Available online at www.sciencedirect.com
ScienceDirect
Chemico-Biological Interactions 171 (2008) 294–305
Chemico-Biological Interactions
www.elsevier.com/locate/chembioint

Mitochondrial toxicity and antioxidant activity of a prenylated flavonoid isolated from *Dalea elegans*

Igal Elingold [a], M. Paula Isollabella [b], Marta B. Casanova [a], Ana M. Celentano [c], Cristina Pérez [d], José Luis Cabrera [e], Roberto A. Diez [b], Marta Dubin [a,*]

[a] *Centro de Estudios Farmacológicos y Botánicos (CEFYBO), Facultad de Medicina, UBA-CONICET, Paraguay 2155, piso 16, C1121 ABG Buenos Aires, Argentina*
[b] *Departamento de Farmacología, Facultad de Medicina, UBA, Argentina*
[c] *Departamento de Microbiología, Facultad de Medicina, UBA, Argentina*
[d] *Departamento de Farmacología, Facultad de Odontología, UBA, Argentina*
[e] *Farmacognosia, Departamento de Farmacia, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Córdoba, Argentina*

Received 19 July 2007; received in revised form 4 October 2007; accepted 15 October 2007
Available online 25 October 2007

Abstract

The prenylated flavanone 2′-4′-dihidroxy-5′-(1‴-dimethylallyl)-6-prenylpinocembrin) (6PP), isolated from the roots of *Dalea elegans*, shows antimicrobial activity. The aim of this study was to evaluate mitochondrial toxicity and antioxidant properties of 6PP. Addition of micromolar concentrations of 6PP to rat liver mitochondria, stimulated O_2 uptake in state 4 and inhibited it in state 3 when malate–glutamate was the respiratory substrate, and inhibited O_2 uptake in state 3 when succinate was the substrate. Highest concentration of 6PP also inhibited O_2 uptake in state 4 in the latter case; in both conditions, respiratory control index values were decreased. This flavanone collapsed the mitochondrial membrane potential in a concentration-dependent manner. 6PP also inhibited F0F1-ATPase activity in coupled mitochondria and in submitochondrial particles. In the latter, this compound also inhibited NADH oxidase and succinate dehydrogenase activities. HEp-2 cells were incubated for 24 h with 6PP in presence or absence of 0.5% albumin. As measured by reduction of the mitochondrial-related probe MTT, in the albumin-free condition, 6PP was cytotoxic in a concentration-dependent manner; on the other hand, albumin decreased 6PP effect. In addition, in rat liver microsomes 6PP: (1) inhibited the enzymatic lipid peroxidation, (2) exhibited significant scavenging activity, measured by DPPH reduction assay and (3) demonstrated significant antioxidant activity by decreasing the reduction of Mo(VI) to Mo(V). We suggest that 6PP impairs the hepatic energy metabolism by acting as mitochondrial uncoupler and by inhibiting enzymatic activities linked to the respiratory chain. 6PP also exerts both antioxidant and antiradical activities. Due to its cytotoxicity, this molecule, and its future structure developments, can be considered as a potentially promising therapeutic agent, for instance in cancer chemotherapy.

Keywords: Flavonoids; Mitochondria; Liver; Antioxidant; Toxicity

1. Introduction

It is well known that diets rich in fruit and vegetables protect against several diseases [1]. These effects have been attributed, in large part, to several antioxidants

* Corresponding author. Tel.: +54 11 4508 3680x107; fax: +54 11 4508 3680x107.
E-mail address: dubin@mail.retina.ar (M. Dubin).

0009-2797/$ – see front matter © 2007 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.cbi.2007.10.005

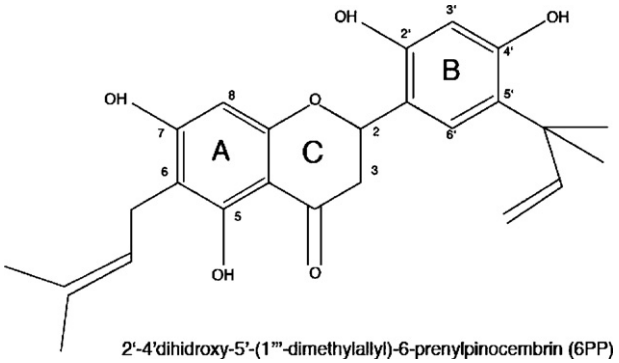


Fig. 1. Structure of 6PP.

present in this kind of aliments. Among them flavonoids may play an important role. Flavonoids are a large class of compounds with phenolic hydroxyl groups attached to ring structures, which confer antioxidant activity [2–4]. These antioxidant properties are related to their ability to chelate metal ions and scavenge singlet oxygen, superoxide, peroxyl, hydroxyl and peroxynitrite radicals [3,5,6]. *In vivo*, further antioxidant activities have been found, such as the increase in the total radical antioxidant parameters [7]. In addition to their free radical scavenging activity, flavonoids have been reported to possess many important biological activities: anticarcinogenic, anti-inflammatory, antimutagenic, antimicrobial and vasodilatory, as well as several toxic effects [8,9]. Many of them, most of natural origin, are currently under prospective scrutiny for therapeutic application. Antiproliferative properties of prenylated flavonoids have been demonstrated in several cancer cell lines [10].

Prenylated flavonoids have a relatively narrow distribution in the plant kingdom and are constitutively expressed in only some plants, unlike prenylated isoflavonoids [11]. In previous studies, the prenylated flavanone 2′-4′-dihidroxy-5′-(1‴-dimethylallyl)-6-prenylpinocembrin (6PP, shown in Fig. 1), extracted from the roots of the Argentine plant *Dalea elegans*, has demonstrated an antibacterial effect against *Staphylococcus aureus* as well as antifungal action on multi-resistant nosocomial strains of *Candida glabrata*, *C. krusei* and *C. albicans*, among others [12]. Some of these pathogens were isolated from immunocompromised patients, mainly AIDS cases. Whereas several antibacterial mechanisms have been described for different flavonoids – including inhibition of DNA and RNA synthesis, anti-folate activity and unselective disruption of cell membranes – [13–15], the target of 6PP is still unknown. The same is true for most flavonoids [16].

Since prenylation of the flavonoids increases lipophilicity, and confers a strong affinity to biological membranes [17], this compound was considered an interesting candidate for further development, into an eventual clinical option compromised patients like these. This development requires the initial study of its pharmacological and toxic properties. Based on the known effects of flavonoids upon oxidative activities, we performed an evaluation of several antioxidant mechanisms and targets, in order to test whether 6PP is active on mitochondria and whether it possesses antiradical/antioxidant properties. We now report a partial characterization of the *in vitro* 6PP effects on rat liver mitochondrial respiration, NADH oxidase, succinate dehydrogenase, ATPase activities and mitochondrial membrane potential, as well as cytotoxicity against human tumor cells, and its scavenger/antioxidant activity.

2. Materials and methods

2.1. Chemicals

6PP was isolated as previously described [18]. Compound was provided as a lyophilized powder and diluted in dimethylformamide (DMFA) for all assays. The same volume of solvent, as test compound, was used as control and never exceeded 0.5% (v/v). The different incubation times used are presented in the text. HCl, NaOH, KCl, H_2SO_4, trichloroacetic acid (TCA) and ammonium molybdate were provided by Merck Química Argentina S.A., Buenos Aires, Argentina. EDTA and EGTA were obtained from BHD Chemicals Ltd., England; tris (hidroxymethyl) aminomethane (Trizma base), thiobarbituric acid (TBA), Na_2HPO_4, KH_2PO_4, sucrose, $MgCl_2$, $FeCl_3$, glucose-6 phosphate (G6P), glucose 6 phosphate dehydrogenase (G6PD), $NADP^+$, ADP, NADH, malic acid, glutamic acid, malonic acid, succinic acid, rotenone, 2,2-diphenyl-1-picryl-hydrazyl (DPPH), 3-[4,5-dimethyl thiazol-2-y1]-2,5diphenyl tetrazolium bromide (MTT), rhodamine 123, *N*,*N*-dimethyl-formamide (DMFA), glutamine and serum bovine albumin (BSA) were acquired from Sigma Chemical Co., St. Louis, MO, United States. Methanol and ethanol were provided by Riedel de Haen AG, Germany. Sterile saline solution was acquired from Roux-Ocefa S.A. (Buenos Aires, Argentina). All the other chemicals used in this study were of analytical grade.

2.2. Cell culture

HEp-2 cells kindly provided by Dr. A.S. Mistchenko (Hospital de Niños R. Gutiérrez, Buenos Aires,

Argentina) were cultured in Earle-MEM (Gibco BRL, USA), with 10% inactivated fetal calf serum (FCS) from Bioser, Buenos Aires, Argentina. The cell line derives from a laryngeal epidermoid carcinoma and was chosen mainly because it is widely available and has been frequently used for the screening of cytotoxic drugs, including natural products such as ginger extract [19]. Though the selection was not based on this information, free radical metabolism has been suggested to be involved in the pathogenesis of laryngeal cancer [20].

2.3. Liver mitochondria, submitochondrial particles and microsomal preparations

Liver mitochondria were isolated from adult male Wistar rats by conventional differential centrifugation [21,22]. The liver of overnight fasted adult animals was removed and placed quickly in an ice-bath. The tissue was homogenized in 250 mM sucrose, 1 mM EDTA and 5 mM HCl–Tris buffer (pH 7.4). The 600 g pellet was discarded and mitochondria were pelleted at $6800 \times g$, washed twice with the same solution, and the final pellet suspended in 240 mM sucrose, 34 mM KCl, 5 mM $MgCl_2$, 9 mM HCl–Tris, 1 mM EDTA, 6 mM Na_2HPO_4–KH_2PO_4 (pH 7.4) (1 ml buffer/g liver). All experiments were performed within 3 h following mitochondrial isolation. Submitochondrial particles were prepared with the same procedure except that the final pellet was kept dry overnight at −20 °C. The pellet was suspended in 100 mM tetrasodium pyrophosphate (pH 7.4). The suspension was sonicated three times at 50 W and centrifuged at $26{,}000 \times g$ for 15 min, sediments discarded and the supernatant centrifuged at $105{,}000 \times g$ for 45 min. Pelleted submitochondrial particles were washed twice and resuspended in 250 mM sucrose, 1 mM EDTA and 5 mM HCl–Tris buffer (pH 7.4) at roughly 20 mg protein/ml, and kept at −70 °C (up to 6 months). NADH oxidative activity of these preparations was 100–190 nmol NADH/(min mg protein), at 30 °C and remained unchanged for at least 6 months. Microsomes were obtained by differential centrifugation [23,24]. Briefly, the liver tissue was homogenized in 50 mM HCl–Tris, 150 mM KCl (pH 7.4) (4 ml buffer/g liver). Centrifugation at $600 \times g$ for 10 min and then at $10{,}800 \times g$ for 15 min was performed to eliminate nuclear and mitochondrial fractions, respectively. The microsomal fraction was obtained by centrifugation of the supernatant at $105{,}000 \times g$ for 60 min. The microsomal pellet was washed with 150 mM KCl and either used immediately or stored in liquid nitrogen, for 3 months.

2.4. Mitochondrial respiration rates

Respiration rates were measured, with a model 5/6 Oxygraph (Gilson Medical Electronics, Middleton, WI, USA) fitted with a Clark oxygen electrode, at 30 °C. Reactions were conducted in a 1.8 ml closed thermostatic and magnetically stirred glass chamber containing mitochondria (2–3 mg protein/ml) suspended in a respiration buffer containing 240 mM sucrose, 34 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 9 mM HCl–Tris, and 6 mM Na_2HPO_4–KH_2PO_4 (pH 7.4). For respiration experiments with L-malate and L-glutamate as substrates, 5 mM L-malate, 5 mM L-glutamate (MG) and 2.5 mM malonate were added. For respiration with succinate as substrate, 10 mM succinate and 3 μM rotenone were added. The latter was used to prevent electron transport from and to Complex I. The rate of mitochondria respiration was measured in metabolic state 3, after adding 0.5 mM ADP, or in metabolic state 4, without ADP. The respiratory control index (RCI) was calculated as the ratio of state 3/state 4 respiration [25].

2.5. Enzyme assays

F0F1-ATPase activity was measured at 30 °C in an incubation mixture (final volume 1 ml) containing 150 mM HCl–Tris (pH 7.6), 3 mM ATP, 4 mM $MgCl_2$ and 0.25 mM EGTA. The reaction was started by adding the mitochondria or submitochondrial particles to a final concentration of 0.125 mg protein/ml. After 20 min of incubation, 0.1 ml of 50% (w/v) TCA was added. Inorganic phosphate (Pi) released from ATP was determined by the Fiske–Subbarow method [26]. Values were expressed as percentage of ATPase activity versus DMFA. Succinate dehydrogenase (SDH) activity [27] was measured at 30 °C in an incubation mixture (final volume 1 ml) containing 100 mM Na_2HPO_4–KH_2PO_4, 1 mM EDTA, 0.1% BSA (pH 7.6), 1 mM KCN and 1 mM potassium ferricyanide. The reaction was started by adding 18 mM succinate to a preparation containing submitochondrial particles (0.6 mg/ml). Enzyme activity was followed by the rate of reduction of potassium ferricyanide at 455 nm ($\varepsilon = 150\ M^{-1}\ cm^{-1}$). NADH oxidase activity was measured at 30 °C in an incubation mixture (final volume 1.5 ml) containing 50 mM Na_2HPO_4–KH_2PO_4 (pH 7.4). The reaction was started by adding 0.125 mM NADH to a preparation containing submitochondrial particles (0.2 mg/ml). Enzyme activity was followed by the oxidation of NADH at 340 nm ($\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$).

2.6. Measurement of mitochondrial membrane potential

Mitochondrial membrane potential was measured by rhodamine 123 fluorescence [28]. The reaction mixture contained: mitochondria (1–2 mg protein/ml), 0.5 mM ADP, 5 mM L-malate, 5 mM L-glutamate and 2.5 mM malonate or 10 mM succinate and 3 μM rotenone in respiration buffer, as was described in Section 2.4. Mitochondria were preincubated or not as indicated in each case, with 6PP for 15 min aerobically in a shaker (90 cycles/min) at 37 °C. Rhodamine 123 (1 μM) was added and, after 10 min incubation, the reaction mixture was centrifuged at 6800 × *g*. Rhodamine 123 accumulation in mitochondria was determined by the difference between the fluorescence in the supernatant and in a solution of rhodamine 123 (1 μM). Fluorescence was measured in an Aminco SLM 8000C spectrofluorometer, at 498 nm excitation and 525 nm emission.

2.7. Cell viability

Two methods were used:

(a) Trypan blue dye exclusion method [29]. To determine initial conditions of cytotoxic assay, HEp-2 cell cultures (10^5 cells/well) were grown on 24-well tissue culture plates (Corning Cell Wells, Corning, New York, NY, USA) at 37 °C in a 5% CO_2 humidified atmosphere. At confluence, medium was discarded and replaced by fresh medium without FCS, containing or not 6PP at different concentrations, as described in the text. After 24 h incubation, cells were trypsinized and the cell suspension was diluted 1:10 with 1.6% (w/v) trypan blue solution and examined in a phase contrast microscope (C. Zeiss, Germany) to count cells able to exclude the dye. Controls included untreated cells (culture medium alone) or cells incubated with DMFA. Cytotoxicity values were graphically estimated from plots of the percentage of viable cells yielded by the different concentrations of 6PP, compared to untreated controls.

(b) Cell viability test by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method [30]. HEp-2 cell cultures (1.5×10^4 cells/well) were prepared on 96-well tissue culture plates (Nunc, Denmark). At confluence, medium was discarded and replaced by fresh medium without FCS, containing the different concentrations of 6PP, with or without 0.5% BSA, in a final volume of 100 μl per well. After 24 h incubation, medium was discarded and an aliquot of 10 μl of MTT, 5 mg/ml in aqueous solution, was added to each well; then the plates were incubated for 90 min at 37 °C in 5% CO_2 atmosphere. The reaction was stopped by adding isopropanol (100 μl to each well) to dissolve formazan crystals. To allow complete dissolution of formazan, plates were further incubated at 37 °C for 15 min, followed by reading on a multiwell spectrophotometer (Bio-Tek, Elx 800, USA) at 540 nm. In some experiments, the effect of albumin on 6PP cytotoxicity was assessed by adding BSA in a final concentration of 0.5% (w/v). The percentage of cytotoxicity was calculated as $[(A - B)/A \times 100]$, where A and B are the absorbance of control and treated cells, respectively. The IC_{50} (inhibitory concentration 50%) was defined as the concentration that reduced the absorbance of treated cells to 50% when compared to control cells.

2.8. Assay of microsomal lipid peroxidation

To assay of NADPH-dependent lipid peroxidation, the incubation mixture consisted of liver microsomes (1 mg protein/ml), the NADPH-generating system (0.55 mM $NADP^+$, 5.5 mM G6P, 1.4 units/ml G6PD, 5.5 mM $MgCl_2$), as the reducing system for the microsomal electron transfer, 1.7 mM ADP, 0.1 mM $FeCl_3$ as the iron catalyst, 130 mM KCl and 23 mM Na_2HPO_4–KH_2PO_4 buffer, pH 7.4. After thermal equilibration at 37 °C, the reaction was started by adding G6PD and incubated for 60 min aerobically in a water bath with shaker (90 cycles/min) at 37 °C. Aliquots (1.0 ml) were withdrawn at the times indicated and chilled to 0 °C. The malondialdehyde (MDA) content of these samples was measured by the TBA method [31]. Absorbance was measured spectrophotometrically at 535 nm and the amount of MDA was calculated from $\varepsilon = 1.56 \times 10^5\ M^{-1}\ cm^{-1}$.

2.9. Antiradical activity determination

Two methods were used to test the antioxidant activity of 6PP. One is based on the evaluation of free radical scavenging [32] and the other is based on the reduction of a phosphomolybdenum complex [33]. Scavenging of free radicals was tested in a methanolic solution of DPPH [32]. The degree of decoloration of the solution indicates the scavenging efficiency of the substance added. DPPH is a stable free radical and accepts an electron or hydrogen to become a stable diamagnetic molecule [34]. For each compound, aliquots of 1 ml of the drugs in methanol were added to 2 ml of DPPH solution (10 mg/l). Fifteen

minutes later, the reduction capability of DPPH radicals was determined by the decrease in its absorbance at 517 nm. A reference sample was prepared with 1 ml of methanol. Antiradical activity was calculated as a percentage of DPPH decoloration using the following equation:

$$\text{antiradical activity} = 100 \times \left(\frac{1 - \text{absorbance of sample}}{\text{absorbance of reference}} \right)$$

The antioxidant power of the 6PP was also evaluated with the molybdate reduction assay [33]. The reagent solution contained 4 mM ammonium molybdate, 28 mM sodium phosphate and 600 mM sulfuric acid, to which 6PP (dissolved in methanol) was added, at the final concentrations indicated in the text. The samples were incubated for 60 min at 95 °C and the absorbance of the green phosphomolybdenum complex was measured at 695 nm. A typical blank solution contained 1 ml of reagent solution and the appropriate volume of the same solvent used for the sample, and it was incubated in the same conditions as the other samples.

2.10. Statistical analysis

The effect of 6PP was calculated taking the result in the sample containing DMFA as the control value. GraphPad InStat and GraphPad Prism version 4.00 software (GraphPad Software, San Diego, CA, USA) was used to calculate the standard errors of independent experiments involving duplicate analyses for each sample condition. Statistical analysis was done with either one-way analysis of variance (ANOVA) test for repeated measurements and Dunnett post-test. Significance was accepted at $p < 0.05$.

3. Results

3.1. Effect of 6PP, on electron transfer and oxidative phosphorylation in coupled mitochondria

Table 1 shows the effect of 6PP on the respiration rate of coupled mitochondria, with MG site I substrate, or succinate site II substrate. It can be seen that with MG as substrate and mitochondria in metabolic state 3 (active respiration, the maximal physiological rate of O_2 uptake and ATP synthesis), 6PP produced a significant inhibition: 27 and 56.5%, at 50 and 100 μM, respectively; with the same substrate mixture and mitochondria in metabolic state 4 (resting or controlled respiration), 6PP activated respiration to a significant degree 2.1- and 2.4-fold at 50 and 100 μM, respectively; as a result RCI significantly decreased. With succinate as substrate and mitochondria in state 3, 6PP produced significant inhibition 50, 68, 79 and 92% at 12.5, 25, 50 and 100 μM, respectively; the state 4 respiration was significantly decreased only at the highest concentration of 6PP, as a result of the last variations RCI significantly decreased.

3.2. Effect of 6PP on the activities of membrane-bound enzymes

Considering the results shown in Table 1, it seemed of interest to study the effect of 6PP on ATPase, SDH and NADH oxidase activities.

ATP synthetase (F0F1-ATPase) is an essential component of the mitochondrial energy-transducing system, which can show ATP synthetase or ATPase activity. Fig. 2 shows that 100 μM 6PP inhibited about 50% of ATPase activity both in coupled mitochondria and in submitochondrial particles; in the absence of inhibitor the ATPase activity was 108 ± 26 and 276 ± 38 mU/mg protein, respectively.

Table 1
Effect of 6PP on mitochondrial oxygen uptake

6PP (μM)	Malate–glutamate (nmol O_2/(min mg protein))			Succinate (nmol O_2/(min mg protein))		
	State 3	State 4	RCI	State 3	State 4	RCI
0	16.35 ± 3.49	2.18 ± 0.49	7.18 ± 0.45	27.43 ± 3.12	5.26 ± 0.50	4.94 ± 0.36
0.1	14.88 ± 2.93	2.33 ± 0.49	6.48 ± 0.87	25.36 ± 2.50	5.02 ± 0.47	5.08 ± 0.40
1.0	15.18 ± 2.77	2.54 ± 0.42	5.77 ± 0.51	24.67 ± 2.35	5.03 ± 0.48	4.95 ± 0.37
12.5	14.26 ± 2.74	2.65 ± 0.40	5.09 ± 0.47**	13.77 ± 1.50**	5.05 ± 0.52	2.74 ± 0.19**
25.0	14.81 ± 3.24	3.14 ± 0.49	4.31 ± 0.61**	8.68 ± 1.16**	5.97 ± 0.91	1.66 ± 0.12**
50.0	11.96 ± 3.06**	4.60 ± 0.57*	2.29 ± 0.45**	5.73 ± 0.80**	5.50 ± 0.64	1.27 ± 0.08**
100.0	7.11 ± 2.54**	5.15 ± 1.47**	1.06 ± 0.07**	2.09 ± 0.30**	2.09 ± 0.30**	1.00 ± 0.00**

Values are mean ± S.E.M. for four rats (malate–glutamate plus malonate) or for seven rats (succinate plus rotenone), in independent, duplicate measurements. Experimental conditions are described in Section 2. *$p < 0.05$, **$p < 0.01$ vs. control in absence of 6PP.

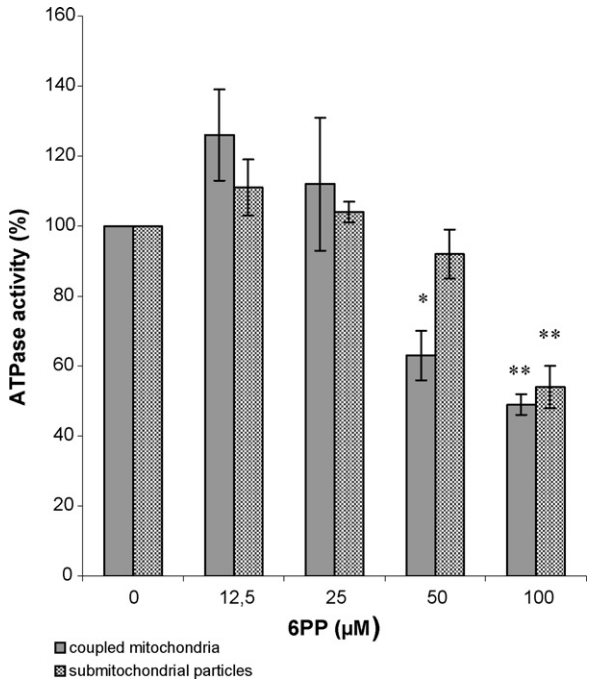


Fig. 2. Effect of 6PP on mitochondrial ATPase activity. F0F1-ATPase activity was assayed by rate of ATP hydrolysis as indicated in Section 2. Values are mean ± S.E.M. of four duplicate, independent measurements $*p < 0.05$, $**p < 0.01$ vs. control in absence of 6PP.

Fig. 3 shows that addition of 6PP promoted significant inhibition of SDH (Fig. 3A) and NADH oxidase (Fig. 3B) activity in a concentration-dependent manner (IC_{50} of 25 and 19 μM, respectively). DMFA, even in amounts present in the highest concentration of 6PP used, had no significant effect on any of the activities assayed.

3.3. Effect of 6PP on mitochondrial membrane potential

The effect of 6PP on mitochondrial membrane potential was investigated in coupled mitochondria. Using MG (Fig. 4A) as substrate, 6PP (100 μM) significantly decreased by 47.5 and 60% mitochondrial membrane potential, after 10 or 25 min incubation, respectively. With succinate (Fig. 4B) as substrate, 6PP (100 μM) decreased mitochondrial membrane potential by 51.5% after 10 min incubation. On the other hand, 6PP (25, 50 and 100 μM) decreased mitochondrial membrane potential after 25 min incubation (34.4, 55 and 56%, respectively). These effects were not observed in the absence of ADP (data not shown).

3.4. Dose-dependent cytotoxicity of 6PP on HEp-2 cells

Panel A of Fig. 5 illustrates the cytotoxic effect of 6PP on HEp-2 cells monolayers after overnight incubation, compared to control (untreated) cultures (Panel B). On the contrary, DMFA did not affect HEp-2 cells (data not shown). The effect of 6PP was dose-dependent, in the micromolar range (between 10 and 400 μM, data not shown), by microscopic evaluation of the morphology and trypan blue exclusion.

The MTT reduction method was used to further characterize the cytotoxic effect of 6PP. Fig. 6 shows a dose-dependent decrease in the percentage of viable cells was obtained, with an IC_{50} of 20 μM. Since 6PP is a rather lipophilic molecule, this experiment was also run in the presence of albumin (Fig. 6). In this condition, 6PP cytotoxicity was markedly decreased, reaching statistical significance only at 400 μM.

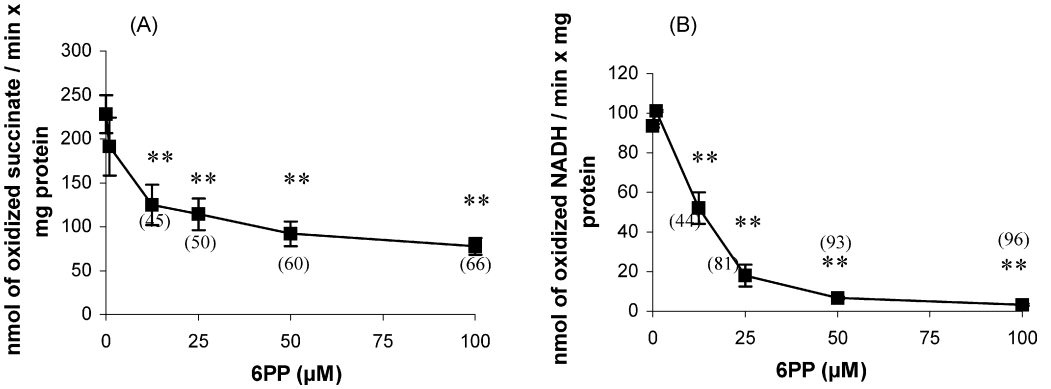


Fig. 3. Effect of 6PP on mitochondrial enzymatic activities. (A) SDH activity and (B) NADH oxidase activity. Values are mean ± S.E.M. of three duplicate, independent measurements. $**p < 0.01$ vs. control in absence of 6PP. Percentage of enzymatic inhibition is shown in parentheses.

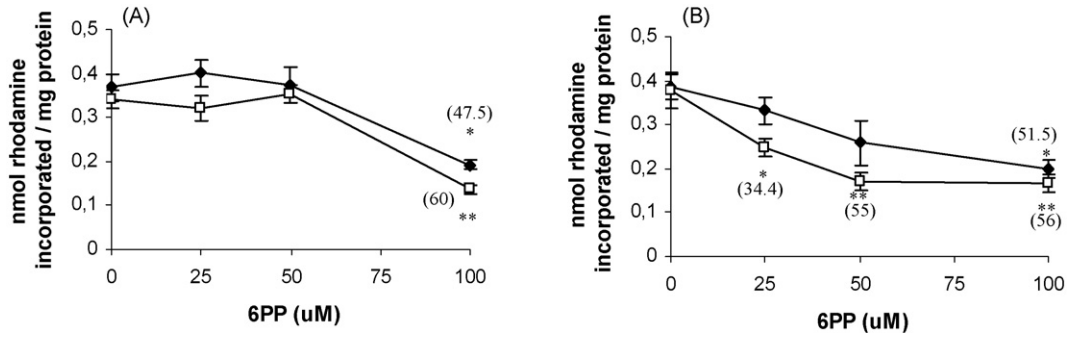
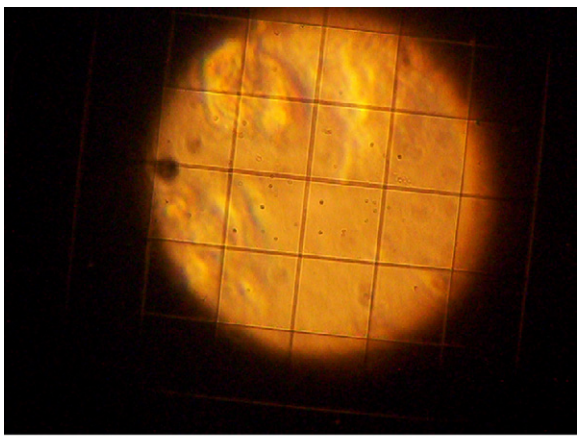
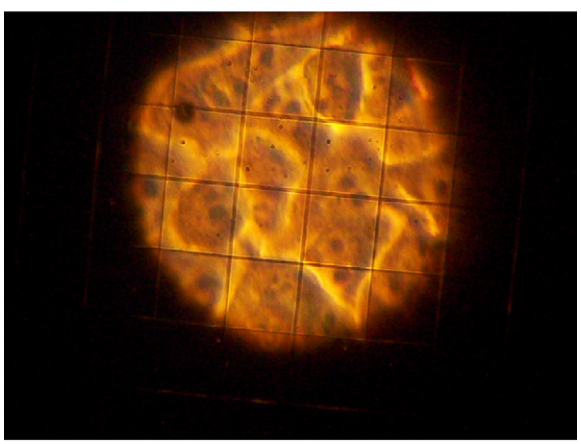


Fig. 4. Effect of 6PP on mitochondrial membrane potential. 6PP was incubated with submitochondrial particles for 10 min (♦) or 25 min (□). (A) With malate–glutamate or (B) succinate, as respiratory substrates. Values are mean ± S.E.M. of four independent, duplicate measurements. $*p < 0.05$; $**p < 0.01$ vs. control in absence of 6PP. Percentage of inhibition is shown between parentheses.



Panel A (with 6PP)



Panel B (with medium alone)

Fig. 5. Effect of 24 h incubation with 6PP on fresh HEp-2 cells, analyzed by phase contrast microscopy (original, 400×). Panel A shows the toxic effect on the monolayer, whereas the control cells, in the absence of 6PP, display a normal appearance (Panel B).

3.5. Effect of 6PP on lipid peroxidation

In microsomes, NADPH-cytochrome P-450 reductase is involved in NADPH-dependent enzymatic lipid peroxidation [35,36]. Lipid peroxidation, which can be measured by the TBA method, occurs when rat liver microsomes are incubated with Fe^{+3}-ADP/NADPH. Fig. 7 shows that 6PP inhibited lipid peroxidation in a concentration-dependent manner, by 82–88% at 50 and 100 μM, respectively.

3.6. Antioxidant activity evaluation

Antioxidant activity of the 6PP was evaluated by the DPPH method. Fig. 8 shows that 6PP, in the range of 12.5–100 μM, produced a very significant dose-dependent decrease in the concentration of DPPH, thus demonstrating its scavenging activity. In the same conditions, quercetin was more effective as scavenger than 6PP, whereas the effect of naringenin was negligible.

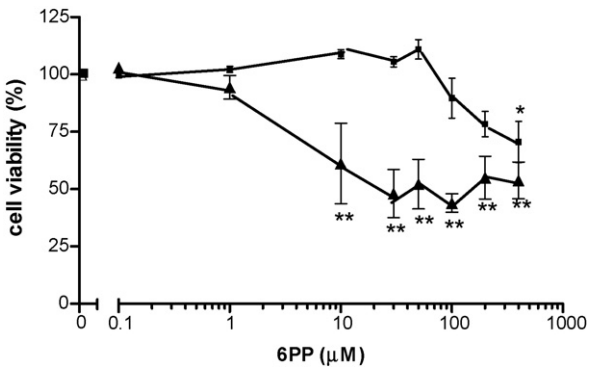


Fig. 6. Effect of 6PP on cellular viability. Experimental conditions are described in Section 2; (▲) medium, (■) medium + albumin. Values are mean ± S.E.M. of at least three triplicate, independent measurements, and were compared to the DMFA-containing sample at the same incubation time. $**p < 0.01$, $*p < 0.05$.

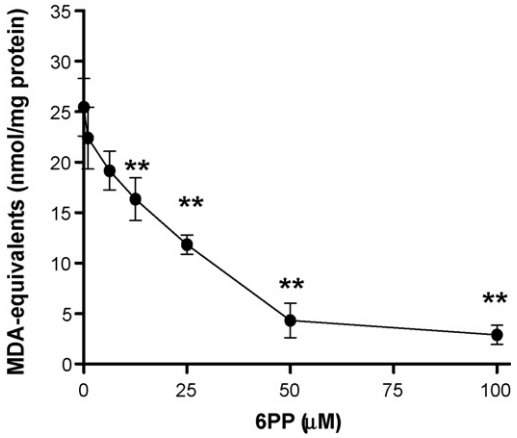


Fig. 7. Effect of 6PP on MDA production by liver microsomes. Experimental conditions are described in Section 2. Values are mean ± S.E.M. of at least three duplicate, independent measurements, and were compared to the DMFA-containing sample at the same time of incubation $^{**}p < 0.01$.

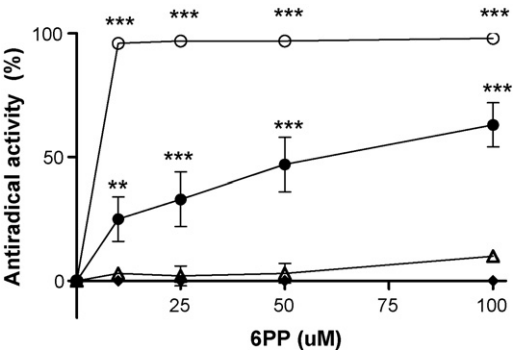


Fig. 8. Antiradical activities of 6PP, quercetin and naringenin in a methanol solution of DPPH. The antiradical activity of 6PP (●), quercetin (◯) and naringenin (▲) was calculated as a percentage of DPPH decoloration compared to DMFA (♦). Values are mean ± S.E.M. of at least three duplicate, independent measurements.

Also, antioxidant activity, measured by the reduction of a phosphomolybdenum complex (Fig. 9), significantly increased in a concentration-dependent manner after adding 6PP (25–100 μM); quercetin exhibited stronger activity than 6PP and the effect of naringenin was negligible (data not shown).

4. Discussion

The hydrophobic compound 6PP, a prenylated flavonoid, is currently under pharmaceutical development aimed to explore some of its potential activities. Results herein demonstrate that 6PP has an intrinsic antioxidant activity, but also inhibits several mitochondrial enzymes and induces cytotoxicity.

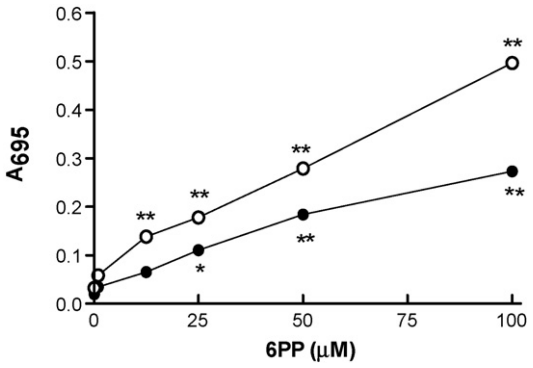


Fig. 9. Antioxidant activity of 6PP and quercetin. The formation of the phosphomolybdenum complex was measured for 60 min by measuring absorbance at 695 nm. Experimental conditions are described in Section 2. 6PP (●) and quercetin (◯). Values are mean ± S.E.M. of at least three duplicate, independent measurements $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control in absence of 6PP.

Pharmaceutical development of flavonoids usually involve several potential activities. Perhaps the most common is related to its antioxidant property. Flavonoids, like other phenolic compounds with free hydroxyls, are considered to possess some degree of antioxidant activity. Phenoxy radicals are readily formed by donation of phenolic hydrogens and subsequently react with peroxy radicals involved in lipid peroxidation. In fact, a range of flavonoid-enriched plant extracts is widely marketed for their antioxidant properties and putative health benefits [37,38]. However, their use as dietary supplements should be considered with caution as they could also exhibit mutagenic and/or pro-oxidant effects [39].

Under physiologic conditions, most electron-transfer processes leading to reactive oxygen species (ROS) production are localized in the mitochondria [40,41]. However, an additional candidate is the electron-transfer chain of the CYP450 system found in the endoplasmic reticulum [42]. The CYP450 family can be a very important source of oxidative damage and, under specific circumstances, its activity plays a critical role for ROS-dependent toxicity of drugs such as cisplatin [43]. Natural flavonoids have been shown to modulate the CYP450 system [44], including the induction of specific CYP isoenzymes, and the activation or inhibition of these enzymes.

In our experimental conditions we cannot exclude a 6PP interaction with the CYP450 system. Indeed, as shown in this paper, 6PP exhibited dose-dependent antiradical and antioxidant activities, including an effect on microsomal lipid peroxidation. Evidence for the ability of 6PP to scavenge free radicals was provided by the DPPH assay and probably by the inhibition

of microsomal lipid peroxidation. The phosphomolybdenum reduction assay demonstrated the antioxidant power of 6PP. The introduction of a prenyl group into a flavonoid structure, as is the case for 6PP, usually increases its hydrophobicity, which would be expected to enhance affinity for lipophilic membranes and the related pharmacological and biochemical properties [11]. Since incorporation of chemical compounds into the cell is a function of their lipophilicity, the antioxidant and/or toxic effects of 6PP could be dictated by the chemical structure of this compound and subsequently its orientation in biomembranes. Flavonoids and their metabolites have one-electron reduction potentials lower than those of highly oxidising reactive oxygen species (ROS) [45], thus they are capable to reduce these species. We propose that this property contributes to the *in vitro* free radical-scavenging effect of 6PP. In addition, like other flavonoids [46], the prenylated flavonoid 6PP contains phenolic hydroxyl groups that might likely act as hydrogen donors in the scavenging of free radicals. In addition to these potentially protective effects, 6PP, within the same concentration range, also inhibited and/or uncoupled mitochondrial respiration. This effect is consistent with previous reports for other flavonoids [47]. Since flavonoids are weak, hydrophobic acids, it is likely that they are potentially capable of causing mitochondrial uncoupling [47–49], although the structural features associated with this property have not yet been established. With MG as substrate, 6PP stimulated mitochondrial state 4 respiration (indicating that it uncouples mitochondria) and inhibited state 3, suggesting that 6PP would also affect the mitochondrial respiratory chain. On the other hand, addition of 6PP inhibited state 3 respiration of succinate-energized mitochondria, indicating an interference either in the respiratory chain or in the ATP-synthase. In this condition, state 4 was inhibited only by 100 μM 6PP. A similar finding has been reported for quercetin [49]. Parallel to the inhibitory effects of 6PP on the mitochondrial electron transport chain and/or uncoupling of oxidative phosphorylation, an inhibition of mitochondrial membrane potential was observed. Unproductive electron transport results in damage to the mitochondria, thus impairing their capacity to meet cellular energy requirements.

We explored some of the possible targets for the effect of 6PP on electron transfer. These targets could be SDH, and NADH oxidase, among other enzymes associated with the electron transport chain. SDH also contains a number of cystein-rich sulfur clusters and can be inhibited by a number of agents that modify sulfhydryl groups. 6PP might have directly interacted with sulfhydryl groups on SDH and/or NADH oxidase resulting in the decline of their activity and thereby limiting the ability of the mitochondria to meet the energy demands of the cell and disrupting cellular energy homeostasis [50]. In fact, 6PP inhibited SDH and NADH oxidase activities, which in turn would decrease respiration rate and consequently transmembrane potential. Another candidate was F0F1-ATPase/ATP synthase (Complex V), an enzyme present on the inner membrane of the mitochondria that uses the energy of the proton gradient across the membrane to synthesize ATP. In certain conditions, it can also act in the reverse direction, hydrolysing ATP and pumping protons. The inhibition by 6PP of F0F1-ATPase in both mitochondria and submitochondrial particles suggests a direct inhibitory effect on ATPase enzyme subunits. Similar results have been reported for other phytochemical agents [51,52]. 6PP showed relatively low inhibitory ATPase activity (IC_{50} close to 100 μM) when compared to reported values for other flavonoids, such as piceatannol (8 μM) and quercetin (50 μM) [52]. Nevertheless, the inhibitory effect of 6PP on F0F1-ATPase we observed could in part contribute to the alterations we found in the respiration of isolated mitochondria. Inhibition of SDH, NADH-oxidase and ATPase activities in mitochondria by 6PP would explain the decreased flux of electrons.

On whole cells, within the same range of concentration, 6PP decreased HEp-2 cell viability in several assays, including one (MTT) that depends on mitochondrial function (namely, SDH activity), suggesting that at least the effects on mitochondria can negatively affect cell survival. Flavonoids like sanggenol and sanggenons have also shown cytotoxicity against other human tumour cell lines [53]. The location of the prenyl group can be relevant to biological activities of prenylflavonoids. 6PP is prenylated at C6 and C5′, showing cytotoxicity as well as antiradical/antioxidant activity. By contrast, flavonoids prenylated at C8, such as 8-prenylapigenin and 8-prenylliquiritigenin, though they exert cytotoxicity on rat H4IIE hepatoma and C6 glioma cells, failed to exhibit radical scavenging activity in the DPPH assay [10].

Alike other lipophilic compounds, 6PP is supposed to enter cell by freely diffusing through the cell membranes. As for other drugs the lipophilic character of 6PP also impacts on *in vivo* pharmacokinetics, determining usually a high fraction of the compound to be bound to plasma proteins, such as albumin, with a paralell reduction in the concentration of the free compound which is supposed to be the molecular species that diffuses. Interestingly, the cytotoxic effect of 6PP was markedly decreased when albumin was present in the medium, probably reflecting the lowering of free 6PP

concentration as a result of albumin binding capacity. While preliminary, this observation may have profound consequences for the *in vivo* effect of 6PP. It is noteworthy that, in previous studies, DL50 of oral 6PP was found to be 350 mg/kg of body weight in male mice and 250 mg/kg of body weight in females.[1] Since this effect can also place a limit on the use of 6PP, related to systemic toxicity, further studies are required to determine whether the cytotoxic effect is also found against normal human cells or preferentially involves cancer cells, such as the HEp-2 cell line. A study in this line could orient the selection of models to further develop 6PP as a potential pharmaceutical. Interestingly, several members of the prenylated flavonoid family are important inhibitors of multidrug resistance proteins, which are largely responsible for the failure of anticancer chemotherapy [54–56]. Plasma membrane ATP-binding cassette transporters mediate cancer cell resistance to chemotherapy, and are inhibited by several prenylated flavonoids, which bind with high affinity to these transporters and inhibit both drug export and nucleotide hydrolysis. Structure–activity relationship studies have shown that prenylation is a key chemical modification that underlies the inhibitory activity of these compounds [57].

The alterations observed in isolated mitochondria and submitochondrial particles provide evidence that 6PP is able to uncouple oxidative phosphorylation and to inhibit enzymatic activities linked to the respiratory chain. These results indicate a potentially toxic action of 6PP. Up to present, 6PP has been subjected to limited exploration, since available quantities are small. Thus, *in vivo* data are scarce and include the already mentioned DL50 study and a preliminary testing against *Toxoplasma gondii* in the NMRI strain of mice, with gavage administration of 6PP (35 mg/(kg d)) without finding evidence of drug toxicity (Pérez et al., manuscript in preparation). Moreover, according to our knowledge, no folk use of *D. elegans* or its extracts has been reported. If 6PP development progresses, further testing for antioxidant/prooxidant activity should be required, including reactive oxygen species production and *in vivo* well-characterized methods such as total radical-trapping antioxidant parameter (TRAP).

In summary, we have partially characterized the activity of the prenylated flavonoid 6PP, demonstrating its toxic effects on isolated rat liver mitochondria and human tumour cells, as well as anti-oxidant and antiradical activity. Whether these effects are mainly a shortcoming for its potential use or the beginning of its development as a chemotherapeutic agent will require additional studies.

[1] Pérez et al., Evaluación de la letalidad y otros efectos tóxicos agudos de un flavonoide prenilado obtenido de *D. elegans*, XXXVI Reunión Anual de la Sociedad Argentina de Investigación Odontológica, San Luis (Argentina), 2003.

Acknowledgements

We are indebted to G. Ortega, PhD, and M. Peralta, PhD, for isolation of 6PP, Prof. J. Cannata for helpful discussion, and Prof. S. Gallego for allowing us to use the spectrofluorometer. MD and JLC are members of the Research Career of CONICET (Argentina's National Research Council). IE and MPI received partial support from Fundación CONAMED. This research received financial support from FONCYT PICT 2002, expte. 12642 (JLC) and CONICET PIP 6100 (MD).

References

[1] J.B. Harbone, Nature, distribution and function of plant flavonoids, in: B. Cody, E. Middleton, J.B. Harbone (Eds.), Plant Flavonoids in Biology and Medicine, Alan Liss, NY, 1986, pp. 15–24.
[2] G. Block, A role for antioxidants in reducing cancer risk, Nutr. Rev. 50 (1992) 207–213.
[3] C. Yuting, Z. Rongliang, J. Zhong Jian, J. Yong, Flavonoids as superoxide scavengers and antioxidants, Free Radical Biol. Med. 9 (1990) 19–21.
[4] C.A. Rice-Evans, N.J. Miller, G. Paganga, Structure–antioxidant activity relationships of flavonoids and phenolic acids, Free Radical Biol. Med. 20 (1996) 933–956.
[5] Y. Hanasaki, S. Ogawa, S. Fukui, The correlation between active oxygens scavenging and antioxidative effects of flavonoids, Free Radical Biol. Med. 16 (1994) 845–850.
[6] D. Boveris, S. Puntarulo, Free radical scavenging actions of natural antioxidants, Nutr. Res. 18 (1998) 1545–1557.
[7] P. Pietta, P. Simonetti, C. Gardana, A. Brusamolino, P. Morazzoni, E. Bombardelli, Relationship between rate and extent of catechin absorption and plasma antioxidant status, Biochem. Mol. Biol. Int. 46 (1998) 895–903.
[8] J. Duarte, F. Pérez-Vizcainom, P. Utrilla, J. Jimenez, J. Tanargo, A. Zarzuelo, Vasodilatory effects of flavonoids in rat aortic smooth muscle. Structure–activity relationships, Gen. Pharmacol. 24 (1993) 857–862.
[9] J.J. Lee, H.C. Chen, Flavonoid-induced acute nephropathy by *Cupressus funebris* Endl (Mourning Cypress), Am. J. Kidney Dis. 48 (2006) 81–85.
[10] W. Watjen, N. Weber, Y.J. Lou, Z.Q. Wang, Y. Chovoulou, A. Kamkotter, R. Kahl, P. Proksch, Prenylation enhances cytotoxicity of apigenin and liquiritigenin in rat H4HE hepatoma and C6 glioma cells, Food Chem. Toxicol. 45 (2007) 119–124.
[11] B. Botta, A. Vitali, P. Menendez, D. Misiti, G. Delle Monache, Prenylated flavonoids: pharmacology and biotechnology, Curr. Med. Chem. 12 (2005) 713–739.
[12] C. Perez, L.N. Tiraboschi, M.G. Ortega, A.M. Agnese, J.L. Cabrera, Further antimicrobial studies of 2′-4′-dihidroxy-5′-(1‴-

dimethylallyl)-6-prenylpinocembrin from *Dalea elegans*, Pharm. Biol. 41 (2003) 171–174.
[13] K. Ulanowska, A. Tkaczyk, G. Konopa, G. Węgrzyn, Differential antibacterial activity of genistein arising from global inhibition of DNA, RNA and protein synthesis in some bacterial strains, Arch. Microbiol. 184 (2006) 271–278.
[14] M.D. Navarro-Martínez, E. Navarro-Perán, J. Cabezas-Herrera, J. Ruiz-Gómez, F. García-Cánovas, J.N. Rodríguez-López, Antifolate activity of epigallocatechin gallate against *Stenotrophomonas maltophilia*, Antimicrob. Agents Chemother. 49 (2005) 2914–2920.
[15] S.F. Nielsen, M. Larsen, T. Boesen, K. SchØnning, H. Kromann, Cationic chalconi antibiotics. Design, synthesis, and mechanism of action, J. Med. Chem. 48 (2005) 2667–2677.
[16] T.P. Cushnie, A.J. Lamb, Antimicrobial activity of flavonoides, Int. J. Antimicrob. Agents 27 (2005) 343–356.
[17] C.L. Miranda, J.F. Stevens, V. Ivanov, M. McCall, B. Frei, M.L. Deinzer, D.R. Buhler, Antioxidant and prooxidant actions of prenylated and nonprenylated chalcones and flavonones *in vitro*, J. Agric. Food Chem. 48 (2000) 3876–3884.
[18] M. Caffaratti, M.G. Ortega, M.E. Scarafia, L. Ariza-Espinar, H.R. Juliani, Prenylated flavanones from *Dalea elegans*, Phytochemistry 36 (1994) 1083–1084.
[19] V. Vijaya Padma, S. Arul Diana Christie, K.M. Ramkuma, Induction of apoptosis by ginger in HEp-2 cell line is mediated by reactive oxygen species, basic, Clin. Pharmacol. Toxicol. 100 (2007) 302–307.
[20] Y.F. Yilmaz, F. Akbiyik, U. Tuncel, A. Unal, Lipid peroxidation and antioxidant levels in patients with laryngeal carcinoma, Kulak Burun Bogaz Ihtis Derg. 17 (2007) 81–84.
[21] M. Dubin, P.H. Carrizo, A.M. Biscardi, S.H. Fernández Villamil, A.O.M. Stoppani, Effect of 5-nitroindole on adenylate energy charge, oxidative phosphorylation, and lipid peroxidation in rat hepatocytes, Biochem. Pharmacol. 48 (1994) 1483–1492.
[22] N. de Witte, A.O.M. Stoppani, M. Dubin, 2-Phenyl-β-lapachone can affect mitochondrial function by redox cycling mediated oxidation, Arch. Biochem. Biophys. 432 (2004) 129–135.
[23] M. Dubin, S.H. Fernandez Villamil, A.O.M. Stoppani, Inhibition of microsomal lipid peroxidation and cytochrome P-450-catalyzed reactions by β-lapachone and related naphthoquinones, Biochem. Pharmacol. 39 (1990) 1151–1160.
[24] S. Fernandez Villamil, A.O.M. Stoppani, M. Dubin, Redox Cycling of β-lapachone and structural analogues in microsomal and cytosol liver preparations, Meth. Enzymol. 378 (2004) 47–67.
[25] R.W. Estabrook, Mitochondrial respiratory control and the polarographic measurement of ADP: O ratios, Meth. Enzymol. 10 (1967) 41–47.
[26] C.H. Fiske, Y. Subbarow, The colorimetric determination of phosphorus, J. Biol. Chem. 66 (1925) 375–400.
[27] C. Veeger, D.V. DerVartanian, W.P. Zeylemaker, Succinate dehydrogenase, Meth. Enzymol. 13 (1969) 81–90.
[28] R.K. Emaus, R. Grunwald, J.J. Lemasters, Rhodamine 123 as a probe of transmembrane potential in isolated rat-liver mitochondria: spectral and metabolic properties, Biochim. Biophys. Acta 850 (1986) 436–448.
[29] R.L.P. Adams, Cell culture for biochemists, in: R.H. Burdon, P.H. van Knippenberg (Eds.), Laboratory Techniques in Biochemistry and Molecular Biology, Amsterdam, The Netherlands, 1990, pp. 1–95.
[30] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, J. Immunol. Meth. 65 (1983) 55–63.
[31] J.A. Buege, S.D. Aust, Microsomal lipid peroxidation, Meth. Enzymol. 52 (1978) 302–310.
[32] S. Burda, W. Oleszek, Antioxidant and antiradical activities of flavonoids, J. Agric. Food Chem. 49 (2001) 2774–2779.
[33] P. Prieto, M. Pineda, M. Aguilar, Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E, Anal. Biochem. 269 (1999) 337–341.
[34] L. Mathiesen, K.E. Malterud, R.E. Sund, Hidrogen bond formation as basis for radical scavenging activity: a structure–activity study of C-methylated dihydrochalcones from *Myrica gale* and structurally related acetophenones, Free Radical Biol. Med. 22 (1997) 307–311.
[35] S.D. Aust, B.A. Svingen, The role of iron in enzymatic lipid peroxidation, in: W.A. Pryor (Ed.), Free Radicals in Biology, Academic Press, New York, 1982, pp. 1–28.
[36] T.C. Pederson, J.A. Buege, S.D. Aust, Microsomal electron transport, J. Biol. Chem. 248 (1973) 7134–7141.
[37] K. Briviba, H. Sies, Nonenzymatic antioxidant defense systems, in: B. Frei (Ed.), Natural Antioxidants in Human Health and Disease, Academic Press, San Diego, CA, 1994, pp. 107–128.
[38] W. Bors, C. Michel, K. Stematter, Antioxidant effects of flavonoids, Biofactors 6 (1997) 399–402.
[39] E. Joubert, P. Winterton, T.J. Britz, W.C.A. Gelderblom, Antioxidant and pro-oxidant activities of aqueous extracts and crude polyphenolic fractions of Rooibos (*Aspalathus linearis*), J. Agric. Food Chem. 53 (2005) 10260–10267.
[40] B. Chance, H. Sies, A. Boveris, Hydroperoxide metabolism in mammalian organs, Physiol. Rev. 59 (1979) 527–605.
[41] A. Boveris, E. Cadenas, Mitochondrial production of superoxide anions and its relationship to the antimycin insensitive respiration, FEBS Lett. 54 (1975) 311–314.
[42] S.C. Bondy, S. Naderi, Contribution of hepatic cytochrome P450 systems to the generation of reactive oxygen species, Biochem. Pharmacol. 48 (1994) 155–159.
[43] H. Liu, R. Baliga, Cytochrome P450 2E1 null mice provide novel protection against cisplatin-induced nephrotoxicity and apoptosis, Kidney Int. 63 (2003) 1687–1696.
[44] A.W. Wood, D.S. Smith, R.L. Chang, M.T. Huang, A.H. Conney, Effects of flavonoids on the metabolism of xenobiotics, Prog. Clin. Biol. Res. 213 (1986) 195–210.
[45] S.V. Jovanovic, S. Stecnken, M. Tosic, B. Marjanovic, M.G. Simic, Flavonoids as antioxidants, J. Am. Chem. Soc. 116 (1994) 4846–4851.
[46] J. Neuzil, S.R. Thomas, R. Stocker, Requirement for, promotion, or inhibition by alpha-tocopherol of radical-induced initiation of plasma lipoprotein lipid peroxidation, Free Radical Biol. Med. 22 (1997) 57–71.
[47] P. Ravanel, Uncoupling activity of a series of flavones and flavonols on isolated plant mitochondria, Phytochemistry 25 (1986) 1015–1020.
[48] C. van Dijk, A.J.M. Driessen, D. Recourt, The uncoupling efficency and affinity of flavonoids for vesicles, Biochem. Pharmacol. 60 (2000) 1593–1600.
[49] D.J. Dorta, A.A. Pigoso, F.E. Mingatto, T. Rodríguez, I.M.R. Prado, A.F.C. Helena, S.A. Uyemura, A.C. Santos, C. Curti, The interaction of flavonoids with mitochondria: effects on energetic processes, Chem. -Biol. Interact. 152 (2005) 67–78.
[50] K. Vijayavel, C. Anbuselvam, M.P. Balasubramanian, Free radical scavenging activity of the marine mangrove *Rhizophora apiculata* bark extract with reference to naphthalene induced mitochondrial dysfunction, Chem. -Biol. Interact. 163 (2006) 170–175.

[51] J. Zheng, V.D. Ramirez, Inhibition of mitochondrial proton F0F1-ATPase/ATP synthase by polyphenolic phytochemicals, Br. J. Pharmacol. 130 (2000) 1115–1123.
[52] J. Zheng, V.D. Ramirez, Piceatannol, a stilbene phytochemical, inhibits mitochondrial F0F1-ATPase activity by targeting the F1 complex, Biochem. Biophys. Res. Commun. 261 (1999) 499–503.
[53] Y.Q. Shi, T. Fukai, H. Sakagami, W.J. Chang, P.Q. Yang, F.P. Wang, T. Nomura, Cytotoxic flavonoids with isoprenoid groups from *Morus mongolica*, J. Nat. Prod. 64 (2001) 181–188.
[54] A. Ahmed-Belkacem, A. Pozza, F. Muñoz-Martinez, S.E. Bates, S. Castanys, F. Gamarro, A. Di Pietro, J.M. Pérez-Victorica, Flavonoid structure–activity studies identify 6-prenylchrysin and tectochrysin as potent and specific inhibitors of breast cancer resistance protein ABCG2, Cancer Res. 65 (2005) 4852–4860.
[55] F. Cerqueira, A. Cordeiro-Da-Silva, N. Araújo, H. Cidade, A. Kijjoa, M.S.J. Nascimento, Inhibition of lymphocyte proliferation by prenylated flavones: artelastin as a potent inhibitor, Life Sci. 73 (2003) 2321–2334.
[56] M. Bobrowska-Hagerstrand, A. Wrobel, L. Mrowczynska, T. Soderstrom, Y. Shirataki, N. Motohashi, J. Molnar, K. Michalak, H. Hagerstrand, Flavonoids as inhibitors of MRP1-like efflux activity in human erythrocytes. A structure–activity relationship study, Oncol. Res. 13 (2003) 463–469.
[57] B. Botta, G. Delle Monache, P. Menendez, A. Boffi, Novel prenyltransferase enzymes as a tool for flavonoid prenylation, Trends Pharmacol. Sci. 26 (2005) 606–608.