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Hispidulin: Antioxidant properties and effect on mitochondrial energy metabolism†

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Abstract
Hispidulin (6-methoxy-5,7,4′-trihydroxyflavone) and eupafolin (6-methoxy-5,7,3′,4′-tetrahydroxyflavone), are flavonoids found in the leaves of *Eupatorium litoralle*. They have recognized antioxidant and antineoplastic properties, although their action mechanisms have not been previously described. We now report the effects of hispidulin on the oxidative metabolism of isolated rat liver mitochondria (Mit) and have also investigated the prooxidant and antioxidant capacity of both flavonoids. Hispidulin (0.05–0.2 mM) decreased the respiratory rate in state III and stimulated it in state IV, when glutamate or succinate was used as oxidizable substrate. Hispidulin inhibited enzymatic activities between complexes I and III of the respiratory chain. In broken Mit hispidulin (0.2 mM) slightly inhibited ATPase activity (25%). However, when intact Mit were used, the flavonoid stimulated this activity by 100%. Substrate energized mitochondrial swelling was markedly inhibited by hispidulin. Both hispidulin and eupafolin were able to promote iron release from ferritin, this effect being more accentuated with eupafolin with the suggestion of a possible involvement of H_2O_2 in the process. Hispidulin was incapable of donating electrons to the stable free radical DPPH, while eupafolin reacted with it in a similar way to ascorbic acid. The results indicate that hispidulin as an uncoupler of oxidative phosphorylation, is able to release iron from ferritin, but has distinct prooxidant and antioxidant properties when compared to eupafolin.

Keywords: *Flavone, hispidulin, eupafolin, mitochondrial respiratory chain, mitochondrial swelling, free radicals*

Abbreviations: *BSA, bovine serum albumin; ATP, adenosine 5′ triphosphate; ADP, adenosine 5′diphosphate; DCPIP, 2,6-dichloroindophenol, sodium salt; DMSO, dimethylsulfoxide; DPPH, 1,1-diphenyl-2-picrylhydrazyl radical; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis[β-aminoethyl ether]-N, N, N′, N′-tetraacetic acid; ROS, reactive oxygen species; FCCP, p-trifluoro-methoxycarbonylcyanide phenylhydrazone; Glut, glutamate; HEPES, (N-2-hydroxyethyl) piperazine-N′-(2-ethanosulfonic acid); Hisp, hispidulin; Mit, mitochondria; NADH, nicotinamide adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate; PEP, phosphoenolpyruvate; PK, pyruvate kinase; PMS, phenazine methasulfate; RCC, respiratory control coefficient; Rot, rotenone; SOD, superoxide dismutase*

Introduction

Flavonoids are polyphenols that have innumerous biological effects, namely: antioxidant, mutagenic, carcinogenic, cardioprotector, anticarcinogenic, cytotoxic, antineoplastic, anti-inflammatory, among others [1,2]. Inhibition of a broad spectrum of enzymes, including human neutrophile NADPH-oxidase,

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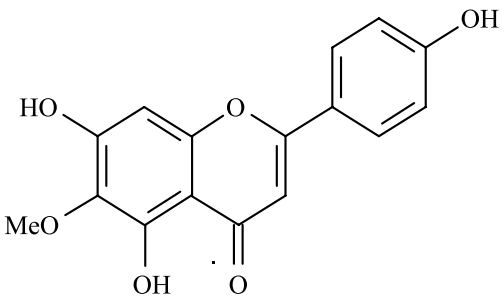
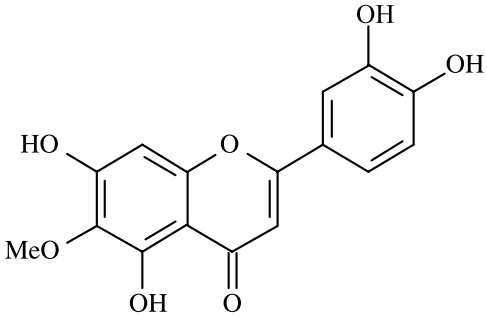
aldose reductase and several other oxido-reductases is also described [2]. Studies comparing the structure and activity of flavonoids that inhibit NADH oxidase and succinooxidase enzyme systems, showed that the C-2 to C-3 double bond and the C-4 keto and 3′, 4′ 5′ hydroxyl groups are important for inhibiting NADH oxidase [3]. The flavones hispidulin (5,7,4′-trihydroxy-6-methoxy-flavone) and eupafolin (5,7,3′, 4′-tetrahydroxy-6-methoxy-flavone) (Figure 1) are now studied. They are found in the leaves of *Eupatorium litoralle* and most of these required structural characteristics. For hispidulin, there have been reports of a definite antiatheromatous effect when orally administered to rats [4], an effective antitumor activity against mouse S180 and ascite hepatoma cell cultures [5], ability to inhibit the human platelet aggregation [6], hepatoprotection [7,8] and antineoplastic activity [9] among others. Eupafolin has antineoplastic and antioxidant activities [10–13]. Coleman et al. [14], using a single dose of hispidulin (50 μM), on a mitochondrial preparation, observed a mild respiratory inhibition of 14 and 10% with sodium glutamate or sodium succinate as substrates, respectively. However, other studies on mitochondrial functions were not carried out. Despite the innumerous effects reported for these flavonoids, their mechanisms of action have not yet been studied.



HISPIDULIN



EUPAFOLIN

Figure 1. Chemical structure of flavones.

Accordingly, detection of effects of flavonoids on the activities of enzymes of the respiratory chain are important and should contribute to the understanding of their mechanisms especially their cytotoxic effects.

Total antioxidant assays can also be used to compare the antioxidant activities of different flavonoids, the scavenging activity of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals being a valuable tool [15]. Since eupafolin has a catechol ring which has been indicated to be important for biological and antioxidant activity [13,16–19], we now compare antioxidant activity of eupafolin and hispidulin, using the DPPH assay.

It is important that, under some reaction conditions, the flavonoids can also have prooxidant effects, so that they cannot be classified simplistically as antioxidants [20]. Some flavonoids may contribute to reactive oxygen species (ROS) generation by an autoxidation process or stimulation of superoxide production in the respiratory chain. This would thus promote iron release of non-heme proteins and by the Fenton reaction give rise to increased amounts of free radicals, especially the hydroxyl radical, which damages mitochondria (Mit). Ferritin is the major protein that binds non-heme iron inside cells and the liberation of iron from ferritin has been used to compare the reducing properties of molecules and radicals [21].

The present study was thus undertaken to evaluate the effects of hispidulin on the oxidative metabolism of isolated rat liver Mit and its antioxidant and prooxidant properties when compared with those of eupafolin.

Materials and methods

Chemicals

Glutamic acid, succinic acid, NADH, ATP, ADP, EGTA, EDTA, FCCP, DPPH, rotenone, D-mannitol, sucrose, HEPES, BSA, phosphoenolpyruvate (PEP), pyruvate kinase, valinomycin, oligomycin, cytochrome *c*, bathophenanthroline sulfonate, horse spleen ferritin and Tris were products of Sigma® (St Louis, MO, USA). Potassium hydroxide, potassium chloride, chloroform, potassium dihydrogen phosphate, di-sodium hydrogen phosphate, hydrochloric acid, sulfuric acid, ammonium heptamolybdate and ferrous sulfate were products of Merck® (Brazil). Other reagents were of analytical grade. Solutions were prepared with distilled water and Millipore Milli Q®deionized water.

Animals

The animals used were male albino rats (Wistar strain; 220–300 g), which received a standard laboratory diet

(Purina®) and water *ad libitum*. All animals were starved for 12 h prior to being sacrificed. All recommendations of the Brazilian Law (no. 6.638.05 November 1979) for scientific management of animals were respected.

Preparation of hispidulin and eupafolin solutions

Hispidulin and eupafolin were extracted from leaves of *E. litoralle* and their structures confirmed as described [22]. The flavonoids were dissolved in DMSO and then further diluted with the assay medium. Hispidulin and eupafolin were added to the system at varying concentrations (0.05–0.2 mM). Solvent controls with DMSO were carried out in each assay. Flavone stock solutions were stored at 4°C and warmed to 25°C before use.

Preparation of rat liver mitochondria

Rat liver Mit were prepared according to the procedure of Voss et al. [23] with slight modifications. The extraction medium contained: 250 mM mannitol, 10 mM HEPES, pH 7.2, 1 mM EGTA and 0.1 g % BSA. For the polarographic and swelling experiments only mitochondrial preparations with RCC $\geq$ 4.0 were used. When evaluating the respiratory chain enzyme complex, the mitochondrial suspension was frozen in liquid N_2 and at the time of use each aliquot was disrupted by freeze-thawing treatments ($\times$ 3). During enzymatic assay the mitochondrial suspension was mantained at 4°C.

Oxygen uptake

Oxygen uptake and oxidative phosphorylation were carried out using a Clark type oxygen electrode (Yellow Springs Instruments Co.), with a Gilson Oxygraph and evaluated at 30°C in a 1.3 ml thermostatically controlled, water jacketed, closed chamber with magnetic stirring. The standard respiratory medium was used according to Braguini et al. [24]. The respiratory rate was expressed as nmol of oxygen consumed per minute per milligram of mitochondrial protein, according to Estabrook [25]. The ADP/O ratio was calculated according to Chance & Williams [26]. Respiratory control coefficient (RCC) values were obtained from the ratio between the rate of mitochondrial oxygen consumption in state III and that of in state IV.

Measurement of activities of enzymatic complexes of the respiratory chain

NADH oxidase and succinate oxidase were assayed polarographically. NADH dehydrogenase (NADH/ubiquinone oxidoreductase) and succinate dehydrogenase (succinate/ubiquinone oxidoreductase) activities were assayed spectrophotometrically, as described by Singer [27]. NADH-cytochrome *c* reductase (NADH: cytochrome *c* oxidoreductase) and succinate cytochrome *c* reductase (succinate/cytochrome *c* oxidoreductase) activities were measured as described by Somlo [28]. The activity of cytochrome *c* oxidase was determined at 550 nm, according to Mason et al. [29]. ATPase activity in disrupted and intact Mit was evaluated as described by Pulmann et al. [30], but with modifications. In disrupted Mit, the enzyme was assayed at 37°C, using a system consisting of 250 mM sucrose, 50 mM Tris–HCl, pH 8.4, 3.0 mM magnesium sulfate, 2.5 mM PEP, 10 U pyruvate kinase, 4 mM ATP and 100 μg mitochondrial protein. The ATPase activity of intact mitochondria was assayed at 30°C in the presence or absence of 1 μM FCCP. Liberated orthophosphate was measured according to Sumner [31] and results expressed as nmols of Pi liberated per min^{-1} mg of protein.

Mitochondrial swelling experiments

Swelling in energized (Mustafa et al. [32] and Sepalla et al. [33]) or de-energized Mit (Moreno & Madeira [34]) was measured at 546 nm using a HITACHI (mod U-2001 UV/VIS) spectrophotometer at 546 nm. The reactions were carried out at 28°C in 1 ml of each medium using a mitochondrial suspension (1 mg protein).

Determination of iron release from ferritin by hispidulin and eupafolin

Determination of iron release from ferritin was carried out as described by Oteiza et al. [35]. Iron release from 0.3 mg ml^{-1} ferritin by hispidulin and eupafolin was followed spectrophotometrically. The increase in absorbance due to the chelation of Fe^{++} by 1 mM bathophenanthroline sulfonate after 30 min (total volume: 1 ml), was measured at 530 nm ($\varepsilon_{530\,nm} = 22.140\,mM^{-1}\,cm^{-1}$). The reaction was initiated by the addition of flavonoids. Analysis was also carried out in the presence of the antioxidant enzymes, catalase (10 and 25 U ml^{-1}) and superoxide dismutase (40 and 80 U ml^{-1}).

Reactivity with the DPPH radical

The reactivity between hispidulin and the free radical DPPH was measured spectrophometrically at 517 nm, according to Blois [36]. The medium was supplemented with hispidilin or eupafolin, in

Table I. Effects of hispidulin on respiratory parameters using sodium glutamate as oxidizable substrate.

Hispidulin (mM)	State III (%)	State IV (%)	RCC (%)	ADP/O (%)
0	100	100	100	100
0.05	82.8 ± 9.7*	101.4 ± 11	84.26 ± 12.2*	120.5 ± 24.7
0.075	84.4 ± 5.7*	111.6 ± 10.5	78.0 ± 7.3*	102.5 ± 14.9
0.10	75.8 ± 7.9*	107.7 ± 9.8	69.8 ± 9.5*	101.4 ± 9.9
0.15	67.9 ± 8.4*	131.9 ± 9.4*	52.5 ± 9.5*	85.9 ± 12.1*
0.20	58.1 ± 9.1*	165.1 ± 3.1*	38.6 ± 7.4*	69.3 ± 16.1*

Conditions for oxygen uptake measurements are described in "Materials and methods" section. The reaction medium at final volume 1.3 ml and 28°C consisted of: 125 mM D-mannitol, 65 mM KCl, 10 mM HEPES buffer (pH 7.2), 0.1 mM EGTA, 0.1 g % BSA, 5 mM sodium glutamate, 0.8 mM KH_2PO_4, 0.16 mM ADP and 2 mg mitochondrial protein. Control (100%) corresponds to: 58.36 nmol of oxygen consumed $min^{-1} mg^{-1}$ protein in state III, 12.4 nmol of oxygen consumed $min^{-1} mg^{-1}$ protein in state IV, 3.17 nmol of oxygen consumed $min^{-1} mg^{-1}$ protein in ADP/O ratio and RCC 4.9. Each value represents the means ± SD of six different experiments was expressed as percentage of control. *Significantly different from control ($p \leq 0.05$).

concentrations that ranged from up to 200 μM, in order to determine its I_{50}. Ascorbic acid (0.025–0.2 mM) was used as a positive control.

Protein determination

Mitochondrial protein was assayed by the method of Lowry et al. [37], calibrated with bovine serum albumin.

Statistical analysis

Data are presented as mean ± SD. Statistical analysis of the data was carried out as analysis of variance and test Tukey for average comparison. Results were considered significant as a $p < 0.05$ level.

Results

Effects on oxygen consumption

Tables I and II show the effects of hispidulin on the respiratory parameters of functionally intact Mit, when glutamate (Table I) or succinate (Table II) were the oxidizable substrates. The analysis parameters were: (i) rates of mitochondrial oxygen consumption in the presence of ADP (state III), (ii) after ADP exhaustion (state IV), (iii) the RCC and (iv) the ADP/O ratio. Hispidulin inhibited oxygen consumption during state III in the presence of both substrates in all doses used. At 0.2 mM, the inhibition was ~42 and 26.5% when glutamate or succinate were the oxidizable substrates, respectively (Tables I and II). When glutamate was the substrate, the rate of oxygen consumption after ADP exhaustion (state IV) was stimulated by ~65 and ~31% when 0.2 and 0.15 mM of hispidulin were used, respectively. With succinate as substrate, this parameter was stimulated by ~47, ~55, ~53 and ~65% by 0.075, 0.10, 0.15 and 0.2 mM hispidulin, respectively during its oxidation. Hispidulin (0.2 mM) decreased the RCC value (~60%) and also the ADP/O ratio (~40%) for both substrates. These results suggest that hispidulin, besides acting as an uncoupler due to the stimulus of state IV, exerts inhibitory effects on enzymatic complexes of the respiratory chain, as visualized by the decreased rates of state III in the presence of this flavonoid.

Table II. Effects of hispidulin on respiratory parameters using sodium succinate as oxidizable substrate.

Hispidulin (mM)	State III (%)	State IV (%)	RCC (%)	ADP/O (%)
0	100	100	100	100
0.05	85.5 ± 5.8*	125.7 ± 9.4	67.4 ± 10.9*	93.8 ± 4.8
0.075	86.9 ± 6.9*	147.4 ± 11.6*	60.2 ± 5.5*	93.4 ± 4.9
0.10	86.1 ± 5.6*	155.9 ± 22.6*	63.2 ± 1.8*	82.2 ± 10.1*
0.15	74.8 ± 7.1*	153.4 ± 1.9*	54.5 ± 9.1*	77.7 ± 9.4*
0.20	73.5 ± 12.0*	165.5 ± 1.6*	41.4 ± 4.6*	60.4 ± 6.4*

Conditions for oxygen uptake measurements are described in "Materials and methods" section. The reaction medium at a final volume 1.3 ml and 28°C consisted of: 125 mM D-mannitol, 65 mM KCl, 10 mM HEPES buffer (pH 7.2), 0.1 mM EGTA and 0.1 g% BSA, supplemented with 3 mM potassium succinate, 0.8 mM KH_2PO_4, 0.08 mmol l^{-1} ADP, 1 μM rotenone and 0.5 mg mitochondrial protein. 100% (control) corresponds to: 135.4 nmol of oxygen consumed $min^{-1} mg^{-1}$ protein in state III, 31.84 nmol of oxygen consumed $min^{-1} mg^{-1}$ protein in state IV, 1.17 nmol of oxygen consumed $min^{-1} mg^{-1}$ protein in ADP/O ratio and RCC 4.3. Each value represents the media ± SD of six different experiments and was expressed as percentage of control. *Significantly different from control ($p \leq 0.05$).

Table III. Effects of hispidulin on enzymatic activities of the mitochondrial respiratory chain.

Hispidulin (mM)	NADH oxidase (%)	NADH cytochrome *c* reductase (%)	NADH dehydrogenase (%)	Succinate oxidase (%)	Succinate cytochrome *c* reductase (%)	Succinate dehydrogenase (%)	Cytochrome *c* oxidase (%)
0	100	100	100	100	100	100	100
0.05	90.0 ± 6.9*	86.5 ± 12.5*	89.5 ± 6.6*	98.3 ± 6.5	92.5 ± 7.4	101.5 ± 7	96.2 ± 4.4
0.075	77.5 ± 7.7*	82.7 ± 10.6*	88.4 ± 8.0*	96.4 ± 5.4*	93.9 ± 6.4	98.1 ± 3.4	93.2 ± 8.3
0.1	71.2 ± 7.5*	83.1 ± 13.3*	87.8 ± 6.8*	91.1 ± 4.6*	81.9 ± 8.4*	98.9 ± 6.7	97.1 ± 4.8
0.15	59.9 ± 5.0*	70.0 ± 9.1*	85.8 ± 8.2*	88.7 ± 3.0*	83.4 ± 7.8*	95.4 ± 4.8	98.5 ± 5.9
0.2	57.1 ± 3.8*	59.9 ± 5.9*	84.4 ± 7.9*	82.1 ± 4.9*	78.2 ± 8.3*	92.5 ± 7	96.8 ± 6.0

Experimental conditions are described in "Materials and methods" section. Control values (100%) were NADH oxidase: 22.2 nmol of O_2 consumed $min^{-1} mg^{-1}$ of mitochondrial protein; NADH cytochrome *c* reductase: 14.44 nmol of cytochrome *c* reduced $min^{-1} mg^{-1}$ of mitochondrial protein; NADH dehydrogenase: 220 μmol of ferricyanide reduced $min^{-1} mg^{-1}$ of mitochondrial protein; succinate oxidase: 20 nmol O_2 consumed $min^{-1} mg^{-1}$ of mitochondrial protein; succinate cytochrome *c* reductase: 40 nmol of cytochrome *c* reduced $min^{-1} mg^{-1}$ of mitochondrial protein; succinate dehydrogenase: 258 μmol of PMS reduced $min^{-1} mg^{-1}$ of mitochondrial protein; Cytochrome *c* oxidase: 145.36 μmol cytochrome *c* oxidized $min^{-1} mg^{-1}$ of mitochondrial protein. Results (mean ± SD of four independent experiments) are expressed as % of control activities. *Significantly different from control (100%), $p \leq 0.05$.

Hispidulin effects on enzymatic activities

In order to clarify possibility of effects of hispidulin on enzymatic complexes of the respiratory chain assays were carried out using broken Mit under experimental conditions, which allowed the evaluation of electron transport through respiratory chain complexes, independent of the membrane barrier to substrate and drugs, phosphorylation activity, or any other process dependent on the inner membrane electrical potencial ($\Delta\psi$). Table III shows that the presence of hispidulin ($0.2\ mmol\ l^{-1}$) in these systems significantly inhibited the activities of components of the respiratory chain, namely NADH oxidase (~43%), NADH cytochrome *c* reductase (~40%), NADH dehydrogenase (~15%), succinate oxidase (~18%), succinate cytochrome *c* reductase (~22%). The activities of succinate dehydrogenase and cytochrome *c* oxidase were not significantly affected by the presence of hispidulin. These results suggests that hispidulin acts between complexes I and III, decreasing the activities of the mitochondrial respiratory chain by ~40%.

Effects of hispidulin on ATPase activity

Having in mind, the stimulation of state IV caused by hispidulin, suggested its action as an uncoupler, the effects of the flavonoid on ATPase was investigated in order to confirm this possibility. Table IV shows the effect of hispidulin on the ATPase mitochondrial activity under three different experimental conditions, namely with disrupted, intact and FCCP-uncoupled Mit.

At all doses, hispidulin caused only a slight inhibition (20%) of ATPase activity when disrupted Mit were used. Since, under this experimental condition there is no integrity of the membrane, the flavonoid would have full access to the enzyme. The results therefore suggest that hispidulin does not exert direct drastic effects on ATPase. However, when evaluating the enzyme with intact Mit, hispidulin promoted an accentuated stimulus, which was dose-dependent. In this sense, it is widely recognized that uncouplers increase the membrane permeability to protons, causing a decrease in the proton motive force (Δp). This causes both increased rates of oxygen consumption during substrate oxidation in the absence of ADP (state IV) and ATP hydrolysis by ATPase of intact Mit, these effects occurring as an attempt to restore the Δp [38]. Therefore, our observation confirms hispidulin as an uncoupling agent [38].

Must to be emphasized that hispidulin did not promote alterations in ATPase activity when intact Mit were incubated in the presence of the classic uncoupler FCCP (Table III). The absence of effect on

Table IV. Effects of hispidulin on ATPase activities.

Hispidulin (mM)	Disrupted Mit (%)	Intact Mit (%)	Uncoupled Mit (%)
0	100	100	100
0.05	82.5 ± 8.1*	144.4 ± 8.6*	97.1 ± 7.9
0.075	79.6 ± 8.7*	171.3 ± 18.5*	99.0 ± 2.6
0.1	88.6 ± 9.6*	167.8 ± 18.6*	99.0 ± 4.9
0.15	77.5 ± 7.7*	181.5 ± 19.9*	99.6 ± 6.0
0.2	74.9 ± 7.5*	207.23 ± 22.2*	95.46 ± 7.9

Experimental conditions are described in "Materials and methods" section. Control values (100%) correspond to: 466.87 ± 50 nmol Pi liberated to disrupted Mit, 22.17 ± 6.3 nmol Pi liberated to intact Mit and 71.14 ± 4 nmol Pi liberated to uncoupled Mit. Results (means ± SD of four independent experiments in triplicate) are expressed as percentage of control activities. *Inhibition considered statisticately significant ($p \leq 0.05$).

ATPase activity in the presence of FCCP could suggest that hispidulin did not exert additional effects to another uncoupler. The result suggest that hispidulin not affected the ADP^{+3}/ATP^{+4} transporter, considering that when in such an experimental condition (intact Mit) the ADP^{+3}/ATP^{+4} transporter is still functional, the flavonoid does not affect its activity.

Effects of hispidulin on mitochondrial swelling

The actions of hispidulin on energy-linked functions of isolated Mit motivated us to investigate its effects on osmotic volume changes of liver Mit, to provide further information on its possible effects on the mitochondrial membrane. In terms of matrix volume changes occurring in respiring Mit, one has to consider the contribution of the protons pumped across the membrane in the respiratory chain. Respiration-dependent swelling occurs in the presence of an electrically permeant cation, which is accumulated due to the membrane potential [38]. Thus, an increase in the matrix volume, owing to the influx of a permeable solute, results in a decrease in light scattering as the refractive index of the matrix approaches that of the medium during the swelling process.

Trace A of Figure 2 shows a typical mitochondrial swelling experiment, where the addition of glutamate to Mit, incubated in a medium containing sodium acetate, gave rise to accentuated swelling, as observed by a decrease in absorbance. The addition of rotenone promoted shrinkage of the organelles, which occurs as a result of inhibition of electron transfer, reinforcing the concept that swelling is dependent on electron transfer through NADH dehydrogenase. Traces C–F which were obtained in the presence of varying concentrations of hispidulin, shows that it, at all concentrations tested (Figure 2), strongly decreases swelling of the organelle. Both the initial velocity and total amplitude were affected. With 0.2 mM hispidulin, the decrease was ~90% for both parameters. Since, under these conditions the swelling is dependent on the substrate oxidation, the inhibition of mitochondrial swelling by hispidulin can be explained by the effects of flavonoids on enzymatic complexes of the respiratory chain, as demonstrated in Table III.

Change in the mitochondrial volume has also been observed due to K^+ ion flux across the inner membrane [39], in the presence of the mobile carrier valinomycin. Therefore, swelling driven by the K^+ diffusion potential can be evaluated in intact mitochondrial preparations, where neither the respiratory chain nor ATP synthase was functional due to the absence of substrates. Under this condition, hispidulin did not promote alterations in the swelling parameters.

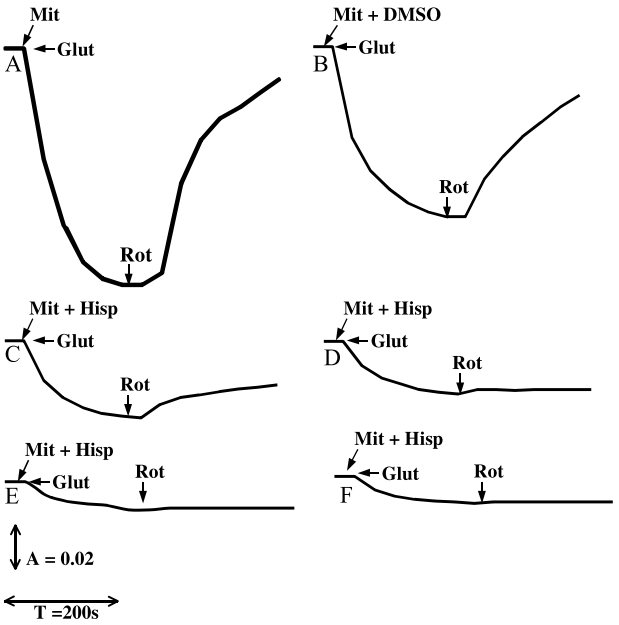


Figure 2. Effects of hispidulin on the amplitude and rate of swelling of liver Mit energized with glutamate. Mit (3 mg) were suspended in a medium containing 100 mM sucrose, 30 mM Tris–HCl, pH 7.2, 0.5 mM EDTA, 50 mM sodium acetate and hispidulin AT the following concentratrions: (A) none (control); (B) control plus 20 μl DMSO; (C) 0.05 mM (D) 0.1 mM; (E) 0.15 mM and (F) 0.2 mM. Swelling was induced by addition of 15 mM glutamate (Glut) and shrinkage by 4 $\mu mol\,l^{-1}$ rotenone (Rot), as indicated, in a final volume of 3 ml. Traces are representative of recordings of five independent experiments. Swelling is indicated by a downward, and shrinkage by upward deflection of the spectral trace.

These effects on mitochondrial swelling, in the presence and absence of the oxidizable substrate, may be interpreted partly as a consequence of inhibition of the enzymatic activities of the mitochondrial respiratory chain, as previously demonstrated (Table III).

Effects of hispidulin and eupafolin on iron release from ferritin

Ferritin is an iron storage protein that contributes to protect the cell against toxicity by ROS, because the iron within ferritin is not available to catalyze free radical reactions. This iron is readily liberated when necessary [40]. The Fe^{3+} liberated from ferritin can be reduced by several radicals such as $O_2^{\bullet -}$ and semiquinone, and released as Fe^{2+}, and thus promotes oxidative injury, leading to lipid peroxidation and oxidation of DNA and protein damage, and possibly playing a role in iron-induced carcinogenesis [41,42]. Considering the protective role of flavonoids in such a process, an evaluation of hispidulin on iron liberation from ferrritin is important. In these experiments, another flavonoid structurally distinct in only one OH position, eupafolin (see Figure 1) was included for comparison.

Tables V and VI show that both hispidulin and eupafolin significantly stimulate release of iron from ferritin at all concentrations used, this effect being more accentuated with eupafolin. Such results corroborate the proposition that the catechol ring is important in the liberation of iron [43].

In order to evaluate if ROS were involved in this process, experiments were carried out in the presence of different concentrations of the antioxidant enzymes catalase or SOD. With hispidulin (0.2 mM), the iron release did not significantly decrease in the presence of catalase (10 and 25 U) and SOD (40 and 80 U) (data not shown). With eupafolin (0.2 mM), when catalase (25 U) was present, a drastic decrease (~75%) occurred of mobilized iron (Table VII). With catalase (10 U), the iron release decreased by ~13%, when compared to that promoted by eupafolin in the absence of the antioxidant enzyme. When SOD (40–80 U) was added in the presence of eupafolin no significant difference was observed when compared with the iron released by the action of eupafolin in the absence of SOD (data not shown).

These results suggest that for eupafolin, but not for hispidulin, iron release from ferritin is a process associated with H_2O_2 production that could be generated as a consequence of auto-oxidation of the former. This kind of reaction commonly occurs in molecules that have a catechol ring in the presence of transition metals, such as iron [44].

Reactivity with the DPPH radical

The possibility that hispidulin and eupafolin could act as a radical scavenger was evaluated by DPPH radical reduction. DPPH is a stable free radical which can accept an electron or hydrogen radical, converting it into a stable, diamagnetic molecule [36]. Ascorbic acid, a known antioxidant, was used in this experiment as a positive control. Hispidulin did not react with DPPH at concentrations of up to 0.2 mM, demonstrating that it is not able to donate electrons or act as a radical scavenger. However, eupafolin strongly reacts with the DPPH radical, as does ascorbic acid, which has a strong reducting power. With eupafolin and ascorbic acid (0.2 mM), the absorbance values decreased by ~90% due to reduction of DPPH, confirming the importance of the catechol ring in eupafolin, which is absent of hispidulin. This is in accord with Ohkawa et al. [45,46], who observed no reactivity with the DPPH radical for isovitexin, a flavonoid structurally similar to hispidulin (with an identical hydroxylation pattern, but containing a glucose molecule at C-6 of ring A), with DPPH at concentrations up to 1 mM.

Table V. Effect of hispidulin on iron released from ferritin.

Hispidulin (mM)	Iron released (μM)
0	0.48 ± 0.07
0.05	0.99 ± 0.16*
0.075	1.04 ± 0.11*
0.1	1.15 ± 0.11*
0.15	1.18 ± 0.14*
0.2	1.26 ± 0.16*

Reaction mixture: 20 mM Tris–HCl buffer, 140 mM NaCl, pH 7.3, 1 mmoll^{-1} bathophenantrolin sulfonate, 300 μg ferritin and hispidulin in indicated concentrations in a final volume of 1 ml. Results were obtained by determination of formation of the Fe^{+2}/batophenantrolin complex ($\varepsilon_{530\,nm} = 22.14\,cm^{-1}\,mM^{-1}$) at 530 nm. Results are expressed as means ± SD of four independent assays at 28°C and expressed as released iron (μM) in 30 min. *Significantly different at $p \leq 0.05$.

Table VI. Effect of eupafolin on iron released from ferritin.

Eupafolin (mM)	Iron released (μM)
0	0.57 ± 0.13
0.05	2.86 ± 0.64*
0.075	2.8 ± 0.71*
0.1	2.87 ± 0.80*
0.15	3.12 ± 0.70*
0.2	3.23 ± 0.82*

Reaction mixture: 20 mM Tris–HCl buffer, NaCl 140 mM, pH 7.3, bathophenantrolin sulphonate 1 mmoll^{-1}, ferritin 300 μg and eupafolin at indicated concentrations in final volume 1 ml. Results were obtained by the determination of the Fe^{+2}/batophenantrolin complex ($\varepsilon_{530\,nm} = 22.14\,cm^{-1}\,mM^{-1}$) at 530 nm. Results are expressed as means ± SD of four independent assays at 28°C and expressed as iron released (μM) in 30 min. *Significantly different at $p \leq 0.05$.

Discussion

The effects of hispidulin on Mit are complex with a wide spectrum of responses. In addition, hispidulin

Table VII. Effect of hispidulin and eupafolin on iron released from ferritin in the presence of catalase.

	Iron released (μM)
Control	0.54 ± 0.16
Eupafolin (0.2 mM)	2.69 ± 0.5*
Eupafolin (0.2 mM) plus Catalase 10 U	2.33 ± 0.13*
Eupafolin (0.2 mM) plus Catalase 25 U	0.65 ± 0.05*

Reaction mixture: Tris–HCl buffer 20 mM, NaCl 140 mM, pH 7.3, bathophenantrolin sulfonate 1 mmoll^{-1}, ferritin 300 μg and eupafolin or eupafolin plus catalase at indicated concentrations in final a volume of 1 ml. Results were obtained by determination of the formation of the Fe^{+2}/batophenantrolin complex ($\varepsilon_{530\,nm} = 22.14\,cm^{-1}\,mM^{-1}$) at 530 nm. Results are expressed as means ± SD of four independent assays at 28°C and expressed as iron released (μM) in 30 min. *Significantly different at $p \leq 0.05$.

satisfies the criteria for an uncoupling agent as deduced by stimulus of both oxygen consumption during state IV and ATPase in intact Mit (Tables I, II and IV) and also acting as an electron transport inhibitor, as deduced by the decreased rate of oxygen consumption in state III and inhibition of enzymatic activities in the mitochondrial respiratory chain (Table III) and stimulating of ATPase activity when intact Mit were used (Table IV). The results on enzymatic activities indicated that the main hispidulin inhibition site is between complexes I and III (Table III).

These results are in agreement with those of Coleman et al [14] who used only a single dose of hispidulin (50 μM) and verified inhibition of state III of 14 and 10% when glutamate and succinate were used as substrate, respectively, and a decrease in the ADP/O ratio, suggesting a possible uncoupling effect. In addition, the present results confirm that the presence of the C-4 keto group and the C-2,3 double bond are structural features of flavonoids that are important for inhibition of NADH-oxidase [47]. Such structural characteristics are present in the flavones now investigated (Figure 1).

These effects on the mitochondrial function could play a role in the cytotoxicity of hispidulin observed against human carcinoma of the nasopharyn in cell culture [9], as suggested by Hodnick et al. [3].

Hispidulin is a flavonoid that has hydroxyl groups in the C-5 and C-7 position (Figure 1). Other flavonoids that have hydroxyl groups in positions C-5 and C-7, and whose effects as an uncoupler are recognized, are platanetin (3,5,7,8-tetrahydroxy-6-dimethylallylflavone), platanin (3,5,7,8-tetrahydroxy-6-methylflavone) e 3 hydroxywogonin (3,5,7-trihydroxy-8-methoxyflavone) [48]. Their uncoupling activity was explained using the scheme of Terada [49], but taking into account protein-binding effects, which can interfere with direct transmembrane proton transport. Ravanel [50] observed that apigenin (5,7,4′-trihydroxyflavone), which has a similar lipophilic and hydroxyl structure as hispidulin, was classified as a weak uncoupler.

Having in mind that some flavonoids such as quercetin (3,3′,4′,5,7 pentahydroxyflavone) are able to strongly inhibit F_1F_0 ATPase, this possibility was also evaluated for hispidulin. The effect of hispidulin differs from that caused by quercetin since in broken Mit all doses of hispidulin promoted only a slight decrease (20%) of ATPase activity [51]. In addition, as hispidulin did not affect ATPase activity when intact Mit treated with FCCP were used, the possibility of effects on the ADP/ATP translocator can be excluded.

The effects caused by hispidulin on parameters of mitochondrial swelling in the presence of substrate and sodium acetate were in agreement with the inhibition of the mitochondrial respiratory chain. In addition, the absence of effects on mitochondrial swelling induced by valinomycin indicates that a membrane parameter such as fluidity, which directly interferes in valinomycin transit through the membrane, is not affected by hispidulin.

Flavonoids, as polyphenols, have antioxidant activity arising from a free radical scavenging mechanism, with the formation of less reactive flavonoid phenoxyl radicals. A hydroxyl group in ring B (Figure 1) preferably a catechol moiety, is required for good scavenging activity and a C-2,3 double bond in combination with a hydroxyl at C-3 can further increase scavenging activity [52]. Both hispidulin and eupafolin were able to promote iron release from ferritin, the effect of the latter being three times greater than that caused by hispidulin at a same molar concentration (0.2 mM) suggesting that the catechol ring in the eupafolin structure can be responsible for such differences. Other phenols, such as 6-hydroxydopamine (6-OHDA) that has a catechol ring, can undergo autooxidation and promote iron release from ferritin [44]. The levels of iron mobilization promoted by eupafolin did not undergo alterations on addition of SOD, but decreased in the presence of catalase. This indicates the involvement of H_2O_2, as well as with the presence of redox active metals. A possibility is that the autoxidation may be occurring by inner sphere electron transfer, presumably within a ternary complex mechanism that does not involve free $O_2^{\bullet -}$ and is not inhibited by superoxide dismutase, as observed in the autoxidation of the 6-OHDA [53]. Superoxide dismutase failed to inhibit 6-OHDA autoxidation, except in the presence of the metal chelators EDTA or DETAPAC [53].

Iron released by eupafolin can undergo the Fenton reaction and generate ROS. However, eupafolin ($I_{50} = 15$ μM) is a good reducing agent, comparable to ascorbic acid ($I_{50} = 20–25$ μM), using the reaction with the stable free radical DPPH. The presence of an *o*-hydroxylation on the B-ring of the flavonoid molecule and the number of free hydroxyl groups, a C-2,3 double bond in the C ring, or the presence of a 3-hydroxyl group is usually considered as a necessity for antioxidant and antiradical activities [54]. However, hispidulin was incapable of reacting with the stable free radical DPPH at concentrations of up to 0.2 mM. These results indicate that eupafolin is a better antioxidant than hispidulin.

Mitochondrial toxicity has been involved in mechanism of action of drugs that can be useful as chemotherapeutic agents [55–57], which include some flavonoids [47]. The effects of hispidulin on mitochondrial metabolism may be therefore related

with the cytotoxic properties described by Kupchan et al. [9], who described values for ED_{50} (i.e. doses inhibiting growth to 50% of control growth) against a KB cell culture.

Several groups have identified novel mitochondrial uncoupling proteins (UCPs), which can promote increased oxygen consumption and heat production by uncoupling mitochondrial electron transport from ATP synthesis. UCP-1 and UCP-3 are expressed predominately in adipose tissue and muscle. UCP-2, besides being expressed in these tissues is also more ubiquitous, with low levels of expression in several other tissues including those of heart, liver, lung, pancreas, kidney and brain [58]. Recently, up-regulated UCP-2 mRNA expression in liver was described [59,60]. Considering possible UCP expression and that some products of the lipid peroxidation are UCP activators, the observed flavonoid effects could be mediated by uncoupling proteins such as UCP-2, which have been recently proposed to modulate oxidative stress [61,62]. Our results now show that interference in respiratory chain electron transport where occurs in the superoxide production. It is also shown that it promotes iron release from non-heme protein (ferritin). Therefore, it is possible that this flavone is involved in lipoperoxidation through the mechanism proposed by Brand et al. [61].

Since flavonoids, and more recently their metabolites, have been reported to act on kinase signaling cascades, a clear understanding of the mechanisms of action of flavonoids, either as antioxidants or modulators of cell signaling is important [63].

In addition, the influence of their metabolism on these properties is a key for evaluation of these potent biomolecules as anticancer agents. Such studies are under progress in our laboratory.

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