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Assessment of the antimicrobial activity of *Casearia sylvestris* extract against oral pathogenic microorganisms

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ABSTRACT

An ethanolic extract of leaves from the tree *Casearia sylvestris*, known as guaçatonga in Brazil, was tested for *in vitro* activity against oral pathogenic bacteria and fungi. The results showed susceptibility of all the microorganisms tested. This study suggests a potential use of ethanolic extract of *C. sylvestris* as a novel treatment of oral infectious conditions, such as denture stomatitis, periodontitis and dental caries.

Keywords: *Casearia sylvestris*; guaçatonga; oral microorganisms; antimicrobial activity.

INTRODUCTION

Medicinal plants have been used for many years to cure a great variety of diseases. Recently, according to the World Health Organization, the use of traditional herbal medicines has spread not only in developing countries, but also in the industrialized ones, as a complementary way to treat and prevent illnesses (Phillipson, 2003). Plant extracts constitute rich sources of novel compounds with a variety of pharmacological activities. In many countries, plant extracts have been traditionally used for the treatment of oral mucosal lesions and periodontal diseases without any scientific validation (Basile et al., 1990).

Casearia sylvestris (Flacourtiaceae) is an American shrub or small tree known popularly in Brazil by names such as guaçatonga or porangaba, words originating from the indigenous tupi-guarani language, indicating an age-old usage of this species by native Brazilian communities (Oberlies et al., 2002; Esteves et al., 2005). This plant species has been widely used in popular medicine as an antiseptic, topical anesthetic, anti-tumor, anti-ulcer and anti-ophidian agent (Basile et al., 1990) and, more recently, as an adjuvant to weight-loss treatments. It has been reported that the extract obtained by boiling the plant is used by popular communities against fever, as well as for treating herpes, diarrhea and snakebites (Esteves et al., 2005).

The development of new treatments for diseases of the oral cavity is of great relevance, since the systemic administration of antimicrobials has been found to cause the development of multiresistant microorganisms, intermicrobial transfer of resistance factors, and side effects (Walker, 1996).

There has been little research on the antimicrobial activity of *C. sylvestris* extracts against pathogenic microorganisms. The aim of this study was to investigate the *in vitro* susceptibility of oral pathogenic bacteria and fungi to *C. sylvestris* extracts.

MATERIAL AND METHODS

Plant material

C. sylvestris leaves were collected in Sabará (23º 43' 7.8" S, 50º 45' 23.5" W, elevation 926m, Minas Gerais State, Brazil), in July 2005. A voucher specimen was deposited at the Herbarium of the Laboratory of Pharmacognosy, Faculty of Pharmacy, Universidade Federal de Minas Gerais (UFMG), in Belo Horizonte, Brazil.

Preparation of plant extract

Leaves of *C. sylvestris* were washed and homogenized with deionized water (500 g/250 mL) in a Waring blender, for 15 min at room temperature, and then filtered through a fine sieve. The filtrate was centrifuged at 30,000×*g* for 20 min and the supernatant was lyophilized (*Cs*) and the dry extract stored at −20°C. The twigs were washed, dried at 40°C and then homogenized and processed as described elsewhere (Sertie et al., 2000). Leaf and twig extract was weighed and dissolved in phosphate-buffered saline (PBS) before use (50 mg/mL).

Microbial strains

The strains of the microorganisms used in this study were from the Institute of Biological Sciences,

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UFMG: *Streptococcus mutans* (ATCC 70069), *Tannerella forsythia* (ATCC 700191), *Staphylococcus aureus* (ATCC 12692), *Porphyromonas gingivalis* (ATCC 33277), *Fusobacterium nucleatum* (ATCC 31647), *Actinobacillus actinomycetemcomitans* (ATCC 33384), *Lactobacillus casei* (ATCC 14435), *Candida albicans* (ATCC 18804), *C. tropicalis* (ATCC 750), *C. dubliniensis* (ATCC MYA-179). All strains were stored at 20°C in the appropriate medium containing 10% glycerol and regenerated twice before use in the manipulations.

The yeasts *C. albicans, C. tropicalis* and *C. dubliniensis* were grown initially in Sabouraud dextrose broth for 24h at 35°C in aerobic conditions. The anaerobic strains *T. forsythia, A. actinomycetemcomitans, F. nucleatum* and *P. gingivalis* were grown in brain heart infusion broth supplemented with yeast extract (5%), hemin (1%) and menadione (1%) for 48h at 37°C in anaerobic conditions [CO_2 (10%) N_2 (90%)]. The aerobic bacteria *S. aureus* was grown initially in thioglycollate broth and on BHI agar for 24h at 37°C in aerobic conditions. *S. mutans* and *L. casei* were grown in BHI broth and on BHI agar for 19h at 37° C in microaerophilic conditions.

Minimal Inhibitory Concentration (MIC) by the Pour Plate Method

The MIC was defined as the lowest in a series of concentrations at which no microbial growth was observed after incubation at 37ºC for 48h. The Pour Plate Method was used to measure growth inhibition by disk diffusion of the toxic substance, as described by DiFiore et al. (1983). Serial dilutions of *C. sylvestris* extract were tested in plates seeded with each organism, as follows: 0.0625, 0.125; 0.25; 0.5; 0.7; 0.8; 0.9, 1 µg/mL. Each antimicrobial test also included plates containing the culture medium plus ethanol as the solvent control. All sensitivity tests were done in Muller Hinton agar plates, in triplicate. Tetracycline and nystatin were used as positive controls and compared. The inhibition zone diameters were measured after 48h incubation of plates at 37ºC.

Agar Dilution Method

The antibacterial activity of *C. sylvestris* extract dilutions was also determined by the agar dilution method, in Muller Hinton agar (Oxoid-USA). The aerobic and anaerobic bacterial strains were maintained individually in suitable growth media. The obligate anaerobes were kept in pre-reduced medium in an anaerobic atmosphere (10% CO_2, 90% N_2). The density of the inoculum was adjusted to a turbidity of 0.5 on the McFarland scale (1.5 x 10^8 bacteria/mL). 100 µL of each bacterial suspension was spread onto Muller Hinton agar plates containing 1/128-1/2048 dilutions of *C. sylvestris* extract, which were prepared on the same day. The results (concentrations inhibiting 50% and 90% of tested bacteria) were read after incubation of plates at 37ºC for 48h.

The antifungal activity of *C. sylvestris* solutions, containing 1/64-1/8000 dilutions, against *Candida* spp., was estimated in RPMI 1640 medium (Sigma-USA) with L-glutamine and phenol red and without sodium bicarbonate. *Candida* spp. suspensions were prepared from colonies of the strains that had grown on Sabouraud dextrose agar (Difco-USA) after 24h of incubation. Yeast suspensions were inoculated into microplate wells that contained 1/64-1/8000 dilutions of *C. sylvestris* extract. Microplates were examined after incubation at 37ºC for 48 h.

A positive control was performed for each pathogen on a separate plate containing tetracycline and nystatin at the same concentration as the test drug mixed with liquid agar. Growth of microorganism was monitored visually. Agar plates without solutions were also prepared and used as growth controls. All tests were performed in triplicate.

Statistical analysis

The diameters of the inhibition zones were reported as mean ± standard deviation (M±SD). The inhibitory activity of the tested solutions against the oral pathogenic microorganisms was compared by nonparametric Kruskal-Wallis tests. Differences were considered to be significant when $p<0.05$.

RESULTS

In this study, all microorganisms showed sensitivity to *C. sylvestris* extracts. The results are shown in Table 1. MIC values were higher for anaerobic gram-negative periodontopathogenic strains (>1 µg/mL), with the exception of *F. nucleatum*. Among the bacteria, the lowest MICs were observed for *S. mutans* and *L. casei*, which were also the most sensitive bacteria according to the inhibition zones. MICs and inhibition zones for *C. albicans* and *C. dubliniensis* were similar, while *C. tropicalis* was the most sensitive fungus of the strains tested. For all strains tested, statistical differences were observed between the inhibition zones produced by the *C. sylvestris* extracts and the positive control.

DISCUSSION

C. sylvestris is a perennial medicinal plant distributed throughout Brazil, also growing in Mexico, Antilles islands and elsewhere in Central and South America (Lorenzi & Matos, 2002). The plant used in this study was selected on the basis of interviews with healers and individuals with experience in traditional medicine.

Table 1 - Diameters of inhibition zones (mean ± standard deviation) and MIC of ethanolic extracts of *C. sylvestris* for oral bacteria and fungi.

Microorganism	MIC (µg/mL)				Casearia sylvestris Eth 1:10 w/v	Nys	Tetr
	50%[a]	90%[a]	Nys	Tetr	Inhibition zone (mm)		
C. albicans	0.5	0.8	0.5	-	12.8±0.31	21.0±0.60	-
C. tropicalis	0.25	0.5	0.5	-	14.0±0.00	20.3±1.52	-
C. dubliniensis	0.5	0.8	0.5	-	13.6±0.13	22.0±0.00	-
S. aureus	0.25	0.5	-	0.125	11.9±0.90	-	21.3±1.64
S. mutans	0,125	0.5	-	0.8	14.00±0.00	-	22.0±1.50
A. actinomycetemcomitans	0.75	>1	-	0.8	10.6±0.18	-	16.5±0.50
P. gingivalis	0.5	>1	-	0.125	9.88±0.10	-	18.0±0.50
T. forsythia	0.8	>1	-	0.5	10.9±1.02	-	20.2±1.00
F. nucleatum	0.5	0.80	-	0.8	9.00±0.00	-	15.6±1.57
L. casei	0.125	0.5	-	0.25	13.00±0.00	-	23.0±0.00

Key: Eth=ethanolic, Nys=nystatin, Tetr=tetracycline. MIC=Minimum Inhibitory Concentration; [a]=50% and 90%, MIC that inhibits/kills 50% and 90% of the tested strain; *mean of three experiments

In Brazil, *C. sylvestris* leaves are prepared for topical application for anti-inflammatory, antiviral, antiulcer, anaesthetic and hemostatic purposes, especially for injuries of the mucosae and skin (Sato, 1998; Sertie et al., 2000). The chemical make-up of *C. sylvestris* is complex; its leaves contain phytochemical (diterpene) molecules considered similar to piroxicam and meloxicam (Almeida, 1999; Oberlies et al., 2002). Esteves et al. (2005) determined the composition of *C. sylvestris* as follows: caryophyllene (13.8%), thujopsene (5.2%), humulene (3.7%), acoradiene (20.8%), germacrene-D (1.9%), bicyclogermacrene (40.9%), calamenene (1.5%), germacrene B (3.9%), spathulenol (12.6%) and globulol (2.2%). The major terpene detected in essential oil of *C. sylvestris* was bicyclogermacrene (sesquiterpene) (Esteves et al., 2005). According to de Abreu Gonzaga et al. (2003), this compound possesses anti-inflammatory and antibacterial activities.

Several studies have described *C. sylvestris* as a source of casearins, which are diterpenes known for their cytotoxic activity (Morita et al., 1991; Oberlies et al., 2002). A new clerodane diterpene isolated from *C. sylvestris* showed pronounced activity against *Trypanosoma cruzi* (Espindola et al., 2004).

The resistance to antimicrobials detected in oral pathogenic bacteria and fungi in clinical cases has stimulated the search for natural agents as alternative treatments for infectious conditions of the oral cavity (Stamatis et al., 2003; Filoche et al., 2005). In this study, we showed the antimicrobial efficacy of *C. sylvestris* extract against bacteria and fungi associated with oral diseases, such as dental caries, periodontitis and candidosis (MICs<1 µg/mL).

MIC levels of up to 1.0 µg/mL for *A. actinomycetemcomitans, P. gingivalis* and *T. forsythia;* 0.8 µg/mL for *C. albicans, C. dubliniensis* and *F. nucleatum*, and 0.5 µg/mL for *L. casei, C. tropicalis, S. aureus* and *S. mutans,* were able to inhibit the growth of 90% of these microorganisms.

It is difficult to compare our results with those reported in the literature because of the naturally varying chemical composition of the extract, due to differences in chemotypes, harvest times and extraction methods. Furthermore, it is important to consider the different microbiological tests used in different studies and the variable sensitivities of the strains (Concha et al., 1998; Chao et al., 2000).

As indicated, we tested important oral pathogens for several oral diseases*: S. aureus* (dental abscesses); *S. mutans* and *L. casei* (dental caries); *A. actinomycetemcomitans, T. forsythia, P. gingivalis* and *F. nucleatum* (gingivitis and periodontitis) and *Candida* spp. (candidosis). There is no previous mention in the literature of *C. sylvestris* activity against oral microorganisms.

This study suggests the potential role of ethanolic extracts of *C. sylvestris* as an alternative treatment of oral infectious conditions. Further studies are needed to investigate the clinical efficacy of *C. sylvestris* solutions in patients with oral infections. Chemical studies are also needed in order to characterize the molecules responsible for the antimicrobial activity of *C. sylvestris* extract.

RESUMO

Avaliação da atividade antimicrobiana do extrato de Casearia sylvestris contra microorganismos patogênicos da cavidade bucal

A atividade antimicrobiana do extrato etanólico de *Casearia sylvestris* (guaçatonga), planta endêmica no Brasil, foi analisada contra bactérias e fungos patogênicos da cavidade bucal. Os resultados demonstraram sensibilidade de todos os microorganismos estudados. Este trabalho sugere o potencial uso do extrato etanólico de *Casearia sylvestris* como uma forma de tratamento alternativo de condições infecciosas da cavidade bucal, como estomatite protética, periodontite e cárie dentária.

Palavras-chave: *Casearia sylvestris*; guaçatonga; microorganismos orais; atividade antimicrobiana.

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