Effects of Copaifera duckei Dwyer oleoresin on the cell wall and cell division of Bacillus cereus

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The aim of this work was to evaluate the antibacterial activity of Copaifera duckei oleoresin and to determine its possible mechanism of action against bacteria of clinical and food interest. The antibacterial activity was determined by agar diffusion and dilution methods; the mechanism of action by transmission electron microscopy and by SDS-PAGE; the bioactive compounds by bioautography; and the chemical analysis by GC/MS. Oleoresin showed activity against nine of the 11 strains of bacteria tested. Bacillus cereus was the most sensitive, with a MIC corresponding to 0.03125 mg ml−1 and with a bactericidal action. Oleoresin acted on the bacterial cell wall, removing proteins and the S-layer, and interfering with the cell-division process. This activity probably can be attributed to the action of terpenic compounds, among them the bisabolene compound. Gram-negative bacteria tested were not inhibited. C. duckei oleoresin is a potential antibacterial, suggesting that this oil could be used as a therapeutic alternative, mainly against B. cereus.

INTRODUCTION

Bacterial resistance to antimicrobials is a problem that persists worldwide, representing a threat to public health (Zhang et al., 2011). Thus, the search for new substances able to inhibit species of resistant micro-organisms has favoured research to obtain more effective antibacterial agents of natural origin, either semi-synthetic or synthetic (Silveira et al., 2006).

Plants have been the main target of these studies, as they represent a great source of substances, many of which may have pharmacological potential. In vitro studies have described the activity of plants and their products for the treatment of microbial diseases, among them species of the genus Copaifera, which have shown antibacterial and antifungal activities in their oil (Santos et al., 2008b; Pieri et al., 2011).

Copaiba oil is an oleoresin extracted from the trunk of species from the genus Copaifera (Leguminosae, Caesalpinioidea), popularly known as ‘copaibeiras’ or ‘pau d’óleo’, which has been reported to be used in popular medicine to treat throat, urinary and pulmonary infections, to heal cutaneous ulcers and to serve as an antiseptic (Santos et al., 2008b). In addition to these activities, other pharmacological properties have been attributed to oil extracted from this genus, such as its

Abbreviations: TEM, transmission electron microscopy; VS, vanillin-sulphuric acid.

Ten supplementary figures are available with the online version of this paper.
gastroprotective, antitumour and cytotoxic, antinociceptive, antioxidant, leishmanicidal and larvicidal properties (Paiva et al., 2004; Veiga et al., 2007; Gomes et al., 2007; Santos et al., 2008a; Geris et al., 2008).

Copaiba oleoresin from different species is used in popular medicine and compounding and homeopathic pharmacies, due to its pharmacological properties, including its antimicrobial activity. However, there are no reports on this activity for Copaifera duckei. Other than the research conducted in Brazil, few other studies have presented scientific data (Santos et al., 2008b) referring to the antimicrobial mechanisms of action of derived from these plants. Thus, this study aimed to evaluate the antibacterial activity of C. duckei oleoresin against bacteria of clinical and food interest and to determine its possible mechanism of action.

METHODS

Micro-organisms. Nine reference strains were used as test organisms: Staphylococcus aureus (ATCC 29213, 25923 and 33591), Enterococcus faecalis (ATCC 29212), Listeria monocytogenes (ATCC 15313), Bacillus cereus (ATCC 11778), Salmonella Typhimurium (ATCC 14028), Escherichia coli (ATCC 25922) and Pseudomonas aeruginosa (ATCC 27853). There were also two clinical isolates of Staphylococcus epidermidis and Shigella sonnei maintained at the Oral Microbiology and Anaerobes Laboratory of the Department of Microbiology at Instituto de Ciências Biológicas Universidade Federal de Minas Gerais. Stock cultures were kept at 4 °C on nutrient agar and at −80 °C in brain–heart infusion broth supplemented with 10 % glycerol.

Plant material. Copaiba oleoresin was extracted from the trunk of C. duckei Dwyer and collected in October 2004, in the town of Moju, Pará, Brazil, and a sample specimen was deposited at the Herbarium of the Embrapa Amazonia Oriental, under registration number 178851. Botanical identification was made by Regina Célia Viana Martins da Silva, Embrapa Amazonia Oriental, Belém, Pará.

Antibacterial activity assays. Antibacterial activity assays were performed by the agar-well diffusion method on Mueller–Hinton agar inoculated with a sterile swab. Wells were made in the MHA with 1 ml sample was removed from each test suspension, serially diluted in sterile saline (from 10−1 to 10−8) and plated on MHA plates (100 μl) for colony count determination, incubated at 37 °C and counted after 24 h. A bactericidal effect was established as a 3 log10 c.f.u. ml−1 killing (99.9 %) of the initial inoculum. Time–kill curves were constructed by plotting the log c.f.u. ml−1 versus time (h). Control of viability was performed. Vancomycin (4 μg ml−1; Vancocin) and a suspension of B. cereus without added oleoresin were used as positive and negative controls, respectively (NCCLS, 1999).

Transmission electron microscopy (TEM). B. cereus was treated with copaiba oleoresin at a subinhibitory concentration corresponding to 0.015625 mg ml−1. The sample was then processed for TEM. Samples were fixed in 2.5 % glutaraldehyde (Sigma) in 0.1 M sodium cacodylate buffer at room temperature for 6 h, post-fixed in 1 % tetroxide osmium (Sigma) in cacodylate buffer for 1 h at room temperature, dehydrated in an increasing ethanol gradient (30–100 %, 15 min each; Merck), immersed in pure acetone (Merck) and embedded in Epon resin mixture: acetone (1:1) and Epon resin (EMS). Ultrathin sections (60 nm) were obtained in a LEIC UC6 ultramicrotome, stained with uranyl acetate and lead citrate, and images were obtained in a Tecnai G2 FEI TEM at 80 keV.

Analysis of copaiba oleoresin activity on cell-wall proteins by SDS-PAGE. The assay of copaiba oleoresin activity on cell-wall proteins of B. cereus was based on the method of Rundegren et al. (1995). An extract of the cellular wall was prepared starting from a bacterial suspension equivalent to 0.5 on the McFarland scale. Aliquots of 10 ml of this suspension were added to sterile tubes and centrifuged (16 000 g for 10 min). The supernatant was removed, and the pellet washed twice with phosphate buffer (0.1 M, pH 7.4). After washing, a 2 ml aliquot of 6.4 mM SDS, copaiba oleoresin (2 μg and 10 μg) and 4 μg vancomycin (Vancocin) ml−1 and 2 % DMSO aqueous solution was added to each pellet. Proteins were removed at 200 r.p.m. at 37 °C in a shaking water bath (Duhnon), at 10, 30 and 90 min. After incubation, 600 μl of each suspension was transferred to microtubes and centrifuged (16 000 g for 10 min).

The test was also performed at 24 h, and analysed, in addition to other substances, using copaiba oleoresin at a concentration of 1 μg and pure oleoresin, without the test bacterium as the negative control. SDS, DMSO and vancomycin were used as controls.

Supernatants were treated with sample buffer (1:1; glycerol, 0.5 M Tris–HCl pH 6.8, β-Mercaptoethanol, SDS, 0.05 % w/v bromophenol blue) and denatured at 100 °C for 5 min. An aliquot of 20 μl of sample was added to the polyacrylamide gel at 12 %. The protein marker (BenchMark Protein Ladder; Invitrogen) was not submitted to the same treatment as the sample, with 0.8 μl added to the gel. Electrophoresis was performed for 2.5 h, in an EPS 301 electrophoresis device power supply (Amersham Biosciences) at a constant voltage of 120 V and at 80 A. All gels were silver stained (Silver Staining kit, Protein; Amersham Biosciences).

Chemical analysis. C. duckei oleoresin was analysed using a THERMO DSQII GC–MS instrument (Thermo Fisher Scientific),
equipped with a DB-5ms fused capillary column (30 m × 0.25 mm; 0.25 μm film thickness). Helium (32 cm s⁻¹ at 100 °C) was used as the carrier gas, the oven temperature program was 60–240 °C at 3 °C min⁻¹, and 2 μl of the oil solution in hexane was injected. Injector and detector temperatures were 240 °C. All mass spectra were acquired in electron impact mode with an ionization voltage of 70 eV. The temperature of the ion source and connection parts was 200 °C. The filter quadrupole swept the range of 39 to 450 Da s⁻¹. Individual components were identified by comparison of both MS and GC retention data with authentic compounds previously analysed and stored in the data system. Other identifications were made by comparison of mass spectra with those existing in the data system libraries and cited in the literature (Adams, 2007). The retention index was calculated using an n-alkanes homologous series.

Bioautography agar-overlay assay. Aliquots of C. duckei oleoresin (1:1, v/v), β-caryophyllene (1:1, v/v, purity ≥80%; Sigma) and bisabolene, a mixture of isomers (1:1, v/v, Santa Cruz Biotechnology), solutions in methanol were applied in aluminium plates containing silica gel G60 F254 (6.0 × 10.0 cm; Merck). Compounds were separated by the eluent system chloroform : toluene (75:25, v/v). After drying, chromatoplates were transferred to sterile Petri dishes, in which MHA had been transferred containing Petro dishes, in which MHA had been transferred containing previously standardized B. cereus inoculum. Plates were incubated at 37 °C for 24 h. After detection using 2,3,5-triphenyltetrazolium chloride (Sigma) to verify the presence of zones of inhibition, vancomycin was used as a positive control of growth inhibition, whereas β-caryophyllene and bisabolene isomers were used as standards. Bioautography was used in duplicate. Plates that were not submitted to the antibacterial activity test were detected with vanillin-sulphuric acid (VS) reagent in order to verify the main chemical groups responsible for the zone of inhibition, with bands seen after heating at 105 °C for 10 min (Wagner & Bladt, 2001; Rakotoniriana et al., 2010).

Statistical analysis. Results were analysed using analysis of variance followed by Tukey's multiple comparison test using the GraphPad Instat software (version 5.0). Results with P<0.05 were considered statistically significant.

RESULTS AND DISCUSSION

C. duckei oleoresin showed inhibitory activity against all Gram-positive bacteria tested, particularly L. monocytogenes 15313, Staphylococcus aureus 25923 and B. cereus 11778. The susceptibility of these bacteria treated with oleoresin differed significantly from that of the other bacteria, except for Enterococcus faecalis 29212, which presented a susceptibility profile similar to that of B. cereus. The Gram-negative bacteria tested were not inhibited (data not shown). Our results are similar to those of Santos et al. (2008b), who observed the antibacterial action of the species Copaifera reticulata, Copaifera martii, Copaifera cearensis, Copaifera paupera, Copaifera langsdorffii, Copaifera officinalis, Copaifera multiugia and Copaifera lucens against the bacteria Staphylococcus aureus, methicillin-resistant Staphylococcus aureus, Staphylococcus epidermidis and Enterococcus faecalis. Pacheco et al. (2006) also verified the activity of different species of Copaifera against Staphylococcus aureus and B. subtilis, but not against Gram-negative (Escherichia coli and P. aeruginosa) bacteria. However, studies with C. langsdorffii and C. officinalis oleoresins have shown inhibitory action against Escherichia coli strains recovered from mastitic milk (Pieri et al., 2011), P. aeruginosa, and Shigella flexneri (Pieri et al., 2012). Mendonça & Onofre (2009) also observed action of C. multiugia oleoresin against Gram-positive (Staphylococcus aureus) and Gram-negative (Escherichia coli and P. aeruginosa) bacteria.

These inter- and intraspecific differences of copaiba oleoresin with regard to its spectrum of antimicrobial action may be related to the variation in its chemical constitution, influenced by environmental factors such as time of year, dry and rainy seasons and age of the plant, among others (Cascon & Gilbert, 2000; Lameira et al., 2009).

As shown in Table 1, using agar dilution, B. cereus 11778 was shown to be more susceptible to C. duckei oleoresin, with a MIC corresponding to 0.03125 mg ml⁻¹, which is considered a strong antibacterial activity. The other bacteria that were susceptible to the oleoresin tested had MICs ranging from 2.0 to >16.0 mg ml⁻¹, showing a weak inhibitory action. One study suggests that an inhibitory action for plant extracts with MICs of up to 0.5 mg ml⁻¹ is classified as strong, from 0.6 to 1.5 mg ml⁻¹ as moderate and over 1.6 mg ml⁻¹ as weak (Duarte et al., 2007).

Activity of oleoresins from different species (C. martii, C. reticulata and C. officinalis) has been observed against another Bacillus species, B. subtilis, with a MIC ranging from 15.62 to 31.25 μg ml⁻¹ (Pieri et al., 2011).

B. cereus is a pathogen of great importance in the food industry, responsible for toxin production, which causes a diarrhoeal and emetic syndrome. Interest in B. cereus has increased recently as a result of opportunistic infections (in skin and soft tissues) in immunocompromised individuals, in addition to the occurrence of bacteraemia, pneumonia, meningitis, endophthalmitis, salpingitis and endocarditis (Budzik et al., 2007).

Fig. 1 graphically represents the death curve of B. cereus (inoculum of about 10⁵ c.f.u. ml⁻¹) treated with C. duckei oleoresin, showing the bactericidal effect against B. cereus, in less than 4 h, at concentrations ranging from 0.03125 to 0.125 mg ml⁻¹ (1× to 4× MIC). Copaiba oleoresin

<table>
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<tr>
<th>Strain</th>
<th>Copaiba oleoresin (mg ml⁻¹)</th>
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<tr>
<td>Staphylococcus aureus 29213</td>
<td>4.0</td>
</tr>
<tr>
<td>Staphylococcus aureus 33591</td>
<td>4.0</td>
</tr>
<tr>
<td>Staphylococcus aureus 25923</td>
<td>&gt;16.0</td>
</tr>
<tr>
<td>Staphylococcus epidermidis</td>
<td>16.0</td>
</tr>
<tr>
<td>Enterococcus faecalis 29212</td>
<td>2.0</td>
</tr>
<tr>
<td>L. monocytogenes 15313</td>
<td>2.0</td>
</tr>
<tr>
<td>B. cereus 11778</td>
<td>0.03125</td>
</tr>
</tbody>
</table>
presented a profile similar to that of 4 μg vancomycin ml⁻¹, inhibiting bacterial growth before 4 h of exposure. DMSO did not affect the results.

Analysis of B. cereus ultrastructure shows the bacterial cell with its preserved S-layer and cell wall (Fig. 2a). After exposure to C. duckei oleoresin at a subinhibitory MIC of 0.015625 mg ml⁻¹, alterations were seen in its cell structure, such as material loss from the S-layer and thickening of the cell wall (Fig. 2b, c), as well as an increased number of centres of septum formation (Fig. 2c). This finding corroborates the ideas of Santos et al. (2008b), who suggested that C. martii oleoresin acts on the Staphylococcus aureus cell wall, causing disruption to the bacterial cell and leading to its death. Morphological changes and a decrease in the number of B. cereus cells were also observed in previous experiments in our group, after exposure to copaiba oleoresin, close to the inhibitory halo (supplementary material, available in JMM Online).

C. duckei oleoresin was able to extract proteins from the B. cereus cell wall at a concentration of 2.0 × and 4.0 × MIC, in 10, 30 and 90 min (Fig. 3a), except for oleoresin at 10 min (Fig. 3a, lane 1). Fig. 3(b) shows the extraction capacity of the cell-wall proteins by SDS, the substance used as a control. We observed that the treatment with oleoresin showed an electrophoretic profile similar to that of vancomycin, in 10–90 min (Fig. 3c). In the test of exposure for 24 h, removal of the protein was observed at all concentrations of oleoresin, with extraction of more protein at a concentration of 4 × MIC (Fig. 3d, lane 15), evidenced by multiple bands in the gel, also observed with vancomycin (Fig. 3d, lane 16). Thus, we concluded that one of the sites of action of C. duckei oleoresin is the cell wall of B. cereus.

These results confirmed the data observed by electron microscopy, in which changes were seen in the cell wall of B. cereus, with the formation of filaments in the S-layer, suggesting material release. It was believed that the antibacterial activity was due to the presence of the terpenic compounds found in copaiba oleoresin. Table 2 shows the sesquiterpenic compounds of C. duckei oleoresin, listed in sequence according to their retention time. Oleoresin is composed mainly of β-bisabolene (40.94 %), trans-α-bergamotene (20.47 %) and β-caryophyllene (9.16 %). In their studies with oleoresin from trees of the species C. duckei in different periods of the year, Cascon & Gilbert (2000) and Lameira et al. (2009) observed the presence of these compounds, in addition to β-selinene, α-selinene and α-bisabolene.

Maistro et al. (2005) performed a phytochemical analysis of C. duckei oleoresin and verified the presence of sesquiterpene hydrocarbons, neutral diterpene and diterpene acids. The

Fig. 1. Time–kill curves of copaiba oleoresin against B. cereus. Results are from experiments in triplicate.

![Fig. 1](image1)

Fig. 2. Ultrastructural effect of copaiba oleoresin after incubation of B. cereus for 24 h, observed by TEM. (a) Untreated; (b–c) B. cereus treated with 0.015625 mg copaiba oleoresin ml⁻¹. Bars, 500 nm. Arrows indicate changes in the cell wall.

![Fig. 2](image2)
sesquiterpene β-caryophyllene was attributed to insecticidal, anaesthetic, anticarcinogenic, anti-inflammatory and anti-microbial activities (Leandro et al., 2012). An inhibitory activity of diterpene copalic acid was observed against bacteria responsible for periodontal diseases (Souza et al., 2011).

Data obtained by bioautography (Fig. 4) show the inhibitory action of terpenic compounds present in C. duckei oleoresin against B. cereus, and showed an inhibition zone of 35 mm (Fig. 4a, lane 1). As shown in Fig. 4(a), the bisabolene isomers (compound 2) showed inhibitory activity, with an inhibitory halo corresponding to 18 mm [retention factor (Rf), the distance traveled by a compound divided by the distance traveled by a solvent in the eluent system: 0.29], presenting bands similar to oleoresin. In the region of the spot corresponding to β-caryophyllene (Fig. 4b, lane 3), inhibitory activity was not observed, these data are not in agreement with previous work published in the literature, which report an antimicrobial activity for this compound (Huang et al., 2012; Leandro et al., 2012). Thus, it is believed that several compounds are involved in this inhibitory activity, among them bisabolene.

Studies with species of the genus Psidia showed activity against certain bacteria, among them B. cereus, and this activity was attributed to sesquiterpenic compounds, mainly the β-bisabolene (Govinden-Soulange et al., 2004). Nascimento et al. (2007) observed the inhibitory activity of β-bisabolene against resistant strains of S. aureus, as well as its ability to restore the effectiveness of ampicillin on these bacteria.

C. duckei oleoresin showed considerable antibacterial activity against B. cereus, with a fast bactericidal action in which its active compounds acted on the bacterial cell wall, removing proteins from the S-layer and interfering with the cell-division process. These data suggest the possibility of using this oleoresin in the food industry, and even as an alternative therapy in infectious diseases. However, further studies are required to better understand its antibacterial mechanism of action, as well as to identify the bioactive substances.

**Table 2.** Major constituents of C. duckei oleoresin identified by GC/MS

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<tr>
<th>Retention time (min)</th>
<th>Component</th>
<th>Composition (%)</th>
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<tr>
<td>24.37</td>
<td>β-Caryophyllene</td>
<td>9.16</td>
</tr>
<tr>
<td>25.08</td>
<td>Trans-α-bergamotene</td>
<td>20.47</td>
</tr>
<tr>
<td>25.78</td>
<td>α-Humulene</td>
<td>3.47</td>
</tr>
<tr>
<td>26.98</td>
<td>Sesquisabinene</td>
<td>2.33</td>
</tr>
<tr>
<td>27.76</td>
<td>(Z)-α-bisabolene</td>
<td>2.38</td>
</tr>
<tr>
<td>28.26</td>
<td>β- Bisabolene</td>
<td>40.94</td>
</tr>
<tr>
<td>28.65</td>
<td>β-Sesquiphellandrene</td>
<td>2.04</td>
</tr>
<tr>
<td>29.30</td>
<td>Cis-α-bisabolene</td>
<td>3.39</td>
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</tbody>
</table>

Fig. 4. Detection of the antibacterial activity of C. duckei oleoresin by bioautography assay against B. cereus. (a) Formation of an inhibition halo by copaiba oleoresin (lane 1, 35 mm), bisabolene isomers (lane 2, 18 mm) and β-caryophyllene standard (lane 3). (b) Compounds detected by VS reagent, using absorbent silica gel G_{60F254} with solvent chloroform:toluene 75:25 (v/v).
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