

Antimicrobial activity of *Aegiphila sellowiana* Cham., Lamiaceae, against oral pathogens

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RESUMO: “Atividade antimicrobiana de *Aegiphila sellowiana* Cham., Lamiaceae, contra patógenos orais”. A atividade antimicrobiana de *Aegiphila sellowiana* Cham., Lamiaceae, contra patógenos da cavidade bucal foi avaliada empregando-se o método de microdiluição em caldo, segundo o protocolo CLSI M7-A7, utilizando-se clorexidina como controle positivo. O extrato etanólico bruto das partes aéreas de *A. sellowiana*, obtido por maceração, exibiu atividade contra os microrganismos testados neste trabalho, entretanto, os valores de CIM (concentração inibitória mínima) aumentaram após a partição com *n*-hexano, diclorometano e acetato de etila. Entre as frações testadas, a fração *n*-hexânica foi a mais efetiva contra os patógenos bucais avaliados, com valores de CIM entre 140 e 350 µg/mL⁻¹. Dados obtidos por CG-EM revelaram que ésteres de ácidos graxos, esteróides e hidrocarbonetos sesquiterpênicos alifáticos são os constituintes majoritários desta fração. Embora esses compostos possam ser os responsáveis pela atividade da fração *n*-hexânica, outros constituintes químicos presentes nas frações em diclorometano, acetato de etila e hidroalcoólica podem potencializar suas atividades no extrato bruto.

Unitermos: *Aegiphila sellowiana*, atividade antimicrobiana, CG-EM, patógenos orais, Lamiaceae.

ABSTRACT: The antimicrobial activity of *Aegiphila sellowiana* Cham., Lamiaceae, against oral pathogens is reported. The Minimal Inhibitory Concentrations (MICs) for inhibiting the microorganisms growth were determined using the broth microdilution method from the CLSI M7-A7 protocol. Chlorhexidine was used as the positive control. The ethanol crude extract of the aerial parts of *A. sellowiana* exhibited activity against the microorganisms tested in this work; however, the activity decreased after partition with *n*-hexane, dichloromethane, and ethyl acetate. Among the tested fractions, the *n*-hexane fraction was found to be the most effective against the evaluated oral pathogens. GC-MS analysis of this latter fraction revealed that fatty acids esters, steroids, and aliphatic sesquiterpene hydrocarbons are its major constituents. These compounds may be responsible for the activity of the *n*-hexane fraction, but other chemical constituents of the dichloromethane, ethyl acetate, and hydroalcoholic fraction may potentialize their activities in the crude extract.

Keywords: *Aegiphila sellowiana*, antimicrobial activity, GC-MS, oral pathogens, Lamiaceae.

INTRODUCTION

Dental caries and periodontal diseases are associated with oral pathogens, mainly bacteria (Botelho et al., 2007). Both of the cariogenic and periodontopathic bacteria present in the oral cavity form an adherent, structurally- and functionally-organized biofilm (Marsh, 2006). Strategies to control caries include inhibition of the

biofilm development (e.g. prevention of the attachment of cariogenic bacteria, delivery of effective antimicrobials etc.) or enhancement of the host defenses (Gilbert et al., 2002). Mechanical methods of oral hygiene such as brushing and flossing are the most commonly applied approaches for routine denture biofilm control (Sharma et al., 2004; Barnett, 2006). However, studies have indicated that most people fail to maintain a sufficient level of

biofilm control by brushing only, so that chemotherapeutic mouth rinses may play a key role as adjuncts to daily home care (Santos, 2003).

Several plant derivatives, including crude extracts (Wu et al., 2001; AbdElRahman et al., 2002), essential oils (Takarada et al., 2004; Prabuseenivasan et al., 2006), and pure compounds (Silva et al., 2007) have been evaluated with respect to their antimicrobial effects against oral pathogens. These derivatives have attracted the interest of many research groups, since they may be employed in the development of new mouth rinses for oral hygiene.

As part of our ongoing research on the antimicrobial activities of Brazilian plants and bioactive natural compounds (Oliveira et al., 2007; Silva et al., 2007; Cunha et al., 2007), and because previous reports documenting the antimicrobial activity of Verbenaceae species against oral pathogens (Chung et al., 2004; Botelho et al., 2007), we have investigated the *in vitro* antimicrobial potential of *Aegiphila sellowiana* Cham., Lamiaceae, popularly known as “tamanqueira”. This species is an arboreous (or sometimes arbustive) species, which naturally occurs in the tropical and subtropical regions of America, mainly in Brazil. Despite its use in Brazilian folk medicine as an anti-inflammatory agent and against snake venom (Leitão et al., 1992), there are no previous phytochemical or pharmacological investigations regarding *A. sellowiana*.

MATERIAL AND METHODS

Plant material

Aerial parts of *A. sellowiana* were collected near Usina Mascaranhas, Peixoto-MG, Brazil (20°16'S, 47°05'W G) by Wilson R. Cunha in December 2004 and identified by Prof. Alba R. de Araújo of the Botany Department of Universidade de Franca, Franca-SP, Brazil. A voucher specimen (# UEC 15320) has been deposited at the Herbarium of the Biology Institute, UNICAMP, Campinas – SP, Brazil.

Extraction and fractionation

Aerial parts of the plant (1.2 kg) were dehydrated, powdered, and exhaustively extracted by maceration with ethanol (Merck, Darmstadt, Germany) at 25 °C. A sample (25 g) of the resulting extract was dissolved with a minimum volume of a mixture of ethanol and distilled water (9:1 v/v), and it was successively partitioned with *n*-hexane, CH₂Cl₂, and ethyl acetate (3 x 500 mL) from Merck (Darmstadt, Germany).

Antimicrobial assays

The minimum inhibitory concentration (MIC) values of the crude extract and its fractions were determined

by using the broth microdilution method (CLSI, 2006) in 96-well microplates. 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 10.0 mL of tryptic soy broth (Difco). The standardization of each microorganism suspension was carried out using spectrophotometer (Femto, São Paulo, Brazil) at wavelength (λ) of 625 nm to match the transmittance of 81, equivalent to 0.5 McFarland scale (1.5×10^8 CFU/mL) and dilution at final concentration of the 5×10^5 CFU/mL. The samples were dissolved in DMSO (Merck, Darmstadt, Germany), at 1 mg/mL, and they were then diluted in tryptic soy broth (Difco) so that concentrations in the range 400 to 20 μ g/mL would be achieved. The final DMSO concentration was 4% (v/v), and this solution was used as negative control (concentrations ranging from 4 to 1%). 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 (Sigma) were made in tryptic soy broth (Difco) to achieve concentrations ranging from 5.9 to 0.0115 μ g/mL and were used as positive control. The microplates (96 well) were sealed with parafilm and incubated at 37 °C for 24 h. After that, 30 μ L of 0.02% resazurin (St. Louis, MO, USA) aqueous solution was poured in each microplate reservoir, to indicate the microorganism viability (Palomino et al., 2002). The MIC was determined as the lowest concentration of the extract or of its fractions capable of inhibiting microorganism growth. Three replicates were made for each microorganism.

Gas chromatography-mass spectrometry

Gas chromatography-mass spectrometry analysis was performed on a Shimadzu GCMS model QP2010 apparatus. Helium (99.999%) was used as the carrier gas at a constant flow rate of 1.62 mL/min. A DB5-MS (30 m x 0.25 mm i.d, film thickness 0.25 μ m, 5% crosslinked phenyl-methylpolysiloxane) column was employed. The column temperature was programmed to rise from 105 °C (0 min hold) to 200 °C at 13 °C/min, from 200 °C (0 min hold) to 240 °C at 5 °C/min, from 240 (20 min hold) to 280 °C at 10 °C/min, and it was finally maintained at 280 °C for 10 min. The injector temperature was set at 250 °C, with a split ratio of 1:60. The column outlet was inserted directly into the electron ionization source block, operating at 70 eV. The scan range was 40-500 Da. The identification of the individual components was carried out by comparing the mass spectra data with those of the spectra library Wiley.

RESULTS

The results of the *in vitro* antimicrobial activity of the crude ethanol extract of *A. sellowiana* (AS) and its *n*-hexane (AS₁), dichloromethane (AS₂), ethyl acetate (AS₃), and ethanol:water (AS₄) fractions are shown in Table 1.

Table 1. Antimicrobial activity of *A. sellowiana* determined by the broth microdilution method.

	Minimum inhibitory concentration (µg/mL)					
	<i>Chlorelidine</i>	AS	AS ₁	AS ₂	AS ₃	AS ₄
<i>Enterococcus faecalis</i>	0.3688	200	*	*	*	*
<i>Lactobacillus casei</i>	0.0230	*	150	*	*	*
<i>Streptococcus mitis</i>	0.3688	50	140	400	400	*
<i>Streptococcus mutans</i>	0.3688	200	250	400	*	*
<i>Streptococcus sanguinis</i>	0.0922	70	140	*	400	*
<i>Streptococcus sobrinus</i>	0.0115	80	250	*	*	*
<i>Streptococcus salivarius</i>	0.0461	80	350	*	*	*

*Inactive in the evaluated concentrations. AS refers to the crude ethanol extract of *A. sellowiana*; AS₁, AS₂, AS₃ and AS₄ refer to the *n*-hexane, dichloromethane, ethyl acetate and ethanol:water

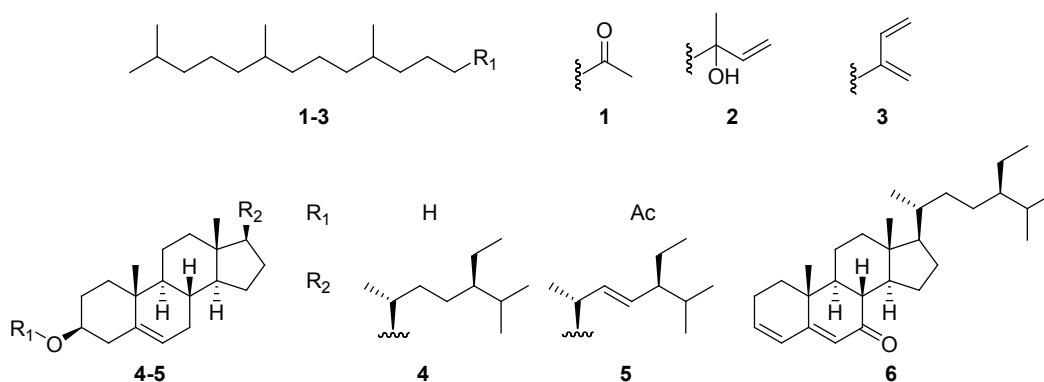
fractions of AS, respectively.

The GC-MS analysis of AS₁, the most active fraction, revealed the presence of the following major components: four fatty acid esters (methyl palmitate, 21.1%; ethyl palmitate, 8.3%; methyl oleate, 5.9%; methyl stearate, 2.1%; and ethyl oleate, 2.0%); three aliphatic sesquiterpene hydrocarbons (6,10,14-trimethyl-pentadecan-2-one (**1**), 7.7%; 3,7,11,15-tetramethyl-hexadec-1-en-3-ol (**2**), 5.3%; and 2,6,10-trimethyl-14-ethylenyl-pentadec-14-ene (**3**), 11.6%); and three steroids (stigmast-5-en-3-ol (**4**), 0.5%; stigmast-5,22-dien-3-ol acetate (**5**), 2.4%; and stigmast-3,5-dien-7-one (**6**), 1.3%).

DISCUSSION

At concentrations below 100 µg/mL, the crude extract of *A. sellowiana* (AS) displayed activity against most of the oral pathogens tested in this work, except for *S. mutans* and *E. faecalis*. However, the extract was found to be inactive against *L. casei*. The most sensitive microorganisms were *S. mitis* (crude extract concentration = 50 µg/mL) and *E. sanguinis* (crude extract concentration = 70 µg/mL). Considering the previous statements of Rios and Recio (2005) on the antimicrobial activity of medicinal plants, the MIC values of AS against *S. mitis*, *S. sanguinis*, *S. sobrinus* and *S. salivarius* were considered interesting.

Fractions AS₁, AS₂, AS₃ and AS₄ displayed activity at higher concentrations than the corresponding crude extract (AS), as evidenced by the MIC values. The



most active fraction was AS₁. On the other hand, at the evaluated concentrations, some fractions did not exhibit any activity against some bacteria and the MIC values were higher than 400 µg/mL. These results clearly demonstrate that AS fractionation results in a decrease of the *in vitro* antimicrobial activity.

The *n*-hexane fraction (AS₁) was the most active one. Its activity may be related to the presence of lipophilic compounds, mainly long chain fatty acids, which are the major constituents of this fraction. Lipophilicity is known to be closely related to permeation through a lipidic coating of bacteria (Tokuyama et al., 2001). These compounds are

often identified in natural extracts that exhibit antimicrobial properties (Petschow et al., 1996; Gyles et al., 2002). However, it is likely that the effects of these compounds on oral pathogens are potentialized by synergistic/additive effects of other minor chemical constituents present in AS₁, as well as by other compounds present in AS₂, AS₃, and AS₄.

In summary, the results obtained in this study demonstrate the antimicrobial potential of the crude ethanol extract of the aerial parts of *A. sellowiana* against oral pathogens.

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