Identification of Triterpenoids from *Schefflera systyla*, *Odontadenia puncticulosa* and *Conostegia speciosa* and In Depth Investigation of Their *in vitro* and *in vivo* Antifungal Activities

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As a part of a broad screening of antifungal agents from plant origin, crude extracts from Panamanian plants having related types of constituents displayed significant activities in an agar overlay thin layer chromatography assay against a susceptible strain of *Candida albicans*. These were the methanolic extract of the leaves of *Schefflera systyla* and *Odontadenia puncticulosa* and of the stems of *Conostegia speciosa*, that are species not previously investigated from a phytochemical viewpoint. For all plants, high-performance liquid chromatography (HPLC) antifungal activity based profiling allowed the rapid localization of antifungal agents that were further obtained by targeted isolation procedure by semi-preparative HPLC or medium pressure liquid chromatography (MPLC) after LC gradient transfer. Different hederagenin saponins and one aglycone were found to be responsible for the antifungal activities of the extracts. Alpha-hederin was the antifungal of *S. systyla*, pulsatilla saponin D and 3β-O-[β-D-xylpyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→4)]-α-L-arabinopyranosylhederagenin of *O. puncticulosa* and arjunolic acid of *C. speciosa*. Their minimal inhibitory concentration (MIC) against planktonic and biofilm cells of *C. albicans* were determined. Alpha-hederin was the most potent compound with a MIC of 4 µg mL⁻¹. Structurally related compounds (hederagenin, medicagenic acid 3-O-β-D-glucopyranoside and medicagenic acid) were used as standards and tested for comparison purposes. In order to better estimate the potential of these triterpenoids as antifungal agents, their cytological effects on *C. albicans* were determined by transmission electron microscopy (TEM) and the *in vivo* activity of alpha-hederin, medicagenic acid 3-O-β-D-glucopyranoside and medicagenic acid was evaluated for the first time in the *Galleria mellonella* larvae model.

Keywords: triterpenoids, microfractionation, antifungal, *Candida* species, *Galleria mellonella*

Introduction

Fungal infections have recently increased worldwide and cause high morbidity and mortality rates among different groups of human patients.¹ The development of drug resistance in fungal pathogens compromises the efficacy of the limited number of therapeutic agents.²

The main fungal opportunistic human pathogens are *Candida* spp., and among them, the most common species is *Candida albicans*.³ In the USA, *Candida* species are the fourth cause of nosocomial bloodstream infections.⁴,⁵ Over 400,000 candidiasis infections are declared per year worldwide.⁶ Moreover, Candida species display a tendency
Identification of Triterpenoids from *Schefflera systyla*, *Odontadenia puncticulosa* and *Conostegia speciosa* J. Braz. Chem. Soc.

Results and Discussion

The minimal inhibitory quantities (MIQ) measured by bioautography without elution at the screening step were 15 and 20 µg for the *S. systyla* and *O. puncticulosa* extracts, respectively. Interestingly, for the extract of *C. speciosa* the antifungal activity was observed only when the extract was separated on the plate and therefore no MIQ values could be determined. For *S. systyla* and *O. puncticulosa* the active zones could not be correlated to an UV detectable spot in the high-performance thin-layer chromatography-ultraviolet (HPTLC-UV) analysis suggesting the presence of non-UV active compounds (Figure 1A; Figure S1, Supplementary Information).

HPLC-PDA-ELSD metabolite profiling of the active extracts

Each extract was analysed by HPLC-photo diode array- evaporative light scattering detector (PDA-ELSD) using a reverse phase C18 column. Since the HPTLC analysis suggested that the active compounds did not possess an UV chromophore, ELSD was used in order to detect these compounds. The comparison between the PDA and ELSD traces showed the presence of major compounds only detected with ELSD (Figures 1B and S1).

HPLC antifungal based profiling by at-line microfractionation and bioautography

In order to localize and isolate the compounds responsible for the antifungal activities, the active extracts were fractionated by semi-preparative HPLC on a 10 mm i.d. C18 column. The gradient time and flow rate for this micro-fractionation were adapted from the analytical HPLC using a gradient transfer method. This procedure provided a satisfactory separation of the crude extract constituents, and most microfractions corresponded to single LC peaks. The microfractions obtained in this step were dried and submitted to the antifungal bioautography assay with the susceptible strain of *C. albicans* without any further elution step. Following this procedure the methanolic extract of *S. systyla* (25 mg) yielded 42 microfractions. The antifungal activity was found in three consecutive fractions, corresponding to the major LC peak detected by ELSD (Figure 1). Similarly the extract of *O. puncticulosa* yielded 57 fractions and two presented antifungal activities corresponding to a minor LC-peak in the UV chromatogram (Figure S1, Supplementary Information). The extract of *C. speciosa*, 39 mg yielded 50 fractions. Among these fractions, three presented antifungal activity (Figure S1, Supplementary Information). The procedure provided thus a rapid and efficient way to rapidly localize and isolate the bioactive LC-peaks of interest.
Identification of the active compounds

Nuclear magnetic resonance (NMR) and high-resolution mass spectrometry (HRMS) analyses were performed on each active microfraction (Figures 1 and S1). The active compound of *S. systyla* was identified as the triterpenoid saponin (α)-hederin (1), which has been already described in some species of the *Schefflera* genus.\(^{26}\) Similarly, the active principle of *C. speciosa* was identified as arjunolic acid (4) which was previously isolated from *Terminalia arjuna* (Figure 2).\(^{27,28}\)

The NMR and MS analyses of the first fraction of *O. puncticulosa* allowed the identification of the active pulsatilla saponin D (2), a compound previously described in *Pulsatilla cemum* and *P. koreana*.\(^{29}\) The structure of the compound present in the second active fraction could...
not be achieved due to the small amount available. Its targeted isolation was performed by medium pressure liquid chromatography (MPLC) after direct transfer of the analytical HPLC conditions to MPLC from 4 g of crude dichloromethane extract according to a previously reported protocol. This yielded 33.8 mg of the minor active principle that was identified as 3β-O-[β-D-xylopyranosyl-(1→3)]-α-L-rhamnopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→4)]-α-L-arabinopyranosylhederagenin (3), a saponin previously described on Anemone taipaiensis (Figure 2).\(^{31}\)

Antifungal activities of the isolated triterpenoids

The isolated compounds were tested against wild-type C. albicans cells (CAF2-1) with both bioautography and standard broth dilution susceptibility assays.\(^{13}\)

Figure 2. Structures of the triterpenoids isolated (1-4) and commercially obtained (5-7).
Compound 1 was the most active with a MIQ value of 2.5 µg with bioautography and a minimal inhibitory concentration (MIC) of 4 µg mL\(^{-1}\) against the susceptible strain (DSY2621) (Table 1). Antifungal activities against Microsporum canis, Coccidioides immitis, Trichophyton mentagrophytes, Cryptococcus neoformans and C. albicans of this compound have been previously described in the literature.\(^{32}\) Compound 2 was active against C. albicans with a MIQ of 15 µg and a MIC of 8 µg mL\(^{-1}\), which is consistent with published data indicating an antifungal activity at similar concentration range for this compound.\(^{33}\) Compound 3 was less active with a MIQ equal to 20 µg and a MIC equal to 16 µg mL\(^{-1}\) and it was not previously been reported for its antifungal activity. No activity was detected using broth dilution susceptibility assay (MIC > 32 µg mL\(^{-1}\)) for 4 despite the MIQ of 10 µg. This compound was previously described as active against C. albicans at higher concentration (50 µg mL\(^{-1}\)).\(^{34}\)

All compounds were then evaluated against different Candida species (C. glabrata, C. krusei, C. tropicalis and C. parapsilosis) in broth dilution assays in order to determine the bioactivity profile against various strains of the genus (Table 2). All compounds showing activities against C. albicans exhibited similar activities against the different species tested.

As most C. albicans cells do not exist as free planktonic cells but rather as groups of organized cells called biofilms that display increase resistance to antifungal drugs,\(^{35}\) compounds were tested with mature C. albicans biofilms according to standard protocol.\(^{36}\) Compounds 1 and 2 inhibited biofilm metabolic activity at the highest concentration tested (50 µg mL\(^{-1}\)) whereas compounds 3 and 4 did not have any effect.

In order to verify the importance of the sugars moiety for the antifungal activity, the common aglycone of compounds 1, 2 and 3, named hederagenin (5), was commercially obtained and evaluated in the antifungal assays. This compound was found completely inactive. A comparison of the activity between 1, 2, and 3 suggests that the antifungal activity decreases as the number of sugars present in the molecule increases. According to MIC values obtained, it seems clear that

### Table 1. Bioautography and broth dilution assay of the isolated compounds and reference compound

<table>
<thead>
<tr>
<th>Compound</th>
<th>Bioautography assay</th>
<th>Dilution assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C. albicans (DSY2621) / µg</td>
<td>C. albicans (CAF2-1) / µg</td>
</tr>
<tr>
<td>1</td>
<td>1.5</td>
<td>2.5</td>
</tr>
<tr>
<td>2</td>
<td>3.2</td>
<td>15</td>
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<tr>
<td>3</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td>d</td>
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</tr>
<tr>
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<td>5</td>
</tr>
<tr>
<td>7</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>Miconazole(^c)</td>
<td>0.0006</td>
<td>0.005</td>
</tr>
</tbody>
</table>

\(^a\)Minimum amount required for antifungal activity on TLC plate; \(^b\)minimum inhibitory concentration (MIC); \(^c\)reference compound; \(^d\)inactive: MIQ > 20 µg; MIQ > 32 µg mL\(^{-1}\). MIQ: minimal inhibitory quantities.

### Table 2. Broth dilution assay of the isolated compounds and reference compound on Candida species

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dilution assay / (µg mL(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C. glabrata (DSY562)(^e)</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>4</td>
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<td>5</td>
<td>c</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>Caspofungin(^e)</td>
<td>0.0625</td>
</tr>
</tbody>
</table>

\(^e\)Minimum inhibitory concentration (MIC); \(^f\)reference compound; \(^g\)inactive: MIC > 32 µg mL\(^{-1}\).
one or two sugars at C-3 of the aglycone hederagenin are ideal for the antifungal activity. Indeed, some monodesmosidic glycosides (with only one sugar chain at C-3) of hederagenin have been showed to be active against *C. albicans* whereas their bidesmosides forms (with an additional sugar chain at C-28) were inactive.\(^3\)\(^2\) In addition, it has been described that rhamnosyl-arabinosyl moiety at C-3 increased the anti-*Candida* activity of hederagenin glycosides compared to other sugar chain.\(^3\)\(^7\) It might be due to variances in the molecular conformation of the saponins that would induce differential membrane curvature and membrane permeabilization.\(^3\)\(^8\)

In order to compare the activities and cytological effects of the isolated compounds, one additional antifungal saponin, medicagenic acid 3-O-β-D-glucopyranoside (6) and its aglycone, medicagenic acid (7) were included in the different biological assays. In the broth dilution assay, 6 and 7 showed as expected strong antifungal activity with MIC value of 2 µg mL\(^{-1}\). The activity spectrum was similar to the isolated compound. The MIC values were 1 or 2 µg mL\(^{-1}\) on every *Candida* species tested. Compound 6 has been reported to have antifungal effects against *Trichoderma viride*, *Aspergillus niger* and against several medically important yeasts such as *C. albicans*.\(^3\)\(^9\)-\(^4\)\(^2\) Its mode of action against *Saccharomyces cerevisiae* seems to be different from that described for polyene antibiotics.\(^4\)\(^1\) 7 has reported antifungal activities against *C. tropicalis*, *C. albicans*, *Blastomyces capitatus* and *Saccharomyces cerevisiae*.\(^4\)\(^4\) As compared to other saponins aglycones that are inactive, the low MIC value of 7 might be rationalised by the presence of the carboxylic acid function in position 4 that gives polar properties to the molecules.

Examination of cytological alterations caused by antifungal compounds using transmission electron microscopy evaluation (TEM)

Although interference with the integrity of biological membranes is the main effect of saponin activities, saponins are not limited to this type of action. As a matter of fact, saponins show significant haemolytic activity.\(^4\)\(^5\) Solely based on their structures it is difficult to predict their activities. To get a better understanding of the action of isolated saponins, the cytological effects of the most active compounds on wild type strain of *C. albicans* were investigated by transmission electron microscopy analysis (TEM). Yeast were grown on YNB (yeast nitrogen base) medium at pH 4.6 and treated with these compounds at the MIC concentration including controls. Non-treated *C. albicans* cells showed intact cell walls, cell membranes and organelles, such as mitochondria, nuclei and nuclear membranes. A high number of ribosomal particles could be observed across the cytoplasm. Contrary to negative controls (Figures 3A and 3B), *C. albicans* cells treated by 1, 2, and 3 exhibited alterations of the cell wall architecture with the appearance of dark disorganized material into the cell wall space (Figures 3C and 3D). This is consistent with previous studies on the cytotoxic effect of (α)-hederin on *C. albicans* cells.\(^4\)\(^8\) Important disorganization, darkening and retraction of the cytoplasmic content were observed, where mitochondria, nucleus, nuclear membranes, Golgi apparatus and ribosomes were not visible. In addition, the plasma membrane exhibited significant degradation and several breakage zones.

Yeast cells treated with 6 showed similar effects to those exposed to 2 with an important retraction and distortion of the plasma membrane and a thickening of the cell wall (Figures 3E and 3F). These results are consistent with the cytotoxic effects observed on plant cell suspension cultures.\(^4\)\(^9\) Contrary to what was shown after treatment with the reference drug miconazole,\(^1\) no accumulation of lipid-like bodies was observed in vacuoles, neither highly contrasted membranes, with the exception of dark material in the parietal zone of cell walls.

*In vivo* assays using the *Galleria mellonella* model

Compounds 1, 6 and 7 were selected for further investigation to determine their *in vivo* activity on the *G. mellonella* infection model.\(^1\)\(^3\) This *in vivo* model is used to study the virulence of microbial pathogens and the efficacy of antimicrobial agents using insect larvae. Several studies have demonstrated the efficacy of known antifungal agents in this model for *Candida* infection.\(^4\)\(^8\) A direct correlation has been demonstrated between the virulence of *C. albicans* in murine and wax moth larvae models of infection.\(^4\)\(^9\) Moreover, this model is inexpensive and easy to handle.

To assess the potential toxicity of 1, several doses were first administered through the last pro-leg and the survival of the larva was monitored. Unexpectedly, larvae exhibited an important and significant increase in mortality from concentrations equal or superior to 50 mg kg\(^{-1}\) (n = 10, log-rank (Mantel-Cox) test, p < 0.0001) and thus, the *in vivo* efficacy was tested at lower concentrations. The larvae were infected with wild-type *C. albicans* and one hour post-infection 1 was administered at 25 mg kg\(^{-1}\). The survival rate was monitored for eight days. While fluconazole, a well-known antifungal azole drug, caused a dramatic increase in the survival rate of larvae compared to the infected controls (Mantel-Cox test, p < 0.0001),
1 showed no significant effects on survival as compared to controls (Mantel-Cox test, p > 0.05) (data not shown). Contrary to 1, compounds 6 and 7 did not show toxicity effect on larvae up to 50 mg kg\(^{-1}\), however, similarly to 1, and even at higher doses, they did not exhibit significant effect on survival as compared to non-treated controls (Mantel-Cox test, p > 0.05).

Conclusions

HPLC activity-based metabolite profiling enable a rapid and efficient identification of triterpenoids from Panamanian plants not previously investigated for their bioactive constituents. From a chemotaxonomic viewpoint, the presence of these compounds are in agreement with...
Identification of Triterpenoids from *Schefflera systyla*, *Odontadenia puncticulosa* and *Conostegia speciosa* J. Braz. Chem. Soc.

Among isolates compounds, (α)-hederin (I) was the most interesting by its large spectral profile against *Candida* species and its activity against biofilms. It induces modifications of cellular contents and alterations of cell envelope with degradation and death of the yeast cells. A recent study demonstrated that the haplo-insufficiency profile of (α)-hederin was similar to that reported for drugs such as caspofungin that inhibit synthesis of the fungal cell wall. It has been shown that I daily orally administrated in mice infected with *C. albicans* was able to cure the mice at 100 mg kg\(^{-1}\) over 10 days. In our assay (α)-hederin did not show antifungal activity in *G. mellonella*, suggesting the limitation of this assay for such class of compounds. The absence of in vivo antifungal activity of I in *G. mellonella* could be explained by the poor membrane permeability of the triterpene glycosides due to its large molecular mass (> 500 Da), high hydrogen-bonding capacity (> 12) and high molecular flexibility (> 10).

Saponins are known to possess many biological activities such as haemolytic, antiviral, fungicidal, molluscicidal or cytotoxic activities, partially due to their interaction with the cell membrane. Recently, some saponins have shown in vivo antifungal activity comparable to amphotericin B against *Candida* species in the *C. elegans* nematode assay. In this work no hemolysis of erythrocytes (common cause of toxicity) was observed suggesting that some saponins have preference to bind to fungal ergosterol instead of human cholesterol. For all these reasons the saponin scaffold may represent an opportunity to expand the available limited class of antifungal agents. The mode of action as well as their potential hemolytic and or cytotoxic effect must be investigated more in depth in order to take into account their bioavailability.

**Experimental**

**General experimental procedures**

UV spectra were measured on a PerkinElmer Lambda 20 spectrophotometer. LC-PDA-MS data were obtained with an Agilent 1100 series system (Santa Clara, CA, USA) consisting of an auto sampler, a high-pressure mixing pump and a PDA detector connected to a Finnigan MAT LCQ ion trap mass spectrometer equipped with a Finnigan electrospray (ESI) interface (San Jose, CA, USA). HRESIMS data were obtained on a Micromass-LCT Premier Time of Flight (TOF) mass spectrometer (Waters, MA, USA) with an electrospray interface. ¹H and ¹³C NMR data were recorded on a Varian Inova 500 spectrometer (Palo Alto, CA, USA) (499.87 and 125.70 MHz, respectively) in DMSO-\(d_6\) with TMS as an internal standard. Complete assignment was performed using 2D experiments such as gradient COSY (correlation spectroscopy), gradient HSQC (heteronuclear single quantum correlation spectroscopy), gradient HMBC (heteronuclear multiple bond correlation spectroscopy) and NOESY (nuclear Overhauser effect spectroscopy). UHPLC-TOF-HRMS analysis of the isolated compounds was performed according to standard procedures. Analytical HPLC was carried out on an HP 1100 system equipped with a photodiode array detector (Agilent Technologies, Santa Clara, CA, USA). The standard of compound 5 (hederagenin, > 90%, cas. 465-99-6) was obtained from Extrasynthese, Genay, France. Compound 6 (3-β-D-glucose medicagenic acid, > 95%, cas. 49792-23-6) was obtained from the Phytochemistry and Bioactive Natural Products database, University of Geneva. Standard of compound 7 (medicagenic acid, > 95%, cas. 599-07-5) was obtained from Phytolab, Vestenbergsgreuth, Germany.

**Plant material**

All the plant material was collected in the Panama Channel Zone, Panama, and identified by De Gracia, J. The steams of *Conostegia speciosa* Naudin were collected in August 1991, at Carretera Llano Cartí. The leaves of *Odontadenia puncticulosa* (Rich.) Pulle were collected in March 1992, at Cerro Azul, La Eneida. The leaves of *Schefflera systyla* (Donn. Sm.) R. Vig. were collected in July 2001, at Parque Nacional Altos de Campana. A voucher of each species was deposited at the National Herbarium of Panama (FLORPAN (No. 1673, 0705 and 5223, respectively)) and at the Phytochemistry & Bioactive Natural Products and Pharmacognosy Laboratory, University of Geneva, Switzerland (No. 7715, 6317 and 8275, respectively).

**Preparation of the crude extracts**

The air-dried plant material was pulverized in a Wiley Mill and extracted at room temperature successively with dichloromethane (CH\(_2\)Cl\(_2\)) and methanol (MeOH). The extracts were concentrated under pressure and later lyophilized. From 580 g of *S. systyla* leaves, 18.8 g of CH\(_2\)Cl\(_2\) extract and 65 g of MeOH extract were obtained. From 500 g of *O. puncticulosa* leaves, 14 g of CH\(_2\)Cl\(_2\) extract and 78 g of MeOH extract were obtained. From 32.5 g of *C. speciosa* stems, 1.4 g of CH\(_2\)Cl\(_2\) extract and 5 g of MeOH extract were obtained.
HPTLC analysis

The HPTLC analyses were performed with an Automatic TLC Sampler (4) and an Automatic Developing Chamber (ADC 2) (CAMAG, Muttenz, Switzerland). Plant extract (200 µg) was deposited onto the HPTLC plate (10 × 10 cm, silica gel 60, Merck, Darmstadt, Germany). Methanolic extract of \( O. \) pusticulosa was eluted with the solvent system \( CH_2Cl_2/MeOH/ethyl acetate \) (EtOAc) 87:12:1. \( C. \) speciosa was eluted with \( CH_2Cl_2/MeOH/EtOAc \) 70:15:15. The methanolic extract of \( S. \) systila was eluted with \( CH_2Cl_2/MeOH/EtOAc \) 50:30:20. TLC profiles were revealed by ultraviolet (UV) detection at 254 and 366 nm with a TLC Visualizer (CAMAG), and images were obtained with visionCATS version 1.4.0 software. The HPTLC analyses were performed in duplicate for the bioautography and for the chemical detection with specific reagents.

Semi preparative HPLC-microfractionation for the antifungal assay and isolation of the major antifungal compounds

200 µL of each extract were injected in a semi preparative HPLC (Spotprep Liquid Chromatography, Armen instrument) with an X-Bridge C\(_18\) column (250 × 10 mm i.d.; 5 µm, Waters) for the fractionation of the methanolic extracts of \( S. \) systyla and \( O. \) pusticulosa, while the extract of \( C. \) speciosa was fractionated with a X-Bridge C\(_18\) column (150 × 19 mm i.d.; 5 µm, Waters). Fractions were collected every 10 mL. After collection, each fraction was evaporated to dryness using a SpeedVac (HT-4X Genevac\(^s\), Stone Ridge, NY, USA). The dried fractions were evaluated in the bioautography antifungal assay. The methanolic extract of \( S. \) systyla leaves (25 mg) was eluted with a flow rate of 4.7 mL min\(^{-1}\) with the following solvent system: A = \( H_2O \) with 0.1% formic acid (FA), B = MeOH with 0.1% FA; gradient: 5 to 100% B from 0-60 min. The detection was performed at 280 nm. The active fractions 30-32 yielded compound 1 (2.1 mg). The methanolic extract of \( O. \) pusticulosa stems (45 mg) was eluted with a flow rate of 14 mL min\(^{-1}\) with the following solvent system: A = \( H_2O \) with 0.1% FA, B = MeOH with 0.1% FA; gradient: 2 to 45% B from 0-25 min, then to 45 to 100% B from 25-31 min, 100% B between 31-40 min. The detection was performed at 254 nm. The active fraction 54 yielded compound 2 (4.3 mg). The methanolic extract of \( C. \) speciosa stems (38.65 mg) was eluted with a flow rate of 4.7 mL min\(^{-1}\) with the following solvent system: A = \( H_2O \) with 0.1% FA, B = MeOH with 0.1% FA; gradient: 5 to 100% B from 0-80 min. The detection was performed at 254 nm. The active fraction 35 yielded compound 4 (2.5 mg).

Isolation of the antifungal compounds from the methanolic extract of \( O. \) pusticulosa using medium pressure liquid chromatography (MPLC-UV)

The MeOH (5 g) extract of \( O. \) pusticulosa was fractionated using MPLC with a \( C_18 \) reverse stationary phase (Zeoprep\(^s\) 60, 15-25 µm, (Zeochem, Uetikon am See, Switzerland) column (460 × 70 mm, i.d.; Buchi, Flawil, Switzerland) with a linear gradient MeOH and H\(_2\)O from 5 to 100% MeOH with 0.1% formic acid at a flow rate of 6 mL min\(^{-1}\) yielded 31 fractions. Fractions F28 yielded compound 2 (33.8 mg), while F30 yielded compound 3 (127.4 mg).

Identification of the isolated compounds

\( (\alpha)-\)Hederin (1)

Amorphous powder; HR-ESI-MS \( m/z : \) 749.4447 \( [M - H]^+ \) (calcld. for \( C_{31}H_{43}O_{17} : \) 749.4476, \( \Delta = 3.9 \) ppm). For the \( ^1H \) and \( ^13C \) NMR, see Supplementary Information, Shao et al.\(^{37}\) and Panov et al.\(^{38}\)

Pulsatilla saponin D (2)

Amorphous powder; HR-ESI-MS \( m/z : \) 913.5292 \( [M + H]^+ \) (calcld. for \( C_{35}H_{53}O_{17} : \) 912.5362, \( \Delta = 3.4 \) ppm). For the \( ^1H \) and \( ^13C \) NMR, see Supplementary Information and Bang et al.\(^{39}\)

\( 3\{\beta\}-O-[(\beta-D-Xylopyranosyl-(1\rightarrow3)]-\alpha-L-rhamnopyranosyl-(1\rightarrow2)-[\beta-D-glucopyranosyl-(1\rightarrow4)]-\alpha-L-arabinopyranosylhederagenin \) (3)

Amorphous powder; HR-ESI-MS \( m/z : \) 1045.5612 \( [M + H]^+ \) (calcld. for \( C_{42}H_{63}O_{21} : \) 1044.5539, \( \Delta = 2.8 \) ppm). For the \( ^1H \) and \( ^13C \) NMR, see Supplementary Information and Wang et al.\(^{41}\)

Arjunolic acid (4)

Amorphous powder; HR-ESI-MS \( m/z : \) 487.3419 \( [M - H]^+ \) (calcld. for \( C_{39}H_{47}O_{18} : \) 487.3424, \( \Delta = 1 \) ppm). For the \( ^1H \) and \( ^13C \) NMR, see Supplementary Information and Ghosh et al.\(^{42}\)

Yeast strains

The strains used in the study are: \( C. \) albicans DSY2621,\(^{43}\) \( C. \) albicans CAF2.1, \( C. \) krusei ATCC 6258, \( C. \) tropicalis ATCC 75, \( C. \) parapsilosis ATCC 22019, \( C. \) glabrata DSY562.\(^{44}\)

Bioautography

The assay used was an optimized version of a method
published by Rahalison et al. Briefly, the *C. albicans* strains were cultivated overnight at 36 °C in Sabouraud broth medium. A dilution was made in order to obtain an inoculum of 10⁵ cells mL⁻¹ (an optical density (OD) equal to 1 at 630 nm corresponding to approximately 10⁷ cells mL⁻¹) in malt agar (malt extract, 30.0 g L⁻¹; peptone from soymeal, 3.0 g L⁻¹; agar-agar, 15.0 g L⁻¹; Merck). The molten medium was maintained in a water bath at 45 °C. The OD at 630 nm of the *C. albicans* culture was measured with an UV-Vis spectrophotometer (Synergy H1, Biotek, equipped with the software Gen 5.2 software). Approximately 20 mL of the inoculum (either DSY2621 or CAF2-1) were distributed rapidly over the HPTLC plate with a sterile pipet. After solidification of the medium, the plates were incubated overnight at 36 °C in polyethylene boxes lined with moist chromatography paper. The bioautograms were sprayed with an aqueous solution (2.5 mg mL⁻¹) of thiazolyl blue (methyl thiazoly tetrazolium chloride; MTT; Fluka), and incubated for 6 h at 36 °C. Clear inhibition zones were observed against a purple background. To calculate the MIQ, 10 µL aliquots of different concentrations (from 0.01 to 10 mg mL⁻¹ in MeOH) of the pure compounds were spotted manually on the HPTLC plate as well as 10 µL of only MeOH. Then, the HPTLC plate without elution was submitted to the same procedure published by Favre-Godal et al. The MIQ was defined as the test compound quantity at which inhibition was observed.

**Antifungal susceptibility testing**

Antifungal susceptibility testing on planktonic cells was carried out on the basis of EUCAST protocols with slight modifications. Briefly, *C. albicans* strains were cultivated overnight at 30 °C under constant agitation in yeast extract peptone dextrose (YPD). Cultures were diluted to a density of 0.5-2 × 10⁸ cells mL⁻¹ in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma) with L-glutamine, without bicarbonate, and with phenol red as the pH indicator. RPMI 1640 medium was buffered to pH 7 with 0.165 M morpholinepropanesulfonic acid (MOPS) and was supplemented with glucose to a final concentration (m/v) of 2% and with 1% dimethyl sulfoxide (DMSO). Compounds were dissolved in DMSO to 1 mg mL⁻¹ as final concentration. First, 50 µL of RPMI was distributed on each well of the 96-well plate. Two-fold serial dilutions were prepared from 32 to 0.0162 µg mL⁻¹. Since the yeast inoculum was fixed at a volume of 150 µL with a density of 2 × 10⁸ cells mL⁻¹, 50 µL of an eight-fold concentrated compound stock was first dispensed to the well corresponding to the highest drug concentration. Two-fold dilutions were next performed by serially transferring half volumes of each well up to the last well of the microplate row. Finally, 150 µL of the yeast inoculum were added to each well. Drug-free cultures and sterility controls were included in each 96-well plate. Plates were incubated at 35 °C for 24 h and then MICs were read with a spectrophotometer plate reader set at 450 nm. The MIC was defined as the drug concentration at which the optical density was equal or decreased more than 50% from that of the drug-free culture. Antifungal susceptibility assays on biofilms were conducted according to a published protocol. Briefly, an aliquot of a 100 µL solution (1 × 10⁶ cells mL⁻¹ density) per well prepared in the RPMI medium 0.2% glucose (pH 7) was deposited in a 96 well plate and incubated at 37 °C for 48 h to allow biofilm formation. Wells were then washed twice with phosphate-buffered saline (PBS). Two-fold serial dilutions of the compounds were prepared from 50 to 1.56 µg mL⁻¹ and added to the wells containing the biofilms. Plates were incubated again for 48 h at 37 °C and then washed twice with PBS. A measurement of the metabolic activity of the sessile cells was performed using a colorimetric assay with 2H-tetrazolium,2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[phenylamino] carbonyl]-hydroxide salt (XTT) (X4626, Sigma Aldrich). Plates were read with a spectrophotometer plate reader set at 492 nm. The MIC was defined as the drug concentration at which the optical density value was equal or less than 50% of the one of the drug-free biofilm.

**Electron microscopy**

The *C. albicans* CAF2-1 strain was grown in 10 mL YNB liquid cultures (50 mL plastic tubes, 37 °C, 2 h). At this time, pulsatilla saponine D (2) and medicagenic acid 3-O-β-D-glucopyranoside (6) were added at a concentration of respectively 8 and 2 µg mL⁻¹ in DMSO and the cultures were grown for 18 h to evaluate their cytotoxic effect. The samples were centrifuged (10 min, 1300 × g, room temperature), the supernatant was discarded, and the resulting pellet was prepared according to Roland and Vian. Briefly, the pellets were prefixed with a solution of 3% glutaraldehyde-2% paraformaldehyde in 0.07 M phosphate buffer (pH 7), embedded in 2% agarose and postfixed with a solution of 1% OsO₄. They were then dehydrated in a graded series of ethanol solutions [30-50-70-95-100% (v/v)] and embedded in LR white resin (14381-UC; London Resin Company). After polymerization for 24 h at 60 °C, thin (0.08 µm) sections were cut and stained with a 2% uranyl acetate followed by lead citrate according to Reynolds. The thin sections were observed with a transmission electron microscope.
Supplementary Information

Supplementary information (representation of the isolation process of antifungal saponins from *Odontadenia puncticulosa* and *Conostegia speciosa* and 1D and 2D NMR spectra) is available free of charge at http://jbcs.sbq.org.br.

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