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Antioxidant activity of isocoumarins isolated from *Paepalanthus bromelioides* on mitochondria

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Abstract

The isocoumarins (1–50 μM) paepalantine (9,10-dihydroxy-5,7-dimethoxy-1H-naptho(2,3c)pyran-1-one), 8,8′-paepalantine dimer, and vioxanthin isolated from *Paepalanthus bromelioides*, were assessed for antioxidant activity using isolated rat liver mitochondria and non-mitochondrial systems, and compared with the flavonoid quercetin. The paepalantine and paepalantine dimers, but not vioxanthin, were effective at scavenging both 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\bullet}$) and superoxide ($O_2^{\bullet-}$) radicals in non-mitochondrial systems, and protected mitochondria from *tert*-butylhydroperoxide-induced H_2O_2 accumulation and Fe^{2+}-citrate-mediated mitochondrial membrane lipid peroxidation, with almost the same potency as quercetin. These results point towards paepalantine, followed by paepalantine dimer, as being a powerful agent affording protection, apparently via $O_2^{\bullet-}$ scavenging, from oxidative stress conditions imposed on mitochondria, the main intracellular source and target of those reactive oxygen species. This strong antioxidant action of paepalantine was reproduced in HepG2 cells exposed to oxidative stress condition induced by H_2O_2.

Keywords: *Paepalanthus bromelioides*; Eriocaulaceae; Isocoumarins; Paepalantine; Mitochondria; Reactive oxygen species; Oxidative stress; Membrane lipid peroxidation

1. Introduction

Mitochondria are important intracellular sources of reactive oxygen species (ROS). During the oxidative phosphorylation process, mitochondria reduce O_2 to H_2O via the respiratory chain. However, a small fraction of the O_2 available to this system may undergo incomplete reduction giving rise in the complex I and ubisemiquinone formed at the complex III of the respiratory chain, to the superoxide radical ($O_2^{\bullet-}$) (Brookes, 2005), which is scavenged by the antioxidant defences of the organelle. When $O_2^{\bullet-}$ is additionally formed or not adequately removed, H_2O_2 accumulates (Boveris and Chance, 1973; Turrens et al., 1985; St-Pierre et al., 2002). In the presence of iron, a hydroxyl radical ($^{\bullet}OH$) is produced via the Fenton/Haber–Weiss reaction, leading to membrane lipid peroxidation (Halliwell and

Abbreviations: *P. bromelioides*, *Paepanthus bromelioides*; CM-H_2DCFDA, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester; DPPH, 2,2-diphenyl-1-picrylhydrazyl; EGTA, ethylene glycol bis (-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, *N*-(2-hydroxyethyl) piperazine-*N*′-(2-ethanesulfonic acid); ROS, reactive oxygen species; TRIS, tris(hydroxymethyl)-aminomethane.

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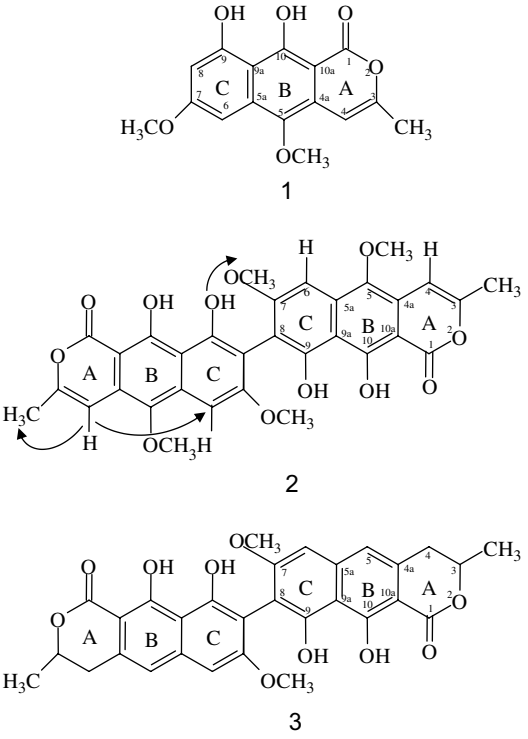

Fig. 1. Structure of the isocoumarins used in this study: paepalantine (**1**), 8,8′-paepalantine dimer (**2**), vioxanthin (**3**).

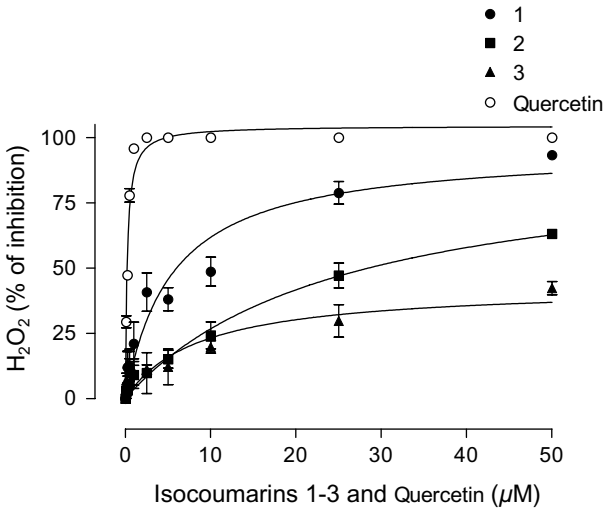

Fig. 2. Concentration–response curves for the effects of paepalantine (**1**), 8,8′-paepalantine dimer (**2**), vioxanthin (**3**) and quercetin (Quer) on H_2O_2 accumulation in isolated rat liver mitochondria, incubated in a standard medium containing 125 mM sucrose, 65 mM KCl, 10 mM HEPES-KOH, pH 7.4, as described in Section 4. Values are means ± SEM of the results of three experiments with different mitochondrial preparations and relative to a control in absence of isocomarins.

Gutteridge, 1999). Therefore, mitochondria may be regarded also as important intracellular targets of ROS.

Isocoumarins comprise a class of polyphenolic natural products present in a variety of plant species (Hill, 1986), displaying several pharmacological activities, including anti-tumor action (Cañedo et al., 1997; Devienne et al., 2002; Di Stasi et al., 2004; Kostova, 2005; Devienne et al., 2005; Okamoto et al., 2005). We previously isolated the isocoumarins, paepalantine (**1**) (9,10-dihydroxy-5,7-dimethoxy-1H-naptho(2,3c)pyran-1-one) (Vilegas et al., 1990), 8,8′-paepalantine dimer (**2**) (Coelho et al., 2000) and vioxanthin (**3**) (Fig. 1) from *Paepalanthus bromelioides*, a brazilian plant known as "sempre viva" and commonly found in the region of the Cipó Mountain range, Minas Gerais State, Brazil. In the present study, we have addressed the interaction of these isocoumarins with mitochondria regarding their antioxidant activities, compared to the flavonoid quercetin, and in parallel, assessed their ROS scavenging properties in non-mitochondrial systems.

2. Results and discussion

The antioxidant activity of paepalantine (**1**), its dimer (**2**), and vioxanthin (**3**) isolated from *P. bromelioides*, on isolated rat liver mitochondria was evaluated and compared with quercetin, a well-known flavonoid with strong antioxidant activity. Within the 1–50 μM range, paepalantine (**1**), and to a lesser extent its dimer (**2**), were effective antioxidants protecting the organelles from the *tert*-butylhydroperoxide-induced H_2O_2 accumulation (Fig. 2) and from Fe^{2+}-citrate-mediated mitochondrial membrane lipid peroxidation, LPO (Fig. 3), while vioxanthin (**3**) was a far less effective antioxidant; the IC_{50} values are presented in Table 1. According to our previous report (Calgaro-Helena et al., 2006), the isocoumarins did not caused a significant inhibition of state 3 respiration of mitochondria, indicating that they do not affect respiratory chain components of the organelle nor F_1F_o-ATP syntase. This was even though vioxanthin (**3**) significantly stimulated state 4 respiration, indicating that it causes mitochondrial uncoupling; the latter effect was lower for either paepalantine (**1**) or the paepalantine dimer (**2**). Therefore, it seems unlikely any

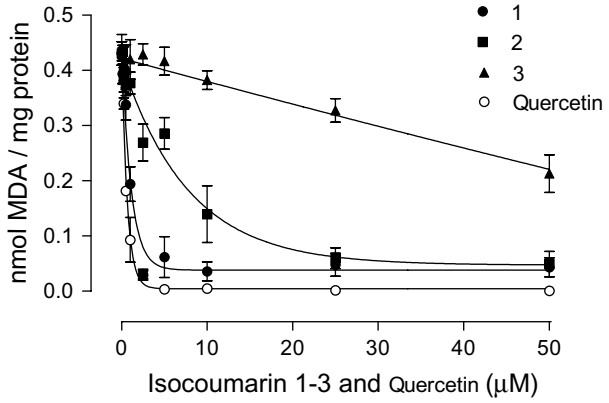

Fig. 3. Concentration–response curves for the effects of paepalantine (**1**), 8,8′-paepalantine dimer (**2**), vioxanthin (**3**) and quercetin (Quer) on LPO in isolated rat liver mitochondria, incubated in the standard medium described in legend to Fig. 2, as described in Section 4. Values are means ± SEM of the results of three experiments with different mitochondrial preparations.

Table 1
IC_{50} values for the effects of paepalantine (**1**), paepalantine dimer (**2**), vioxanthin (**3**) and quercetin on H_2O_2 accumulation and LPO in mitochondria, as well as Fe^{2+}chelation and $DPPH^{\cdot}/O_2^{\cdot-}$ scavenging in non-mitochondrial systems

	Inhibition		Fe^{2+}chelation	Scavenging	
	H_2O_2 accumulation	LPO		$DPPH^{\bullet}$	$O_2^{\cdot-}$
Paepalantine (**1**)	6.00 ± 1.42	0.92 ± 0.06	ND	3.66 ± 0.97	55.17 ± 3.14
Paepalantine dimer (**2**)	28.44 ± 3.51	6.09 ± 1.12	ND	1.85 ± 0.28	ND
Vioxanthin (**3**)	ND	48.97 ± 4.18	ND	22.72 ± 4.71	ND
Quercetin	0.24 ± 0.06	0.51 ± 0.13	13.34 ± 3.84	5.76 ± 1.04	26.47 ± 2.66

IC_{50} – Concentration (μM) producing 50% of the maximum effect, calculated from the concentration–responses curves (Figs. 2–6).
ND – not determined.

eventual interference of this aspect of the mitochondrial function in the high antioxidant activity presented by paepalantine (**1**) on mitochondria.

It is worth mentioning that within the 1–50 μM range, the iron chelating/oxidation capacity of paepalantine (**1**) or its dimer (**2**), that could prevent the formation of $^{\bullet}OH$ mediated by the Fenton/Haber–Weiss reaction, was not significant (Fig. 4); this ruled out any substantial contribution of this mechanism to the antioxidant activity of these compounds on mitochondria. Then, to check on the mechanism involved in this action, we evaluated the ROS scavenging activity of isocoumarins **1**–**3** by using non-mitochondrial systems. Fig. 5 shows the concentration–response curves for the capacity of isocoumarins **1**–**3** and quercetin to scavenge $DPPH^{\bullet}$, a stable free radical potentially reactive with all compounds capable of donating a hydrogen atom; Fig. 6 shows their capacities to scavenge $O_2^{\cdot-}$. Paepalantine (**1**) and its dimer (**2**) were effective at scavenging both $DPPH^{\bullet}$ and $O_2^{\cdot-}$, while vioxanthin (**3**) was a poor scavenger of these radicals; the IC_{50} values are given in Table 1. These results correlate well with those obtained on isolated mitochondria (Figs. 2 and 3). Therefore, we can consider $O_2^{\cdot-}$ scavenging as being the most probable cause for protection afforded by paepalantine (**1**) and its dimer (**2**) against oxidative stress conditions imposed on the mitochondria by Fe^{2+}-citrate, and also by *tert*-butylhydroperoxide, which is a generator of alkoxyl or peroxyl radicals depressing the antioxidant defences of those organelles. Concerning LPO, the $O_2^{\cdot-}$ scavenging, at

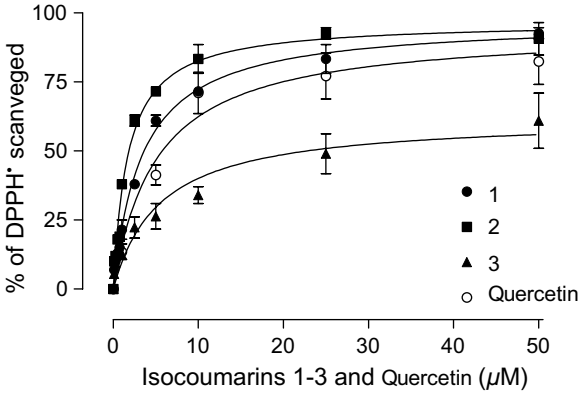

Fig. 5. Concentration–response curves for the effects of paepalantine (**1**), 8,8′-paepalantine dimer (**2**), vioxanthin (**3**) and quercetin (Quer) on $DPPH^{\bullet}$ scavenging, as described in Section 4. Values are means ± SEM of the results of three different experiments and relative to a control in absence of isocoumarins.

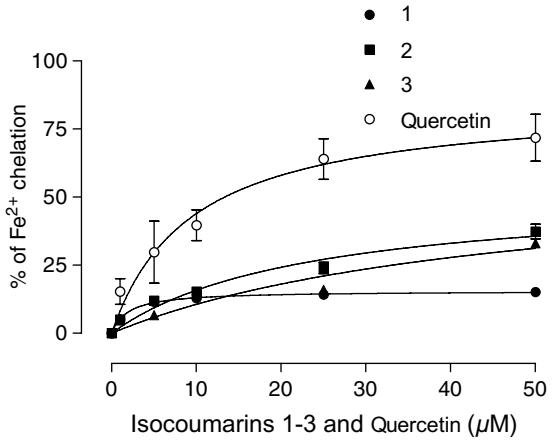

Fig. 4. Concentration–response curves for the effects of paepalantine (**1**), 8,8′-paepalantine dimer (**2**), vioxanthin (**3**) and quercetin (Quer) on iron chelation, as described in Section 4. Values are means ± SEM of the results of three different experiments and relative to a control in absence of isocoumarins **1**–**3**.

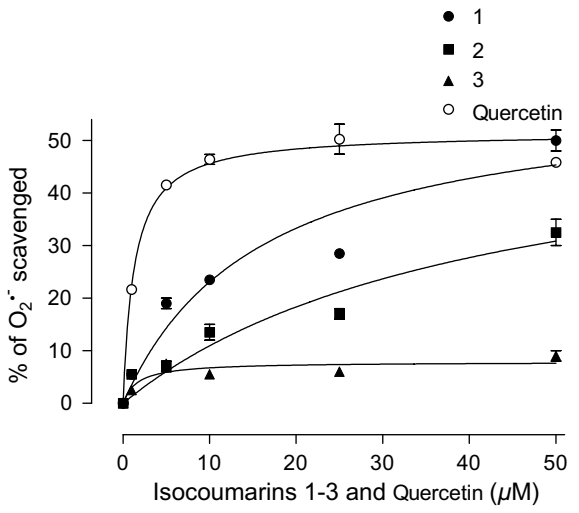

Fig. 6. Concentration–response curves for the effects of paepalantine (**1**), 8,8′-paepalantine dimer (**2**), vioxanthin (**3**) and quercetin (Quer) on $O_2^{\cdot-}$ scavenging, as described in Section 4. Values are means ± SEM of the results of three different experiments and relative to a control in absence of isocoumarins.

the moment when this radical is generated in the respiratory chain, would decrease its availability for the $^{\bullet}OH$ formation *via* the Fenton/Haber–Weiss reaction. It is worth mentioning that paepalantine (**1**) was almost of the same potency as quercetin, thereby, affording protection against the above oxidative stress-related processes on mitochondria.

The ROS scavenging capacity of organic compounds depends mainly on formation of stable free radicals. which in turn, depends on delocalization of the electrons inside these molecules; the planarity of some structures thus becomes a pre-requisite for an orbital arrangement capable of stabilizing these radicals; the adjacency of two hydroxyl groups (catechol), as in the *ortho*-diphenolic arrangement on the B-ring of flavonoids, is a well established requirement for the ROS-scavenging activities of polyphenols (Rice-Evans et al., 1996; Silva et al., 2002). In this connection, paepalantine (**1**) possesses 9-OH and 10-OH groups providing a catecholic-like arrangement as well as a planar structure, both of which are highly favourable for electron delocalization. While this molecular arrangement would explain the high antioxidant activity of paepalantine (**1**), the fact that the two units of the compound in the 8,8′-paepalantine dimer (**2**) are orthogonal to each other, would in principle influence this activity: this molecular arrangement would thus hinder formation of a planar catechol-like system between 9-OH and 10-OH, rendering electron delocalization less favourable than in paepalantine (**1**) itself. Additionally, the lack of the 3,4-double bond in the A-ring of vioxanthin (**3**) would hinder electron delocalization, either per se, or by an additional interference in the planarity of molecule. It therefore seems conceivable that a planar catechol-like system, involving 9-OH/10-OH and/or a 3,4-double bond, constitutes important structural requirements for the antioxidant activity of isocoumarins, including that on mitochondria.

By means of the above-mentioned structural features, our results point towards paepalantine (**1**), as well as to a lesser extent its dimer (**2**), as beging powerful agents for the protection, apparently via $O_2^{\bullet-}$ scavenging, of oxidative stress conditions imposed on mitochondria, the main intracellular source and target of ROS. Although, we were unable to detect any protection of isocoumarins 1–3 against HepG2 cell death induced by 200 μM H_2O_2 (Chernyak et al., 2006), evaluated by the trypan blue assay (data not shown), paepalantine (**1**), and to a lesser extent, paepalantine dimer (**2**) and vioxanthin (**3**), strongly protected these cells from the oxidative stress condition elicited under this same experimental conditions. The percent of protections were $94.75 \pm 3.82\%$, $48{,}51 \pm 4.23\%$ and $61{,}48 \pm 1.97\%$, respectively for paepalantine (**1**), paepalantine dimer (**2**) and vioxanthin (**3**).

3. Concluding remarks

It has been proposed that the modulatory effects in cells by some natural compounds such as flavonoids may be independent of their classical antioxidant capacities. That is, via selective action to a number of protein and lipid kinase signalling cascades and/or modulation of gene expression, improving the cellular redox status (Williams et al., 2004). In this context, despite a previous study demonstrating that rats treated during 3 days with paepalantine (**1**) significantly increased the colonic glutathione content (Di Stasi et al., 2004), the antioxidant action of this compound in our study, as well as of the dimer (**2**) and vioxantin (**3**), took place on isolated organelles. This apparently ruled out any involvement of modulation of gene expression or activation of signal transduction pathways involving the whole cell. In addition, even in HepG2 cells exposed to oxidative stress induced by H_2O_2, the antioxidant action of paepalantine (**1**) was already observed after a short time period of incubation.

4. Experimental

4.1. Chemicals

All reagents were of the highest commercially available grade. For all experiments, maximum volume of DMSO and isocoumarins used was 5 μl/ml, which had no effect on assays. All stock solutions were prepared using glass-distilled deionized water.

4.2. Plant material

Paepalantine (**1**), the 8,8′-paepalantine dimer (**2**) and vioxanthin (**3**) were isolated from *Paepalanthus bromelioides* by chromatographic procedures and compared to spectroscopic data previously published (Vilegas et al., 1990; Coelho et al., 2000). *Paepalanthus bromelioides* Silv. was collected during November 2000 at Serra do Cipó, State of Minas Gerais, Brazil, and identified by Dr. Paulo T. Sano, from IB-USP. A voucher specimen was deposited at the Herbarium of the Departamento de Botânica of the Instituto de Biociências of USP, under the number CFSC 13839.

4.3. Extraction and isolation

Powdered capitula from *Paepalanthus bromelioides* (580 g) were extracted with CH_2Cl_2 (4000 ml final volume) during one week, at room temperature. The combined CH_2Cl_2 extracts were evaporated in vacuo affording a crude extract (20 g), which was submitted to Si-gel CC, thus being eluted with mixtures of increasing polarity, starting with toluene–HOAc 99.5:0.5, and ending with toluene–EtOAc–HOAc 70.0:29.5:0.5. Polarity of the elution mixture was increased by the introduction of 5% EtOAc in the second step of the elution. From this point, increasing amounts (5%) of EtOAc were added, whereas the concentration of toluene was decreased at the same amount (5%) up to the final mixture. The initial fractions contained

paepalantine (**1**) according to Vilegas et al (1990) (Si-gel TLC, toluene–EtOAc–HOAc 99.0:0.5:0.5; R_F 0.6) and subsequent fractions contained the 8,8′-paepalantine dimer (**2**) according to Coelho et al (2000) (Si-gel TLC, toluene–EtOAc–HOAc 95.0:4.5:0.5; R_F 0.4). The fractions collected subsequently contained another substance, which showed a R_F 0.35 in Si-gel TLC, toluene–EtOAc–HOAc 95.0:4.5:0.5. This substance (50 mg) was obtained as a yellow-green powder. The structure of this compound was elucidated as vioxanthin on the basis of NMR, UV and IR spectroscopic analyses and by comparison of spectroscopic data with those previously reported (Blank et al., 1966; Saito et al., 1979). The purity of compounds **1**–**3**, was (~97%), by HPLC analyses, using column chromatography on C_{18} reversed phase and MeCN–H_2O (90:10 v/v) as the eluting solvent.

4.4. Isolation of rat liver mitochondria

Mitochondria were isolated by standard differential centrifugation (Pedersen et al., 1978). Male Wistar rats weighing approximately 200 g were sacrificed by cervical dislocation; livers (10–15 g) were immediately removed, sliced in medium (50 ml) containing 250 mM sucrose, 1 mM EGTA and 10 mM HEPES-KOH, pH 7.2, and homogenized three times for 15 s at 1 min intervals with a Potter-Elvehjem homogenizer. Homogenates were centrifuged (580*g*, 5 min) with the resulting supernatant further centrifuged (10,300*g*, 10 min). Pellets were next suspended in medium (10 ml) consisting of 250 mM sucrose, 0.3 mM EGTA and 10 mM HEPES-KOH, pH 7.2, and centrifuged (3400*g*, 15 min). The final mitochondrial pellet was suspended in medium (10 ml) containing 250 mM sucrose and 10 mM HEPES-KOH, pH 7.2, and used within 3 h. Mitochondrial protein contents were determined by the biuret reaction (Cain and Skilleter, 1987).

4.5. Standard incubation procedure

Mitochondria (1.5 mg protein) incubated at 30 °C with 2.5 μM rotenone in a standard medium (125 mM sucrose, 65 mM KCl, 10 mM HEPES-KOH, pH 7.4) plus 0.5 mM EGTA and 10 mM K_2HPO_4, at a final volume of 1.5 ml, were energized by addition of 5 mM potassium succinate. Isocoumarins **1**–**3** were individually dissolved in DMSO and added to the mixture assay at the beginning of the incubation periods.

4.6. Determination of H_2O_2

The H_2O_2 levels in mitochondria (2 mg protein, final volume 2 ml) were determined in the standard medium following reaction of H_2O_2 with homovanillic acid in the presence of horseradish peroxidase to form a dimer which fluoresced at 312 nm excitation and 420 nm emission, by using a Model F-4500 Hitachi fluorescence spectrophotometer (Tokyo, Japan) (Barja, 2002).

4.7. Fe^{2+}/citrate-mediated lipid peroxidation of mitochondrial membrane (LPO) assay

LPO was estimated from malondialdehyde (MDA) generation (Buege and Aust, 1978). The mitochondrial suspension (1 ml, 1 mg protein) was incubated with 50 μM $FeSO_4$ plus 2 mM sodium citrate at 37 °C. After 30 min, 1 ml of 1% thiobarbituric acid (TBA, prepared in 50 mM NaOH), 0.1 ml of 10 M NaOH and 0.5 ml of 20% H_3PO_4 were added, followed by incubation for 20 min at 85 °C. The MDA–TBA complex was extracted with 2 ml of *n*-butanol with absorbance measured at 535 nm. MDA concentration was calculated from $\varepsilon = 1.56 \times 10^5\ M^{-1}\ cm^{-1}$.

4.8. Assays of isocoumarins (1–50 μM) in non- mitochondrial systems

Reduction of 100 μM $DPPH^{\bullet}$ by isocoumarins **1**–**3** was individually monitored by changes in absorbance at 517 nm, 5 min after their incubation with 40 mM acetate, pH 5.5, and 1 ml ethanol (Blois, 1958), respectively. The O_2^{-} scavenging activity was determined by measurement of the product of the reduction of nitro blue tetrazolium (600 μM), monitored at 560 nm, by using the xanthine (100 μM)/xanthine oxidase (0.07 U/ml) system as a source of the radical, in 0.1 M phosphate buffer, pH 7.6 (Robak and Gryglewski, 1988). Iron chelation was monitored through prevention of formation of the $FeII(BPS)_3$ complex. Isocoumarins **1**–**3** were individually added to the standard medium in the presence of 50 μM Fe^{2+} plus 200 μM bathophenanthroline disulfonic acid (BPS) with absorbance at 530 nm measured after 30 min (Bolanm and Ulvik, 1987).

4.9. Culture and treatment of HepG2 cells and determination of H_2O_2

HepG2 cells were obtained from the American Type Culture Collection, No. HB 8065 and maintained in Dulbecco's Modified Eagle medium (DMEM) supplemented with 10% bovine fetal serum plus 100 IU/ml penicillin G and 100 μg/ml streptomycin. The logarithmically growing HepG2 cells were detached using 2.5 g/l trypsin-EDTA solution, washed twice with supplemented DMEM at (1000 rpm) 300*g*/5 min. The cells were incubated at 37 °C with 5% CO_2 in air during the required time, in the presence of 50 μM of isocoumarins **1**–**3** previously dissolved in DMSO. Control cells were cultured in the presence of medium plus DMSO. After the incubation period, the cells were washed with buffered saline solution.

For induction of oxidative stress, the cells previously incubated during 1 h with the isocoumarins **1**–**3** were individually treated during the same period with 200 μM H_2O_2 in the complete medium. ROS production was measured after loading the cells with 5 μM CM-DCF-DA for 15 min, at 37 °C, immediately after washing out H_2O_2. Fluorescence was analyzed using a F-4500

spectrofluorometer (Hitachi, Tokyo, Japan) at excitation and emission wavelengths of 503 and 529 nm, respectively (Chernyak et al., 2006).

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