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Artigo

Purification of an antibacterial compound from *Lantana lilacina*

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RESUMO: "Purificação de um composto antibacteriano de *Lantana lilacina*". Observou-se, em estudo preliminar, que o extrato metanólico das folhas de *L. lilacina*, coletadas no município de Lavras (MG, Brasil), apresentava atividade antibacteriana. Em decorrência, buscou-se purificar e identificar a substância responsável por tal efeito, através de fracionamento do referido extrato direcionado por testes de difusão em agar com *Aeromonas hydrophila*, *Bacillus subtilis*, *Pseudomonas aeruginosa* e *Staphylococcus aureus*. Após partições com solventes e vários processos cromatográficos, isolou-se o [β-3,4-diidroxifenil)etil]-(3'-*O*-α-L-ramnopiranosil)-(4'-*O*-cafeoil)-β-D-glicopiranosídeo, que é conhecido como acteosídeo. A concentração inibitória mínima e a concentração bactericida mínima desta substância para *A. hydrophila, B. subtilis, P. aeruginosa* e *S. aureus* foram de 0,12, 1,00, 1,00 e 0,25 mg/mL, respectivamente.

Unitermos: *Lantana lilacina*, Verbenaceae, atividade antibacteriana, acteosídeo.

ABSTRACT: Since the methanol extract of *Lantana lilacina* leaves collected in the city of Lavras (MG, Brazil) showed antibacterial properties in a preliminary study, a fractionation process guided by agar diffusion assays with *Aeromonas hydrophila*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* was carried out to purify and identify the active compounds. After solvent partition and several chromatographic steps, [β-3,4-dihydroxyphenyl)-ethyl]-(3'-*O*-α-L-rhamnopyranosyl)-(4'-*O*-cafeoyl)-β-D-glycopyranoside, known as acteoside, was isolated. The minimal inhibition concentration and the minimal bactericidal concentration of such substance against *A. hydrophila, B. subtilis, P. aeruginosa* and *S. aureus* were 0.12, 1.00, 1.00 and 0.25 mg/mL, respectively.

Keywords: *Lantana lilacina*, Verbenaceae, antibacterial activity, acteoside.

INTRODUCTION

Although pharmaceutical industries have produced a large number of new antimicrobial agents, the emergence of resistant bacterial strains has become a public health problem all over the world. In the United States of America, for instance, each year, 70% of the two million cases of bacterial infections acquired in hospitals consist of strains resistant to at least one antibacterial compound (Alexandria, 2004). Moreover, adverse effects and the high cost of the antimicrobial substances make the search for new effective drugs extremely necessary.

As the biological activity of plants has been known since antiquity (Rios and Recio, 2005), a preliminary evaluation of local plant extracts was carried out to identify those with antimicrobial properties (Oliveira et al., 2007). During such study, a pronounced *in vitro* antibacterial property was observed for the aerial parts of *Lantana lilacina* Desf. (Verbenaceae), a 50-120 cm height native Brazilian shrub, which produces pink or purple flowers. Known as a weed and an ornamental plant (Lorenzi, 2000), it has been used in the traditional medicine to treat cold and bronchitis (Balbach, 1986). Although several substances have been identified in other species of the *Lantana* genus (Begum et al., 2000; Siddiqui et al., 1995; Barbosa-Filho et al., 2006), only monoterpene glucoside esters were isolated from *L. lilacina* leaves (Dembitsky, 2004). No further studies concerning the chemical composition of this plant species have been found. Therefore, this research was aimed to purify and identify the antibacterial compounds present in the methanol extract of *L. lilacina* leaves.

MATERIAL AND METHODS

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Plant material

Leaves from *Lantana lilacina* Desf. (Verbenaceae) were collected in the city of Lavras, State of Minas Gerais (Brazil). Voucher specimens were identified by Prof. Valéria E. G. Rodrigues and deposited in the Herbarium ESAL (ESAL 15.172), at Universidade Federal de Lavras, Lavras.

General experimental procedures

All reagents used were of recognized analytical grade. Acetic acid, acetonitrile, and methanol were HPLC-grade (Vetec, Brazil). During the purification steps, solvent concentration was carried out in a rotatory evaporator at 35 ºC, followed by 24 h in a freeze-drier. Except when mentioned otherwise, all fractions were submitted to antibacterial diffusion assays to direct purification. Column chromatography was carried out on silica gel 60 (230-400 mesh, Merck). Mass spectra were obtained on an Agilent 1100 LC/MS Trap equipped with an electrospray interface. Samples (1.0 mg) were dissolved in MeOH (1.0 mL) and 20 µL were directly injected into the interface at a flow rate of 5 µL/min. Deuterated dimethylsulphoxide (DMSO-*d6*) and deuterated methanol (CD_3OD) were used as solvents for nuclear magnetic resonance (NMR) analyses performed on a Varian Unit 500 instrument using solvent peak as reference. ^{1}H and ^{13}C NMR spectra were obtained in both solvents, while HMBC, HMQC and TOCSY analyses were carried out only in DMSO-*d6*.

Extraction and isolation

Fresh leaves of *L. lilacina* were exhaustively extracted with methanol at room temperature. Part of the crude extract (0.5 mg) was dissolved in 0.5 mL of an ethanol:H_2O (7:3; v/v) solution and submitted to the antibacterial diffusion assay. Another part (0.5 mg) of such extract was dissolved in 0.5 mL of an aqueous 1% (g/mL) Tween 80 solution and used in the broth microdilution assay.

The crude extract (10.0 g) was subsequently washed with hexane (10 x 100 mL), ethyl acetate (AcOEt; 10 x 100 mL) and methanol (MeOH; 10 x 100 mL). Part (3.5 g) of the MeOH fraction (M1) was successively eluted with MeOH, H_2O and HCl 0.1 M through a silica gel column (4 x 15 cm). Part (1.8 g) of the resulting MeOH fraction (M2) was eluted with MeOH through a C-18 column (1 x 5 cm), yielding fraction M3 (1.0 g). Then, M3 was fractionated on a C-18 column (Luna C-18, 200 x 21.2 mm, 10 µM, Phenomenex, USA), using aqueous 0.1% acetic acid solution:MeOH (5% MeOH during 5 min, 5-100% MeOH during 60 min, 100% MeOH for 21 min), at a flow rate of 30 mL/min, as eluent. An UV detector set at 254 nm was employed to monitor the fractionation. One of the resulting fractions (F11; 169 mg; eluted between 18-19 min) was purified on the same column employing aqueous 0.1% acetic acid solution:MeOH (62:38) as eluent, at a flow rate of 20 mL/min. In this case the UV detector was set at 320 nm. This procedure yielded fraction 6 as a pure compound (F6; 15 mg; eluted between 10.2-12.5 min), which was identified as acteoside (Figure 1) by NMR and mass spectrometry analyses.

Antibacterial assays

Antibacterial activity was evaluated with four standard bacterial strains acquired from the American Type of Culture Collection (ATCC, USA): *Bacillus subtilis* ATCC 6633 and *Staphylococcus aureus* ATCC 25923 (Gram-positive), *Aeromonas hydrophila* ATCC 7966 and *Pseudomonas aeruginosa* ATCC 27853 (Gram-negative). Agar diffusion assays (NCCLS, 2003a) were carried out in duplicates. Briefly, after bacterial growth in triptic soy agar (TSA, Acumedia, USA) during 24 h at 37 ºC, the resulting cultures were used to prepare cell suspensions in an aqueous 0.85% (g/mL) NaCl solution, at 0.5 turbidity according to MacFarland scale. Such suspensions were inoculated with a swab on the surface of Mueller-Hinton agar (Merck, Germany) Petri dishes and 40 µL of each sample were deposited into 6 mm diameter holes made on the inoculated medium. After 24 h at 37 ºC, samples causing 7 mm or larger inhibition zone diameters around the holes were considered active.

Minimal inhibitory and minimal bactericidal concentrations (MIC and MBC) were determined by a broth microdilution assay (NCCLS, 2003b). A twofold serial dilution of the reference (chloramphenicol succinate: Sigma, USA; 400 µg/mL) and samples were prepared using Mueller-Hinton broth (MHB: Biolife, Italy) supplemented with calcium and magnesium cations (Alderman and Smith, 2001). The crude extract was dissolved in an aqueous 1% (g/mL) Tween 80 solution at a concentration of 10 mg/mL and filtered through a 0.22 µm membrane (GV Durapore PVDF, Milipore, USA). 7.5 x 10^4 CFU were poured into each well and the initial extract concentration was 5.0 mg/mL. Aqueous 1% Tween 80 and chloramphenicol solutions were employed as negative and positive control, respectively. After 24 h at 37 ºC, 10 µL were withdrawn from each well with no bacterial growth and subcultured in TSA during 24 h at the same temperature. MIC was considered the lowest concentration of the extract that prevented visible growth in the well during 24 h and MBC was defined as the lowest concentration yielding negative subcultures during 24 h. The isolated compound (acteoside, Figure 1) was also submitted to a broth microdilution assay. It (2 mg) was dissolved in 100 µL of DMSO (P.A.) and diluted with MHB. The highest acteoside's concentration in the well was 1.0 mg/mL.

RESULTS AND DISCUSSION

As preliminarily observed (Oliveira et al., 2007), *L. lilacina* methanol extract was able to inhibit the growth of Gram-positive and Gram-negative bacteria strains (Table 1).

During all purification steps, only one active fraction was observed. Consequently, it seems that the antibacterial property of the crude extract was due only to the isolated substance, which amounted to 15 mg of a hygroscopic viscous oil that could get dark easily when exposed to light and air. In the ^{1}H NMR spectrum (Table 2), obtained in DMSO-*d6*, it was clear that most signals belonged to groups linked to heteroatoms (2.7-5.0 ppm) or unsaturated carbons (6.2-7.5 ppm). Such result suggested aromatic rings linked to carbohydrate units. The ^{13}C NMR spectrum (Table 2) pointed to the same direction, since most signals were in the carbon sp^3-heteroatom and carbon sp^2 regions. Signals at 140 and 150 ppm suggested carbon sp^2 - heteroatom groups, while the one at 165 ppm seemed due to a carbonyl group, probably an ester (Silverstein and Webster, 1998; Pretsch et al., 1989). The

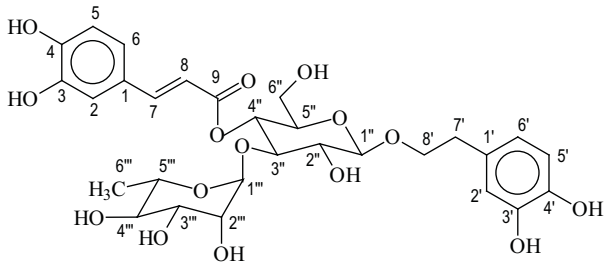

Figure 1. Structure of the acteoside.

Table 1. Inhibition zone diameter (IZD), minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) for the crude methanol extract of *Lantana lilacina* aerial part: (-) no inhibition zone; (x) not performed.

Crude extract	*A. hydrophila*	*B. subtilis*	*P. aeruginosa*	*S. aureus*
IZD (mm)	12.5	10.0	-	8.0
MIC (mg/mL)	1.25	1.25	x	0.62
MBC (mg/mL)	1.25	1.25	x	2.50

Table 2. ^{13}C- and ^{1}H- NMR data for the isolated compound (acteoside) in DMSO-*d6* and CD_3OD: δ ppm (mult; *J* = Hz; H).

Position	^{13}C; 125 MHz		^{1}H; 500 MHz	
	DMSO-*d6*	CD_3OD	DMSO-*d6*	CD_3OD
1	125.8	126.5		
2	114.6	114.1	7.08 (ls;1H)	7.05 (d; *J*=2.0; 1H)
3	145.5	146.8		
4	148.4	149.2		
5	113.9	115.9	6.82 (d; *J*=8.2; 1H)	6.77 (d; *J*=8.1; 1H))
6	121.3	122.0	7.03 (dd; J_1=8.2; J_2=1.5; 1H)	6.95 (dd; J_1=8.1; J_2=2.0; 1H)
7	145.5	148.5	7.51(d; *J*=16.0; 1H)	7.58 (d; *J*=16.0; 1H)
8	113.5	115.1	6.25 (d; *J*=16.0; 1H)	6.26 (d; *J*=16.0; 1H)
9	165.5	167.1		
1’	129.6	130.3		
2’	116.2	115.3	6.68 (ls; 1H)	6.69 (d; *J*=2.0; 1H)
3’	144.9	145.9		
4’	143.5	145.6		
5’	115.5	115.3	6.69 (d; *J*=6.5; 1H)	6.67 (d; *J*=8.3; 1H)
6’	119.5	120.0	6.55 (dd; J_1=6.5; J_2=1.5; 1H)	6.56 (dd; J_1=8.3; J_2=2.0; 1H)
7’	34.9	35.3	2.78 (ddd; J_1=13.0; J_2=J_3=7.5; 1H) 2.73 (dd; J_1=13.0; J_2=7.5; 1H)	2.79 (m; 2H)
8’	70.2	72.3	3.94 (ddd; J_1=J_2= 7.5; J_3=9.1; 1H) 3.66 (ddd; J_1=7.5; J_2=J_3=9.1; 1H)	4.03 (dd; J_1=7.5; J_2=13.0; 1H) 3.72 (dd; J_1=9.5; J_2=13.0; 1H)
1’’	102.2	101.8	4.41(d; *J*=8.0; 1H)	4.37 (d; *J*=8.0; 1H)
2’’	74.4	75.0	3.27 (dd; J_1=J_2= 8.5; 1H)	3.38 (dd; J_1=8.0; J_2=7.7; 1H)
3’’	79.6	80.4	3.76 (dd; J_1=J_2=8.5; 1H)	3.81 (dd; J_1=7.7; J_2=9.3; 1H)
4’’	69.1	70.9	4.77 (dd; J_1=J_2=8.5; 1H)	4.90 (dd; J_1=6.0; J_2=9.3; 1H)
5’’	74.4	74.8	3.53 (m)	3.53 (m; 1H)
6’’	61.0	61.2	3.47 (m; 1H) 3.36 (m; 1H)	3.62 (m; 1H) 3.52 (m; 1H)
1’’’	101.1	103.0	5.09 (s; 1H)	5.18 (d; *J*=1.5;1H)
2’’’	70.3	71.1	3.74 (s; 1H)	3.91 (m; 1H)
3’’’	70.4	71.0	3.30-3.58 (m; 1H)	3.55 (m; 1H)
4’’’	72.0	72.6	3.16 (dd; J_1=9.0; J_2=9.5; 1H)	3.27 (m; 1H)
5’’’	68.8	69.2	3.32 (m; 1H)	3.54 (m; 1H)
6’’’	18.1	17.2	1.01(d; *J*=6.5; 3H)	1.08 (d; *J*=6.0; 3H)

Table 3. Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of acteoside and chloramphenicol: Values in mg/mL; (x) not performed.

Substance	*A. hydrophila*		*B. subtilis*		*P. aeruginosa*		*S. aureus*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Acteoside	0.12	0.12	>1.00	x	1.00	1.00	0.25	0.25
Chloramphenicol	0.02	0.05	0.10	0.10	>0.20	x	0.20	>0.20

interpretation of TOCSY 1D, ^{1}H and ^{13}C NMR spectra, as well as heteronuclear correlations at short (HMQC) and long distances (HMBC), permitted to attribute the structure of the [β-3,4-dihydroxyphenyl)-ethyl]-(3'-*O*-α-L-rhamnopyranosyl)-(4'-O-cafeoyl)-β-D-glycopyranoside, known as acteoside or verbascoside (Figure 1), to the isolated compound. It is worth to mention that the stereochemistry of the caffeoyl moiety was clear in the ^{1}H spectrum, since the coupling constant (*J*) between H7 and H8 was 16 Hz, which is characteristic of a *trans* arrangement (Silverstein and Webster, 1998)

Although the spectra obtained in DMSO-*d6* were in accordance with NMR data reported for the acteoside dissolved in the same solvent (Tanaka et al., 2004), CD_3OD was also used, since it could afford a simpler ^{1}H NMR spectrum. As a consequence, the *α*-L-rhamnose moiety with OR (linked to C1''') and OH (linked to C2''') in the axial positions was confirmed by the low *J* between H1''' and H2''' (*J* = 1.5 Hz, equatorial-equatorial). In the β-D-glucose unit, the *trans*-diaxial couplings (6.0 - 9.3 Hz) became clear for all hydrogen atoms linked to the ring. Moreover, NMR spectra obtained in CD_3OD were totally in agreement with the findings of Wu et al. (2004) and Owen et al. (2003) for the acteoside.

The mass spectrometry analysis of the isolated compound in the negative mode showed peaks at *m/z* 623 $[M-H]^-$ and 659 $[M+Cl]^-$. Experiments inducing *m/z* 623 to fragmentation resulted in *m/z* 461 $[M-H-162]^-$ (MS^2) and *m/z* 315 $[M-H-162-146]^-$ (MS^3), corresponding to caffeoyl and rhamnose units loss, respectively. In the positive mode, as observed by Plaza et al. (2005), peaks were detected at *m/z* 647 $[M+Na]^+$ and 501 $[M+Na-146]^+$ (MS^2), which was due to the rhamnose unit loss.

Once there was no doubt that the isolated compound was the acteoside, a microdilution assay was carried out to evaluate its antimicrobial activity. Specifically with *A. hydrophila* and *S. aureus,* MIC and MBC values were very close to those obtained with chloramphenicol (Table 3). These results are in agreement with those reported by Didry et al (1999), who observed *Proteus mirabilis* and *S. aureus* growth inhibition by the acteoside at 0.128 mg/mL, during an agar dilution assay. Similarly, Lima et al. (2003) observed that a mixture of acteoside and isoacteoside showed MIC of 0.6 mg/mL against *S. aureus* and *B. subtilis*. According to Avila et al. (1999), acteoside inhibits *S. aureus* leucine admission, which stops protein synthesis and kills such bacterium. Unfortunately, *P. aeruginosa*, a bacterium resistant to several drugs (Barros, 2001), was able to grow in the presence of *L. lilacina* crude extract. Moreover, acteoside's MIC and MBC against this microorganism were both 1 mg/mL, which is a high value when compared to other substances (Ng et al., 1996; Ogundipe et al., 2001; Lima et al., 2003).

It is worth to mention that other biological activities have also been attributed to acteoside: protein kinase C inhibitor (Herbert et al.,1991); antitumor and immunosuppressive (Zhang et al., 2002; Ohno et al., 2002); antioxidant (Ono et al., 2005; Owen et al., 2003; Aligiannia et al., 2003); and antiinflammatory (Diaz et al., 2004).

In conclusion, for the first time it is shown that the antibacterial activity of *L. lilacina* leaves methanol extract is due to the presence of acteoside, a compound largely distributed in the plant kingdom. As observed in this study and by other research groups, such substance should be better evaluated by pharmacological and chemical assays aiming at the pharmaceutical use, either in humans or animals.

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