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Article

Antifungal Piperolides from *Piper malacophyllum* (Prels) C. DC.

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O estudo fitoquímico biomonitorado do extrato em CH_2Cl_2 das folhas de *Piper malacophyllum* através da técnica de bioautografia frente a fungos, resultou no isolamento de dois piperolidos bioativos: 4,6-dimetóxi-5-*E*-fenilbutenolido (**1**) e 4,6-dimetóxi-5-*Z*-fenilbutenolido (**2**). Essas substâncias foram identificadas com base na análise dos espectros de IV, EM e RMN, incluindo técnicas bidimensionais, e comparação com dados descritos na literatura. A configuração *E* da ligação dupla confere maior atividade para **1** considerando-se que as quantidades mínimas de **1** e **2** necessária para inibir o crescimento dos fungos *Cladosporium cladosporioides* e *C. sphaerospermum* foram de 1,0/1,0 μg (**1**) e de 5,0/10,0 μg (**2**), respectivamente.

Bioactivity-guided fractionation of the CH_2Cl_2 extract from leaves of *Piper malacophyllum* using bioautography assay against fungi, led to the isolation of two known bioactive piperolides: 4,6-dimethoxy-5-*E*-phenylbutenolide (**1**) and 4,6-dimethoxy-5-*Z*-phenylbutenolide (**2**). IR, MS and NMR spectroscopic data were used for the identification of these compounds including comparison with previously reported data. The *E* configuration is associated to higher activity for compound **1**, since the minimum amount required for the growth inhibition of *Cladosporium cladosporioides* and *C. sphaerospermum* were 1.0/1.0 μg (**1**) and 5.0/10.0 μg (**2**), respectively.

Keywords: *Piper malacophyllum*, piperolides, antifungal activity

Introduction

The genus *Piper* is a source of several classes of bioactive secondary metabolites including alkaloids, amides, flavonoids, benzoic acid derivatives, terpenes, and cyclopentanediones.[1-3] In the course of our search aiming to unravel new antifungal metabolites from plants in Brazilian Atlantic Forest and Cerrado,[4,5] the crude CH_2Cl_2 extract from leaves of *Piper malacophyllum* was selected for bioactivity-guided phytochemical investigation due to the strong activity observed against *Cladosporium cladosporioides* and *C. sphaerospermum.* After several chromatographic steps, this extract yielded two structurally related piperolides, which had their structures identified by analysis of spectrometric data.

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Results and Discussion

Bioactivity-guided fractionation of the CH_2Cl_2 extract from leaves of *P. malacophyllum* by silica gel and Sephadex LH-20 chromatography yielded two butenolides of the piperolide series.

Compound **1** had its molecular formula indicated as $C_{13}H_{12}O_4$ by analysis of LREIMS (70 eV, $[M]^{+\bullet}$ at *m/z* 232), and ^{13}C NMR (BBD and DEPT 135°). The IR spectrum exhibited bands at 1748 (conjugated lactone carbonyl group) and at 1592, 1493, and 1446 (aromatic) cm^{-1}. The ^1H NMR (Table 1) spectrum of **1** showed multiplets at δ 7.43-7.45 (2H) and δ 7.73 (3H), a pattern of a monosubstituted aromatic ring. This spectrum also exhibited two methoxyl signals at δ 3.99 (s) and δ 3.65 (s) besides one singlet at δ 5.27 (1H). These information, associated to the ^{13}C NMR resonances at δ 171.7 (C), 168.1

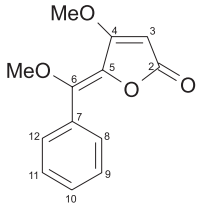
Table 1. ^{1}H and ^{13}C NMR (500 and 125 MHz) spectral data for piperolides **1** and **2** (δ, $CDCl_3$) isolated from *P. malacophyllum*

	1			**2**		
position	δ_H	δ_C[a]	HMBC (H → C)	δ_H	δ_C[a]	HMBC (H → C)
2	-	168.1 (C=O)	-	-	167.8 (C=O)	-
3	5.27 (s)	88.8 (CH)	C-2, C-4, C-5	5.11 (s)	87.5 (CH)	C-2, C-4, C-5
4	-	171.7 (C)	-	-	170.8 (C)	-
5	-	135.0 (C)	-	-	129.0 (C)	-
6	-	144.6 (C)	-	-	144.0 (C)	-
7	-	131.2 (C)	-	-	130.7 (C)	-
8	7.73 (m)	129.0 (CH)	C-6, C-7, C-10, C-12	7.42 (m)	130.1 (CH)	C-6, C-10, C-12
9	7.43 (m)	128.6 (CH)	C-7, C-11	7.37 (m)	128.0 (CH)	C-7, C-11
10	7.73 (m)	129.9 (CH)	C-8, C-12	7.42 (m)	130.0 (CH)	C-8, C-12
11	7.45 (m)	128.6 (CH)	C-7, C-9	7.37 (m)	128.0 (CH)	C-7, C-9
12	7.73 (m)	129.0 (CH)	C-6, C-7, C-8, C-10	7.42 (m)	130.1 (CH)	C-6, C-8, C-10
4-OCH_3	3.99 (s)	59.5 (CH_3)	C-4	3.59 (s)	58.9 (CH_3)	C-4
6-OCH_3	3.65 (s)	60.5 (CH_3)	C-6	3.74 (s)	58.8 (CH_3)	C-6

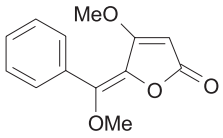
[a]Multiplicities obtained from ^{13}C NMR DEPT 135° spectrum.

(C=O), 135.0 (C), 88.8 (CH) indicated a piperolide type structure containing one methoxyl group at C-4.[6] The resonance signals at δ 135.0 (C) and δ 144.6 (C) are indicative of a tetrasubstituted double bound, and thus the second methoxyl group was placed at C-6. Finally, the signals at δ 129.0 (CH), 128.6 (CH), and 131.2 (C) were assigned to C-8/C-12, C-9/C-11, and C-7 carbon atoms of the aromatic ring, respectively. The HMBC spectrum of **1** showed long-range ^{1}H-^{13}C correlations between δ 3.99 (OCH_3) and δ 171.7 (C-4) and between δ 3.65 (OCH_3) and δ 144.6 (C-6) confirming the assignments of the methoxyl groups at C-4 and C-6. Additionally, the correlations between H-3 (δ 5.27) and C-2 (δ 168.1) / C-5 (δ 135.0) and between H-8/H-12 (δ 7.73) and C-6 (δ 144.6) confirmed the piperolide moiety and its connectivity with the aromatic ring as well. The configuration of the double bond was determined based on nOe experiments. Irradiation of δ 5.27 (H-3) gave a nOe enhancement for the hydrogens at δ 3.99, confirming the positioning of this methoxyl group at C-4. On the other hand, irradiation at δ 3.99 (4-OCH_3) gave a positive nOe for the hydrogens at δ 3.65 (6-OCH_3) and δ 5.27 (H-3), indicating the *E* configuration to double bond. Therefore, the compound **1** was identified as 4,6-dimethoxy-5-*E*-phenylbutenolide (5,6-*E*-fadyenolide), previously isolated from roots of *Piper fadyenii*. [6,7]

The LREIMS spectrum of **1** and **2** showed the same molecular ion-peak and fragment ions, suggesting similar structures. The IR spectrum with bands at 1745, 1592, 1493, and 1443 cm^{-1} confirming the presence of conjugated lactone group and aromatic ring in the structure of compound **2**. The ^{13}C NMR spectra (Table 1) exhibited signals of two methoxyl groups (δ 58.9 / 58.8), two olefinic carbons [δ 144.0 (C) and 130.7 (C)], four carbons of the butenolide ring [δ 170.8 (C), 167.8 (C=O), 129.0 (C), 87.5



1



2

(CH)], and six aromatic carbons [δ 130.7 (C), 130.1 (2 CH), 130.0 (CH), 128.0 (2 CH)] similarly to those signals observed to the piperolide **1**. The ^{1}H NMR spectrum showed a multiplet at δ 7.37-7.42 (5H), indicative of a monosubstituted aromatic ring, and three singlets at δ 5.11 (1H), 3.59 (3H) and 3.74 (3H), which confirmed the structural similarity between **1** and **2**. The cross-peaks between δ 3.59 (OCH_3) / 170.8 (C-4) and δ 3.74 (OCH_3) / 144.0 (C-6) in its HMBC spectrum determined the position of the methoxyl groups at C-4 and C-6, as depicted to compound **1**. This spectrum showed also cross peaks between H-3 (δ 5.11) and C-2 (δ 167.8) / C-5 (δ 129.0) and between H-8/H-12 (δ 7.37-7.42) and C-6 (δ 144.0). Thus the connectivity between the butenolide and aromatic rings and the same planar structure of **1** was confirmed. However, irradiation of signal at δ 3.59 (4-OCH_3) gave a nOe enhancement for the hydrogens at δ 5.11 (H-3) and δ 7.42 (H-8/H-12) while the signal at δ 3.74 (6-OCH_3) were not affected, indicating the *Z* configuration for the double bound. Therefore, the compound **2** was identified as 4,6-dimethoxy-5-*Z*-phenylbutenolide, as previously defined to 5,6-*Z*-fadyenolide, also isolated from roots and aerial parts of *Piper fadyenii*.[6,7]

There are only few report of piperolides in Piperaceae species and to date they have been described from *Piper fadyenii*[6,7] and *P. sanctum*[8,9] which occur from Jamaica and

Mexico, respectively. In such reports[8] the NMR data of compounds **1** and **2** were assigned only to the butenolide moiety. Thus herein we present an unambiguous assignment of their NMR data based on several 2D spectra including HMBC experiment (Table 1).

Both compounds were isolated in a bioactivity-guided fractionation procedure. The antifungal activity of compounds **1** and **2** was determined by means of direct bioautography on TLC plate[11]. The detection limits of these compounds required to inhibit growth of the fungus *C. cladosporioides* and *C. sphaerospermum* (Table 2) were obtained according to methodology described elsewhere.[5,11,12] Compound **1** presented stronger antifungal activity than that observed for compound **2**, suggesting that the configuration of the double bond in the butenolide ring could be associated to their fungitoxic potential. There was no previous report describing the fungitoxic potential of these compounds, mainly **1** which showed a similar activity of the positive control (nystatin and miconazole).

Table 2. Antifungal activity of compounds **1** and **2** against *Cladosporium cladosporioides* and *C. sphaerospermum*

Compound	Antifungal activity[a] (μg)	
	C. cladosporioides	*C. sphaerospermum*
1	1.0	1.0
2	5.0	10.0

Positive control: nystatin (1.0 μg) and miconazole (1.0 μg); [a]Minimum amount required for the inhibition of fungal growth on thin-layer chromatographic plates (TLC).

Experimental

Instrumental and chromatography materials

Silica gel (Merck 230-400 mesh) and Sephadex LH-20 (Pharmacia) were used for column chromatographic separation. Silica gel PF_{254} (Merck) was used to TLC preparative purification. The NMR spectra were recorded on Bruker DRX-500 operating at 500 MHz to ^{1}H and at 125 MHz to ^{13}C, in $CDCl_3$ with TMS as internal standard. IR spectra were obtained on a FT-IT 510 Nicolet spectrometer. LREIMS were measured at 70 eV on a HP 5990/5988A spectrometer. UV spectra were recorded on a UV/Visible Hitachi U-3000 spectrophotometer.

Plant material

The leaves of *Piper malacophyllum* (Prels) C. DC. was collected in December 2000 in the Parque Estadual Intervales, São Paulo State, Brazil and was identified by Dr. Elsie F. Guimarães (Jardim Botânico – Rio de Janeiro). The voucher specimen (Kato-0100) has been deposited at Herbarium of Instituto de Botânica, SMA, São Paulo, SP, Brazil.

Antifungal assays

The microorganisms used in the antifungal assays *C. sphaerospermum* (Penzig) SPC 491 and *C. cladosporioides* (Fresen) de Vries SPC 140 have been maintained at the Instituto de Botânica, São Paulo, SP, Brazil. Ten microliters of the solutions of the crude extracts, fractions and pure compounds were prepared, in different concentrations, corresponding to 20, 10, 5 and 1 μg for pure compounds and 100 μg for the crude extracts or fractions. The samples were applied to TLC plates, these being eluted with $CHCl_3$-MeOH 99:1 followed by complete removal of the solvent at room temperature. The chromatographic plates were sprayed with spores suspension of *C. sphaerospermum* and *C. cladosporioides* in a nutritive medium (glucose and salt solution[11]) and incubated for 48 h and 37 °C. After incubation, clear inhibition zones appeared against a dark background chromatogram. Nystatin and miconazole were used as positive controls whereas ampicillin and chloramphenicol were used as negative controls.[5,11]

Extraction and isolation of constituents

The dried and powdered leaves of *P. malacophyllum* (200g) were extracted with CH_2Cl_2 (3 × 1.5 L), for two days, at room temperature. The resulting extract was filtered and concentrated in vacuum to afford 11.3 g of the crude extract. Bioactivity-guided fractionation of this extract by flash silica gel column chromatography using hexane (300 mL), hexane-CH_2Cl_2 (1:1 - 300 mL), CH_2Cl_2 (450 mL), CH_2Cl_2-EtOAc (1:1 - 300 mL), EtOAc (150 mL), EtOAc-MeOH (1:1 - 200 mL) and MeOH (200 mL) as eluents, afforded thirteen fractions (150 mL each), whose bioactivity was detected only in two of them (5 and 7). Fraction 5 (380 mg) was submitted to Sephadex LH-20 exclusion chromatography using hexane-CH_2Cl_2 (1:4 – 150 mL), CH_2Cl_2-Me_2CO (3:2 and 1:4 – 150 mL each) as eluent to give twelve sub-fractions (25 mL each). Antifungal assay on all these sub-fractions indicated that the bioactivity was concentrated on the sub-fractions 3 and 4. These sub-fractions were pooled (70 mg) and submitted to preparative silica-gel TLC purification, using hexane-EtOAc 4:1 as eluent. This purification procedure yielded 10 mg of **1** (yielding 0.09%). Fraction 7 (238 mg) was subjected to Sephadex LH-20 exclusion chromatography, using hexane-CH_2Cl_2 (1:4 – 100 mL), CH_2Cl_2-Me_2CO (3:2 and 1:4 – 100 mL each) as eluent, to

give four sub-fractions (50 mL each). Compound **2** (8 mg – yielding 0.07%) was isolated from the bioactive sub-fraction 2 (112 mg), by further purification over column chromatography on silica-gel eluted with hexane-EtOAc 7:3 (150 mL).

*4,6-Dimethoxy-5-E-phenylbutenolide (5,6-E-fadyenolide - **1**)*

Colorless crystals. mp 129-131°C. IR(film) ν_{max} / cm^{-1}: 2936, 1748, 1592, 1493, 1446, 1378, 1216, 1135; UV λ_{max} MeOH nm (log ε): 248 (4.05), 320 (4.06); LREIMS *m/z* (rel. int.): 232 (58) [$M^{+\bullet}$], 217 (7), 202 (4), 189 (11), 161 (14), 145 (3), 131 (2), 115 (7), 105 (100), 91 (4), 77 (51), 69 (26), 51 (25); ^1H and ^{13}C NMR spectra (see Table 1).

*4,6-Dimethoxy-5-Z-phenylbutenolide (5,6-Z-fadyenolide - **2**)*

Colorless crystals. mp 130-132°C. IR (film) ν_{max} / cm^{-1}: 2941, 1745, 1592, 1493, 1443, 1385, 1218, 1135; UV λ_{max} MeOH nm (log ε): 245 (2.72), 313 (3.15); LREIMS *m/z* (rel. int.): 232 (67) [$M^{+\bullet}$] 217 (7), 202 (4), 189 (14), 161 (13), 145 (4), 131 (3), 115 (8), 105 (100), 91 (5), 77 (53), 69 (27), 51 (26); ^1H and ^{13}C NMR spectra (see Table 1).

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References

1. Parmar, V.S.; Jain, S.C.; Bisht, K.S.; Jain, R.; Taneja, P.; Jha, A.; Tyagi, O.D.; Prasad, A.K.; Wengel, J.; Olsen, C.; Boll, P.M.; *Phytochemistry* **1997**, *46*, 597.
2. Orjala, J.; Erdelmeier, C.A.J.; Wright, A.D.; Rali, T.; Sticher, O.; *Planta Med.* **1993**, *59*, 546.
3. Facundo, V. A.; Sa, L. A.; Silva, S.A.; Morais, S. M.; Matos, C.R.R.; Braz-Filho, R. *J. Braz. Chem. Soc.* **2004**, *15*, 140.
4. Navickiene, H.M.B.; Alécio, A.C.; Kato, M.J.; Bolzani, V. da S.; Young, M.C.M.; Cavalheiro, A.J.; Furlan, M.; *Phytochemistry* **2000**, *55*, 621.
5. Danelutte, A.P.; Lago, J.H.G.; Young, M.C.M.; Kato, M.J.; *Phytochemistry* **2003**, *64*, 555.
6. Pelter, A.; Al-Bayati, R.; Hänsel, R.; Dinter, H.; Burke, B.; *Tetrahedron Lett.* **1981**, *22*, 1545.
7. Nair, M.G.; Mansingh, A.P.; Burke, B.A.; *Agric. Biol. Chem.* **1986**, *50*, 3053.
8. Ganzera, M.; Khan, I.A.; *Forst. Chromatographia* **1999**, *50*, 649.
9. Ranjith, H.; Dharmaratne, W.; Nanayakkara, N.P.D.; Khan, I.A.; *Phytochemistry* **2002**, *59*, 429.
10. Singh, Y.M.; Blumenthal, M.; *Herbalgram* **1997**, *39*, 33.
11. Homans, A.L.; Fuchs, A.; *J. Chromatogr.* **1970**, *51*, 327.
12. Rahalison, L.; Hamburger, M.; Monod, M.; Frenk, E.; Hostettmann, K.; *Planta Med.* **1994**, *60*, 41.

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