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Artigo

Effects of natural flavones on membrane properties and citotoxicity of HeLa cells

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RESUMO: "Efeitos de flavonas naturais em propriedades de membranas e em citotoxicidade de células HeLa". O objetivo deste estudo foi avaliar se eupafolina e hispidulina, flavonas extraídas do *Eupatorium littorale* Cabrera, Asteraceae, possuíam a capacidade de alterar propriedades das membranas biológicas e promover efeitos citotóxicos. Eupafolina (50-200 µM) reduziu em aproximadamente 30% a velocidade e amplitude do inchamento mitocondrial induzido por valinomicina e 60-100% o inchamento mitocondrial dependente de substrato. Além disso, eupafolina na dose de 200 µM reduziu a transição de permeabilidade mitocondrial em 35% entretanto, a hispidulina não alterou este parâmetro em todas as doses testadas. A avaliação da transição de fase dos lipossomas de DMPC com a sonda DPH demonstrou que ambas as flavonas afetam a fase gel e fluida. Quando lipossomas de membranas mitocondriais e a sonda DPH-PA foram utilizados, houve aumento da polarização de fluorescência promovido pela hispidulina. Eupafolina e hispidulina, na dose de 100 µM, promoveram 40% de redução da viabilidade de células HeLa em 24 h. Nossos resultados sugerem que eupafolina e hispidulina têm efeitos citotóxicos que podem ser explicados em parte pelas alterações promovidas por estas flavonas sobre propriedades de membranas biológicas e sobre a bioenergética mitocondrial.

Unitermos: Eupafolina, hispidulina, células HeLa, inchamento mitocondrial.

ABSTRACT: The aim of this study was to determine whether eupafolin and hispidulin, flavones extracted from *Eupatorium littorale* Cabrera, Asteraceae, have the ability to change properties of biological membranes and promote cytotoxic effects. Eupafolin (50-200 µM) decreased approximately 30% the rate and total amplitude of valinomycin induced swelling and 60-100% the energy-dependent mitochondrial swelling. Moreover, eupafolin (200 µM) reduced 35% the mitochondrial permeability transition, and hispidulin did not change this parameter in any of the doses tested. The evaluation of phase transition of DMPC liposomes with the probe DPH demonstrated that hispidulin and eupafolin affect gel and fluid phase. With mitochondrial membrane as model, hispidulin increased the polarization of fluorescence when used DPH-PA probe. Eupafolin and hispidulin (100 µM) promoted a reduction of 40% in cellular viability of HeLa cells in 24 h. Our results suggest that eupafolin and hispidulin have cytotoxic effects that can be explained, in part, by alterations promoted on biological membranes properties and mitochondrial bioenergetics.

Keywords: Eupafolin, hispidulin, HeLa cells, mitochondrial swelling.

INTRODUCTION

Flavonoids are compounds of vegetal origin present in many medicinal plants and foods. They may affect many cellular functions including growth, differentiation and proliferation (Weng et al., 2005; Chen et al., 2005; Rusak et al., 2005) and they also have antioxidant properties (Torel et al., 1986). However, many flavonoids, especially those with catechol ring, have pro-oxidant effects and promote inhibition of mitochondrial respiration (Hodnick, 1986).

We have studied the biological effects of eupafolin (6-methoxy 5,7,3',4'-tetrahydroxyflavone) (**1**), and hispidulin (6-methoxy-5,7,4'-trihydroxyflavone) (**2**). These flavones were extracted from *Eupatorium littorale* Cabrera, Asteraceae, a shrub of up to 1 m high, common in many parts of Brazil, but very little is known about their chemistry (Oliveira et al., 2001). These flavones are also

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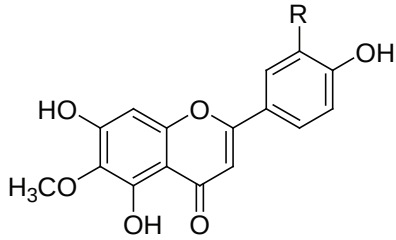
found in several others plants as: *Baccharis trimera* (Soicke & Leng, 1987), *Artemisia vulgaris*, (Jun et al., 1998) and *Arnica montana* L (Santos et al., 1998), all widely used by Brazilians for treatment of various diseases, however, little is known about its effects on mitochondrial bioenergetics and cytotoxic properties. Eupafolin had demonstrated antioxidant properties (Sanz et al., 1994; Kim et al., 2002) and anti-inflammatory activity (Sala et al., 2000). Hispidulin had promoted inhibition of human platelet aggregation (Bourdillat et al., 1988) and cytotoxicity in nasopharynx human carcinoma cells (Kupchan et al., 1969).



1 R = OH
2 R = H

We have shown that eupafolin and hispidulin are able to inhibit enzymes of the mitochondrial respiratory chain (Dabaghi-Barbosa et al., 2005; Herrerias et al., 2008); however, the mechanisms involved in this action are not clear. Considering the potential therapeutic significance of these effects, it becomes important a better understanding of their mechanism of action. In this study, we performed assays of mitochondrial swelling and analysis of fluidity of natural and artificial membranes to evaluate the interaction of eupafolin and hispidulin with the mitochondrial membrane in order to clarify if this interaction could be involved in the previously observed effects of these flavonoids on mitochondrial bioenergetics. Also, we evaluated the cytotoxic effect on human cervix adenocarcinoma cell line (HeLa) and discussed its relationship with the effects in biological membranes.

MATERIAL AND METHODS

Chemicals

Bovine serum albumin (BSA), valinomycin, rotenone, HEPES, EDTA, dimyristoylphosphatidylcholine (DMPC), sodium glutamate and sucrose were acquired from Sigma (St. Louis, MO, USA). The 1,6-diphenyl-1,3,5-hexatriene (DPH) and 3-[*p*-(6-phenyl)-1,3,5-hexatrienyl] phenylpropionic acid (DPH-PA) were obtained from Molecular Probes. All others reagents were commercial products of the highest available purity grade.

Flavones

Eupafolin and hispidulin were extracted from leaves of *Eupatorium litoralle* Cabrera from the Asteraceae family and their structures confirmed as described in Oliveira et al. (2001). The aerial parts were collected in Piraquara (Metropolitan Region of Curitiba-Brazil) and identified by Prof. Olavo Guimarães, the curator of UFPR Herbarium, where a voucher specimen has been deposited (# UPCB12400) (Oliveira et al., 2001). The flavones were dissolved in dimethylsulfoxide (DMSO) and then further diluted with the assay medium (25-200 µM). Solvent controls with DMSO were carried out in each assay.

Animals

Male Wistar rats (180-200 g) were obtained from the Central Animal House of the Federal University of Paraná (PR, Brazil). The experiments were conducted following the recommendation of the Brazilian Law 6638, 05/11/1979 for the scientific management of animals and the procedures were approved by the Institutional Animal Ethics Committee.

Preparation of rat liver mitochondria

Rat liver mitochondria were prepared according to Voss et al. (1961) with slight modifications. Male Wistar rats were sacrificed by decapitation; liver was immediately removed, sliced in the extraction medium and homogenized three times with a Potter-Elvehjem homogenizer. Homogenates were centrifuged at 2500 x g for 5 min and the resulting supernatant was further centrifuged at 12500 x *g* for 10 min. Pellets were suspended in medium and centrifuged twice at 10000 x *g*. Pellets were suspended in 0.5 mL medium free of EDTA.

Mitochondrial swelling experiments

Swelling in energized (Mustafa et al., 1966; Sepalla et al., 1973) or de-energized mitochondria (Moreno & Madeira, 1990) was monitored by absorbance using a HITACHI (mod U-2001 UV/VIS) spectrophotometer at 546 nm. In both experiments, eupafolin was added to the medium 2 min before initiating swelling measurements.

Mitochondrial permeability transition (MPT)

Calcium induced-swelling was determined by the turbidity decrease in mitochondrial suspension at 546 nm in a Hitachi U-2001 UV/VIS spectrophotometer (Bernardi, 1992; Gunter & Pfeifer, 1990). CsA (1 µM) or EGTA (2 mM) were used as controls.

Preparation of membranes

Dimyristoylphosphatidylcholine (DMPC) membranes were prepared as described by Antunes-Madeira & Madeira (1984) and Cadena et al. (2001). Native mitochondrial membranes were prepared as described by Antunes-Madeira & Madeira (1989).

Incorporation of probes and flavonoids into membranes

Aliquots of 1,6-diphenyl-1,3,5-hexatriene (DPH) or 3-[*p*-(6-phenyl)-1,3,5-hexatrienyl] phenylpropionic acid (DPH-PA), dissolved in tetrahydrofuran or dimethylformamide, respectively, were added (1.73 µM) into membrane suspensions. Flavone (10 µM) was then added and incubated for 2 h.

Fluorescence polarization measurements

Fluorescence measurements were performed with a RF-5301PC spectrofluorophotometer (Shimadzu Scientific Instruments, Inc.), equipped with a thermostatic cell holder. The excitation was set at 365 nm and the emission at 450 nm (slit width of excitation and emission = 3). The degree of fluorescence polarization (*P*) was calculated according to the equation of Shinitzky & Barenholz (1978). A high degree of polarization (*P*) represents a high structural order or low membrane fluidity.

Cell viability

Human cervix adenocarcinoma cell line (HeLa) were obtained from Institute Adolfo Lutz (São Paulo). HeLa cells were grown in MEM medium containing 7.5% fetal bovine serum and maintained at 37 °C in a humidified incubator containing 5% CO_2. Cell viability was assessed by MTT staining as described by Reilly et al. (1998).

Protein determination

Mitochondrial protein was assayed by the method of Lowry et al. (1951), using BSA as standard.

Statistical analysis

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

RESULTS AND DISCUSSION

Mitochondrial swelling

The effects of eupafolin on energy-dependent mitochondrial swelling using glutamate as substrate are represented in Figure 1A. Eupafolin promoted a dose-dependent decrease in rate and amplitude of swelling and in the shrinkage of the organelles (Figure 1A). At lower concentrations (25-50 µM), eupafolin decreased amplitude and velocity of the swelling by approximately 60%, reaching up approximately 100% at higher doses (75-200 µM). We could explain such effects on energized swelling to some extent by enzymatic inhibition (Herrerias et al., 2008) and a possible fluidity reduction of the mitochondrial membrane. Under de-energized conditions, eupafolin (50-200 µM) decreased the initial rate and total amplitude of valinomycin-induced swelling by approximately 30% (Figure 1B). This result suggests that eupafolin interacts with the lipid bilayer promoting fluidity modifications of the inner membrane.

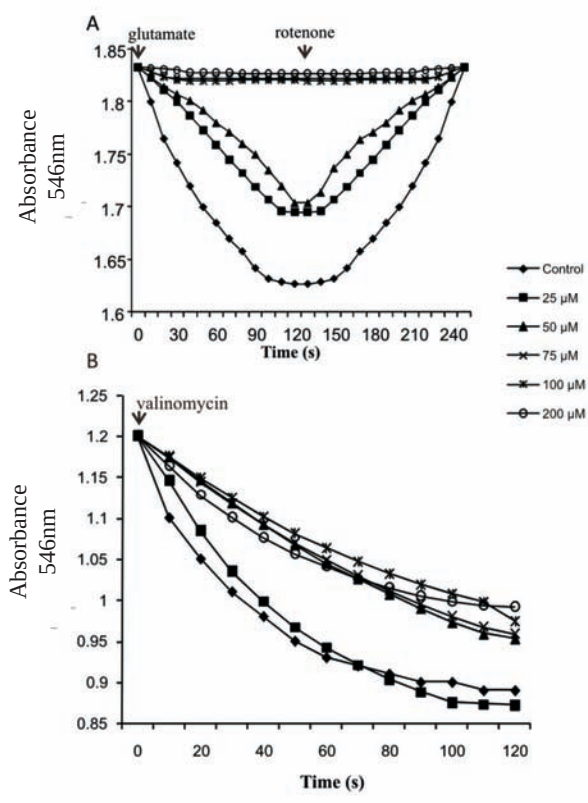


Figure 1. Effect of eupafolin on swelling and shrinkage of rat liver mitochondria (a) energized and (b) de-energized. (A) Mitochondrial suspension (1 mg protein) in a medium containing: 100 mM sucrose, 30 mM Tris-HCl, pH 7.2, 0.5 mM EDTA, 50 mM sodium acetate. Swelling was induced by addition of 15 mM of glutamate and shrinkage by addition of 4 µM of rotenone in a final volume of 1 mL. (B) Mitochondria suspension (1 mg protein) was incubated in medium containing: 135 mM KNO_3, 5 mM HEPES buffer (pH 7.4), 0.1 mM EDTA, 4 µM rotenone, 2 µg/mL antimycin A, the reaction was started by addition of 4 µg/mL valinomycin. In both experiments eupafolin was incubated for 2 min before of the swelling at different concentrations as indicated. Traces are representative of recordings of three independent experiments in triplicate.

Fluorescence polarization measurements

In order to evaluate the effects of eupafolin and hispidulin on membrane fluidity, we employed models consisting of liposomes (DMPC) and native membranes (mitochondrial). The probe DPH evaluates interactions of flavonoids with the hydrophobic core, while DPH-PA interacts with outer regions of the bilayer. The effect of eupafolin and hispidulin on the cooperative phase transitions of DMPC liposomes with DPH and DPH-PA is represented in Figure 2A and B, respectively. Hispidulin (10 µM) increased *P* values in both, fluid and gel phases with DPH probe (Figure 2A) suggesting that hispidulin decreases the fluidity of DMPC membrane in these conditions. In turn, eupafolin (10 µM) modified the phase transition profile of DMPC in a different way. The *P* values in the gel phase were lower, while they were higher in fluid phase (Figure 2A). The modifications of phase transition profile of DMPC caused by eupafolin and hispidulin did not change the midpoint temperature of thermotropic phase transition (Tm), which remained at 23.5 ºC (Figure 2A). With DPH-PA probe, both drugs affected slightly the thermotropic profile of DMPC liposomes, increasing the *P* values only at the higher temperatures (32 to 40 ºC). The Tm value was not altered by flavones (Figure 2B).

For a better correlation with experiments on which were used isolated mitochondria, assays were carried using mitochondrial membrane as model. With DPH probe, eupafolin did not affect the *P* values, while hispidulin promoted a slight decrease in response to temperature increase (Figure 3A). However, when the probe was DPH-PA, hispidulin increased the polarization of fluorescence in relation to control, indicating that it causes a diminution of membrane fluidity. Eupafolin also slightly increased the *P* values (Figure 3B). Comparing the structure of the flavones used in this study, the difference is the presence of a catechol ring in eupafolin, which could explain, in part, the different interaction with mitochondrial membrane and DMPC liposome (Scheidt et al., 2004). Our results suggest that incorporation of hispidulin preferentially affects the hydrophobic core, and it is in agreement with the hydrophobic characteristic of this flavonoid structure.

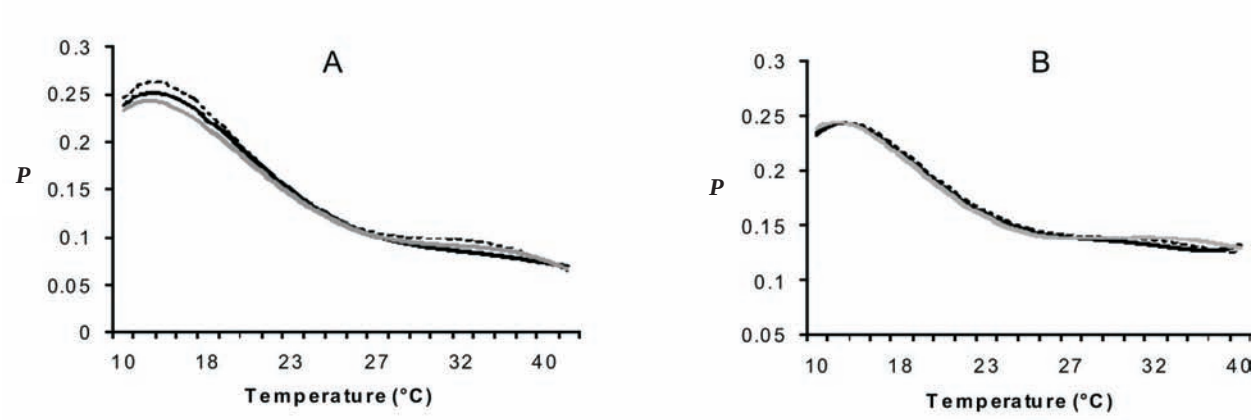


Figure 2. Effects of hispidulin and eupafolin on fluorescence polarization (*P*) of DPH (a) and DPH-PA (b) in dmpc bilayers as a function of temperature. DMPC membranes (345 µM in total lipid) prepared in 10 mM of Tris-Maleate buffer (pH 7.0) containing 50 mM KCl were incubated with eupafolin (10 µM) or hispidulin (10 µM) or none (control) in a final volume of 3 mL. The excitation was set at 365 nm and emission at 450 nm. The traces represent typical recordings of three independent experiments.

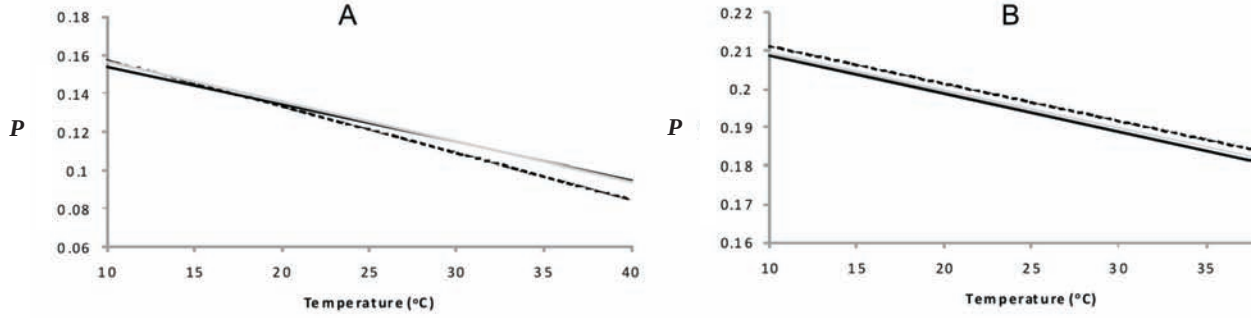


Figure 3. Effects of hispidulin and eupafolin on fluorescence polarization (*P*) of DPH (a) and DPH-PA (b) in mitochondrial native membranes as a function of temperature. Mitochondrial membranes (345 µM in total lipid) prepared in 10 mM of Tris-Maleate buffer (pH 7.0) containing 50 mM KCl and 10 µM PMSF were incubated with eupafolin (10 µM) or hispidulin (10 µM) or none (control) in a final volume of 3 mL. The excitation was set at 365 nm and emission at 450 nm. The traces represent typical recordings of three independent experiments.

Mitochondrial permeability transition (MPT)

In this study, we evaluate the effects of eupafolin and hispidulin on swelling induced by calcium. Mitochondrial swelling was observed in the presence of calcium and Pi, in contrast with its full inhibition after addition of the calcium chelator EGTA or the specific pore opening inhibitor CsA (Figure 4A). Eupafolin promoted alteration in the amplitude of the calcium induced mitochondrial swelling only in the highest concentration (200 µM) with a reduction of approximately 35% in this parameter (Figure 4A). Hispidulin did not affect this process in all tested doses (Figure 5B).

Cell viability

Eupafolin and hispidulin had demonstrated a similar behavior in cellular viability of HeLa cells.

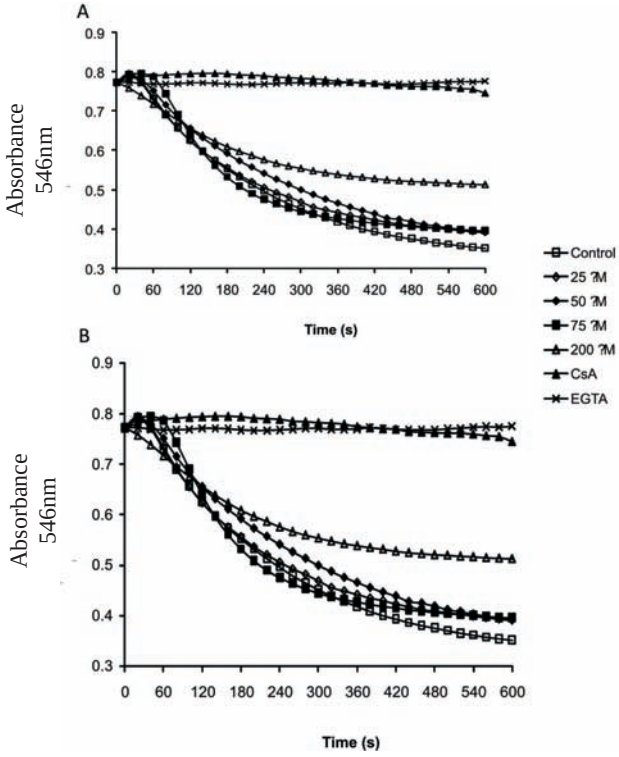


Figure 4. Effect of eupafolin (a) and hispidulin (b) on calcium-induced swelling. Mitochondrial protein (0.3 mg) suspended in a medium containing 250 mM sucrose, 10 mM HEPES, pH 7.2, 0.3 mM KH_2PO_4, 75 µM $CaCl_2$, 6 µM rotenone, 3 mM succinate supplemented by 1 µM CsA, 2 mM EGTA and eupafolin or hispidulin as indicated. Traces are representative of recordings of three independent experiments in triplicate.

They promoted a reduction of approximately 40% in viability only in the highest dose (Figure 5), what suggest an important anticancer activity effect. These results demonstrate that both flavones, despite the structural differences have similar cytotoxic effects on HeLa cells.

In summary, the results obtained with swelling and fluorescence polarization analysis suggest a fluidity reduction of the mitochondrial membrane promoted by hispidulin and eupafolin. This effect together with the enzymatic inhibitions, which promote an alteration of the electron flux in the respiratory chain and increase the generation of reactive oxygen species (ROS) (Herrerias et al., 2008; Dabaghi-Barbosa et al., 2005), could result in the cytotoxic effects observed in the HeLa cells. These results are important because they contribute to a better understanding of hispidulin and eupafolin action in biological systems and indicate a potential effect against tumoral cells."

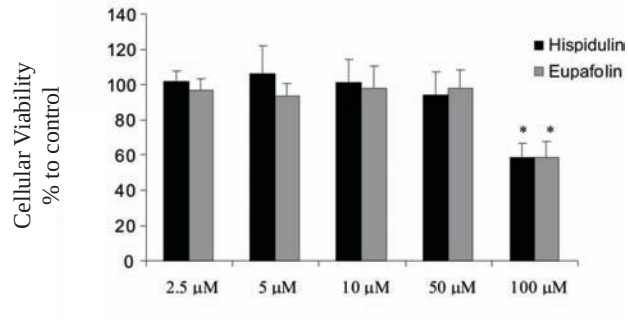


Figure 5. Effects of eupafolin and hispidulin on cellular viability of hela cells. Experimental conditions are described in the Materials and Methods. HeLa cells were plated into 96-well plates and treated with different concentrations of the eupafolin or hispidulin dissolved in DMSO 0.7% (v/v) as indicated for 24 h at 37 °C. Each value represents the media±SD of three different experiments in triplicate. *Significantly different from the control (100%), $p<0.05$.

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