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Screening of selected plant-derived extracts for their antimicrobial activity against oral pathogens

Abstract

Background: Dental caries is a major public health concern that affects the populations of many countries worldwide. In this paper, we screened twenty-four plant-derived extracts for their antimicrobial activity against a representative panel of cariogenic bacteria.

Methods: The leaves of each species were dried, powdered and sequentially extracted with *n*-hexane, dichloromethane and methanol under Sonication for 5min. The minimum inhibitory concentration (MIC) values of the resulting extracts were determined by using the broth micro dilution method in 96-well micro plates. Chlorhexidine was used as positive control. The chemical composition of the most active extract was determined by gas chromatography –mass spectrometry (GC–MS).

Results: The *n*-hexane extract of *P. neochilus* (PN-Hex) afforded the lowest MIC values against *S. mitis* (MIC =31.2µg/mL), *S. mutans* (MIC=31.2µg/mL), *S. sanguinis* (MIC=31.2µg/mL), *S. salivarus* (MIC= 62.5µg/mL), *S. sobrinus* (MIC=62.5µg/mL), *E. faecalis* (MIC= 62.5µg/mL) and *L. casei* (MIC = 250µg/mL). GC-MS analysis of this extract revealed that spathulenol (46.1%), trans-caryophyllene (19.0 %), caryophyllene oxide (10.7 %) and germacrene D (7.8 %) were the major constituents in PN-Hex.

Conclusion: The *n*-hexane extract of *P. neochilus* (PN-Hex) displays promising antimicrobial activity against some cariogenic bacteria. Our results suggest that this extract might be promising for the development of new oral care products.

Keywords: oral pathogens, antibacterial activity, *plectranthus neochilus, streptococcus mutans,* dental caries

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Abbreviations: GC-MS, gas chromatography-mass spectrometry; CHD, chlorhexidine

Introduction

Dental caries and other periodontal diseases are a major public health concern that affects the populations of many countries worldwide.[1] These pathologies are associated with acidogenic and aciduric bacteria that adhere to the tooth surface as a structurally and functionally organized biofilm (dental plaque) and can destroy dental hard tissues.[2] These bacteria can reach the bloodstream and trigger other diseases such as endocarditis, brain abscesses, throat infections, respiratory and gastrointestinal system infections, and bacteraemia.[3,4]

The most efficient way to prevent caries and other periodontal diseases is to remove the biofilm by brushing and flossing the teeth and conducting periodic dental cleaning or prophylaxis.[5] Unfortunately, most people fail to maintain a sufficient level of oral control through mechanical removal only, which has called for the use of oral products containing antimicrobial ingredients as a complementary measure to diminish biofilm formation on the tooth surface.[6] Therefore, the use of chemicals as a complementary measure to diminish the tooth surface biofilm is necessary and has proven to be a valuable tool to diminish the tooth surface biofilm.[7,8] Currently, chlorhexidine has been the most effective antiplaque agent tested to date; but some reversible local side effects have led dentists to recommend its use for short periods only.[9] Several other antimicrobial agents including fluorides, phenol derivatives, ampicillin, erythromycin, penicillin, tetracycline, and vancomycin can also inhibit bacterial growth. Nevertheless, excessive use of these chemicals can disturb the oral and intestinal flora and cause microorganism susceptibility, vomiting, diarrhea, and tooth staining.[9] To find an alternative to the substances currently employed to prevent caries and to control plaques, researchers have investigated the antimicrobial activities of natural products and their potential as new chemotherapeutic agents for incorporation in dental products.[10]

As part of our ongoing research on the antibacterial activities of natural products as potential leads for use in dental products,[1,11,12] this paper reports on the evaluation of the antibacterial activity of twenty-four plant-derived extracts against a panel of oral pathogenic bacteria.

Materials and methods

Plant material and extraction

The plants used in this study were collected in May 2010 at "Sítio 13 de Maio" (20o26'S 47o27'W 977m) near Franca, State of São Paulo, Brazil. Voucher specimens of each species were deposited at the Herbarium of Departamento de Biologia, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, São Paulo, Brazil (Herbarium SPFR), as shown in Table 1.

Firstly, fresh leaves of each species were submitted to hydrodistillation in a Clevenger-type apparatus for 3h, as previously reported.[13,17] After the essential oil extraction, the leaves of each species were dried at room temperature, powdered and sequentially extracted with *n*-hexane, dichloromethane and methanol (1mL/10mg of powder) under Sonication for 5min. Samples were concentrated under reduced pressure to result in the *n*-hexane (HEX), dichloromethane (DCM) and methanol (MeOH) extracts.

Antimicrobial assays

The minimum inhibitory concentration (MIC) values of the plant-derived extracts were determined by using the broth microdilution

method in 96-well microplates.[18] The following standard strains from the ATCC were used: *Enterococcus faecalis* (ATCC 4082), *Streptococcus salivarius* (ATCC 25975), *Streptococcus sobrinus* (ATCC 33478), *Streptococcus mutans* (ATCC 25175), *Streptococcus mitis* (ATCC 49456), *Streptococcus sanguinis* (ATCC 10556) and *Lactobacillus casei* (ATCC 11578). Individual 24 h colonies from blood agar (Difco Labs, Detroit, Mich, USA) were suspended in 10.0mL of tryptic soy broth (Difco). The standardization of each microorganism suspension was carried out using spectrophotometer (Femto, São Paulo, Brazil) at wavelength (λ) of 625nm to match the transmittance of 81, equivalent to 0.5 McFarland scale (1.5 x 108 CFU/mL) and dilution at final concentration of the 5 x 105 CFU/mL. The samples were dissolved in DMSO (Merck, Darmstadt, Germany) at 4mg/mL, and they were then diluted in tryptic soy broth (Difco) so that concentrations in the range of 3.9 to 4000μg/mL were achieved. After dilutions, the DMSO concentrations were between 4% and 0.0039% (v/v). Three inoculated wells containing DMSO at concentrations ranging from 4% to 1% were used as negative controls. One inoculated well was included, so as to control the adequacy of the broth for organism growth. One non-inoculated well free of antimicrobial agent was also included to ensure medium sterility. Two-fold serial dilutions of chlorhexidine digluconate (Sigma) were made in tryptic soy broth (Difco) to achieve concentrations ranging from 59 to 0.115μg/mL. These dilutions were used as positive control. The microplates (96 well) were sealed with parafilm and incubated at 37°**C** for 24h. After that, 30mL of 0.02% reassuring (Sigma, St. Louis, MO, USA) aqueous solution was poured in each microplate reservoir, to indicate the microorganism viability.[19] The MIC (i.e., the lowest concentration of a sample capable of inhibiting microorganism growth) was determined as the lowest concentration of the extracts capable of preventing a colour change of the resazurin solution.[19] Three replicates were conducted for each microorganism.

Table 1 Plant species selected for this study and their respective voucher number and code

Plant	Family	C Code	Voucher number
Artemisia absinthum Vill.	Asteraceae	AA	12417
Bidens sulphurea(Cav.) Sch. Bip.	Asteraceae	BS	12020
*Ocimum gratissimum*L.	Lamiaceae	OG	12420
*Plectranthus neochilus*Schltr.	Lamiaceae	PN	12323
Syzygium aromaticum(L.) Merr. & L.M.Perry	Myrtaceae	SA	12418
Stachytarpheta cayennensis (Rich.) Vahl.	Verbenaceae	SC	10005
Tagetes erecta L.	Asteraceae	TE	10009
Tetradenia riparia (Hochst.) Codd.	Lamiaceae	TR	12421

Table 2 In vitro antibacterial activity (MIC) of the selected plant-derived extracts against oral pathogens

	S. mitis	*S. mutans*	*S. sanguinis*	*S. salivarus*	*S. sobrinus*	*E. faecalis*	*L. casei*
AA-Hex	≥4000	4000	4000	4000	≥4000	≥4000	≥4000
AA-DCM	4000	≥4000	≥4000	≥4000	≥4000	≥4000	4000
AA-MeOH	4000	4000	4000	4000	4000	≥4000	4000
BS-Hex	2000	4000	4000	4000	4000	4000	4000
BS-DCM	2000	4000	4000	4000	4000	≥4000	4000
BS-MeOH	1000	2000	1000	2000	2000	≥4000	4000
OG-Hex	≥4000	≥4000	≥4000	≥4000	≥4000	≥4000	≥4000
OG-DCM	≥4000	≥4000	≥4000	≥4000	≥4000	≥4000	≥4000
OG-MeOH	4000	≥4000	2000	4000	4000	≥4000	≥4000
PN-Hex	31.2	31.2	31.2	62.5	62.5	62.5	250
PN-DCM	62.5	62.5	62.5	250	250	250	1000
PN-MeOH	62.5	500	125	1000	1000	1000	4000
SA-Hex	2000	≥4000	4000	4000	4000	1000	≥4000
SA-DCM	2000	4000	4000	4000	4000	≥4000	500
SA-MeOH	≥4000	2000	≥4000	≥4000	2000	≥4000	≥4000
SC-Hex	250	125	500	4000	250	≥4000	4000
SC-DCM	250	250	500	2000	250	≥4000	≥4000
SC-MeOH	250	125	500	4000	250	≥4000	4000
TE-Hex	1000	4000	4000	4000	4000	≥4000	4000
TE-DCM	2000	4000	4000	4000	4000	4000	4000
TE-MeOH	2000	4000	4000	2000	4000	4000	2000
TR-Hex	125	62.5	1000	4000	250	4000	1000
TR-DCM	125	62.5	1000	4000	250	4000	1000
TR-MeOH	2000	4000	2000	2000	2000	4000	2000
CHD	14.7	1.8	7.3	7.3	1.8	14.7	3.6

Gas chromatography-mass spectrometry analysis

Gas chromatography–mass spectrometry (GC–MS) analysis was carried out on a ShimadzuQP2010 Plus (Shimadzu Corporation, Kyoto, Japan) system equipped with an AOC-20i autosampler. The column consisted of Rtx-5MS (Restek Co., Bellefonte, PA, USA) fused-silica capillary (length = 30m, i.d. = 0.25mm, and film thickness = 0.25μm). Helium (99.999%) at a constant flow of 1.0mL/min was the carrier gas. The injection volume was 0.1 μL (split ratio of 1:10). The injector and the ion source temperatures were set at 240 and 280 °C, respectively. The oven temperature was programmed to increase from 60 °C to 240 °C at 3 °C /min, then hold at 240°C for 20min. The

electron ionization (EI-MS) mode at 70eV was employed. The mass spectra were registered with a scan interval of 0.5s in the mass range of 40 to 600Da.

The chemical components of the *n*-hexane extract of *Plectranthus neochilus* were identified on the basis of their retention indices relative to a homologous series of *n*-alkanes (C8–C40) on a Rtx-5MS capillary column under the same operating conditions and computer matching with the Wiley 7, NIST 08 and FFNSC 1.2 spectral libraries of the GC-MS system, as well as by comparison of their mass spectra with those reported in the literature.[20]

Table 3 Chemical composition of the n-hexane extract of Plectranthus neochilus (PN-Hex)

Compound	RT	RI_{exp}	RI_{lit}	RA %	Identification
Trans-caryophyllene	23.84	1416	1418	19.0	RI, MS, Co
Alloaromadendrene	25.52	1457	1461	0.7	RI, MS
Germacrene D	26.47	1480	1480	7.8	RI, MS
a–amorphene	26.63	1484	1485	0.6	RI, MS
Valencene	26.88	1490	1491	0.2	RI, MS
g–cadinene	27.76	1512	1512	0.3	RI, MS
Caryophyllene oxide	30.12	1572	1573	10.7	RI, MS, Co
Sphatulenol	30.24	1575	1576	46.1	RI, MS
Neophytadiene	39.71	1834	1836	3.2	RI, MS
Ledane	40.63	1841	1844	3.4	RI, MS
trans-phytol	43.36	1943	1949	3.0	RI, MS
Squalene	53.96	2784	2790	1.7	RI, MS
a–tocopherol	59.08	305	3100	3.3	RI, MS
Total identified				99.9	

Results and discussion

This work investigated the antimicrobial activity of twenty-four extracts from the leaves of eight herbaceous or arbustive plant species against a representative panel of cariogenic bacterial strains. These species were selected on the basis of reports on their uses as antimicrobial in the folk medicine or previous studies on their antibacterial or antifungal activities.[1,11,12,21,22]

The antimicrobial activity was evaluated in terms of their minimum inhibitory concentration (MIC). Classification of the antibacterial activity was based on MIC values, as follows: MIC < 100mg/mL, good; 100 < MIC < 500mg/mL, moderate; 500 < MIC < 1000mg/mL, weak; MIC > 1000mg/mL, inactive.[23] Most of the evaluated extracts were inactive or weakly active against the panel of selected oral bacteria (Table 2). The *n*-hexane extract of *P. neochilus* (PN-Hex) afforded the lowest MIC values against *S. mitis* (MIC=31.2 µg/mL), *S. mutans* (MIC = 31.2µg/mL), *S. sanguinis* (MIC= 31.2µg/mL), *S. salivarus* (MIC=62.5µg/mL), *S. sobrinus* (MIC= 62.5µg/mL), *E. faecalis* (MIC=62.5µg/mL) and *L. casei* (MIC=250µg/mL). The dichlorometane (PN-DCM) and methanol (PN-MeOH) extracts of *P. neochilus* was also active against *S. mitis* (MIC = 62.5µg/mL). Moreover, the dichlorometane extract of *P. neochilus* (PN-DCM) and the *n*-hexane and dichloromethane extracts of *Tetradenia riparia* (TR-Hex and TR-DCM, respectively) were also active against *S. mutans* ((MIC=62.5µg/mL). The activity of PN-Hex, PN-DCM, TR-Hex and TR-DCM extracts against *S. mutans* is an interesting result because very few natural compounds can inhibit these bacteria, one of the primary causative agents of dental caries.[24] All the extracts of *Stachytarpheta cayenensis* displayed moderate activity against *S. mitis*, *S. mutans*, *S. sanguinis* and *S. salivarus*. None of the tested EOs was significantly active against *Lactobacillus casei* (Table 2).

The chemical composition of PN-Hex, the most active extract against the selected panel of cariogenic bacteria was determined by gas chromatography–mass spectrometry (GC-MS). A total of thirteen compounds were detected, with predominance of sesquiterpenes (88.8 %), as given in Table 3. The major constituents were identified as being spathulenol (46.1 %), *trans*-caryophyllene (19.0 %), caryophyllene oxide (10.7 %) and germacrene D (7.8 %). *Trans*-caryophyllene, caryophyllene oxide and germacrene D were previously detected in the essential oil from the leaves of *P. neochilus*.[1,15]

Crevelin and co-workers demonstrated that *trans*-caryophyllene and caryophyllene oxide, when tested alone against the same selected cariogenic bacteria as we used here, displayed MIC values higher than 4000µg/mL).[1] Aguiar and co-workers reported germacrene D as being the major constituent in the essential oil from the leaves of *Bidens sulphurea*, which displayed moderate activity against *Streptococcus mutans* (MIC=250µg/mL) and significant activity against *Streptococcus mitis* (MIC=31.2µg/mL).[11] On the other hand, although the antimicrobial activity of spathulenol against *Staphylococcus aureus* and *S. epidermidis* was previously reported,[25] it has been identified only as minor constituent in some essential oils with antimicrobial activity against cariogenic bacteria.[12]

Non-polar compounds are usually assumed to diffuse across the cell membranes easily and to kill microorganisms by effecting the metabolic pathways or organelles of the bacteria. Additionally, these compounds could interact with the bacteria membrane and elicit drastic physiological changes, causing loss of membrane permeability, which ultimately leads to cell death.[1] This hypothesis is consistent with the fact that of the *n*-hexane and dichloromethane extracts of *P. neochilus*, *S. cayennensis* and *T. riparia* were the most active against the selected panel of cariogenic bacteria, whereas most of the methanol extracts were inactive against these bacteria. In the case of PN-Hex, which was the most active extract against the cariogenic bacteria tested in this study, the sesquiterpenes spathulenol and germacrene D could be the responsible for its activity. Alternatively, the antimicrobial activity of PN-Hex may also be related to the other minor chemical constituents identified in the oil, which may underlie or even increase the activity of the major chemical constituents of PN-Hex.

Conclusion

The *n*-hexane extract of *P. neochilus* (PN-Hex) displays promising antimicrobial activity against some cariogenic bacteria, including *Streptococcus mutans*, which is one of the main causative agents of dental caries. Taken together, our results suggest that this

extract might be promising for the development of new oral care products. Further studies to isolate and to identify the chemical constituents of PN-Hex are underway.

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Conflicts of interest

Author declares there are no conflicts of interest.

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