Antimicrobial Activity of *Copaifera langsdorffii* Oil and Evaluation of its Most Bioactive Fraction against Bacteria of Dog’s Dental Plaque

FA Pieri1,2,3, VO Silva1, FS Vargas3, VF Veiga Junior3 and MAS Moreira1*

1Bacterial Diseases Laboratory, Sector of Preventive Veterinary Medicine and Public Health, Veterinary Department, Federal University of Viçosa; Av. P.H. Rolfs, s/n, CEP: 36570-000, Viçosa, MG, Brazil; 2Leônidas and Maria Deane Institute, Oswaldo Cruz Foundation. Rua Terezina, 476, Adrianópolis, CEP: 69.057-070, Manaus, AM, Brazil; 3Exact Sciences Institute, Federal University of Amazonas. Av. Rodrigo Otávio, 6200, Coroado, CEP: 69.080-005, Manaus, AM, Brazil

*Corresponding author: masm@ufv.br

**ARTICLE HISTORY** (13-067)

Received: February 09, 2013
Revised: August 16, 2013
Accepted: October 07, 2013

**Key words:**
Copaiba
Dogs
Medicinal plants
Periodontal disease
Plaque inhibition

**Abstract**

The aim of this study was to evaluate the inhibitory effect of copaiba oil and its resinous and volatile fractions against 20 bacterial isolates from dental plaque of dogs. The antimicrobial activities of the oil and its fractions were evaluated by the agar diffusion test with solutions at 10% concentration. The results showed antimicrobial activity for the copaiba oil solution on 16 isolates. The volatile fraction was considered statistically similar (P>0.05) to copaiba oil intact on the size of inhibition zones inhibiting 17 isolates. The resinous fraction inhibited only eight isolates, with smaller haloes when compared with those of the volatile fraction and intact oil (P<0.05). It is concluded from these results that copaiba oil is a potential phytotherapic to be used in dental plaque antimicrobial therapy of dogs and suggests that its activity is due to sesquiterpenes of the volatile fraction.

**INTRODUCTION**

Periodontal disease, which involves support and protective structures of the teeth, is a disease that affects most dogs, reaching about 85% prevalence in animals older than four years (Kyllar and Witter, 2005). The aetiological agent is the dental plaque that develops on the tooth surface, and the dog’s immune system reaction. Plaque is formed with the initial colonisation of the tooth surface by autochthonous bacteria, which promote the adhesion of other more virulent strains (Niemiec, 2008a). Thus, combat to microorganisms of the initial phase of dental plaque formation is a preventive action to periodontal disease (Pieri et al., 2010).

Among several ways to combat dental plaque, the most appropriate is dental brushing (Niemiec, 2008b). Another means of preventing plaque formation would be the administration of antibacterial substances that prevent the proliferation or adhesion of bacteria to the tooth surface, and thus also inhibit the development of periodontal disease (Pieri et al., 2010). A substance that is widely used by veterinarians and dentists in dental treatment is chlorhexidine, usually commercialised at a concentration of 0.12% (Hennet, 2002). However, the use of chlorhexidine should be restricted to a few days, in short treatments, due to its side effects such as darkening of the tooth enamel, loss of taste, burning sensation in the oral cavity and oral mucosa ulcerations in longer treatments (Dantas et al., 2007).

For this reason, researchers have studied new substances with antimicrobial activity against the most important oral bacteria in the formation of dental plaque for the establishment of a new preventive therapy to be used in association with brushing for both humans and dogs (Pieri et al., 2010; Pieri et al., 2012).

Traditional medicine, based on natural products, has served as a source of alternative drugs for therapeutic use in several diseases (Kim et al., 2012). The copaiba (*Copaifera sp.*) is a tree that has been highlighted, especially in Brazil, to provide oil that has several different therapeutic indications (Leandro et al., 2012). Many studies have confirmed its properties, including most importantly the inflammatory, neuroprotective, healing, antitumour, antibacterial and analgesic activities (Lima et al., 2003; Gonçalves et al., 2005; Kobayashi, et al., 2011; Pieri et al., 2011; Bah et al., 2012; Guimarães-Santos et al., 2012).
**MATERIALS AND METHODS**

**Obtaining the oleoresin:** The oil of *C. langsdorffii* was extracted by adapting the technique described by Rigamonte Azevedo et al. (2006), with the perforation of the trunk of the copaiba tree, using a chainsaw with a 5/8-inch drill, in two holes. The first hole made 1m above the base of the tree and the second 1m above this. PVC pipes were placed to facilitate the flow of oil and prevent predation of the tree by parasites and microorganisms. The tree was located in the city of Alfenas-MG, Brazil, under the geographical coordinates 21º26'33"S and 46º0'55"W, and a specimen is deposited in the VIC Herbarium of Federal University of Viçosa with record number 34,894.

**Chromatographic procedures:** To obtain fractions in which activity is concentrated, oil was fractionated by chromatography on an ion-exchange column eluted with dichloromethane and methanol, using methods adapted from Pinto et al. (2000). Following experiments were performed with open-column chromatography eluted with hexane and ethyl acetate. The fractions were esterified with diazomethane and analysed by gas chromatography using flame ionisation detection (GC-FID) and mass spectrometry (GC-MS).

The chromatographic analyses of oil and fractions were performed on a gas chromatograph Shimadzu® Model 2010 GC with flame ionisation detector (GC-FID) using a CP-Sil 5 CB column (100% dimethylpolysiloxane) from Varian®, and a chromatograph model QP-2010 from Shimadzu®, with mass spectrometry detector (GC-MS) using a VF-1MS column from Varian®.

For analysis of GC-FID, helium gas was used for drag with a flow rate of 2.0 mL/min, and the injection was performed on Split 1:10, the injector temperature at 250°C and the detector at 290°C. The programming of the oven was 60 to 240°C (3°C/min). For qualitative analysis, GC-MS was used with the same conditions as GC-FID, and for detecting technique has been applied to electron impact at 70 eV.

The analysed compounds were identified by comparing the mass spectrum with those of Wiley 7.0 (GC-MS) and by calculating the retention indices (RI) (GC-FID), which were compared with the rates available in the literature (Adams, 2007). To obtain the RI, a hydrocarbons pattern of a homologous series of *n*-alkane hydrocarbons (C8-C20) under the same analysis conditions was used.

**Antimicrobial assays:** The copaiba oil-resin (*C. langsdorffii*), its resinous acid fraction, and the volatile no acid fraction were tested. All solutions were formulated at 100 mg/mL, solubilized with Tween 80 at 100 mg/mL. A negative control solution was used, consisting of sterile distilled water and tween 80 (100 mg/mL). Tests were performed in triplicate with two replicates.

20 bacterial isolates obtained from initial dental plaque of dogs between one- and two-years old, belonging to the bacterial collection of the Laboratory of Bacterial Diseases, Department of Veterinary Medicine, Federal University of Viçosa were used. The genera of the isolates were *Aerococcus* (1/20), *Enterococcus* (3/20), *Lactobacillus* (1/20), *Lactococcus* (1/20), *Leuconostoc* (3/20), *Pasteurella* (1/20), *Staphylococcus* (4/20) and *Streptococcus* (6/20). Adapting the technique of plate cylinder diffusion in agar (Breier et al., 2002), 100 µL of bacterial culture adjusted to 0.5 MacFarland scale were seeded with the aid of Drigalski spatula in 9 cm disposable petri dishes previously prepared with a bottom layer of 10 mL of bacteriological agar from Himedia®, and a top layer of 20 mL of Brain Heart Infusion agar (BHI agar) from Himedia®. With the use of sterile 1mL pipette tips were produced, only BHI agar, orifices, one per treatment, equidistantly distributed on the plates. The volume used of each solution and negative control in wells followed 100 µL.

After preparation, the plates were incubated at 37°C for 24 h. Antimicrobial activity was represented by inhibition haloes that were measured in millimetres for later comparison. It was considered a positive result if haloes of any size were present around the orifice. Tests were performed in triplicate with two replicates.

**Statistical analysis:** Comparison of the fractions and the oil activities was made by analysis of variance (ANOVA). Homoscedastic of variances was analysed by Bratlett’s test to verify the normal distributions of results and multiple comparison between the means of inhibition haloes of each treatment were analysed by Tukey-Kramer test. Two-Way ANOVA was used to compare the treatments within each isolate with Bonferroni test. All analysis was made with the use of the software Prism statistical package 5 for Windows from Graphpad®. Results were accepted as significant at P<0.05.

**RESULTS**

The fractionation of copaiba oil obtained in this study yielded 18.75% of an acid resinous fraction and 81.25% volatile non acid fraction. The acid fraction presented high levels of kaurenoic acid. The non acid fraction obtained is composed of α-trans-bergamotene (21.3%), β-bisabolene (20.1%), δ-amorphene (12.9%), carophyllene oxide (5.6%), kaurene (2.6%), ylangene <α> (1.7%) and δ-elemene (0.6%) (Fig. 1).
Fig. 1: GC-FID spectrum showing the chemical composition of non acid fraction obtained from the *Copaifera langsdorffii* oil.

The inhibition haloes of intact copaiba oil and the acid and non acid fractions against dental plaque bacterial isolates from dogs are shown in table 1 and figure 2. The negative control with Tween 80 showed no inhibition on any isolate. This study showed antimicrobial activity *in vitro* by copaiba solution on dental plaque bacteria in dogs, including: one isolate of the genera *Aerococcus* sp., *Lactobacillus* sp., *Lactococcus* sp. and *Pasteurella* sp.; two isolates of *Enterococcus* sp., *Leuconostoc* sp.; three *Staphylococcus* sp.; and five of *Streptococcus* sp. An isolate of *Enterococcus* sp. *Staphylococcus* sp., *Streptococcus* sp. and *Leuconostoc* sp. were resistant (Tab. 1). The inhibition zones were similar for most of the isolates: between 10 mm and 15 mm (Fig. 2 and Tab. 1). However, two isolates, one of *Lactococcus* sp. and one *Leuconostoc* sp. were more sensitive, with larger haloes (Tab. 1). To isolate *Lactococcus* sp. the haloes were 16mm and 20mm for the intact oil and non acid fraction respectively (Tab. 1). As for the isolated *Leuconostoc* sp., the haloes were 16mm for the acid fraction and 30mm for the other solutions tested (Tab. 1).

All data were considered not significant in Bratlett’s test (P=0.05), showing no difference in standard deviation, indicating the results for parametric analysis. In Tukey-Kramer test, the non-acid fraction showed superior results to the acid fraction (P=0.0015) (Fig. 2). The results of this fraction were considered statistically similar (P=0.05) to intact copaiba oil based on the size of inhibition zones, but as well as inhibiting all isolates that the intact oil inhibited, this fraction also inhibited one *Enterococcus* isolate which was resistant to the oil (Tab. 1). The other three isolates that were resistant to oil were not inhibited by the non acid fraction. The acid fraction was able to inhibit only eight isolates with smaller haloes compared to the non acid fraction and intact oil (P=0.0015) (Fig. 2), being three *Streptococcus* sp., one *Staphylococcus* sp., one *Lactobacillus* sp. and two *Leuconostoc* sp. (Tab. 1). The results of comparison between the treatments within each bacterial isolate are presents in Table 1.

**DISCUSSION**

As reported in the literature, copaiba oil resin is commonly characterised as consisting solely of a mixture of sesquiterpenes and diterpene acids (Barbosa et al., 2012). This distribution of fractions identified in this work, with high percentages of sesquiterpenes and high kaurenoic acid presence in the composition of the resinous fraction is expected according to the species *C. langsdorffii* (Paiva et al., 2002; Cavalcanti et al., 2006).

Similar to some studies of antimicrobial activity that used copaiba oil against reference strains derived from human dental plaque, this study also presented inhibition of bacterial isolates from dental plaque of dogs (Vasconcelos et al., 2008; Pieri et al., 2010; Souza et al., 2011). Pieri et al. (2012) presented results that confirm *S. mutans* susceptibility to copaiba. In this study was showed the activity of *C. officinalis* oil tested, with the MIC average of 0.78 µl/ml. This activity, however, at all concentrations tested did not show the death of bacteria being bacteriostatic.

Vasconcelos et al. (2008) evaluated the ability of antibacterial root canal sealers with copaiba oil (*C. multijuga* Hayne) as active principle against dental strains of *Streptococcus mutans* (ATCC 25175) and *Streptococcus sanguinis* (ATCC 15300), important components of human dental plaque. The oil used had as main components the sesquiterpenes β-caryophyllene (9.2%), α-humulene (1.8%), germacrene D (3.5%), caryophyllene...
Table 1: Antimicrobial activity (inhibition zones; mm) of Copaifera langsdorffii oil-resin and its acid and non acid fractions against bacterial isolates (n=20) obtained from dogs dental plaque

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Copaiba oil-resin (10%)</th>
<th>Non-acid fraction (10%)</th>
<th>Acid fraction (10%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerococcus viridans</td>
<td>11.33±0.58a</td>
<td>12.83±0.29a</td>
<td>0.00±0.00a</td>
</tr>
<tr>
<td>Enterococcus sp - 1</td>
<td>12.17±0.29b</td>
<td>12.00±1.00a</td>
<td>0.00±0.00a</td>
</tr>
<tr>
<td>Enterococcus sp - 2</td>
<td>0.00±0.00a</td>
<td>12.00±0.00a</td>
<td>0.00±0.00a</td>
</tr>
<tr>
<td>Enterococcus sp - 3</td>
<td>11.0±0.00e</td>
<td>11.0±0.50g</td>
<td>0.00±0.00e</td>
</tr>
<tr>
<td>Lactobacillus sp</td>
<td>11.17±0.29g</td>
<td>11.83±0.29g</td>
<td>10.83±0.29g</td>
</tr>
<tr>
<td>Lactococcus sp</td>
<td>20.0±0.10e</td>
<td>16.33±0.29g</td>
<td>0.00±0.00e</td>
</tr>
<tr>
<td>Leuconostoc sp - 1</td>
<td>0.00±0.00a</td>
<td>0.00±0.00e</td>
<td>0.00±0.00e</td>
</tr>
<tr>
<td>Leuconostoc sp - 2</td>
<td>30.00±1.00b</td>
<td>30.00±0.00e</td>
<td>16.0±1.00b</td>
</tr>
<tr>
<td>Leuconostoc sp - 3</td>
<td>11.33±0.58h</td>
<td>11.83±0.29g</td>
<td>10.0±0.00e</td>
</tr>
<tr>
<td>Pasteurella sp</td>
<td>11.67±0.58h</td>
<td>12.50±0.50h</td>
<td>0.00±0.00h</td>
</tr>
<tr>
<td>Staphylococcus sp - 1</td>
<td>10.67±0.58h</td>
<td>13.00±1.00h</td>
<td>0.00±0.00h</td>
</tr>
<tr>
<td>Staphylococcus sp - 2</td>
<td>11.50±0.50h</td>
<td>10.33±0.58j</td>
<td>9.50±0.50j</td>
</tr>
<tr>
<td>Staphylococcus sp - 3</td>
<td>0.00±0.00a</td>
<td>0.00±0.00h</td>
<td>9.67±0.29g</td>
</tr>
<tr>
<td>Staphylococcus sp - 4</td>
<td>15.00±0.00c</td>
<td>14.00±1.00c</td>
<td>0.00±0.00h</td>
</tr>
<tr>
<td>Streptococcus sp - 1</td>
<td>0.00±0.00a</td>
<td>0.00±0.00e</td>
<td>0.00±0.00e</td>
</tr>
<tr>
<td>Streptococcus sp - 2</td>
<td>11.50±0.50h</td>
<td>12.67±0.58g</td>
<td>11.0±0.00e</td>
</tr>
<tr>
<td>Streptococcus sp - 3</td>
<td>11.50±0.00e</td>
<td>11.33±0.58g</td>
<td>0.00±0.00h</td>
</tr>
<tr>
<td>Streptococcus sp - 4</td>
<td>11.00±0.00e</td>
<td>11.67±0.58g</td>
<td>10.0±0.00f</td>
</tr>
<tr>
<td>Streptococcus sp - 5</td>
<td>7.83±0.29f</td>
<td>12.17±0.29g</td>
<td>9.5±0.50j</td>
</tr>
<tr>
<td>Streptococcus sp - 6</td>
<td>12.00±1.00b</td>
<td>14.00±0.00c</td>
<td>0.00±0.00e</td>
</tr>
</tbody>
</table>

Values (Mean±SD) bearing different letters in a row differ significantly (P<0.05).

In the study by Souza et al. (2011) seven diterpenes and four sesquiterpenes from copaiba oil were tested against several strains of the standard of human dental plaque, getting more efficient inhibition by three diterpenes, with special reference to copallic acid with MIC values ranging between 2 and 6 mg/mL. The results of this study show that there was inhibition of only eight bacterial isolates from dogs by the acid resinous fraction, suggesting a different susceptibility profile of dental bacteria in dogs and humans. Still this minor inhibition of acid fraction may be due the absence of copallic acid, since this fraction was composed almost exclusively by kaurenoic acid that is commonly the main acid in oil-resins of C. langsdorffii.

The mechanism of action of terpenes contained in vegetable oil over microorganisms is not yet fully understood; however, it is speculated that the disruption of cell membranes by these substances is the most likely mechanism, because of its lipophilic characteristics, increasing the permeability of microbial cells and leading to overflow of cellular contents (Cowan, 1999; Deuschle et al., 2007). This mechanism of action was confirmed by Santos et al. (2008) who showed, with electron microscopy, the action of copaiba oil on Staphylococcus aureus, demonstrating the cellular destruction and overflow of cytoplasmic material of bacteria. It should be noted that the antimicrobial activity of terpene compounds from natural products probably cannot be attributed to a specific mechanism of action or just one compound due to the large number of different substances in their composition. This represents an important advantage to the use of copaiba oil as an alternative to conventional antimicrobial agents, since this diversity of compounds and mechanisms of action could act additively and synergistically against bacteria, which would hinder the emergence of resistant bacteria to treatment.

Regarding the use of copaiba oil in reducing dental plaque in dogs, Pieri et al. (2010) evaluated the ability of copaiba (C. officinalis) to inhibit dental plaque formation in an in vivo assay, showing a significant reduction in plaque formation that was statistically equal to the chlorhexidine results. The results of the present study are in accordance with the results of these researchers, since the copaiba oil had an inhibitory effect on most of the bacterial isolates from dog dental plaque with particular reference to the volatile fraction of the oil that showed higher results than the resin, suggesting that this is the main fraction involved in this plaque reduction in dogs.

Conclusion: It was concluded in this study that the copaiba oil is a potential substitute to chlorhexidine to combat the bacteria in the dental plaque of dogs and consequently the prevention and treatment of periodontal disease, with potential benefits by the supposed synergistic action of various substances, and that the volatile fraction of the oil appears to be the most bioactive against these bacteria.

Acknowledgement: The authors thank the Foundation for Research Support of the State of Minas Gerais (FAPEMIG), the Foundation for Research Support of the State of Amazonas (FAPEAM), the Brazilian Coordination of Improvement of Higher Education.
Personnel (CAPES) and the Brazilian National Council for Scientific and Technological Development (CNPq) for financial support.

REFERENCES


