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Bioautography to assess antibacterial activity of *Ottonia martiana* Miq. (Piperaceae) on the human oral microbiota

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ABSTRACT

***Ottonia martiana* Miq. (Piperaceae), a plant known popularly in southern Brazil as "anestésia" and used in the treatment of odontalgia for its anesthetic action on the oral mucosa, was investigated for antibacterial activity by paper disc agar diffusion and bioautographic methods, against microorganisms present in the human oral cavity [*Streptococcus mutans* (ATCC 25175), *Streptococcus mitis* (ATCC 49456), *Streptococcus pyogenes* (ATCC 19615), *Streptococcus salivarius* (ATCC 25975), *Escherichia coli* (ATCC 11229 and 25922), *Pseudomonas aeruginosa* (ATCC 27853) and *Enterobacter aerogenes* (ATCC 27853). The crude extract of *O. martiana* (32.9 mg mL^{-1}) had antibacterial potential against all Gram-positive bacteria tested. Analysis of the bioautograms led to the detection of bioactive substances, among which it was possible to identify piperovatine (Rf 0.35), piperlonguminine (Rf 0.52) and isopiperlonguminine (Rf 0.52). The piperovatine and isopiperlonguminine were isolated from the roots of *O. martiana*, guided by a bioautographic antibacterial bioassay.**

Keywords: Amides. Antibacterial activity. Bioautography. Toothache. *Ottonia martiana*.

INTRODUCTION

Although antimicrobial activity has already been demonstrated in many plant extracts, few studies on bacteria of clinical relevance in dentistry have been conducted in Brazil.

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Despite scientific advances, we are still constantly affected by caries and periodontal disease. Microorganisms of the human oral biota, such as *Streptococcus mutans*, are associated with the formation of dental plaque and are a determining factor in the development and progression of caries and periodontal disease (Pereira et al., 2009). Gram-negative bacteria, such as *Escherichia coli, Enterobacter* spp. and *Pseudomonas aeruginosa*, are opportunistic microorganisms colonizing the oral cavity, and have a role in the formation of oral biofilm and development of nosocomial pneumonia (Amaral et al., 2009). Studies on *Salmonella* bacteria revealed a family of proteins related to the formation of biofilms, promoting the colonization of new surfaces, and prevention, by oral hygiene and control of the oral biofilm, is of extreme importance (Amaral et al., 2009). Some of these microorganisms have acquired antibiotic resistance and this has led to a search for potential alternative antimicrobial substances in plants (Sarac & Ugur, 2007; Firas & Al-Bayati., 2008; Pereira et al., 2009).

Amongst some plant species popularly used to treat diseases of the oral cavity is *Ottonia martiana* Miq. (Piperaceae), a local shrub of the Brazilian Atlantic Forest (Cunico et al., 2006). In Brazil, *O. martiana* is known as "anesthesia" among the coastal community of Paraná state. The roots and aerial parts of *O. martiana* are largely employed to relieve toothache, in ethanolic mouthwashes, due to its anesthetic action on the oral mucosa (Cunico et al., 2005; Cunico et al., 2006). In South and Southeast Brazil, it is popularly known as "jaborandi", "jaguarandi" and "taburutá" (Cunico et al., 2005).

Phytochemical investigations of the roots and aerial parts of *O. martiana* resulted in the isolation of the isobutylamides piperlonguminine, isopiperlonguminine and piperovatine, bioactive substances of medical interest (Cunico et al., 2006). Seventy-seven compounds have been identified in three essential oils extracted from the leaves, fruits and roots of this species (Cunico et al., 2007). These chemical substances were previously isolated from a number of closely-related Piperaceae species (Cunico et al., 2005).

Recent phytochemical investigations of the Piperaceae species have traced the piscicidal, oral 'local anesthetic' and saliva-producing (sialogogic) properties of piperovatine and the insecticidal potential of piperlonguminine (Cunico et al., 2005).

Biological studies of the crude extract of roots and aerial parts of *O. martiana* show antimicrobial activity against both human and plant pathogens (Cunico et al., 2005; Cunico et al., 2007).

Although the antibacterial activity of "anestésia" has already been demonstrated with some microorganisms, no study has been conducted under bacterial conditions of clinical relevance to dentistry. Therefore, the principal aim of the present study is to evaluate the antibacterial activity of *O. martiana* against pathogenic microorganisms in the human oral microbiota.

EXPERIMENTAL

General

All reagents were of analytical grade. M.p. determined on a Kofler hot-stage (uncorr). Column chromatography and TLC were performed on silica gel 60 F_{254} (Merck). Mass spectra were collected with a Varian 3800 GC-EIMS system (70 eV), equipped with a CP-SIL 5 CB capillary column (60 m x 0.25 mm i.d. x 1.00 µm film thickness) with helium as the carrier gas flowing at 1.6 mL min^{-1}; injector temperature 300°C, detector temperature at 300°C, using a temperature program of 100-300°C at a heating rate of 20°C min^{-1}, standing 10 min at 300°C; injection in the split mode (1:100).

The bacterial strains were provided by the Laboratory of Basic Pathology, Federal University of Paraná. Tests were carried out in triplicate with strains of *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228), *Streptococcus mutans* (ATCC 25175), *Streptococcus mitis* (ATCC 49456), *Streptococcus pyogenes* (ATCC 19615), *Streptococcus salivarius* (ATCC 25975), *Escherichia coli* (ATCC 11229 e 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Enterobacter aerogenes* (ATCC 27853), *Salmonella choleraesuis* (ATCC 10708) and *Salmonella typhimurium* (ATCC 14028). Fresh cultures of each strain were standardized by dilution in sterile saline solution to 0.5 on the MacFarland scale (Bier, 1994). *Brain Heart Infusion agar* (BHI agar; Difco Laboratories) is an established medium for the culture of streptococci, pneumococci, meningococci and enterobacteria (ANVISA, 2004) and it was used in this experiment as the culture medium. The plates with *Streptococcus* spp. were incubated at 37ºC for 24 h in an aerobic atmosphere with 5% CO_2. Other plates were incubated at 37ºC for 24 h in air. We scored bacterial growth inhibition zones of diameter ≥ 7 mm (Haida et al., 2007). Bacterial tests were performed in triplicate and the developing inhibition zones were compared with those of the reference discs.

Plant material

Roots and aerial parts of *O. martiana* were collected in Paraná state, Southern region of Brazil, during April 2002. Dr. Gerdt Hatschbach of the Museu Botânico Municipal da Prefeitura de Curitiba, Paraná (MBM) identified these plant materials and voucher specimens are deposited at the MBM Herbarium under number 259,057.

Extraction, isolation and identification of the constituents

The dried and powdered total organs of *O. martiana* (700 g) were exhaustively extracted in three steps of 7 days maceration with 2L of 95% ethanol at ambient temperature. Extracts were pooled and the solvent evaporated under reduced pressure (40^{0}C), yielding 180 mL of dark, viscous extract (32.9 mg mL^{-1}). A part of the crude extract was partitioned with hexane (H), dichloromethane (D), ethyl acetate (A) and methanol (M), respectively. The crude extract (CE) and fractions obtained (FH – 0.86 g, FD – 0.42 g, FA – 0.1 g, FM – 0.5 g) were subjected to antimicrobial assay.

The air-dried and powdered roots of the *O. martiana* (470 g) were extracted with 95% ethanol in a Soxhlet extractor for 5 h on a hot stage [470 g /3L]. This extract was filtered and, after removal of the ethanol under reduced pressure, partitioned with 250 mL of hexane, dichloromethane, ethyl acetate and methanol, consecutively. The hexane fraction [RFHex (4.02 g)] obtained by evaporation of the solvent under vacuum at 40ºC was chromatographed on a column of silica gel and eluted first with hexane, then with solvent mixtures of increasing polarity (5%), through hexane, ethyl acetate and methanol, yielding 2.9 mg compound **1** (RFHex 68-72.1) and 7.0 mg compound **2** (RFHex 68-72.2), respectively, which were isolated by monitoring with a TLC procedure, direct comparison with an authentic sample and guided by bioautographic antibacterial bioassay.

Antimicrobial activity assay

The crude extract of *O. martiana* was tested for antimicrobial activity by the paper disc agar diffusion method, adapted from Pereira et al. (2009) and Amorim et al. (2006). Sterile paper discs (6 mm diameter) impregnated with 10 µL test sample (CE) were dried and placed on plates of previously inoculated BHI agar, whose surface had been spread with the previously standardized bacterial culture suspension. Negative control discs contained 10 µL of ethanol. In addition, chloramphenicol (Newprov-30 µg) and chlorhexidine gluconate (0.2%) were used as positive controls in the assay. The plates with *Streptococcus* spp. were incubated at 37ºC for 24 h in an aerobic atmosphere with 5% CO_2 (other plates at 37ºC for 24 h, in air). The results were recorded by measuring the zones of growth inhibition surrounding the discs.

The bioautography method (Valgas et al., 2007), adapted from Cunico et al. (2004), was based on the loading of 3 µL of test samples of CE (32.9 mg mL^{-1}), FH (1 mg mL^{-1}), FD (1 mg mL^{-1}), FA (1 mg mL^{-1}), FM (1 mg mL^{-1}), piperovatine (0.1 mg mL^{-1}) and isopiperlonguminine (0.1 mg mL^{-1}) on a TLC plate. The samples were first applied to a GF_{254} TLC plate (2.5 x 5.0 cm) and developed with hexane/EtOAc (7:3) as eluent. The plate was transferred to a Petri dish and overlaid with BHI agar containing 1%

triphenyltetrazolium chloride (TTC), which was inoculated with a standard bacterial suspension. Inhibition zones after 24 h of incubation (37ºC) indicated the presence of active compounds. Chloramphenicol (Newprov - 30 µg) and chlorhexidine gluconate were used as positive controls of growth inhibition.

To assess the chemical profile of the samples, a replicate TLC plate was developed simultaneously and visualized with Dragendorff's coloring reagent. By scraping samples from the TLC plates in zones with similar *Rf*s to the bioactive zones and analyzing the samples by GC-MS, it was possible to reveal the identity of the bioactive compounds.

RESULTS

The experiment was performed with two different extraction procedures. In the first process, by maceration, a crude extract of the whole plant of *O. martiana* was subjected to antimicrobial assay. In the second process, by Soxhlet extraction, a crude extract of the roots was prepared for isolation of bioactive compounds.

The bioautography assay confirmed the results obtained by disc agar diffusion and allowed the detection of several growth inhibition zones on TLC plates carrying both crude extract (CE) and hexane (FH) and dichloromethane (FD) fractions of *O. martiana*, against all the Gram-positive bacteria tested, indicating the presence of several bioactive compounds (Table 1).

Table 1 – Antibacterial activity of crude extract (32.9 mg mL^{-1}) and fractions (1 mg mL^{-1}) from roots and aerial parts of *Ottonia martiana* Miq., Piperaceae.

		*Inhibition zone (Disc agar diffusion) (mm)		Inhibition zones (IZ) (Bioautography)											
Microorganisms	Gram	CE 10 µL	CO (30µg) CG (0.2%)	CE 3µL		FH 3µL		FD 3µL		FA 3µL		FM 3µL		A/ B	
				IZ	Rfs	IZ	Rfs	IZ	Rfs	IZ	Rfs	IZ	Rfs	IZ	Rfs
Staphylococcus aureus (ATCC 25923)	G+	8.0	18.0 20.0	+ + +	0.35 0.52 0.81	+ + +	0.35 0.52 0.81	+ + +	0.35 0.52 0.81	-	-	-	-	+/ +	0.35/ 0.52
Staphylococcus epidermidis (ATCC 12228)	G+	8.0	18.0 20.0	+ + +	0.35 0.52 0.81	+ + +	0.35 0.52 0.81	+ + +	0.35 0.52 0.81	-	-	-	-	+/ +	0.35/ 0.52
Streptococcus mutans (ATCC 27175)	G+	9.0	18.0 20.0	+ + +	0.35 0.52 0.81	+ + +	0.35 0.52 0.81	+ + +	0.35 0.52 0.81	-	-	-	-	+/ +	0.35/ 0.52
Streptococcus mitis (ATCC 49456)	G+	9.0	18.0 20.0	+ + +	0.35 0.52 0.81	+ + +	0.35 0.52 0.81	+ + +	0.35 0.52 0.81	-	-	-	-	+/ +	0.35/ 0,52
Streptococcus pyogenes (ATCC 19615)	G+	7.0	18.0 20.0	+ + +	0.35 0.52 0.81	+ + +	0.35 0.52 0.81	+ + +	0.35 0.52 0.81	-	-	-	-	+/ +	0.35/ 0.52
Streptococcus salivarius (ATCC 25975)	G+	7.0	18.0 20.0	+ + +	0.35 0.52 0.81	+ + +	0.35 0.52 0.81	+ + +	0.35 0.52 0.81	-	-	-	-	+/ +	0.35/ 0.52
Escherichia coli (ATCC 25922)	G-	-	15.0 24.0	-	-	-	-	-	-	-	-	-	-	-/ -	-/ -
Pseudomonas aeruginosa (ATCC 27853)	G-	-	00.0 06.0	-	-	-	-	-	-	-	-	-	-	-/ -	-/ -
Enterobacter aerogenes (ATCC 13048)	G-	-	00.0 12.0	-	-	-	-	-	-	-	-	-	-	-/ -	-/ -
Salmonella choleraesuis (ATCC 10708)	G-	-	12.0 23.0	-	-	-	-	-	-	-	-	-	-	-/ -	-/ -
Salmonella typhymurium (ATCC 14028)	G-	-	12.0 23.0	-	-	-	-	-	-	-	-	-	-	-/ -	-/ -

ATCC (American Type Culture Collection) / **CE** (crude extract) / **CO** (Chloramphenicol) **CG** (chlorhexidine gluconate) / G+ (Gram positive) / G- (Gram-)
FH (hexane fraction) / **FD** (dichloromethane fraction) / **FA** (ethyl acetate fraction) /
FM (methanol fraction) / **A** [piperovatine (0.1 mg mL^{-1})] / **B** [isopiperlonguminine (0.1 mg mL^{-1})]
+(inhibition zone) / **-** (no inhibition zone) /dpm ± 0.01

Active spots at *Rf* 0.81 on the bioautographed TLC plates suggested the presence of a mixture of antibacterial compounds, whose identity remains to be elucidated.

Inhibition zones with similar *Rf*s, 0.35 and 0.52, respectively, were seen on the TLC plate bioautograms loaded with CE, FH, FD, isopiperlonguminine and piperovatine, against *S. mutans, S. mitis* and *S. pyogenes*, proving the identity of three of the substances responsible for this bioactivity. It should be clarified that, by scraping the TLC plates in zones with similar *Rf*s to the bioactive zones and analyzing these zones by GC-MS, it was possible to reveal the identity of the bioactive isobutylamides

(Figure 1): piperovatine (**1**), isopiperlonguminine (**2**) and piperlonguminine (**3**).

Piperovatine (1): White crystals. $C_{17}H_{23}NO_2$. MS *m/z* (rel. int): 273 ($[M]^{\bullet+}$, 28%), 201 (14%), 173 (100%), 159 (35%), 152 (46%), 139 (28%), 121 (22%), 115 (10%), 96 (23%).

Isopiperlonguminine (2): White-yellow crystals. $C_{16}H_{19}NO_3$. MS *m/z* (rel.int): 273 ($[M]^{\bullet+}$, 92%), 216 (26%), 201 (97%), 173 (77%), 172 (22%), 159 (20%), 152 (9%), 143 (39%), 139 (3%), 135 (12%), 121 (5%),115 (100%), 96 (5%).

Piperlonguminine (3): Yellow crystals. $C_{16}H_{19}NO_3$. MS *m/z* (rel. int): 273 ($[M]^{\bullet+}$, 80%), 216 (21%) 201 (100%), 173 (82%), 172 (17%), 159 (2%), 152 (20%),143 (20%), 139 (10%), 135 (15%), 121 (10%), 115 (65%), 96 (10%).

These results indicated an effective *in vitro* activity in the *O. martiana* root extract, piperovatine, piperlonguminine and isopiperlonguminine, which inhibits bacterial growth, encouraging further studies for its application in bacteria-associated disease of the oral cavity.

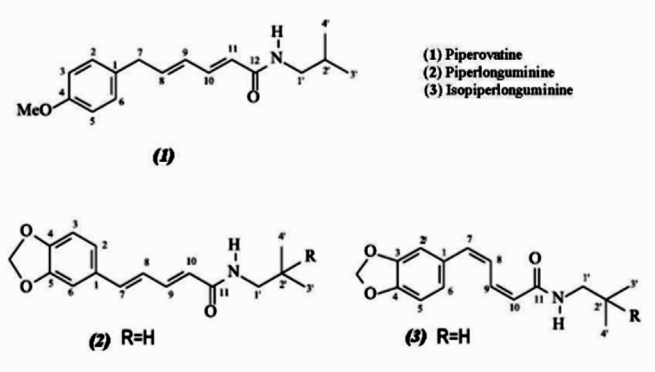

Figure 1. Chemical structures of the isobutylamides of Ottonia martiana.

DISCUSSION

Antibacterial screening by paper-disc agar diffusion revealed the activity of the crude extract of *O. martiana* against all Gram-positive bacteria tested. The apparently highest antimicrobial activity was observed in the crude extract against *S. mutans* and *S. mitis*, which was assessed by comparing the growth inhibition of bacteria by the sample disc with that by the control disc; however, the biggest inhibition halos were not assumed to show the most effective action of the extract. This method was used only as a qualitative preliminary assay, owing to its limitation for compounds with low diffusibility in the culture medium. Usually, in the methods proposed by the Clinical and Laboratory Standards Institute (*CLSI*), standard substances can be used for comparison, but for plant extracts there is a lack of standardization because they are a mixture of polar and nonpolar substances (Carelli et al., 2011). Diffusion tests with extracts of *O. martiana*, at 20.5 mg mL^{-1} against *Staphylococcus aureus* and *Staphylococcus epidermidis* on Mueller Hinton Agar (Cunico et al., 2003), and at 32.9 mg mL^{-1} against *Enterococcus faecium*, *Enterobacter aerogenes* and *Pseudomonas aeruginosa* on *Plate Count Agar* (Cunico et al., 2004), showed antimicrobial activity against both Gram-positive and Gram-negative bacteria and revealed the existence of interference, since *Staphylococcus epidermidis* did not exhibit inhibition. In addition to the culture medium used and the concentration of the extract, other factors may have affected the response, such as extraction methods and genetic strains of the microorganisms. Therefore, it is recommended that a preliminary factorial design study be done, when it is intended to prospect antimicrobial activity (Migliato et al., 2011).

Bioautography is a very convenient and simple test for plant extracts and pure substances, to assess their effects on both human and plant pathogenic microorganisms (Weltzien, 1958; Cunico et al., 2005). It was also employed here in the target-directed isolation of compounds **1** (2.9 mg) and **2** (7.0 mg) in the extract of the roots of *O. martiana* (RFHex 68-72).

The presence of isobutylamides in the extract of *O. martiana* (Cunico et al., 2007) confirms the expectation of antimicrobial activity in these extracts, but the possible existence of other bioactive compounds and synergy between them has not been disregarded.

Further studies on both the identity and the structure-activity relationship of these antibacterial substances from *O. martiana* remain to be performed.

RESUMO

Bioautografia para avaliação da atividade antibacteriana da Ottonia martiana Miq. (Piperaceae) sobre a microbiota oral humana

***Ottonia martiana* Miq. (Piperaceae), planta conhecida popularmente por "anestésia" e empregada no tratamento de odontalgias devido à sua ação anestésica sobre a mucosa oral, foi investigada por meio de ensaios antibacterianos de difusão em disco de papel e de bioautografia frente a microorganismos presentes na microbiota oral humana [*Streptococcus mutans* (ATCC 25175), *Streptococcus mitis* (ATCC 49456), *Streptococcus pyogenes* (ATCC 19615), *Streptococcus salivarius* (ATCC 25975), *Escherichia coli* (ATCC 11229 e 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Enterobacter aerogenes* (ATCC 27853). Os resultados dos bioensaios mostraram que o extrato bruto de *O. martiana* (32.9 mg mL^{-1}) apresenta potencial antibacteriano frente às bactérias Gram-positivas testadas. Dentre as substâncias bioativas detectadas foram identificadas a piperovatina (Rf 0.35), piperlonguminina (Rf 0.52) e a isopiperlonguminina (Rf 0.52). A piperovatina e isopiperlonguminina foram isoladadas do extrato das raízes de *O. martiana*, guiadas pelo teste de bioautografia.**
Palavras-Chave: Amidas. Atividade antibacteriana. Bioautografia. Odontalgia. *Ottonia martiana*.

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