Atividade antimicrobiana do óleo-resina de copaíba (*Copaifera langsdorffii)* em bactérias de significância clínica em úlceras cutâneas

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RESUMO: Este trabalho avaliou a atividade antimicrobiana *in vitro* do óleo-resina da *Copaifera langsdorffii,* o qual vem sendo utilizado há muitos anos na medicina tradicional popular, principalmente devido às suas propriedades antiinflamatórias, antibacterianas, cicatrizante entre outras. O óleo-resina foi testado em bactérias Gram-positivas (*Staphylococcus aureus, Streptococcus pyogenes, Enterococcus faecalis*) e Gram-negativas (*Pseudomonas aeruginosa* e *Escherichia coli*) relacionadas com infecções de úlceras cutâneas. A atividade antimicrobiana foi determinada pelos testes da Concentração Inibitória Mínima (CIM) e Concentração Bactericida Mínima (CBM). O óleo-resina apresentou atividade antimicrobiana *in vitro* apenas para as bactérias Gram-positivas, com valores de CIM de 200 µg/mL, 400 µg/mL e 1100 µg/mL para *S. aureus*, *S. pyogenes* e *E. faecalis,* respectivamente. Os valores de CBM foram os mesmos que os valores de MIC para *S. aureus* e *S. pyogenes.* O valor de CBM para o microrganismo *E. faecalis* foi de 1200 µg/mL. Considerando que a presença de infecção significativamente impede o processo normal de cicatrização de úlceras cutâneas, acreditamos que o óleo-resina de copaíba, utilizado como componente de formulações tópicas, poderia ser um adjunto importante no tratamento de úlceras cutâneas infectadas, principalmente nos casos de infecção por microrganismos Gram-positivos.

Palavras-chave: *Copaifera langsdorffii*, óleo-resina de copaíba, atividade antimicrobiana, cicatrização.

ABSTRACT: Antimicrobial activity of copaíba *(Copaifera langsdorffii)* oleoresin on bacteria of clinical significance in cutaneous wounds. The present study was designed to evaluate the *in vitro* antimicrobial activity of *Copaifera langsdorffii* oleoresin, which has been used in folk medicine as an anti-inflammatory, antibacterial, healing among others. The oleoresin was tested against Gram-positive (*Staphylococcus aureus, Streptococcus pyogenes, Enterococcus faecalis*) and Gram-negative (*Pseudomonas aeruginosa* and *Escherichia coli*) bacteria related to infections in cutaneous wounds. Antimicrobial activity was determined by the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays. Copaiba oleoresin showed antimicrobial activity only against the Gram-positive bacteria with MIC of 200 µg/mL, 400 µg/mL and 1100 µg/mL for *S. aureus*, *S. pyogenes* and *E. faecalis,* respectively. MBC values were the same as MIC for *S. aureus* and *S. pyogenes* and for *E. faecalis* it was 1200 µg/mL. Considering that infection significantly impairs the wound healing process, we believe that the use of copaiba oleoresin as a component of a topical formulation could be a valuable adjunct in the treatment of infected wounds, mainly in the case of wounds infected by Gram-positive microorganisms.

Keywords: *Copaifera langsdorffii*, copaiba oleoresin, antimicrobial activity, wound healing.

INTRODUCTION

From a microbiological perspective, the primary function of normal, intact skin is to control microbial populations that live on the skin surface and to prevent underlying tissue from becoming colonized and invaded by potential pathogens. Exposure of subcutaneous tissue following a loss

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of skin integrity (*i.e*., a wound) provides a moist, warm, and nutritious environment that is conducive to microbial colonization and proliferation (Bowler et al., 2001). Bacteria, their metabolites and microbial interactions to wound environment may significantly impair the process of normal wound healing, and contribute to the antibiotic resistance (Bowler et al., 2001; Wall et al., 2002). Staphylococci, streptococci, enterococci, and facultative Gram-negative bacilli are the most frequent bacterial groups recovered from chronic venous leg ulcers (Davies et al., 2007; Stephens et al., 2003). In the development of infection, a wound fails to heal, and it has deleterious effects on patients by causing increased pain and discomfort that can result in life-threatening illness or even death. Additionally, treatment costs rise, and general wound management practices become more resource demanding (Bowler et al., 2001; Cooper et al., 2002).

The indiscriminate use of antimicrobial drugs has led to multiple bacterial resistance and side effects (Fu et al., 2007). As the development of antibiotics continues and controversy regarding the use of topical antiseptics persists, the need for identification and development of new agents that are safe and broadly effective becomes increasingly critical (Bowler et al., 2001). Thus, efforts have been made to develop new compounds outside conventional antibiotic therapy and more natural antimicrobial substances such as plants are desired (Rodrigues et al., 2005).

Brazil is well known for the variety of its tropical plants and, many of them are used as natural medicines by native population. Plants of the *Copaifera* genus (Leguminosae, Caesalpinioideae), popularly known as copaiba, are large trees that grow in northern Brazil. The exuded material from these trees, traditionally obtained by tapping the trunk of the tree is called copaiba oil, copaiba balsam or oleoresin, a transparent liquid whose color varies from yellow to light brown (Carvalho et al., 2005; Castro-e-Silva et al., 2004; Paiva et al., 2002; Santos et al., 2008a).

Phytochemical studies carried out on the oleoresin from *Copaifera langsdorffii* revealed the presence of a mixture of diterpenes and sesquiterpenes. The biological activities of *Copaifera* spp. have been attributed to these groups of compounds. The main sesquiterpene is β-Caryophyllene and the main diterpenes are kaurenoic acid and copalic acid (it is considered a characteristic diterpene of the genus *Copaifera*). Some plant extracts containing diterpenes were shown to exhibit antimicrobial, anti-inflammatory and wound healing effects (Paiva et al., 2002; Santos et al., 2008).

The oleoresin of several species of *Copaifera* is used in the Amazonian region mainly as a topical anti-inflammatory and healing agent (Carvalho et al., 2005). It has been also used in folk medicine as an antineoplastic, and urinary antiseptic, as well as to treat bronchitis, skin diseases, ulcers and syphilis (Brandão et al., 2008). It is also important for its gastroprotective, analgesic, antinociceptive, insect repellant and antimicrobial properties. In cosmetic industry copaiba oleoresin is a component in capillary lotions, shampoos, soaps, creams and bathing foams (Gomes et al. 2008; Paiva et al., 2002; Pieri et al., 2009; Santos et al., 2008b; Veiga Júnior et al. 2001).

Thus, the purpose of this study was to investigate the *in vitro* antimicrobial activity of *Copaifera langsdorffii* oleoresin on standard strains: *Staphylococcus aureus, Streptococcus pyogenes, Enterococcus faecalis, Pseudomonas aeruginosa and Escherichia coli*, which are important microorganisms related with cutaneous wounds infections.

MATERIAL AND METHOD

Plant Material

Copaiba oleoresin was collected from the trunk of the *Copaifera langsdorffii* tree in Tarauacá, Acre, Brazil (latitude 9º41'0" South, altitude 72º5'0" West), during 1999 and 2000, during the dry season (most likely between May and October). It was obtained from the Central de Cooperativas de Rio Branco by Prof Dr Osvaldo de Freitas (School of Pharmaceutical Sciences of Ribeirão Preto - University of São Paulo), who kindly supplied the oleoresin for this study.

Stock solutions preparation

Specific amounts (0.04; 0.05; 0.1 and 0.15 g) of copaiba oleoresin were solubilized in dimethyl sulfoxide (DMSO), and then appropriate volumes of Brain Heart Infusion (BHI) medium were added in order to obtain final concentrations of 4 mg/mL, 5 mg/mL, 10 mg/mL and 15 mg/mL, respectively (Valdevite, 2007).

Microorganisms and culture conditions

Gram-positive bacteria: *S. aureus* (ATCC 6538), *S. pyogenes* (ATCC 19615) and *E. faecalis* (ATCC 10541) and Gram-negative bacteria: *E. coli* (ATCC 10538), *P. aeruginosa* (ATCC 2327) were evaluated. Stock cultures of *S. aureus, P. aeruginosa* and *E.coli* were maintained in Muller-Hinton (MH) agar slant at 25°C; stock cultures of *S. pyogenes* were stored in freeze medium at -20°C and *E. faecalis* was maintained in thioglycollate medium without dextrose or indicator broth at 25ºC.

A volume of 300 µL of *S. pyogenes* and *E. faecalis* were firstly inoculated into 10 mL thioglycollate medium without dextrose or indicator broth, and then these cultures were incubated at 37ºC for 24 hours. Secondly, from these first cultures, 100 µL were subcultured into 5.0 ml thioglycollate medium and incubated at 37ºC for 24 hours. Thirdly, from these previous cultures, 100 µL was cultured into 10 mL thyoglicollate medium, incubated at 37ºC for 24 hours.

S. aureus, E.coli and *P. aeruginosa* were firstly seeded into a fresh MH agar slant and incubated at 25ºC for 24 hours. Secondly, each microorganism was cultured into 5.0 mL MH broth and incubated at 37ºC for 24 hours. Thirdly, 100 µL was cultured into 10 mL MH broth, incubated at 37ºC for 24 hours.

All bacteria were cultured for three days because they were obtained from cultures stored in different conditions. Performing this procedure allowed all microorganisms to be reactivated and in the same growth conditions.

Bacterial growth curves

Overnight cultures, described above, were added (2.5 % v/v) to a final volume of 200 mL of: MH broth (MHb), BHI and Tryptic Soy Broth (TSB) (*S. aureus*); BHI and TSB (S. *pyogenes*); BHI supplemented with glucose and BHI (*E. faecalis*)*;* MH broth, BHI and TSB (*E.coli*); MHb, BHI, TSB, Peptone water and Nutrient broth (*P. aeruginosa*). Before adding the cultures, the absorbance (at 660 nm) of the liquid media (blank), as well as the initial inocula, was measured. All flasks were incubated at 37ºC, under agitation of 100 rpm (Shaker Marconi – Mod. MA420, Brazil). The absorbance of each culture medium was measured at 1 hour intervals throughout the incubation period (Spectronic® 20 Genesys, USA). The bacterial growth and the exponential growth phases were determined from data of absorbance (at 660 nm) *versus* incubation time (Gabrielson et al., 2002; Leitão et al., 2004).

Inoculum preparation

All bacteria were cultivated for three days, as previously described, and then the most suitable medium was chosen to prepare the inoculum of each microorganism. At half of log phase from exponential growth curves, 1.0 mL was collected in microcentrifuge tubes and centrifuged (Centrifuge Eppendorf 5810-R) at 11500 x g for 2 minutes. The supernatants were discarded and the inocula were prepared by diluting cell mass in 0.9% NaCl solution, adjusted to McFarland scale 0.5 by turbidity reading (Densimat - *BioMerieux*). Suspensions were further diluted to provide a final inoculum of 10^5 CFU mL^{-1} to be used in the antimicrobial activity assays as the standard inoculum (Duarte et al., 2007; NCCLS, 2003).

Minimum Inhibitory Concentration

MICs were carried out in 96-well microplates. The concentrations of copaiba oleoresin ranged from 1.56 to 3000 µg/mL, depending on the microorganism. BHI broth containing 2% DMSO was the first solution to be added into wells, this media was chosen because it allows a suitable growth of all tested microorganisms. The stock solutions of copaiba oleoresin were transferred and diluted into the wells. The standard inoculum was added to the corresponding wells and the microplates were incubated at 37˚C for 15 hours, under agitation (40 rpm). Control wells with cultures without copaiba oleoresin, *i.e.*, wells with culture medium (BHI containing 2% DMSO) and inoculum: negative control, and wells with the culture medium only, without the inoculum: sterility control, were also included (Valdevite, 2007). Tests were performed in triplicate. To overcome the turbidity problem because of the emulsified oil, and to allow visual identification of metabolic activity, antimicrobial activity was detected by adding 50 µL of 0.125% (w/v) nitrotetrazolium blue chloride solution (Loughlin et al. 2008). The final concentration of nitrotetrazolium in each well was 0.025% (v/v). All microplates were again incubated at 37ºC for 2 hours under agitation (40 rpm). Then, they were centrifuged at 2000 x g for 10 minutes and images of all microplates were taken by using a digital scanner. MIC was defined as the lowest concentration of copaiba oleoresin that inhibited visible growth, as indicated by the nitrotetrazolium staining (Tortora et al., 2005).

Minimum Bactericidal Concentration

MBCs were carried out by inoculating 250 µL of each well from microplates where there was no visible growth. Solutions from the three closest wells to MIC values were used. BHI agar plate was used for *S. pyogenes* and *E. faecalis*, and MH agar plate was used for *S. aureus*. All Petri plates were incubated at 37˚C for 48 hours. MBC was defined as the lowest concentration of copaiba oleoresin at which incubated microorganisms were completely killed (Fu et al., 2007).

RESULT AND DISCUSSION

Growth profiles of all tested microorganisms are shown in Figure 1, and the required time for each microorganism to attain maximum growth, *i.e.*, half of log phase, is indicated by the arrows. The most suitable medium for *S. aureus, S. pyogenes, E. faecalis* and *E.coli* was BHI, and for *P. aeruginosa*

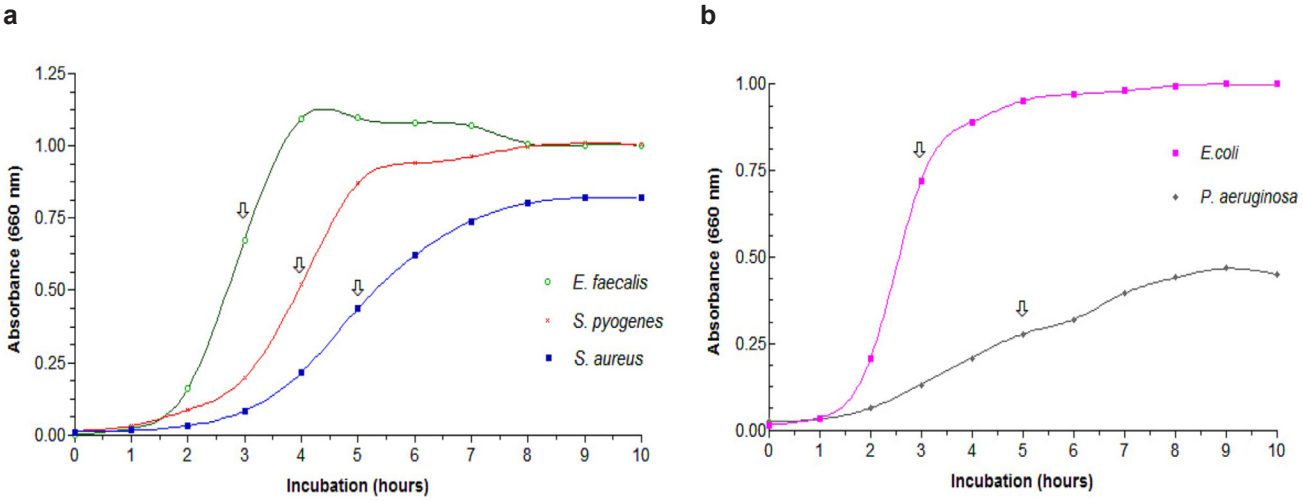

FIGURE 1. Bacterial growth curves to (a) *S. aureus*, *S. pyogenes*, *E. faecalis*; (b) *E. coli* and *P. aeruginosa* . The arrows represent half of log phase.

was Nutrient broth.

Assessment of bacterial growth is important in the investigation of antimicrobial effects of a compound (Gabrielson et al., 2002). Before assessing MICs and/or MBCs it is valuable to select a suitable medium and determine the exponential growth phase. During this phase, the cell division is extremely active (binary fission) and cell metabolism is increased, justifying the use of this phase as preferred for research and industrial purposes (Tortora et al. 2005).

Copaifera langsdorffii oleoresin exhibited a broad inhibition spectrum only toward Gram-positive bacteria (Table 1). The solvent DMSO showed no effect on the microorganisms growth (data not shown). MIC values of copaiba oleoresin on each microorganism were visualized by coloration development (tetrazolium salt). Wells that showed a blue-colored precipitate indicated microbial growth, and those wells without coloration indicated killed bacteria (Andrews, 2001; Gabrielson et al., 2002). MBC values were the same as MICs to *S. aureus* and *S. pyogenes*, but different to *E. faecalis.* MBC assay was not carried out for *P. aeruginosa* and *E. coli,* because these microorganisms were resistant to copaiba oleoresin in the concentrations studied.

High resolution gas chromatography-mass spectrometry (HRGC-MS) analyses on this same oleoresin found a mixture of sesquiterpenes (75%) and diterpenes (25%). The main compounds among sesquiterpenes were β-caryophyllene (51%), followed by α-humulene (8.52%); among diterpenes, the main compounds were 11-acetoxy-copalic acid (5.23%), 11-hydroxy-copalic acid (4.8%), copalic acid (4.69%) and agatic acid (3.32%) (Ramos, 2006). Since sesquiterpenes have been reported to have potent antimicrobial activity and play a critical role in plant defense mechanisms (Teng et al. 2010), they could be related to the bacteriostatic and bactericidal activities observed against the Gram-positive bacteria evaluated in this study.

Our results are in agreement with a previous study that investigated the antimicrobial activity of oleoresins from *Copaifera martii*, *Copaifera officinalis* and *Copaifera reticulata* against Gram-positive and Gram-negative microorganisms. This study showed that these species of copaiba did not present antimicrobial activity against Gram-negative

TABLE 1. MIC and MBC values of copaiba oleoresin against *S. aureus, S. pyogenes*, *E. faecalis*, *E. coli* and *P. aeruginosa.*

Bacteria	**MIC** (µg/mL)		**MBC** (µg/mL)	
	Range	**MIC**	**Range**	**MBC**
Staphylococcus aureus (ATCC 6538)	3.12 - 1500	200	200 - 400	200
Streptococcus pyogenes (ATCC 19615)	1.56 - 1000	400	400 - 1000	400
Enterococcus faecalis (ATCC 10541)	1.56 - 2000	1100	1100 - 1300	1200
Escherichia coli (ATCC 10538)	1000 - 3000	*	*	*
Pseudomonas aeruginosa (ATCC 2327)	1000 - 3000	*	*	*

MIC and MBC > 3000 µg/mL

bacteria. Due to its property of causing morphological and ultra-structural changes, such as cell wall disruption and release of cytoplasmatic content and decreased cellular volume, copaiba oleoresin might affect the bacteria cell wall (Santos et al., 2008a).

Pacheco et al. (2005) also conducted a study on the antimicrobial activity of oleoresins from different trees against Gram-positive and Gram-negative bacteria. They found inactivity of all the samples against Gram-negative bacteria up to 1000 µg/ml.

Gram-positive and Gram-negative bacteria differ on their walls, where Gram-negative bacteria walls represent a stronger barrier than Gram-positive. Copaiba oleoresin seems to modify bacteria cell wall; therefore, a reasonable explanation for no activity against Gram-negative bacteria could be due to their complex walls morphophysiology. Gram-negative walls have a bilayer (containing proteins, phospholipids, and lipopolysaccharides), which is usually considered to be their outermost layer situated above a thin peptidoglycan layer. Together, plasma membrane and the cell wall (outer membrane, peptidoglycan layer, and periplasm) constitute the Gram-negative envelope (Beveridge, 1999).

There is not a consensus on the acceptable bacterial inhibition level for plant materials when compared with standards. A previous study (Duarte et al., 2007) described a classification of plant materials based on MIC results (strong inhibitors: MIC up to 500 µg/mL; moderate inhibitors: MIC between 600 and 1500 µg/mL; weak inhibitors: MIC above 1600 µg/mL). This classification was based on antibiotic resistant bacteria, *i.e*., clinical isolates. Another study (Aligiannis et al., 2001) suggested a classification based on ATCC strains (strong activity: MIC between 280 and 1270 µg/mL; weak activity: MIC between 1810 and 8850 µg/mL). Thus, *Copaifera langsdorffii* oleoresin showed better inhibition levels against *S. aureus* (MIC 200 µg/mL) and *S. pyogenes* (MIC 400 µg/mL) than against *E. faecalis* (MIC 1100 µg/mL), but still effective.

Our results showed that *Copaifera langsdorffii* oleoresin has antimicrobial activity against Gram-positive bacteria (*S. aureus*, *S. pyogenes* and *E. faecalis*) ATCC strains, and it didn't have any activity on Gram-negatives (*P. aeruginosa* and *E. coli*). In view of the significant healing impairment due to bacterial infection, the use of copaiba oleoresin as a component of a topical formulation could be a valuable adjunct in the treatment of infected wounds, mainly in case of those infected by antibiotic resistant Gram-positive strains.

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