Revista Brasileira de Farmacognosia 26 (2016) 497–501

Brazilian Journal of Pharmacognosy
REVISTA BRASILEIRA DE FARMACOGNOSIA
www.elsevier.com/locate/bjp

Original Article

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

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ARTICLE INFO

Article history:
Received 10 October 2015
Accepted 1 March 2016
Available online 21 May 2016

Keywords:
Dentistry
Emulsions
Cytotoxicity
Fabaceae
Phytotherapy

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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http://dx.doi.org/10.1016/j.bjp.2016.03.010

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)