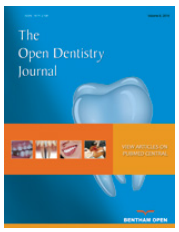

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CLINICAL TRIAL STUDY

Antibacterial Activity of Phytochemical Extracts and Endophytic Fungi of *Carapa Guianensis* Against *Enterococcus Faecalis* in Endodontic Infections An *In Vitro* Study

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Abstract:

Objective:

The objective of this study was to evaluate the antibacterial activity of phytochemical extracts and endophytic fungi of *Carapa guianensis* against *Enterococcus faecalis. Carapa guianensis* leaves and stems were collected to obtain phytochemical extracts and fungal metabolites and evaluated for *in vitro* antibacterial activity against *E. faecalis* using the disc diffusion method and dentin blocks with bacterial biofilm.

Methods:

Thirty dentin blocks were prepared and contaminated for 60 days with *E. faecalis*. The specimens were randomly divided into 6 experimental groups according to the test solution used: G1 – hexane stem extract of *Carapa guianensis*; G2 – methanol stem extract of *Carapa guianensis*; G3 – methanol leaf extract of *Carapa guianensis*; G4 – ethyl acetate extract of the endophytic fungus *Penicillium* isolated from *Carapa guianensis*; G5 – negative control, with no addition of bacterial inoculum; G6 – positive control.

Results:

Bacterial growth was analyzed by spectrophotometry after 14 days of direct contact between the extracts and dentin blocks. The hexane-stem, methanol-stem, methanol-leaf, and ethyl-acetate endophytic fungus *Penicillium* extracts inhibited bacterial growth in 100% of the samples.

Conclusion:

The present study demonstrated the antibacterial potential of phytochemical extracts and endophytic fungi of *Carapa guianensis* against *E. faecalis.*

Keywords:: Endodontics. *Enterococcus faecalis*. Phytochemicals, *Carapa guianensis*, Endophytic fungi, *Penicillium*.

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1. INTRODUCTION

The success of endodontic treatment is related to the elimination of microorganisms from the root canal system [1, 2]. Persistent infection after endodontic treatment, resulting from remnants of bacteria in root canal systems in areas of anatomical complexity and difficult access, continues to present a challenge in endodontics [1]. Thus, recurrent periapical infections are mainly associated with *Enterococcus faecalis* [2]. Some factors allow *E. faecalis* to survive in root canal systems for long periods, such as resistance to antimicrobials, ability to survive for long periods without nutrients, capability to adhere to dentin, invasion of dentinal tubules, and ability to bind to collagen and form a biofilm [3].

Several intracanal medicaments have been proposed for the

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treatment of endodontic infections, of which calcium hydroxide is the most important due to its antimicrobial and anti-inflammatory properties [4]. Associated with induction of mineralized tissue formation, dissolution of organic matter, and biocompatibility, calcium hydroxide acts on the cell walls of bacteria, causing damage to the bacterial cytoplasmic membrane, protein denaturation, and DNA damage [4]. However, several studies have indicated that calcium hydroxide is ineffective in inhibiting the growth of *E. faecalis*, because the buffering effect of dentin neutralizes the action of this medicament at deeper layers of dentinal tubules, resulting in the survival of this microorganism [5].

Biological activity of some plant species has been raising interest in research due to the production of bioactive substances [6 - 9]. *Carapa guianensis* Aublet, known as Andiroba, presents applications in Brazilian medicine and in other countries of the Amazonian forest [7]. Studies evaluating its biological activity have reported antimicrobial activity, cicatrization, anti-inflammatory [7 - 9]. However, there is no report in the literature about its use in the endodontic therapy.

Endotoxic microorganisms, which inhabit the interior of the plant, without causing any damage, produce secondary metabolites that also generate bioactive principles [7 - 9]. Endophytic fungi are important and can produce toxins, antibiotics, drugs, growth factors and many products of biotechnological interest, being also a natural source of new drugs that can control microorganisms that cause infections [7 - 9]. Therefore, the purpose of this *in vitro* study was to evaluate the efficacy of phytochemical extracts and endophytic fungi of *Carapa guianensis* against *E. faecalis* biofilm on dentin.

2. MATERIALS AND METHODS

2.1. Production of Phytochemical Extracts From *Carapa Guianensis*

The leaves and stems were washed with tap water, dried at room temperature and grounded to a fine powder using an electric mill. Extracts were obtained according to the technique described by Celeghini *et al*. (2001) [10], where 100 g of powered plant material was extracted with hexane and methanol (100 g of samples for 400 mL of solvent) under sonication for 20 minutes.

2.2. Production of Endophytic Fungal Extracts From *Carapa Guianensis*

The *Carapa guianensis* endophytic fungus no. 2477, identified as *Penicillium sp*., was grown on a larger scale for metabolite extraction. This endophytic fungus was cultured in 30 Petri dishes (80 × 15 mm) containing potato dextrose agar medium for 14 days at 28 °C. Five plates served as an inoculum for each 1000-mL Erlenmeyer flask containing 500 mL of potato dextrose broth. Six Erlenmeyer flasks were inoculated and incubated without shaking for 14 days at 28 °C. The fungal mycelium was separated from the liquid medium by filtration using filter paper. The filtrate was extracted 3 times by liquid-liquid extraction with ethyl acetate and concentrated in a rotary evaporator under reduced pressure in a water bath at 40 °C.

2.3. Bioassay of *In Vitro* Antibacterial Activity

The phytochemical and endophytic fungus *Penicillium* extracts were dissolved in Dimethyl Sulfoxide (DMSO) and analyzed at the initial concentration of 1 g mL^{-1} using the disc diffusion test with *E. faecalis* (ATCC 4083) as the test microorganism. To prepare the inoculum, three well-isolated bacterial colonies of the same morphological type were selected from a Mueller-Hinton agar plate culture. The top of each colony was touched with a loop, and the growth was transferred into a tube containing 3 mL of Luria-Bertani broth and incubated at 37 °C for 4 hours. The turbidity of the actively growing broth culture was adjusted with 0.9% saline to obtain turbidity optically comparable to that of the 0.5 McFarland standard, resulting in a suspension containing approximately 1.5 × 108 Colony-Forming Units (CFU) per mL.

After adjusting the turbidity, a sterile cotton swab was dipped into the suspension and inoculated into Mueller-Hinton agar. The procedure was repeated 2 more times, rotating the plate approximately 60° each time to ensure an even distribution of the inoculum, and the rim of the Petri dish was swabbed as a final step. After inoculation, the lid of the plate was left ajar for 5 minutes to allow for any excess surface moisture to be absorbed before applying the paper discs.

Sterile paper discs were dispensed onto the surface of the inoculated agar plate, and 20 µL of the extracts under analysis were placed onto the discs. The plates were stored for 2 hours at 4 °C to allow diffusion of the analyzed samples and subsequently incubated at 37 °C for 24 hours. After the 24-hour incubation, each plate was examined for the presence of an inhibition halo, which was measured in millimeters using a digital caliper. DMSO served as a negative control. All antibacterial activity analyses were performed in triplicate. The extracts with positive antagonism were dissolved in DMSO and analyzed at concentrations of 1g mL^{-1}, 500 mg mL^{-1}, 250 mg mL^{-1}, 125 mg mL^{-1}, 62.5 mg mL^{-1}, and 31.25 mg mL^{-1} for the Minimum Inhibitory Concentration (MIC).

2.4. Preparation of Dentin Blocks

Thirty dentin blocks were prepared from extracted single-rooted human teeth. All teeth used in the study had an indication for extraction for periodontal or prosthetic reasons. Teeth with root canal obliteration, root dilaceration, or root canal filling were excluded. The teeth were sectioned to obtain dentin blocks of 4 mm height and 4 mm length. Two layers of nail polish were applied to the external surfaces of all dentin blocks to prevent infiltration. The specimens were washed twice under sonication with 17% EDTA for 5 minutes and then rinsed with sterile distilled water under sonication for 10 minutes. The dentin blocks were autoclaved at 120 °C for 30 minutes and subsequently incubated in 7 mL of Brain Heart Infusion (BHI) broth at 37 °C for 48 hours to ensure sterilization, so that, after this incubation period, no bacterial growth was observed.

2.5. Contamination of Dentin Blocks

For contamination, *E. faecalis* (ATCC 29212) was inoculated into 7 mL of BHI broth, incubated at 37 °C for 24 hours and subsequently inoculated onto the surface of the BHI

agar plate under the same incubation conditions. Microbial cells were suspended in saline solution to a concentration of 3 x 108 cells mL^{-1}, matched to 1 McFarland standard.

The dentin blocks were inoculated with 0.01 mL of the bacterial inoculum using a pure culture of *E. faecalis* grown for 24 hours and adjusted to 1 McFarland standard. The contaminated blocks were incubated for 60 days at 37 °C in a humid chamber. Five uncontaminated specimens (negative control group) were incubated at 37 °C during the contamination period to monitor sample sterility, while the positive control group consisted of 5 contaminated specimens that were incubated at 37 °C throughout the experimental period to determine the viability of the biological indicator. After 60 days, under aseptic conditions, each dentin block was removed from the inoculated suspension and rinsed with 5 mL of sterile distilled water to remove the culture and non-adherent cells. Bacterial growth was determined by turbidity of the culture medium.

2.6. Application of Phytochemical Extracts and Endophytic Fungi of *Carapa guianensis* to Dentin Blocks

The selected extracts were distributed in 30 Eppendorf tubes for each experimental group taking into account the MIC previously determined in the disc diffusion test. The contaminated dentin blocks were randomly selected and immersed in the test solutions, accounting for 6 experimental groups, for 14 days, as described in Table **1**, and an initial spectrophotometer reading was taken. After 14 days, the dentin blocks were removed from the experimental solutions, rinsed with 5 mL of sterile distilled water, transferred individually to 7 mL of BHI broth, with the addition of two neutralizers (sodium thiosulfate and Tween 80), and incubated at 37 °C for 48 hours.

2.7. Microbiological Analysis

The tubes containing dentin blocks were incubated at 37°C for 48 hours, and microbial growth was analyzed. Visual readings were taken over the 48-hour period for the absence (negative) or presence (positive) of microbial growth in the 6 groups, as determined by turbidity of the culture medium. After 48 hours, 0.1 mL of BHI medium was inoculated into 20 tubes containing 5 mL of Letheen broth, which were then incubated under the same conditions for an additional 48 hours, when a spectrophotometer reading was taken. Microbial concentration was analyzed using a UV spectrophotometer (Model NI-1600UV; Nova Instruments) adjusted for reading at a wavelength of 600 nm.

2.8. Statistical Analysis

Distribution of random errors around the mean (normality) and presence or absence of homogenous variances were tested, respectively, by the Shapiro-Wilk and Lévene tests. Sequentially, Student's T-test was used to compare the data between the means obtained in the initial and final analyzes. Level of significance was set at 5%.

3. RESULTS

The results of the disc diffusion test indicated that the methanol-leaf, hexane-stem, and methanol-stem extracts and the ethyl-acetate endophytic fungus *Penicillium* extract showed antibacterial activity against *E. faecalis* (Table **2**). The MICs of the phytochemical and ethylacetate endophytic fungus *Penicillium* extracts that showed activity against *E. faecalis* are shown in Table **2**.

Table 1. Experimental groups for antibacterial analysis of *Carapa guianensis* endophytic fungi against *Enterococcus faecalis* biofilm.

Group	*Carapa guianensis*	Dentin Blocks
1	Stem - Hexane	5
2	Stem - Methanol	5
3	Leave - Methanol	5
4	Endofitic fungi *Penicilium* sp. – Ethyl acetate	5
5	Negative control	5
6	Positive control	5

Table 2. Evaluation of minimum inhibitory concentration of the phytochemical extracts and endophytic fungi of *Carapa guianensis* against to *Enterococcus faecalis* (ATCC 29212).

Carapa guianensis Extracts	Inhibition Hale (mm)	MIC ($mg\ mL^{-1}$)
Stem - Hexane	16	62.50
Stem - Methanol	17	62.50
Leave - Methanol	18	62.50
Endofitic fungi *Penicilium sp*. – Ethyl acetate	20	31.25
Negative control	0	--
Positive control	0	--

Table 3. Evaluation of bacterial growth after the application of phytochemical extracts and endophytic fungi of *Carapa guianensis* against to *Enterococcus faecalis biofilm.*

Experimental Group		Extract	Spectrophotometer Readings		Inhibition (%)
Group	Concentration (mg mL^{-1})		Initial	Final	
1	62.5	Stem - Hexane	0.967 (±0.116)[a*]	0.000 (±0.000)[b]	100%
2	62.5	Stem - Methanol	1.034 (±0.136)[a]	0.000 (±0.000)[b]	100%
3	62.5	Leave - Methanol	1.052 (±0.111)[a]	0.000 (±0.000)[b]	100%
4	31.25	*Penicillium Endofitic fungi*	0.985 (±0.121)[a]	0.000 (±0.000)[b]	100%
Negative control	–	–	0.000 (±0.000)[a]	0.000 (±0.000)[a]	–
Positive control	–	–	1.319 (±0.283)[a]	1.208 (±0.007)[a]	–

*Different letters indicate statistically significant differences in lines ($p<0.05$).

Regarding the action of the phytochemical extracts and endophytic fungi of *Carapa guianensis* against contamination of dentin blocks with *E. faecalis*, showed that all extracts in the experimental groups 1, 2, 3, and 4 had antibacterial effects presenting the elimination of *E. faecalis* grown on dentin blocks ($p<0.05$) (Table **3**). In group 5 (negative control), no bacterial growth was observed ($p>0.05$), confirming the sterility of the culture medium. In group 6 (positive control), the viability of the strains used in the experiment was confirmed.

4. DISCUSSION

The viability of the remaining microorganisms after instrumentation and disinfection of root canals contributes significantly to endodontic treatment failure [1]. In the absence of clinical evidence that intracanal medicaments are sufficiently effective against persistent bacterial infections, there is a need for further investigation of new medicaments for use in periradicular conditions [2].

The hexane-stem, methanol-stem, methanol-leaf, and ethyl-acetate endophytic fungus *Penicillium sp.* extracts of *Carapa guianensis* analyzed in the present study showed antibacterial activity against *E. faecalis* in disc diffusion and dental biofilm tests. Several methods have been proposed to evaluate the antibacterial potential of medicaments used in the treatment of endodontic infections, such as disc diffusion method [11], dentin infection and disinfection test [12], and dentin block biofilm models [13]. All these methods allow sufficient time for satisfactory colonization of bacterial species with virulence and adherence properties [11 - 13]. Additionally, Estrela *et al*. (2009) [13] regarding colonization in dentin blocks validated the methodology presented in this study concluding that this biofilm model is viable for studies on antimicrobial strategies and allows satisfactory colonization of the selected bacterial species with virulence and adherence properties.

The biotechnological potential of several plant species against microorganisms present in root canals has been reported in the literature [9 - 13]. However, there are currently no reports on the biological activity of extracts obtained from the leaves, stems or endophytic fungi of *Carapa guianensis* against *E. faecalis*. Some studies were found that analyzed the antibacterial activity of phytochemical extracts against *E. faecalis*, such as the study conducted by Costa *et al*. (2010) [14], who found that ethanol extracts of the plant species *Schinus terebinthifolius, Astronium urundeuva, Ximenia americana,* and *Syderoxylum obtusifolium* showed antimicrobial activity. Nath *et al*. (2012) [15] in an attempt to evaluate the antimicrobial activity of fungal endophytes inhabiting *Emblica officinalis* assessed their effect against four bacteria namely, *Escherichia coli*, *Enteroccocus faecalis*, *Salmonella enterica* ser. *paratyphi* and *Streptococcus pyogenes*, and the fungus *Candida albicans*. In general, the fungal extracts inhibited the growth of all tested organisms except *Escherichia coli*.

Fungi of the genus *Penicillium* can produce several secondary metabolites, and 132 different metabolites have been reported to be produced by fungi of this genus, including pigments and antimicrobial compounds [16]. *Penicillium sp*. are characterized by the abundant production of spores and have been widely investigated in the search for bioactive compounds [17], with endophytic fungal strains showing neuroprotective [18], antitumor [19], and antibacterial [20] activities. In this study the endophytic fungus *Penicillium sp* demonstrated promising results corroborating the idea of its application against *E. faecalis* in order to overcome deficiencies of the current intracanal medications.

Studies have demonstrated the antimicrobial activity of *Carapa guianensis* against several microorganisms [8, 9, 21, 22], but none of them investigated *E. faecalis*, thereby hindering a proper comparison with the results obtained in the present study. Costa-Silva *et al.*, (2007) [22] found that the use of *Carapa guianensis* seed oil in pregnant rats produced no toxic effect. Miranda-Junior *et al*., (2012) [23] evaluated the antispasmodic effects and toxicity activity of *Carapa guianensis* in mice, and no toxic limonoids were identified. Nevertheless, studies evaluating the antimicrobial actions of *Carapa guianensis* phytochemical compounds still show promising perspectives [24,25]. *Carapa guianensis* demonstrated effects against protozoa of the genera *Plasmodium* and *Trypanosoma*, due to the limonoids that exist in its compound [25]. As the chemical constituents of the *Carapa guianensis* exhibited anti-*Leishmania* activity [24] due to the limonoids 11β-hydroxygedunin and 6α,11 β-diacetoxygedunin, identified in the active limonoid-rich fractions [24].

Only one study was found considering the endophytic fungal community associated with *Carapa guianensis* as well as its potential to produce bioactive compounds. In this study, a total of 162 fungi were isolated from the leaves of *Carapa guianensis*, and some of these fungi showed biological activity, such as *Colletotrichum sp*. and *Pilidiella wangiensis*, which showed antibacterial activity; however, the activity against *E.*

faecalis was not evaluated [26]. Most bioactive substances obtained from fungal extracts can be used directly as medications or even modified to obtain a new molecule with different properties, such as increased activity, selective toxicity, and reduced undesirable side effects [16 - 26]. Even when these substances do not yield the expected activity, they may serve as prototypes for the planning and development of new molecules, which further highlight the importance of studying endophytic fungi as a source of new substances against recurrent endodontic infections [16 - 26]. Substances tested against *Enterococcus faecalis* demonstrated promising *in vitro* results. New studies should be designed to evaluate other biological properties of these compounds.

CONCLUSION

The present study demonstrated the antibacterial potential of phytochemical extracts and endophytic fungi of *Carapa guianensis* against *E. faecalis*. Nevertheless, further antimicrobial, biocompatibility, and toxicity assays of the active compounds are warranted to confirm these findings.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the Human Research Ehtics Committee of Fedral University of Goiás- number 1.214.

HUMAN AND ANIMAL RIGHTS

No animals were used in this research. All research procedures followed were in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national), and with the Helsinki Declaration of 1975, as revised in 2013 (http://ethics.iit.edu/ecodes/node/3931).

CONSENT FOR PUBLICATION

The participants provided written informed consent to be involved in this study.

AVAILABILITY OF DATA AND MATERIALS:

The data supporting the findings of the article is available in the [Federal University of Acre] at [https://bionorte.org.br/programa-de-pos-graduacao/corpo-discente.htm?idp=1&pgn=6].

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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