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Artigo

Antimicrobial activity of *Trembleya laniflora*, *Xyris platystachia* and *Xyris pterygoblephara*

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RESUMO: "Atividade antimicrobina de *Trembleya laniflora*, *Xyris platystachia* e *Xyris pterygoblephara*". As espécies *Trembleya laniflora* (Melastomataceae), *Xyris platystachia* (Xyridaceae) e *Xyris pterygoblephara* foram coletadas na Serra do Cipó, região considerada *hotspot* para conservação de biodiversidade. A atividade antimicrobiana dessas espécies foi avaliada em ensaios *in vitro* de difusão em ágar frente a linhagens padronizadas de *Staphylococcus aureus* e *Micrococcus luteus*. Todos os extratos, avaliados na concentração de 2000 µg/disco, foram ativos contra *M. luteus*, enquanto a inibição de crescimento de *S. aureus* somente foi observada para os extratos de *T. laniflora* (folhas) e *X. platystachia* (partes aéreas). A partição dos extratos brutos entre solventes imiscíveis resultou na obtenção de frações ativas, oriundas de extratos originalmente inativos frente a *S. aureus*, observando-se atividade principalmente para as frações de baixa e média polaridade. O extrato de folhas de *T. laniflora* foi adicionalmente fracionado por cromatografia em coluna de sílica gel e as frações resultantes apresentaram atividade antimicrobiana e perfis por CLAE distintos daquelas obtidas pela partição entre solventes imiscíveis.

Unitermos: *Trembleya laniflora*, *Xyris platystachia*; *Xyris pterygoblephara,* atividade antimicrobiana.

ABSTRACT: *Trembleya laniflora* (D. Don) Cogn. (Melastomataceae), *Xyris platystachia* Alb. Nilss. (Xyridaceae) and *Xyris pterygoblephara* Kunth., Brazilian species collected from a biodiversity hotspot for conservation priority, had their antimicrobial activity evaluated against standardized strains of *Staphylococcus aureus* and *Micrococcus luteus*, by the agar diffusion assay. All extracts, assayed in the concentration of 2000 µg/disc, were active against *M. luteus*, whereas *S. aureus* growth was inhibited only by *T. laniflora* leaves and *X. platystachia* aerial parts. Fractionation of the extracts by partition between immiscible solvents resulted in active fractions from extracts originally inactive against *S. aureus*. Activity was mainly found in low and medium polar fractions. The extract of *T. laniflora* leaves was also fractionated by silica gel column chromatography and both the HPLC fingerprint and antimicrobial activity of the obtained fractions were distinct of those originated from the partition process.

Keywords: *Trembleya laniflora*, *Xyris platystachia*, *Xyris pterygoblephara,* antimicrobial activity.

INTRODUCTION

Brazil is recognized as one of the megadivesity countries, concentrating around 10 to 20% of all plant species in the world (Mittermeier et al., 1997). A total of 10,000 plant species are estimated to occur in Minas Gerais (Mendonça; Lins, 2000). *Serra do Cipó* is a national park located in this state, in a region classified as a biodiversity hotspot for conservation priority (Myers et al., 2000). The area presents exceptional concentrations of endemic species and is experiencing loss of habitats, contributing to drive many species into extinction. We have previously investigated the antifungal and antibacterial activity of 20 plant species from *Serra do Cipó* (Cota et al., 2002). Among the active species, three were selected for study in the present work.

Trembleya laniflora (Melastomataceae) is a shrub popularly named "flor-de-lã" (wool flower), used as ornamental species (Pio Corrêa, 1969). *T. laniflora* grows mainly in rocky soils from *campos rupestres*, an altitudinal ecosystem covered by open vegetation, being the genus endemic in Brazil. A chemotaxonomic study carried out for the leaves of Melastomataceae species, belonging to the closely related genera *Lavoisiera*, *Microlicia* and *Trembleya*, resulted in the identification of 116 flavonoids, comprising 69 flavonol and 47 flavone glycosides, including kaempferol 3-*O*-glycosides and quercetin 3-*O*-glycosides in *T. laniflora* (Bomfim-Patrício et al., 2001). A literature search indicated no ethnomedical use or biological activity other than antimicrobial described for *T. laniflora* (Cota et al., 2002).

Xyris species are small shrubs, popularly known as "sempre-vivas" (everlasting plants). Some are collected for ornamental purposes or for medicinal

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uses, to treat eczemas and dermatitis (Pio Corrêa, 1969). The identification of *Xyris* species based solely on morphological characters is rather limited (Varanda et al., 2002). Around 90% of the *Xyris* found in Brazil are endemic (Sajo et al., 1997) and over harvesting has put several species in risk of extinction.

The chemistry and biological activity of *Xyris* have been poorly investigated. Metabolites obtained from this genus include isocoumarins from *X. indica* (Ruangrungsi et al., 1995), anthraquinones from *X. semifuscata* (Fournier et al., 1975) and flavonoids from *X. itatiayensis*, *X. longiscapa* and *X. obtusiuscula* (Varanda et al., 2002). Recently, we reported the isolation of a new anthraquinone from *X. pilosa*, active against *Fusarium oxysporum* (Cota et al., 2004). Except the antimicrobial effect (Cota et al., 2002; Cota et al., 2004), no other biological activity or any chemical data has been described for *X. platystachia* and *X. pterygoblephara*, species selected for the study.

The diversity of compounds found in plant species make these organisms promising sources of new antimicrobial agents, with general or specific effects (Amaral et al., 2006; Leitão et al., 2006). The interest in plant secondary metabolites with antimicrobial properties has revived as a consequence of microbial resistance development against the antibiotics in clinical use (Rocha et al., 2004; Lima et al., 2006; Oliveira et al., 2006), especially in the case of opportunistic infections affecting immunocompromised patients (Klausmeyer et al., 2004). Considering that the species selected for the study occur in an ecosystem with a high degree of endemism, it is feasible to infer that they are a potential source of bioactive compounds. Hence, the main goal of this work was to assay the antimicrobial activity of extracts and fractions from *T. laniflora*, *X. platystachia* and *X. pterygoblephara*.

MATERIAL AND METHODS

Plant materials

The species *Trembleya laniflora* (D. Don) Cogn., *Xyris pterygoblephara* Kunth. and *Xyris platystachia* Alb. Nilss. were collected in Minas Gerais state, Brazil, at Serra do Cipó National Park and APA Morro da Pedreira. The plants were identified by botanists from the Fundação Zoo-Botânica, Belo Horizonte, Brazil, where voucher specimens are deposited, under numbers BHZB 2340, BHZB 2496 and BHZB 2492, respectively.

Plant material extraction and preliminary fractionation

The plants were dried separately, at 40 ºC, for 72 h. The extracts of *T. laniflora* (leaves, stems), *X. pterygoblephara* (aerial parts) and *X. platystachia* (aerial parts) were prepared by exhaustive percolation with ethanol. The extracts were concentrated to residue by removing the solvents in a rotavapor, at 50 ºC. Data obtained for the dry extracts are shown in Table 1. Portions (2 g) of the dry extracts were suspended in MeOH/H_2O (1:1; 120 mL) and sequentially partitioned with equal volumes (120 mL) of *n*-hexane, CH_2Cl_2 and EtOAc. MeOH was removed in a rotavapor, before partitioning the extract suspension with CH_2Cl_2 and EtOAc. Solvents were removed in a rotatory evaporator, at maximum temperature of 50 °C, and the obtained residues are displayed in Table 2. Emulsions were generated during the partition of the extract from *T. laniflora* leaves between *n*-hexane (Emulsion 1), dichloromethane (Emulsion 2) and ethyl acetate (Emulsions 3 and 4). HPLC analysis carried out for the emulsions indicated distinct profiles from the obtained fractions and for this reason they were concentrated separately and had their residues evaluated in the antimicrobial assays.

Chromatographic fractionation of the extract from *T. lanifora* leaves

The crude extract of *T. laniflora* leaves (22.8 g) was chromatographed on a silica gel column (70-230 mesh, Merck), employing a gradient elution of *n*-hexane (79.6 mg, TL1), *n*-hexane:CH_2Cl_2 (1:1) (361.7 mg, TL2), CH_2Cl_2 (689.4 mg, TL3), CH_2Cl_2:EtOAc (1:1) (1639.3 mg, TL4; 1239.7 mg, TL5), EtOAc (2205.2 mg, TL6), MeOH (80.9 mg, TL7; 11589.8 mg, TL8) and MeOH: H_2O (1:1) (1201.6 mg, TL9).

Bacterial cultures and growth conditions

Staphylococcus aureus ATCC 25923 and *Micrococcus luteus* ATCC 9341 were employed as test organisms. The cultures were grown in agar medium, in tubes kept in a slating position, at 36 ºC, for 24 h. Cultures were maintained in plates, at 4 °C, in nº 1 antibiotic agar.

Antimicrobial assay

The antibacterial activity of extracts and fractions was evaluated by the disk diffusion method. For the assays, solutions of the extracts and fractions were prepared in MeOH to concentrations of 100 and 50 mg/mL, respectively. Suspensions of microorganisms were prepared in peptone saline solution. The transmittance of the inoculum suspension was adjusted to 50 ± 1%, at 580 nm. Seeded agar plates were prepared by pouring 20 mL of nº 1 antibiotic agar into each plate. After medium solidification, each plate was overlaid with 5 mL medium containing 0.05% of the inoculum suspension. Sterile paper discs (6 mm diameter) were impregnated with 20 µL of the extracts (2000 µg/disc) or fractions (1000 µg/disc). The discs were placed in duplicate onto the plates and incubated for 24 h, at 37 ºC. The experiments were carried out in six replicates. The results (mean value

Table 1. Ethanol extractives obtained from the plants in study.

Plant name	Part	Dry vegetal material (g)	Dry extract (g)	Extractive (%)
Xyris pterygoblephara	Aerial parts	43.3	4.30	9.93
Xyris platystachia	Aerial parts	100.0	13.31	13.31
Trembleya laniflora	Leaves	200.0	28.50	14.25
	Stems	100.0	15.34	15.34

Table 2. Fractions resulting from partition of plant extracts between immiscible solvents.

Plant extract (2 g)	Fractions (mg)								
	Part	Hex	*Em1	DCM	*Em2	EtOAc	*Em3	*Em4	Water
Xyris pterygoblephara	Aerial parts	365.3		146.3		518.5			1016.5
Xyris platystachia	Aerial parts	164.5		142.8		467.8			1051.3
Trembleya laniflora	Leaves	158.1	98.7	164.7	338.4	83.9	65.0	55.8	716.7
	Stems	80.0		44.6		67.6			1392.8

*Emulsions formed during the partition process. See experimental for details.

Table 3. Antimicrobial activity of plant ethanol extracts and fractions obtained by partition of crude extract between immiscible solvents, assayed by the agar diffusion method.

Plant extract	Part	Extract / fractions	Microbial inhibition (mm diameter zone ± rsd)	
			M. luteus	*S. aureus*
Trembleya laniflora	stems	[a]crude extract	9.2 ± 0.3	[b]–
		n-hexane	12.8 ± 1.0	9.0 ± 0.0
		DCM	11.5 ± 0.5	9.3 ± 0.3
		EtOAc	13.5 ± 0.9	10.2 ± 0.6
		water	–	–
	leaves	crude extract	12.8 ± 0.6	9.5 ± 0.5
		n-hexane	21.0 ± 0.9	14.3 ± 0.8
		Emulsion 1	13.0 ± 0.9	10.2 ± 0.3
		DCM	16.2 ± 0.8	14.2 ± 0.8
		Emulsion 2	12.7 ± 0.8	8.3 ± 0.8
		EtOAc	13.2 ± 0.6	12.7 ± 0.3
		Emulsion 3	–	8.2 ± 0.3
		Emulsion 4	7.3 ± 0.3	–
		water	9.2 ± 0.6	–
Xyris platystachia	aerial parts	crude extract	10.3 ± 0.8	7.7 ± 0.6
		n-hexane	–	–
		DCM	12.2 ±0.8	11.2 ± 0.7
		EtOAc	9.7 ± 0.3	–
		water	-	–
Xyris pterygoblephara	aerial parts	crude extract	10.0 ± 0.5	–
		n-hexane	–	–
		DCM	9.2 ± 0.6	8.3 ± 0.3
		EtOAc	11.0 ± 0.5	7.7 ± 0.6
		water	–	–
Chloramphenicol			21.0 ± 0.9	12.6 ± 0.7

[a]Paper discs were impregnated with 2000 µg of the extracts or 1000 µg of the fractions.
[b](–) no detected activity at the assayed concentrations. MeOH (control) did not show any inhibitory activity.

Table 4. Antimicrobial activity of chromatographic fractions from the extract of *Trembleya laniflora* leaves, assayed by the agar diffusion method

Fractions	Microbial inhibition (mm diameter zone ± rsd)	
	M. luteus	*S. aureus*
TL1 (*n*-hexane)[a]	[b]–	–
TL2 (DCM:*n*-hexane, 1:1)	–	–
TL3 (DCM)	–	–
TL4 (DCM:EtOAc, 1:1)	14.0 ± 0.9	–
TL5 (DCM:EtOAc, 1:1)	20.0 ± 0.5	9.2 ± 0.8
TL6 (EtOAc)	12.7 ± 1.0	8.5 ± 0.0
TL7 (MeOH)	8.6 ± 1.0	7.8 ± 0.6
TL8 (MeOH)	–	–
TL9 (MeOH:water, 1:1)	–	–
Chloramphenicol	21.4 ± 0.9	10.5 ± 0.7

[a]Paper discs were impregnated with 1000 µg of the fractions.
[b](–) no detected activity at the assayed concentrations. MeOH (control) did not show any inhibitory activity.

plus standard deviation) were recorded by measuring the zones of growth inhibition surrounding the discs. Chloramphenicol (3 µg/disc) was included in the assays as positive control, whereas control disks contained solvent only (MeOH) as negative control.

HPLC characterization of fractions

Analysis were carried out on a Merck-Hitachi apparatus (Germany) composed of pump L-6200A, automatic injector AS-2000A, UV-VIS detector L-4250 and integrator D-2500. An ODS column (150 × 4.0 mm I.D., 5 µM) was employed (Merck, Germany) at a temperature of 40 °C and flow rate of 1.0 mL/min. Analysis were performed at 220 nm. A linear gradient of H_2O (A) and CH_3CN (B) was employed: 0 min 90% A, 10% B; 60 min 10% A, 90% B, followed by 10 min of isocratic elution. Solvents used were of HPLC grade (Merck, Germany) and were degassed by sonication before use. Fractions were dissolved in MeOH to a concentration of 5 mg/mL. After centrifugation at 10,000 r.p.m, the sample solutions (30 µL) were automatically injected.

Phytochemical analysis

The presence of saponins, alkaloids, coumarins, anthraquinones, flavonoids, triterpenes and tannins was evaluated in the ethanol extracts, by TLC analysis, according to Wagner et al. (1984).

RESULTS AND DISCUSSION

The ethanol extracts of *Trembleya laniflora*, *Xyris platystachia* and *Xyris pterygoblephara* have been previously evaluated in antimicrobial assays, against different strains of bacteria and fungi (Cota et al., 2002; Cota et al., 2004). The three species were particularly active against *S. aureus* and *M. luteus*, and for this reason both were selected as test organism in the present work. Aliquots from the crude ethanol extracts were initially fractionated by partition between immiscible solvents and the obtained results are displayed in Table 2.

All assayed extracts were active against *M. luteus*, whereas *S. aureus* growth was inhibited only by the extracts from *T. laniflora* leaves and *X. platystachia* aerial parts. In the previous work (Cota et al., 2002), the extracts from *T. laniflora* stems and *X. pterygoblephara* aerial parts were active against *S. aureus*, while *X. platystachia* did not show inhibitory effect against this microorganism. Such contradictory results may be explained by differences in extract compositions, since the plant materials were collected in distinct locations.

Phytochemical analysis (Wagner et al., 1984) of the ethanol extracts gave positive results for saponins, triterpenes and tannins. Flavonoids were also detected in the three species, except in the stems of *T. laniflora*, whereas coumarins were solely present in the aerial parts of *X. platystachia* and *X. pteryglobephara*. Several flavonoids (Harborne; Williams, 2002), coumarins (Borges et al., 2005), tannins (Chung et al., 1998), saponins (Wallace, 2004) and triterpnes (Katerere et al., 2003) have been reported to posses antimicrobial activity. Therefore, the presence of metabolites from these classes in the assayed species might explain their antimicrobial activity here reported.

The four extracts were submitted to preliminary fractionation by partition between immiscible solvents and the resulting fractions had their antimicrobial

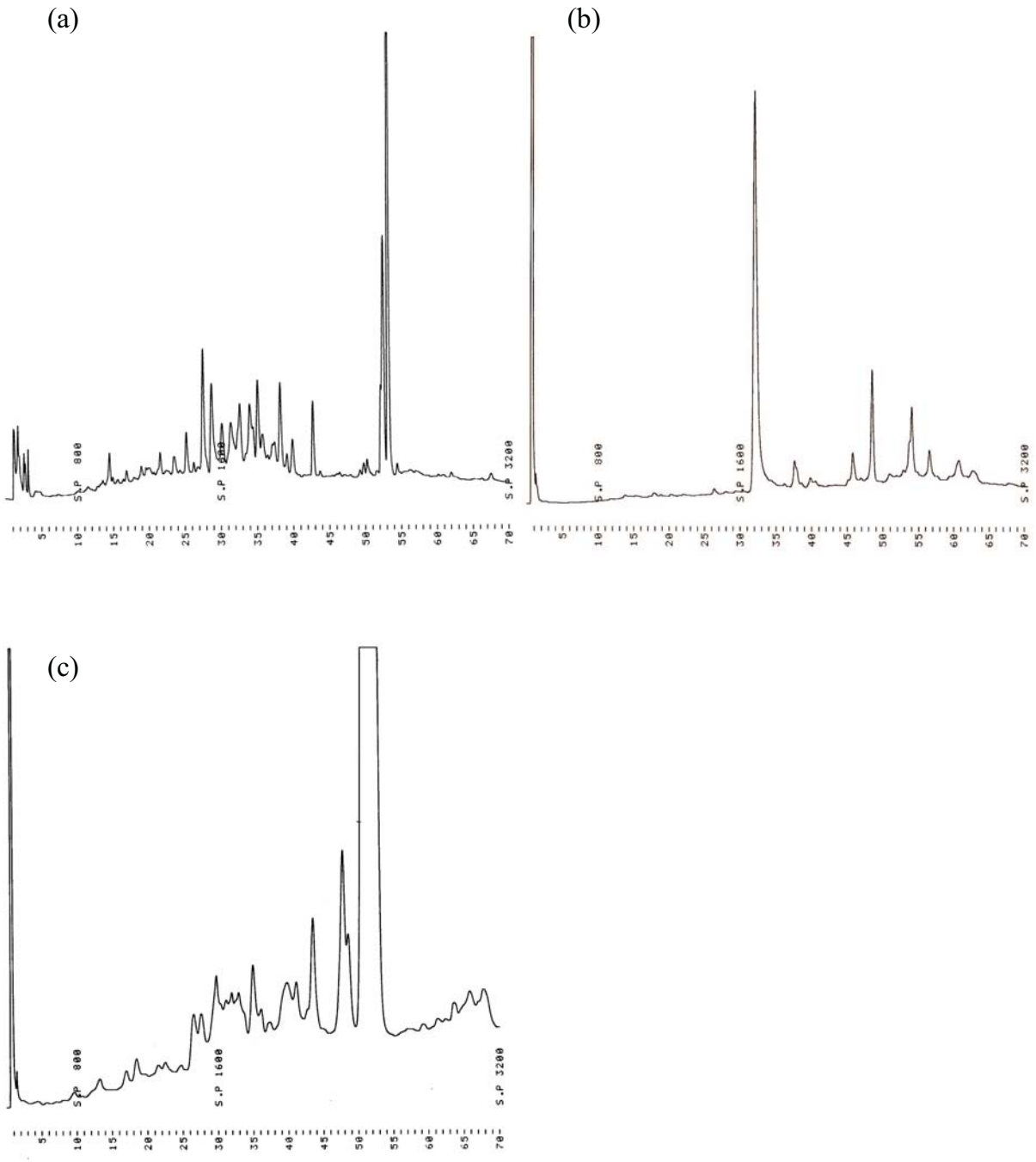

Figure 1. HPLC profiles of fractions from *Trembleya laniflora* leaves. (a) Dichloromethane fraction obtained by partition between immiscible solvents; (b) dichloromethane (TL3) and (c) dichloromethane / ethyl acetate (1:1) (TL4) fractions from silica gel column chromatography. HPLC conditions: see experimental.

activity evaluated. Active fractions were obtained from extracts originally inactive against *S. aureus* (Table 3). This result demonstrates the importance of a preliminary fractionation when assaying the antimicrobial activity of plant extracts, once the low concentration of the active compounds may impair their detection in crude extracts.

Partition between immiscible solvents is an adequate approach for the preliminary separation of complex matrices, such as vegetal extracts. However, scaling up this procedure to obtain quantities of material for further studies is frequently time consuming and production of emulsions is almost impossible to avoid. Fractionation by silica gel column chromatography constitutes an alternative to overcome these limitations. It should be reminded, however, that different physical phenomena are involved in these procedures, namely

solubility in the first and adsorption in the second. Hence, applying these methods to the same matrix might result in fractions with distinct compositions and activities.

In order to confirm this supposition, fingerprint profiles were registered by HPLC for fractions of the extract from *T. laniflora* leaves obtained by partition between immiscible solvents and by fractionation on a silica gel column. Besides, the antimicrobial activity of the chromatographic fractions was also assayed.

Dichloromethane fractions originated from both approaches showed distinct HPLC profiles (Figure 1) and antimicrobial effects: while the fraction obtained by partition was significantly active against *M. luteus* and *S. aureus* (Table 3), the chromatographic one (TL3) showed no activity against both microorganisms (Table 4). On the other hand, HPLC analysis of the dichloromethane / ethyl acetate (1:1) fraction (TL4), originated from the chromatographic fractionation, showed a more related profile to that of the dichloromethane fraction obtained by partition between immiscible solvents (Figure 1). However, TL4 was active solely against *M. luteus*. These results clearly confirm our hypothesis that fraction constitution, and therefore biological activity, depends on the procedure adopted for fractionation.

In conclusion, the results here reported corroborate the popular use of the species to treat microbial diseases and also demonstrate the relevance of a preliminary fractionation for detecting active fractions, when assaying the antimicrobial effect of plant extracts.

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