Antibacterial activity of *Pinus elliottii* and its major compound, dehydroabietic acid, against multidrug-resistant strains

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Antibiotic-resistant bacteria have emerged from the widespread use of antibiotics worldwide and have prompted the search for new sources of antimicrobial substances. *Pinus* spp. contain several bioactive compounds consisting mainly of terpenes, terpenoids and some other aromatic and aliphatic constituents. These compounds exert important biological effects, and pine oils have found wide application in the industry. In the present study, we have evaluated the potential activity of the resin-oil of *P. elliottii* and its major compound dehydroabietic acid (DA) against multiresistant bacteria by MIC, minimum bactericidal concentration and time-kill assays. The MIC of the resin-oil of *P. elliottii* varied between 25 and 100 \( \mu \text{g} \text{ ml}^{-1} \). As for DA, the MIC and minimum bactericidal concentration varied between 6.25 and 50 and between 6.25 and 100 \( \mu \text{g} \text{ ml}^{-1} \), respectively. The time-kill assay conducted with DA at 6.25 \( \mu \text{g} \text{ ml}^{-1} \) evidenced bactericidal activity against *Staphylococcus epidermidis* (American Type Culture Collection 14990) within 24 h. On the basis of these results, the resin-oil of *P. elliottii* and its major compound DA play an important part in the search for novel sources of agents that can act against multiresistant bacteria.

INTRODUCTION

The widespread use of antibiotics worldwide has culminated in a high prevalence of infections by antibiotic-resistant bacteria. Indeed, exchangeable genetic elements such as plasmids, transposons and integrons disseminate antibiotic resistance in many bacteria (Nikokar et al., 2013). The slower introduction of new chemotherapeutic drugs into the market and clinical practice over the last few years may yet be another reason for the growing number of resistant bacteria (Russell, 2002; Coelho et al., 2013). This slow-down may have caused a number of antibiotics to lose their efficacy against antibiotic-resistant organisms, making the use of drugs that are often reserved for last-line treatment necessary (Coelho et al., 2013). The overall result of the emergence of human pathogenic organisms exhibiting multidrug resistance has been the search for new antimicrobial substances from other sources, including plants (Ahmad & Beg, 2001).

During the 1950s, exotic, fast-growing trees such as *Pinus* spp. were introduced in southern Brazil to supply wood for the paper industry and cellulose for energy production. Today, Brazil holds one of the largest planted areas in the world (Pacheco et al., 2009). A number of compounds displaying anti-inflammatory, antiparasitic, antioxidant, antimutagenic and antitumour activities, as well as the ability to regulate cancer-related proteins, have been isolated from different *Pinus* spp. (Tóro et al., 2003; Karonen et al., 2004; Kwak et al., 2006; Li et al., 2007; Politeo et al., 2011). Some species are frequently used for their medicinal benefits, such as diuretic and expectorant actions, to treat diseases that include pulmonary, urinary, hepatic and hypertensive disorders (Lawless, 1992; Politeo et al., 2011). Moreover, pine oils are widely employed as fragrances in cosmetics, flavouring additives in food, and intermediates in the synthesis of perfume chemicals (Sticher, 1977; Politeo et al., 2011). They consist mainly of terpenes, terpenoids and some other aromatic and...
aliphatic constituents, which usually have a strong smell and low molecular mass (Marriott et al., 2001; Burt, 2004; Politeo et al., 2011; Zeng et al., 2012).

The current literature contains no reports on the biological activities of the resin-oil of Pinus elliottii and its bioactive compounds against antibiotic-resistant bacteria. Therefore, the present study aimed to investigate the in vitro antibacterial activity of the oleoresin of P. elliottii and its major compound dehydroabietic acid (DA) against multi-resistant bacteria.

**METHODS**

**Tested micro-organisms.** The bacteria were acquired from the American Type Culture Collection (ATCC) and National Collection of Type Cultures (NCTC); the Hospital das Clínicas de Ribeirão Preto (São Paulo, Brazil) kindly supplied seven bacteria from clinical isolates, which were maintained in the culture collection of the Laboratory of Research in Applied Microbiology at the University of Franca, São Paulo, Brazil, at −80 °C. The following micro-organisms were used: Staphylococcus epidermidis (ATCC 14990), S. epidermidis (clinical isolate), Staphylococcus aureus (ATCC 29213), S. aureus (clinical isolate), Enterococcus faecium (NCTC 717), E. faecium (clinical isolate), Staphylococcus capsici (ATCC 27840), S. capitis (clinical isolate), Enterococcus faecalis (ATCC 14990), E. faecalis (clinical isolate), Staphylococcus haemolyticus (ATCC 29970), S. haemolyticus (clinical isolate), Klebsiella pneumoniae (ATCC 700603) and K. pneumoniae (clinical isolate).

**Oil-resin and isolation of the major compound.** The Associação dos Resinadores do Brasil, located in the city of Avaré, São Paulo, Brazil, donated the original oil-resin of P. elliottii var. elliottii (OPe) tested here. First, the oil-resin (40.0 g) was partitioned by vacuum liquid chromatography using a solvent system of increasing polarity (n-hexane/ethyl acetate). This procedure furnished five fractions (2 l each) that were named OPe1 (100 % n-hexane), OPe2 (20 % ethyl acetate), OPe3 (40 % ethyl acetate), OPe4 (60 % ethyl acetate) and OPe5 (100 % ethyl acetate). The MIC values of the five fractions were determined against the tested bacteria. MIC results revealed that fraction OPe2 was the most active against multiresistant bacteria (Table 1).

The major compound in OPe2 was isolated from 1.0 g of this fraction by classic chromatography using 500 ml of the mixture n-hexane/ethyl acetate (8:2, v/v) as the mobile phase. The obtained DA was dissolved in DMSO (Synth) at 1.0 mg ml⁻¹ and then, by dilution in tryptic soy broth (Difco); concentrations ranging from 100.0–1.0 μg ml⁻¹ were achieved. The final DMSO content was 5 % (v/v) and this solution was used as a negative control. The inoculum was adjusted for each organism to yield a cell concentration of 5 × 10⁸ c.f.u. ml⁻¹, according to previous standardization by the Clinical Laboratory Standards Institute (CLSI, 2009).

One inoculated well, free of antimicrobial agent, was also employed to ensure medium sterility. Vancomycin and imipenem were used as positive controls. The microtitre plates (96 wells) were sealed with plastic film and incubated at 37 °C for 24 h. After that, resazurin (30 μl) in aqueous solution (0.02 %) was added to the microplates to indicate micro-organism viability (Sarker et al., 2007; Ambrosio et al., 2008; Porto et al., 2009, 2013). Before addition of resazurin and to determine the MBC, an aliquot of the inoculum was aseptically removed from each well presenting no apparent growth and then plated onto tryptic soy agar. The plates were incubated as described previously.

**Time-kill curves.** Time-kill assays were performed in triplicate based on the method of D’Arrigo et al. (2010). DA was chosen to conduct the time-kill curve assays because, according to Rios & Recio (2005), isolated compounds that inhibit the growth of the micro-organisms at concentrations below 10 μg ml⁻¹ are considered very promising in the search for new anti-infection agents. DA displayed the highest antibacterial activity (Table 1, MIC and MBC were 6.25 μg ml⁻¹) only against S. epidermidis (ATCC14990). This micro-organism is considered as one of the most important pathogens involved in hospital-associated bloodstream infections related to vascular catheters because of its virulence factors and ability to produce biofilm (Otto, 2009; Widerström et al., 2012). A tube containing 1 ml of sterile tryptic soy broth and DA at a final concentration of 6.25 μg ml⁻¹ (1 × MBC of DA for S. epidermidis) was inoculated with the tested micro-organism, resulting in an initial bacterial density of 1.35 × 10⁸ c.f.u. ml⁻¹, and then incubated at 37 °C. Aliquots (100 μl) were removed for enumeration of viable colonies at 30 min and 6, 12 and 24 h after incubation, followed by serial dilution, until final dilution (10⁻⁶) in sterile tryptic soy broth. The diluted samples (50 μl) were spread onto tryptic soy agar plates, incubated at 37 °C, and viable colonies were counted after 24 h. The data were analysed and time-kill curves were constructed by plotting log, c.f.u. ml⁻¹ versus time using Prism software (version 5.0; GraphPad). The assays were performed in triplicate as were the positive (vancomycin) and negative controls (suspension of S. epidermidis without added DA). Vancomycin was used at its MBC (0.74 μg ml⁻¹).

**RESULTS**

The resin-oil of P. elliottii furnished satisfactory MIC values (between 25 and 100 μg ml⁻¹) when tested against the multiresistant bacteria S. epidermidis (ATCC 14990), S. epidermidis (clinical isolate), S. aureus (clinical isolate), E. faecium (NCTC 717), E. faecium (clinical isolate), S. capitis (clinical isolate), E. faecalis (ATCC 27840), E. faecalis (NCTC 14990), E. faecalis (clinical isolate), S. haemolyticus (ATCC 29970) and S. haemolyticus (clinical isolate). In the case of the bacteria K. pneumoniae (ATCC 700603), K. pneumoniae (clinical isolate), S. capitis (clinical isolate), and S. aureus (ATCC 29213), the MIC values were equal to 200 μg ml⁻¹ or higher (Table 1).

Assays revealed bactericidal activity against S. epidermidis (ATCC 14990) only – both MIC and MBC were equal to 6.25 μg ml⁻¹ (Table 1).

The time-kill assay helped to assess the kinetic behaviour of DA. MBC values were lower than 10 μg ml⁻¹. The best activity was obtained against S. epidermidis (ATCC 14990).

The results achieved with S. epidermidis (ATCC 14990) demonstrated that DA, at a concentration of 6.25 μg ml⁻¹,
started to progressively diminish the number of microorganisms at 12 h of incubation; bactericidal action was evident at 24 h. Vancomycin (0.74 μg ml⁻¹), used as a positive control, reduced the number of micro-organisms by over 2 log₁₀ at 12 h and exhibited bactericidal activity at 24 h of incubation (Fig. 2).

**DISCUSSION**

The development of antibiotic resistance by bacteria that threaten human health has become a major clinical problem worldwide. This has prompted the search for new antimicrobial substances from other sources, including plants (Ahmad & Beg, 2001; Bennett, 2008). In this context, the present study evaluated the antibacterial action of the resin-oil of *P. elliottii* and its major compound DA against multiresistant bacteria.

In a study conducted by Zeng et al. (2012), the authors determined the MIC and MBC for the essential oil of pine needles from *Cedrus deodara* against the causative agents of food poisoning such as *Escherichia coli* (ATCC 25922), *S. aureus* (ATCC 25923), *Bacillus subtilis* (ATCC 21216), *Bacillus cereus* (ATCC 14579), *Saccharomyces cerevisiae* (ATCC 9763), *Aspergillus niger* (ATCC 16404), *Penicillium citrinum* (ATCC 14994), *Rhizopus oryzae* (ATCC 9363) and *S. epidermidis* (ATCC 14990). Positive control: vancomycin.

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**Table 1.** *In vitro* antibacterial activity (MIC and MBC) of the resin-oil of *P. elliottii*, fraction OPe2 and DA against multidrug-resistant bacteria

<table>
<thead>
<tr>
<th>Bacterium</th>
<th><em>P. elliottii</em> resin-oil (μg ml⁻¹)</th>
<th>Fraction OPe2 (μg ml⁻¹)</th>
<th>DA (μg ml⁻¹)</th>
<th>Positive control (μg ml⁻¹)</th>
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<tbody>
<tr>
<td></td>
<td>MIC</td>
<td>MIC</td>
<td>MIC</td>
<td>MBC</td>
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<tr>
<td><em>S. epidermidis</em> ATCC 14990</td>
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<td>6.25</td>
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<td>50</td>
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<tr>
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<td><em>K. pneumonia</em> clinical isolate</td>
<td>400</td>
<td>400</td>
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−MIC or MBC value >400 μg ml⁻¹, corresponding to a lack of antibacterial activity.
*Positive control, μg ml⁻¹ vancomycin.
†Positive control, μg ml⁻¹ imipenem.

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**Fig. 1.** Chemical structure of DA.

**Fig. 2.** Time-kill curve profiles for DA against *S. epidermidis* (ATCC 14990). Positive control: vancomycin.
Aspergillus flavus (ATCC 204304). The MIC and MBC values of the oil ranged from 0.2 to 1.56 µg ml⁻¹ and from 0.39 to 6.25 µg ml⁻¹, respectively. R. oryzae was the most susceptible bacterium among the tested micro-organisms; the essential oil of the pine needle inhibited the growth of R. oryzae at 0.2 µg ml⁻¹ and exerted a sterilizing effect at 0.39 µg ml⁻¹. Among the assayed micro-organisms, B. subtilis was the most susceptible to the essential oil of the pine needle; the MIC and MBC values were 0.39 and 0.78 µg ml⁻¹, respectively. The present study provided promising results: the MIC values of the resin-oil of P. elliottii varied from 25 to 100 µg ml⁻¹ against the Gram-positive, multiresistant bacteria S. epidermidis (ATCC 14990), S. epidermidis (clinical isolate), S. aureus (clinical isolate), E. faecium (NCTC 717), E. faecium (clinical isolate), S. capitis (ATCC 27840), E. faecalis (NCTC 14990), E. faecalis (clinical isolate), S. haemolyticus (ATCC 29970) and S. haemolyticus (clinical isolate). According to Rios & Recio (2005), a promising result arises when the crude extracts and essential oils of plants exhibit antibacterial activity at concentrations equal to or lower than 100 µg ml⁻¹. Because the resin-oil of P. elliottii contains many substances, the observed activity probably originated from a synergistic action among the resin-oil constituents. Concerning the tested Gram-negative multiresistant bacteria (K. pneumoniae ATCC 700603 and K. pneumoniae clinical isolate), the MIC of the resin-oil of P. elliottii was 400 µg ml⁻¹. In contrast to the Gram-negative bacteria, the Gram-positive micro-organisms were more sensitive to certain compounds. Antimicrobials act on the cell wall, and the different composition of the cell walls of these classes of bacteria may account for these results. In fact, Gram-positive bacteria present a thick cell wall consisting mainly of peptidoglycan, whereas Gram-negative bacteria display a stratified cell wall consisting of an outer membrane and a thin peptidoglycan layer (Beveridge, 1999; Navarre & Schneewind, 1999; Araujo et al., 2010). The unique structural properties of the cell wall of the Gram-negative bacteria may have hindered penetration of the resin-oil of P. elliottii; the external membrane contains lipopolysaccharides which determine surface properties and alter cell permeability and susceptibility to the resin-oil of P. elliottii.

DA was only active against S. epidermidis (ATCC14990); both MIC and MBC were 6.25 µg ml⁻¹. According to Rios & Recio (2005), this is a promising result for a pure isolated compound because it was below 10 µg ml⁻¹. The MIC and MBC for the other tested micro-organisms ranged from 25 to 50 µg ml⁻¹ and from 50 to 100 µg ml⁻¹, respectively.

S. epidermidis is one of the species that is most frequently isolated from the human epithelium. It is an important opportunistic pathogen that often occurs in hospital infections (NNIS, 2004; Otto, 2009). This micro-organism is the commonest source of infection in medical devices such as intravenous catheters (Otto, 2009; Uğkay et al., 2009; Widerström et al., 2012). The following factors complicate treatment: specific genes for antibiotic resistance, biofilm formation, multicellular agglomerations and host defence mechanisms (Costerton et al., 1999; Otto, 2009). In the United States, annual costs involved in the treatment of hospital infections associated with catheters contaminated by S. epidermidis are around $2 billion (Kloos & Musselwhite, 1975; Costerton et al., 1999; Otto, 2009). Hence, it is essential to search for new substances that can fight and eliminate this pathogenic micro-organism. DA proved to be a promising compound in this sense.

We conducted time-kill assays for DA only against S. epidermidis (ATCC 14990) which, as already mentioned, is one of the most important pathogens involved in hospital-associated bloodstream infections related to vascular catheters due to its ability to produce a biofilm (Widerström et al., 2012).

In a study conducted by Mun et al. (2013), the authors used time-kill assays to assess the action of curcumin isolated from the species Curcuma longa Linné against S. aureus resistant to metillin (metillin-resistant S. aureus, ATCC 33591). In isolation, curcumin was not active against metillin-resistant S. aureus. In combination with the antibiotic oxacillin, it presented activity. Our study demonstrated that DA at 6.25 µg ml⁻¹, not in combination with antibiotics, exerted a bactericidal effect against S. epidermidis within 24 h.

In the present scenario regarding the emergence of human pathogens that are resistant to multiple drugs, the resin-oil of P. elliottii and its major compound DA have become important assets in the fight against multiresistant micro-organisms. Further studies to unveil their action mechanisms are therefore essential.

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REFERENCES
Antibacterial activity of Pinus elliottii compounds


