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Antioxidant activity of prenylated hydroquinone and benzoic acid derivatives from *Piper crassinervium* Kunth

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Dedicated to Professor Rod Croteau on the occasion of his 60th birthday.

Abstract

Growing evidence suggests that RNOS (reactive nitrogen and oxygen species) are involved in the damage of biomolecules, contributing to the aetiology of several human diseases. Thus, the demand for antioxidants has stimulated the search for new compounds with potential use in this field. The in vitro antioxidant potential of prenylated hydroquinones and prenylated 4-hydroxy-benzoic acids from fruits of *P. crassinervium* was evaluated in terms of their capacity to suppress both DPPH (2,2-diphenyl-1-picrylhydrazyl) radical and chemiluminescence produced from luminol, using 2,2′-azo-bis(2-amidinopropane) (ABAP) as a peroxyl radical source. The inhibition of lipid peroxidation was assessed using liposomes from phosphatidylcholine as a membrane model. The prenylated hydroquinones had higher antioxidant activity than the benzoic acids and, among the hydroquinones, the *E* isomer was more efficient than the *Z* isomer.

Keywords: *Piper crassinervium*; Piperaceae; Prenylated hydroquinones; Prenylated benzoic acids; Antioxidants; Lipoperoxidation; DPPH; Chemiluminscence

1. Introduction

Hydroquinones bearing prenyl moieties are natural products common to marine organisms such as Ascidiaceae (*Aplidium* sp.) and brown algae (Howard et al., 1979; Fadli et al., 1991) and have rarely been found in other organisms. The derivatives isolated from leaves of *Piper crassinervium* are among the few cases of their occurrence in land plants (Danelutte et al., 2003; Lago et al., 2004). The biological activities for such compounds include antitumoral, antileukemic and mitosis inhibition (Muller et al., 1985a,b). Furthermore they have analgesic, relaxant (De Pasquale et al., 1991) and antioxidant effects (Cotele et al., 1991).

Piper species have also been described to contain structurally similar compounds to prenylated benzoic acids, and in the case of *P. aduncum* (Baldoqui et al., 1999) antimicrobial and molluscicidal activities were evaluated (Okunade et al., 1997; Orjala et al., 1993), *P. arieianum, P. tabogatum* and *P. dilatatum* also contain further prenylated benzoic acids with antifungal activity (Green et al., 1991; Roussis et al., 1990; Terreaux et al., 1998).

The antioxidant potential of the compounds from *P. crassinervium* (**1**–**6**) were evaluated through the capacity to inhibit the luminescence from luminol induced by 2,2′-azo-bis (2-amidinopropane) (ABAP) radical liberation. Additionally, the ability to scavenge 1,1-diphenyl-2-picryl-hydrazyl (DPPH), a stable radical was analyzed, as well. Finally, protection against lipoperoxidation was evaluated using Fe^{3+}/EDTA and ascorbic acid induced-peroxidation in liposome from phosphatidylcholine.

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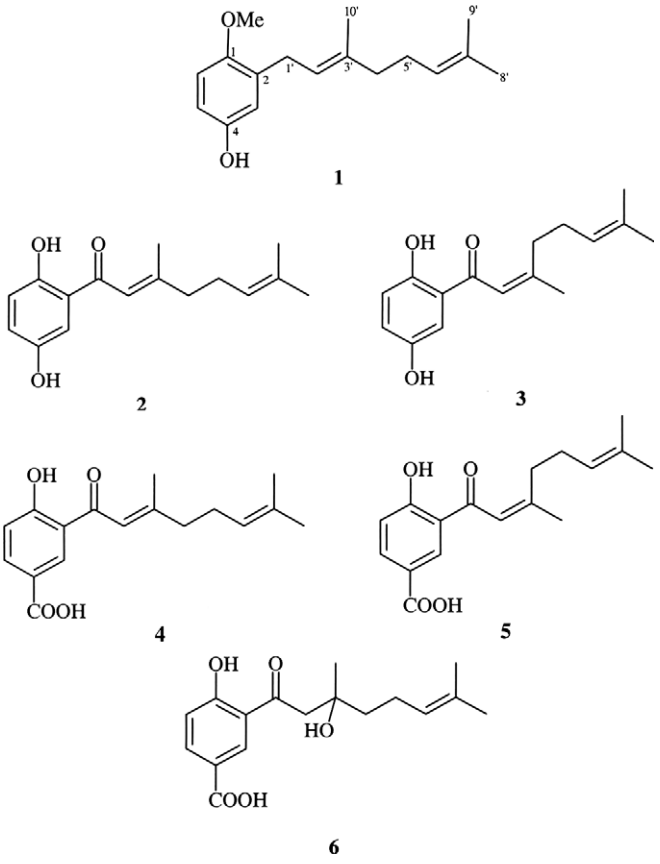

2. Results and discussion

2.1. Characterization of the compounds

The compounds **2–6** were previously isolated and characterized as potent antifungal agents and were identified by direct comparison with authentic standards (Danelutte et al., 2003; Lago et al., 2004). Compound **1** was isolated as a pale yellow amorphous solid. Its molecular formula was determined as $C_{17}H_{24}O_2$ by ^{13}C NMR spectroscopy and HREIMS, which showed a molecular ion peak at *m/z* 260.1780. The ^{13}C NMR spectrum revealed 17 carbon signals, 10 of which were sp^2 carbons [δ 111.5 (CH), 112.5 (CH), 116.6 (CH), 121.9 (CH), 124.3 (CH), 131.6 (C), 131.4 (C), 136.5 (C), 149.3 (C), 151.6 (C)], and seven aliphatic carbons [δ 56.1 (CH_3), 39.8 (CH_2), 28.0 (CH_2), 26.7 (CH_2), 25.2 (CH_3), 17.7 (CH_3), 16.1 (CH_3)]. These data suggested a geranyl-hydroquinone derivative as for compounds **2** and **3**, previously isolated from leaves of *P. crassinervium* (Danelutte et al., 2003; Baldoqui et al., 1999). The ^1H NMR spectrum exhibited three aromatic proton resonances at δ 6.62 (*dd*, $J = 8.5$ and 3.5 Hz), 6.65 (*d*, $J = 3.5$ Hz) and 6.72 (*d*, $J = 8.5$ Hz), which were indicative of a 1,2,4-trisubstituted aromatic ring, and two broad-triplets at δ 5.27 ($J = 7.2$ Hz) and 5.09 ($J = 6.8$ Hz) assignable to olefinic protons in the side-chain. This spectrum also showed two broad-singlets at δ 1.58 (3 H), 1.67 (6 H), characteristic of methyl groups in a double bond, and a singlet at δ 3.76 (3H) of an aromatic methoxyl group. The doublet at δ 3.26 ($J = 7.2$ Hz), associated to the absence of carbonyl groups absorptions as revealed by IR spectrum, indicated the presence of a geranyl moiety linked to the aromatic ring and not an oxo-geranyl moiety, as observed in compounds **2–6** previously isolated from leaves of *P. crassinervium* (Lago et al., 2004).

The positions of the substituents were confirmed through analysis of the HMBC spectrum. The correlation between the methoxyl signal at δ 3.76 (s) and the carbon at δ 151.6 (C-1) associated to the cross-peaks between δ 3.26 (H-1′) and δ 151.6 (C-1), 131.6 (C-2), 116.6 (C-3), 149.3 (C-4), and 136.5 (C-3′), indicated that the methoxyl group and geranyl moiety were connected to C-1 and C-2 positions in the aromatic ring, respectively. Other important correlations are given in Table 1. Therefore, from

Table 1
^1H and ^{13}C NMR data ($CDCl_3$) for the prenylated hydroquinone **1** from *P. crassinervium*

No.	C[a]	H	DQ-COSY	HMBC (H → C)
1	151.6 (C)	–	–	–
2	131.6 (C)	–	–	–
3	116.6 (CH)	6.65 *d* (3.5)	H-5	C-1, C-5, C-1′
4	149.3 (C)	–	–	–
5	112.5 (CH)	6.62 *dd* (8.5, 3.5)	H-3, H-6	C-1, C-3
6	111.5 (CH)	6.72 *d* (8.5)	H-5	C-2, C-4
1′	39.8 (CH_2)	3.26 *d* (7.2)	H-2′	C-1, C-2, C-3, C-2′, C-3′
2′	121.9 (CH)	5.27 *br. t* (7.2)	H-1′, H-10′	C-2, C-1′, C-4′, C-10′
3′	136.5 (C)	–	–	–
4′	28.0 (CH_2)	2.02 *m*	H-5′, H-10′	C-2′, C-3′, C-6′, C-10′
5′	26.7 (CH_2)	2.11 *m*	H-4′, H-6′	C-3′, C-6′, C-7′
6′	124.3 (CH)	5.09 *br.t* (6.8)	H-5′, H-9′	C-5′, C-8′, C-9′
7′	131.4 (C)	–	–	–
8′	25.2 (CH_3)	1.58 *br.s*	H-9′	C-6′, C-7′, C-9′
9′	17.7 (CH_3)	1.67 *br.s*	H-6′, H-8′	C-6′, C-7′, C-8′
10′	16.1 (CH_3)	1.67 *br.s*	H-2′	C-2′, C-3′, C-4′
OMe	56.1 (CH_3)	3.76 *s*	–	C-1

[a] Multiplicity determined by DEPT 135°.

the above evidence, the structure of **1** was determined as the new 1-methoxy-4-hydroxy-2-(3′,7′-dimethyl)-2′*E*, 6′-octadienylbenzene.

2.2. Antioxidant activity

DPPH, a stable free radical, has been used to evaluate free radical scavenging capacities of compounds (Blois, 1958). The scavenging activity of the compounds is shown in Table 2. Addition of compounds **1**–**3**, Trolox® or hydroquinone to the DPPH radical cation solution elicited a progressive decrease in the concentration of free radicals (Fig. 1). The compound **2** ($EC_{50} = 22.08$ μM) had the highest hydrogen-donating capacity. The compounds **1** and **3** were weakly active ($EC_{50} > 40.00$ μM), while compounds **4**, **5** and **6** of the benzoic acids derivatives and even *p*-hydroxybenzoic acid were inefficient as antioxidants.

The luminescence of luminol can be induced by alkylperoxyl free radicals generated from pyrolysis of ABAP (Lissi et al., 1992). The addition of the compounds to reaction mixture (luminol and ABAP) can delay the emission of luminescence. The dependence of induction time upon antioxidants is shown in Fig. 2, and the relative efficiency of the different antioxidants was obtained by slopes of the curve (Table 2).

Table 2
Antioxidant properties of the prenylated derivatives **1**–**6**, hydroquinone and Trolox® as determined by the quenching of the free-radical induced luminescence of luminol, the DPPH radical assay and the inhibition of lipoperoxidation

Compounds	Inhibition of luminescence[a] Slope	DPPH radical assay[a] EC_{50} (μM)	Inhibition of lipoperoxidation[a] IC_{50} (μM)
1	2.16 ± 0.04	>40.00	14.48 ± 0.38
2	2.34 ± 0.04	22.08 ± 0.34	26.43 ± 0.43
3	1.15 ± 0.04	>40.00	63.11 ± 0.45
4	0.09 ± 0.11	–	69.54 ± 0.40
5	0.05 ± 0.15	–	81.16 ± 0.39
6	0.05 ± 0.12	–	89.74 ± 0.47
p-Hydroxybenzoic acid	–	–	–
Hydroquinone	2.46 ± 0.06	1.20	45.00 ± 0.51
Diacetoxyhydroquinone	–	–	–
Trolox®	4.49 ± 0.04	10.00 ± 1.93	3.70 ± 0.42

[a] Mean values (±SD); $n = 3$.

Prenylated hydroquinones (**1**–**3**) were found to be more potent luminescence inhibitors as compared to benzoic

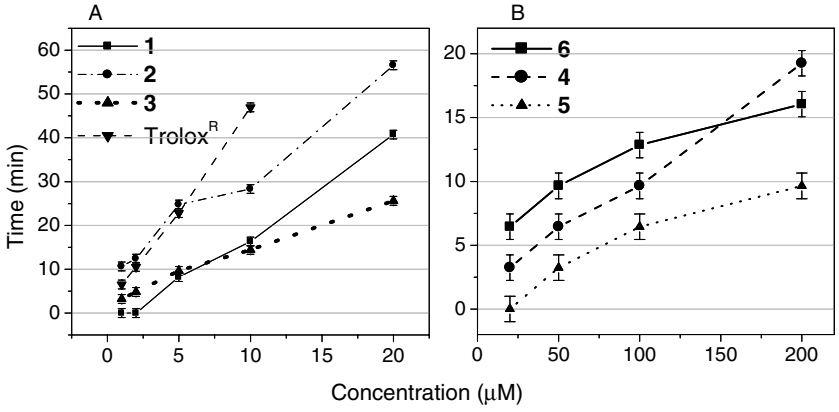

Fig. 1. The effect of the prenylated hydroquinones **1**–**3**, of the prenylated benzoic acids **4**–**6**, and of Trolox® on the induction time of emission of the radical-induced luminescence of luminol as a function of the concentration of additive.

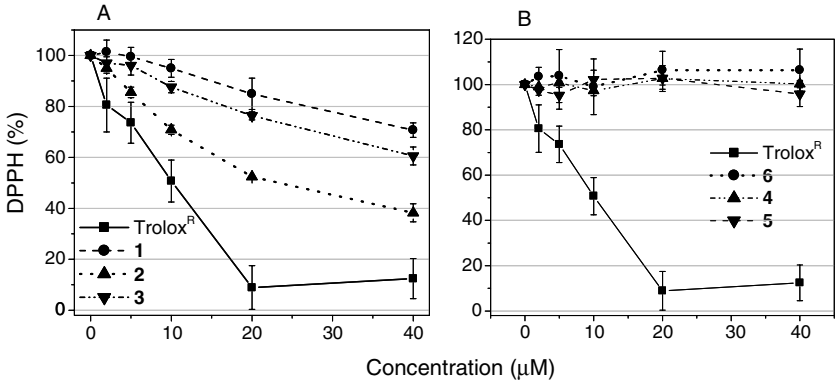

Fig. 2. Inhibition of the stable DPPH radical by different concentrations of the prenylated hydroquinones **1**–**3**, of the prenylated benzoic acids **4**–**6**, and of Trolox®.

acids (**4**–**6**) (Table 2, Figs. 2A and B). A preliminary structure–activity relationship indicated that compound **2**, the *E* isomer, has a higher activity than its *Z* isomer **3**.

The ability of the compounds to suppress peroxidation was evaluated using a Fe^{3+}/EDTA, ascorbic acid-induced, peroxidation in liposomes of phosphatidylcholine (Zhao and Jung, 1995). The concentration of an antioxidant needed to decrease the lipoperoxidation by 50% (IC_{50}) is a parameter used to measure antioxidant activity. The IC_{50} of prenylated hydroquinone **1** (14.48 μM) was higher than others except for Trolox® (IC_{50} = 2.59 μM) (Table 2).

A broad evaluation in the structure–activity relationship including hydroquinone, diacetoxyhydroquinone, and *p*-hydroxybenzoic acid was carried out. Hydroquinone was a very efficient suppressor of DPPH and chemiluminescence but was not as Trolox® or prenylated hydroquinone to inhibit lipoperoxidation. The diacetoxyhydroquinone and *p*-hydroxybenzoic acid were not able to act as antioxidants.

3. Conclusions

The prenylated hydroquinones studied have considerable radical scavenger capacities in the DPPH, chemiluminescence and lipoperoxidation assays as compared to benzoic acids. The presence of the hydroxyl group at C-4 confers the antioxidant activity for compounds **1**–**6** since the prenylated benzoic acids, diacetoxyhydroquinone and *p*-hydroxybenzoic acid, did not display any activity in chemiluminescence and DPPH assays. Compound **1** had a higher activity for lipoperoxidation analyses. The higher efficiency of prenylated hydroquinones **2** and **3** to inhibit lipoperoxidation, as compared to hydroquinone, should be due to the presence of the lipophylic prenylated group that could be inserted into the liposome. The linear hydroquinone **2**, with an *E*-configuration, showed higher activity than **3**, probably due to its better insertion in the liposome. The benzoic acid derivatives **4**–**6** were not as efficient as hydroquinone derivatives, and the *E*-geometries had a higher capacity to suppress the chemiluminescence than the *Z*-form, as suggested by higher conjugation in the *E*-configuration.

4. Materials and methods

4.1. General experimental procedures

MeOH, CH_3CN, *n*-hexane, EtOH, BuOH, HCl, and $HClO_4$ were purchased from Merck (Darmstadt, Germany). Trolox®, whereas sodium phosphate, Tris, EDTA, thiobarbituric acid, DPPH, $FeCl_3$, ascorbic acid, luminol, glycine, NaOH, defeoxamine, *p*-hydroxybenzoic acid and hydroquinone were from Sigma (CA, USA). ABAP was purchased from Polyscience (PA, USA).

Silica gel (Merck 230–400 mesh) and Sephadex LH-20 (Sigma) were used for all CC separations, while silica gel 60 PF_{254} (Merck) was used for analytical (0.50 mm) and preparative TLC (1.0 mm). ^1H and ^{13}C NMR spectra were recorded at 500 and 125 MHz, respectively, using a Bruker DRX-500 spectrometer, with $CDCl_3$ (Aldrich, CA, USA) as solvent and TMS as internal 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.

4.2. Plant material

Fresh fruits of *P. crassinervium* (Kunth) were collected in Iporanga city in the region of Vale do Ribeira, Atlantic Forest (São Paulo State, Brazil) and identified by Dr. Guillermo E. Delgado (Universidad Nacional Pedro Ruiz Gallo, Peru). The voucher specimen (Kato-0084) was deposited in the Herbarium of Instituto de Botânica, São Paulo - SP, Brazil.

4.3. Extraction and isolation

Dried fruits (50 g) were extracted with MeOH (three times, 350 mL, at room temperature, for one day) to yield a crude extract (4.16 g), which was partitioned between *n*-hexane and MeOH/H_2O (4:1). The *n*-hexane phase was dried (anhydr. Na_2SO_4) and concentrated in vacuo to give the corresponding extract (2.48 g). This fraction was subjected to silica gel CC eluted with *n*-hexane:EtOAc of increasing polarity to yield 10 fractions. Fraction 2 (121 mg) was applied to a silica gel column eluted with increasing amounts of EtOAc in *n*-hexane to yield 6 subfractions (I–VI). Subfraction V (23 mg) was purified by prep. TLC (*n*-hexane:CH_2Cl_2:EtOAc 5:9:1) to give **1** (10 mg). Fraction 3 (381 mg) was submitted to TLC (*n*-hexane:EtOAc 4:1, two elutions) affording compounds **2** (307 mg) and **3** (22 mg). Compound **4** (38 mg) was obtained after purification by prep. TLC (*n*-hexane:EtOAc 7:3) of fraction 6 (144 mg). Fraction 7 (56 mg) was purified by prep. TLC (CH_2Cl_2:EtOAc 7:3, two elutions) yielding **5** (6 mg). Fraction 9 (57 mg) was subjected to column chromatography on Sephadex LH-20 (*n*-hexane:CH_2Cl_2 1:4 and CH_2Cl_2:Me_2CO 3:2) to yield **6** (8 mg).

4.4. 1-Methoxy-4-hydroxy-2(3′,7′-dimethyl)-2E′,6′-octadienylbenzene (*1*)

Amorphous solid. IR (KBr) νmax cm^{-1}: 3389, 2925, 1499, 1452, 1216, 1037, 804 cm^{-1}. UV λmax (MeOH) nm (log ε): 262 (3.71), 320 (3.30) nm; LREIMS (70 eV) *m*/*z*. (rel. int.): 260(25), 217(7), 191(52), 176(15), 161(20), 149(10), 137(39), 123(59), 107(26), 91(14), 77(20), 69(53), 55(17), 41(100).

HREIMS (70 eV) *m/z*: 260.1780 (calcd. for C_{17} $H_{24}O_2$ 260.1777). For ^1H and ^{13}C NMR spectra: see Table 1.

4.5. Diacetoxyhydroquinone

Hydroquinone (60 mg) was acetylated with Ac_2O (7.0 mL) and pyridine. The work-up, followed by silica-gel chromatography of the crude product furnished the diacetylated derivative (62 mg, yield 78 %) as a white amorphous solid, which was characterized by NMR spectroscopic analysis.

4.6. Suppression of luminol luminescence induced by 2,2′-azo-bis(2-amidinopropane) (ABAP) thermolysis

The luminescence of luminol was induced by thermolysis of ABAP, which produce the alkylperoxyl radical species. Five millimolar of ABAP dissolved in 0.1 M Glycine/NaOH, pH 8.6 with 0.2% MeOH were added to the solutions of prenylated hydroquinones, benzoic acids and Trolox® at 1, 2, 5, 10 and 20 μM in the same buffer, followed by 50 μM of luminol in 0.1 M glycine/NaOH, pH 8.6 buffer (Lissi et al., 1992). Luminescence was measured in a luminometer (MicroLuminat Plus, Microplate LuminometerLB 96 V, EG & G Berthold, MA, USA).

4.7. Scavenging effect on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical

Solutions of prenylated hydroquinones **1**, **2** and **3**, benzoic acids **4**, **5** and **6** and Trolox® in methanol (1–40 μM) were individually added to a 0.1 mM of DPPH radical in MeOH. The mixture was incubated for 20 min at 37 °C, with the absorbance of the resulting solution measured at 517 nm.

4.8. Effect on peroxidation in liposomal membranes

The protective effect of prenylated hydroquinones, benzoic acids and Trolox® against peroxidation induced by Fe^{3+}/EDTA and ascorbic acid were evaluated using phosphatidylcholine (PC) liposomes (Mandal and Chatterji, 1980). Liposomes were incubated with 4 mM of ascorbic acid and 0.4 mM of Fe^{3+}/EDTA for 30 min at 37 °C, with the reaction halted by addition, 35% $HClO_4$ (10 μl). Samples were heated with 200 μl of TBA reagent (HCl 0.25 M, 0.375% of thiobarbituric acid and 2 mM defeoxamine) at 100 °C for 15 min, were the TBARS subsequently extracted with butanol (400 μL) and then analyzed using a fluorescence spectrophotometer ($\lambda_{exc.}$ 515 and $\lambda_{emiss.}$ 550 nm) (Hitachi F-2000, Japan).

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