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Antifungal flavanones and prenylated hydroquinones from *Piper crassinervium* Kunth

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Dedicated to the memory of Professor Jeffrey B. Harborne

Abstract

Bioactivity-guided fractionation of the EtOAc extract from leaves of *Piper crassinervium* yielded three prenylated hydroquinones together with two known flavanones naringenin and sakuranetin. Their structures were determined by means of spectroscopic analysis (NMR, IR, UV and MS) including two-dimensional NMR spectroscopy experiments (^{1}H–^{1}H COSY, HMQC, HMBC and NOESY). The antifungal activity was determined by direct bioautography against *Cladosporium cladosporioides* and *C. sphaerospermum*.

Keywords: *Piper crassinervium*; Piperaceae; Prenylated hydroquinones; Flavanones; Antifungal activity

1. Introduction

The Piperaceae family comprises 14 genera and ca. 1950 species (Mabberley, 1997). Among these, *Piper* and *Peperomia* are the more abundant with approximately 700 and 600 species, respectively (Joly, 1985). Phytochemical investigations carried out on *Piper* species have revealed many bioactive compounds such as amides, alkaloids, lignans, benzoic acids and chromenes (Parmar et al., 1997; Alécio et al., 1998; Wu et al., 1997; Ruangrungsi et al., 1992, Navickiene et al., 2000, Silva et al., 2002).

Considering the availability of Piperaceae species in Brazil, our bioprospecting studies are directed towards the discovery of new antifungal agents based on a simple bioautographic assay. Thus, the EtOAc extract from leaves of *Piper crassinervium* was selected for dereplication due to its potent activity against *Cladosporium cladosporioides* and *C. sphaerospermum*. Additionally, *P. crassinervium* is quite abundant in the Atlantic Forest of Brazil and also occurs in Colombia, Equador and Peru (Yuncker, 1972), but no previous phytochemical investigations have been carried out so far. Thus, the bioactivity-guided fractionation of an EtOAc extract from leaves of *P. crassinervium* yielded three new prenylated hydroquinones: 1,4-dihydroxy-2-(3′,7′-dimethyl-1′-oxo-2′-*E*,6′-octadienyl)benzene (**1**), 1,4-dihydroxy-2-(3′,7′-dimethyl-1′-oxo-2′-*Z*,6′-octadienyl)benzene (**2**), and 1,4-dihydroxy-2-(7′-methyl-3′-methylene-1′-oxo-4′,7′-peroxide-octyl)benzene (**3**) together with two known flavanones, naringenin (**4**) and sakuranetin (**5**).

2. Results and discussion

The EtOAc extract from leaves of *P. crassinervium* was submitted to bioactivity-guided fractionation by column chromatography on silica gel followed by prep. TLC to afford compounds **1**–**5**. The flavanones naringenin (5,7-dihydroxy-4′-methoxyflavanone-**4**) and sakuranetin (5,4′-dihydroxy-7-methoxyflavone-**5**) were identified by comparison of their physical and spectral data with those previously reported (Bohlmann et al., 1981; McCormick et al., 1986) while the new compounds were determined as follows.

Compound **1** was obtained as a white amorphous powder and had a molecular ion peak at *m*/*z* 260.1392 in HREIMS, in agreement with the molecular formula

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$C_{16}H_{20}O_3$. The IR spectrum indicated the presence of an α,β-unsaturated carbonyl system (1642 cm^{-1}), an aromatic ring (1588 cm^{-1}) and hydroxyl group (3389 cm^{-1}). The ^1H NMR spectrum exhibited three aromatic proton resonances at δ 6.84 (d, $J=8.9$ Hz), δ 6.98 (dd, $J=8.9$ and 3.0 Hz) and δ 7.21 (d, $J=3.0$ Hz) which were indicative of a 1,2,4-trisubstituted phenyl ring. In addition, the ^1H NMR spectrum showed signals assignable to three methyl at δ 1.63, 1.71 and 2.17 (s, 3H each), two methylene groups at δ 2.30 (m) and 2.20 (m), besides two olefinic hydrogens at δ 5.12 (t, $J=7.3$ Hz) and 6.66 (s). The ^{13}C NMR spectra (BBD and DEPT 135°) showed 16 signals belonging to three methyls, two methylenes, five methines and six quaternary carbons. The signals at δ 119.2, 119.7, 120.6, 123.0, 147.3, and 157.4 were indicative of a hydroquinone function bearing an α,β-unsaturated carbonyl system (δ 196.1, 161.1, and 115.0). Such connectivity was determined based on the correlations of signals at δ 7.21 (H-3) and 196.1 (C=O) as observed in the HMBC spectrum. Indeed, this spectrum also showed correlations between the H-2′ (δ 6.66) and C-4′ (41.6), C-10′ (20.0), and C-2 (120.6), and between H-6′ (δ 5.12) and C-8′ (25.7), C-9′ (17.8), and C-4′ (41.6). Therefore, an oxo-geranyl moiety was determined as the side chain (Fig. 1) of compound **1**. Analysis of the ^1H–^1H COSY, HMQC and HMBC spectra (Table 1) allowed the complete assignment of all hydrogens and carbons of compound **1** determined as 1,4-dihydroxy-2-(3′,7′-dimethyl-1′-oxo-2′-*E*,6′-octadienyl)benzene.

Compound **2** was isolated as a white amorphous powder. The HREIMS of this compound showed the molecular ion peak at m/z 260.1396, corresponding to the molecular formula $C_{16}H_{20}O_3$. IR absorptions at 1587, 1645, and 3390 cm^{-1} suggested the presence of an aromatic system, an α,β-unsaturated carbonyl and a hydroxyl group, respectively. The ^{13}C NMR spectra (BBD and DEPT 135°) revealed 16 signals: three methyls, two methylenes, five methines and six quaternary carbons. On the basis of these spectroscopic data, compounds **1** and **2** were found to have similar structures. Analysis of their ^{13}C NMR spectra indicated a variation in the chemical shifts of C-4′ and C-10′, suggesting the occurrence of a $\Delta^{2'}$ geometric isomer derivative. The cross-peaks observed between the signals at δ 6.66 (H-2′) and δ 2.00 (H-10′) in the 2D NOESY spectrum suggested the *Z* configuration of the double bond of the α?β-unsaturated system (Moreira et al., 1998). Therefore, the structure of **2** was defined as 1,4-dihydroxy-2-(3′,7′-dimethyl-1′-oxo-2′-*Z*,6′-octadienyl)benzene.

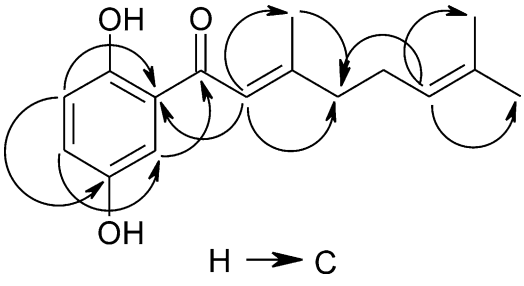


Fig. 1. Important ^1H–^{13}C couplings observed in HMBC spectrum of **1**.

Compound **3** has the molecular formula $C_{16}H_{20}O_5$ determined by analysis of LREIMS associated to the ^1H and ^{13}C NMR spectral data. The IR spectrum indicated carbonyl and hydroxyl function at 1690 and 3390 cm^{-1}, respectively. The signals in the ^1H NMR spectrum showed chemical shifts and splitting pattern similar to those observed to compounds **1** and **2**. This spectrum also showed two doublets at δ 3.37 ($J=15.5$ Hz) and 3.58 ($J=15.5$ Hz), characteristic of an allylic α-carbonylic methylene group. Two broad singlets at δ 4.96 (1H) and 4.99 (1H) indicated the presence of one non-conjugated terminal methylene group in the side chain. The ^{13}C NMR spectra also displayed signals at δ 113.5 (CH_2) and 142.4 (C), which were assigned to C-3′/C-10′ double bond, and at δ 83.7 (CH) and 79.9 (C), assigned to carbinolic carbons C-4′ and C-7′. These data associated to LREIMS, which showed a molecular ion peak at m/z 292, suggested the presence of an endoperoxide in the side chain. The mass spectrum showed a fragment ion peak at m/z 177 [M^+–$C_6H_{11}O_2$] that resulted from a loss of the six-member endoperoxide moiety. All hydrogen and carbon signals of compound **3** could be assigned by analysis of the HMQC and HMBC spectra and its structure could be defined as 1,4-dihydroxy-2-(7′-methyl-3′-methylene-1′-oxo-4′,7′-peroxide-octyl)benzene.

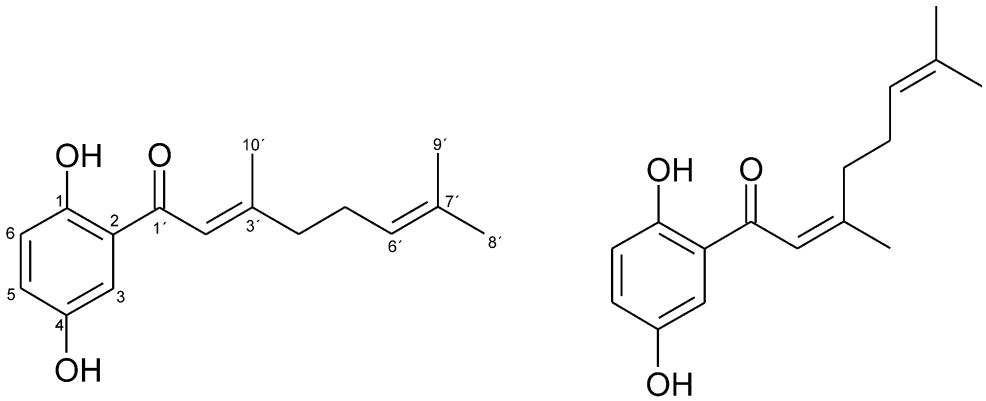
The antifungal activity of compounds **1**–**5** was evaluated by means of direct bioautography in a TLC bioassay (Homans and Fuchs, 1970). As can be seen by the detection limits of these compounds (Table 2) the prenylated hydroquinone **1** and sakuranetin (**5**) were the most potent with activities comparable to the controls. Detailed structure–activity relationship should be further investigated.

This work represents the first report for the occurrence of prenylated hydroquinone derivatives in Piperaceae species. The closest structurally related compounds are the prenylated 4-hydroxy-benzoic acids isolated from *Piper lhotzkyanum* (Moreira et al., 1998), in which a pair of *E*/*Z* isomers was described. Interestingly, prenylated hydroquinone compounds were described from the marine tunicate *Aplidium californicum* (Cotelle et al., 1991; Howard and Clarkson, 1979), in which the antioxidant properties were detected and suggested to act similarly as tocopherols.

3. Experimental

3.1. General

Silica gel (Merck, 230–400 mesh) was used for all column chromatographic separations and silica gel 60 PF_{254} (Merck) for analytical (0.50 mm) and prep. TLC (1.0 mm).



1 **2**

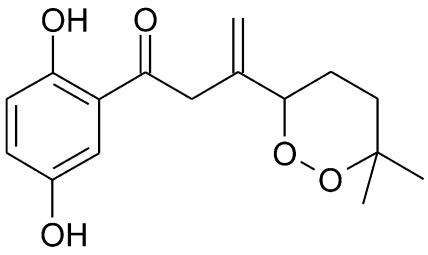


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Table 1
^{1}H and ^{13}C NMR spectral data (δ, 500 and 125 MHz, $CDCl_3$) of hydroquinones **1**–**3** isolated from *Piper crassinervium*.

Position	**1**		**2**		**3**	
	^{1}H [m, *J* (Hz)]	^{13}C[a]	^{1}H [m, *J* (Hz)]	^{13}C	^{1}H [m, *J* (Hz)]	^{13}C
1	–	157.4	–	157.4	–	157.0
2	–	120.6	–	120.5	–	119.9
3	7.21 (*d*, 3.0)	119.7	7.23 (*d*, 2.8)	120.3	7.35 (*d*, 3.0)	115.7
4	–	147.3	–	147.2	–	147.6
5	6.98 (*dd*, 8.9; 3.0)	123.0	6.99 (*dd*, 9.0; 2.8)	123.5	7.04 (*dd*, 8.9; 3.0)	125.3
6	6.84 (*d*, 8.9)	119.2	6.87 (*d*, 9.0)	119.2	6.87 (*d*, 8.9)	119.3
1′	–	196.1	–	195.6	–	204.7
2′	6.66 (*s*)	115.0	6.66 (*s*)	115.0	3.37 (*d*, 15.5) 3.58 (*d*, 15.5)	43.1
3′	–	161.1	–	161.5	–	142.4
4′	2.30 (*m*)	41.6	2.62 (*m*)	34.5	4.48 (*d*, 9.6)	83.7
5′	2.20 (*m*)	26.2	2.21 (*m*)	26.8	1.89 (*m*)	33.2
6′	5.12 (t, 7.3)	124.2	5.13 (t, 7.2)	124.1	1.76 (*m*)	24.8
7′	–	132.8	–	132.6	–	79.9
8′	1.71 (*s*)	25.7	1.64 (*s*)	25.6	1.28 (*s*)	24.7
9′	1.63 (*s*)	17.8	1.62 (*s*)	17.7	1.79 (*s*)	19.6
10′	2.17 (*s*)	20.0	2.00 (*s*)	26.1	4.96, 4.99 (*br*, *s*)	113.5
1-OH	12.3 (*s*)	–	12.3 (*s*)	–	12.3 (*s*)	–

^{1}H and ^{13}C NMR spectra were recorded at 500 and 125 MHz, respectively, in a Bruker DRX-500 spectrometer. $CDCl_3$ (Aldrich) was used as solvent and TMS as int. standard. Chemical shifts were reported in δ units (ppm) and coupling constants (*J*) in Hz. LREIMS and HREIMS were measured at 70 eV, respectively, on a HP 5990/5988 A and a VG Autospec spectrometers. IR spectra were measured in KBr pellets in a Perkin-Elmer Infrared Spectrometer model 1750. UV spectra were recorded in a HP 8452 A spectrophotometer using $CHCl_3$ as solvent.

Table 2
Minimum amount of compounds **1**–**5** isolated from *Piper crassinervium* required for the inhibition of fungal growth on thin-layer chromatography plates (TLC)

Compounds	Antifungal activity/μg	
	Cladosporium cladosporioides	*C. sphaerospermum*
1	1.0	1.0
2	5.0	10.0
3	5.0	10.0
4	1.0	5.0
5	1.0	1.0
Nystatin	1.0	1.0
Miconazole	1.0	1.0

3.2. Plant material

Leaves of *P. crassinervium* Kunth were collected in the region of Vale do Ribeira, Atlantic Forest (São Paulo State, Brazil) and identified by Dr. Guillermo E. D. Paredes (Universidad Nacional Pedro Ruiz Gallo, Peru). A voucher specimen (KATO-0084) was deposited in the Herbarium of Instituto de Botânica, São Paulo, SP, Brazil.

3.3. Antifungal assay

The microorganisms used in the antifungal assays *C. cladosporioides* (Fresen) de Vries SPC 140 and *C. sphaerospermum* (Perzig) SPC 491 have been maintained at the Instituto de Botânica, São Paulo, SP, Brazil. For the antifungal assay—10.0 μl of solutions corresponding to 100.0 μg of crude extract and 10.0, 5.0 and 1.0 μg of pure compounds were applied to precoated Si-gel TLC plates. TLC plates were developed with hexane:EtOAc (7:3) and dried for complete removal of solvents. The chromatograms were sprayed with a spore suspension of *C. cladosporioides* or *C. sphaerospermum* in glucose and salt solution (Rahalison et al., 1994) and incubated for 72 h in darkness in a moistened chamber at 25 °C. Clear inhibition zone appeared against a dark background indicating the minimal amount of **1**–**5** required for it (Table 2). Nystatin and miconazole were used as positive controls whereas ampicillin and chloramphenicol were used as negative controls.

3.4. Extraction and isolation of the constituents

Dried leaves (750 g) were milled and extracted three times with EtOAc at room temperature. The combined EtOAc solution was conc. in vacuo yielding 10.6 g of crude extract. This extract was applied to a silica-gel column (250 g) and eluted with a gradient mixture of hexane:EtOAc (98:2, 95:5, 9:1, 4:1, 7:3, 3:2, 1:1, 3:7). A total of 50 fractions (50 ml each) were collected and combined into 29 groups on the basis of similarities in TLC profiles. The activity against *C. cladosporioides* and *C. sphaerospermum* was found in fractions 14, 15, 18, 24, and 27, which were then submitted to further purification procedures. Fraction 14 (326 mg) was purified by prep. TLC [hexane:CH_2Cl_2:$(CH_3)_2CO$ (20:79.6:0.4)] to yield 18 mg of **2**. Fraction 15 (136 mg) was subjected to CC on Si gel, eluted with gradient mixtures of EtOAc in hexane yielding 23 fractions which were pooled in three groups (I–III). Prep. TLC of group I (75 mg) [hexane:EtOAc (7:3)] yielded 32 mg of **1** and 15 mg of **3**. Fraction 18 (228 mg) was purified by prep. TLC [hexane:*i*-PrOH:EtOAc (7:3:0.1)] to give 50 mg of **1** and 25 mg of **2**. Flavanone **4** (56 mg) was isolated from fraction 24 (467 mg), after prep. TLC purification [hexane:*i*-PrOH:EtOAc (7:3:0.1)]. Fraction 27 (263 mg) was purified by prep. TLC [hexane:*i*-PrOH:EtOAc (7:3:0.1), two elutions] to yield 22 mg of **5**.

3.5. HPLC analysis

Crude EtOAc extract was filtered on a Sep-Pak column using MeOH as eluent. Samples containing 1 μl of the crude extract and pure compounds **1**, **2** and **3** were analyzed by HPLC using a Luna C-18 (Phenomenex) column (5 μm, 250×4 mm), with a gradient from MeOH:H_2O 65:35 (0 min) to MeOH 100% (30 min), flow rate 1.0 ml min^{-1}, and detection at 254 nm.

3.6. 1,4-Dihydroxy-2-(3′,7′-dimethyl-1′-oxo-2′-E,6′-octadienyl)benzene (*1*)

White amorphous powder. RR_t (HPLC): 16.6 min. IR (KBr) ν_{max} cm^{-1}: 3389, 2922, 1642, 1588, 1487, 1291, 1172, 789. UV λ_{max} ($CHCl_3$) nm (log ε): 276 (4.14), 378 (3.69). For ^1H and ^{13}C NMR spectra: see Table 1. LREIMS *m*/*z* (rel. int.): 260 $[M]^+$ (3), 178 (11), 177 (100), 137 (22), 109 (4), 69 (12). HREIMS *m*/*z* 260.1392 (calcd. for $C_{16}H_{20}O_3$, 260.1413).

3.7. 1,4-Dihydroxy-2-(3′,7′-dimethyl-1′-oxo-2′-Z,6′-octadienyl)benzene (*2*)

White amorphous powder. RR_t (HPLC): 17.7 min. IR (KBr) ν_{max} cm^{-1}: 3390, 2925, 1645, 1587, 1488, 1293, 1175, 790. UV λ_{max} ($CHCl_3$) nm (log ε): 276 (4.29), 376 (3.91). For ^1H and ^{13}C NMR spectra: see Table 1. LREIMS *m*/*z* (rel. int.): 260 $[M]^+$ (3), 242 (10), 199 (13), 177 (100), 137 (44), 109 (11), 69 (27). HREIMS *m*/*z* 260.1396 (calcd. for $C_{16}H_{20}O_3$, 260.1413).

3.8. 1,4-Dihydroxy-2-(7′-methyl-3-methylene-1′-oxo-4′,7′-peroxide-octyl)benzene (*3*)

White amorphous powder. RR_t (HPLC): 10.7 min. IR (KBr) ν_{max} cm^{-1}: 3390, 2925, 1708, 1690, 1484, 1449, 1366, 1264, 1187, 790. UV λ_{max} ($CHCl_3$) nm (log ε): 256

(2.85), 362 (2.41). For ^{1}H and ^{13}C NMR spectra: see Table 1. LREIMS m/z (rel. int.): 292 $[M]^{+}$ (2), 177 (35), 137 (100), 109 (11), 81 (17), 55 (12).

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