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Original Article

Amazon emulsions as cavity cleansers: antibacterial activity, cytotoxicity and changes in human tooth color

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ABSTRACT

The copaiba oleoresin, *Copaifera multijuga* Hayne, Fabaceae, is a phytotherapeutic agent with antimicrobial activity. This study evaluated the antibacterial activity and cytotoxicity of, and tooth color changes caused by four copaiba oil emulsions (Emulsion 1, 10% CM; Emulsion 2, 10% *C. multijuga* + 1% biotech product; Emulsion 3, 30% *C. multijuga*; and Emulsion 4, 30% *C. multijuga* + 1% biotech product). The antibacterial activities against microorganisms causing dental caries (*Streptococcus mutans* ATCC25175, *S. oralis* ATCC10557, *S. salivarius* ATCC7073, and *Lactobacillus casei* ATCC7469) were tested using three parameters: minimum inhibitory concentration, minimum bactericidal concentration, and cell viability by fluorescence microscopy. The emulsions were assessed for cytotoxicity by means of the hemolytic assay and cell culture (murine fibroblast cells NHI3T3) using Alamar Blue™. The dentin color change caused by the emulsions was examined at 10 s, 30 s, and 10 min. The emulsions showed antibacterial activity against the microorganisms tested with an MIC of 125 μl/ml. The minimum bactericidal concentration was higher than minimum inhibitory concentration for the tested microorganism and the fluorescence confirmed that the cells were viable at minimum inhibitory concentration values. The emulsions had a hemolytic activity of 71.16% (Emulsion 3) and 44.67% (Emulsion 4) at a concentration of 30 μl/ml. In cell culture assay, NHI-3T3 cells treated with the emulsions showed 6–16% viability. Emulsion 1 caused clinically imperceptible color change in dentin at 10 s ($\Delta E = 3.21$), Emulsion 2 at 30 s ($\Delta E = 2.70$) and 10 min ($\Delta E = 3.08$), and Emulsion 4 at 10 min ($\Delta E = 3.03$). Emulsion 3 caused color change at all times tested. This research documented positive data regarding antibacterial activity, cytotoxicity, and tooth color changes when using copaiba oleoresin emulsions, showing its potential for use in dentistry.

Introduction

Copaiba (*Copaifera multijuga* Hayne, Fabaceae) oleoresin has great social and economic value in phytotherapy especially in the Amazon region, where it is native and widely used as an antibacterial, anti-inflammatory, anesthetic, and antitumor agent, and for healing wounds (Bandeira et al., 1999; Veiga Junior et al., 2007; Vasconcelos et al., 2008).

Caries is one of the main dental diseases affecting humanity. It is considered a multifactorial disease that involves behavioral and social factors coupled with interactions among microorganisms, host, and diet (Fejerskov and Kidd, 2011).

The process of caries occurs when microorganisms like *Streptococcus mutans*, *S. oralis*, *S. salivarius*, and *Lactobacillus casei*, present in biofilm or dental plaque, produce metabolites that cause fluctuations in pH. The result is mineral tooth loss and formation of dental cavity (Kidd, 2011).

The use of rotary tools during the restoration procedures and after establishment of the cavity lesion, results in the formation of a smear layer. The layer consists of saliva, blood, bacteria, and residue oils of rotary instruments. The smear layer is removed by the application of cleaning agent, which substantially decreases the cariogenic microorganisms and prevents the recurrence of caries. The procedure reduces the occurrence of microleakage restorations and thus, reduces the possibility of postoperative

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sensitivity, marginal staining, marginal fractures, and injuries to the pulp–dentin complex (Reis and Loguercio, 2007).

The goal of the study was to evaluate the use of copaiba oleoresin emulsions as cavity cleansers and determine their antibacterial activity and cytotoxicity as reported in the literature for different bioactive Amazonian products that have important dentistry functions against periodontal diseases and dental caries (Souza et al., 2011a, 2011b; Pieri et al., 2012).

Materials and methods

Collection of copaiba oleoresin and biotech product (BP)

Copaifera multijuga Hayne, Fabaceae (CM), was collected at the Adolph Ducke Reserve, at Manaus, Amazonas state (Brazil), and cataloged under N° 69 by the Instituto Nacional de Pesquisas da Amazônia (INPA). Once the plant material was identified, a voucher specimen was deposited in the INPA herbarium under the registration N° 270709. The biotech product (BP) was extracted from a natural pitch of the Burseraceae family.

Identification of chemicals and emulsions formulation

Identification of copaiba components was performed by gas chromatography coupled with a flame ionization detector (GC-FID) and gas chromatography coupled with mass spectrometric detector (GC-MSD) (Vasconcelos et al., 2008).

Four test emulsions with different concentrations (Emulsion [EM] 1, 10% CM; EM2, 10% CM + 1% BP; EM3, 30% CM; and EM4, 30% CM + 1% BP) and a pH ≅ 4.598 were formulated at the School of Dentistry, Federal University of Amazonas, following the requirements of the Brazilian Pharmacopoeia (Brasil, 2010). The emulsions, which are currently under the patent registration process, were composed of distilled water (DW), Tween® 80 (Merck, Germany), CM, and BP.

The experiments were carried out after approval from the Federal University of Amazonas/Institutional Ethical Committee (number 0312.0.115.000-08/2009).

Antibacterial activity

S. mutans (ATCC25175), *S. oralis* (ATCC10557), *S. salivarius* (ATCC7073), and *L. casei* (ATCC7469) were used for assessing antibacterial activity. The microorganisms were reactivated in Brain Heart Infusion broth (BHI, Himedia, Mumbai, IN), at 37 °C for 24 h in aerophilia for *S. oralis*, *S. salivarius*, and *L. casei*, and in a microaerophilia for *S. mutans.* Inoculants were standardized at #0.5 McFarland scale (Probac of Brazil, São Paulo, SP, BR).

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC of test emulsions was determined according to the method described by CLSI (2007), Andrews (2001), and Sampaio et al. (2009). The emulsions were diluted in 1:1 ratio with dimethyl sulfoxide (DMSO) (Vetec, Germany) to form a stock solution (SS). The 2% chlorhexidine digluconate (FGM – Joinville, SC, Brazil) was used as a positive control and the emulsion vehicle (Tween® 80) was used as a negative control.

Copaiba emulsions were diluted and added to 96-well plates with each well containing 100 μl of differing concentrations (400–125 μl/ml). A volume of inoculum (20 μl) standardized at 10^8 CFU/ml and complemented with BHI was added. The 1st plate column represented the sterility test substance (medium + emulsion). The 12th column represented the bacteria viability (bacteria + medium). The antibacterial activity of each emulsion was tested in triplicate. The well plates were sealed with parafilm after filling and incubated at 37 °C for 24 h. After 24 h, 30 μl of 0.01% resazurin (Sigma Aldrich, USA) was added and the plates were incubated for another 30 min. Absence of color change was interpreted as microorganism sensitivity to the tested emulsion during the reading of the plates. For MBC, 100 μl of copaiba oil emulsions was placed in the wells at different concentrations, plated on BHI agar, and incubated at 37 °C under microaerophilic and aerophilic conditions. Testing of microorganisms was performed after 24 h.

Cell viability analysis by fluorescence technique

The effect on cell viability for emulsions that showed better MBC (EM3 and EM4) was determined according to fluorescence technique against *S. mutans* (ATCC25175) and *L. casei* (ATCC7469) (Filoche et al., 2007).

Initially, the dilution of the test emulsions (DTE) was performed by adding 1 ml of emulsion + 1 ml of Tween® 80 + 1 ml of DMSO. The DTE was further diluted forming three solutions: SS1: 500 μl of DTE + 500 μl of BHI; SS2: 500 μl of DTE + 2500 μl of BHI; and SS3: 500 μl of DTE + 4500 μl of BHI. The MIC of the solutions was between 4.5 and 120 μl/ml.

The reading of cell viability was performed by fluorescence technique using the Live/Dead® BacLight™ Bacterial Viability Kit L13152 (Molecular Probes, Eugene, USA). In this system, viable cells without wall damage are stained green (Component A: SYTO 9) and cells with damage to the cell membrane are stained red (Component B: propidium iodide).

For the preparation of bacterial suspensions, tubes were used to identify viable and non-viable bacteria and 3 ml of BHI broth containing the inoculant according to the test microorganisms was added. The preparations were centrifuged for 15 min and the supernatant was removed. The infranatant was resuspended in 240 μl of sodium chloride (NaCl) and homogenized. NaCl (4.8 ml) and 4.8 ml of 70% isopropyl alcohol were added to the tubes containing dead bacteria. Both tubes were incubated at room temperature for 60 min, stirring every 15 min, and then were centrifuged again for 15 min. The supernatant was removed and the infranatant was resuspended in 2.4 ml of NaCl.

The pattern of bacterial cells was prepared in proportions of known concentrations of viable and non-viable bacteria, as follows: 0:100; 20:80; 50:50; 80:20, and 100:0. Construction of standard curves used 96-well plates with black color (Greiner-Bio-One). Concentration ratios of viable and non-viable bacteria (100 μl) were inserted into wells A1–A5. In wells B1–B5 were placed 100 μl of the rates of MIC results considering two concentrations forward and two after.

Equal volumes of SYTO9 and propidium iodide were prepared and homogenized using 3 min of vortex. The mixture (30 μl) was added to a fluorescence plate and the wells were incubated under light at room temperature for 15 min.

The reading was evaluated as fluorescence intensity read using the microplate reader multimode type (FluorStar Optima, BMGLab Tech, Germany) under an excitation filter of 485 nm and emission wavelength of 520 and 620 nm for the detection of green and red color, respectively.

Cytotoxicity evaluation of C. multijuga *oil emulsions*

Hemolytic assay

The test was performed in 96-well plates using a 2% human erythrocyte suspension in 0.85% NaCl containing 10 mM calcium chloride ($CaCl_2$) (Jimenez et al., 2003). The substances EM1, EM2, EM3, EM4, fresh copaiba oleoresin, Tween® 80 (emulsion vehicle)

and chlorhexidine (control) were tested at concentrations ranging from 0.234 to 30 μl/ml. The supernatant was removed and the released hemoglobin was measured spectrophotometrically at an absorbance at 540 nm after incubation at room temperature for 1 h and centrifugation.

Cell culture assay

The NHI-3T3 cell line of mouse fibroblasts was grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin, and incubated at 37 °C with a 5% atmosphere of carbon dioxide (CO_2). The groups tested were EM1, EM2, EM3, EM4, fresh copaiba oleoresin, Tween® 80 (emulsion vehicle), and chlorhexidine (control). The cells were plated in 96-well plates (2.5×10^4 cells per well) and the Alamar Blue™ assay was performed using previously described procedures of Ahmed et al. (1994). After 24 h, the compounds were dissolved in DMSO and added to each well to give final concentrations of 5 μl/ml. Control groups had final well concentrations of 0.1% DMSO. Plates were incubated for 72 h and 3 h before the end of the incubation, 10 μl of Alamar Blue™ was added to each well. The fluorescent signal was monitored with a multiplate reader at excitation wavelength of 530–560 nm and emission wavelength of 590 nm.

Influence of the time of test emulsion application on human tooth color change

The fabrication of 150 test specimens (TS) of 75 human teeth extracted for orthodontic and/or surgical reasons was performed according to the methodology proposed by Araújo et al. (1998) and the rulings of the Ethics Committee. After extraction, the teeth were stored in 2% thymol for a minimum of 24 h for decontamination. The crown was separated from the root at the cementum enamel junction with the aid of a cutter (Presi Mecatome, Grenoble, France, p100) and fitted with a double-sided diamond disk. The crown was sectioned in a mesiodistal direction to obtain two test specimens of each sectioned tooth. Each specimen was stored in individual vials containing deionized water and kept at a temperature of 37 °C.

Ten TS were used for each test emulsion, which were immersed in the test emulsions for the time intervals of 10 s, 30 s, and 10 min. The control used in the study was 2% chlorhexidine (FGM, Joinville, SC, Brazil).

The color measurement of TS was performed with the Vita Easyshade (Easyshade®, Vivadent, Brea, CA, USA) spectrophotometer at the initial time (*T0*) without treatment and experimental time intervals. Color was determined in accordance with the parameters of the CIE lab system (L^* a^* b^*), in which L^* indicates the brightness where the average ranges from 0 (black) to 100 (white). The a^* and b^* represent the hue with a^* as the saturation red–green axis and b^* the saturation in blue–yellow axis. Comparison of the color change of teeth before and after immersion in the test substances was observed and represented by the equation: $\Delta E^* ab = ([\Delta L^*]^2 + [\Delta a^*]^2 + [\Delta b^*]^2)^{0.5}$.

Statistical analysis

The antibacterial activity was analyzed in descriptive form. The hemolytic assay was performed using the average percentage standard deviation and linear sigmoid regression to calculate the effective concentration of 50% of cell lysis (EC_{50}). The cell culture assay was evaluated by one-way analysis of variance using Dunnett's test ($p < 0.05$) for multiple comparisons adjustment. The median and quartiles (Q_i) were used to summarize the chromatic change of the TS. For comparison of the medians, Kruskal–Wallis tests were applied for comparison of the different groups, and Mann–Whitney tests were used for the comparison of two medians ($p < 0.05$).

Results and discussion

Chromatographic analysis of CM revealed sesquiterpenes, especially β-caryophyllene (9.2%), α-humulene (1.8%), germacrene d (3.5%), caryophyllene oxide (11.5%), bisabolol (7.2%), labdane diterpene skeleton-containing compounds such as copalic acid (2.1%), 3β-hydroxy-copalic (1.7%), and pinipholic (1.3%) as the major components. These results were similar to the results of Bandeira et al. (1999), Cascon and Gilbert (2000), Veiga Junior and Pinto (2002), Lima et al. (2003), Vasconcelos et al. (2008), Souza et al. (2011a, 2011b), and Barbosa et al. (2013), who reported the sesquiterpenes β-caryophyllene and caryophyllene oxide as the main components.

The MIC (125 μl/ml) of all tested emulsions showed antibacterial activity against *S. mutans*, *S. oralis, S. salivarius*, *and L. casei.* Bandeira et al. (1999), Vasconcelos et al. (2008), and Pieri et al. (2012) also reported the antibacterial activity of copaiba oil against microorganisms in the oral cavity.

The antibacterial activity observed in emulsion tests is possibly related to the CM components, including the diterpenes, a fact corroborated by Vasconcelos et al. (2008) and Souza et al. (2011a, 2011b). The MBC and MIC analysis results are shown in Table 1.

Table 1 shows the different concentrations of emulsions that showed bactericidal activity against *S. mutans*, *S. oralis*, *and L. casei*. However, no bactericidal activity against *S. salivarius* could be detected. Pieri et al. (2012) demonstrated the bacteriostatic activity of copaiba oil solutions at concentrations up to 100 μl/ml, whereas the results of this work showed bactericidal activity at concentrations above 100 μl/ml.

All of the 2% chlorhexidine concentrations tested showed bactericidal activity, similar to the results of Souza et al. (2011b), which showed that the MBC of chlorhexidine was 3.5-fold higher than that of the extracted copalic acid oil of *C. langsdorffi*, confirming that chlorhexidine is the gold standard for antibacterial action against oral pathogens.

Cell viability analysis of EM3 at 9 and 12 μg/ml by fluorescence technique showed 8.59% viable *S. mutans* cells and 6.59% viable *L. casei* cells, respectively. The MIC of and cell viability upon treatment with EM4 were 45 μl/ml and 11.22% for *S. mutans*, respectively; the MIC for and cell viability of *L. casei* were 60 μl/ml and 11.20%, respectively. Cell viability can be less than the MIC found in the first

Table 1
Minimum bactericidal concentration (MBC) and Minimum inhibitory concentration (MIC) of *Copaifera multijuga* oil emulsions against bacteria.

Tested emulsions	MBC and MIC of emulsions (μl/ml) against bacteria							
	S. mutans		*S. oralis*		*S. salivarius*		*L. casei*	
	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC
EM1	175	125	125	125	Not known	125	350	125
EM2	150	125	125	125	Not known	125	300	125
EM3	125	125	125	125	Not known	125	300	125
EM4	125	125	125	125	Not known	125	275	125

Table 2
Median score distribution and quartiles of final color change in human teeth at 10 s, 30 s and 10 min immersion in chlorhexidine and *Copaifera multijuga* oil emulsions.

Groups	Score						p^a
	10 s		30 s		10 min		
	Med	Q1/Q3	Med	Q1/Q3	Med	Q1/Q3	
Chlorhexidine	3.60[b]	1.93/4.32	3.81[b]	1.51/4.68	1.22[c]	0.85/1.80	*0.009*
EM 1	3.21	2.24/6.11	10.00	3.98/12.72	7.41	2.66/14.69	0.059
EM 2	3.88	1.96/5.66	2.70	1.97/3.57	3.08	2.20/5.52	0.703
EM 3	13.73	7.28/20.12	8.35	4.05/17.87	7.49	5.12/21.86	0.532
EM 4	3.48	2.08/6.19	4.39	2.12/5.62	3.03	2.04/4.06	0.802

Med, median; Q, quartile.
Italicized value indicate statistical difference ($p < 0.05$).
[a] Kruskal–Wallis test.
[b] and c – statistically different.

test owing to dilution. The presence of viable cells, suggests bacteriostatic activity at concentrations that induce bactericidal activity, 125 μl/ml.

Cytotoxicity assessment of C. multijuga *oil emulsions*

Hemolytic assay

The fresh copaiba oleoresin, the vehicle of the emulsions, and 2% chlorhexidine showed 0.72%, 0.71%, and 1.87% cell lysis, respectively.

The results of hemolytic activity indicated that at 30 μl/ml, all formulations showed high hemolytic activity with 70.82%, 47.58%, 71.16%, and 44.67% cell lysis with EM1, EM2, EM3, and EM4, respectively. However, owing to the high hemolytic activity of these substances and as a safety precaution, it is recommended to use these emulsions at low EC_{50} values, 11.15 μl/ml for EM1 and 16.55 μl/ml for EM3.

The test emulsions showed hemolytic activity, similar to that reported by Costa-Lotufo et al. (2002), who evaluated kaurenoic acid from *C. langsdorffi* oleoresin. Kaurenoic acid showed an EC_{50} of 54.6 μM for human erythrocytes.

The fresh copaiba oleoresin showed no significant hemolytic activity, which is in disagreement with the research of Costa-Lotufo et al. (2002), who tested kaurenoic acid, a compound of copaiba oil diluted in DMSO and water. This result may be attributable to the absence of a detergent to lyse the CM macromolecules, prevent the contact of CM with the solution of erythrocytes, and thus, induce cell lysis.

Cell culture assay

Cell culture assay showed that all test emulsions and fresh copaiba oleoresin were toxic to NHI3T3 cells, causing a decrease in cell viability (Fig. 1).

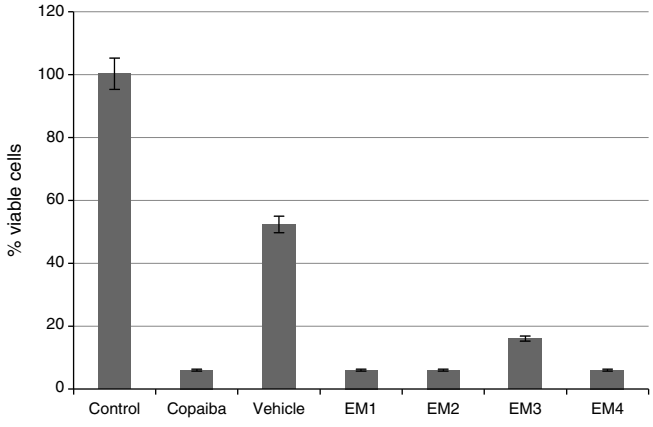

Fig. 1. Cell viability testing of *Copaifera multijuga* oil emulsions in the cell line NHI3T3.

The decreased cell viability of NHI3T3, non-neoplastic murine fibroblasts, validate the results of Costa-Lotufo et al. (2002), Lima et al. (2003), and Veiga Junior et al. (2007), who demonstrated a decrease in viability of tumor cells. Cell viability without copaiba was 52.33% indicating that the high cytotoxicity in this study may be related to the vehicle used in the emulsion.

The vehicle used in the formulation of emulsions was Tween® 80, which is a nonionic surfactant that is toxic to biological membranes and consists of the fatty acid ester, polyoxyethylene. Toxicity of Tween® 80 as a vehicle in cell culture is possibly related to its ability to alter the surface morphology and cell wall via its detergent action (Domingues et al., 2000).

Influence of the time of test emulsion application on human tooth color change

Influence of time of test emulsion application on human teeth color change is shown in Table 2.

Results of ΔE values equal to or lower than 3.3 are considered clinically insignificant from the daily clinical perspective of visual appearance; that is to say, the color change of human teeth of this magnitude would be clinically imperceptible (Vichi et al., 2004). Based on these facts, the test emulsions for which the color changes of teeth would not be clinically perceptible were EM1 at 10 s, EM2, at 30 s, and 2% chlorhexidine, EM2, and EM4 at 10 min.

Chlorhexidine was used in the present experiment as a solution for cavity and tooth surface cleaning and for short-term non-staining procedures of the tooth structure.

Use of copaiba oleoresin emulsions, a biotechnological product from the Amazonian region, contributed significantly to the reduction of color change (expressed in ΔE values); however, no studies reported in the literature support the data obtained in this research.

The copaiba emulsion tests demonstrated the potential of copaiba emulsions for use in dentistry, especially EM1 and EM2. Further research on the clinical application is necessary.

Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Authors' contributions

CNCDB, FCS, CT, and NCOC contributed to the development of the experimental phase for the emulsion formulations and antibacterial activity. VVJ contributed to the chemical analysis of the raw material. MCV and GSB contributed to the cytotoxicity assay. MFCLB coordinated the progress of all experimental phases and data analysis. CNCDB, LGM, and GNV drafted the article. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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