

Assessment of the antibacterial, cytotoxic and mutagenic potential of the phenolic-rich hydroalcoholic extract from *Copaifera trapezifolia* Hayne leaves

Luís Fernando Leandro,¹ Thaís da Silva Moraes,¹
Pollyanna Francielli de Oliveira,² Jacqueline Morais Alves,²
Juliana Marques Senedese,² Saulo Duarte Ozelin,²
Flávia Aparecida Resende,³ Rone Aparecido De Grandis,³
Eliaana Aparecida Varanda,³ Jairo Kenupp Bastos,⁴
Denise Crispim Tavares² and Carlos Henrique Gomes Martins¹

Correspondence

Carlos Henrique Gomes Martins
carlos.martins@unifran.edu.br

¹Laboratory of Research in Applied Microbiology, University of Franca – UNIFRAN, Franca, 14404-600 São Paulo, Brazil

²Laboratory of Mutagenesis, University of Franca – UNIFRAN, Franca, 14404-600 São Paulo, Brazil

³School of Pharmaceutical Sciences, State University of São Paulo, Araraquara, 14801-902 São Paulo, Brazil

⁴School of Pharmaceutical Sciences, University of São Paulo, Ribeirão Preto, 14040-903 São Paulo, Brazil

Copaifera trapezifolia Hayne occurs in the Atlantic Rainforest, which is considered one of the most important and endangered tropical forests on the planet. Although literature works have described many *Copaifera* spp., their biological activities remain little known. In the present study, we aimed to evaluate (1) the potential of the hydroalcoholic extract from *C. trapezifolia* leaves (CTE) to act against the causative agents of tooth decay and apical periodontitis and (2) the cytotoxicity and mutagenicity of CTE to ensure that it is safe for subsequent application. Concerning the tested bacteria, the MIC and the minimum bactericidal concentration of CTE varied between 100 and 400 µg ml⁻¹. The time-kill assay conducted at a CTE concentration of 100 µg ml⁻¹ evidenced bactericidal activity against *Porphyromonas gingivalis* (ATCC 33277) and *Peptostreptococcus micros* (clinical isolate) within 72 h. CTE at 200 µg ml⁻¹ inhibited *Porphyromonas gingivalis* and *Peptostreptococcus micros* biofilm formation by at least 50 %. A combination of CTE with chlorhexidine dichlorohydrate did not prompt any synergistic effects. The colony-forming assay conducted on V79 cells showed that CTE was cytotoxic at concentrations above 156 µg ml⁻¹. CTE exerted mutagenic effect on V79 cells, but the micronucleus test conducted on Swiss mice and the Ames test did not reveal any mutagenicity. Therefore, the use of standardized and safe extracts could be an important strategy to develop novel oral care products with antibacterial action. These extracts could also serve as a source of compounds for the discovery of new promising biomolecules.

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Abbreviations: ATCC, American Type Culture Collection; BW, body weight; CHD, chlorhexidine dichlorohydrate; CI, cytotoxicity index; CTE, hydroalcoholic extract from *Copaifera trapezifolia* leaves; FIC, fractional inhibitory concentration; MBC, minimum bactericidal concentration; MI, mutagenic index; MIC₅₀, minimum inhibitory concentration of biofilm; MMS, methyl methanesulfonate; MN, micronucleus; MNPCE, micronucleated polychromatic erythrocyte; NCE, normochromatic erythrocyte; NDI, nuclear division index; PCE, polychromatic erythrocyte.

INTRODUCTION

Oral diseases affect millions of people worldwide. More than 750 bacterial species occur in the oral cavity. Some of these bacteria underlie oral diseases such as caries and apical periodontitis, which are among the most common bacterial infections in humans (Aas *et al.*, 2005; Palombo, 2011).

Tooth decay is normally associated with *Streptococcus* spp., especially *Streptococcus mutans* and *Streptococcus sobrinus*, and *Lactobacillus* spp. Biofilm formation on the tooth surface (dental plaque) is one of the causes of this disease (Hirasawa & Takada, 2002; Chung *et al.*, 2006). Apical periodontitis is an inflammatory reaction to the presence of bacteria within the root canal system. Inflammation emerges in the periapical tissues and may result in tooth loss if left untreated (Peculiene *et al.*, 2008). Bacterial species frequently detected in acute and chronic apical periodontitis belong to diverse genera of Gram-negative (*Fusobacterium*, *Dialister*, *Porphyromonas*, *Prevotella*, *Tannerella*, *Treponema*, *Campylobacter* and *Veillonella*) and Gram-positive (*Parvimonas*, *Filifactor*, *Pseudoramibacter*, *Olsenella*, *Actinomyces*, *Peptostreptococcus*, *Streptococcus*, *Propionibacterium* and *Eubacterium*) bacteria (Siqueira & Rôças, 2009).

Oral diseases such as tooth decay and apical periodontitis impact people's health negatively – they diminish the mastication function, alter the psychosocial development and the facial aesthetics, cause phonetic disturbances and pain and generate local and systemic infectious complications (Melo *et al.*, 2008). The effects that tooth decay and apical periodontitis exert on humans have intensified the search for new antimicrobial compounds from other sources, including plants. These novel compounds could aid in controlling oral diseases.

Plants belonging to the genus *Copaifera* L. (Fabaceae-Caesalpinioideae), popularly known as 'copaiba', are native to the tropical regions of Latin America and Western Africa. These trees display a wide range of pharmacological properties, including anti-inflammatory, analgesic, antileishmanial, antimutagenic, gastroprotective and antimicrobial activities (Leandro *et al.*, 2012).

Among the 20 or more *Copaifera* spp. found in the Brazilian territory (Leandro *et al.*, 2012), *Copaifera trapezifolia* Hayne occurs in one of the most devastated and endangered regions on the planet, the Atlantic Rainforest, where only 12.5 % of the primary vegetation remains [SOS Mata Atlântica & INPE (Instituto Nacional de Pesquisas Espaciais), 2015]. Although the Atlantic Rainforest is under major threat, it still contains biologically important areas that deserve protection and expansion to ensure preservation of the species (Milani *et al.*, 2012).

A phytochemical study on the composition of the essential oil of *C. trapezifolia* leaves by GC/MS revealed that β -caryophyllene (33.5 %) and germacrene D (10.9 %) are the major constituents of this oil (Veiga *et al.*, 2006). *C. trapezifolia* constitutes a large and promising source of bioactive compounds for use in the preventative therapy or treatment of various disorders, including oral diseases. Although

literature papers have described the biological action of other *Copaifera* spp. (Santos *et al.*, 2008; Alves *et al.*, 2013b), there are no reports on the antimicrobial activity of *C. trapezifolia* against micro-organisms that cause oral diseases or on the safety of this plant.

The use of medicinal plants, crude plant extracts and isolated compounds for therapeutic purposes requires evaluation of their cytotoxic and mutagenic potential. This is because plant-synthesized substances generally serve as chemical defences against micro-organisms and herbivores and can therefore be toxic to other organisms (Cavalcanti *et al.*, 2006).

The present study has investigated the antibacterial activity of the hydroalcoholic extract from *C. trapezifolia* leaves (CTE) against micro-organisms that cause dental caries and apical periodontitis; it has also evaluated the cytotoxicity and mutagenicity of CTE to ensure that it is safe for subsequent application.

METHODS

Plant material

Preparation of the CTE. *C. trapezifolia* Hayne leaves were collected in the botanical garden of Rio de Janeiro, Brazil, in July 2012. Dr José Silva Costa of the Botanical Garden Herbarium in Rio de Janeiro, Brazil, authenticated the plant material. A voucher specimen was deposited in this same herbarium under number NY 00470875. The leaves were dried at 40 °C in an oven operating with circulating air for 36 h. The drying procedure furnished 500 g of dried plant material. The dried leaves were powdered in a knife mill, and 100 g of the leaves was macerated three times in 500 ml of aqueous ethanol 7:3 (water/ethanol, v/v), at room temperature, for 48 h. The extracts were combined, concentrated below 40 °C under vacuum and lyophilized, to yield 72 g of the crude CTE.

HPLC chromatographic analysis of CTE

CTE was analysed on a Shimadzu SCL-10Avp multisolvent delivery HPLC system equipped with a Shimadzu SPD-M10Avp photodiode array detector, a Shimadzu Synergi Polar-RP 80 A (150×4.60 mm, 4 µm) column and a Shimadzu pre-column. An Intel Celeron computer helped to control the analytical system.

The mobile phase consisted of (A) 0.01 % trifluoroacetic acid in water and (B) methanol. The elution program was as follows: from 15 to 50 % of B within 45 min, from 50 to 90 % of B within 65 min and from 90 to 15 % of B within 70 min. Then, the system was eluted with 15 % of B for 5 min to return it to the initial conditions and to re-equilibrate the column. The flow rate was maintained at 1.0 ml min⁻¹; detection was set at 280 nm. HPLC-grade solvents were acquired from the Tedia Company. Water was purified on the Milli-Q-plus filter system (Millipore). The flavonoids afzelin and quercitrin were isolated in our laboratory with purities above 95 % and identified by NMR.

Tested micro-organisms

Bacteria were acquired from the American Type Culture Collection (ATCC) maintained in the culture collection of the Laboratory of Research in Applied Microbiology (LaPeMA) of the University of Franca, state of São Paulo, Brazil, at –80 °C. The following micro-organisms, causative agents of dental caries, were used: *Streptococcus salivarius* (ATCC 25975 and clinical isolate), *Streptococcus sobrinus* (clinical

isolate), *Streptococcus mutans* (ATCC 25175 and clinical isolate), *Streptococcus mitis* (ATCC 49452 and clinical isolate), *Streptococcus sanguinis* (ATCC 10556 and clinical isolate), *Lactobacillus casei* (ATCC 11578 and clinical isolate) and *Enterococcus faecalis* (ATCC 4082 and clinical isolate). The following micro-organisms, causative agents of apical periodontitis, were used: *Porphyromonas gingivalis* (ATCC 33277 and clinical isolate), *Prevotella intermedia* (clinical isolate), *Prevotella nigrescens* (ATCC 33563), *Fusobacterium nucleatum* (ATCC 25586 and clinical isolate), *Bacteroides fragilis* (ATCC 25285), *Actinomyces naeslundii* (ATCC 19039 and clinical isolate), *Actinomyces viscosus* (clinical isolate), *Prevotella buccae* (clinical isolate) and *Peptostreptococcus micros* (clinical isolate).

Antimicrobial assays

Determination of the MIC and minimum bactericidal concentration. MIC was determined by the broth microdilution method, conducted in 96-well microplates; the methodology described by CLSI (2007, 2009) was used. Briefly, CTE samples were dissolved in DMSO (Merck) at 1.0 mg ml⁻¹. Then, the samples were diluted in brain–heart infusion broth (Difco) or in Schaedler broth (Difco) supplemented with hemin (5.0 mg ml⁻¹) and vitamin K (10.0 mg ml⁻¹) in the case of aerobic and anaerobic micro-organisms, respectively. The CTE samples were tested at concentrations ranging from 0.195 to 400 mg ml⁻¹. The final DMSO content was 5% (v/v). DMSO at 5% (v/v) was the negative control. The inoculum was adjusted at 625 nm in a spectrophotometer to give a cell concentration of 5 × 10⁵ and 1 × 10⁶ c.f.u. ml⁻¹ for aerobic (CLSI, 2009) and anaerobic (CLSI, 2007) micro-organisms, respectively. One inoculated well was included to control broth adequacy for micro-organism growth. One non-inoculated well free of antimicrobial agent was also used to ensure medium sterility. Chlorhexidine dichlorohydrate (CHD; Sigma-Aldrich) at concentrations ranging from 0.1 to 59 µg ml⁻¹ and metronidazole (Sigma-Aldrich) at concentrations ranging from 0.01 to 5.9 µg ml⁻¹ were the positive controls for aerobic micro-organisms and for the anaerobic micro-organisms *Bacteroides fragilis* (ATCC 25285) and *Bacteroides thetaiotaomicron* (ATCC 29741), respectively.

The microplates were incubated at 37 °C for 24 h for aerobic micro-organisms and for 72 h for anaerobic micro-organisms in an anaerobic workstation (Don Whitley Scientific). After that, 30 µl of aqueous resazurin (Sigma-Aldrich) solution at 0.02% was added to each well. Resazurin is a redox probe that allows immediate observation of microbial growth. Blue and red colours represent absence and presence of microbial growth, respectively (Sarker *et al.*, 2007).

To determine minimum bactericidal concentration (MBC), an aliquot of the inoculum was aseptically removed from each well without apparent micro-organism growth before resazurin addition. This aliquot was plated onto brain–heart infusion agar supplemented with 5% sheep blood or onto Schaedler agar in the case of aerobic and anaerobic micro-organisms, respectively. The plates were incubated as described above. MBC was determined for all the investigated bacterial strains.

Time-kill curves

Triplicate time-kill assays were performed according to D'Arrigo *et al.* (2010). CTE displayed the highest antibacterial activity (MIC and MBC, 100 µg ml⁻¹) against the anaerobic micro-organisms *Porphyromonas gingivalis* (ATCC 33277) and *Peptostreptococcus micros* (clinical isolate), important causative agents of apical periodontitis (Grenier & Bouclin, 2006; Grenier & La, 2011). These micro-organisms were chosen for the time-kill curve assays. Tubes containing CTE at a final concentration of 100 µg ml⁻¹ (one time the CTE MIC determined for *Porphyromonas gingivalis* and *Peptostreptococcus micros*) were inoculated with the tested micro-organisms at an initial bacterial density of 2.12 × 10⁵ and 17.2 × 10⁵ c.f.u. ml⁻¹ for *Porphyromonas gingivalis* and *Peptostreptococcus*

micros, respectively. After that, the plates were incubated in 5–10% H₂, 10% CO₂ and 80–85% N₂ atmosphere inside an anaerobic workstation operating at 36 °C for 72 h. Aliquots were removed (50 µl) at 0 and 30 min and at 6, 12, 24 and 72 h to count the number of viable colonies after incubation. Next, the aliquots were serially diluted until final dilution of 10⁻⁴ in sterile Schaedler broth. The diluted samples (50 µl) were spread onto Schaedler agar plate and incubated at 36 °C; viable colonies were counted after 72 h. Time-kill curves were constructed by plotting log₁₀ c.f.u. ml⁻¹ versus time with the aid of the Prism software (version 5.0; GraphPad). Assays were performed in triplicate for CTE and also for the positive (CHD) and negative controls (suspension of *Porphyromonas gingivalis* and *Peptostreptococcus micros* without added CTE). CHD was used at its MIC (3.688 µg ml⁻¹ for *Porphyromonas gingivalis* and 7.375 µg ml⁻¹ for *Peptostreptococcus micros*).

Antibiofilm activity evaluation

The MIC of biofilm (MICB₅₀) was determined based on the lowest CTE concentration that was able to inhibit at least 50% of biofilm formation. The anaerobic micro-organisms *Porphyromonas gingivalis* (ATCC 33277) and *Peptostreptococcus micros* (clinical isolate), the bacteria that were the most susceptible to CTE, were used in the assays. A microtitre plate assay based on CLSI (2007) previously described by Caetano da Silva *et al.* (2014) was used, with some modifications. This method was similar to the MIC assay conducted for planktonic cells. To this end, twofold serial dilutions of each sample were prepared in the wells of a 96-well polystyrene tissue culture plate (Costar) containing 200 µl of Schaedler broth (Difco) per well. The final concentration of CTE ranged from 0.195 to 400 µg ml⁻¹. CHD at concentrations between 0.115 and 59 µg ml⁻¹ was the negative control; the bacterial strains in the absence of the antibacterial agent were the positive control. The *Porphyromonas gingivalis* or *Peptostreptococcus micros* cell suspension was added at concentrations of 1 × 10⁶ c.f.u. ml⁻¹. To assess culture growth, absorbance readings were accomplished after 72 h incubation in 5–10% H₂, 10% CO₂ and 80–85% N₂ atmosphere inside an anaerobic chamber (Don Whitley Scientific) operating at 36 °C. Next, the culture supernatant of each well was decanted, and planktonic cells were removed by washing with PBS (pH 7.2). The biofilms were fixed with methanol for 15 min and air dried at room temperature. They were then stained with 0.2% (w/v) crystal violet (Sigma) for 20 min and rinsed thoroughly with water until control wells became colourless. Biofilm formation was quantified by addition of 150 µl of acetic acid at 33% to each crystal-violet-stained well. The plates were shaken at room temperature for 30 min; the absorbance at 595 nm (A₅₉₅) was determined by means of a microplate reader (ASYS; Eugendorf). The percentages of inhibition were calculated by using the equation (1 – A₅₉₅ of the test well/A₅₉₅ of non-treated control) × 100 (Wei *et al.*, 2006).

The antibiofilm activity was also measured by counting the number of micro-organisms. The procedures were the same as the ones described above, but they were conducted on another microplate. After the incubation period, the micro-organisms were counted. Initially, the volume of each well was gently aspirated to remove planktonic cells. To remove non-adhered cells, 200 µl of sterilized Milli-Q water was added and aspirated. Next, 200 µl of supplemented Schaedler broth (Difco) was added, and the microplate was submitted to sonication for 15 min to shed the biofilms. After sonication, the wells were homogenized with the aid of a micropipette. To enable counting, decimal dilutions were accomplished by removing 20 µl aliquots from each well and transferring them to another well containing 180 µl of supplemented Schaedler broth (Difco) in another microplate. Twenty microlitres from this 10⁻¹ dilution was also transferred to another well to afford a 10⁻² dilution. The same sequence was adopted repeatedly until 10⁻⁷ dilution was achieved. Then, 50 µl of each dilution was seeded with *Drigalski spatula* in supplemented Schaedler agar plates. This procedure was carried out in triplicate for *Porphyromonas gingivalis* (ATCC 33277) and *Peptostreptococcus micros* (clinical isolate). The plates were incubated in anaerobic chamber

at 36 °C for 72 h. Colonies were counted after incubation. The results are expressed as $\log_{10}$ in c.f.u. ml^{-1} . The best inoculum concentration and incubation time for the antibiofilm activity assay were selected by standardizing the biofilm formation (data not shown) (Caetano da Silva *et al.*, 2014).

Synergistic antimicrobial activity

Chequerboard assays were derived from the standard procedure established by CLSI (2007). These assays helped to investigate the *in vitro* antimicrobial efficacy of a combination of CHD and CTE against the anaerobic micro-organisms *Porphyromonas gingivalis* (ATCC 33277) and *Peptostreptococcus micros* (clinical isolate), which were the most susceptible to CTE. The synergy tests were evaluated in triplicate. The combination was algebraically calculated to determine the fractional inhibitory concentration (FIC) index. FIC_A was calculated as MIC of drug A in the combination/MIC of drug A alone; FIC_B was determined as MIC of drug B in the combination/MIC of drug B alone. The FIC summation ($\sum \text{FIC}$) was calculated as: $\sum \text{FIC} = \text{FIC}_A + \text{FIC}_B$ (Chaturvedi *et al.*, 2008). The $\sum \text{FIC}$ index values were interpreted according to Lewis (2002). $\text{FIC} \leq 0.5$ and $0.5 < \text{FIC} < 1$ corresponded to synergism and additivity, respectively; $1 \leq \text{FIC} < 4$ and $\text{FIC} \geq 4$ referred to indifference and antagonism, respectively.

Salmonella mutagenic assay

The mutagenic activity was also evaluated by the *Salmonella*/microsome assay also known as the Ames test. This assay used the *Salmonella typhimurium* tester strains TA98, TA100, TA97a and TA102 with (+S9) and without (–S9) metabolism, provided by Dr B.N. Ames (Berkeley, CA, USA). These strains, which were grown from frozen cultures in Oxoid Nutrient Broth No. 2 for 12–14 h overnight, were assessed by the pre-incubation method (Maron & Ames, 1983).

The metabolic activation mixture (S9 fraction), prepared from livers of Sprague–Dawley rats treated with the polychlorinated biphenyl mixture Aroclor 1254 (500 mg kg^{-1}), was purchased from the Molecular Toxicology and freshly prepared before each test. The metabolic activation system consisted of 4 % S9 fraction, 1 % MgCl_2 at 0.4 M, 1 % KCl at 1.65 M, 0.5 % D-glucose-6-phosphate disodium at 1 M, 4 % NADP at 0.1 M, 50 % phosphate buffer at 0.2 M and 39.5 % sterile distilled water (Maron & Ames, 1983).

To determine the mutagenic activity, five different CTE concentrations (1.56–12.50 mg plate^{-1}), diluted in DMSO, were assayed. The CTE concentrations were selected based on a preliminary toxicity test. In all the subsequent assays, the upper limit of the tested dose range was either the highest non-toxic dose or the lowest toxic dose determined in this preliminary assay. Toxicity was detected either as reduction in the number of histidine revertants (His+) or as thinning of the auxotrophic background (i.e. background lawn). DMSO (100 $\mu\text{l plate}^{-1}$) was the negative (solvent) control.

The various tested CTE concentrations were added to 0.5 ml of phosphate buffer at 0.2 M or to 0.5 ml of an S9 mixture at 4 %, which contained 0.1 ml of bacterial culture. Then, the samples were incubated at 37 °C for 20–30 min. Next, 2 ml of top agar was added, and the mixture was poured onto a plate containing minimal agar. Subsequently, the plates were incubated at 37 °C for 48 h, and His+ revertant colonies were counted manually. All the experiments were carried out in triplicate. The results were analysed with the statistical software package Salanal 1.0 (U.S. Environmental Protection Agency, Monitoring Systems Laboratory, from the Research Triangle Institute); the model of Bernstein *et al.* (1982) was adopted. The data (revertants/plate) were assessed by ANOVA, followed by linear regression. The mutagenic index (MI) was calculated for each tested concentration. MI corresponded to the average number of revertants per plate containing the test compound

(CTE) divided by the average number of revertants per plate containing the negative control (solvent). A test solution was considered mutagenic when a dose–response relationship and a twofold increase in the number of mutants ($\text{MI} \geq 2$) were detected for at least one concentration (Resende *et al.*, 2012).

Cytotoxicity and mutagenicity assays

DNA damage-inducing agents. Methyl methanesulfonate (MMS) was dissolved in PBS (pH 7.4) and used at concentrations of 110 and 44 $\mu\text{g ml}^{-1}$ for the colony-forming assays and micronucleus (MN) tests on V79 cells, respectively. For the bone marrow MN assays, the animals were treated with 40 mg kg^{-1} of body weight (BW). In the case of the Ames test, the following standard mutagens were used as positive controls in experiments without the S9 mixture: 4-nitro-*o*-phenylenediamine (10 $\mu\text{g plate}^{-1}$) for TA98 and TA97a, sodium azide (1.25 $\mu\text{g plate}^{-1}$) for TA100 and mitomycin C (0.5 $\mu\text{g plate}^{-1}$) for TA102. In experiments with S9 activation, 2-amino-anthracene (1.25 $\mu\text{g plate}^{-1}$) was used for TA98, TA97a and TA100; 2-amino-fluorene (10 $\mu\text{g plate}^{-1}$) was used for TA102. The substances were purchased from the Sigma Chemical.

Cell culture conditions

The V79 cells (fibroblasts, Chinese hamster lung) were maintained as monolayers in plastic culture flasks (25 cm^2) containing HAM-F10 (Sigma–Aldrich) and DMEM (Sigma–Aldrich, 1 : 1) supplemented with 10 % foetal bovine serum (Nutricell), antibiotics (streptomycin at 0.01 mg ml^{-1} and penicillin at 0.005 mg ml^{-1} , Sigma–Aldrich) and HEPES (Sigma–Aldrich) at 2.38 mg ml^{-1} , at 37 °C, in a Biochemical oxygen demand (BOD)-type chamber. Under these conditions, the average cell cycle time was 12 h. The experiments were conducted by using V79 cells between the 6th and 12th culture passage after thawing. The V79 cells were used for the cytotoxicity and mutagenicity assessments.

Clonogenic efficiency assay

The clonogenic efficiency assay previously described by Leandro *et al.* (2013) was used to determine CTE cytotoxicity. The V79 cells were treated with CTE concentrations ranging from 9.70 to 5000 $\mu\text{g ml}^{-1}$ for 3 h. Positive (MMS, 110 $\mu\text{g ml}^{-1}$) and negative (no treatment) controls were included. Next, the cultures were trypsinized, and 300 cells were seeded per culture flask (three flasks per concentration). The experiments lasted 10 days. The culture medium was removed, and the colonies were washed with PBS and stained with Giemsa (1 : 20 in phosphate buffer, pH 7.0) for 20 min. The colonies were counted under a magnifying glass. The survival fraction (SF) was calculated for the different treatments based on the following formula:

$$\text{SF}(\%) = \frac{A}{B} \times 100$$

where A is the number of colonies found in the different treatments, and B is the number of colonies found in the negative control (Franken *et al.*, 2006).

In vitro MN assay

The MN assays were performed according to Resende *et al.* (2010) and as previously described by Leandro *et al.* (2013), with modifications. For the tests, 0.5×10^6 cells were seeded in tissue culture flasks and incubated in 5 ml of complete HAM-F10/DMEM for two cycles (24 h), followed by washing with PBS (pH 7.4). The cells were then submitted to one of the following treatments in serum-free medium for 3 h: (a) negative control, (b) solvent control (DMSO at 1 %), (c) positive control (MMS at 44 $\mu\text{g ml}^{-1}$) and (d) CTE (at 40, 80, 120 and 160 $\mu\text{g ml}^{-1}$). The CTE

concentrations were chosen based on the results obtained during the colony-forming assay; cytotoxicity was used as a selection criterion. At the end of the treatment period, the cells were washed twice with PBS. Next, fresh serum-supplemented medium containing cytochalasin-B (Sigma-Aldrich) at $3 \mu\text{g ml}^{-1}$ was added, and incubation was accomplished for additional 17 h. At harvest, the cells were trypsinized (0.025 %) and treated with a hypotonic sodium citrate solution at 1 % at 37°C . The cells were fixed in methanol/acetic acid (3:1), and the slides were stained with Giemsa at 3 % for 5 min. The criteria established by Fenech (2000) were used to analyse MNs and binucleated cells. A total of 3000 binucleated cells were scored per treatment, which corresponded to 1000 cells per treatment per repetition. The nuclear division index (NDI) was determined for the 1500 cells analysed per treatment, for a total of 500 cells per repetition. The cells with well-preserved cytoplasm and containing 1–4 nuclei were scored. The NDI was calculated according to Eastmond & Tucker (1989); the following formula was used:

$$\text{NDI} = \frac{M1 + 2(M2) + 3(M3) + 4(M4)}{N}$$

where M1–M4 is the number of cells with 1, 2, 3 and 4 nuclei, respectively, and N is the total number of viable cells.

Additionally, the CTE cytotoxicity index (CI) was calculated according to Kirsch-Volders *et al.* (2003):

$$\text{CI} = 100 - 100 \left[\frac{\text{NDIT} - 1}{\text{NDIC} - 1} \right]$$

where NDIT is the NDI found for the different treatments, and NDIC is the NDI of the negative control. The mutagenicity assay comprised three totally independent experiments.

In vivo MN assay

Male Swiss mice (7–8 weeks old) weighing approximately 25 g were provided by the Faculty of Pharmaceutical Sciences, University of São Paulo, Ribeirão Preto, São Paulo, Brazil. The animals were kept in plastic boxes in an experimental room under controlled temperature ($22 \pm 2^\circ\text{C}$) and humidity ($50 \pm 10\%$) in a 12 h light/dark cycle, with standard mouse chow and water available *ad libitum*. The Ethics Committee for Animal Care of the University of Franca (protocol 001/14) approved the study protocol.

The study animals were divided into different experimental groups of six animals each. The different CTE doses (125, 250, 500, 1000 and 2000 mg kg^{-1} BW) were administered by gavage (0.5 ml per animal). Negative (water), solvent (DMSO at $0.5 \mu\text{g ml}^{-1}$) and positive (intraperitoneal MMS at 40 mg kg^{-1} of BW, 0.3 ml per animal) control groups were included. The bone marrow samples were collected 24 h after treatment.

The bone marrow MN assays were performed according to the protocol described by MacGregor *et al.* (1987). A total of 2000 polychromatic erythrocytes (PCEs) were analysed per animal to determine the frequency of micronucleated polychromatic erythrocytes (MNPCEs). The PCE/PCE+NCE (normochromatic erythrocytes) ratio was calculated by analysis of 400 erythrocytes for determination of the cytotoxicity (Merish-Sundermann *et al.*, 2004). The slides were scored blindly by using a light microscope with a $\times 100$ objective immersion.

Statistical analysis

The results obtained during the MN assays were evaluated by ANOVA for completely randomized experiments, with calculation of F statistics and the respective P values. When $P < 0.05$, the treatment means were

compared by the Tukey test, and the minimum significant difference was calculated for $\alpha = 0.05$.

RESULTS

HPLC chromatographic analysis of the crude CTE

Comparison with authentic standards showed that the compounds with retention times of 41 and 47 min corresponded to quercitrin and afzelin, respectively. The profiles of the other major compounds detected in CTE were also compatible with the profile of phenolic compounds (Fig. 1).

MIC and MBC

We considered that MIC values higher than $400 \mu\text{g ml}^{-1}$, the highest CTE concentration used in this study, corresponded to lack of antibacterial action. The assays against causative agents of dental caries provided MIC and MBC values of 200 and $400 \mu\text{g ml}^{-1}$ against *Streptococcus salivarius* (clinical isolate), respectively, which demonstrated that CTE exerted a bacteriostatic effect on this micro-organism. CTE displayed bactericidal activity against *Streptococcus sanguinis* (clinical isolate) only. Both MIC and MBC were equal to $400 \mu\text{g ml}^{-1}$ for *Streptococcus sobrinus* (ATCC 33478 and clinical isolate), *Streptococcus mitis* (ATCC 49456 and clinical isolate), *Streptococcus mutans* (ATCC 25175 and clinical isolate), *Streptococcus salivarius* (ATCC 33478), *Streptococcus sanguinis* (ATCC 10556) and *L. casei* (ATCC 11578 and clinical isolate); therefore, at the concentrations used in this study, CTE was not active against these bacteria (Table 1).

Table 1 lists the results obtained for the assays against causative agents of apical periodontitis. CTE afforded MIC and MBC values of $400 \mu\text{g ml}^{-1}$ against *F. nucleatum* (ATCC 25586 and clinical isolate) and *A. naeslundii* (clinical isolate), respectively, which characterized a bactericidal effect. CTE also exhibited satisfactory bactericidal effect against *Porphyromonas gingivalis* (ATCC 33277) and *Peptostreptococcus micros* (clinical isolate) – MIC and MBC values were equal to $100 \mu\text{g ml}^{-1}$, respectively. At the concentrations used in this study, CTE had no antibacterial action against *Porphyromonas gingivalis* (clinical isolate), *Prevotella intermedia* (clinical isolate), *A. naeslundii* (ATCC 19039), *A. viscosus* (clinical isolate), *Prevotella nigrescens* (ATCC 33563), *Bacteroides fragilis* (ATCC 25285) or *Prevotella buccae* (clinical isolate).

We determined the MIC value for CHD against *Porphyromonas gingivalis* (ATCC 33277) and *Peptostreptococcus micros* (clinical isolate), the bacteria that were the most susceptible to CTE. These results were important for the time-kill assay and for evaluation of the synergistic antimicrobial activity (Table 1).

Time-kill assay

Regarding *Porphyromonas gingivalis* (ATCC 33277), CTE at a concentration of $100 \mu\text{g ml}^{-1}$ started to progressively diminish the number of micro-organisms at 48 h

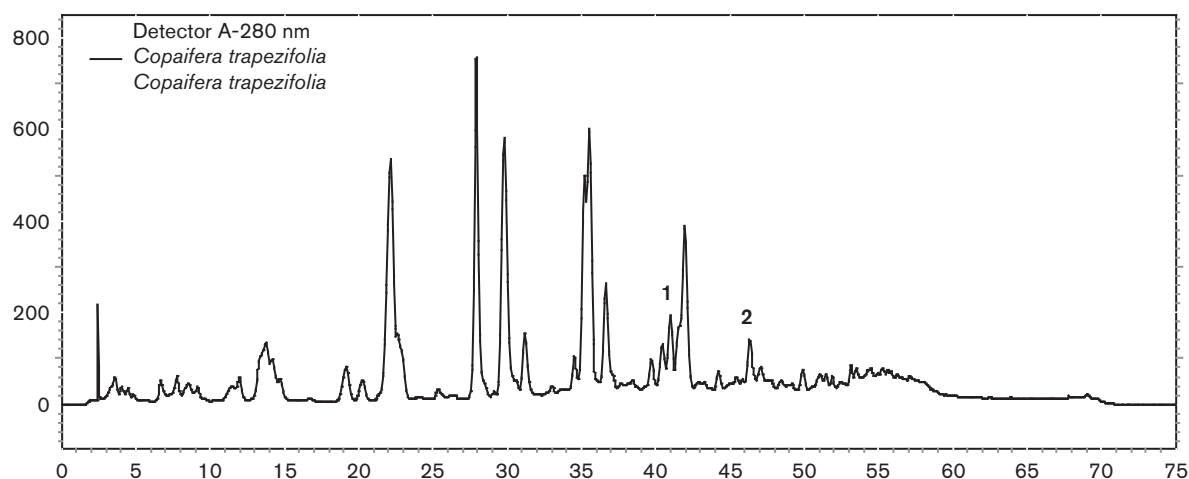


Fig. 1. Chromatographic profile of the CTE obtained by HPLC-UV-DAD on a Synergi Polar-RP 80 A column (150×4.60 mm, 4 µm), at a flow rate of 1 ml min⁻¹ and detection at 280 nm. 1, quercitrin; 2, afzelin.

incubation; the bactericidal action was evident at 72 h (Fig. 2b). As for *Peptostreptococcus micros* (clinical isolate), CTE at a concentration of 100 µg ml⁻¹ reduced the number of micro-organisms by over 3 log₁₀ at 30 min. The number of micro-organisms continued to decrease progressively until 48 h incubation, and the bactericidal activity clearly emerged at 72 h incubation (Fig. 2a).

CHD (3.688 µg ml⁻¹), used as positive control for *Peptostreptococcus micros* (clinical isolate) and *Porphyromonas gingivalis* (ATCC 33277), reduced the number of micro-organisms at 48 h incubation and exhibited bactericidal activity at 72 h incubation (Fig. 2a, b).

Antibiofilm activity

Analysis of the OD and of the number of micro-organisms (log₁₀ c.f.u. ml⁻¹) demonstrated the antibiofilm activity of CTE and CHD. The MIC₅₀ helped us to evaluate the CTE antibiofilm activity. The graphs in Fig. 3(a, c) illustrate the MIC₅₀ results for CTE and CHD against *Peptostreptococcus micros* (clinical isolate) and *Porphyromonas gingivalis* (ATCC 33277), respectively. At a concentration of 29.5 µg ml⁻¹, CHD inhibited *Peptostreptococcus micros* and *Porphyromonas gingivalis* biofilm formation by at least 50 % (Fig. 3b, d, respectively). At a concentration of 200 µg ml⁻¹, CTE inhibited *Peptostreptococcus micros* and *Porphyromonas gingivalis* biofilm formation by at least 50 % (Fig. 3a, c, respectively).

Synergistic antimicrobial activity

The combination of CTE with CHD did not elicit any synergistic effects. The ΣFIC indices of 2.24 and 11.98 for *Porphyromonas gingivalis* (ATCC 33277) and *Peptostreptococcus micros* (clinical isolate) indicated an indifferent effect and an antagonistic effect, respectively.

Salmonella mutagenic assay

Table 2 presents the mean number of revertants/plate (M), the SD and the MI obtained after treatment of the *Salmonella typhimurium* strains TA98, TA100, TA102 and TA97a with CTE in the presence (+S9) and absence (–S9) of metabolic activation. Compared with the negative control, CTE did not increase the number of revertant colonies, which attested to lack of mutagenic activity.

Cytotoxicity assay

Fig. 4 displays the dose-dependent changes in the viability of V79 cells treated with CTE as evaluated by the colony-forming assay. The cultures treated with CTE at 9.7, 19.5, 39.0 and 78.1 µg ml⁻¹ did not differ significantly from the negative control. In contrast, the cultures treated with CTE at 156.2, 312.5, 625, 1250, 2500 and 5000 µg ml⁻¹ were significantly different from the negative control, which revealed a cytotoxic effect. Therefore, we used CTE at 40, 80, 120 and 160 µg ml⁻¹ for the *in vitro* MN test (Table 3).

In vitro MN test

Table 3 contains the mean MN frequency and NDI in the V79 cells treated with different CTE concentrations. The cell cultures treated with CTE at 120 and 160 µg ml⁻¹ exhibited significantly higher MN frequencies as compared with the negative control. Hence, CTE was mutagenic at these concentrations. Compared with the negative control, NDI was not significantly different in any of the treated groups. Therefore, cytotoxicity did not emerge under the experimental conditions of the treatments applied herein.

In vivo MN assay

Table 4 lists the MNPCE frequencies in the bone marrow of Swiss mice treated with different CTE and/or MMS doses.

Table 1. *In vitro* antibacterial activity (MIC and MBC) of the CTE against micro-organisms causing dental caries and apical periodontitis

Growth requirements	Bacteria	Origin	CTE ($\mu\text{g ml}^{-1}$)		CDH ($\mu\text{g ml}^{-1}$)		Metronidazole ($\mu\text{g ml}^{-1}$)	
			MIC ($\mu\text{g ml}^{-1}$)	MBC ($\mu\text{g ml}^{-1}$)	MIC ($\mu\text{g ml}^{-1}$)	MBC ($\mu\text{g ml}^{-1}$)	MIC ($\mu\text{g ml}^{-1}$)	MBC ($\mu\text{g ml}^{-1}$)
Aerobic	<i>Streptococcus sobrinus</i>	Clinical isolate	>400	>400	3.688	3.688	–	–
	<i>Streptococcus mitis</i>	ATCC 49456	>400	>400	3.688	3.688	–	–
	<i>Streptococcus mitis</i>	Clinical isolate	>400	>400	7.375	7.375	–	–
	<i>Streptococcus mutans</i>	ATCC 25175	>400	>400	0.922	0.922	–	–
	<i>Streptococcus mutans</i>	Clinical isolate	>400	>400	0.922	0.922	–	–
	<i>Streptococcus salivarius</i>	ATCC 25975	>400	>400	7.375	7.375	–	–
	<i>Streptococcus salivarius</i>	Clinical isolate	200	400	7.375	7.375	–	–
	<i>Streptococcus sanguinis</i>	ATCC 10556	>400	>400	3.688	3.688	–	–
	<i>Streptococcus sanguinis</i>	Clinical isolate	400	400	7.375	7.375	–	–
	<i>L. casei</i>	ATCC 11578	>400	>400	3.688	3.688	–	–
	<i>L. casei</i>	Clinical isolate	>400	>400	14.75	14.75	–	–
	<i>E. faecalis</i>	ATCC 4082	>400	>400	7.375	7.375	–	–
	<i>E. faecalis</i>	Clinical isolate	>400	>400	14.75	14.75	–	–
Anaerobic	<i>Porphyromonas gingivalis</i>	ATCC 33277	100	100	3.688	3.688	–	–
	<i>Porphyromonas gingivalis</i>	Clinical isolate	>400	>400	–	–	–	–
	<i>Prevotella intermedia</i>	Clinical isolate	>400	>400	–	–	–	–
	<i>F. nucleatum</i>	ATCC 25586	400	400	–	–	–	–
	<i>F. nucleatum</i>	Clinical isolate	400	400	–	–	–	–
	<i>A. naeslundii</i>	ATCC 19039	>400	>400	–	–	–	–
	<i>A. naeslundii</i>	Clinical isolate	400	400	–	–	–	–
	<i>A. viscosus</i>	Clinical isolate	>400	>400	–	–	–	–
	<i>Bacteroides fragilis</i>	ATCC 25285	>400	>400	–	–	–	–
	<i>Peptostreptococcus micros</i>	Clinical isolate	100	100	7.375	7.375	–	–
	<i>Prevotella nigrescens</i>	ATCC 33563	400	>400	–	–	–	–
	<i>Prevotella buccae</i>	Clinical isolate	>400	>400	–	–	–	–
	* <i>Bacteroides fragilis</i>	ATCC 25285	–	–	–	–	1.475	1.475
	* <i>Bacteroides thetaiotaomicron</i>	ATCC 29741	–	–	–	–	2.95	2.95

MIC or MBC value >400 $\mu\text{g ml}^{-1}$, corresponding to a lack of antibacterial activity.

CHD, positive control.

*Strain controls.

The animals treated with distinct CTE doses did not have significantly different MNPCE frequency as compared with the negative control, which demonstrated the absence of mutagenic activity.

Table 4 also summarizes the results of PCE/PCE+NCE ratio. The PCE percentage in the bone marrow cells did not decrease significantly in any of the treatment groups as compared with the negative control. This confirmed that the different treatments were not cytotoxic.

DISCUSSION

Phytochemical studies on species belonging to the genus *Copaifera* have demonstrated that *Copaifera* leaves are rich in phenolic compounds (Brancalion *et al.*, 2012; Sousa *et al.*, 2012; de Oliveira *et al.*, 2013). In this work, HPLC

analysis identified two flavonols, quercitrin and afzelin, in CTE. Other authors have isolated these compounds from the hydroalcoholic extract of *Copaifera langsdorffii* leaves (Brancalion *et al.*, 2012; Sousa *et al.*, 2012; Alves *et al.*, 2013b; de Oliveira *et al.*, 2013; Senedese *et al.*, 2013). Quercitrin and afzelin are natural phenolic compounds that display antioxidant, anti-inflammatory and antimicrobial capacities (Shin *et al.*, 2013; Flores *et al.*, 2013; Gómez-Flores *et al.*, 2015; Lee *et al.*, 2014). The presence of this class of compounds in CTE might contribute to the antibacterial activity observed in this study against *Streptococcus salivarius* (clinical isolate), *Streptococcus sanguinis* (clinical isolate), *F. nucleatum* (ATCC 25586 and clinical isolate), *A. naeslundii* (clinical isolate), *Porphyromonas gingivalis* (ATCC 33277) and *Peptostreptococcus micros* (clinical isolate) at concentrations ranging from 100 to 400 $\mu\text{g ml}^{-1}$.

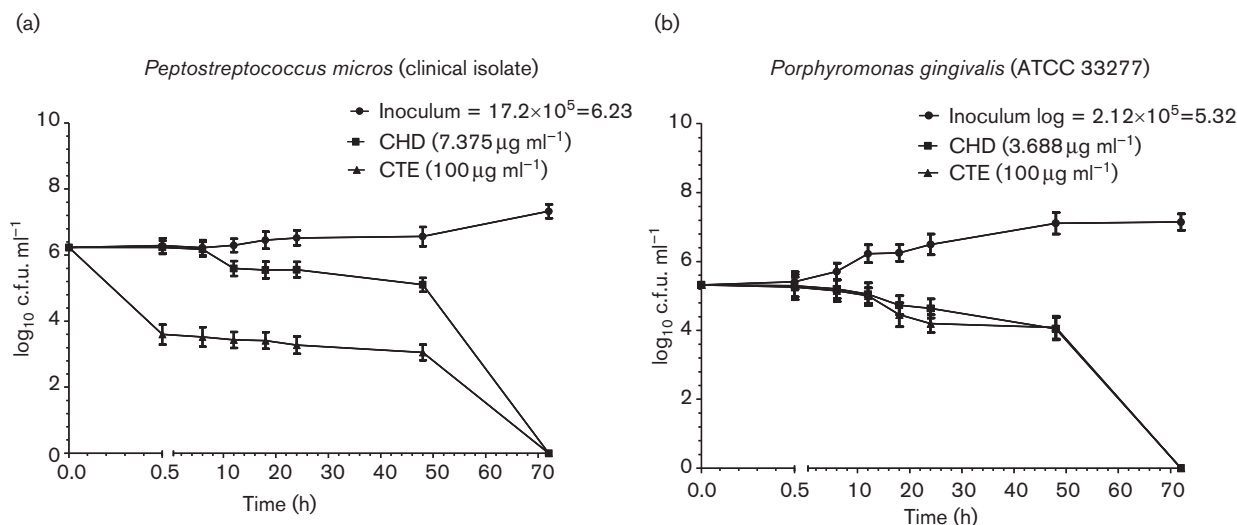


Fig. 2. Time-kill curve profiles for the CTE against *Peptostreptococcus micros* (clinical isolate) (a) and *Porphyromonas gingivalis* (ATCC 33277) (b). Positive control: CHD.

According to Leandro *et al.* (2012), although many species of *Copaifera* have been described, only nine of these species have been the object of biological studies, which have evaluated their traditional uses in folk medicine. Some researchers have not specified which *Copaifera* spp. they investigated. *C. langsdorffii*, *Copaifera lucens*, *Copaifera martii*, *Copaifera multijuga*, *Copaifera officinalis*, *Copaifera paupera*, *Copaifera reticulata*, *Copaifera duckei* and *Copaifera cearensis* are the most often evaluated species. To our knowledge, there are no literature reports on the biological action of *C. trapezifolia*. Therefore, the present study aimed to evaluate the antimicrobial activity of *C. trapezifolia* CTE against causative agents of dental caries and apical periodontitis.

Oral diseases affect 3.9 billion people worldwide (Marcenes *et al.*, 2013). Dental treatment is usually very expensive and not readily accessible, especially in developing countries (More *et al.*, 2008). *Copaifera* spp. constitute a rich and promising source for the discovery of novel biologically active compounds with potential use in preventative therapies and in the treatment of oral diseases (Pieri *et al.*, 2010; Souza *et al.*, 2011a, b). In this sense, the search for new antimicrobial substances from other sources (including plants) for incorporation into dental products has intensified.

It is now widely accepted that a myriad of bacteria, and not a single micro-organism, underlie dental caries and periodontal diseases. On the tooth surface, for example, the main early or primary colonizers are streptococci and actinomyces, which underlie dental caries. CTE exhibited antibacterial activity at concentrations ranging from 200 to 400 µg ml⁻¹ against some of these colonizers. In turn, the onset of periodontal tissue inflammation is triggered by colonization of the subgingival region by periodontal bacteria. Over time, the proportions of Gram-positive facultative anaerobic bacteria decrease, and Gram-negative anaerobes

eventually become more established, especially at the interface between the teeth and gums (Jenkinson & Lamont, 2006; How *et al.*, 2016). *F. nucleatum*, which plays a crucial role as bridging micro-organism between early and late colonizers, and *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia*, which are late colonizers, represent a portion of the climax community in the biofilms at sites that exhibit the disease (Kolenbrander *et al.*, 2002; Holt & Ebersole, 2005; Park *et al.*, 2014). Alone, none of these microbial species cause the destructive events involved in periodontal disease progression. This is because the aetiology requires concerted interaction among these members so that they can establish their niches in the oral cavity (Marcotte & Lavoie, 1998; Maiden *et al.*, 2003; Paster *et al.*, 2006; How *et al.*, 2016). CTE exhibited antibacterial activity against some of these colonizers, e.g. *F. nucleatum* (ATCC 25586 and clinical isolate), *A. naeslundii* (clinical isolate), *Porphyromonas gingivalis* (ATCC 33277) and *Peptostreptococcus micros* (clinical isolate), with MIC and MBC ranging from 100 to 400 µg ml⁻¹.

CTE displayed different antimicrobial activities against the *Porphyromonas gingivalis* strains (ATCC 33277 and clinical isolate). Experiments in laboratory use pure cultures with well-known characteristics, which greatly contrasts with real-world environments. *In vivo*, bacteria have developed elaborate strategies to cope with particular habitats, which has resulted in significant genetic diversity within one species. In the course of sequential *in vitro* passage, laboratory reference strains might have significantly differentiated features as compared to non-passaged clinical samples. Therefore, any research conducted based on current laboratory strains and their genome sequence could miss important pathophysiological mechanisms that are only present in clinical strains (Fux *et al.*, 2005). Another issue is that wild

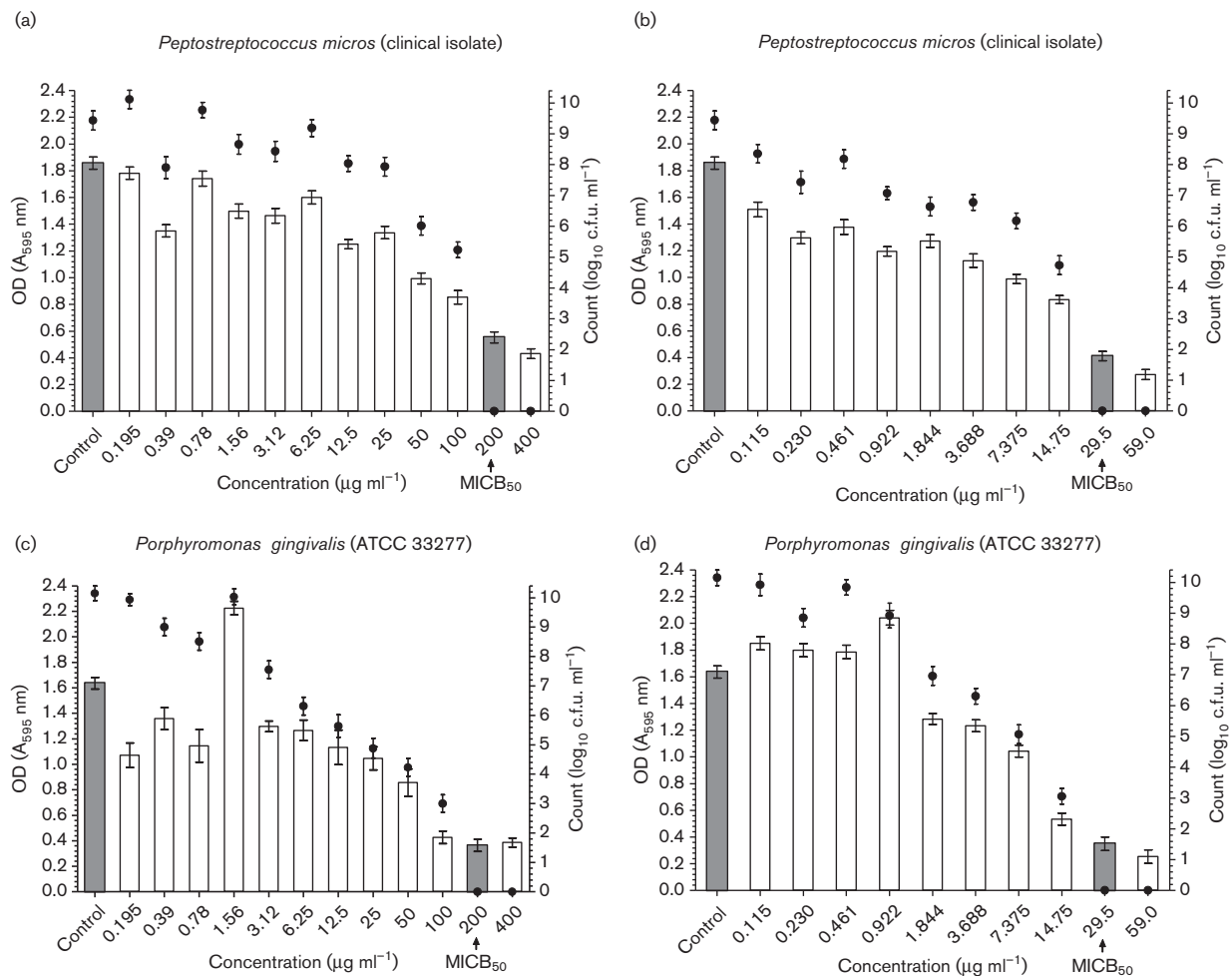


Fig. 3. Antibiofilm activity of CTE against *Peptostreptococcus micros* (clinical isolate) (a) and *Porphyromonas gingivalis* (ATCC 33277) (c) and CHD (b and d) by analysis of the OD and counting of the number of micro-organisms ($\log_{10}$ c.f.u. ml^{-1}). Highlight: MIC₅₀.

strains (clinical isolates) are generally more resistant to antimicrobials because they are more likely to have contact with different compounds (selective pressure). In contrast, standard strains do not undergo this type of selective pressure and thus experience fewer genetic alterations, which confer them with higher sensitivity to antimicrobials. Here, the *Porphyromonas gingivalis* (ATCC) standard strain confirmed these assumptions: they were more sensitive to CTE than the clinical isolate.

In the present study, we did not detect CTE antibacterial activity against *Streptococcus sobrinus* (ATCC 33478 and clinical isolate), *Streptococcus mitis* (ATCC 49456 and clinical isolate), *Streptococcus mutans* (ATCC 25175 and clinical isolate), *Streptococcus salivarius* (ATCC 33478), *Streptococcus sanguinis* (ATCC 10556), *L. casei* (ATCC 11578 and clinical isolate), *Porphyromonas gingivalis* (clinical isolate), *Prevotella intermedia* (clinical isolate), *A. naeslundii* (ATCC 19039), *A. viscosus* (clinical isolate), *Prevotella nigrescens* (ATCC 33563), *Bacteroides fragilis* (ATCC 25285) or

Prevotella buccae (clinical isolate), which could be due to interactions between phenolic compounds present in CTE and these micro-organisms. Only the isolation of these compounds and new antibacterial assays could clarify these results and the mechanisms of action of the compounds.

As for the results obtained for CHD against the cariogenic bacteria evaluated herein, this gold standard appears to be the most effective antimicrobial agent as judged from its bactericidal and bacteriostatic effects and its long-lasting action within the oral cavity (8 h after rinsing). However, the various adverse effects of CHD limit the long-term use of this antiseptic agent including taste alteration, excess formation of supragingival calculus, soft-tissue lesions in young patients, allergic responses and staining of teeth and soft tissues (Najafi *et al.*, 2012). These problems, therefore, denote that finding new effective and safe antibacterial compounds is essential. CTE constitutes a large and promising source of bioactive compounds that may be used to develop new oral products. In the future, the CTE could be applied

Table 2. Revertants/plate, SD and MI (in parentheses) for strains TA98, TA100, TA102 and TA97a of *Salmonella typhimurium* after treatment with various doses of the CTE, with (+S9) and without (–S9) metabolic activation

Treatment (mg plate ⁻¹)	No. of revertants (M±SD) per plate and MI					
	TA98		TA100		TA102	
	–S9	+S9	–S9	+S9	–S9	+S9
0.0*	16±5	18±1	79±9	113±32	340±43	361±12
1.56	21±4 (1.3)	18±2 (1.0)	101±8 (1.3)	95±4 (0.8)	336±36 (1.0)	388±21 (1.1)
3.12	18±5 (1.1)	18±1 (1.0)	90±8 (1.1)	97±15 (0.9)	334±34 (1.0)	384±83 (1.1)
6.25	16±3 (1.0)	16±3 (0.9)	100±10 (1.3)	99±22 (0.9)	339±33 (1.0)	399±27 (1.1)
9.37	16±5 (1.0)	16±2 (0.9)	96±18 (1.2)	116±17 (1.0)	295±35 (0.9)	383±12 (1.1)
12.50	15±4 (1.0)	15±3 (0.8)	99±17 (1.2)	110±12 (1.0)	278±33 (0.8)	286±32 (0.8)
C+	847±17†	1322±63	1333±36‡	1488±77	1265±191§	1151±239¶
					887±49†	1352±89

C+, positive control.

*Negative control: DMSO (100 µl plate⁻¹).†4-Nitro-o-phenylenediamine (10.0 µg plate⁻¹ – TA98, TA97a).‡Sodium azide (1.25 µg plate⁻¹ – TA100).§Mitomycin (0.5 µg plate⁻¹ – TA102), in the absence of S9 and||2-Anthrane (1.25 µg plate⁻¹ – TA97a, TA98, TA100), in the presence of S9.¶2-Amino-fluorene (10.0 µg plate⁻¹ – TA102), in the presence of S9.

to the oral cavity in isolation or associated with CHD to fight against potentially pathogenic micro-organisms.

Santos *et al.* (2008) used the microdilution technique to test the antimicrobial activity of copaiba oils against Gram-positive bacteria, namely *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (ATCC 6623), *Staphylococcus epidermidis* (ATCC 12228), methicillin-resistant *Staphylococcus aureus* ATCC 33591 and *E. faecalis* (ATCC 29212). The oils of *C. martii*, *C. officinalis*, *C. reticulata*, *C. cearensis*, *C. paupera*, *C. langsdorffii*, *C. multijuga* and *C. lucens* exhibited antibacterial activity against Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus*, with MIC ranging from 62.5 to 1000 µg ml⁻¹. It is noteworthy that most studies on *Copaifera* spp. concern the trunk resin oil; few studies have dealt with their aerial parts. The hydroalcoholic extract from *C. langsdorffii* leaves has potential application in the treatment of urolithiasis (Brancalion *et al.*, 2012), exerts a protective effect against doxorubicin-induced chromosome damage (Alves *et al.*, 2013b), potentially prevents kidney stone formation (de Oliveira *et al.*, 2013) and displays chemopreventative action against colon cancer (Senedese *et al.*, 2013). These data denote the importance of investigating other *Copaifera* extracts, including CTE.

By means of time-kill assays, Santos *et al.* (2008) examined the action of *C. martii* resin oil against *Staphylococcus aureus*. Time-kill curves evidenced that no viable cells remained after 3 h of treatment with copaiba oil at twice the MIC (125 µg ml⁻¹) value. The time-kill assays conducted for CTE against anaerobic micro-organisms revealed that *Porphyromonas gingivalis* (ATCC 33277) and *Peptostreptococcus micros* (clinical isolate) were the most susceptible to CTE (Table 1). At 100 µg ml⁻¹, CTE exerted a bactericidal effect against *Porphyromonas gingivalis* within 72 h. As for *Peptostreptococcus micros*, CTE at 100 µg ml⁻¹ reduced the number of micro-organisms by over 3 log₁₀ at 30 min and exhibited bactericidal activity at 72 h incubation. According to Petersen *et al.* (2004), bactericidal activity corresponds to a reduction of 99.9 % (≥3 log₁₀) in the total number of c.f.u. ml⁻¹ in the original inoculum, whereas bacteriostatic activity refers to maintenance of the original inoculum concentration or reduction of less than 99.9 % (<3 log₁₀) in the total number of c.f.u. ml⁻¹ in the original inoculum.

Dental biofilm formation is the most important factor in the aetiology of caries and apical periodontitis. These biofilms are more resistant to antimicrobial agents as compared with planktonic cells (Stewart & Costerton, 2001). Antibiofilm substances can inhibit biofilm formation (preventative effect) or act on biofilms that have already emerged (therapeutic effect) (Alves *et al.*, 2013a). OD analysis and counting of the number of micro-organisms (log₁₀ c.f.u. ml⁻¹) provided reliable results for evaluation of the antibiofilm activity of CTE and CHD. At 200 µg ml⁻¹, CTE inhibited *Porphyromonas gingivalis* (ATCC 33277) and *Peptostreptococcus micros* (clinical isolate) biofilm formation by at least 50 %. Bearing in mind that biofilms tend to be

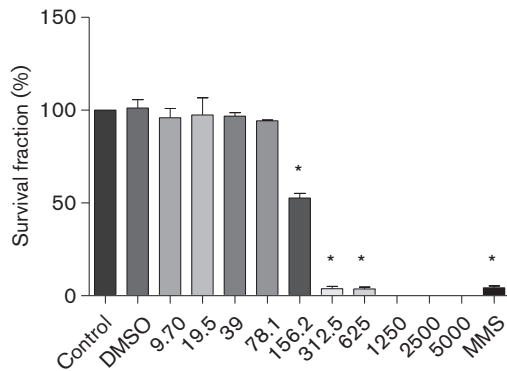


Fig. 4. Percentage of survival fraction in V79 cells treated with different concentrations ($\mu\text{g ml}^{-1}$) of CTE and their respective controls. MMS ($110 \mu\text{g ml}^{-1}$) and DMSO ($0.5 \mu\text{g ml}^{-1}$). *Significantly different from the negative control group ($P < 0.05$).

10–1000 times more resistant to antimicrobial agents as compared with the planktonic state (Ramage *et al.*, 2001), the assay conducted in the present study, which used biofilms consisting of a single species, may have had its limitations. According to Park *et al.* (2014), multi-species biofilms mimic the condition of the oral cavity more closely as compared to single-species biofilms. Therefore, multi-species biofilms offer many advantages. First, they minimize the possibility of contamination and increase reproducibility. Second, they form a thicker biofilm and more complex networks than single-species biofilms. Third, they are more resistant to antibiotics than single-species biofilms. However, even though our assay had limitations because it only evaluated single-species biofilms, we understand that eliminating micro-organisms present as planktonic cells is necessary and important to avoid biofilm formation. In this sense, the results reported herein are extremely relevant: they demonstrate the potential use of *Copaifera* spp. as antibiofilm agents incorporated into dental products (Cai &

Table 3. MN frequency, NDI and CI for V79 cell cultures treated with the CTE

Treatment ($\mu\text{g ml}^{-1}$)	MN frequency*	NDI†	CI (%)
Control	8.67±1.53	1.78±0.04	–
DMSO	12.00±1.00	1.75±0.09	3.84
40	6.67±1.15	1.73±0.01	6.41
80	13.67±1.15	1.72±0.02	7.69
120	18.67±3.06‡	1.61±0.11	21.79
160	28.33±4.04‡	1.57±0.03	26.92
MMS	40.00±6.56‡	1.70±0.06	10.25

DMSO, $0.5 \mu\text{g ml}^{-1}$; MMS, $44 \mu\text{g ml}^{-1}$.

Values are expressed as mean±SD.

*A total of 3000 binucleated cells were analysed per treatment group.

†A total of 1500 cells were analysed per treatment group.

‡Significantly different from the negative control group ($P < 0.05$).

Table 4. MNPCE ratio and PCE/PCE+NCE ratio in Swiss mice bone marrow collected 24 h after treatment with different doses of the CTE and their respective controls

Treatment ($\mu\text{g ml}^{-1}$)	MNPCE frequency*	PCE/PCE+NCE†
Control	3.33±2.07	0.69±0.07
DMSO	2.47±1.47	0.63±0.04
125	2.50±0.84	0.67±0.04
250	2.33±1.75	0.66±0.05
500	2.83±1.72	0.68±0.04
1000	5.33±1.03	0.65±0.08
2000	2.83±0.85	0.61±0.05
MMS	23.33±3.14‡	0.62±0.05

DMSO, $0.5 \mu\text{g ml}^{-1}$; MMS, 40 mg kg^{-1} of BW.

Values are expressed as mean±SD.

*A total of 2000 PCEs analysed per animal.

†A total of 400 erythrocytes analysed per animal.

‡Significantly different from the control group ($P < 0.05$).

Wu, 1996; Ambrosio *et al.*, 2008) as a means to complement mechanical biofilm removal from the oral cavity and to reduce the incidence of oral diseases in humans (Allaker & Douglas, 2008; More *et al.*, 2008).

Because of lack of papers on other *Copaifera* spp., we had difficulty comparing the results from the assessment of the synergistic antimicrobial activity of CTE and CHD with data from other works. However, in a study with (–)-copalic acid, a diterpene that serves as biomarker of the genus *Copaifera* (Leandro *et al.*, 2012), Souza *et al.* (2011a) determined the synergistic antimicrobial activity of (–)-copalic acid isolated from *C. langsdorffii*. Together, CHD and (–)-copalic acid had indifferent effect on *Streptococcus mutans*, a bacterium that underlies the start of the caries process; the ΣFIC index was 2.02. Here, CTE did not act synergistically with CHD. The ΣFIC indices of 2.24 and 11.98 obtained for *Porphyromonas gingivalis* (ATCC 33277) and *Peptostreptococcus micros* (clinical isolate) characterized an indifferent effect and an antagonistic effect, respectively. To our knowledge, this is the first work that has investigated the synergistic antimicrobial action of *C. trapezifolia*.

According to Palombo (2011), plant extracts, essential oils and purified phytochemicals can be potentially applied in preventative therapy or treatment of oral diseases. Nevertheless, assessment of the safety and efficacy of these agents is crucial to establish whether they offer therapeutic benefits. Therefore, another aim of this study was to evaluate the cytotoxic and mutagenic potential of CTE.

The colony-forming assay showed that CTE was cytotoxic at concentrations equal to or higher than $156.2 \mu\text{g ml}^{-1}$. A study by Masson-Meyers *et al.* (2013) provided data on the cytotoxicity of the *C. langsdorffii* resin oil. Increasing copaiba resin oil concentrations decreased the viability of fibroblasts. However, even at the highest tested resin oil

concentrations (500 $\mu\text{g ml}^{-1}$ and 1000 $\mu\text{g ml}^{-1}$), 70 % of the cells remained viable as compared with the control, which attested that copaiba resin oil has low toxicity. Herein, CTE was cytotoxic at concentrations equal to or higher than 156.2 $\mu\text{g ml}^{-1}$. This may be attributed to the fact that the chemical composition of *Copaifera* leaves differs from the chemical composition of the trunk resin oil, which are rich in phenolic compounds and terpenoids, respectively (Alves *et al.*, 2013a).

The *Salmonella* mutagenic assay revealed no gene mutations in the presence of CTE, regardless of metabolic activation. The fact that CTE had no mutagenic effects on *Salmonella typhimurium* bacterial strains during the Ames test is a positive step towards ensuring its safe use in medicine. Considering the popular use of *Copaifera* plants, the lack of mutagenic effects in bacteria is highly relevant. The *Salmonella* mutagenic assay is used worldwide for initial screening of the mutagenic potential of new chemicals and drugs (Adzu *et al.*, 2015; Uysal *et al.*, 2016). In this assay, a mutagenic response has high predictive value for rodent carcinogenicity. Over the years, the scientific community as well as government agencies and corporations (McCann *et al.*, 1975; Sugimura *et al.*, 1976; Zeiger, 1985; Zeiger *et al.*, 1990) have recognized the value of this assay, which constitutes an excellent tool to assess the safety of extracts, oils and phytochemicals isolated from *Copaifera* spp.

MN tests on V79 cells evidenced a mutagenic effect for CTE at the two highest tested concentrations. However, *in vivo* MN tests did not show any mutagenicity at the tested CTE concentrations. *Copaifera* leaves are rich in phenolic compounds (Alves *et al.*, 2013b). According to Ferguson (2001), even phenolic compounds that test negative in bacterial assays may be clastogenic in the case of mammalian cells. Indeed, CTE displayed no mutagenicity in the *Salmonella* mutagenic assay, but it was mutagenic in the MN test conducted on V79 cells. This mutagenicity may be at least partly due to the presence of phenolic compounds in *Copaifera* leaves.

Phenolic compounds occur naturally in a range of plants, many of which have antioxidant, antimutagenic, antiestrogenic, anticarcinogenic and anti-inflammatory properties that might prevent disease and protect genome stability. Unfortunately, not all these compounds, and not all the actions of individual phenolic compounds, are necessarily beneficial. In fact, some of these compounds have mutagenic and/or pro-oxidant effects (Ferguson, 2001).

Additionally, CTE was not mutagenic as revealed by the *in vivo* MN test. Alves *et al.* (2013b) did not find any mutagenic effect for the hydroalcoholic extract of *C. langsdorffii* leaves in the *in vivo* MN test, either. CTE metabolism may underlie the absence of CTE mutagenicity during *in vivo* assays. Several studies have shown that phenolic compounds are extensively metabolized *in vivo*, mainly in the liver. These compounds also undergo metabolism by colonic microflora during transfer across the small intestine. These metabolic processes result in significant

alteration in redox potentials (Day & Williamson, 2003; Donovan & Waterhouse, 2003). After undergoing phase I deglycosylation, phenolic aglycones are converted to glucuronides, sulfates and *o*-methylated derivatives during phase II metabolism (Schroeter *et al.*, 2002).

Because very few natural products are known to inhibit the growth of oral pathogens (Saleem *et al.*, 2010) and studies on the safety of these extracts are scarce (Palombo, 2011), this is an important report on the antibacterial action of the CTE against causative agents of dental caries and apical periodontitis.

In conclusion, our study has demonstrated that CTE exhibits antibacterial activity against some of the tested microorganisms under the experimental conditions used. Although CTE at higher concentrations displayed *in vitro* cytotoxicity and mutagenicity, it was not mutagenic during the *Salmonella* mutagenic assay and the MN test *in vivo*. Hence, this plant species can be an important source of biologically active compounds and aid in the search for new effective and safe agents that act against oral pathogens.

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