



## Memórias do Instituto Oswaldo Cruz

ISSN 0074-0276 versão impressa

Mem. Inst. Oswaldo Cruz v.103 n.3 Rio de Janeiro maio 2008

### ARTICLES

## Antimicrobial activity of Brazilian copaiba oils obtained from different species of the *Copaifera* genus

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
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### ABSTRACT

The antimicrobial activity of copaiba oils was tested against Gram-positive and Gram-negative bacteria, yeast, and dermatophytes. Oils obtained from *Copaifera martii*, *Copaifera officinalis*, and *Copaifera reticulata* (collected in the state of Acre) were active against Gram-positive species (*Staphylococcus aureus*, methicillin-resistant *S. aureus*, *Staphylococcus epidermidis*, *Bacillus subtilis*, and *Enterococcus faecalis*) with minimum inhibitory concentrations ranging from 31.3-62.5 µg/ml. The oils showed bactericidal activity, decreasing the viability of these Gram-positive bacteria within 3 h. Moderate activity was observed against dermatophyte fungi (*Trichophyton rubrum* and *Microsporum canis*). The oils showed no activity against Gram-negative bacteria and yeast. Scanning electron microscopy of *S. aureus* treated with resin oil from *C. martii* revealed lysis of the bacteria, causing cellular agglomerates. Transmission electron microscopy revealed disruption and damage to the cell wall, resulting in the release of cytoplasmic compounds, alterations in morphology, and a decrease in cell volume, indicating that copaiba oil may affect the cell wall.

**Key words:** copaiba oil - yeast - dermatophyte - bacteria - *Copaifera* - leguminosae

The bacteria responsible for severe post-surgery infections are predominantly Gram-positive (Okdakowska-Jedynak et al. 2003). *Staphylococcus aureus* is the major cause of community- and hospital-acquired infections. It is responsible for skin or soft-tissue infections, urinary tract infections, gastrointestinal abscesses, peritonitis, lower respiratory tract infections, osteomyelitis, tropical myositis, endocarditis, and bacteremia (Fung et al.

2001). *S. aureus* has a formidable ability to rapidly acquire resistance to any antibiotic. The multi-resistance of Gram-positive bacteria to antimicrobial agents is increasing at a alarming rate for many commonly prescribed classes of antimicrobial agents, including the penicillinase-resistant penicillins, other  $\beta$ -lactams, fluoroquinolones, and macrolides (Zetola et al. 2005). The increasing resistance of bacteria to conventional antibiotics has encouraged intensive efforts to develop new antimicrobial agents that are effective against resistant bacteria. Plants provide great chemical diversity and bioactivity, the exploration of which has led to the development of hundreds of pharmaceutical drugs (Shu 1998).

Copaiba oils are produced by exudation from the trunks of trees belonging to the genus *Copaifera*. The exuded material is a transparent, yellow to light-brown liquid (Veiga-Junior & Pinto 2002). 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-Junior et al. 2001). The effects attributed to copaiba oils in folk medicine include anti-inflammatory, anti-tetanus, anti-tumour, anti-blenorrhagea, and urinary antiseptic activities. In addition, it has been used to treat bronchitis, skin diseases, ulcers, and syphilis, as well as for healing wounds (Gomes et al. 2007). Pharmacological studies have demonstrated its properties as an anti-inflammatory; gastroprotective; analgesic; wound-cicatrisatinal (Paiva et al. 2004a, b, Araújo-Junior et al. 2005, Brito et al. 2005, Carvalho et al. 2005, Veiga-Junior et al. 2006, 2007); anti-nociceptive (Gomes et al. 2007); anti-tumour (Lima et al. 2003); insect repellent (Gilbert et al. 1999); and antimicrobial (Costa-Lotufo et al. 2002, Tincusi et al. 2002). The cosmetic industry uses copaiba oils in shampoos, capillary lotions, and bathing foams. Previous studies have demonstrated that the oleoresin did not show cytotoxicity in mammalian cells, induce behavioural alterations, or cause lesions or bleeding in the stomach of treated mice (Gomes et al. 2007, Veiga-Junior et al. 2007).

Many of these studies were performed with commercial oils without botanical identification. Oils from more than 20 species of *Copaifera* are used in folk medicine in Brazil, and significant differences in chemical composition occur among them (Veiga-Junior et al. 2007). Here, we report the antimicrobial activity of oils from eight species of this genus: *Copaifera multijuga*, *Copaifera martii*, *Copaifera cearensis*, *Copaifera paupera*, *Copaifera langsdorffii*, *Copaifera officinalis*, *Copaifera lucens*, and *Copaifera reticulata*, collected in the states of Acre and Pará, Brazil.

## MATERIALS AND METHODS

**Plant material** - The copaiba oils used here were exuded directly from the trunks of the trees. *C. multijuga* Hayne was collected at Manaus, Amazonas (INPA 82.418); *C. officinalis* Jacq. was collected at Boa Vista, Rondonia (INPA 191.211); *C. reticulata* Ducke was collected at Belém, Pará (INPA 61.212); specimens of all three were deposited in the Herbarium of INPA (Manaus). *C. reticulata* Ducke was collected at Tarauacá, Acre (RB 359.606); *C. lucens* was collected in Rio de Janeiro, state of Rio de Janeiro (RB 348.717); *C. langsdorffii* Desf. was collected at Campinas, São Paulo (RB 344.931); *C. paupera* Dwyer was collected at Tarauacá, Acre (RB 359.599); specimens of these four species were deposited in the Herbarium of the Jardim Botânico in Rio de Janeiro. *C. martii* was collected at Taparará, Pará (DC 349) and the sample was deposited in the Herbarium Chico Mendes (Rio de Janeiro). *C. cearensis* Huber was collected in Minas Gerais (SP 55.425) and the sample was deposited in the Herbarium of the Instituto de Botânica da Universidade de São Paulo.

**Microorganisms** - The test organisms included Gram-positive bacteria: *S. aureus* ATCC 25923, *Bacillus subtilis* ATCC 6623, *Staphylococcus epidermidis* ATCC 12228, methicillin-resistant *S. aureus* (MRSA) ATCC 33591, and *Enterococcus faecalis* ATCC 29212; and Gram-negative bacteria: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Proteus mirabilis* ATCC 25933, *Klebsiella pneumoniae* ATCC 13883, *Shigella flexnerii* ATCC 12022, and *Enterobacter cloacae* ATCC 13047. Bacteria were grown in Mueller-Hinton broth (Difco Becton Dickinson Co., Sparks, Maryland, USA) at 37°C for 24 h and maintained on slopes of nutrient agar (Difco). The yeasts *Candida albicans* ATCC 10231, *Candida tropicalis* ATCC 28707, and *Candida parapsilosis* ATCC 22019 were stored in water suspensions at room temperature and subcultured in Sabouraud-dextrose broth before being used in each test. The filamentous fungi *Trichophyton rubrum* ATCC 28189, *Trichophyton mentagrophytes* ATCC 4481, *Microsporium canis* ATCC 32903, and *Microsporium gypseum* ATCC 14683 were subcultured every 15 days to prevent pleomorphic transformations. The yeast and dermatophyte fungi were grown on Sabouraud-dextrose agar (Merck SA, São Paulo, Brazil) at 37°C or 28°C, respectively. All standard microorganisms were kindly provided by the Instituto Oswaldo Cruz, Rio de Janeiro, Brazil.

**Antibacterial assay** - The minimum inhibitory concentrations (MIC) of copaiba oils and reference antibiotics (tetracycline, vancomycin, penicillin, and oxacillin from Sigma Chemical Co., St. Louis, Missouri, USA) were determined by microdilution techniques in Mueller-Hinton broth (Difco) for Gram-positive and Gram-negative bacteria (CLSI 2005a). The assay was carried out in 96-well microtitre plates. Each oil was mixed with an inoculum prepared in the same medium at a density adjusted per tube to 0.5 of the McFarland scale ( $10^8$  bacterial cells) and diluted 1:10 for the broth microdilution procedure. Microtitre 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 produced 80% reduction in visible growth compared with the control. The minimum bactericidal concentration was defined as the lowest concentration yielding negative subcultures or only one colony, when subcultured in Mueller-Hinton agar (MHA) without test compound. These tests were performed in duplicate on

separate occasions.

**Antifungal assay** - The antifungal assay was performed by microdilution techniques in sterile flat-bottom microplates (CLSI 2005b, c). The MICs of copaiba oils and reference drugs (nystatin, fluconazole, and amphotericin B) were determined by microdilution techniques in RPMI 1640 (Sigma Chemical Co., Missouri, USA) and approximately  $10^4$  spores or  $10^5$  yeast were inoculated in a total volume of 100  $\mu$ l. The plates were incubated at 28°C for 48 h for yeast and 72 h for dermatophytes. MIC was defined as the lowest concentration of oil at which the microorganism tested did not show visible growth. Each experiment was performed twice on different occasions.

**Time-kill curve methodology** - The antibacterial activity of *C. martii* against *S. aureus* and MRSA was studied over a range of multiples of the MIC (62.5  $\mu$ g/ml), from 0.12 to 2.0 X MIC. Tests were performed in triplicate and incubated at 37°C. At predetermined time points (0, 3, 6, 9, 12, 24, 48 h), a 100  $\mu$ l sample was removed from each test suspension, serially diluted in sterile saline, and plated on MHA plates for colony count determination. Data from triplicate runs were averaged and plotted as log CFU/ml versus time (h) for each time point (CLSI 2005a).

**Scanning electron microscopy** - *S. aureus* treated with *C. martii* (31.5 and 50  $\mu$ g/ml) was collected by centrifugation after 3 h incubation at 37°C, washed in PBS, and fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer containing 1.0 mM  $\text{CaCl}_2$ . After fixation, small drops of the sample were placed on a specimen support with poly-L-lysine. Subsequently, the samples were dehydrated in graded ethanol, critical-point dried in  $\text{CO}_2$ , coated with gold and observed on a Shimadzu SS-550 scanning electron microscope.

**Transmission electron microscopy** - The bacterium *S. aureus*, collected from exponential phase, was cultured with *C. martii* (31.5 and 50  $\mu$ g/ml) for 3 h at 37°C, washed in PBS, and fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer containing 1.0 mM  $\text{CaCl}_2$ . Cells were then washed with 0.1 M sodium cacodylate buffer and post-fixed for 1 h at room temperature in 1% osmium tetroxide plus 0.8% potassium ferrocyanide and 5 mM calcium chloride. After rinsing, cells were dehydrated in acetone and embedded in Epon<sup>®</sup> resin. Ultrathin sections obtained in a Reichert Ultracut E ultramicrotome were stained with uranyl acetate and lead citrate, and observed in a Zeiss CEM-900 electron microscope.

## RESULTS

**Antimicrobial susceptibility** - The activity of the copaiba oils against Gram-positive bacteria using the microdilution technique is reported in [Table](#). The in vitro antimicrobial activity was classified according to MIC values as follows: MIC  $\leq$  100  $\mu$ g/ml, good;  $100 \leq$  MIC  $\leq$  500  $\mu$ g/ml, moderate;  $500 \leq$  MIC  $\leq$  1,000  $\mu$ g/ml, weak; MIC  $\geq$  1,000  $\mu$ g/ml, inactive. Copaiba oils obtained from *C. martii*, *C. officinalis* and *C. reticulata* (collected in Acre) exhibited good antibacterial activity against Gram-positive bacteria, including MRSA, with a MIC range of 62.5-125  $\mu$ g/ml. In contrast, all oils tested were inactive against Gram-negative bacteria. Results of antifungal activity showed that oils from *C. paupera* and *C. lucens* exhibited moderate activity against *T. rubrum* and *M. canis*, and those from *C. cearensis*, *C. langsdorffii* and *C. multijuga* showed moderate activity only against *T. rubrum* (MIC = 250-500  $\mu$ g/ml). Copaiba oils were inactive against *T. mentagrophytes*, *M. gypseum* or yeast. The solvent DMSO and the Petrolatum oil (NUJOL), used as an indifferent oil, showed no effect on the growth of microorganisms (data not shown). The MICs of the reference drugs used in this study (tetracycline, vancomycin, penicillin, oxacillin, nystatin, fluconazole, and amphotericin B) were similar to those reported elsewhere. The times needed by the copaiba oil from *C. martii* to kill *S. aureus* are shown in [Fig. 1](#). Analysis of the time-kill curves showed that after 3 h of treatment with copaiba oil at 2.0 X MIC, no viable cells remained. Furthermore, a steep decline in CFU/ml was observed after 6 h exposure when treated with 1.0 X MIC treatment. The concentration 0.5 X MIC showed a bacteriostatic effect and a regrowth after 9 h of treatment. Concentrations equal to 0.25 and 0.12 X MIC did not show activity. The time-kill curve of the same oil against MRSA was similar to [Fig. 1](#).

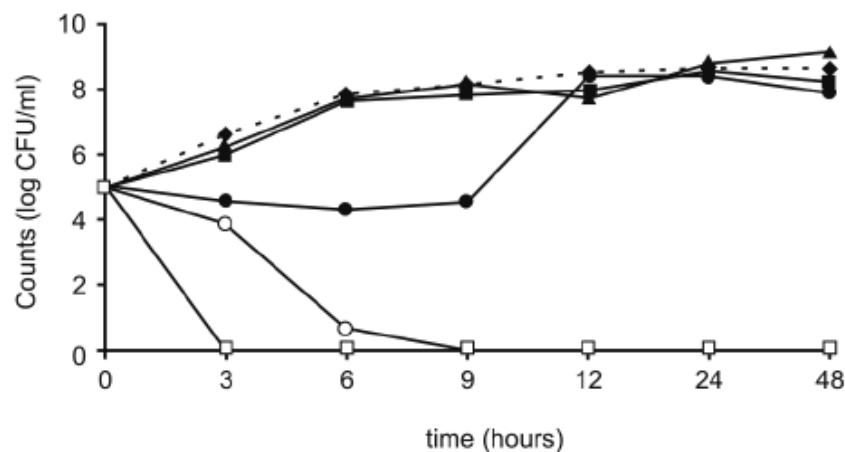


Fig. 1: time-kill curve of *Staphylococcus aureus* treated with copaiba oil obtained from *Copaifera martii*. ♦: control; ▲: 0.12 MIC; ■: 0.25 MIC; ●: 0.5 MIC; ○: 1.0 MIC; □: 2.0 MIC.

*Effect of copaiba oil from C. martii on the morphology and ultrastructure of S. aureus* - Scanning and transmission electron microscopy analyses of untreated and treated *S. aureus* were performed in order to determine morphological and ultra-structural changes caused by at sub-inhibitory concentrations (31.5 and 50  $\mu\text{g/ml}$ ) *C. martii* oil for 3 h (Figs 2, 3). In scanning electron microscopy, untreated *S. aureus* appeared to have a regular, smooth surface and was spherical in grape-like clusters (Fig. 2A). Bacteria treated with sub-inhibitory concentrations of the copaiba oil at 31.5 (Fig. 2B) and 50  $\mu\text{g/ml}$  (Fig. 2C-D) for 3 h showed lysed and agglomerated cells. These morphological features in bacterial cells may be due to the action on the cell wall followed by loss of cell volume. These data were confirmed by transmission electron microscopy (Fig. 2F). Control cells demonstrated their structural integrity with a thicker cell wall (Figs 2E, 3A-B). In addition, transmission electron microscopy analyses of *S. aureus* treated with oil from *C. martii* showed the cell wall being disrupted and damaged, resulting in a release of cytoplasmic compounds (Fig. 3C), loss of cell walls (Fig. 3D), alterations in morphology, and decrease in cell volume (Fig. 3E-H).

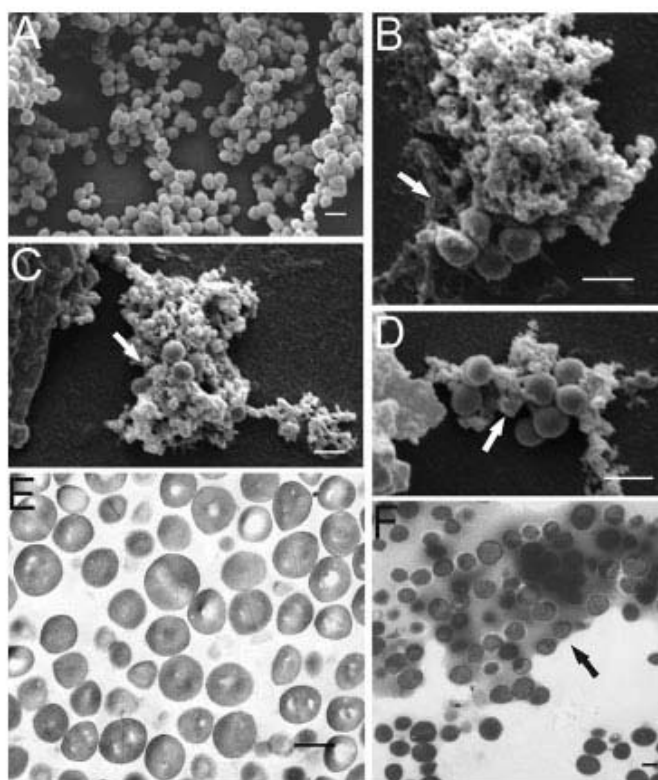


Fig. 2: scanning electron microscopy and transmission electron microscopy of *Staphylococcus aureus* treated with copaiba oil obtained from *Copaifera martii* for 3 h at 37°C. A and E: control; B and F: 31.5 µg/ml; C and D: 50 µg/ml. Bars = 1 µm.

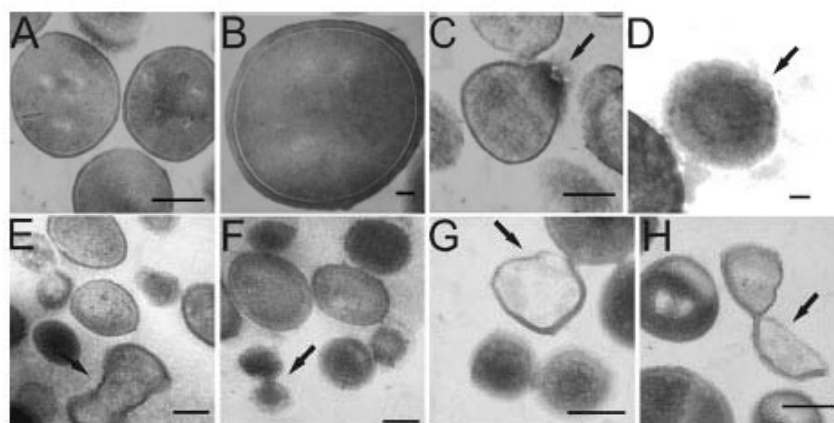


Fig. 3: transmission electron microscopy of *Staphylococcus aureus* treated with copaiba oil obtained from *Copaifera martii* for 3 h at 37°C. A and B: control; C, E, F, G, H: 31.5 µg/ml; D: 50 µg/ml. Bars A, C, E, F = 1 µm; B and D = 0.1 µm.

## DISCUSSION

There are more than 20 species of *Copaifera* genus in Brazil, which provide oils that differ significantly in chemical composition. The main constituents of these oils are sesquiterpenes and diterpenes (Braga et al. 1998, Veiga-Júnior et al. 2001). Although different activities have been reported and a variety of substances have been identified, little is known about the relationship between the structures and activity of the components (Tincusi et al. 2002). Sesquiterpenes comprise about 80% of the oils; the most common are  $\alpha$ -copaene,

b-cariofilene, b-bisabolene, a and b-selinene, a-humulene, and d and g-cadidene (Veiga-Júnior & Pinto 2002). The present study was designed to evaluate the antimicrobial activities of copaiba oils obtained from several species of *Copaifera*. Copaiba oils obtained from *C. martii*, *C. officinalis* and *C. reticulata* (collected in Acre) showed in vitro bactericidal activity against a wide spectrum of Gram-positive organisms. This spectrum includes MRSA, for which there are very few therapeutic alternatives (Samuelsen et al. 2005). Despite many recent advances in antimicrobial therapy, resistance among Gram-positive pathogens continues to pose a serious and growing clinical problem. Increasing resistance has created a need for new antimicrobial agents. The major compounds of *C. martii*, *C. officinalis*, and *C. reticulata* (Acre) are 3- $\alpha$ -hydroxy-copalic acid, hardwickiic acid, and a-copaene, respectively (Veiga-Junior & Pinto 2002, Oliveira et al. 2006).

Variation in chemical composition is common among different species and even among individual trees (Cascon & Gilbert 2000). For example, resin oils from *C. reticulata* collected in Acre and Pará showed differences in activity against bacteria because of differences in chemical composition.

Both prokaryotes (bacteria) and eukaryotes (yeast and dermatophytes) were included in the present study. Copaiba oils showed good activity only against Gram-positive bacteria, and moderate activity against dermatophytes. *B. subtilis* was the most sensitive organism. Interestingly enough, copaiba oils were active not only against a wild strain of *S. aureus*, but also against MRSA. Morphological and ultrastructural alterations of the bacterium treated with copaiba oils for 3 h were revealed by scanning and transmission electron microscopy. Cell wall disruption, release of cytoplasmic compounds, and decreased cellular volume were observed, indicating that the copaiba oil from *C. martii* might affect the cell wall. Additionally, for the time-kill curve, copaiba oils showed good and rapid bactericidal activity within 3 h. The observations from electron microscopy seemed to be well correlated with the effects observed in the time-kill experiment.

In conclusion, the results of the present study suggest that copaiba oils may be potential sources of new and selective agents for the treatment of important infectious diseases. Further laboratory and clinical studies of these oils are required in order to understand their antibacterial principles.

## ACKNOWLEDGEMENTS

To Dr José Andrés Morgado-Díaz, INCA - Rio de Janeiro, for performing the transmission electron microscopy.

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Received 4 January 2008

Accepted 28 April 2008

Financial support: CNPq, Capes, Programa de Pós-Graduação em Microbiologia, Universidade Estadual de Londrina

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