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Original Article

Antibacterial activity of (−)-cubebin isolated from *Piper cubeba* and its semisynthetic derivatives against microorganisms that cause endodontic infections

Karen C.S. Rezende[a], Rodrigo Lucarini[a], Guilherme V. Símaro[a], Patricia M. Pauletti[a], Ana H. Januário[a], Viviane R. Esperandim[a], Carlos H.G. Martins[a], Mislaine A. Silva[a], Wilson R. Cunha[a], Jairo K. Bastos[b], Márcio L.A.E. Silva[b,*]

[a] *Núcleo de Ciências Exatas e Tecnológicas, Universidade de Franca, Franca, SP, Brazil*
[b] *Department of Pharmaceutical Sciences, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brazil*

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ABSTRACT

Recent publications have highlighted the numerous biological activities attributed to the lignan (−)-cubebin (**1**), *Piper cubeba* L. f., Piperaceae, and ongoing studies have focused on its structural optimization, in order to obtain derivatives with greater pharmacological potential. The aim of this study was the obtainment of (**1**), its semisynthetic derivatives and evaluation of antibacterial activity. The extract of the seeds of *P. cubeba* was chromatographed, subjected to recrystallization and was analyzed by HPLC and spectrometric techniques. It was used for the synthesis of: (−)-*O*-methylcubebin (**2**), (−)-*O*-benzylcubebin (**3**), (−)-*O*-acetylcubebin (**4**), (−)-*O*-(*N*,*N*-dimethylamino-ethyl)-cubebin (**5**), (−)-hinokinin (**6**) and (−)-6.6′-dinitrohinokinin (**7**). The evaluation of the antibacterial activity has been done by broth microdilution technique for determination of the minimum inhibitory concentration and the minimum bactericidal concentration against *Porphyromonas gingivalis*, *Prevotella nigrescens*, *Actinomyces naeslundii*, *Bacteroides fragilis* and *Fusobacterium nucleatum*. It was possible to make an analysis regarding the relationship between structure and antimicrobial activity of derivatives against microorganisms that cause endodontic infections. The most promising were minimum inhibitory concentration =50 μg/ml against *P. gingivalis* by (**2**) and (**3**), and minimum inhibitory concentration =100 μg/ml against *B. fragilis* by (**6**). Cytotoxicity assays demonstrated that (**1**) and its derivatives do not display toxicity.

Introduction

In the last decade, there has been intensification in the search of anti-bactericidal compounds from natural sources, mainly from plants, which continue to be a major source of biologically active metabolites that may provide lead structures for the development of new drugs (Ayres and Loike, 1990).

The biological properties of lignans are closely related to the stereochemistry of the various stereogenic centers present in their chemical structure, which makes these compounds interesting synthetic targets (Silva et al., 2009). In this context, many biological activities have been reported in the literature (Saleem et al., 2005), such as antitumor (Ayres and Loike, 1990; Li et al., 2006), antiviral (Rimando et al., 1994), anti-PAF (Shen et al., 1985; Braquet and Godfroid, 1986), antidepressant (Deyama et al., 2001), anti-inflammatory (Souza et al., 2004; Kassuya et al., 2006) and cardiovascular (Rimando et al., 1994), and antiviral (Charlton, 1998; Piccinelli et al., 2005), trypanocidal (De Souza et al., 2005), analgesic (Da Silva et al., 2005).

The literature reports the accumulation of lignoids and other metabolites that possess significant pharmacological activity in species of the genus *Piper*, Piperaceae family, and especially in *P. cubeba* L. f. (Parmar et al., 1997; Arruda et al., 2005).

An example of these substances is (−)-cubebin (**1**), a dibenzylbutyrolactolic lignan found in high concentrations in seeds of *Piper cubeba* (Elfahmi, 2006). Due to its well-known biological activities, **1** has become the object of study of many research groups over the last decade. These works have culminated in the discovery of new and important biological actions for **1** (De Souza et al., 2005; Da Silva et al., 2005; Silva et al., 2007, 2009; Usia et al., 2005; Hirata et al., 2007a,b, 2008; Saraiva et al., 2007; Yam et al., 2008).

* Corresponding author.
E-mail: mlasilva@unifran.br (M.L.A.E. Silva).

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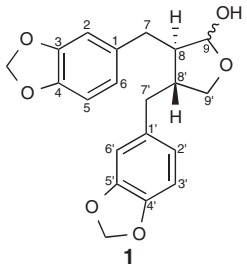

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The establishment of a inflammatory process leads the major forms of endodontic disease, gingivitis and periodontitis. The intensity of the inflammatory response varies with the stage of evolutionary development of infection, as it has in the beginnings of a pathological process a minor reaction that tends to increase in proportion to the spread of microbial invasion, and may lead total necrosis of the pulp tissue providing an opportunity for the development of severe apical periodontitis (Luisi and Fachin, 1990).

The oral micro-organisms are found in saliva within a complex matrix composed of microbial extracellular products and salivary compounds on the surfaces of growing enamel, known as biofilms (Marsh, 2005). The pathogenesis of pulpal injury is due to bacterial invasion in this tissue (Bergenholtz and Crawford, 1989).

The harmful effect of microorganisms in the pulp and periapical tissues and its installation has been the subject of ongoing studies to show their presence, to correlate the association between these micro-organisms and oral infections (Kakehashi et al., 1965; Wittgow and Sabiston, 1975; Tani-Ishii et al., 1994; Fine, 2000; Socransky and Haffajee, 2002; De Lillo et al., 2004) and develop methods that lead to its destruction with the development of new antimicrobials.

Ideally, in addition to having antimicrobial activity, the endodontic formulation should not harm the host's tissues (Baumgartner, 1997). In this regard, the use of an orabase formulation containing 0.1% fluocinolone acetonide and the active lignin derivative would enhance the effectiveness against oral infections. However, there is yet no drug or substance capable of bringing together these two requirements, which justifies the research and development of new antimicrobial agents. Additionally, he literature reports the possibility of isolation of lignans from *P. cubeba* and other species such as *Chamaecyparis obtusa* (Marcotullio et al., 2014), *Linum* (Schmidt et al., 2010). However, although these compounds can be isolated from plants, they are obtained in low yields in extractions, also resulting in difficult purification process with several steps. Therefore, partial synthesis from (−)-cubebin is the most viable way, with reactions that allow yields above 98% (Da Silva et al., 2005) and simpler purification steps.

Taking account the various biological properties attributed to lignans, the previous studies obtained by our research group describing satisfactory results concerning these compounds, this work aims to expand knowledge of the relationship between the chemical structures of lignans and their biological activities and thus the search for bioactive molecules.

Materials and methods

Solvents and reagents

Anhydrous sodium sulfate (Merck Co.), dry dichloromethane (Acros Co.), magnesium sulfate (Merck Co.), pyridinium chlorochromate (Acros Co.), hexane (Mallinckrodt Co.), ethyl acetate (Mallinckrodt Co.), ethanol (Merck Co.), acetic anhydride (Merck Co.), dry tetrahydrofuran (THF) (Merck Co.), sodium bicarbonate (Merck Co.), sodium chloride (Merck Co.), dry pyridine (Acros Co.), toluene (Mallinckrodt Co.), chloroform (Merck Co.), hexane (Merck Co.), sodium hydride (NaH) (Acros Co.), methyl iodide (Merck Co.), dimethylethylamine chloride (Merck Co.), metallic sodium bars (Merck Co.), fuming nitric acid (Merck Co.) and ethyl acetate (Mallinckrodt Co.).

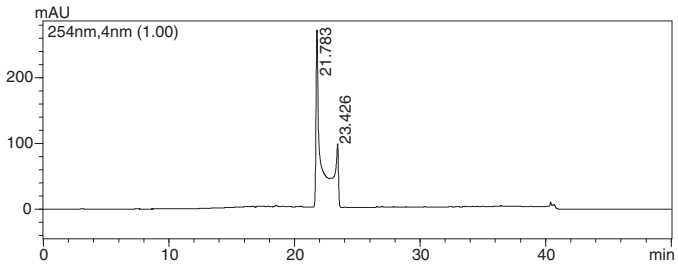


Fig. 1. Chromatogram of the epimers of **1** obtained using a. Shim-pack ODS, 250 × 4.20 mm, 5 μm Column, volume of injection: 20 μl, flow: 1 ml/min, detection at λ 254 nm and linear gradient: MeOH-H_2O (50%)/MeOH (100%), in 48 min.

*Plant material collection and isolation of **1***

The seeds of *Piper cubeba* L. f., Piperaceae, used in this study were imported from Floral Seed Company, Dehradun, India (Proc. FAPESP 05/01550-8). The process of seeds of *P. cubeba* extraction was carried out according to literature (Souza et al., 2004). It was used 1 kg of seeds, which yielded the crude ethanolic extract. The partitions originated a hydroalcoholic extract, 285 g. A chromatographic column chromatography was undertaken and the fractions were collected and combined according to their chromatographic profiles.

HPLC analysis of fraction 10, obtained from the filtration column chromatography, showed the presence of **1**. The fraction was subjected to the crystallization process, solubilizing it with acetone, and adding hexane until turbidity of the medium. After crystallization, the supernatant was removed, thus furnishing crystals of **1**.

The recrystallization process was repeated five consecutive times, which made it possible to obtain 13.9 g **1**. This method allowed excellent purification, which was confirmed by analysis using a binary system chromatograph Shimadzu CBM-20A, -Prominence LC-6AD equipped with a manual injector with 20 ml loop, a "degasser" DGU-20A5 coupled to a UV–vis detector model SPD-20A and data acquisition by a microcomputer and an analytical column of Shimadzu, Shim-pack ODS, 250 mm × 4.20 mm, 5 μm, equipped with pre-column. The solvent system consisted of a mixture of methanol and water in a linear gradient 50–100% in 48 min. The chromatographic conditions defined for compound HPLC analysis were: λ 254 nm, injection volume of 20 μl and flow rate of 1 ml/min.

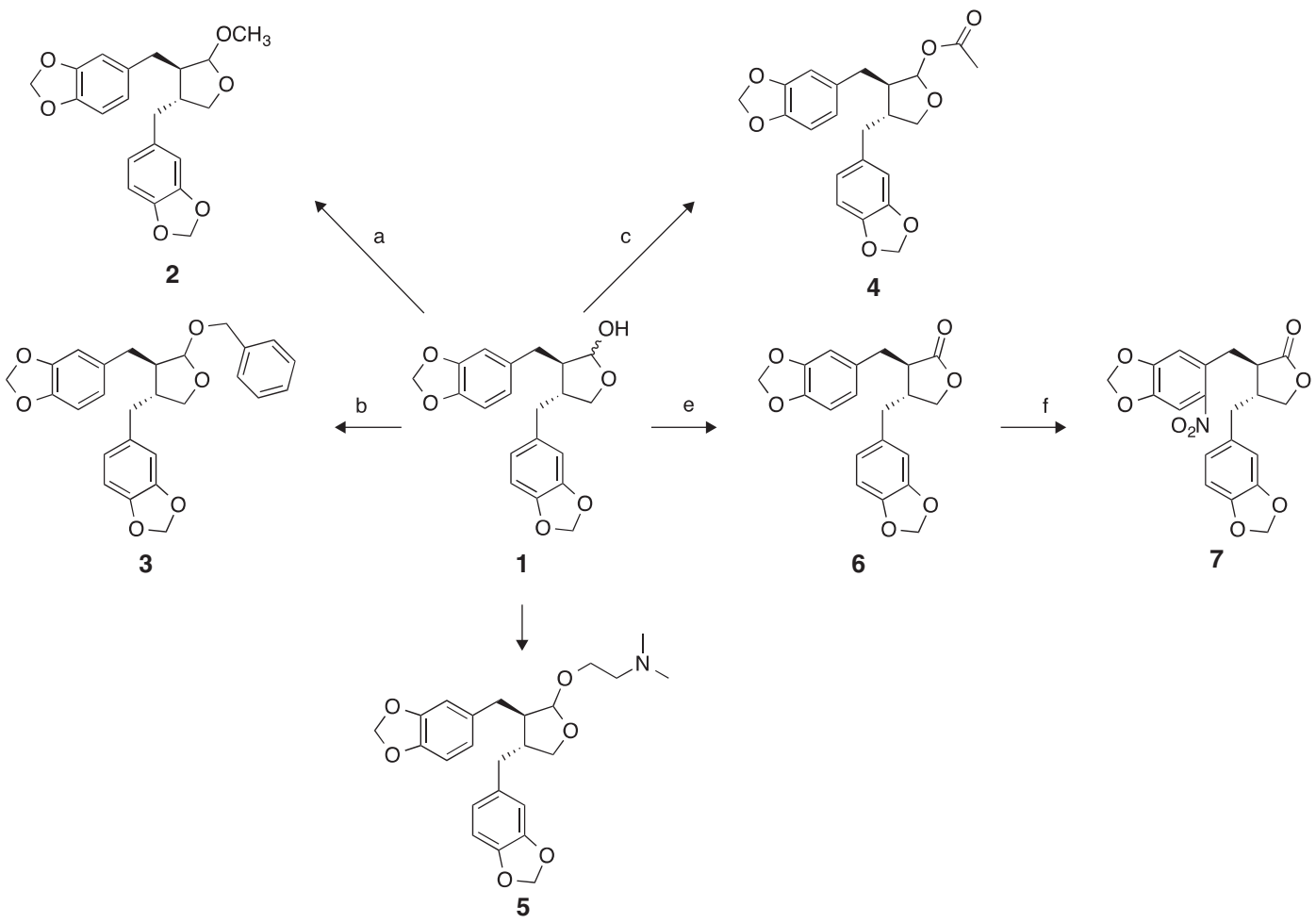
These crystals were analyzed using nuclear magnetic resonance techniques of hydrogen (^{1}H) and carbon (^{13}C) and HPLC (Fig. 1), confirming its identification as (−)-cubebin.

Obtainment of semi-synthetic derivatives

Scheme 1 represents a summary of the reactions for obtainment of the derivatives of **1**.

*Obtaining the derivate **2** (a)*

It was used the method described by Venkateswarlu et al. (1999), in which **1** (0.5 g) in 5 ml of THF was added to a dry suspension of NaH (1 g) in 50 ml of dry THF and the mixture was left for 30 min at room temperature, stirring and atmosphere of N_2. Then, 1 ml of methyl iodide was added and the mixture was left reacting for 24 h under N_2 atmosphere. After this period, excess NaH was decomposed by adding slowly solution methanol/water (1:1,



Scheme 1. Obtaining semisynthetic derivatives of **1**: **2**, where: CH_3I, NaH, dry THF, 30 min, TA atm N_2; **3**, where b: benzyl bromide, NaH, THF, 6 h, TA; **4**, where c: acetic anhydride, dry pyridine, toluene, dichloromethane; **5**, where d: dimethylethylamine chloride, sodium ethoxide, reflux for 8 h, **6** where e: PCC, dry DCM, atm N_2,-60C, 24 h; **7**, where f: HNO_3 6 equiv, $CHCl_3$, 10 °C, 4 h.

v/v). The reaction was neutralized with dilute HCl and subjected to partition with ethyl acetate (3 × 30 ml). The organic fraction was washed successively with 5% aqueous $NaHCO_3$ (3 × 20 ml) and 10% saline (3 × 20 ml), dried with $MgSO_4$ and filtered.

*Obtaining the derivate **3** (b)*

To obtain the benzyl derivative (Souza et al., 2004), a solution of **1** (300 mg) and 10 ml of dry THF was added to a mixture of NaH and dry THF (5 ml), and it was allowed under reflux for 30 min. Benzyl bromide (0.12 ml) was added and left under the same conditions for 2 h. To complete the reaction, water was slowly added to the flask and became extractions with ether.

*Obtaining the derivative **4** (c)*

To obtain the derivative **4** (Souza et al., 2004), 50 mg of **1** and 3 ml of acetic anhydride were added to 0.8 ml dry pyridine. After 5 h of reaction at room temperature, the mixture was placed in a vial containing toluene and evaporated under the reduced pressure to remove pyridine. Subsequently, dichloromethane was added and evaporated under reduced pressure to remove traces of toluene.

*Obtaining the derivate **5** (d)*

To obtain **5** (Souza et al., 2004), 300 mg of **1** was added a 10 ml of sodium ethoxide and left it under reflux for 2 h. Later, it was added 120 mg of dimethylethylamine chloride. The reaction was maintained under the same conditions for 6 h. Water (5 ml) was added and partitions were made with ethyl acetate (3 × 10 ml). The organic phase was washed with 10% NaCl aqueous solution (3 × 10 ml), dried with $MgSO_4$ and filtered. The solvent was evaporated and the residue was purified by column chromatography on silica gel 60 (0.063–0.200 mm, Merck Co.) eluted with dichloromethane.

*Obtaining the derivative **6** (e)*

In a three-burner reaction flask protected from light and maintained at 0 °C, was added to 10 ml of dichloromethane and 50 mg of **1**. Later, under inert atmosphere, was added 2 equivalents of **1** to 1 equivalent of PCC (pyridinium chlorochromate), and the reaction was maintained under stirring for 12 h (Souza et al., 2004; De Souza et al., 2005).

*Obtaining the derivate **7** (f)*

To obtain a nitrated derivative (Da Silva et al., 2005; De Souza et al., 2005), 200 mg of **6** was subjected to reaction with nitric acid (6 EqM) for 2 h under magnetic stirring and 0 °C. Later, it was added a solution of sodium carbonate, and partitions were made with chloroform (5 × 30 ml). The product was subjected to repeated recrystallization from methanol.

Determination of minimum inhibitory concentration (MIC)

To obtain the inoculum, the strains were stored at −20 °C and resuspended in liquid medium broth Schaedler supplemented with hemin (5 mg/ml) and menadione (1 mg/ml). Then all test bacteria were subcultured in Schaedler agar plus defibrinated sheep blood (5%), hemin (5 mg/ml) and menadione (1 mg/ml) were subsequently incubated in temperature of 36 °C in an anaerobic chamber for 72 h in an atmosphere containing 10.5% H_2, 10% CO_2 and 80–85% N_2 to confirm the purity of the strains. After confirmation, the strains were used in the tests for determination of MIC.

The density of the inoculum was prepared to scale of 0.5 Mc Farland second Bier (1981). All bacterial suspensions were adjusted in the same transmittance. Compounds were dissolved in dimethyl sulfoxide (DMSO) using an ultrasonic bath which were used for the broth microdilution technique.

The following "American Type Culture Collection" (ATCC) microorganisms were used in this study: *Porphyromonas gingivalis* (33277), *Prevotella nigrescens* (33563), *Actinomyces naeslundii* (19039), *Bacteroides fragilis* (25285) and *Fusobacterium nucleatum* (25 586).

Method of broth microdilution

The technique was performed according to methodology recommended by the Clinical and Laboratory Standards Institute (CLSI, 2007), with adaptations.

In a sterile plate of 96 holes were deposited a total of 200 μl of the mixture of Schaedler broth, microbial suspension and test substance in order to obtain concentrations from 20 to 400 μg/ml. It was made the culture control, the control of sterility of the broth and compounds. The chlorhexidine digluconate was used as positive control and DMSO as negative control.

The plate was incubated at 36 °C in an anaerobic chamber for 72 h. Later were added to each hole of 30 μl resazurin 0.02% prepared in aqueous solution. The plate was reincubated for 30 min. There was, then, changing the color blue (no bacterial growth) and pink (bacterial growth). This procedure was performed in triplicate.

Determination of minimum bactericidal concentration (MBC)

To determine the MBC we used the quantitative technique based on the method of Lifeng et al. (2004). The series used for MIC determination were performed in duplicate and one was used to evaluate the MBC of the substances obtained. 10 ml of the solution which showed inhibition of bacterial growth in the MIC method were plated in Schaedler agar, and subsequently incubated for 24 h at 37 °C. The media were analyzed to observe the presence or absence of growth of oral bacteria.

Cytotoxicity assay

The LLCMK2 fibroblast cells were grown in RPMI (Roswell Park Memorial Institute) 1640 medium supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 5% of inactivated fetal calf serum, and maintained at 37 °C in 5% CO_2. A cell suspension was seeded at a concentration of 1 × 106 cells/ml in a 96-well microplate containing RPMI 1640 medium. Thereafter, the cells were treated with PC-EO at different concentrations (200, 100, 50, 25, 12.5, 6.25, and 3.125 μg/ml). The plates were incubated at 37 °C for 24 h, and the biological activity was evaluated by using the MTT colorimetric method [MTT; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] that is cleaved by the mitochondrial enzyme resulting formazan crystals detecting living cells in a 24 h period in a microplate reader at 540 nm. RPMI 1640 medium plus DMSO and RPMI 1640 medium were used as positive and negative controls, respectively. All the experiments were performed in triplicate. The percentage of cytotoxicity was determined by the formula: % cytotoxicity = 1 − [(Y − N)/(N − P)] × 100, where Y = absorbance of wells containing cells and PC-EO at different concentrations; N = negative control; P = positive control (Muellas-Serrano et al., 2000).

Results and discussion

*Isolation of **1***

Regarding the method for extraction by percolation with ethanol, it could be concluded that it is an effective procedure, in which a large amount of substances of different degrees of polarity in the extract could be extracted. The partitions made with organic solvents for the separation of less polar oils, for further fractionation by chromatographic techniques allowed a better separation of substances.

In the analysis by HPLC, compound **1** presented two peaks, showing the existence of β and α epimers in different amounts (Fig. 1). The epimers are diastereoisomers generated by hydroxyl linked to the lactol ring. It is a convertible mixture of both epimers, in which it was possible to assign their signals due to a larger percentage of each conformation. The tested sample is a stable mixture of both isomers.

The crystals were analyzed using techniques of ^1H and ^{13}C NMR. $[\alpha]_D^{25^\circ}$ = −8.12 (*c* 0.46, $CHCl_3$). The purity of (−)-cubebin was estimated to be higher than 95% by both HPLC and ^{13}C NMR analyses, as well as its melting point, 131–132 °C, ^1H NMR [δ, multiplicity (*J* in Hz), 400 MHz, $CDCl_3$]: ArH (6.8–6.4; m; 6 H); H7 (2.8–2.4; m; 4H); H7′ (2.8–2.4; m; 4H); H8 (2.3–1.9; m; 1H); H8′ (2.8–2.4; m; 1H); H9 (5.2; s; 1H); H9′ [4.05 (dd; 6 Hz; 1H); 3.72 (dd; 8 Hz; 1H)]; —O—CH_2—O (5.9; s; 4H). ^{13}C NMR [δ, multiplicity (*J* in Hz), 100 MHz, $CDCl_3$]: C1 (132.3); C2 (108.3); C3 (147.5); C4 (145.9); C5 (109.5); C6 (121.9); C7 (39.5); C8 (53.1); C9 (103.5); C1′ (133.9); C2′ (109.3); C3′ (147.5); C4′ (145.9); C5′ (109); C6′ (121.6); C7′ (38.7); C8′ (45.1); C9′ (72.7); —O—CH_2—O— (101.1).

Obtaining the semi-synthetic derivatives

The purities of the obtained (−)-cubebin derivative compounds were estimated to be higher than 95% by both HPLC and ^{13}C NMR analyses.

*Obtaining the derivative **2***

The compound was successfully obtained using methods described by Venkateswarlu et al. (1999), with a yield of approximately 95% (Fig. 1S).

The reaction occurs via a bimolecular nucleophilic substitution reaction, which there is the formation of the alkoxide ion, negatively charged, in the treatment of **1** with a strongly reactive metal. This intermediate reaction is usually used as a catalyst, so that the center is located in the nucleophilic carbon atom partially negative charge. In general, it is a reaction in which the nucleophile approaches the substrate from the side opposite the halide ion, directly attacking the carbon and at the same time, shifting the leaving group loosing it the molecule. The nucleophile has a pair of electrons that will be used to make the new bond to carbon. The reaction is a process in a single step, without intermediaries, that forms when the carbon-nucleophile – giving rise to the expected compound, **2**, breaks the carbon-halogen.

Of course, "leaving group" out with the pair of electrons from its former connection to the carbon (partially negative charge). A natural consequence of this, but that may not be so obvious is that it

is also a nucleophile, because it also has a pair of electrons to make a substitution reaction.

The compound obtained was subjected to spectroscopic analysis of ^{1}H NMR, ^{13}C and determination of $[\alpha]_D^{25^\circ}$ [−120.4° (*c* 0.0057, $CHCl_3$)]. ^{1}H [multiplicity (*J* in Hz)]: ARH (6.5 m), H7 and H15δNMR at 400 MHz in $CDCl_3$ [(5.8 m)], H2 in a greater proportion of the epimer (*J* = 1.3 Hz) (4.6 d), H2 epimer was lower (*J* = 4.6 Hz) (4.55 d). The remaining signals are indicative of epimers: 3.9 (m, H5a of two epimers), 3.5 (m, H5b of two epimers), 3.2 and 3.1 (two singlets of protons of 16 two epimers), and between 2.7 and 1.9 (m, H3, H8, H6 and H4 of the two epimers).

*Obtaining the derivative **3***

The benzyl derivative (73.78%) was obtained from the reaction between **1** and benzyl bromide. By thin layer chromatography was possible to observe the displacement of the product with R_f than **1**, indicating the possible presence of polarity lower than that of **1** (Fig. 2S).

The reaction occurs via the Williamson synthesis, which consists of an alcoholates by reacting an alcohol with a reactive metal, and subsequent treatment of the alkoxide with an aryl halide. It produces a nucleophilic substitution reaction with formation of ether.

It is also a bimolecular nucleophilic substitution, in which the nucleophile approaches the substrate from the opposite side to the halide ion. The affinity for opposite charges allows the direct attack on the carbon and the simultaneous displacement of the leaving group.

The reaction is a process in a single step, without intermediaries, that forms when the carbon-nucleophile – giving rise to the expected compound, **3**, breaks the carbon-halogen.

This was submitted to spectroscopic analysis of ^{1}H NMR, 13C and determination of $[\alpha]_D^{25^\circ}$ = −2.09 (*c* 0.008, $CHCl_3$). ^{1}H NMR [*δ*, multiplicity (*J* in Hz), 400 MHz, $CDCl_3$]: ArH (6.8–6.4; m; 6H); H7 [2.60–2.10 (m; 4H)]; H7′ [2.60–2.10 (m; 4H)]; H8 [2.15–1.80; (m; 1H)]; H8′ [2.60–2.70 (m; 1H)]; H9 [4.80 (s; 1H)]; H9′ [3.95 (dd; 12 and 6 Hz; 1H) and 3.70 (dd; 12 and 8 Hz; 1H)]; —O—CH_2—O— [5.90 (s; 4H)]; CH_2 [4.70 (d; 1H) and 4.85 (d; 1H)] and Ar [6.40–6.8 (s; 5H) and 7.0 (s)]. ^{13}C NMR [*δ*, multiplicity (*J* in Hz), 100 MHz, $CDCl_3$]: C1 (133.9); C2 (108.2); C3 (142.2); C4 (146.3); C5 (109.3); C6 (121.8); C7 (38.9); C8 (52.5); C9 (103.7); C1′ (134.5); C2′ (108.3); C3′ (148.2); C4′ (146.3); C5′ (109.3); C6′ (121.7); C7′ (39.4); C8′ (46.2); C9′ (72.3); —O—CH_2—O— (100.8); Ar-CH_2 (69.0); Ar (109.7–108.2).

*Obtaining the derivative **4***

The product appeared as a yellowish oil, which can be explained by the presence of two forms (α and β) of lignan modified, and that the product was mixed with **1**. The mass derived from pointed end of the reaction yield (85%) (Fig. 3S).

The acetylation reaction was not developed in a conventional manner as it is for the synthesis of acetylsalicylic acid and other estherification reactions, because they use acid catalysis which leads to disruption of the ring or suffer dehydration cetalic non-characterizing the molecule of the original **1**.

Thus, the acetylation of **1** was performed using pyridine as a catalyst for its ability to mediate redox reactions, acting as "electron transport", which leads to the formation of the nucleophile. The nucleophilic substitution occurs, then, with the attack of the nucleophile to the carbon partially positive charge of acetic anhydride. At the same time, this carbon atom attacks the oxygen atom which has partial negative charge. These reactions occur in one step, without formation of intermediates, giving rise to **4** and acetate ion. This was subjected to NMR spectroscopic analysis of ^{1}H, ^{13}C, and determination of $[\alpha]_D^{25^\circ}$.

$[\alpha]_D^{25^\circ}$ = −123.33 (*c* 0.0057, $CHCl_3$); ^{1}H NMR [δ, multiplicity (*J* in Hz), 400 MHz, $CDCl_3$]: Ar-H (6.65–6.40; m, 6H); C-8 (2.30–1.95; m, 1H); C-9′ (4.04 (dd; 1H) and 4.00 (dd; 1H)); —O—CH_2—O— (5.90; s, 4H); —CH_3 (1.99, s, 3H). ^{13}C NMR [*δ*, multiplicity (*J* in Hz), 100 MHz, $CDCl_3$]: C-1 (132.21); C-2 (107.63); C-3 (147.42); C-4 (145.72); C-5 (108.43); C-6 (121.51); C-7 (38.71); C-8 (51.10); C-9 (102.89); C-1′ (133.29); C-2′ (109.01); C-3′ (147.42); C-4′ (145.92); C-5′ (108.43); C-6′ (120.88); C-7′ (37.40); C-8′ (44.23); C-9′ (73.32); —O—CH_2—O— (100.49); —O—CO— (169.91); —CH_3 (20.89).

*Obtaining the derivative **5***

The synthesis was carried out without causing damage to the lactolic ring of **1** (Fig. 4S). It was obtained in 93% yield.

The technique of Williamson was also utilized, as it is an important process for the preparation of ethers, symmetrical and asymmetric ones. The sodium alkoxide was prepared by direct action of ethoxide, which can make nucleophilic substitutions easily, on alcohol. Replaced alkyl halide reacted with the previously formed. It consists of the nucleophilic substitution of halide ion by the alkoxide ion, with the simultaneous attack on carbon partially loaded, and of the chloride.

This was subjected to NMR spectroscopic analysis of ^{1}H, ^{13}C, and determination of $[\alpha]_D^{25^\circ}$.

$[\alpha]_D^{25^\circ}$ = −4.38° (*c* 0.067, $CHCl_3$); ^{1}H-RMN δ ($CDCl_3$): Ar-H [6.70–6.40 (m; 6H)]; H7 [2.80–2.30 (m; 4H)]; H7′ [2.80–2.30 (m; 4H)]; H8 [2.10–1.90 (m; 1H)]; H8′ [2.80–2.40 (m; 1H)]; H9 [4.75 (s; 1H)]; H9′ [4.05 (m; 1H) and 3.80 (m; 1H)]; —O—CH_2—O— [5.90 (s; 4H)]; O—CH_2— [3.66–3.52 (m; 2H)]; CH_2—N— [1.12 (t; 7.07 Hz; 1H) and 1.09 (t; 7.07 Hz; 1H)]; —N—$(CH_3)_2$ [1.09 (s; 6H)]. ^{13}C-RMN δ ($CDCl_3$): C1 (134.5); C2 (108.3); C3 (147.8); C4 (146.2); C5 (109.6); C6 (122.0); C7 (39.5); C8 (52.5); C9 (104.5); C1′ (133.8); C2′ (108.9); C3′ (147.2); C4′ (145.9); C5′ (109.3); C6′ (121.8); C7′ (39.0); C8′ (46.2); C9′ (72.3); —O—CH_2—O— (101.1); —O—CH_2— (63.2); —CH_2—N— (43.7); —N—$(CH_3)_2$ (46.2).

*Obtaining the derivative **6***

The product was purified by providing 98% yield, and its purity was determined by HPLC (Fig. 5S). In an acidic environment, perhaps the **1** suffer lactolic hydroxyl dehydration and elimination. The output of the —OH group leads to formation of a double bond in the furan ring. This effect of acid in order to remove the hydroxyl lactolic of **1** is more difficult to occur. In another possible behavioral mechanism of **1** in an acid medium, protonation of the furan oxygen and subsequent opening of lactolic ring with the formation of an aldehyde and an alcohol.

Thus, the oxidation requires a detailed analysis. Possibly the reaction with PCC led to the formation of an intermediate inorganic ester of an alcohol. The residue contains a metal that can be easily reduced, and in the process the alcohol is oxidized.

In the ^{1}H NMR spectrum showed a marked difference in the structure of **6** with respect to the structure of **1**, which consists mainly in the absence of hydrogen H-9 and may be noted the absence of signal in 5.20 ppm (s, 1H), indicating an oxidation at this position.

$[\alpha]_D^{25^\circ}$ = −30 (*c* 0.99, $CHCl_3$); ^{1}H-RMN δ ($CDCl_3$): Ar-H [6.80–6.40 (m; 6H)]; H7 [2.65–2.40 (m; 4H)]; H7′ [2.65–2.40 (m; 4H)]; C8 [2.10–2.95 (m; 1H)]; H8′ [2.85 (m; 1H)]; H9′ [3.85 (dd; 7.4 Hz; 1H) and 4.12 (dd; 7.0 Hz; 1H)] and —O—CH_2—O— [5.90 (s; 4H)]. ^{13}C-RMN *δ* ($CDCl_3$): C1 (131.6); C2 (108.4); C3 (147.9); C4 (146.3); C5 (108.3); C6 (121.6); C7 (34.8); C8 (46.5); C9 (178.4); C1′ (131.3); C2′ (108.8); C3′ (147.9); C4′ (146.5); C5′ (108.3); C6′ (122.2); C7′ (38.4); C8′ (41.3); C9′ (71.2); —O—CH2—O— (101.0).

Obtaining the derivative **7**

The mechanism of nitration may be through a mixture of nitric and sulfuric acids. In reaction to obtain **7** (Fig. 6S), which had 97.6% of yield, it was used fuming nitric acid which already contains nitroil ion dissolved, which is the electrophilic particle that attacks the benzene ring. Solutions of these salts are stable in certain solvents such as nitromethane or acetic acid, perform the nitration of aromatics, gently and with high yield at room temperature.

Needing electrons, this ion find them particularly available in the π cloud of the benzene ring, so it will be fixed to one of the carbon atoms of the ring by a covalent bond, forming a carbocation, often designated as benzenoid ion (Morrison and Boyd, 1996).

This means that the positive charge is not localized in one carbon atom, but distributed throughout the molecule, and is particularly strong on the carbon atoms in ortho and para position in relation to that which binds the NO_2 group, the dispersion the positive charge throughout the molecule, the effect of resonance, the ion becomes more stable than it would have had a positive charge located. Probably this is the same stabilizing effect that allows the formation of carbocation, taking into account the stability of the original benzene molecule.

The reaction is similar to the addition to alkenes: an electrophile species, drawn electrons, is attached to the benzene molecule forming a carbocation π. The inclusion of a basic group in benzenóico ion with formation of an addition product would destroy the character of the aromatic ring. Instead, the basic ion, HSO_4^-, extracts a hydrogen ion and form benzenoid a substitute product that stays in the ring stabilized by resonance. The loss of hydrogen is one of the typical reactions of carbocations (Morrison and Boyd, 1996).

The electrophilic substitution, such as electrophilic addition, takes place in successive steps, involving the formation of a carbocation intermediate. Both reactions, however, differ from one another, the fate of the carbocation. Although the mechanism of nitration is possibly better than elucidated the mechanisms of other aromatic substitution reactions, it seems almost certain that all of them are processed in the same manner (Morrison and Boyd, 1996).

Aromatic substitution reactions are characterized by the addition of NO_2 group to the aromatic ring leading to the desired product. The aromatic compounds as much as can be nitrated aliphatic, forming important products in organic synthesis. The nitration of aromatic ring deactivates it, in a reaction whose products are easily separated and analyzed, which identifies the proportions of ortho, meta and para products formed (Koul et al., 1983).

This was submitted to spectroscopic analysis of ^{1}H NMR and ^{13}C and $[\alpha]_D^{25^\circ}$. $[\alpha]_D^{25^\circ} = -29.07$ (c 0.008, $CHCl_3$). ^{1}H NMR [δ, multiplicity (J in Hz), 400 MHz, $CDCl_3$]: 7.5 (s, 1H), 7.48 (s, 1H), 6.8 (s, 1H); 6.6 (s, 1H); 6.1 (m, 2H); 4.35 (dd, 1H, J = 7.1 and 9.1 Hz); 4.0 (dd, 1H, J = 7.3 and 9.1 Hz); 3.26 (d, 2H, J = 7.1 Hz); 3.2 (dd, 1H, J = 6.3 and 13.6 Hz); 3.0 (dd, 1H, J = 7.8 and 13.6 Hz); 2.8 (m, 2H). ^{13}C NMR [δ, multiplicity (J in Hz), 100 MHz, $CDCl_3$]: 178.00; 152.3; 152.2; 147.6; 143.1; 142.9; 130.9; 130.7; 112.5; 111.2; 106.6; 106.2; 103.6; 103.5; 71.4; 45.7; 41.7; 37.2; 34.2; EMAR $(M+H)^+$ Calculation for $C_{20}H_{17}N_2O_{10}$: 445.0884; found: 445.0890. Analytic calculation for $C_{20}H_{16}N_2O_{10}$: C, 54.0937; H, 3.6315; N, 6.3081; O, 35.9667; found: C, 53.9173; H, 3.5462; N, 6.2121; O, 36.3244.

Determination of MIC

Silva et al. (2007) studied the antimicrobial activity of derivatives of **1** extracted from *Piper cubeba*, compared to other oral micro-organisms. The antimicrobial activity was tested on seven of the oral cavity pathogens: *Enterococcus faecalis*, *Streptococcus salivarius*, *Streptococcus sanguinis*, *Streptococcus mitis*, *Streptococcus mutans*, *Streptococcus sobrinus* and *Candida albicans*. The compounds were effective on most of the pathogens studied.

In the present work, other microorganisms that cause endodontic infection were tested, to expand knowledge regarding the chemical structure and biological activity. Assays were performed in triplicate, evaluating the compounds separately. The MIC values of compounds are shown in Table 1.

According to Rivers and Recio (2005), among the results obtained in this work, compounds **2** and **3** showed significant activity (50 μg/ml) compared to the microorganism *P. gingivalis* (ATCC 33277) for compounds **1** and **7**, which also showed significant activity (200 μg/ml). However, **5** and **6** showed lower activities, and **4** was not active (MIC > 400 μg/ml).

Compound **6** displayed significant activity against *B. fragilis* (ATCC 25285) (100 μg/ml). On the other hand, no activity was observed for the other evaluated compounds (>400 mg/ml). Compounds **1**, **2**, **3** and **7** showed low activity (400 μg/ml) against *P. nigrescens* (ATCC 33563), and compounds **4**, **5** and **6** were not active MIC values (>400 μg/ml).

The compounds were not active against the microorganisms *Actomyces naeslundii* (ATCC 19039) and *F. nucleatum* (ATCC 25586) showing MIC values >400 μg/ml.

The MIC values displayed by compounds **2** and **3** were significant (50 μg/ml) compared to the values for microorganism *P. gingivalis* (ATCC 33277) and for compound **6** against the microorganism *B. fragilis* (ATCC 25285) (100 μg/ml). The results obtained for antimicrobial activity in this study are in accordance with the activities obtained for the anti-inflammatory and analgesic, according to reports in the literature (Da Silva et al., 2005), in which the anti-inflammatory and analgesic effects of dibenzylbutyrolactone lignans were investigated using different animal models. It was observed that (−)-cubebin and (−)-hinokinin inhibited the edema formation in the rat paw edema assay and that all responses were dose dependent. Also, at the dose of 30 mg/kg, compounds (−)-cubebin, (−)-hinokinin, (−)-6,6′-dinitrohinokinin inhibited the edema formation by 53%, 63% and 54%, respectively, at the third hour of the experiment. In the acetic acid-induced writhing test in mice, (−)-hinokinin produced inhibition level of 97%, while (−)-6,6′-dinitrohinokinin displayed lower effect (75%), which was still higher than (−)-cubebin.

Compounds **2** and **3** differ from **1** by the presence of methyl or benzyl groups replacing the lactolic hydrogen. Compound **6** is a lignan-lactone that differs from **1** by the presence of a carbonyl group at C-9.

The literature reports that compounds having a lactone ring with two methylenodioxyaril groups showed significant anti-inflammatory and analgesic activities, and the introduction of polar groups on the aromatic rings is also advantageous for these activities (Da Silva et al., 2005). However, with respect to trypanocidal activity, the introduction of nitro groups in the aromatic rings is harmful for this activity (Da Silva et al., 2005).

Although chlorhexidine have displayed a considerably lower value of the MIC against all tested micro-organisms in relation to the compounds obtained in this work, the results seem to be satisfactory compared with other previously published works in the literature. In addition, the continual search for new compounds against oral pathogens may be justified by the side effects of chlorhexidine, such as discoloration of the teeth and taste perversion. It should be noted that this study provides additional data that is relevant to show the potential activity against oral pathogens presented by pure compounds obtained from *P. cubeba*, along with their semisynthetic derivatives.

Determination of MBC

Antimicrobial resistance is a serious problem in terms of clinical and public health. Regardless of the origin of resistance, the fact is that the number of new resistant microorganisms in animals and

Table 1
MIC values of evaluated compounds.

Compounds	Minimum inhibitory concentration (μg/ml)				
	P. gingivalis	*A. naeslundii*	*B. fragilis*	*P. nigrescens*	*F. nucleatum*
1	200	>400	>400	400	>400
2	50	>400	>400	400	>400
3	50	>400	>400	400	>400
4	>400	>400	>400	>400	>400
5	400	>400	>400	>400	>400
6	400	>400	100	>400	>400
7	200	>400	>400	400	>400
Control (*Chlorexidine*)	0.922	1.844	7.375	0.922	1.844

Concentrations of the tested substances: 20 μg/ml and 400 μg/ml. Concentrations of positive control: 0.0115 μg/ml and 8 μg/ml.

Table 2
Minimum bactericidal concentration values of the evaluated compounds.

Compounds	Minimum bactericidal concentration (μg/ml)				
	P. gingivalis	*A. naeslundii*	*B. fragilis*	*P. nigrescens*	*F. nucleatum*
1	400	>400	>400	>400	>400
2	200	>400	>400	>400	>400
3	300	>400	>400	>400	>400
4	>400	>400	>400	>400	>400
5	>400	>400	>400	>400	>400
6	400	>400	>400	>400	>400
7	>400	>400	>400	>400	>400
Control (*Chlorexidine*)	0.922	1.844	7.375	0.922	1.844

Concentrations of the tested substances: 20 μg/ml and 400 μg/ml. Concentrations of positive control: 0.0115 μg/ml and 8 μg/ml.

humans grows faster than the development of new drugs. Therefore, the search for alternative therapies is imperative.

An antibiotic may have bactericidal (cause cell death with reduced bacterial growth greater than or equal to 80%), bacteriostatic (inhibits bacterial growth with reduction usually less than 80% since it does not occur cell death), or not present any activity, when the bacterium is resistant to it, according to CLSI (2007).

The tests concerning the minimum bactericidal concentration of the compounds obtained are shown in Table 2. Among it, note that there was some activity of **2** and **3** against *P. gingivalis* with MBC of 200 μg/ml and 300 μg/ml, respectively. **1** and **6** presented the MBC of 400 μg/ml. As for the other compounds, their MBC values was not determined (>400 μg/ml).

However, compared to other microorganisms (*P. nigrescens, A. naeslundii, F. nucleatum* and *B. fragilis*) it was not observed activity, since all the compounds showed MBC values >400 μg/ml.

Determination of cytotoxicity

The viability of the cultures was determined by establishing a relation between the absorbance values obtained in the treated and untreated (control) groups, as shown in Fig. 2. These results revealed that the *in vitro* activity of the tested substances may not be related do cytotoxic effects, since they do not present significant cytotoxicity.

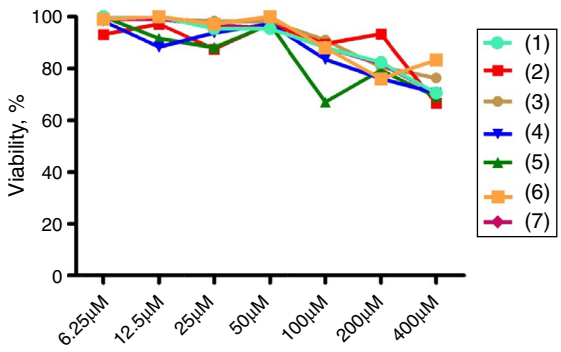


Fig. 2. Effects of the compounds on the viability of LLCMK2 fibroblast cells. Cytotoxicity was determined using MTT assay after 24 h treatment with the indicated concentrations. Values are expressed by mean ± S.D.

Although *in vitro* assays have not been considered suitable to cover all the aspects of the activities of drugs, especially with respect to pharmacological and immunological interactions, they provide first evidence of the effects and insight into the mode of action, thus, they may lead to the development of new therapeutic approaches like drug targeting (Holtfreter et al., 2011).

Conclusions

From this study it was possible to obtain relevant data concerning the relationship between structure and antimicrobial activity for lignans derived from (−)-cubebin (**1**) against microorganisms that cause root canal infections, ie, which substituent groups are important for maintenance or increase of antimicrobial activity for these compounds. Investigations on **1** are always geared toward finding out new biological properties and synthesizing more potent derivatives from a pharmacological viewpoint. It should be highlighted that the derivatives were obtained with high yields, which allowed the completion of all steps of purification, identification and biological evaluation.

According to the current study, the presences of carbonyl group at carbon 9, as well as the introduction of polar groups are beneficial for antimicrobial activity. The analysis of the results also suggests that the lignan **1** and its derivatives, especially **2**, **3** and **6**, constitute an important source of new bioactive natural products, which encourages further studies.

Authors' contributions

KCSR (PhD student) isolated the precursor from the plant, synthesized the derivatives, evaluated the biological activity, running the laboratory work, analyzing data and drafted the paper. RL (PhD student) contributed to biological assays. GVS (PhD student) contributed to chromatographic analysis of plant material. PMP and

AHJ contributed to critical reading of the manuscript. VRE and CHGM contributed to biological assays. MAS contributed to isolation and identification of compounds. WRC contributed to the identification of derivatives. JKB contributed to critical reading of the manuscript. MLAS designed the study and supervised the laboratory. 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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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bjp.2015.12.006.

References

Arruda, D.C., D'alexandri, F.L., Katzin, A.M., Uliana, S.R., 2005. Antileishmanial activity of terpene nerolidol. Antimicrob. Agents Chemother. 49, 1679–1687.

Ayres, D.C., Loike, J.D., 1990. Lignans: Chemical, Biological And Clinical Properties. Chemistry and Pharmacology of Natural Products. Cambridge University Press, Cambridge.

Baumgartner, J., 1997. Microbiologia endodôntica. In: Walton, R.E., Torabinejad, M. (Eds.), Princípios E Prática Em Endodontia. Santos Livraria Editora, São Paulo, pp. 277–291.

Bergenholtz, G., Crawford, J., 1989. Endodontic microbiology. In: Walton, R.E., Torabinejad, M. (Eds.), Principles and Practice of Endodontics. , 1st ed. W.B. Saunders Co., Philadelphia, P.A., USA, pp. 267–282.

Bier, O., 1981. Bacteriologia E Imunologia: Em Suas Aplicações à Medicina E A Higiene. Ed. Melhoramentos, São Paulo.

Braquet, P., Godfroid, J.J., 1986. PAF-acether specific binding sites: 2. Design of specific antagonists. Trends Pharmacol. Sci. 7, 397–403.

Charlton, J.L., 1998. Antiviral activity of lignans. J. Nat. Prod. 61, 1447–1451.

CLSI, 2007. Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria; Approved Standard – Seventh Edition. CLSI document M11-A7 [ISBN 1-56238-626-3]. Clinical and Laboratory Standards Institute, Wayne, Pennsylvania, USA.

Da Silva, R., de Souza, G.H.B., da Silva, A.A., de Souza, V.A., Pereira, A.C., Royo, V.A., Silva, M.L.A., Donate, P.M., Araujo, A.L.S.M., Carvalho, J.C.T., Bastos, J.K., 2005. Synthesis and biological activity evaluation of lignan lactones derived from (−)-cubebin. Bioorg. Med. Chem. Lett. 15, 1033–1037.

De Lillo, A., Booth, V., Kyriacou, L., Weightman, A.J., Wade, W.G., 2004. Culture-independent identification of periodontitis-associated Porphyromonas and Tannerella populations by targeted molecular analysis. J. Clin. Microbiol. 42, 5523–5527.

De Souza, V.A., da Silva, R., Pereira, A.C., Royo, V.A., Saraiva, J., Montanheiro, M., de Souza, G.H.B., da Silva Filho, A.A.S., Grando, M.D., Donate, P.M., Bastos, J.K., Albuquerque, S., Silva, M.L.A., 2005. Trypanocidal activity of (−)-cubebin derivatives against free amastigote forms of *Trypanosoma cruzi*. Bioorg. Med. Chem. Lett. 15, 303–307.

Deyama, T., Nishibe, S., Nakazawa, Y., 2001. Constituents and pharmacological effects of Eucommia and Siberian ginseng. Acta Pharm. Toxicol. 22, 1057–1070.

Elfahmi, Y., 2006. Lignan profile of Piper cubeba, an Indonesian medicinal plant. In: Phytochemical and Biosynthetic Studies of Lignans. Universiy of Groningen, Netherlands (Chapter 4).

Fine, D.H., 2000. Chemical agents to prevent and regulate plaque development. Periodontology 8, 87–107.

Hirata, N., Naruto, S., Ohguchi, K., Akao, Y., Nozawa, Y., Iinuma, M., Matsuda, H., 2007a. Mechanism of the melanogenesis stimulation activity of (−)-cubebin in murine B16 melanoma cells. Bioorg. Med. Chem. 15, 4897–4902.

Hirata, N., Tokunaga, M., Naruto, S., Iinuma, M., Matsuda, H., 2007b. Testosterone 5a-reductase inhibitory active constituents of *Piper nigrum* Leaf. Biol. Pharm. Bull. 30, 2402–2405.

Hirata, N., Naruto, S., Inaba, K., Itoh, K., Tokunaga, M., Iinuma, M., Matsuda, H., 2008. Histamine release inhibitory activity of *Piper nigrum* leaf. Biol. Pharm. Bull. 31, 1973–1976.

Holtfreter, M.C., Loebermann, M., Klammt, S., Sombetzki, M., Bodammer, P., Riebold, D., Kinzelbach, R., Reisinger, E.C., 2011. *Schistosoma mansoni*: schistosomicidal effect of mefloquine and primaquinein vitro. Exp. Parasitol. 127, 270–276.

Kakehashi, S., Stanley, H.R., Fitzgerald, R.J., 1965. The effects of surgical exposures of dental pulps in germ-free and conventional laboratory rats. Oral Surg. Oral Med. Oral Pathol. 20, 340–349.

Kassuya, C.A., Silvestre, A., Menezes-De-Lima, O.J.R., Marotta, D.M., Rehder, V.L., Calixto,J.B., 2006. Antiinflammatory and antiallodynic actions of the lignan niranthin isolated from *Phyllanthus amarus*. Eur. J. Pharmacol. 546, 182–188.

Koul, S.K., Taneja, S.C., Dhar, K.L., Atal, C.K., 1983. Lignans of *Piper clusii*. Phytochemistry 22, 99–1000.

Li, N., Wu, J., Hasegawa, T., Sakai, J., Wang, L., Kakuta, S., Furuya, Y., Tomida, A., Tsuruo, T., Ando, M., 2006. Bioactive dibenzoylbutyrolactone and dibenzoylbutanediol lignanes from *Peperomia duclouxi*. J. Nat. Prod. 69, 234–239.

Lifeng, Q.I., Zirong, X., Xia, J., Caihong, H., Xiangfei, Z., 2004. Preparation and antibacterial activity of chitosan nanoparticles. Carbohyd. Res. 339, 2693–2700.

Luisi, S.B., Fachin, E.V.F., 1990. Revisão e enfoque clínico sobre a bacteriologia das infecções endodônticas agudas. Rev. Fac. Odontol. UFGRS 40, 41–45.

Marcotullio, M.C., Pelosi, A., Curini, M., 2014. Hinokinin, an emerging bioactive lignan. Molecules 19, 14862–14878.

Marsh, P.D., 2005. Dental plaque: biological significance of a biofilm and community lifestyle. J. Clin. Periodontol. 32, 7–15.

Morrison, R., Boyd, R., 1996. Química Orgânica: Hidrogenação, 13ª edição. Fundação Caloustre Gulbenkian, Lisboa, 375, 380, 382 e 384.

Muellas-Serrano, Nogal, S., Martinez-Dias, J.J., Escario, R.A., Martinez-Fernandez, J.Á., Gomes-Barrio., A.R., 2000. A *in vitro* screening of American plant extracts on *Trypanosoma cruzi* and *Trichomonas vaginalis*. J. Ethnopharmacol. 71, 101–107.

Parmar, V.S., Jain, S.C., Bisht, K.S., Jain, R., Taneja, P., Jha, A., Tyagi, O.D., Prasad, A.K., Wengel, J., Olsen, C.E., Boll, P.M., 1997. Phytochemistry of the genus *Piper*. Phytochemistry 46, 597–673.

Piccinelli, A.L., Mahmood, N., Mora, G., Poveda, L., Simone, F., Rastrelli, L., 2005. Anti-HIV activity of dibenzylbutyrolactone-type lignans from *Phenax* species endemic in Costa Rica. J. Pharm. Pharmacol. 57, 1109–1115.

Rimando, A.M., Pezzuto, J.M., Farnsworth, N.R., Santisuk, T.R.V., Kawanishi, K., 1994. New lignans from anogeissus-acuminata with hiv-1 reverse-transcriptase inhibitory activity. J. Nat. Prod. 57, 896–904.

Rivers, J.C., Recio, M.C., 2005. Medicinal plants and antimicrobial activity. J. Ethnopharmacol. 100, 80–84.

Saleem, M., Kim, H.J., Ali, M.S., Lee, Y.S., 2005. An update on bioactive plant lignans. Nat. Prod. Rep. 22, 696–716.

Saraiva, J., Veja, C., Rolon, M., Silva, R., Silva, M.L.A., Donate, P.M., Bastos, J.K., Gomez-Barrio, A., Albuquerque, S., 2007. *In vitro* and *in vivo* activity of lignan lactones derivatives against *Trypanosoma cruzi*. Parasitol. Res. 100, 791–795.

Schmidt, T.J., Hemmati, S., Klaes, M., Konuklugil, B., Mohagheghzadeh, A., Ionkova, I., Fuss, E., Wilhelm Alfermann, A., 2010. Lignans in flowering aerial parts of *Linum* species – chemodiversity in the light of systematics and phylogeny. Phytochemistry 71, 1714–1728.

Shen, T.Y., Hwang, S.B., Chang, M.N., Doebber, T.W., Lam, M.H., Wu, M.S., Wang, X., Han, G.Q., Li, R.Z., 1985. Characterization of a PAF receptor antagonist isolated from haifenteng (*Piper futokadsura*): Specific inhibitor of *in vitro* and *in vivo* PAF induced effects. Proc. Natl. Acad. Sci. U. S. A. 82, 672–676.

Silva, M.L.A., Albuquerque, S., Souza, B.H.B., Bastos, J.K., Silva, R., 2009. Process to obtain dibenzylbutyrolactonic lignans, process to obtain synthetic derivatives from lignans bearing anti-Chagas chemoprophylactic and therapeutical activities. Fundação de Amparo à Pesquisa do Estado de São Paulo. US 7,521,569 B2.

Silva, M.L.A., Silva, R., Rodrigues, V., Pereira Júnior, O.S., Silva Filho, A.A., Donate, P.M., Albuquerque, S., Bastos, J. K. 2007. Process to obtain synthetic and semi-synthetic lignan derivatives, their antiparasitic activities and corresponding pharmaceutical formulations, including the therapeutic method using said lignan for the treatment of parasitosis. Fundação de Amparo à Pesquisa do Estado e São Paulo. WO 2007/009201.

Socransky, S.S., Haffajee, A.D., 2002. Dental biofilms: difficult therapeutic targets. Periodontology 28, 12–55.

Souza, G.H.B., Silva, A.A., Souza, V.A., Pereira, A.C., Royo, V.A., Silva, M.L.A., Silva, R., Donate, P.M., Carvalho, J.C.T., Bastos, J.K., 2004. Analgesic and anti-inflammatory activities evaluation of (−)-*O*-acetyl, (−)-*O*-methyl, (−)-*O*-dimethylethylamine cubebin and their preparation from (−)-cubebin. II Farmaco 59, 55–61.

Tani-Ishii, N., Wang, C.Y., Tanner, A., Stashenko, P., 1994. Changes in root canal microbiota during the development of rat periapical lesions. Oral Microbiol. Immunol. 9, 129–135.

Usia, T., Watabe, T., Kadota, S., Tezuka, Y., 2005. Potent CYP3A4 inhibitory constituents of *Piper cubeba*. J. Nat. Prod. 68, 64–68.

Venkateswarlu, R., Kamakshi, C., Moinuddin, S.G.A., Subhash, P.V., 1999. Transformations of lignans, Part V. Reactions of DDQ with a gmelinol hydrogenolysis product and its derivatives. Tetrahedron Lett. 55, 13087–13108.

Wittgow Jr., W.C., Sabiston Jr., C.B., 1975. Microorganisms from pulpal chambers of intact teeth with necrotic pulps. J. Endod. 1, 168–171.

Yam, J., Kreuter, M., Drewe1, J., 2008. Piper cubeba targets multiple aspects of the androgen-signalling pathway. A potential phytotherapy against prostate cancer growth? Planta Med. 74, 33–38.