RESEARCH ARTICLE

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

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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).

In vitro assays have been developed to evaluate the antimicrobial activity of copaiba oil against important bacterial components of dental plaque in humans, such as *Streptococcus mutans* (Souza *et al.*, 2011; Pieri *et al.*, 2012). However, although one study has evaluated the reduction in the dental plaque formation *in vivo* by this oil in dogs (Pieri *et al.*, 2010), no work has been done yet to confirm the antibacterial activity *in vitro* against bacteria derived from the dental plaque of dogs, which are different to those found in humans.

Due to the high incidence of periodontal disease in dogs, the many undesirable effects resulting from the use of chlorhexidine, the need to find alternative treatments and the absence of data reporting the active fraction of copaiba oil against bacteria of dental in dogs, this study evaluated the antimicrobial activity of *Copaifera langsdorffii* oil and its volatile and resinous fractions against bacterial isolates obtained from the dental plaque of dogs.

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% dimethylpoly-siloxane) 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). Homoscedasticity 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%), caryophyllene oxide (5.6%), kaurene (2.6%), ylangene <α> (1.7%) and δ-elemene (0.6%) (Fig. 1).
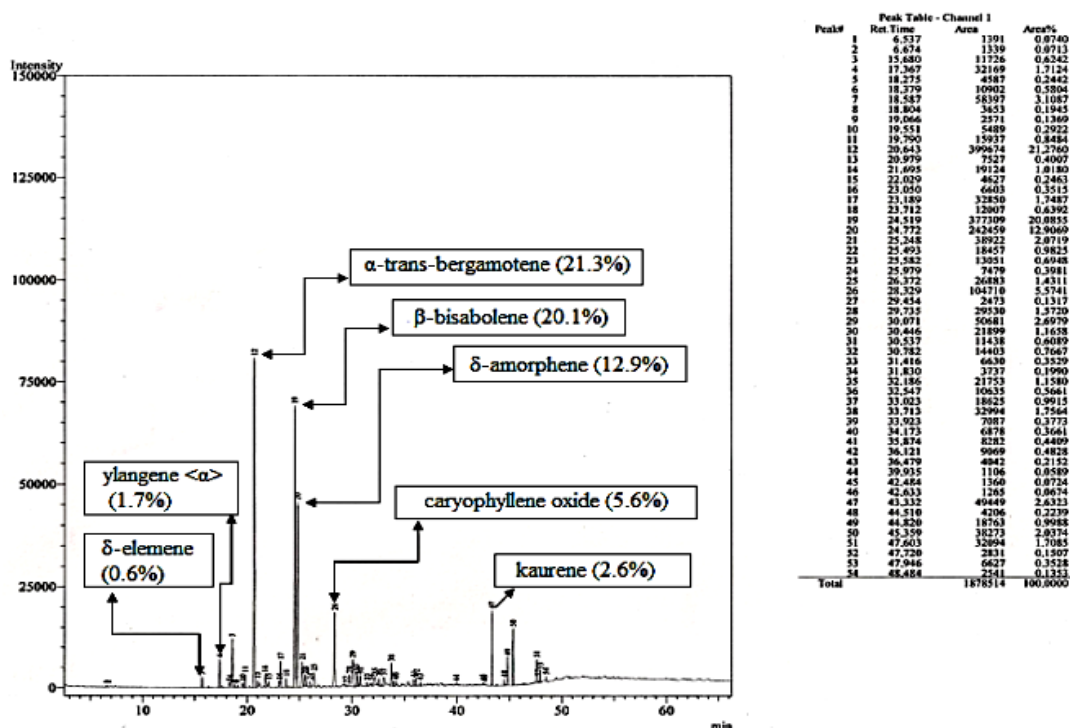
Peak#	Ret.Time	Area	Area%
1	6.537	1391	0.0740
2	6.674	1339	0.0713
3	15.680	11726	0.6242
4	17.367	32169	1.7124
5	18.275	4587	0.2442
6	18.379	10902	0.5804
7	18.587	58397	3.1087
8	18.804	3653	0.1945
9	19.066	2571	0.1369
10	19.551	5489	0.2922
11	19.790	15937	0.8484
12	20.643	399674	21.2760
13	20.979	7527	0.4007
14	21.695	19124	1.0180
15	22.029	4627	0.2463
16	23.050	6603	0.3515
17	23.189	32850	1.7487
18	23.712	12007	0.6392
19	24.519	377109	20.0855
20	24.772	242459	12.9069
21	25.248	38922	2.0719
22	25.493	18457	0.9825
23	25.582	13051	0.6948
24	25.979	7479	0.3981
25	26.372	26883	1.4311
26	28.329	104710	5.5741
27	29.454	2473	0.1317
28	29.735	29530	1.3720
29	30.071	50681	2.6979
30	30.446	21899	1.1658
31	30.537	11438	0.6089
32	30.782	14403	0.7667
33	31.416	6630	0.3529
34	31.830	3737	0.1990
35	32.186	21753	1.1580
36	32.547	10635	0.5661
37	33.023	18625	0.9915
38	33.713	32994	1.7564
39	33.923	7087	0.3773
40	34.173	6878	0.3661
41	35.874	8282	0.4409
42	36.121	9069	0.4828
43	36.479	4042	0.2152
44	39.935	1106	0.0589
45	42.484	1360	0.0724
46	42.633	1265	0.0674
47	43.332	49449	2.6323
48	44.510	4206	0.2239
49	44.820	18763	0.9988
50	45.359	38273	2.0374
51	47.603	32094	1.7085
52	47.720	2831	0.1507
53	47.946	6627	0.3528
54	48.484	2544	0.1353
Total		1878514	100.0000

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 *Leunonostoc* 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

Isolates	Copaiba oil-resin (10%)	Non-acid fraction (10%)	Acid fraction (10%)
Aerococcus viridans	11.33±0.58[b]	12.83±0.29[a]	0.00±0.00[c]
Enterococcus sp - 1	12.17±0.29[a]	12.00±1.00[a]	0.00±0.00[b]
Enterococcus sp - 2	0.00±0.00[b]	12.00±0.00[a]	0.00±0.00[b]
Enterococcus sp - 3	11.00±0.00[a]	11.00±0.50[a]	0.00±0.00[b]
Lactobacillus sp	11.17±0.29[a]	11.83±0.29[a]	10.83±0.29[a]
Lactococcus sp	20.00±1.00[a]	16.33±0.29[b]	0.00±0.00[c]
Leuconostoc sp -1	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]
Leuconostoc sp -2	30.00±1.00[a]	30.00±0.00[a]	16.00±1.00[b]
Leuconostoc sp -3	11.33±0.58[a]	11.83±0.29[a]	10.00±0.00[b]
Pasteurella sp	11.67±0.58[a]	12.50±0.50[a]	0.00±0.00[b]
Staphylococcus sp - 1	10.67±0.58[b]	13.00±1.00[a]	0.00±0.00[c]
Staphylococcus sp - 2	11.50±0.50[a]	10.33±0.58[ab]	9.50±0.50[b]
Staphylococcus sp - 3	0.00±0.00[b]	0.00±0.00[b]	9.67±0.29[a]
Staphylococcus sp - 4	15.00±0.00[a]	14.00±1.00[a]	0.00±0.00[b]
Streptococcus sp - 1	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]
Streptococcus sp - 2	11.50±0.50[ab]	12.67±0.58[a]	11.00±0.00[b]
Streptococcus sp - 3	11.50±0.00[a]	11.33±0.58[a]	0.00±0.00[b]
Streptococcus sp - 4	11.00±0.00[ab]	11.67±0.58[a]	10.00±0.50[b]
Streptococcus sp - 5	7.83±0.29[c]	12.17±0.29[a]	9.50±0.50[b]
Streptococcus sp - 6	12.00±1.00[b]	14.00±0.00[a]	0.00±0.00[c]

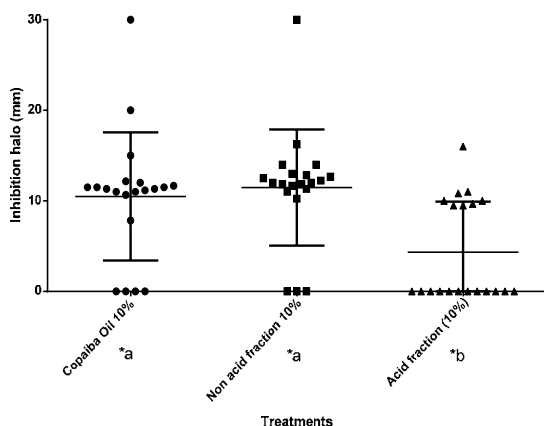Values (Mean±SD) bearing different letters in a row differ significantly (P<0.05).

Fig. 2: Antimicrobial activity of *Copaifera langsdorffii* oil-resin and its acid and non acid fractions against 20 bacterial isolates obtained from dogs dental plaque. Each marker refers to one isolate tested. Regarding Tukey's test copaiba oil-resin and non acid fraction (*a) were statistically different (P=0.0015) of acid fraction (*b).

oxide (11.5%), cubenol (16.7%) and bisabolol (7.2%) and labdane diterpenes as copalic acid (2.1%), 3β-hydroxy-copalic acid (1.7%) and pinifolic acid (1.3%). Our results also presented the non acid fraction as the main constituents of tested copaiba oil.

Pieri *et al.* (2010) reported inhibition of *S. mutans* (ATCC 25175), *S. salivarius* (CDC 262), *S. pyogenes* (ATCC 19615) and *E. faecalis* (ATCC 19433) by *C. officinalis* oil, using the agar diffusion. Their results presented *S. pyogenes* as the most susceptible, with inhibition zones average of 38.8 mm, while 25.8 mm, 24.5 mm and 26.2 mm was presented to *S. mutans*, *S. salivarius* and *E. faecallis* respectively. This study also presented *in vitro* inhibition by the oil of *C. officinalis* of *S. mutans* adherence capacity in concentrations of 6.25 µl/mL. This is a relevant result regarding that the adherence capacity of the bacteria has important role in dental plaque formation and consequently in the installation of periodontal disease.

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 copalic 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 copalic 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.

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