Original Research Article

Antibacterial activity of *calycophyllumspruceanum* leaf extract against *enterococcus faecalis* strains "in vitro" for endodontic purposes

ABSTRACT

Aims:The indiscriminate use of antimicrobials contributed to the selection of multiresistant bacterial strains, mainly Enterococcus faecalis, leading to the search for new strategies for these infections, requiring the identification of new bioactive compounds from medicinal plants, an example is C. spruceanum used in traditional communities in the Amazon.
In this sense, this study aimed to evaluate the antibacterial activity of C. spruceanum leaf extract against E. faecalis "in vitro" for endodontic purposes.
Place and Duration of Study:Sample: the study was carried out at the microbiology laboratory of the Federal University of Acre (UFAC), in the city of Rio Branco, state of Acre, Brazil, from July 2021 to January 2022.
Methodology:For this study, 50mg/mL of the crude extract of C. spruceanum leaf was diluted in Dimethylsulfoxide, as well as 50mg/mL of the extract with 1000mg of calcium hydroxide and 1000mg of calcium hydroxide in 1mL of distilled water. After preparing the extracts, 20µL were deposited in the wells made on Müeler-Hinton agar medium seeded with E. faecalis, incubated for 24 hours at 37°C. After this period, the positive activity of the extracts was obtained from the formation of the inhibition halo in millimeters.
Results:The best results were for extract associated with calcium hydroxide (20mm), followed by calcium hydroxide solution (17.67mm) and with lower activity, C. spruceanum extract (14.33mm). The best results of the Minimum Inhibitory Concentration with values of 6.25 mg/mL for the extract combined with calcium hydroxide and Minimum Bactericidal Concentration that was 25mg/mL for the association of the extract with calcium hydroxide.
Conclusion:The C. spruceanum extract has antibacterial activity against E. faecalis and its association with calcium hydroxide potentiated its effect.

Keywords: Enterococcus faecalis. Antibacterials. Amazon. Plant extracts. Bioactive compounds.

1. INTRODUCTION

Antibacterials are drugs that encompass natural (antibiotics) or synthetic (chemotherapeutic) substances that act on microorganisms to inhibit their growth or to eliminate them. Therefore, they are used in the treatment of various diseases arising from bacterial infections and, for the most part, can reduce the morbidity and mortality rates linked to these diseases[1,2].

However, the indiscriminate use of antimicrobials due to the irrational use of antimicrobials for human and veterinary use, inadequate prescriptions, among others, can contribute to microbial resistance[1]. In this way, treatments become ineffective, infections persistent and most of the time fatal[2,3,4].

Among the microorganisms of clinical interest, *Enterococcus faecalis* stands out, a Gram-positive bacterium, usually present in the mucosa of the gastrointestinal tract, genitourinary tract, and oral cavity[5]. These can survive in complex media with nutritional needs containing serum or blood[6].

***E. faecalis* have the potential for resistance to virtually all clinically useful antibiotics and pose an important public health problem for their resistance[7]. It is an opportunistic pathogen and is part of the group of most resistant microorganisms in the oral cavity and the increasing prevalence of multiresistant strains to synthetic antibiotics raises the search for new strategies to combat these infections[8]**

This facultative bacterium is directly related to cases of refractory lesions in the oral cavity, mainly in endodontics, due to its ability to survive chemical-mechanical instrumentation in root canals[9]. In cases of endodontic infections, severe pulp and periapical changes are observed, with etiology directly related to this microorganism[10].

The only way to eliminate the infectious process in the tooth is through endodontic treatment, with variable success rates[11]. Most dentists perform this treatment in multiple sessions[12], but the success of endodontic treatment depends, above all, on knowledge of pulp anatomy, pathology, and microbiology, in addition to the correct diagnosis of the cases, an adequate ability to perform the treatment phases completely and of an effective medication to eliminate this microorganism[13].

Among them we have sodium hypochlorite and 2% chlorhexidine gluconate with antibacterial properties being used as irrigating solutions, as well as intracanal medications, there is calcium hydroxide that is used as a delay dressing. However, none of these medications is fully effective in combating endodontic infectious processes, in this context, the search for new alternative therapies is necessary[14].

For this reason, medicinal plants present themselves as a viable alternative, as they are a rich source of antimicrobial agents to combat bacterial resistance due to the ineffectiveness of synthetic antibiotics found in the current market[15]. However, the bioprospecting of new drugs from plant extracts becomes a possible alternative to combat microbial resistance, as there are already studies that prove the antimicrobial efficacy of plant extracts from their bioactive components[16].

There is much to discover amid the grandeur of the Amazon region, an example of this is the tree popularly known in Brazil as mulateiro or pau-mulato with the scientific name of *Calycophyllumspruceanum*. There are records of different bark and leaf preparations with anti-aging, antioxidant, antimicrobial, emollient, healing, hemostatic, contraceptive, stimulant, and antidiabetic properties[16].

They are medium to large trees, being considered characteristics of tropical America and, in the Brazilian flora, of the Amazon region. This genus has numerous secondary metabolites, such as alkaloids, iridoids, triterpenes, and anthraquinones that demonstrate important antioxidant activities[17]. In Brazil, it includes more than 2,000 species, in approximately 120 genera[18].

The species occurs throughout the Amazon Region, covering Bolivia, Brazil, Colombia, Ecuador, and Peru[19]. It is especially found along the Amazon River, where it forms almost homogeneous groups, called Matas-de-pau-Mulato (Brazil) or capironais (Peru).

***C. spruceanum* is widely used by traditional communities in the treatment of various diseases, such as mycoses, viruses, various infections, cancer, and skin diseases[20]. It is also used to treat back pain, prostate and kidney problems, urinary tract infection, inflammation, high cholesterol, thyroid problems, skin aging, female reproductive system problems, and body coldness[21,22]. The bark is used in the form of tea to control skin blemishes and to prevent aging, curative treatment of disorders of the genitourinary system, and the bark is used to make immersion baths[22,23]. In general, *C. spruceanum* bark is used to treat inflammation and fungal infections[24].**

***C. spruceanum* has metabolites that are effective in protecting against ultraviolet (UV) rays, functioning as a rejuvenator[24]. It has antioxidant and photoprotective activities through a study with aqueous and ethanolic extracts of the bark, confirming the plant's potential for dermatological use[24,25].**

In the field of Biotechnology, many studies on biological activity against microorganisms are described in the literature, on the other hand, few works on the antibacterial activity of extracts from Amazonian plants are reported[26]. Thus, *C. spruceanum* presents, from its compounds, a high potential for the treatment of several diseases, mainly bacterial.

Given the above, this study aimed to evaluate the antibacterial activity of *C. spruceanum* leaf extract against *E. faecalis* "in vitro" for endodontic purposes.

2. MATERIAL AND METHODS / EXPERIMENTAL DETAILS / METHODOLOGY

2.1 OBTAINING THE GROSS ETHANOL EXTRACT

The crude extract of C. sproceanun was obtained from the Microbiology Laboratory of the Federal University of Acre (UFAC) in glass flasks, weighed for yield analysis, and stored at room temperature.

The obtained extract was solubilized in 10% Dimethylsulfoxide at a concentration of 50 mg/mL for antimicrobial assays. The extract was prepared with calcium hydroxide at a concentration of 50mg/mL of an extract with 1g of Ca.

2.2 BIOASSAY OF ANTIBACTERIAL ACTIVITY

To evaluate the antibacterial activity, the good test was used (CLSI, 2003), with the test bacterium *E. faecalis* from the collection of the microbiology laboratory at the Federal University of Acre (UFAC).

The bacteria were inoculated on Müeler-Hinton (MH) agar and incubated at 37 °C for 24 h. After incubation, Gram staining was performed to verify the viability of the bacteria, then five colonies of the bacteria were inoculated in Luria Bertani broth (LB), incubated for 4-6 h at 37 °C, and the turbidity adjusted to the scale of 0.5 McFarland injection (1.5 x 108 bact/mL) with sterile saline. The bacteria were inoculated on MH agar with a swab, the wells were made in the culture medium and 20 µL of the extracts were deposited. The plates were kept at 4 °C for 24 h, for the diffusion of the extract in the culture medium, and after this period, incubated at 37 °C for another 24 h.

Samples with positive antibacterial activity were those that did not allow bacterial growth around the wells with the formation of inhibition halos measured in millimeters. This assay was performed in triplicate.

2.3 MINIMUM INHIBITORY CONCENTRATION (MIC)

The microdilution technique was performed using sterile 96-well microplates for the Minimum Inhibitory Concentration (MIC) of extracts with antimicrobial activity, pure extract of *C. spruceanum* (E), extract of *C. spruceanum* associated with calcium hydroxide (EH), and with pure calcium hydroxide (H). To perform the antibacterial activity, 100 µL of MH broth were distributed in all wells of the plate, and then 100 µL of extracts at a concentration of 200 mg/mL were added to the test well for serial dilution, obtaining an initial concentration of 100 mg/mL. Continuing the process of serial dilution eight times, being homogenized and transferred 100 µL to the next well, and so on, resulting in final concentrations of 0.78 mg/mL. The control drug, Chloramphenicol 30 mg/mL, was diluted similarly to the extract (Table 1).

Table 1. Experimental groups for carrying out MIC against E. faecalis.

Grouo	Sample	Initial Concentration
N	Negative Control (Environment MH)	100mg/mL
P	Positive Control (BHI infect)	100mg/mL
E	Extract *C. spruceanum*	100mg/mL
EH	Extract *C. spruceanum* + ($Ca(OH)_2$)	100mg/mL
H	($Ca(OH)_2$)	100mg/mL
C	Cloranfenicol	30mg/mL

5 µL of the inoculum corresponding to the tested strain were added, except for the negative control according to the M7-A6 standard. The negative control contained only 100 µL of MH broth and the positive control 100 µL of MH broth and 5 µL of inoculum. The microplate was incubated at 37 °C

for 24 h, after which 20 µL of the Resazurin reagent (0.30 mg/mL) was added to each well, which indicates microbial growth when the color changes from blue to red[27,28].

For the Minimum Microbicide Concentration (MMC), 20 µL of the four concentrations above the MIC were inoculated into a plate containing Müeler-Hinton agar medium and incubated at 37 °C for 24 hours, after which a visual reading of colonies formed was performed, and the result of the CMM was conditioned to the absence of colony formation in the culture medium.

2.4 STATISTICAL ANALYSIS

To compare the means of the extracts between the groups tested, the statistical software Statistical Package for the Social Sciences (SPSS), version 20.0 was used, in which the Student's t-test was performed, with a confidence level of 95% and results were considered p values ≤ 0.05 were statistically significant.

3. RESULTS AND DISCUSSION

The results of the test of antibacterial activity of the extracts of *C. spruceanum* against *E. faecalis* with pure extract, extract with calcium hydroxide and pure calcium hydroxide, with halo results with average 14.33mm, 20.64mm, and 17.67 respectively (Table 2 and Graph 1). These results showed that the extract associated with (Ca (OH)2) and the pure calcium hydroxide paste had more satisfactory results when compared to the pure extract of C. spruceanum, but without statistical significance.

The means of the halos of the extracts were compared using the Student's t-test with a confidence level of 95% and after this comparison, it was shown that there was no statistically significant difference, since all the results were greater than p ≤ 0.05, the which confirms these results.

Table 2. Antimicrobial activity of C. sproceanun extracts against E. faecalis.

Solutions	Concentration	Antibacterial Activity *Efa** Mean±standard deviation	MIC (mg/mL)	MCM	σ^{2***}	*p-value*
Pure Extract	50 mg/mL	14,33±7,506	6,25	50	56,33	0,223
Extract +(Ca $(OH)_2$)	50mg/mL	20,64±1,155	6,25	25	1,333	0,148
(Ca $(OH)_2$)**	1g/mL	17,67±4,933	25	25	24,33	0,408

Efa = E. faecalis, Ca (OH)$_2$ = Calcium Hydroxide, σ^{2***} = variance.*

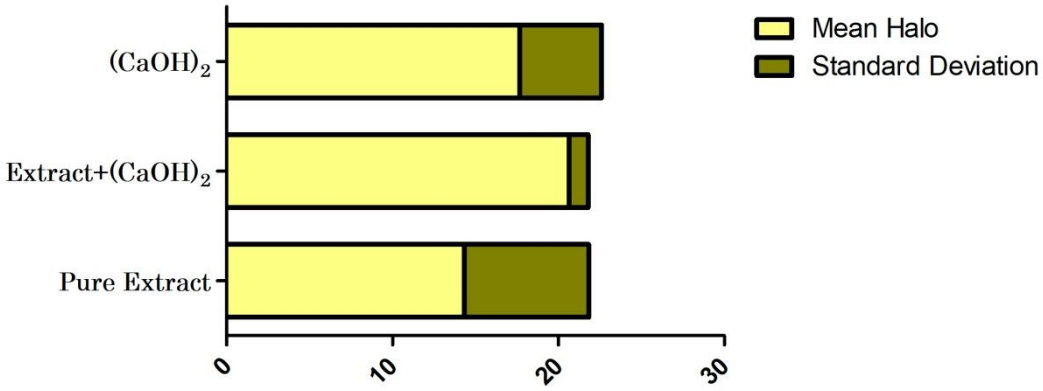
These results are similar to those found in other studies, in which they showed antimicrobial activity of extracts associated with calcium hydroxide[29]. Similar results are described in the association of calcium hydroxide with ethanolic extract of Psidium cattleianum and with pure calcium hydroxide against *E. faecalis*[30].

This effect is due to the high antibacterial activity of calcium hydroxide with bactericidal and bacteriostatic action against bacterial biofilms[31]. On the other hand, a negative point is that its action occurs through the release of its hydroxyl ions, alkalizing the medium and impairing its effect[32,33]. Another factor that can interfere with the effectiveness of this medication is the vehicle used, which can enhance or minimize its results[34].

Differently from what happened in this study of the antibacterial activity of C. spruceanum, some works showed that the action of calcium hydroxide in the control of *E. faecalis* is specifically potentiated when other types of treatments are associated, such as photodynamic therapy with LED[35], incorporation of silver nanoparticles in the short term[34], association with propolis extract36 and natural biopolymers present in endophytic fungi[36].

The antibacterial test against *E. faecalis* of the pure extract of *C. spruceanum*, is similar to other studies that confirm the antibacterial activity against this microorganism, such as plant extracts of *Punica Granatum* against *Staphylococcus aureus*[37]. Among these, we can also highlight bioactive compounds from different plant extracts with antibacterial activity against *E. faecalis* (*Agastache foeniculum, Artemisia absinthium, Evernia prunastri, Humulus lupulus, Laurus nobilis, Origanum vulgare,* and *Vaccinium myrtillus*)[38], Cotula*cinerea extract*[39], and Propolis extract[40].

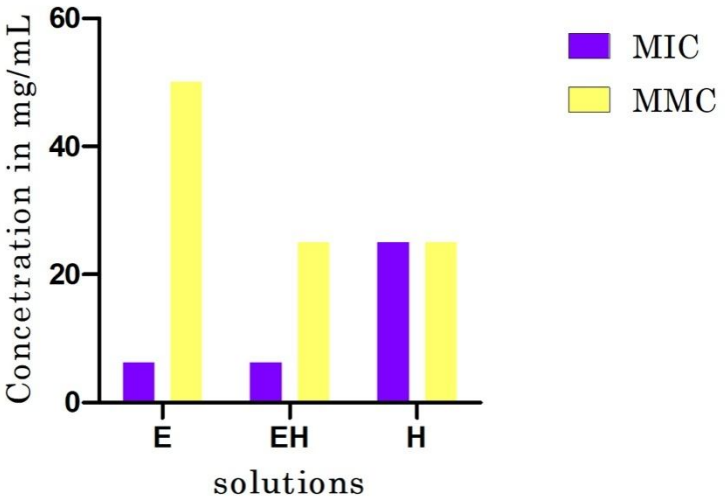
In the Minimum Inhibitory Concentration of C. spruceanum extract, the lowest value capable of inhibiting the growth of *E. faecalis* from the pure extract (E) was 6.25 mg/mL, for the extract associated with calcium hydroxide (EH) it was at 6.25mg/mL and pure calcium hydroxide (H) at 25mg/mL (Graph 1).



Graph 1. Mean and standard deviation of the antibacterial bioactivity test.

The broth microdilution test was performed, as in other similar studies, to determine the Minimum Inhibitory Concentration (MIC), which is the lowest concentration capable of inhibiting bacterial growth[41]. These MIC results are in agreement with other studies on antibacterial activity such as the use of oregano essential oil against *Salmonella enterica*[42], basil essential oil against Salmonella *choleraesuis* with MIC similar to those found in this study[43].

The results of MMC, which is the Minimum Microbicidal Concentration, which in this study for the *C. spruceanum* extract was 50 mg/mL, and for the C. spruceanum extract associated with calcium hydroxide and pure calcium hydroxide, it was 25 mg/mL (Graph 2), which was the lowest concentration that prevented the growth of *E. faecalis*. Other similar studies also evaluated the minimum microbicidal rate, such as rosemary essential oil against *Staphylococcus aureus* and *Escherichia col*[44] and ginger essential oil against *Salmonella enterica*[45].



Graph 2. Microdilution test to evaluate the minimum inhibitory concentrationin triplicate.

4. CONCLUSION

***C. spruceanum* extract can be used as an antibacterial agent against *E. faecalis*, especially when associated with calcium hydroxide. However, new in vitro and in vivo studies should be carried out in this regard, to obtain a new effective medication to combat this microorganism.**

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