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Antimicrobial potential of *Casearia sylvestris* against oral bacteria

Potencial antimicrobiano da Casearia sylvestris frente a bactérias orais

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Resumo

Objetivo: O objetivo deste estudo foi obter extratos por diferentes métodos de extração de *Casearia sylvestris*, incluindo a obtenção de óleo essencial, a fim de comparar suas atividades antimicrobianas em relação aos enxaguatórios bucais convencionais com clorexidina contra bactérias orais. **Material e método:** Para esta avaliação, extratos das folhas foram obtidos por diferentes métodos de extração (infusão, decocção, maceração e percolação) utilizando diferentes sistemas de solventes: 100% de água, 100%, de etanol, metanol 100%, água: etanol 3: 7; água: etanol 7: 3; água: metanol 7: 3 e água: metanol 3: 7. O óleo essencial, que corresponde a uma fração volátil, foi obtido por hidrodestilação usando o aparelho de Clevenger modificado. O método de microdiluição em caldo foi usado para determinar os valores de concentração inibitória mínima (MIC) e a concentração bactericida mínima (MBC) para os seguintes microrganismos: *Streptococcus mutans* ATCC 25175, ATCC 49456 *S. mitis*, *S. sanguinis* ATCC 10556, *S. salivarius* ATCC 25975, *Lactobacillus casei* ATCC 11578 e *Enterococcus faecalis* ATCC4082. Gluconato de clorexidina foi usada como um controle positivo. **Resultado:** Todos os extratos avaliados no protocolo utilizado apresentaram valores de MIC superior a 400 ug/mL e alguns mostraram atividade bactericida. A atividade antimicrobiana do óleo essencial foi maior do que a atividade dos extratos, e a melhor concentração inibitória mínima e valores de concentração bactericida mínima foram obtidos contra *L. casei* (MIC de 0,023 ug/mL e MBC de 0,046 ug/mL) e *S. mutans* (MIC de 25 ug/mL e MBC de 50 ug /mL), respectivamente. **Conclusão:** O óleo essencial de *Casearia sylvestris* tem atividade antimicrobiana significativa contra microrganismos orais.

Descritores: Pesquisa de laboratório; cáries dentárias; microrganismos; fitoterapia.

Abstract

Aim: The aim of this study was to obtain *Casearia sylvestris* leave extracts by different extractive methods, including the obtention of essential oil, in order to compare their antimicrobial activities to conventional mouthwash chlorhexidine against oral bacteria. **Material and method:** For this evaluation, extracts from the leaves were obtained by different methods of extraction (infusion, decoction, maceration and percolation) using different solvent systems: water 100%, ethanol 100%, methanol 100%, water: ethanol 3:7; water: ethanol 7:3; water: methanol 7:3 and water: methanol 3:7. The essential oil, which corresponds to a volatile fraction, was obtained by hydrodistillation using Clevenger modified apparatus. The microdilution broth method was used to determine the values of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for the following microorganisms: *Streptococcus mutans* ATCC 25175, *S. mitis* ATCC 49456, *S. sanguinis* ATCC 10556, *S. salivarius* ATCC 25975, *Lactobacillus casei* ATCC 11578 and *Enterococcus faecalis* ATCC4082. Chlorhexidine gluconate was used as a positive control. **Result:** All extracts evaluated in the used protocol displayed MIC values higher than 400 µg/mL and few showed bactericidal activity. The antimicrobial activity of essential oil was higher than the activity of the extracts, and the best minimum inhibitory concentration and minimum bactericidal concentration values were obtained against *L. casei* (MIC of 0.023 µg/mL and MBC of 0.046 µg/mL) and *S. mutans* (MIC of 25 µg/mL and MBC of 50 µg/mL), respectively. **Conclusion:** The essential oil of *Casearia sylvestris* has significant antimicrobial activity against oral microorganisms.

Descriptors: Laboratory research; dental caries; microorganisms; phytotherapy.

INTRODUCTION

Dental plaque has been considered the main etiologic agent in the initiation of dental caries and periodontal disease, the most common form of which is gingivitis[1]. Periodontal diseases are bacterial infections that affect the supporting structure of the teeth[2]. Among these, dental caries is a localized and transmissible infectious process that ends in the destruction of hard dental tissue[2]. Normally, periodontal diseases are related to anaerobes such as *Treponema denticola* and *Porphyromonas gingivalis*[3]. Dental decay is biofilm dependent; one of the fundamental imbalances that cause the onset of cariogenic biofilm is a rich and frequent diet of fermentable carbohydrates, mainly sucrose[4].

Many bacteria are involved in dental caries, mainly *Streptococcus mutans*[2], which is capable of fermenting carbohydrates resulting in acid production and leading to the demineralization of the tooth. The tooth surface provides the necessary conditions for the adherence and establishment of bacteria, such as *Streptococcus sanguis, Streptococcus mitis, Streptococcus gordonii, Streptococcus mutans* and *Actinomyces* species, which can be considered early colonizers of the enamel surface[5]. Once the bacteria are established on the surface, plaque forms if the surface isn't adequately cleaned. Besides this, *E. faecalis* is also found in the mouth and may cause other diseases[6].

The use of mouthrinses containing anti-plaque or those known to have antimicrobial contents[2] should complement toothbrushing as a potential prophylactic method of reducing plaque-mediated disease[2]. One of the chemical agents widely used against oral bacteria is chlorhexidine. It has higher solubility in water and dissociates to release a cationic component.

Chlorhexidine is the only compound that has substantivity, i.e., its active residence time in the buccal cavity is approximately 12 hours, which can be explained by its chemical structure. The interactions of charges in the film and with the bacteria exert a bactericidal action immediately after the initial rinse, combined with a prolonged bacteriostatic activity[7]. However, when used for an extended period, chlorhexidine causes undesirable side effects, such as stains on teeth and restorations, changes in taste, epithelial desquamation and a burning sensation on the tongue[8].

Casearia sylvestris is considered a promising source of compounds that have an antimicrobial activity[9]. There are already patents to use these Brazilian plant species in a composition of drugs that promotes the healing of herpetic lip lesions, which represents a major breakthrough for herbal medicine[10]. Besides this, some phytotherapics are available in the public health system for medical treatments according to the law of 2006[11]. *Maytenus ilicifolia, Mikania glomerata, Cynara scolymus* and more than 9 plant species are approved until now[12]. However, only a few studies have compared the antimicrobial activity of chlorhexidine with different extracts and essential oil from *Casearia sylvestris*. The aim of this study was to obtain *Casearia sylvestris* leaf extracts by different extractive methods, including the obtention of essential oil, in order to compare their antimicrobial activities with the conventional mouthwash chlorhexidine against *Streptococcus mutans, S. mitis, S. sanguinis, S. salivarius, Lactobacillus casei* and *Enterococcus faecalis*.

MATERIAL AND METHOD

Phytochemical Procedures

Plant material

Dried leaves of *Casearia sylvestris* collected in August 2011 were acquired from Shop Herbs Natural Products Company (CNPJ 08.898.383/0001-79). The leaves were crushed and packed in a glass jar, were properly identified and were covered to avoid contact with air and moisture.

Extracts preparation

Extracts were prepared using the following extractive processes:

I. Infusion

The volume of 30 mL of distilled water was heated to boiling and immediately placed in a glass beaker, which contained one gram of the powdered leaves of *Casearia sylvestris*. This mixture was allowed to stand for 15 minutes and then filtered. The filtrate was transferred to lyophilization flasks and was then frozen and lyophilized.

II. Decoction

One gram of the powdered leaves of *Casearia sylvestris* was placed for heating in 30 mL of distilled water. After the start of boiling, we counted 15 minutes. The mixture was then filtered, and the filtrate was frozen and lyophilized in the same way as previously mentioned.

III. Maceration

a) Static

One gram of the powdered leaves of *Casearia sylvestris* was placed with their respective solvent (water 100%, ethanol 100%, methanol 100%, water: ethanol 3:7; water: ethanol 7:3) into a beaker and allowed to stand for 24 hours. After this period, the suspension was filtered, the filtrate was frozen and a further 30 mL of the same solvent was added to the plant residue. This methodology was repeated three times consecutively over 3 days.

b) Exhaustive

The studied variables in this procedure were the same as in the previous method. However, the plant drug (one gram of powdered leaves of *Casearia sylvestris*) and the solvent were continuously stirred for 2 hours followed by filtration and solvent exchange three consecutive times, totaling an experiment that lasted 6 consecutive hours. After obtaining these statements, they were pooled, frozen and lyophilized.

IV. Percolation

The percolator was filled with one gram of powdered leaves of *Casearia sylvestris* and solvent (water 100%, ethanol 100%, methanol 100%, water: ethanol 3:7; water: ethanol 7:3; water: methanol 7:3 and water: methanol 3:7). This was left to rest (conceptually called maceration) for 6 hours and then the process of percolation was initiated at a rate of 30 drops/minute. Note: The ethanol and methanol were totally evaporated under vacuum conditions, and water was removed by lyophilization.

Essential oil obtention

Fraction of volatile compounds of plant leaves was obtained by hydrodistillation using Clevenger apparatus, following the parameters of the Brazilian Pharmacopoeia edition[13]. The oil obtained was packed in a properly sealed jar and was maintained in a freezer.

Evaluation of Antimicrobial Activity

Bacteria

The following bacteria acquired from the American Type Culture Collection (ATCC) were used: *Streptococcus mutans* ATCC 25175, *Streptococcus mitis* ATCC 49456, *Streptococcus sanguis* ATCC 10556, *Streptococcus salivarius* ATCC 25975 and *Enterococcus faecalis* ATCC 4082.

Antimicrobial activity assay

The minimum inhibitory concentration (MIC) values of the extracts and essential oil from *Casearia sylvestris* leaves were determined in triplicate using the broth microdilution method, according to CLSI (Clinical and Laboratory Standards Institute). The samples were dissolved in DMSO (dimethyl sulfoxide) at 1 mg/mL and were diluted in tryptone soya broth to achieve concentrations in the range 500-20 µg/mL. The final concentration of DMSO was 5% (v/v), and this solution was used as a negative control.

The inoculum was adjusted for each organism after 24h growing in blood agar to yield a cell concentration of 5×10^5 colony-forming units (CFU/mL) using the 0.5 MacFarland scale. One inoculated well was included to control the adequacy of the broth to support the growth of the organisms, and one non-inoculated well, free of antimicrobial agent, was also used to ensure the sterility of the medium. Chlorhexidine was used as a positive control. The microplates (96-well) were incubated at 37 °C for 24h. After that, 30 µL of resazurin (0.02%) in aqueous solution was added to indicate the viability of the microorganisms[14]. The MIC was determined as the lowest concentration of the extract capable of inhibiting microorganism growth. For the determination of either bacteriostatic or bactericidal activities, the entire volume of each well of all the incubated microplates was sub-cultured on blood agar at 37 °C for 24 h. The absence of viable cell growth indicated a bactericidal effect. The minimum bactericidal concentration was defined as the lowest concentration that enabled no growth on blood agar.

Analysis of essential oil by gas chromatography coupled to mass spectrometry

The essential oil sample obtained from the hydrodistillation process was submitted for analysis by gas chromatography coupled with mass spectrometry. Analyses were performed on Shimadzu model GC / MS QP-2010 equipped with a source of electrons by ionization (EI) and quadrupole-type analyzer. The chromatographic conditions used were as follows: ZB-5MS column (0.25mm × 0.25um 30mx)-Phenomenex, injector temperature of 230 °C and hydrogen in a mobile phase (gas flow rate: 1 mL. min^{-1}). The column temperature was kept initially at 60 °C for 1 minute, was increased to 240 °C after 10 minutes and was then kept at this temperature for 60 minutes.

After elution, the flow divider was used to drive the samples into the ionization source with a ratio of 1:40. The source temperature was set at 250 °C and the energy of the electron beam was 70 eV. The analyzer was checked for separating ions of m / z 40 to 400.

RESULT

The yield of each extract was evaluated and the largest was obtained by percolation process using, as the solvent, a mixture of water/methanol (3:7). The smallest was obtained by percolation using water.

The amount of essential oil obtained in the process of hydrodistillation of the dried leaves, which was collected in August 2011, was 0.85 mL/100 g of dry leaves.

All extracts displayed MIC values higher than 400 µg/mL and few showed bactericidal activity. In terms of their activity against *S. mutans*, the three solvent types—ethanol:water (7:3) in exhaustive maceration, 100% ethanol in percolation and water:methanol (7:3) in percolation—displayed values of 400 µg/mL for MBC. In terms of its activity against *S. mitis*, ethanol 100% in percolation also showed MBC values of 400 µg/mL. All the other extracts had MBC levels greater than 400 µg/mL.

All the values of MIC and MBC for the essential oil are presented in Table 1.

Due to the good activity displayed by the essential oil, a sample was analyzed using gas chromatography coupled with mass spectrometry in order to identify the constituents. The mass spectra gave the molecular weight and the fragmentation profile of each component. Therefore, by comparing the data obtained from the Wiley library, it was possible to identify the major constituents: β-caryophyllene, germacrene D, bicyclogermacrene, δ-cadidene, caryophyllene oxide, α-spathulenol and α-elemol. Figure 1 shows the chromatogram and the chemical names of the identified major constituents.

DISCUSSION

Mouth rinses have been used in dentistry as an adjuvant in oral hygiene. Among these, the most used rinse is chlorhexidine, which can be considered a gold standard anticariogenic, although regular use of this rinse is often associated with many negative side effects. Therefore, in order to find another antiplaque agent that can

Table 1. Values of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *Casearia sylvestris* essential oil in µg/mL

Bacteria	Essential oil (µg/mL)		Chlorhexidine (µg/mL)
	MIC	MBC	MIC
S. mutans	25	50	0.365
S. mitis	100	200	2.95
S. salivarius	100	200	1.495
L. casei	0.02	0.05	0.7375
S. sanguinis	100	100	0.7375

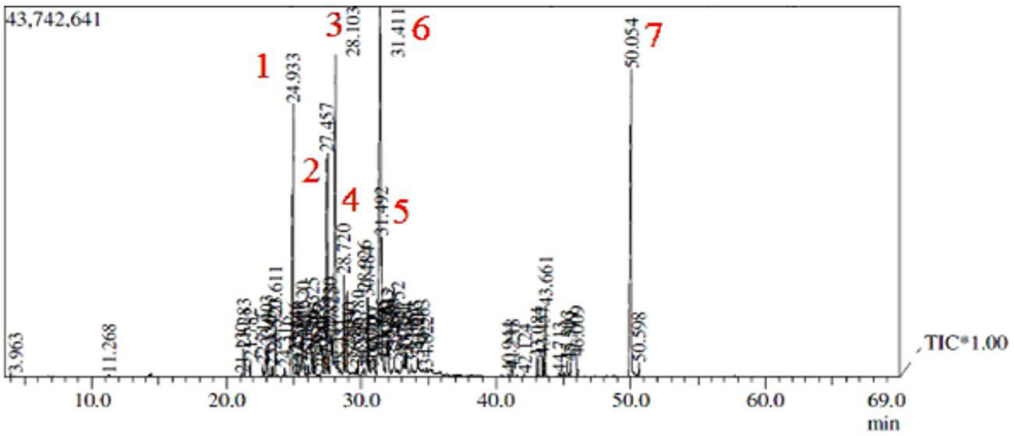

Figure 1. *Casearia sylvestris* essential oil cromatogram 1: β-caryophyllene (RT=24.933); 2: germacrene D (RT=27.457); 3: bicyclogermacrene (RT=28.103); 4: δ-cadidene (RT=28.720); 5: caryophyllene oxide (RT=31.492); 6: α-spathulenol (RT=31.411); and 7: α-elemol (RT=50.054).

be used on a daily basis without the side effects of chlorhexidine, our research group has decided to investigate the antimicrobial potential of a shrub described in the Brazilian pharmacopoeia.

Studies with *C. sylvestris* reported in the literature were carried out with methanol, aqueous, and ethanol extracts. It was shown to have anti-hiperlipidemic, antinoceptive, wound healing and anti-inflammatory activities[15-17]. In this work, different solvents (water, ethanol and methanol in different proportions) were used in different extraction procedures to obtain different types of extracts in order to further analyze the antimicrobial potential. It was also decided to evaluate the essential oil extracted from the leaves through the Clevenger apparatus, because the anti-inflammatory activity of this oil has been described in the literature[18,19]. The MIC values of the obtained extracts were not promising, showing values above 400 µg/mL. Values below 100µg/mL for extracts and fractions can be considered promising[20].

The essential oil displayed minimum inhibitory concentration values and minimum bactericidal concentration values, which is promising in terms of the development of formulations that demonstrate antimicrobial activity. The essential oil did not show activity in the evaluated concentrations against *E. faecalis,* which is an enteric facultative gram-positive bacterium that can grow independently in the root canal without the assistance of other bacteria and is also resistant to other agents such as calcium hydroxide[21] and sodium hipochlorite[22].

Although chlorhexidine displayed lower values of minimum inhibitory concentration for all assayed microorganisms than those of essential oil, the obtained results seem to be satisfactory as the methanol extract of *Hydrastis canadensis* L. showed a minimum inhibitory concentration of 250 µg/mL against *Streptococcus mutans* and has been introduced into the formulation of a number of mouth rinses and toothpastes on the U.S. market[23].

Essential oils are volatile odoriferous oils constituted by a complex mixture of compounds and produced as the secondary metabolites of some plants. Generally, components at a larger percentage determine the biological properties of the oil.

The essential oil composition depends on the stage of a plant's development and the presence of micronutrients, soil quality, temperature and seasonality. This work was carried out with *C. sylvestris* collected in August 2011 and which provided essential oil with a good antimicrobial activity.

Our results were qualitatively similar to others in the literature. They identified about 25 constituents, the most predominant of which was the presence of germacrene D, bicyclogermacrene, δ-cadinene, spathulenol, caryophyllene oxide and β-caryophyllene at different levels[24].

Most of the compounds identified belong to the group of sesquiterpenes, such as bicyclogermacrene, germacrenes D, caryophyllene oxide and β-caryophyllene, which are defined in the literature as possessing anti-inflammatory, anti-swelling, antitumor, antihistaminic, bactericide and repellant properties[16,25].

CONCLUSION

It can be concluded that the essential oil of *Casearia sylvestris* presents significant antimicrobial activity against oral bacteria. The results achieved in this work highlighted the antimicrobial potential of *Casearia sylvestris* essential oil, which could be introduced into the formulation of a number of mouth rinses, toothpastes or other types of phytotherapy for use in dental clinics.

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CONFLICTS OF INTERESTS

The authors declare no conflicts of interest.

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