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ORIGINAL RESEARCH

Antimicrobial Potential of Plant Extracts and Chemical Fractions of *Sideroxylon obtusifolium* (Roem. & Schult.) T.D. Penn on Oral Microorganisms

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ABSTRACT

Aim: The aim of this study is to evaluate the *in vitro* antimicrobial activity of plant extracts and chemical fractions of *Sideroxylon obtusifolium* T.D. Penn on *Streptococcus mutans*, *Streptococcus oralis*, *Streptococcus salivarius*, *Streptococcus parasanguinis*, and *Candida albicans* as well as to identify the chemical classes found in the bioactive extracts possessing better activity.

Materials and methods: Freeze-dried hydroalcoholic extracts of the bark and leaves (LC and LF respectively) and ethanol extracts of bark and leaves (EC and EF respectively) of *S. obtusifolium* were assessed for antimicrobial potential by determining the minimum inhibitory concentration (MIC), minimum bactericidal concentration, and minimum fungicidal concentration (MFC). The tests were performed by microdilution method (in triplicate) in three independent experiments. Phytochemical characterization was performed by quantification of total polyphenols, total flavonoids, and condensed tannins.

Results: The EC extract presented weak antimicrobial potential on the growth of *S. mutans* (MIC = 1000 µg/mL); all extracts showed moderate inhibitory activity on the growth of *C. albicans* (MIC = 500 µg/mL). The dichloromethane and n-butanol fractions of LF extracts showed moderate growth inhibitory activity (MIC = 250 µg/mL) on *C. albicans* and fungicide potential (MFC/MIC = 2). The phytochemical characterization revealed a predominance of total polyphenols (EC = 29.23%; LC = 25.98%) and condensed tannins (LC = 38.84%; LF = 17.78%).

Conclusion: The dichloromethane and n-butanol fractions of *S. obtusifolium* LF extract showed antifungal activity against *C. albicans*, with the potential for bioprospection of phytocompounds for the treatment of periodontal fungal diseases caused by this microorganism. The effect may be related to phytochemical compounds from the polyphenol and condensed tannin classes.

Clinical significance: Research for new oral microbial disease treatment alternatives in bioactive compounds from medicinal plants is of clinical relevance and scientific interest since many therapeutic antifungal agents do not obtain the effectiveness expected due to microbial resistance, or to adverse effects on human tissues.

Keywords: *Candida albicans*, Flavonoids, Laboratory research, Medicinal plants, Polyphenols, *Sapotaceae*, *Streptococcus*, Tannins.

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INTRODUCTION

Biofilms are composed of small colonies of bacterial origin, nonrandomly organized in a matrix composed of water, extracellular polysaccharides, proteins, salts, and cells. It is known that there are specific intrabacterial associations in dental biofilm, which represent the main etiologic agents of several periodontal diseases,[1-3] with *Streptococcus mutans* and *Streptococcus sobrinus* being the principal species associated with dental caries.[3]

Candida albicans are yeast often found in dental biofilms, increasing the production of exopolysaccharides and consequent accumulation of microbial mass,[4] in addition to being responsible for oral candidiasis, the most common fungal infection in humans.[5]

The success of antimicrobial therapy requires the apparently contradictory roles of maintaining biofilms at levels consistent with the oral health, but without disrupting the beneficial properties of the resident microbiota. A major challenge for the future will be the development of products with satisfactory clinical efficacy, which preserves the benefits of the oral microbiota.[6]

In view of the resistance of microorganisms to antimicrobial substances known, experiments with medicinal plants in search of new bioactive compounds present themselves as a promising alternative.[7,8] In addition, *in vitro* studies have demonstrated satisfactory antimicrobial activity from plant extracts.[9]

Sideroxylon obtusifolium (Roem. & Schult.) T.D. Penn, *Sapotaceae*, is known as "*quixaba*" or "*rompe-gibão.*" It extends from the semiarid northeast to the southeastern region of Brazil[10-12] and is a native species from the "Caatinga" biome.[13] The bark of the plant has been used by the population for traumas, pain in general, duodenal ulcer, gastritis, nausea, chronic inflammation, genital lesion, inflammation in the ovaries, kidney problems, heart problems, and diabetes.[14] Infusions of the leaves are used as anti-inflammatories; however, information about the constituents of *S. obtusifolium* remains scarce.[15]

In Northeastern Brazil, because of its medicinal properties, *S. obtusifolium* is a popular plant species. Still, many plants frequently used by local populations have not been studied, and their active principles not identified to validate them as medicines nor to economically explore.[16]

In this perspective, the aim of this study was to evaluate the *in vitro* antimicrobial activity of extracts and chemical fractions of *S. obtusifolium* on *S. mutans*, *Streptococcus oralis*, *Streptococcus salivarius*, *Streptococcus parasanguinis*, and *C. albicans*, as well as identify the classes of chemicals in the bioactive extracts with the best activity.

MATERIALS AND METHODS

Collection and Identification of Plant Material

The bark and leaves of *S. obtusifolium* T.D. Penn were collected in the month of February 2013 in the semiarid region of Paraíba State, Sítio Capim Grande, rural area of the municipality of Campina Grande (7° 22′ 25″ S), Meso region of the Borborema, Paraíba State, Northeast Brazil. According to the current legislation (Provisional Measure No. 2.186/2001), which regulates the collection, access, and transportation/shipping of genetic heritage components, and access to associated traditional knowledge, we performed bark and leaf plant collections so as not to cause death or injury to the vegetal species. The *S. obtusifolium* T.D. Penn examples were deposited in the collection of the Manuel Arruda Câmara (School) Herbarium of the Paraíba State University – UEPB, identified with Voucher number: 583.

Preparation of Plant Extracts

The bark and leaves were cleaned and subjected to drying in an oven for air circulation (FANEM – Model 330/5) at 40°C until final weight stabilization, and subsequently crushed in a knife mill (SOLAB – Model SL 30) with a 10-mesh diameter. The yields of bark and leaves were 41.56 and 48.76% respectively, from their initial weight.

Hydroalcoholic extracts were produced at 70% and ethanol extracts at 96% of the *S. obtusifolium* bark and leaves, in the proportion of 200 gm dried and ground plant to 1 L of solvent, by maceration method. The extracts were subjected to evaporation under reduced pressure in a rotary vaporizer at 40°C and 70 rpm. After this step, the hydroalcoholic extracts were dried at room temperature between −20°C and −40°C. The following obtained extracts were tested as antimicrobials: Freeze-dried hydroalcoholic extracts of leaf and bark (LF and LC) and ethanol extracts of the leaf and bark (EF and EC). The extracts were packed in glass containers, protected from light and stored under refrigeration.

Microorganisms and Susceptibility Testing

We used strains of the American Type Culture Collection from the Fundação Oswaldo Cruz: *S. mutans* (27,175), *S. parasanguinis* (903), *S. salivarius* (7073), *S. oralis* (10,557), and *C. albicans* (10,231). The preparation of the inoculum and susceptibility tests for determination of minimum inhibitory concentration (MIC) was performed according to the recommendations of protocol M7-A6 for bacteria[17] and M27-A3 for yeast.[18] For the reactivation of the microorganisms, preparation of the inoculum and the testing of antimicrobial activity we used brain heart infusion (BHI) broth culture – HIMEDIA® and BHI agar – media HIMEDIA®, plus 5% mutton blood lysate for bacteria, and sabouraud dextrose broth – HIMEDIA® and sabouraud dextrose agar – HIMEDIA® culture media for the yeast.

The freeze-dried hydroalcoholic and ethanol extracts were dissolved in ethanol at 40% yielding test extracts at the concentration of 4000 µg/mL. The tests were performed according to broth microdilution method, in 96-well microplates. We deposited 100 µL of the culture medium in all wells, and then added 100 µL of the extract to be tested; the contents of the wells were homogenized and successive dilutions were performed. We added 100 µL of the microorganism inoculum to be evaluated.

For each concentration of the tested extract, we performed procedures in triplicate in three independent experiments. The concentrations of the extracts in the wells ranged from 1,000 to 15,625 µg/mL. Control of the culture medium and control of growth of the microorganisms were realized with positive controls using chlorhexidine 0.12% for bacteria, and nystatin 100,000 IU/mL for yeast, and solvent control. The plates were incubated for 24 hours at 37°C in a microaerophilous environment for bacteria, and for 48 hours at 37°C for the yeast. After the incubation period was added 20 µL of aqueous resazurin solution (Sigma-Aldrich®) at 0.01% for visual reading of antimicrobial activity.[19-21] The MIC was defined as the lowest concentration of antimicrobial agent, which prevented the visible growth of the microorganism in sensitivity tests,[17] verified through change of the medium color.

For determinations of minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC), a portion of the well corresponding to MIC and previous wells was shown in agar BHI plus sheep blood (5%) for bacteria, and agar sabouraud dextrose for yeast.

The MBC/MFC corresponded to the lowest concentration of the extract that prevented visible microbial growth in a solid culture medium.[7]

All procedures for the characterization of antimicrobial potential were performed in triplicate and in three independent experiments.

Chemical Characterization

The extracts with better antimicrobial activity were quantified for their contents of total polyphenols, total flavonoids, and condensed tannins.

For determination of total polyphenols, we used the Chandra and De Mejia Gonzalez method[22]; for determination of the total flavonoids content, we followed Meda et al method;[23] the content of condensed tannins was quantified using the Makkar and Becker method.[24] All analyses were performed in triplicate.

The freeze-dried hydroalcoholic extracts with better activity were fractionated using organic solvents in increasing order of polarity: Hexane, dichloromethane, ethyl acetate, and n-butanol. The final portion corresponded to the aqueous phase. Each extract fraction was evaporated under reduced pressure in a rotary evaporator at 40°C, with a speed of 70 rpm, and then stored under refrigeration. All fractions were subjected to tests characterizing antimicrobial potential.

RESULTS

Table 1 presents the antimicrobial testing results (MIC, MBC, and MFC) of the *S. obtusifolium* hydroalcoholic freeze-dried and ethanol extracts.

Table 2 presents the phytochemical quantification of the freeze-dried *S. obtusifolium* hydroalcoholic and ethanol extracts for total polyphenols, total flavonoids, and condensed tannins.

Table 3 presents the antimicrobial potential of chemical fractions of the lyophilized hydroalcoholic extracts of *S. obtusifolium* bark and leaves against *C. albicans*.

DISCUSSION

Sideroxylon obtusifolium is a popular plant species in Northeastern Brazil because of its medicinal properties.

Table 1: Antimicrobial potential of chemical fractions of the lyophilized hydroalcoholic and ethanol extracts of bark and leaves of *S. obtusifolium*

	Antimicrobial potential µg/mL									
	S. mutans		*S. oralis*		*S. parasanguinis*		*S. salivarius*		*C. albicans*	
Extracts	*MIC*	*MBC*	*MIC*	*MBC*	*MIC*	*MBC*	*MIC*	*MBC*	*MIC*	*MFC*
LF	>1,000	*	>1,000	*	>1,000	*	>1,000	*	500	>1,000
LC	>1,000	*	>1,000	*	>1,000	*	>1,000	*	500	>1,000
EF	>1,000	*	>1,000	*	>1,000	*	>1,000	*	500	>1,000
EC	1,000	1,000	>1,000	*	>1,000	*	>1,000	*	500	>1,000

*Testing was not performed due to lack of activity in the MIC test

Table 2: Quantification of total polyphenols, total flavonoids, and condensed tannins of the hydroalcoholic freeze-dried and ethanolic bark and leaf extracts of *S. obtusifolium*

	Total polyphenols		*Total flavonoids*		*Condensed tannins*	
Extracts	*mg/gm*	*%*	*mg/gm*	*%*	*mg/gm*	*%*
LF	223.77 ± 37.52	22.37 ± 3.75	17.03 ± 1.09	1.70 ± 0.10	177.88 ± 32.16	17.78 ± 3.21
LC	259.83 ± 24.76	25.98 ± 2.47	2.00 ± −0.10	0.20 ± −0.01	388.44 ± −28.21	38.84 ± −2.82
EF	131.38 ± 2.48	13.13 ± 0.24	14.39 ± 0.49	1.43 ± 0.04	137.87 ± 4.89	13.78 ± 0.48
EC	292.35 ± −6.03	29.23 ± −0.60	2.25 ± −0.02	0.22 ± −0.002	157.05 ± 21.23	15.70 ± 2.12

Each concentration of total polyphenols, total flavonoids, and condensed tannins corresponds to the average of three replicates ± standard deviation

Table 3: Antimicrobial potential of chemical fractions of the lyophilized hydroalcoholic extracts of bark and leaves of *S. obtusifolium* against *C. albicans*

	Antimicrobial potential (µg/mL)									
	Hexane fraction		*Dichloromethane fraction*		*Ethyl acetate fraction*		*N-butanol fraction*		*Aqueous solution fraction*	
Extracts	*MIC*	*MFC*	*MIC*	*MFC*	*MIC*	*MFC*	*MIC*	*MFC*	*MIC*	*MFC*
LF	500	1,000	250	500	500	>1,000	250	500	>1,000	*
LC	500	>1,000	500	>1,000	500	>1,000	500	>1,000	500	>1,000

*Not carried out due lack of results in the MIC test

Many plants are frequently used by local populations that have not been studied for their active principles nor identified to validate them as medicines, nor to leverage them economically.[16]

In this perspective of bioprospecting, the results of this study are presented and were compared with the related literature. According to the classification proposed by Holetz et al,[25] in Table 1, the extract EC presented weak antimicrobial potential on the growth of *S. mutans* (MIC = 1000 µg/mL). The other extracts were inactive for the *Streptococci* strains tested. Under the conditions of this study, the result suggested the infeasibility of prospective research on the extract as a potential antimicrobial agent against the strains of *S. mutans*, *S. oralis*, *S. parasanguinis*, and *S. salivarius*.

In this study, it was observed that all of the extracts tested showed antifungal drug potential against *C. albicans*, demonstrating moderate inhibitory activity on growth (MIC = 500 µg/mL), in line with the Holetz et al[25] classification. The fungistatic or fungicidal potential could not be assigned because the MFC/MIC relationship value was not well defined (>1000/500). We suggest further studies to establish the fungistatic or fungicidal potential of the extracts. The bacterial strains were sensitive to the control substance, chlorhexidine 0.12%, as well as *C. albicans* to nystatin 100,000 IU/mL, in all concentrations tested. Ethanol at 40%, used as a diluent of the extracts, did not affect the microbial growth in the concentrations tested.

Studies describe the action of *S. obtusifolium* bark and leaf extracts. Cruz et al[26] evaluated the antifungal effect, by diffusion in agar method, of lyophilized extract from *Quixabeira* bark on fungal species, among them, *C. albicans*. The authors note that the freeze-dried *Quixabeira* extract showed no antifungal activity against the species analyzed, in disagreement with the findings in this study, where we observed moderate inhibitory activity on the growth of *C. albicans*.

Methodological differences among studies may have influenced the different results for the antifungal effect of the freeze-dried bark extract of *S. obtusifolium*. According to Rocha et al[7] the diffusion in agar method, which presents limitations regarding the diffusion capacity of the substance, may have influenced the antifungal effect in the study of Cruz et al,[26] whereas in this study the action of freeze-dried extract from the *Quixabeira* bark was satisfactory.

Other factors, such as the regional differences as to the place of plant collection and different concentrations of the extract tested between the experiments can interfere in the potential of the antimicrobial activity of plant species under study.[27] The seasonality, type of soil, temperature, and climate can also interfere.[28,29] The method of extract preparation and the part of the plant used are also crucial to unlock the potential of bioactive constituents of studied plant species.[26,30] Differences as to the production method of extracts were also evidenced between the studies. In this study, we used maceration, and in the study by Cruz et al,[26] decoction was used.

The antibacterial potential of hydroalcoholic extracts at 70% of the bark and leaves of *S. obtusifolium* was evaluated on strains of *Staphylococcus aureus*, *Enterococcus faecalis*, and *Escherichia coli* by diffusion in agar and broth microdilution methods. For the diffusion in agar method, none of the extracts presented activity; however, the broth microdilution method for the bark extract showed bactericidal and bacteriostatic activity on *S. aureus* and *E. coli*, while the leaf extract showed the same activities for *S. aureus*.[7] The results do not corroborate the findings of Costa et al,[31] in which a 70% hydroalcoholic extract of *S. obtusifolium* bark presented activity against *E. faecalis* using the agar diffusion method.

The variability of results between the experiments that use similar extracts, microorganisms, and techniques may suggest that the antimicrobial activity evaluation of *S. obtusifolium* hydroalcoholic extracts is influenced by the agar diffusion method.

As to the chemical quantification, in Table 2, the more prevalent compounds in the *S. obtusifolium* bark and leaves extracts are total polyphenols and condensed tannins. Regarding the polyphenols content, the extract EC stood out, followed by the LC extract. For the quantification of condensed tannins, the extract LC stood out followed by the LF extract. The presence of these compounds may be related to the inhibitory action of the EC extract on the growth of *S. mutans,* and with the action of the extracts LC and LF on *C. albicans*, as shown in Table 1.

The abundant presence of polyphenolic compounds was also reported for ethyl acetate bark extract of *S. obtusifolium* and may be linked to the antioxidant potential and antibacterial activity presented against *S. aureus*.[32] This extract corroborates the antimicrobial activity results related to the EC extract in this study, which showed the greatest quantification of total polyphenols, as shown in Table 2, and was the only one that showed inhibitory potential on the growth of *S. mutans* as shown in Table 1. Condensed tannins were quantified in all of the freeze-dried and ethanol extracts, which presented moderate inhibitory activity on the growth of *C. albicans*.

The presence of tannins and flavonoids in *S. obtusifolium* EC extract was also found in a study by Araújo-Neto et al,[33] corroborating the quantification of these compounds in this study.

The ability of tannins to complex with proteins gives them the ability to precipitate these compounds helping, for example, in the inhibition *S. mutans* glycosyltransferase enzymes, as well as other inhibitory effects on fungi.[34] This study suggests that the levels of tannins found in Table 2 may have influenced the antibacterial action presented in this study by the EC extract on *S. mutans*, and the antifungal effects presented by all of the extracts tested on *C. albicans*, as shown in Table 1.

Since the LF and LC extracts showed moderate antimicrobial activity against *C. albicans*, they were fractionated, aiming to verify the antimicrobial activity in their respective fractions, with possibilities for future studies for isolation of compounds responsible for this action.

According to Table 3, the fractions of dichloromethane and n-butanol of the LF extracts showed moderate inhibitory activity against growth (MIC = 250 µg/mL) of *C. albicans* and fungicidal potential (MFC/MIC = 2), with better results as compared with the activity observed in the crude LF extract (Table 1). The hexane fraction showed similar inhibitory activity against fungal growth as compared to that presented by crude LF extract (MIC = 500 µg/mL), however, fungicidal potential (MFC/MIC = 2) was presented, as not previously demonstrated. The increase of the LF antimicrobial potential for dichloromethane and n-butanol fractions may have been influenced by the presence of bioactive compounds with better inhibitory activity on growth, and death of *C. albicans* when separated from crude extract. This result corroborates with Pereira et al,[35] who demonstrated the antifungal activity of n-butanol leaf extracts of *S. obtusifolium* in deleterious effects on the morphology and viability of biofilms treated with this extract, suggesting investigation as a potential alternative for the treatment of *Candida* biofilms.

Höfling et al[36] confirmed the important activity of fractionated plant extracts on clinical isolates of *C. albicans* and inhibition of fungal membrane proteolytic activity, demonstrating an important alternative to control and prevent candidiasis. As for the fractionated bark extracts of *S. obtusifolium* (LC) in this study, the fractioning of the LC extract did not potentiate its action against *C. albicans*.

Studies on the antimicrobial potential of *S. obtusifolium* are scarce in the literature. This study opens the precedent of research to explore its antimicrobial potential (especially antifungal) for the isolation of compounds responsible for this specific action.

CONCLUSION

The *S. obtusifolium* LF extract dichloromethane and n-butanol fractions showed antifungal activity against *C. albicans*, and potential for phytocompound bioprospecting to treat periodontal fungal diseases caused by this microorganism. As for the antibacterial activity of *S. obtusifolium*, satisfactory potential for phytocompound bioprospecting for action against oral diseases on the bacteria tested was not found.

Chemical fractioning revealed polyphenols and condensed tannins as the classes of bioactive compounds with higher contents in the analyzed extracts, which may be related to the antifungal properties demonstrated in this study.

Clinical Significance

The antifungal effect of *S. obtusifolium* on *C. albicans* suggests further study, with the possibility of developing a phytocompound adjuvant therapy to treat oral candidiasis, resistant to conventional therapy. Research for new alternatives to treat fungal diseases of the oral cavity with active ingredients from plants is of clinical relevance and scientific interest, bearing in mind that many antifungal therapeutic agents do not reach expected efficacies due to microbial resistance, or have adverse effects on human tissues.

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