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RESEARCH ARTICLE

Evaluation of the antibacterial activity of the methylene chloride extract of *Miconia ligustroides*, isolated triterpene acids, and ursolic acid derivatives

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Abstract

The methylene chloride extract of *Miconia ligustroides* (DC.) Naudin (Melastomataceae), the isolated compounds ursolic and oleanolic acids and a mixture of these acids, and ursolic acid derivatives were evaluated against the following microorganisms: *Bacillus cereus* (ATCC 14579), *Vibrio cholerae* (ATCC 9458), *Salmonella choleraesuis* (ATCC 10708), *Klebsiella pneumoniae* (ATCC 10031), and *Streptococcus pneumoniae* (ATCC 6305). The microdilution method was used for determination of the minimum inhibitory concentration (MIC) during evaluation of the antibacterial activity. The methylene chloride extract showed no activity against the selected microorganisms. Ursolic acid was active against *B. cereus*, showing a MIC value of 20 µg/mL. Oleanolic acid was effective against *B. cereus* and *S. pneumoniae* with a MIC of 80 µg/mL in both cases. The mixture of triterpenes, ursolic and oleanolic acids, did not enhance the antimicrobial activity. However, the acetyl and methyl ester derivatives, prepared from ursolic acid, increased the inhibitory activity for *S. pneumoniae*.

Keywords: Miconia ligustroides*; antibacterial activity; ursolic acid; oleanolic acid*

Introduction

Because pathogenic microorganisms build up resistance against antibiotics, much attention has recently been paid to extracts and biologically active compounds isolated from plant species (Essawi & Srour, 2000). Antimicrobials of plant origin are effective in the treatment of infectious diseases, and they are able to mitigate many of the side effects often associated with synthetic drugs (Iwu et al., 1999; Silva et al., 2004).

In many parts of Brazil there is a rich tradition of using herbal medicine in the treatment of various infectious diseases (Souza Brito & Souza Brito, 1993; Johann et al., 2007; Oliveira et al., 2007). *Miconia*, a genus bearing approximately 1000 species (Martins et al., 1996), belongs to the Melastomataceae family (Renner, 1993). Previous studies on *Miconia* species have revealed the presence of triterpenes, coumarins, and benzoquinones (Lowry, 1968; Macari et al., 1990; Chan et al., 1992; Gunatilaka et al., 2001). In recent work undertaken in our laboratory, several crude plant extracts from *Miconia* and their isolated compounds were reported to exhibit remarkable biological activities such as trypanocidal (Cunha et al., 2003, 2006), antitumoral (Cunha et al., 2008), antimutagenic (Resende et al., 2006), antimicrobial (Celotto et al., 2003; Cunha et al., 2007), analgesic, and anti-inflammatory activities (Spessoto et al., 2003; Vasconcelos et al., 2006). Ursolic and oleanolic acids are triterpenoid compounds that are widely distributed in the plant kingdom, and they have been frequently isolated from *Miconia* species as mutually isomeric mixtures (Cunha et al., 2006, 2008; Vasconcelos et al., 2006). These triterpene acids display several biological activities (Liu, 1995; Da Silva Filho et al., 2008). Ursolic acid and its 3-keto and 11-keto derivatives have been shown to inhibit the growth of *Staphylococcus aureus* (Zeletova et al., 1986). It was shown that lipophilicity is an important parameter in the development of antimicrobial

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agents because it is related to permeation through a lipid coat of bacteria (Mallavadhani et al., 2004; Urzúa et al., 2008). Although the mechanisms of the antibacterial action may vary, differences of lipophilicity may be explored in the preparation of semisynthetic derivatives in order to improve the antimicrobial activity.

The current study aimed to evaluate the *in vitro* antibacterial activity of the methylene chloride extract of *Miconia ligustroides* (DC.) Naudin, the isolated compounds ursolic acid and oleanolic acid, and the acetyl and methyl ester derivatives prepared from ursolic acid.

Materials and methods

Plant material

M. ligustroides (DC.) Naudin (Melastomataceae) was collected in October, 2005, along the Franca-Claraval highway in São Paulo, Brazil. The plant was identified by Dr. Angela Borges Martins, Instituto de Biologia, UNICAMP (State University of Campinas), Brazil. A voucher specimen (UEC 10821) has been deposited in the Herbarium of the same Institute.

Extraction procedures

The aerial parts of the plant were dehydrated at 40°C, followed by pulverization (0.5 kg), and extracted with methylene chloride (Merck, Darmstadt, Germany) by maceration (5 L, 3 days ×3) at room temperature. The solvent was removed under vacuum in a rotary evaporator, yielding 6.5 g of the dry crude extract.

Isolation of ursolic acid and oleanolic acid

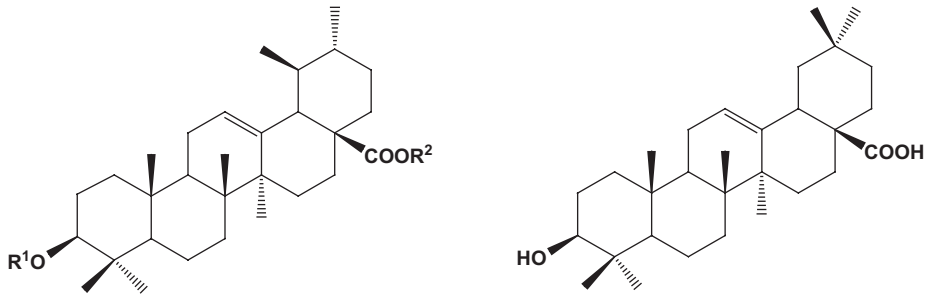
The methylene chloride extract of *M. ligustroides* (6.0 g) was chromatographed over 400 g of a mixture of Celite:Norit (3:1, w/w) by vacuum liquid chromatography (Coll & Bowden, 1986). Elution with methylene chloride afforded 520 mg of a mixture containing 65% ursolic acid (**1**) and 35% oleanolic acid (**2**) (Figure 1). These compounds were separated by high performance liquid chromatography (HPLC) (Cunha et al., 2003). The chemical structure of the two compounds was established by ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) data analysis and by comparison of the data with those of authentic compounds (Kim et al., 2000). Purity of the isolated compounds was determined by HPLC and ^{13}C-NMR, and was considered to be higher than 95%.

Preparation of ursolic acid derivatives

In order to obtain some triterpene acid derivatives, ursolic acid (50 mg) was treated with excess acetic anhydride in pyridine, to give the C-3 acetoxy derivative (45 mg) (**1a**). In another preparation, ursolic acid (about 50 mg) was treated with CH_2N_2 in Et_2O, yielding the respective C-28 methyl ester derivative (40 mg) (**1b**). The derivatives were purified by column chromatography on silica gel 60 (0.063–0.200 mm; Merck, Darmstadt, Germany). The chemical structures of the two semisynthetic derivatives were established by ^{13}C-NMR and electrospray ionization mass spectrometry (ESI-MS) data analysis.

Structure identification

Structures of the compounds were determined by spectroscopic methods. ^{1}H- (400 MHz) and ^{13}C-NMR (100 MHz) spectra were recorded on a Brüker DPX-400 spectrometer in dimethylsulfoxide (DMSO)-d_6 or $CDCl_3$ using tetramethylsilane (TMS) as internal standard. High-resolution ESI-MS was recorded on a Micromass Q-Tof (quadrupole time-of-flight) mass spectrometer.



1	$R^1 = H$	$R^2 = H$
1a	$R^1 = COCH_3$	$R^2 = H$
1b	$R^1 = H$	$R^2 = CH_3$

Figure 1. Chemical structures of ursolic acid (**1**), oleanolic acid (**2**), and ursolic acid derivatives (**1a** and **1b**).

Microorganisms

The following microorganisms were used in this study: *Bacillus cereus* (ATCC 14579), *Vibrio cholerae* (ATCC 9458), *Salmonella choleraesuis* (ATCC 10708), *Klebsiella pneumoniae* (ATCC 10031), and *Streptococcus pneumoniae* (ATCC 6305).

Antimicrobial assay

The minimum inhibitory concentration (MIC) values of the triterpene acids and ursolic acid derivatives were determined in triplicate by using the broth microdilution method (NCCLS, 2008). The samples were dissolved in DMSO (Merck, Darmstadt, Germany) at 0.5 mg/mL, and were then diluted in brain–heart infusion broth (Difco, Detroit, MI, USA) so that concentrations ranging from 1000 to 20 μg/mL were achieved. The final DMSO concentration in the culture medium was 5% (v/v), which was used as a negative control. The inoculum was adjusted to each organism, to yield a cell concentration of 10^5 colony forming units (CFU)/mL. One inoculated well was included 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. Gentamicin, imipenem, and vancomycin were used as positive controls. The microplates (96-well) were incubated at 37°C for 24 h. After that, 30 μL of resazurin (Sigma, St. Louis, MO, USA) in aqueous solution (0.01%) was added to them, to indicate microorganism viability (Palomino et al., 2002). The MIC was determined as the lowest concentration of compound capable of inhibiting microorganism growth.

Results and discussion

The chemical structures of all evaluated compounds are depicted in Figure 1 and their effects on the growth of the selected microorganisms are shown in Table 1.

The antimicrobial activity of the extracts from *Miconia* species has been reported in the literature to be active against several microorganisms (Celotto et al., 2003; Rodrigues et al., 2008). However, the methylene chloride extract obtained from *M. ligustroides* displayed low antibacterial activity toward the selected microorganisms in the case of the undertaken protocol. The best activity of the extract was obtained against *B. cereus* with a MIC of 625 μg/mL. The antimicrobial properties of several triterpene acids have also been studied. In recent work undertaken in our laboratory, ursolic (**1**) and oleanolic (**2**) acids showed an antibacterial effect against microorganisms potentially responsible for the formation of dental caries in humans (Cunha et al., 2007). In this study, ursolic acid (**1**) displayed efficient activity against *B. cereus* with a MIC of 20 μg/mL. Oleanolic acid (**2**) exhibited growth inhibitory activity against *B. cereus* and *S. pneumoniae*, with a MIC of 80 μg/mL for both bacteria. The similar MIC values obtained for compounds (**1**) and (**2**) may be related to their chemical structures because compounds (**1**) and (**2**) are isomers, and they differ only in the position of a methyl group.

In addition, the acetyl and methyl ester derivatives (**1a** and **1b**) prepared from ursolic acid (**1**) were able to increase only inhibitory activity against *S. pneumonieae.* For the other microorganisms, the MIC values were lower than that obtained for the starting material. It may be suggested that the free hydroxyl group, as well as the carboxyl group, may contribute to the inhibitory activity.

On the other hand, the MIC values for the mixture of (**1**) and (**2**) were lower than the MIC values obtained for the isolated compounds. These antimicrobial results are in agreement with the literature (Cunha et al., 2007), which has shown that a mixture of (**1**) and (**2**) displays lower inhibitory activity against cariogenic bacteria than those of the isolated compounds.

Among all evaluated microorganisms in this study, *V. cholerae* was the most resistant to the tested samples. It is important to point out that the negative control used in the antimicrobial assay did not produce any appreciable inhibition against any of the tested bacteria.

Table 1. Minimum inhibitory concentration values obtained for methylene chloride extract of *M. ligustroides*, triterpenes, and ursolic acid derivatives.

	Microorganism				
	B. cereus	*K. pneumoniae*	*S. choleraesuis*	*S. pneumoniae*	*V. cholerae*
Extract	625	>1000	>1000	>1000	>1000
(1)	20	1000	1000	1000	>1000
(1a)	>1000	>1000	>1000	50	>1000
(1b)	>1000	>1000	>1000	50	>1000
(2)	80	1000	1000	80	>1000
(1) + **(2)**	>1000	1000	1000	>1000	>1000
Positive control	0.18 (v)	2.95 (i)	0.36 (g)	0.18 (v)	0.73 (g)

Minimum inhibitory concentration values are expressed in μg/mL.
Positive control: g, gentamicin; i, imipenem; v, vancomycin.

The antimicrobial activity of ursolic acid (**1**) and oleanolic acid (**2**) is not so strong as compared with antimicrobial drugs that are used clinically. However, it has been reported that ursolic and oleanolic acids are not so toxic (Liu, 1995) and possess antimicrobial activity against some multiresistant bacteria (Horiuchi et al., 2007). In conclusion, the antimicrobial activities of ursolic and oleanolic acids, as well as their derivatives, are of particular interest, and they should be investigated in further studies to understand their mechanisms of action.

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***Declaration of interest*:** The authors report no conflicts of interest.

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