* var *hominis* and *Pediculus capitis* (head lice) are also sensitive to TTO. The mechanism of action of TTO in the treatment of the insects is unknown but “suffocation” products are thought to act by blocking the “breathing” spiracles of insects (Barker and Altman, 2011).

The TTO nanoparticles tested presented similar results to the control group (Fig. 2), causing no toxic effects or animal death at tested concentration (higher concentration than that inhibits the growth of *P. larvae* and *M. plutonius*), showing that TTO nanoparticles can be used for the treatment of AFB and EFB. The toxic effect was only observed after TTO exposure. TTO nanoparticles were not able to trigger honeybees' toxic effects, as seen in Fig. 2. This is due to small concentrations of TTO for the manufacture of nanostructures, reflecting a potent antimicrobial activity even in small concentrations. Several studies have reported an enhancement of antimicrobial activity of compounds after its incorporation in nanostructures (Panacek et al., 2006; Pal et al., 2007).

While various hypotheses have been proposed to explain the enhancement of antimicrobial activity of nanoparticles, it is widely believed that these nanoparticles are incorporated in the cell membrane causing leakage of intracellular substances and eventually causing cell death (Sondi and Salopek-Sondi, 2004; Cho et al., 2005). Some of the nanoparticles also penetrate into the cells. It is reported that the bactericidal effect of nanoparticles decreases as the size increases and is also affected by the shape of the particles. Although most studies have utilized spherical particles, truncated triangular shaped particles are reported to have greater bactericidal effect compared to that of spherical and rod-shaped particles (Panacek et al., 2006; Pal et al., 2007). The emergence of nanoscience and nanotechnology in the last decade presents opportunities for exploring the bioactive effects of nanoparticles; however, there are no studies using nanoparticles in the treatment of AFB and EFB and to our knowledge, this is the first study demonstrating the use of nanotechnology to treat such important diseases.

The use of perspective non-toxic compounds could represent a natural alternative to synthetic antibiotics in the control of AFB and EFB. This way, TTO nanoparticles are a potentially useful alternative in suppressing such bacterial diseases that affect honeybee.

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