Screening of 20 Pantanal Wetland Plants for Anti-*Candida* Activity using HPLC-DAD-MS/MS and Bioautography to Characterize Active Compounds

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Key words

Antifungal, bioautography, Candida, podophyllotoxin, Brazilian wetlands

received 04.12.2020
revised 20.04.2021
accepted 27.04.2021

Bibliography

Planta Med Int Open 2021; 8: e96–e103
DOI 10.1055/a-1494-1117
ISSN 2509-9264

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ABSTRACT

The Pantanal wetland harbors a rich flora with uncharted pharmacological potential. This study evaluated 20 Brazilian Pantanal plants against *Candida albicans, C. parapsilosis, C. tropicalis,* and *C. krusei*. Fungal susceptibility was determined by agar diffusion and broth microdilution; active compounds were identified by bioautography and HPLC-DAD-MS/MS. *Sesbania virgata, Polygala molluginifolia, and Cantinoa mutabilis* extracts and their chloroform and ethyl acetate (EtOAc) fractions exhibited the best activity against all *Candida* species tested. The EtOAc fraction of *P. molluginifolia* proved to be more efficient in inhibiting *C. parapsilosis* and *C. krusei* growth (Minimum inhibitory concentration of 125 and 62.5 µg/mL, respectively). Bioautography of this fraction revealed two active bands, characterized by HPLC-DAD-MS/MS as a mixture of podophyllotoxin derivatives blumenol, besides some flavonoids. This work demonstrated antifungal potential of *P. molluginifolia* podophyllotoxin derivatives and the versatility of bioautography with HPLC-DAD-MS/MS to identify the bioactive compounds.

ABBREVIATIONS

Pm-EtOAc	Ethyl acetate fraction of *Polygala molluginifolia*

# Daniela Z. de Brito and Nadla S. Cassemiro have equally contributed to the work and are co-authors.

Introduction

Dermatophytes and yeasts of the genus *Candida* are the primary cause of superficial fungal infections and still among the most prevalent skin, hair, and nail disorders. *Candida* species are also responsible for mucous-membrane infections, affecting several organs and tissues [1, 2]. Recent decades have seen a substantial increase in systemic and bloodstream infections associated with high morbidity and mortality rates, particularly in immunocompromised patients and those undergoing invasive procedures [3, 4].

In general, critically ill patients, such as those with an immature immune system or immune senescence, are at high risk of developing invasive *Candida* infection [2, 3, 5]. Five *Candida* species cause over 80 % of candidemia cases: *C. albicans, C. glabrata, C. parapsilosis, C. tropicalis,* and *C. krusei* [2, 6, 7].

The first-line drugs for the treatment of invasive candidiasis are echinocandins and azoles [3]. One of the most widely used antifungals in medical practice is fluconazole. However, *Candida krusei* is intrinsically resistant to this antifungal, and the resistance to this azole has also been reported in other *Candida* species [2, 3]. More recently, the emergence of multidrug-resistant *Candida auris* in hospitals has caused panic worldwide [3, 4]. In this context, plants as an abundant source of novel active compounds, including antifungal agents, may be used as prototypes to develop new drugs [8].

The tropical wetland Pantanal, located in central-western Brazil, is known for the rich flora with unexplored pharmacological potential [9]. This biome is a unique ecosystem marked by regular seasonal flooding, and vast plant diversity comprises roughly 3500 species across some phytogeographic regions, including vegetation of Seasonal Semideciduous Forest, Amazon Forest, Chaco, and Cerrado [10].

Thus, the present study explores 20 plant species, commonly found in the Pantanal, for their antifungal activity against four *Candida* species using agar diffusion, broth microdilution, bioautography, and HPLC-DAD-MS/MS to find new antifungal agents.

Results

Extracts of all tested species exhibited antifungal activity against at least one *Candida* sp. on the disk diffusion test. Only the crude extracts of *Sesbania virgata*, *Polygala molluginifolia*, and *Cantinoa mutabilis* proved to be active against all *Candida* species tested in both concentrations (▶ **Table 1**). ▶ **Table 2** shows the diameters of inhibition halos promoted by fractions of *S. virgata*, *P. molluginifolia*, and *C. mutabilis* fractions, where we can observe that exclusively $CHCl_3$ and EtOAc fractions were active.

▶ **Table 3** shows the Minimal Inhibitory Concentrations (MIC) of fractions that demonstrated activity against *Candida* species in antifungal susceptibility tests based on microdilution. Minimum fungicidal concentration (MFC) of the EtOAc fraction of *P. molluginifolia* (Pm-EtOAc) was 125 µg/mL against *C. parapsilosis* and 62.5 µg/mL against *C. krusei*, coinciding with MIC values. MFC of the $CHCl_3$ fraction of the same plant species was 500 µg/mL against *C. parapsilosis* and 1000 µg/mL against *C. krusei.*

The Bioautography of the Pm-EtOAc fraction revealed two active bands, which did not separate well, against *C. parapsilosis* and *C. krusei* (▶ **Fig. 1**). These bands were isolated on a preparative TLC and analyzed in the HPLC-DAD-MS/MS. In the bands (▶ **Fig. 1b**), lignans derived from podophyllotoxin, megastigmanes, and flavonoids mixture were identified; these classes of compounds are already reported in some species of *Polygala* [11–18].

The upper and lower bands (▶ **Fig. 1b**) were not well separated by preparative TLC. They revealed two compounds in common (compounds 2 and 12), reflecting the low-resolution power of the chromatographic system. These compounds were identified by HPLC-DAD-MS/MS (▶ **Table 4**). Peaks 1, 2, 3, and 4 did not absorb in the monitored UV region (240-800 nm) and showed a similar fragmentation profile, identified as belonging to the megastigmane class. Compounds 1 and 3 (*m/z* 385 $[M-H]^-$, $C_{19}H_{30}O_8$) were identified as isomers of blumenol hexoside [19]. The *m/z* 205 fragment refers to the loss of a hexosyl group observed for compound 1. Compounds 2 and 4 (*m/z* 387 $[MH]^-$, $C_{19}H_{32}O_8$) displayed the presence of two additional hydrogens in relation to 1 and 3; *m/z* 207 was relative to the loss of a hexosyl group. Based on this data, it was possible to identify them as dihydroblumenol hexoside isomers, according to data reported by Schliemann et al. [20].

Compounds 5, 6, 8, and 10 exhibit a UV spectrum compatible with a flavonol group, compounds 5, 8, and 10 (*m/z* 593 $[M-H]^-$, $C_{27}H_{30}O_{15}$) were identified as a flavonol-*O*-dihexoside, according to the literature [17, 21, 22]. Peak 6 *m/z* 463.0864 $[M-H]^-$is compatible with a quercetin-*O*-hexoside, based on the ion at *m/z* 300.0260 relative to the loss of the hexosyl group [23].

Peaks 11, 12, 13 and 14 showed *m/z* 531.1495 $[M-H]^-$, *m/z* 561.1617 $[M-H]^-$, *m/z* 545.1630 $[M-H]^-$ and *m/z* 575.1756 $[M-H]^-$, respectively. Their UV spectra and fragmentation profile were similar. Fragmentation of *m/z* 561 (12) generated *m/z* 399 $[M-162]^-$, referring to the loss of a hexosyl group. The *m/z* 384, *m/z* 369 fragments are from consecutive losses of methyl groups, and *m/z* 341 fragment refers to the loss of a CO. This compound was identified as 4'-demethylpodophyllotoxin hexoside [24]. Peak 11 has a methoxy group less than compound 12. The *m/z* 354 was formed by losing a hexosyl plus a methyl group; these data are consistent with those reported for podophyllotoxin hexoside derivative NC 370 [25]. Compound 13 has one oxygen less than 12; this was evidenced by the *m/z* 383 fragment relative to the hexosyl group's loss. Compound 14 has one methyl more than 12, which forms the *m/z* 396 by losing a hexosyl plus a water group. These compounds were identified as 4'-demethylpodophyllotoxone hexoside (13) and desoxypodophyllotoxin hexosyl (14) by comparison with the data reported by Zhou et al. and Duan et al. [24, 25]. Finally, compound 15 *m/z* 411.1078 $[M-H]^-$, being identified as podophyllotoxone according to UV spectra and fragmentation reported by Zhou et al. and Meng-long et al. [24, 26].

Discussion

The highly diverse flora of the Pantanal biome remains scarcely studied, despite its potential for discovering pharmaceutical products. Here we demonstrate the potential of these plants to develop new anti-*Candida* drugs, the screening of 20 species led to the detection of antifungal activity against at least one *Candida* species by all crude extracts. Studies have shown that a single plant species can exhibit different activity levels (sensitivity) *in vitro*, depending on the *Candida* species targeted in tests [27].

▶ **Table 1** Diameters (mm) of inhibition halos in disk diffusion tests performed with 20 crude plant extracts against four *Candida* species at concentrations of 10 mg/mL (250 µg/disk) and 40 mg/mL (1000 µg/disk) after 24 h (means ± SD; $n = 3$).

Crude extract	*C. albicans* ATCC 90028		*C. parapsilosis* ATCC 22019		*C. tropicalis* ATCC 750		*C. krusei* ATCC 6258	
	10 mg/mL	40 mg/mL	10 mg/mL	40 mg/mL	10 mg/mL	40 mg/mL	10 mg/mL	40 mg/mL
Negative control (DMSO)	–		–		–		–	
Positive control (fluconazole, 25 µg/disk)	33.33 ± 2.89^{d}		25.67 ± 4.04^{b}		31.67 ± 2.89^{d}		14.67 ± 1.53^{c}	
Astronium fraxinifolium	–	–	–	–	11.00 ± 1.00^{a,b,c}	11.00 ± 1.00^{a,b,c}	11.67 ± 0.58^{b,c}	12.67 ± 0.58^{b,c}
Bidens gardneri	–	8.00 ± 0.00^{a,b}	9.33 ± 0.58^{a}	9.33 ± 0.58^{a}	10.00 ± 0.00^{a}	10.33 ± 0.58^{a}	9.00 ± 0.00^{a}	9.67 ± 1.53^{a}
Centratherum punctatum	8.33 ± 0.58^{a,b}	9.00 ± 0.00^{b}	–	–	10.33 ± 0.58^{a}	10.00 ± 0.00^{a}	10.33 ± 1.15^{a}	11.33 ± 0.58^{b,c}
Croton glandulosus	–	–	–	–	–	–	10.00 ± 0.00^{a}	10.00 ± 0.00^{a}
Diospyros tetrasperma	–	–	–	–	9.00 ± 1.00^{a}	10.33 ± 0.58^{a}	10.00 ± 0.00^{a}	11.00 ± 0.00^{a}
Hyptis brevipes	–	–	–	–	–	10.33 ± 0.58^{a}	12.00 ± 1.73^{b,c}	12.33 ± 1.15^{b,c}
Cantinoa mutabilis	9.00 ± 1.73^{a,b}	12.00 ± 1.00^{c}	10.67 ± 0.58^{a}	11.67 ± 1.15^{a}	10.67 ± 1.15^{a,b,c}	13.33 ± 1.53^{c}	11.67 ± 1.53^{b,c}	11.67 ± 0.58^{b,c}
Lantana canescens	10.00 ± 0.00^{b,c}	10.00 ± 0.00^{b,c}	–	–	12.00 ± 1.00^{b,c}	11.67 ± 1.15^{a,b,c}	10.00 ± 1.00^{a}	11.00 ± 0.00^{a}
Melanthera latifolia	–	–	–	–	10.00 ± 0.00^{a}	10.67 ± 1.15^{a,b,c}	11.00 ± 0.00^{a}	12.00 ± 0.00^{c}
Ocotea diospyrifolia	–	–	–	–	10.00 ± 0.00^{a}	10.67 ± 1.15^{a}	10.33 ± 0.58^{a}	10.33 ± 0.58^{a}
Polygala molluginifolia	9.00 ± 1.00^{b}	12.33 ± 1.53^{c}	9.00 ± 1.00^{a}	10.67 ± 1.15^{a}	9.67 ± 1.53^{a}	12.33 ± 0.58^{b,c}	10.67 ± 0.58^{a}	11.00 ± 2.00^{a}
Randia armata	–	–	–	–	9.00 ± 1.00^{a}	11.00 ± 1.00^{a,b,c}	–	10.67 ± 1.15^{a}
Richardia grandiflora	–	–	–	–	11.33 ± 1.53^{a,b,c}	11.33 ± 0.58^{a,b,c}	10.00 ± 2.00^{a}	10.00 ± 2.00^{a}
Scoparia montevidensis	–	–	8.33 ± 0.58^{a}	9.33 ± 0.58^{a}	10.67 ± 0.58^{a,b,c}	10.67 ± 0.58^{a}	9.33 ± 1.53^{a}	10.67 ± 1.15^{a}
Senna obtusifolia	–	–	–	–	9.67 ± 1.15^{a}	11.00 ± 0.00^{a,b,c}	10.00 ± 1.00^{a}	11.00 ± 1.73^{a}
Sesbania virgata	10.67 ± 0.58^{b}	11.00 ± 0.00^{b}	9.00 ± 1.73^{a}	9.67 ± 1.53^{a}	10.33 ± 0.58^{a}	10.33 ± 0.58^{a}	10.33 ± 0.58^{a}	11.00 ± 1.00^{a}
Smilax fluminensis	–	–	–	–	8.67 ± 0.58^{a}	9.33 ± 0.58^{a}	8.67 ± 1.53^{a}	9.67 ± 0.58^{a}
Stilpnopappus pantanalensis	–	–	9.00 ± 0.00^{a}	9.67 ± 0.58^{a}	10.33 ± 1.15^{a,b,c}	11.33 ± 0.58^{a,b,c}	9.33 ± 0.58^{a}	10.33 ± 0.58^{a}
Tocoyena formosa	–	–	–	–	9.00 ± 1.00^{a}	9.33 ± 0.58^{a}	9.67 ± 0.58^{a}	11.33 ± 1.53^{b,c}
Zanthoxylum rigidum	10.00 ± 0.00^{a,b,c}	10.53 ± 0.58^{a,b,c}	–	10.33 ± 0.58^{a}	10.67 ± 0.58^{a,b,c}	11.67 ± 0.58^{a,b,c}	10.33 ± 0.58^{a}	11.67 ± 1.15^{b,c}

Kruskal–Wallis followed by Dunn's test ($p \leq 0.05$). Means followed by different letters in the same column indicate a significant difference. Dashes indicate the absence of biological activity (no inhibition halo).

▶ **Table 2** Diameters (mm) of inhibition halos in the disk diffusion test performed against all *Candida* species using fraction concentrations of 10 mg/mL (250 µg/disk) and fluconazole at 25 µg/disk after 24 h (means ± SD; $n = 4$).

Extract	Fraction	*C. albicans* ATCC 90028	*C. parapsilosis* ATCC 22019	*C. tropicalis* ATCC 750	*C. krusei* ATCC 6258
Negative control (DMSO)		–	–	–	–
Positive control (fluconazole)		34.75 ± 3.69[b]	26.75 ± 3.95[b]	30.25 ± 3.67[b]	16.50 ± 2.38[b]
Sesbania virgata	Hx	–	–	–	–
	$CHCl_3$	11.00 ± 0.00[a,b]	11.00 ± 0.82[a,b]	10.75 ± 0.50[a,b]	10.75 ± 0.96[a,b]
	EtOAc	10.75 ± 0.50[a,b]	10.75 ± 0.50[a]	10.00 ± 0.00[a]	10.25 ± 0.50[a]
	EtOH	–	–	–	–
	EtOH/H_20	–	–	–	–
Polygala molluginifolia	Hx	–	–	–	–
	$CHCl_3$	10.50 ± 0.58[a]	11.00 ± 0.00[a,b]	11.50 ± 1.29[a,b]	11.00 ± 0.00[a,b]
	EtOAc	11.75 ± 0.96[a,b]	12.00 ± 0.00[b]	12.75 ± 1.71[a,b]	13.00 ± 0.82[b]
	EtOH	–	–	–	–
	EtOH/H_20	–	–	–	–
Cantinoa mutabilis	Hx	–	–	–	–
	$CHCl_3$	11.00 ± 0.00[a,b]	12.00 ± 0.82[a,b]	12.50 ± 0.58[a,b]	11.50 ± 1.29[a,b]
	EtOAc	11.75 ± 0.50[a,b]	11.75 ± 0.50[a,b]	13.50 ± 1.29[b]	11.75 ± 0.96[a,b]
	EtOH	–	–	–	–
	EtOH/H_20	–	–	–	–

Kruskal–Wallis, followed by Dunn's test ($p \leq 0.05$). Means followed by different lettersin the same column indicate a significant difference. Dashes indicate the absence of biological activity (no inhibition halo).

▶ **Table 3** Minimum inhibitory concentrations of fractions exhibiting activity against all *Candida* species.

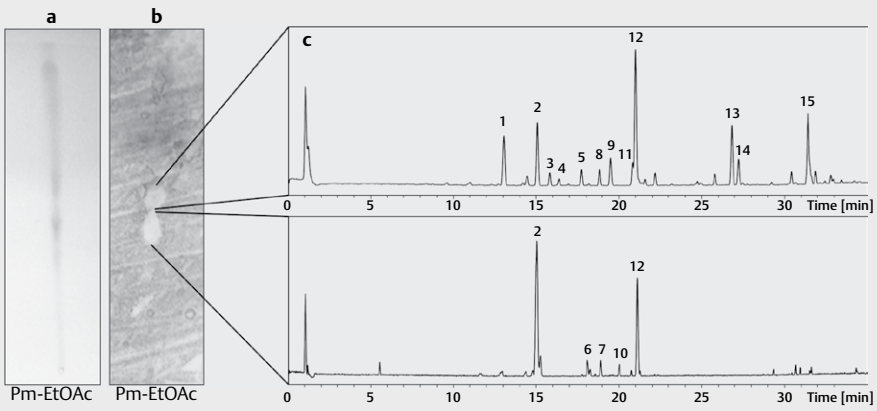
Plant and solvent	*Candida* spp. (MIC, µg/mL)			
	C. albicans	*C. tropicalis*	*C. parapsilosis*	*C. krusei*
Sesbania virgata				
$CHCl_3$	≥ 2000	≥ 2000	≥ 2000	≥ 2000
EtOAc	≥ 2000	≥ 2000	≥ 2000	≥ 2000
Polygala molluginifolia				
$CHCl_3$	≥ 2000	≥ 2000	500	500
EtOAc	≥ 2000	≥ 2000	125	62.5
Cantinoa mutabilis				
$CHCl_3$	≥ 2000	≥ 2000	≥ 2000	≥ 2000
EtOAc	≥ 2000	≥ 2000	≥ 2000	≥ 2000
Fluconazole (control)	0.5	0.5	2	16

Different methodologies have been proposed to determine the antifungal activity of natural products against *Candida* species [28–30], and has been the Disk diffusion the most used. Disk diffusion results showed that almost all crude extracts tested were active against the *Candida* ATCC strains, *C. tropicalis* and *C. krusei*, and seven proved active against *C. albicans* and *C. parapsilosis* (▶ **Table 1**). Extract concentration plays little or no role in antifungal effectiveness, as shown by the small difference between the diameters of inhibition halos obtained using 10 and 40 mg/mL concentrations (▶ **Table 1**).

No information is available on the antimicrobial activity of medium/high polarity secondary metabolites of *S. virgata, P. molluginifolia,* and *C. mutabilis*, which proved effective for inhibiting yeast growth in the disk diffusion test in the present study. *S. virgata* inhibits filamentous fungi through the mediation of FP1-A protein [31].

In the literature, crude extracts of *Hyptis albida* and *H. verticillata* produced, respectively, 4 and 7 mm-wide inhibition halos in agar diffusion tests against *C. albicans* [32]. More recently, low MIC values and good antifungal activity against *C. krusei* and *Crypyococcus neoformans* have been reported for *H. crenata* fractions [32]. In our study, although *C. mutabilis* promoted inhibition halos of 7–14 mm in diameter, depending on extract concentration and *Candida* species MIC was high, suggesting an absence or weak activity. The same difference was also observed for the EtOAc and $CHCl_3$ fractions of *S. virgata* and *C. mutabilis.*

Disk diffusion is commonly used as a pre-test due to its straightforwardness and low cost; however, some factors have to be con-



▶ **Fig. 1** TLC of the EtOAc fraction of *P. molluginifolia.* **b** Bioautography of the EtOAc fraction of *P. molluginifolia* revealed two activity bands against *C. parapsilosis* and *C. krusei*. **b** High-performance liquid chromatography-mass spectrometry chromatographic profile spectra of bioautography active bands. 1: blumenol hexoside; 2: dihydroblumenol hexoside; 3: blumenol hexoside; 4: dihydroblumenol hexoside; 5: flavonol-O-dihexoside; 6: quercetin-O-hexoside; 7: unknown; 8: flavonol-O-dihexoside; 9: unknown; 10: flavonol-O-dihexoside; 11: podophyllotoxin hexoside derivative NC 370; 12: 4'-demethylpodophyllotoxin hexoside; 13: demethylpodophyllotoxone hexoside: 14: podophyllotoxin hexoside; 15: podophyllotoxone

sidered since studies have shown that culture medium composition influences extract activity and those natural compounds with good diffusion coefficients. Thus, weak antimicrobial compounds can penetrate the medium and, even in small amounts, inhibit growth. Additionally, the volume and pH of the medium, type of microorganism, extract volatility, and size and absorption capacity of paper disks may influence test results [29, 33].

Serial broth microdilution yields better results and is regarded as the standard method for evaluating natural products. However, unknown active compounds require more than one method for testing their antimicrobial activity [29].

Polygala is the largest genus of the Polygalaceae family (over 1000 species and worldwide distribution). Antifungal activity in *Polygala spp*. has been the subject of few reports [28, 34], and no studies have been found for the anti-candida activity of *P. molluginifolia*. The low MIC values obtained for the $CHCl_3$ and EtOAc fractions of *P. molluginifolia* against *C. parapsilosis* and *C. krusei* suggest an excellent antifungal activity, a promising result, considering that *C. parapsilosis* is one of the primary agents of bloodstream infection, particularly in children and in patients using intravascular devices [7, 35]. *C. krusei* has been isolated from several immunocompromised patients with cancer or neutropenia [6, 34] and is intrinsically resistant to fluconazole, a drug often used against systemic fungal infection [36].

The bioautography of Pm-EtOAc allowed the isolation of the fraction's active compounds, three main classes of metabolites were identified by HPLC-DAD-MS/MS: lignans, megastigmanes, and flavonoids. The presence of these metabolites has already been reported in the literature in a few species of *Polygala*, such as lignans derived from podophyllotoxin found in *P.macradenia, P. paenea,* and *P. polygama* [11, 12, 15], megastigmane in *P. tenuifolia* and *P. hongkongensis* [14, 16] and flavonoids in *P. hongkongensis* and *P. sibirica* [13, 18].

In our study, glycosylated megastigmane derivatives of blumenol and dihydroblumenol were identified. The absence of UV absorption and the *m/z* 205 fragments for blumenol derivative and *m/z* 207 for dihydroblumenol allowed us to confirm the compounds. Other compounds detected in the two active bands belonged to the class of flavonoids (6, 11, and 13). Anti-*Candida* activity for flavonoids has already been well reported in the literature [37]. On the other hand, there are no reports in the literature of the antifungal activity of blumenol derivatives.

It is essential to highlight that besides the fact that several authors have shown that lignin derivatives have antifungal and antimicrobial activity [38, 39], no reports were found for podophyllotoxin derivatives, broadly known by their cytotoxic, anticancer, and antiviral activity [40].

Thus, this work demonstrated the disk diffusion test's high sensitivity and the importance of performing the MIC for extracts and active fractions; among the twenty plants studied, the best anti-*Candida* activity was observed with the extracts of *S. virgata*, *P. molluginifolia* and *C. mutabilis* in their fractions chloroform (fr-CHCl3) and ethyl acetate (fr-AcOEt) against *C. albicans*, *C. parapsilosis*, *C. tropicalis* and *C. krusei*. *P. molluginifolia* showed better performance *in vitro*, lower minimum inhibitory concentration to *C. parapsilosis* and *C. krusei*. The bioautography along with HPLC-DAD-MS/MS allowed us to identify the bioactive compounds in *P. molluginifolia,* revealing the antifungal potential of podophyllotoxin derivatives, the main compounds of the active bands. The observation of anti-*Candida* activity in *P. molluginifolia* is unprecedented and can be promising for the development of new antifungals, especially for treating infections caused by *C. parapsilosis* and *C. krusei.*

Materials and Methods

Plant material

The plants collected from the Pantanal wetland were identified by Prof. Geraldo A. Damasceno-Junior, and a voucher specimen was

▲ **Table 4** Retention times (Rt), maximal absorption wavelength (UV/VIS), formula, and molecular weight (*m/z*) of compounds of two bands active shown in Bioautography.

Peak	RT (min.)	UV (λ max)	Molecular formula	$[M-H]^-$ (*m/z*)	$[M+H]^+$ (*m/z*)	MS/MS (*m/z*)	MS/MS (*m/z*)	Compound/Class
1	13.0	–	$C_{19}H_{30}O_8$	385.1834	387.2018	205, 153	207	blumenol hexoside
2	15.1	–	$C_{19}H_{32}O_8$	387.1999	–	207, 189	–	dihydroblumenol hexoside
3	15.8	–	$C_{19}H_{30}O_8$	385.1841	387.2027	205	–	blumenol hexoside
4	16.4	–	$C_{19}H_{32}O_8$	387.1989	–	207	–	dihydroblumenol hexoside
5	17.7	280, 330	$C_{27}H_{30}O_{15}$	593.1488	–	–	–	flavonol–*O*–dihexoside
6	18	270, 350	$C_{21}H_{20}O_{12}$	463.0864	465.1024	300, 271, 255		quercetin–*O*-hexoside
7	18.8	–	$C_{25}H_{28}O_{13}$	535.1446*	–	–	–	unknown
8	18.8	280, 340	$C_{27}H_{30}O_{15}$	593.1474	–	–	–	flavonol-*O*-dihexoside
9	19.5	–	$C_{25}H_{26}O_9$	469.1512	471.1642	–	–	unknown
10	19.9	270, 335	$C_{27}H_{30}O_{15}$	593.1487	595.1669	–	–	flavonol-*O*-dihexoside
11	20.8	280	$C_{26}H_{28}O_{12}$	531.1508	533.1695	369, 354, 341, 326	–	podophyllotoxin hexoside derivative NC 370
12	21.0	280	$C_{27}H_{30}O_{13}$	561,1617	563.1737	399, 384, 369, 341, 325	401, 247, 203	4'-demethylpodophy-llotoxin hexoside
13	26.3	290	$C_{27}H_{30}O_{12}$	545.1630	–	383, 369, 353	–	demethylpodophy-llotoxone hexoside
14	27.3	290	$C_{28}H_{32}O_{13}$	575.1756	–	396, 383, 327	–	desoxypodophyllo-toxin hexosyl
15	31.5	270	$C_{22}H_{20}O_8$	411.1078	413.1231	393, 365, 321,233	–	podophyllotoxone

RT: Retation Time; *: $[M-CH_3COOH]^-$

deposited at the CGMS Herbarium (SISBIO license 33165-2). ▶ **Table 5** lists the 20 species, along with coordinates of collection sites, parts used in the experiments, numbers of the exsiccate voucher, and yield of crude extracts.

Preparation of extracts

The samples were oven-dried, grounded, and then extracted with ethanol:water (7:3), as described by Richter et al. [41], in an ASE 150 pressurized solvent extractor (Dionex, USA) (5 mL sample cartridges, 5 min static time program, 100 °C, 60 % rinse volume, 100 s purge time, two cycles), the extracts yields are in ▶ **Table 5** The active extracts on the disk diffusion test were re-extracted with the same apparatus and solvents of gradient polarity (hexane, chloroform, ethyl acetate, ethanol, ethanol:water) to yield fractions.

Antifungal susceptibility test

Candida albicans (ATCC-90028), *C. parapsilosis* (ATCC-22019), *C. krusei* (ATCC-6258), and *C. tropicalis* (ATCC-750) strains from the American Type Culture Collection, were used for *in vitro* susceptibility testing.

Disk diffusion test

Initial screening for antifungal activity was based on disk diffusion [42]. The surfaces of culture plates were seeded with each *Candida* inoculum and covered with sterile filter paper disks (6 mm in diameter) subsequently impregnated with 25 µL of extract previously solubilized in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, France). The concentrations used were 10 mg/mL (250 µg/disk) and 40 mg/mL (1000 µg/disk) for crude extracts and 250 µg/disk for fractions. Fluconazole-impregnated disks (25 µg, Cecon, Brazil) were used as sensitivity controls. The assays were performed at least in triplicate.

Minimum inhibitory concentration (MIC) using microbroth dilution

The fractions with the highest activity in the disk diffusion tests were evaluated for MIC, following the guidelines of CLSI [43]. The extract concentration ranged from 31.25 to 8000 µg/mL and fluconazole from 0.25 to 64 µg/mL (reference control, Sigma Aldrich, France, Purity ≥ 98 %). MIC was defined as the lowest concentration of extract at which no fungal growth was observed after incubation. All testing was conducted in quadruplicate.

Antifungal bioautography

The bioautography assay was carried out as described by Rahalison et al. [44], with minor modifications. 100 µg of *P. molluginifolia* EtOAc fraction (Pm-EtOAc) were applied to the silica gel plate (Macherey-Nagel, Germany) and subsequently eluted with EtOAc:MeOH:H_2O (100:13.5:10). Liquefied SDA was poured onto the eluted TLC plates. After solidification, the yeasts were seeded and incubated at 35 °C for 48 h. Bioautograms were developed with a solution of piodonitrotetrazolium (2 mg/mL).

Isolation of active fractions from Pm-EtOAc by Preparative thin layer chromatography

50 mg of the Pm-EtOAc were applied to the silica gel plate and subsequently eluted with EtOAc: MeOH: H_2O (100:13.5:10). Bands

▶ **Table 5** Taxonomic data coordinates of collection sites, parts used in the experiments, and yield of crude extracts.

Species	Family	Coordinates	Part used	Voucher number	Yield (%)
Astronium fraxinifolium Schott ex Spreng	Anacardiaceae	19°29'2"S 57°2'16"W	T+L	5198	20.02
Bidens gardneri Bak.	Asteraceae	19°28'59"S 57°2'16"W	AP+L	5192	17.68
Centratherum punctatum Cass.	Asteraceae	19°37'5"S 57°2'4"W	AP+FL	5169	15.67
Croton glandulosus L.	Euphorbiaceae	19°34'7"S 57°1'15"W	AP+FL	5186	14.08
Diospyros obovata tetrasperma Sw.	Ebenaceae	19°29'2"S 57°2'16"W	T+L	5203	9.91
Hyptis brevipes Poit.	Lamiaceae	19°34'8"S 57°1'10"W	EP+FL	5177	14.29
Cantinoa mutabilis (Rich.) Harley & J.F.B.Pastore	Lamiaceae	19°29'3"S 57°2'15"W	AP+FL	5191	13.61
Lantana canescens Kunth	Verbenaceae	19°37'5"S 57°2'4"W	AP+FL	5170	23.72
Melanthera latifolia (Gard.) Cabrera	Asteraceae	19°34'36"S 57°1'11"W	AP+FL	5172	14.66
Ocotea diospyrifolia (Meisn.) Mez	Lauraceae	19°36'30"S 57°2'8"W	T+L	5213	18.93
Polygala molluginifolia A. St.-Hil. & Moq.	Polygalaceae	19°34'36"S 57°1'10"W	EP+FL	5171	24.46
Randia armata (Sw.) DC.	Rubiaceae	19°29'2"S 57°2'16"W	T+L+FR	5202	14.58
Richardia grandiflora (Cham. & Schltdl.) Steud.	Rubiaceae	19°34'8"S 57°1'14"W	AP+FL	5188	13.17
Scoparia montevidensis (Spreng.) R.E. Fr.	Plantaginaceae	19°36'18"S 57°3'14"W	AP+FL	5209	14.37
Senna obtusifolia (L.) H.S. Irwin & Barneby	Leguminosae	19°34'9"S 57°1'10"W	AP+FL+FR	5178	18.06
Sesbania virgata (Cav.) Pers.	Fabaceae	19°37'5"S/57°2'4"W	T+L	5168	21.34
Smilax fluminensis Steud.	Smilacaceae	19°29'6"S 57°2'19"W	AP+FL	5190	11.64
Stilpnopappus pantanalensis H. Rob.	Asteraceae	19°29'0"S 57°2'14"W	AP + L	5195	14.97
Tocoyena formosa (Cham. & Schltdl.) K. Schum.	Rubiaceae	19°29'2"S 57°2'16"W	T+L	5205	12.24
Zanthoxylum rigidum Humb. & Bonpl. ex Willd.	Rutaceae	19°29'2"S 57°2'16"W	T+L	5200	12.88

AP: aerial portions (leaves, twigs, stems); EP: entire plant (roots, leaves, twigs); FL: flowers; FR: fruits; L: leaves; R: roots, T: twigs.

were visualized under UV (254 and 365 nm), two bands were removed with Rf: 0.58 e Rf: 0.64.

HPLC-DAD-ESI-QTOF-MS/MS of Pm-EtOAc and active fractions

8 µL of samples (1 mg/mL) was injected into the Shimadzu LC-20AD UFLC chromatograph, coupled to DAD and ESI-QTOF microTOF III (BrukerDaltonics) in line, monitored between 240–800 nm, and operated in negative and positive mode (*m/z* 120–1200 and collision energy 45–65 V). The stationary phase was the Kinetex C-18 chromatography column (Phenomenex, 2.6 µ, 150 × 2.1 mm) and a gradient elution of water (Phase A) and acetonitrile (Phase B) both with 1 % acetic acid: 0–2 min. 3 % Phase B; 2–25 min. 3–25 % Phase B; 25–35 min. 25–62 % Phase B, followed by column washing and reconditioning (11 min). Flow rate: 0.3 mL/min. Data were processed using Data Analysis software version 4.2 (Bruker), and compounds were identified based on ultraviolet spectra, retention time, and fragmentation profile compared to the literature.

Statistical analysis

Statistical significance in the disk diffusion test was determined using Kruskal–Wallis tests and Dunn's post-test ($p \leq 0.05$).

Acknowledgment

The authors thank the Instituto Adolfo Lutz (São Paulo) for the ATCC strains graciously supplied; to the technicians João Roberto Fabri, Amanda Galdi Boaretto, and Fernanda Luíza Espinosa Spositto for the operational laboratory support.

Conflicts of Interest

The authors declare no conflict of interest.

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