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Prunin- and hesperetin glucoside-alkyl (C_4–C_{18}) esters interaction with Jurkat cells plasma membrane: Consequences on membrane physical properties and antioxidant capacity

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ABSTRACT

Prunin (P)- and hesperetin glucoside (HG)-alkyl esters are lipid-soluble compounds with antimicrobial and antioxidant capacities in vitro. The effects of P- and HG-alkyl (C_4–C_{18}) esters (0.1–100 μM) on human leukemia T (Jurkat) cells viability and plasma membrane fluidity were evaluated. After 1 h of exposure, cell viability was not affected in the range 0.1–10 μM. The decrease of cell viability found at 100 μM concentration depended on the length of the alkyl chain and reached a maximum with C_6–C_{12} derivatives. At this concentration, cell hyperpolarization and shrinkage were also observed. Cell plasma membrane fluidity was not affected, regardless the depths of the membrane level evaluated, but mild changes in plasma membrane hydration were found. Esterification did not affect the antioxidant capacity of P and HG (0.1–10 μM) against 1 mM H_2O_2. When exposed to 1 mM AAPH, P-alkyl esters retained P antioxidant capacity, but HG-derivatives acted as pro-oxidants. Together, present experimental evidences suggest that short term exposures to 0.1–10 μM concentrations of P- and HG-alkyl (C_4–C_{18}) esters can be considered safe for cultured human cells, and further studies are required to investigate their long term effects, as well their safety for human consumption.

1. Introduction

Flavanone glycosides constitute a subclass of flavonoids present in *Citrus* fruits. The growing knowledge of their beneficial effects on human health (Benavente-Garcia and Castillo, 2008; Itoh et al., 2009; Gonzalez-Molina et al., 2010; Selvaraj and Pugalendi, 2010; Tsao, 2010; Al-Ashaal and El-Sheltawy, 2011) makes these compounds and their derivatives good candidates to be used in the pharmaceutical and food industries (for a review see Garg et al., 2001).

Aglicons naringenin (4′,5,7-trihydroxyflavanone) and hesperetin (4′-methoxy-3′,5,7-trihydroxyflavanone) (Fig. 1A) can bear a glucose moiety bound to the hydroxyl group in position 7 of the A ring, rendering the glucosyl derivatives named naringenin 7-*O*-glucoside or prunin (P) and hesperetin 7-*O*-glucoside (HG), respectively (Fig. 1B). While P has a hydroxyl group in 4′ of ring B, HG has a hydroxyl group in 3′ and a methoxyl group in 4′ (Fig. 1B). Further chemical modifications of these flavanones can be achieved in vitro. Recently, an enzymatic method that allows the esterification of the hydroxyl group in position 6 of the glucoside moiety in P with alkyl vinyl esters or alkyl acids of different chain length was described (Fig. 1C) (Céliz and Daz, 2011). Using the same methodology, a series of alkyl esters of HG were synthesized (Fig. 1C). All

Abbreviations: 12-AS, 12-(9-anthroyloxy) stearic acid; 16-AP, 16-(9-anthroyloxy) palmitic acid; 6-AS, 6-(9-anthroyloxy) stearic acid; AAPH, 2,2′-azobis-2-methyl-propanimidamide, dihydrochloride; BA, butyric acid; C_{11}-BODIPY, 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid; DA, decanoic acid; DCDCDHF, 5-(and-6)-carboxy-2′,7′-dichloro-dihydrofluorescein-diacetate; DCF, dichlorofluorescein; $DiBaC_4(3)$, bis-(1,3-dibutylbarbituric acid)trimethine oxanol; DMSO, dimethylsulfoxide; GP, generalized polarization; HA, hexanoic acid; HEPES, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); HG, hesperetin glucoside; HGB, hesperetin glucoside 6″-*O*-butyrate; HGD, hesperetin glucoside 6″-*O*-decanoate; HGH, hesperetin glucoside 6″-*O*-hexanoate; HGL, hesperetin glucoside 6″-*O*-laurate; HGO, hesperetin glucoside 6″-*O*-octanoate; HGS, hesperetin glucoside 6″-*O*-stearate; LA, lauric acid; Laurdan, 6-dodecanoyl-2-dimethylamino-naphthalene; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide; NG, naringin; OA, octanoic acid; P, prunin; PB, prunin 6″-*O*-butyrate; PD, prunin 6″-*O*-decanoate; PH, prunin 6″-*O*-hexanoate; PI, propidium iodide; PL, prunin 6″-*O*-laurate; PO, prunin 6″-*O*-octanoate; PS, prunin 6″-*O*-stearate; SA, stearic acid; SDS, sodium dodecyl sulfate.

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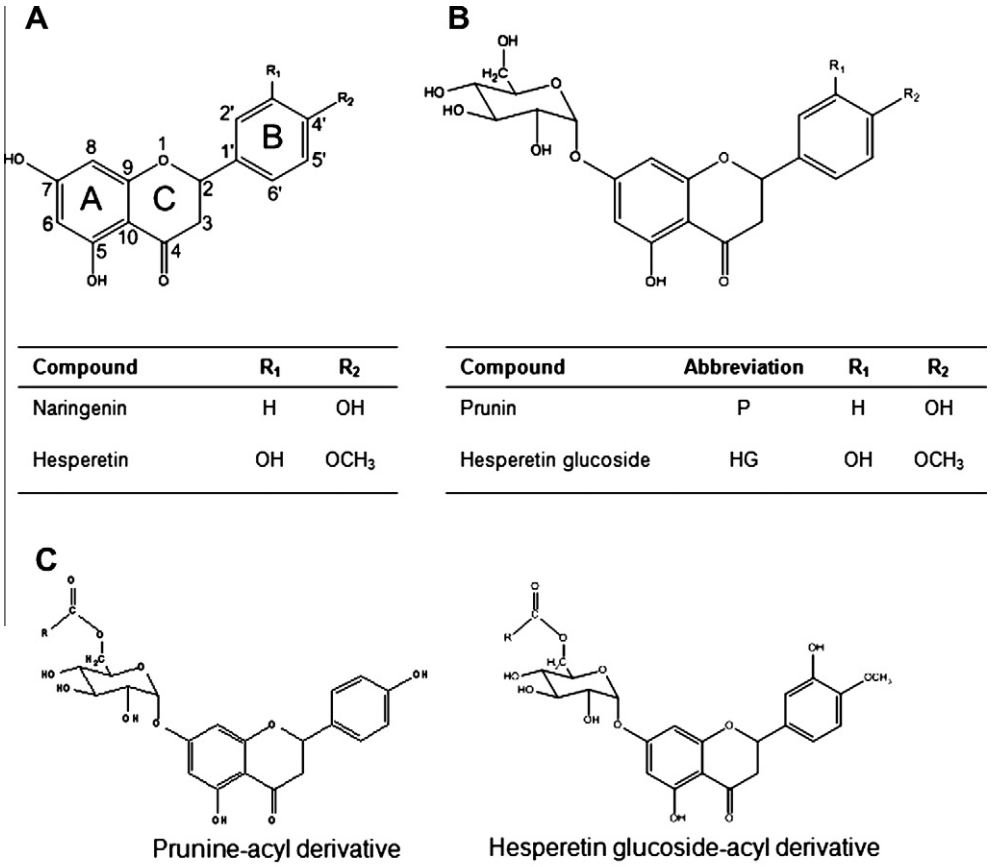

Compound	R_1	R_2
Naringenin	H	OH
Hesperetin	OH	OCH_3

Compound	Abbreviation	R_1	R_2
Prunin	P	H	OH
Hesperetin glucoside	HG	OH	OCH_3

R	Abbreviation	Prunin-alkyl ester	Hesperetin glucoside-alkyl ester
Butyric acid (C_4)	BA	PB	HGB
Hexanoic acid (C_6)	HA	PH	HGH
Octanoic acid (C_8)	OA	PO	HGO
Decanoic acid (C_{10})	DA	PD	HGD
Lauric acid (C_{12})	LA	PL	HGL
Stearic acid (C_{18})	SA	PS	HGS

Fig. 1. Structure of the compounds included in this study. (A) Naringenin (4′,5,7-trihydroxyflavanone); (B) Prunin and hesperetin glucoside; (C) Prunin- and hesperetin glucoside-alkyl esters.

these compounds are hydrophobic, and thus capable to spontaneously incorporate into lipid-reach environments, such as cell plasma membrane. Using a cell-free system, it has been demonstrated that P-alkyl esters retain the antioxidant capacity of the parent flavanone (Céliz and Daz, 2011).

Based on these precedents, we initiated the study of the biological effects of P- and HG-alkyl esters. In the current study we investigated the effects of a short term (1 h) exposure of human leukemia T cells (Jurkat cells) to micromolar concentrations of these compounds on cell viability, plasma membrane physical properties and their potential capacity to prevent the oxidative damage to cells challenged with two different oxidant molecules. Current results suggest that within the 0.1–10 μM range of concentrations, both P- and HG-alkyl esters can be considered safe to cultured human cells when used for short periods of time. However, at higher concentrations (100 μM) these compounds interfered with normal cell metabolism, decreasing mitochondrial activity. In addition, their antioxidant capacity in this experimental system depended on both the concentration of the compounds and the nature of the oxidant used to induce the insult.

2. Materials and methods

2.1. Chemicals

Alkyl acids (C_4, C_6, C_8, C_{10}, C_{12} and C_{18}), naringenin (4′,5,7-trihydroxyflavanone), bis-benzimide H (Hoechst 32258), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) and all other reagents were from the highest quality available and were purchased from Sigma-Aldrich (St. Louis, MO, USA). The fluorescent probes 5-(and-6)-carboxy-2′,7′-dichloro-dihydrofluorescein-diacetate (DCDCDHF), 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid (C_{11}-BODIPY), bis-(1,3-dibutylbarbituric acid) trimethine oxanol ($DiBaC_4(3)$), propidium iodide (PI), 6-(9-anthroyloxy)stearic acid (6-AS), 12-(9-anthroyloxy)stearic acid (12-AS), 16-(9-anthroyloxy) palmitic acid (16-AP) and 6-dodecanoyl-2-dimethylamino-naphthalene (Laurdan) were purchased from Invitrogen/Molecular Probes Inc. (Eugene, OR, USA). 2,2′-azobis-2-methyl-propani-

midamide, dihydrochloride (AAPH) was from Cayman Chemical Co. (MI, USA). RPMI 1640 medium, streptomycin, penicillin, and fetal bovine serum were purchased from Gibco BRL (Grand Island, NY, USA).

2.2. Flavanones and alkyl derivatives

Naringin (NG) (4′,5,7-trihydroxyflavanone-7-[2-*O*-(α-L-rhamnopyranosyl)-β-D-glucopyranoside]) was obtained according to the method described by Geronazzo et al. (2000) from immature aborted grapefruits. Neohesperidin (4′-methoxy-3′,5,7-trihydroxyflavanone-7-[2-*O*-(α-L-rhamnopyranosyl)-β-D-glucopyranoside]) was obtained as previously described (Macoritto et al., 2004). Prunin (P) (4′,5,7-trihydroxyflavanone-7-*O*-β-D-glucopyranoside) and hesperetin glucoside (HG) (4′-methoxy-3′,5,7-trihydroxyflavanone-7-*O*-β-D-glucopyranoside) were obtained by enzymatic hydrolysis of NG and neohesperidin, respectively, as described by Soria and Ellenrieder (2002). Identification and purity of the compounds were assessed by HPLC and their retention times were compared with those of commercial standards (Extrasynthese, Grayne, France). P- and HG-alkyl derivatives were obtained as previously described (Céliz and Daz, 2011).

2.3. Cell culture

Human leukemia T cells (Jurkat) were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Jurkat cells were cultured at 37 °C in a 5% CO_2 atmosphere, in RPMI 1640 medium, supplemented with 2 mM L-glutamine, 10% (v/v) fetal bovine serum, and penicillin (50 units/ml) and streptomycin (50 μg/ml). Immediately before experiments, cells were centrifuged for 5 min at 800*g* and the amount of viable cells was estimated by the Tripan blue exclusion method. Cells were resuspended (1×10^6 cells/ml) in 20 mM HEPES, 1 mM NaH_2PO_4, buffer (pH 7.4) containing 140 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, and 5.5 mM glucose (Verstraeten et al., 2008).

2.4. Incubations

Aliquots containing 0.2 ml of Jurkat cells suspension were poured in 96-well plates apt for fluorescence measurements and added with 0.1–100 μM of the alkyl acids (BA, HA, OA, DA, LA or SA), the flavanones (NG, P or HG), P- or HG-alkyl esters dissolved in dimethylsulfoxide (DMSO). Samples were incubated for 1 h at 37 °C and used for the subsequent determinations. The final concentration of DMSO in the samples was 0.25% (v/v).

2.5. Evaluation of cell viability and integrity

Cell viability was assessed from the MTT reduction assay (Mosmann, 1983). After the incubation in the conditions described in Section 2.4, samples were added with 10 μl of MTT tetrazolium salt solution (0.05 mg/ml) and incubated for 1 h at 37 °C. Formazan crystals were solubilized in 10% (w/v) SDS in HCl 0.01 N and the absorbance at 575 nm (reference: 620 nm) was measured in a Microplate Reader Model 550 (Bio-Rad, Tokyo, Japan).

Cell plasma membrane integrity was evaluated from the binding of propidium iodide (PI) to DNA in the absence of detergents (Verstraeten, 2006). Briefly, after incubating cells in the conditions described in Section 2.4, samples were added with 25 μM PI and incubated for 5 min at room temperature and in the dark. The fluorescence corresponding to PI-DNA complex was evaluated at 590 nm (λ excitation: 538 nm) in a PerkinElmer LS-55 spectrofluorometer (PerkinElmer Ltd., Beaconsfield, United Kingdom). Cells were next disrupted by incubating samples for 30 min at 37 °C in the presence of 0.1% (v/v) Igepal and the fluorescence of PI-DNA complex was recorded again. The amount of cells with intact plasma membrane was calculated from the ratio between PI-DNA fluorescence measured in the absence and in the presence of the detergent.

2.6. Evaluation of plasma membrane potential

After incubating cells in the conditions described in Section 2.4, samples were added with 50 μl of a solution containing 0.5 μM $DiBacC_4(3)$ in 20 mM HEPES, 1 mM NaH_2PO_4, buffer (pH 7.4) containing 140 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, and 5.5 mM glucose, and further incubated for 15 min at 37 °C. $DiBacC_4(3)$ fluorescence was measured at 515 nm (λ excitation: 490 nm). After measurements, samples were added with the fluorescent probe Hoechst 32258 (25 μM final concentration) and incubated for further 10 min in the dark. DNA content was estimated from the fluorescence intensity of DNA-Hoechst 32258 complex at 420 nm (λ excitation: 370 nm). Results were expressed as the ratio between $DiBacC_4(3)$ and Hoechst 32258 fluorescence.

2.7. Evaluation of cell light scattering

After incubating cells in the conditions described in Section 2.4, changes in the average cell size were estimated from their steady-state light scattering (λ excitation and λ emission: 550 nm) (Verstraeten, 2006). Results were normalized by DNA content in the samples using the probe Hoechst 32258, as described in Section 2.6.

To evaluate the relationship between changes in cell light scattering and potential alterations in nuclear cell morphology, after cell incubation in the presence of the flavanones, aliquots of the samples were separated and nuclei were stained with 5 μM Hoechst 32258. Samples were immediately observed through an Olympus BX50 fluorescence microscope coupled with a digital camera (Olympus Optical CO, LTD., Japan).

2.8. Evaluation of membrane fluidity

After incubating cells in the conditions described in Section 2.4, samples were labeled with 0.2 μM of the fluorescent probes 6-AS, 12-AP or 16-AP. Cells were incubated at 37 °C for 15 min to allow the incorporation of the probe into the plasma membrane. Membrane fluidity was evaluated in the samples from the changes in the fluorescence polarization of the probes measured at 435 nm (λ excitation: 384 nm).

2.9. Evaluation of membrane hydration

After incubating cells in the conditions described in Section 2.4, samples were labeled with 0.2 μM of the fluorescent probe Laurdan. Cells were incubated at 37 °C for 15 min to allow the incorporation of the probe into the plasma membrane. Membrane hydration was evaluated in the samples from the changes in Laurdan generalized polarization (GP) corresponding to the excitation spectrum of the probe and was calculated from the following equation:

$$GP = \frac{(I_{380\ nm} - I_{350\ nm})}{(I_{380\ nm} + I_{350\ 9m})} \quad (1)$$

where I_{380nm} and I_{350nm} are the fluorescence intensities measured exciting samples at 380 and 350 nm (λ emission: 430 nm), respectively (Parasassi et al., 1990). GP was calculated from the excitation spectrum of Laurdan since no contributions of cell autofluorescence and flavonoids fluorescence were found in the range 300–400 nm (data not shown), making Laurdan a sensitive probe for the detection of subtle changes in membrane hydration.

2.10. Evaluation of flavanones antioxidant capacity

2.10.1. Evaluation of cell oxidants

The capacity of flavanones to prevent cell oxidative damage was evaluated as previously described (Verstraeten, 2006). Jurkat cells were incubated for 30 min at 37 °C in the presence of 10 μM DCDCDHF which upon oxidation renders a fluorescent molecule, DCF. The excess of non-incorporated probe was eliminated by washing cells twice with warm PBS. Cells were next incubated for 1 h at 37 °C in the presence of the flavanones as described in Section 2.4 and further incubated for 1 h at 37 °C in the presence of 1 mM AAPH or H_2O_2. The production of DCF was evaluated at 525 nm (λ excitation: 475 nm, cut-off: 515 nm). Cells were next disrupted by incubating samples for 30 min at 37 °C in the presence of 0.1% (v/v) Igepal, added with 25 μM PI and the fluorescence of PI-DNA complex was recorded as indicated in Section 2.5. Results were expressed as the DCF to PI fluorescence ratio.

2.10.2. Evaluation of lipid peroxidation

To assess the effects of the flavanones on H_2O_2- and AAPH-mediated lipid peroxidation, Jurkat cells were labeled with the fluorescent probe C_{11}-BODIPY (Verstraeten et al., 2005). Briefly, Jurkat cells were incubated for 20 min at 37 °C in the presence of 2 μM C_{11}-BODIPY to allow the incorporation of the probe into the plasma membrane. Cells were next incubated in the conditions described above, and the oxidation of the probe C_{11}-BODIPY was evaluated at two sets of excitation and emission wavelengths, as described by Drummen et al. (2002). The emissions corresponding to the oxidized (λ excitation, 488 nm; λ emission, 520 nm) and reduced (λ excitation, 545 nm; λ emission, 590 nm) states of the probe were recorded. Lipid peroxidation was estimated by calculating the ratio between the fluorescence of the oxidized and the reduced states.

2.11. Statistics

One-way analysis of variance (ANOVA) followed by Fisher's PLSD (protected least square difference) test was performed using the routines available in StatView 5.0 (SAS Institute, Cary, NC, USA). Two-way ANOVA, non-linear fitting of data and correlations were performed using GraphPad Prism version 5.00 for Windows, GraphPad Software (San Diego, CA, USA). A probability (*P*) value lower than 0.05 was considered as statistically significant.

3. Results

3.1. Effects of flavanones and their alkyl esters on Jurkat cell viability and integrity

The impact of the C_4–C_{18} alkyl chains on Jurkat cells viability was investigated from their capacity to metabolize the dye MTT that evaluates mitochondrial functionality. After 1 h of incubation, BA, HA, OA or DA (0.1–100 μM) did not affect cell viability (Fig. 2A). At 10 μM concentration, SA decreased cell viability in 22% ($P < 0.01$); however, at 100 μM SA no changes in cell viability were observed. On the other hand, LA (0.1–100 μM) caused a concentration-dependent decrease in MTT metabolization, an effect that was significant ($P < 0.05$) at 10 and 100 μM concentrations (Fig. 2A). The concentration of LA necessary to cause a 50% decrease in cell viability (C_{50}) was estimated in 12 μM.

Next, the effects of NG, P, and HG on cell viability were evaluated. None of these compounds affected cell viability (Fig. 2B and C). The incubation of cells in the presence of P-alkyl esters caused marked decrease in cell viability, an effect that was only significant at the highest concentration of the compounds assessed (Fig. 2B). At this concentration, the decrease in cell viability due to PB, PH, PO, PD and PL was significantly higher than the effect caused by alkyl acids at the same concentration ($P < 0.01$, two-way ANOVA). For these compounds, C_{50} values were estimated in PB: 70 μM, PH: 86 μM, PO: 17 μM, PD: 8.5 μM, and PL: 50 μM. A similar pattern of effects were observed for HG-alkyl derivatives (Fig. 2C), with the following calculated C_{50} values: HGB: 82 μM, HGH:

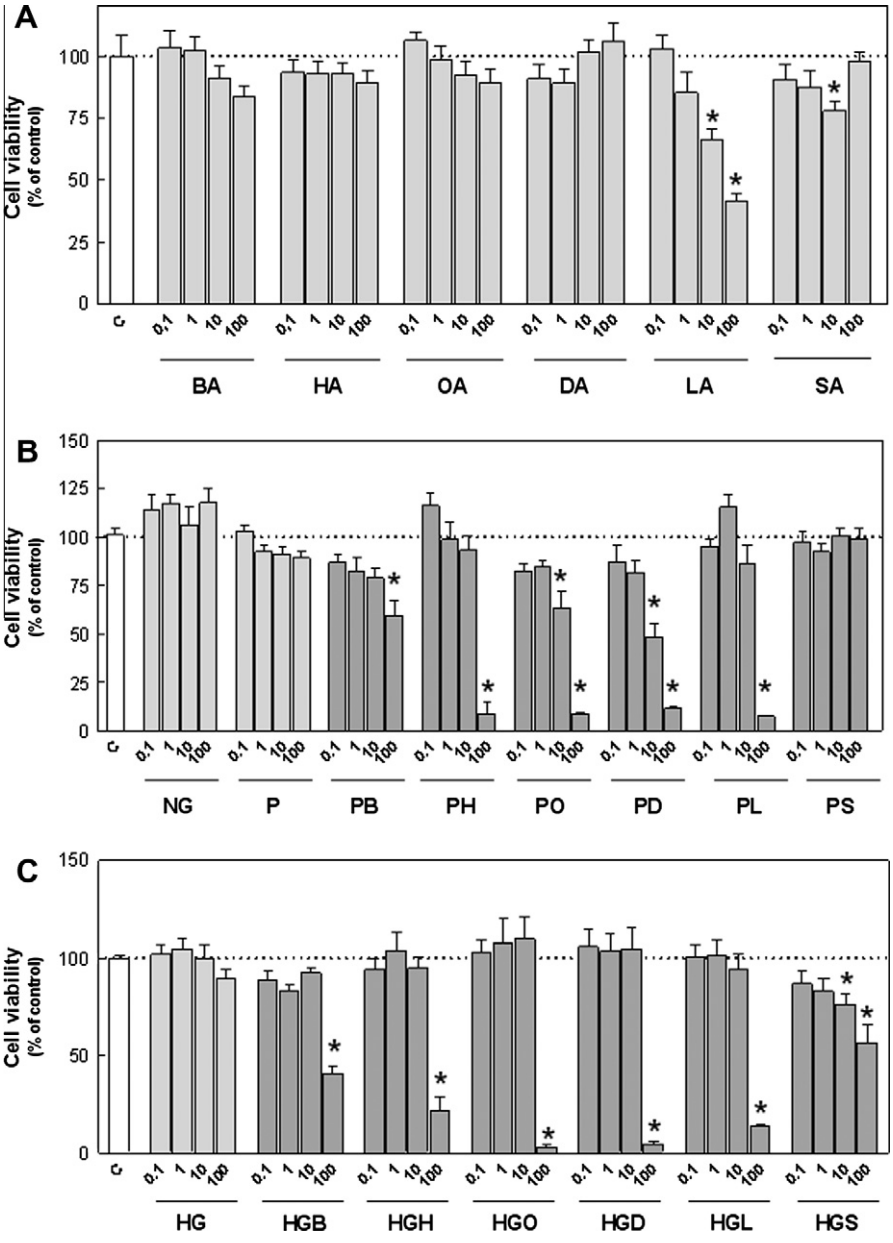

Fig. 2. Flavanones-alkyl esters decrease cell viability. Jurkat cells were incubated for 1 h at 37 °C in the presence of 0.1–100 μM of (A) BA, HA, OA, DA, LA or SA; (B) NG, P or P-alkyl esters; and (C) HG or HG-alkyl esters. After incubation, cell viability was evaluated in the samples from the reduction of MTT, as indicated in Section 2.5. Results are expressed as the percentage of viable cells respect to controls (C), and are shown as the mean ± SEM of 4 independent experiments. * Significantly different from the value measured in control cells ($P < 0.05$, ANOVA).

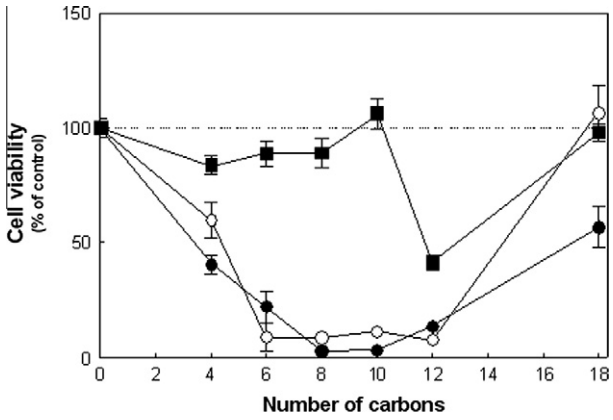

Fig. 3. Influence of alkyl chain length in the loss of cell viability. Graph represents the percentage of viable cells in the samples incubated in the presence of 100 μM free alkyl chains (■), P-alkyl esters (○), or HG-alkyl esters (●) versus the number of carbons present in the alkyl chains. Data taken from Fig. 2.

65 μM, HGO: 55 μM, HGD: 55 μM, HGL: 58 μM and HGS: 125 μM. Surprisingly, PS did not modify cell viability along the range of concentrations assessed (Fig. 2B).

The comparison of the magnitudes of cell viability decrease due to 100 μM of the free alkyl chains indicated that in this experimental model the only toxic specie was LA (C_{12}). On the contrary, at this concentration P- and HG-alkyl esters were more toxic than the corresponding free alkyl acids, even when bearing shorter alkyl chains. Maximal toxicity was reached with alkyl chains between C_6–C_{12} for the P series and C_8–C_{10} for the HG series (Fig. 3).

In order to investigate if the observed impairment of cell capacity to metabolize MTT upon their exposure to the alkyl derivatives was related to alterations in cell plasma membrane integrity, the binding of the intercalating agent PI was quantified. Measurements were performed in the absence of added detergents to avoid further cell rupture. No significant cell damage was observed in samples incubated in the presence of DMSO (vehicle) or the C_4–C_8 free alkyl acids. Though, in 100 μM DA- and LA-treated samples the amount of intact cells decreased in 7.5% and 54%, respectively (Fig 4A, $P < 0.05$ respect to controls). Again, SA did not affect cell integrity (Fig 4A).

None of the flavanones investigated (NG, P, and HG) caused cell lysis *per se*, as evaluated from PI binding to DNA (Fig. 4B and C). Among P-alkyl esters, only PO and PD decreased the number of intact cells in 14% and 10% respectively (Fig 4B, $P < 0.05$ respect to controls). Regarding the HG series (Fig. 4C), only 100 μM HGO, HGD and HGL caused cell lysis, with an effect that corresponded to 19%, 21%, and 14% decrease in the content of intact cells, respectively ($P < 0.05$ respect to controls).

3.2. Effects of flavanones and their alkyl esters on Jurkat cells membrane physical properties

The hypothesis that the interaction of flavanones or their alkyl esters with cell plasma membrane may affect their physical properties was next evaluated.

To evaluate if the assessed compounds may affect the potential of cell plasma membrane, the uptake of the fluorescent probe $DiBacC_4(3)$ was quantified. Results were normalized by DNA content in the samples to minimize differences due to the amount of cells among the experimental conditions. DMSO did not modify $DiBacC_4(3)$ uptake and neither did NG, P and HG (Fig. 5). Among P-alkyl esters, PH, PO, PD and PL at 100 μM concentration, significantly decreased $DiBacC_4(3)$ uptake to ~50% (Fig. 5A). Within the HG-alkyl esters series, 100 μM HGB, HGH, HGO, HGD and HGL also decreased $DiBacC_4(3)$ uptake, with a magnitude that ranged 30–60% decrease ($P < 0.01$ respect to controls) (Fig. 5B). No significant effects on $DiBacC_4(3)$ uptake were observed in PS- or HGS-treated cells (Fig. 5).

Next, the effect of these compounds on the average size of Jurkat cells was evaluated measuring the steady-state light scattering in cell suspensions. Since the amount of light scattered by samples depends on both cell size and concentration, results were normalized by the total amount of DNA present in the samples. In the current model, DMSO did not affect cell size (Fig. 6A). NG caused a slight but significant cell shrinkage ($P < 0.05$ respect to controls) but P did not modify cell size (Fig. 6A). On the other hand, HG caused mild cell enlargement (Fig. 6B), effect that was significant at 100 μM concentration ($P < 0.05$ respect to controls). In PH-, PO-, PD-, PL-, HGO-, HGD-, and HGL-treated cells, a significant decrease in cell size was evidenced, although only at a 100 μM concentration ($P < 0.05$ respect to controls) (Fig. 6). Similarly to the previous findings described in Section 3.1, neither PS nor HGS affected cell size. No alterations in nuclear morphology were evidenced by fluorescence microscopy of nuclei stained with the intercalating agent Hoechst 32258 (Fig. 6C). A significant correlation (r^2: 0.88, $P < 0.0001$) between flavanones-mediated cell shrinkage and plasma membrane hyperpolarization was found (Fig. 7).

The impact of flavanones and their alkyl esters on the fluidity of the plasma membrane was evaluated from the changes in the fluorescence polarization of 6-AS, 12-AS or 16-AP, probes that sense the fluidity at increasing depths of the bilayer. In the 0.1–100 μM range of concentrations none of the flavanones or their alkyl esters significantly affected the fluidity of the plasma membrane, regardless the depth of the membrane region investigated (Supplementary Table 1).

Finally, the hydration state of the plasma membrane was evaluated from the changes in the generalized polarization of the fluorescent probe Laurdan. Using this probe, no changes in GP values were found in 100 μM P-treated cells (Fig. 9A) while higher GP values were found in NG- and HG-treated cells at the same concentration ($P < 0.01$ respect to controls) (Fig. 8). Among P-alkyl esters, 100 μM PO, PD, PL and PS significantly increased Laurdan GP ($P < 0.05$). Overall, HG-alkyl esters did not affect this parameter, except HGS that increased GP value but only at 100 μM concentration ($P < 0.01$).

3.3. Antioxidant capacity of flavanones and their alkyl esters

The capacity of the flavanones to prevent the oxidative damage to cells upon their exposure to external pro-oxidant compounds was next evaluated.

The first oxidant evaluated was the azo-compound AAPH. After 1 h of incubation in the presence of 1 mM AAPH a significant increase in DCF generation was evidenced in samples ($P < 0.001$ respect to controls) (Fig. 9). DMSO did not affect the extent of DCF generation neither in non-treated cells nor in cells incubated in the presence of AAPH alone (Fig. 9). NG (1–10 μM) significantly prevented DCF generation ($P < 0.05$) (Fig. 9A). P and its alkyl esters also prevented DCF formation by AAPH but only at the lowest concentrations assessed (Fig. 9A). At 100 μM concentration, P and its alkyl esters have either no effect or enhanced AAPH-mediated DCF generation (Fig. 9A). Similar results were obtained when the HG series was evaluated. HG significantly prevented DCF formation only at 0.1 μM concentration ($P < 0.01$). At higher concentrations, a tendency towards higher DCF generation was observed in HG-treated cells. HG-alkyl esters partially prevented DCF generation at the lowest concentrations assessed, but displayed a marked pro-oxidant effect at 10 μM concentration ($P < 0.001$) (Fig. 9B).

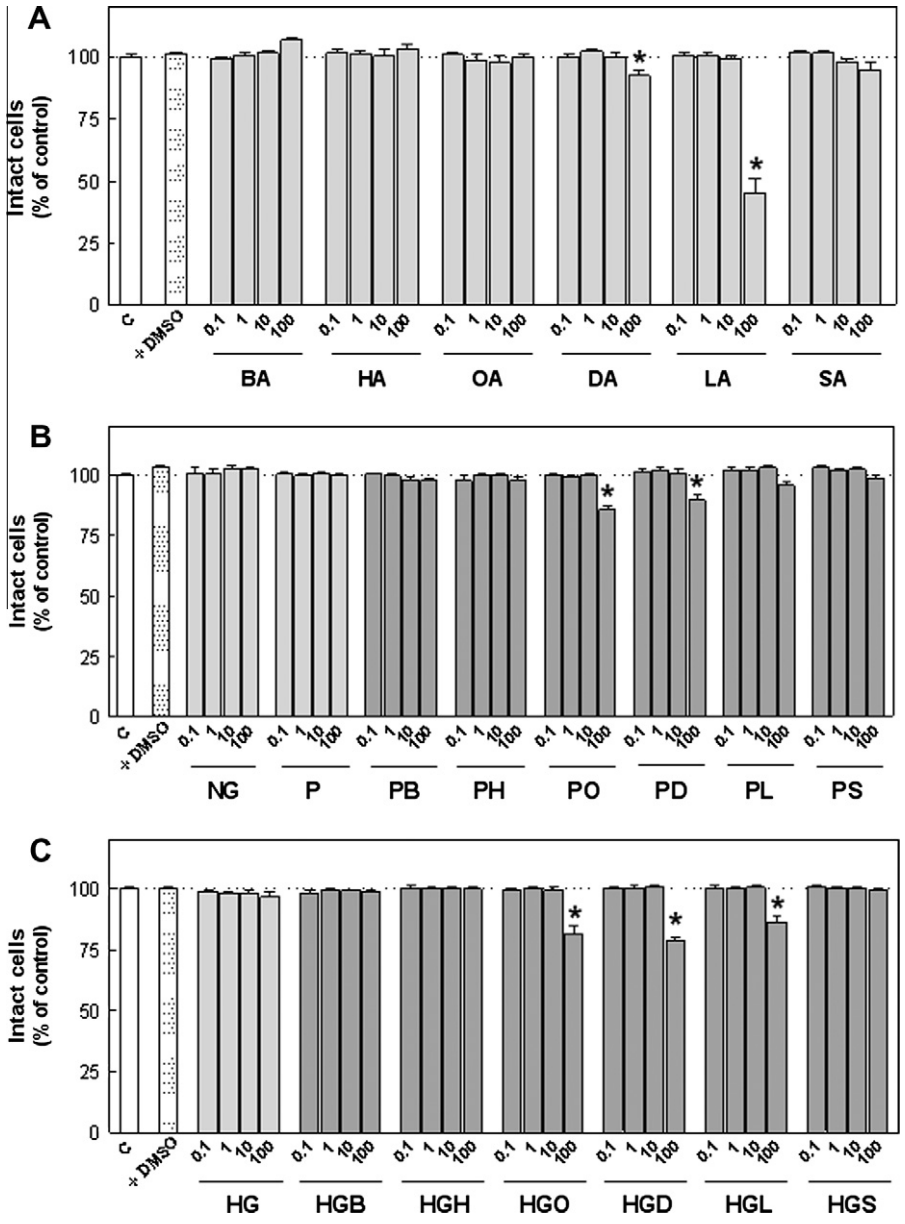

Fig. 4. Flavanones-alkyl esters cause minimal cell lysis. Jurkat cells were incubated for 1 h at 37 °C in the presence of 0.1–100 μM of (A) BA, HA, OA, DA, LA or SA; (B) NG, P or P-alkyl esters; and (C) HG or HG-alkyl esters. After incubation, cell integrity was evaluated in the samples as indicated in Section 2.5. Results are expressed as the percentage of cell integrity in control cells (C), and are shown as the mean ± SEM of four independent experiments. * Significantly different from the value measured in control cells ($P < 0.05$, ANOVA).

The second oxidant investigated was H_2O_2. As expected, H_2O_2 significantly promoted DCF generation in the samples, effect that was prevented by NG and HG. P and its alkyl esters displayed a slight although non-significant effect on H_2O_2-mediated DCF generation (Fig. 10A). On the other hand, all the members of the HG series fully prevented H_2O_2-supported DCF formation ($P < 0.001$) in an extent similar to that of its parent compound, HG (Fig. 10B).

Finally, the capacity of the flavanones to prevent H_2O_2- and AAPH-mediated lipid peroxidation was evaluated by means of the oxidation of C_{11}-BODIPY a probe that is sensitive to lipid hydroperoxides. As expected from the fact that the assessed oxidants are water-soluble, no major changes in lipid peroxidation were observed neither for H_2O_2 nor AAPH *per se* (Fig. 11). In fact, in cells treated with H_2O_2 alone, a decrease in C_{11}-BODIPY oxidation ratio was observed, whereas AAPH increased this ratio in only 10% ($P < 0.05$). This observation was corroborated by measuring the content of 2-thiobarbituric acid-reactive substances (TBARS) a method that quantifies the amount of lipid peroxidation final products (data no shown). Among the assessed alkyl esters, only 10 μM PB, PO, PD, HGB, and HGH protected cells from AAPH-mediated C_{11}-BODIPY oxidation (Fig. 11A). On the other hand, C_{11}-BODIPY oxidation ratio in NG-, P-, PB-, PH-, HG-, HGH-, HGO-, HGL- and HGS-treated cells exposed to H_2O_2 was similar to the value measured in cells exposed to H_2O_2 alone (Fig. 11B), whereas in cells incubated with the remaining alkyl esters the ratio was similar to the value in control cells.

4. Discussion

Flavonoids–membrane interactions emerge as an important mechanism that mediates some of the biological effects of these

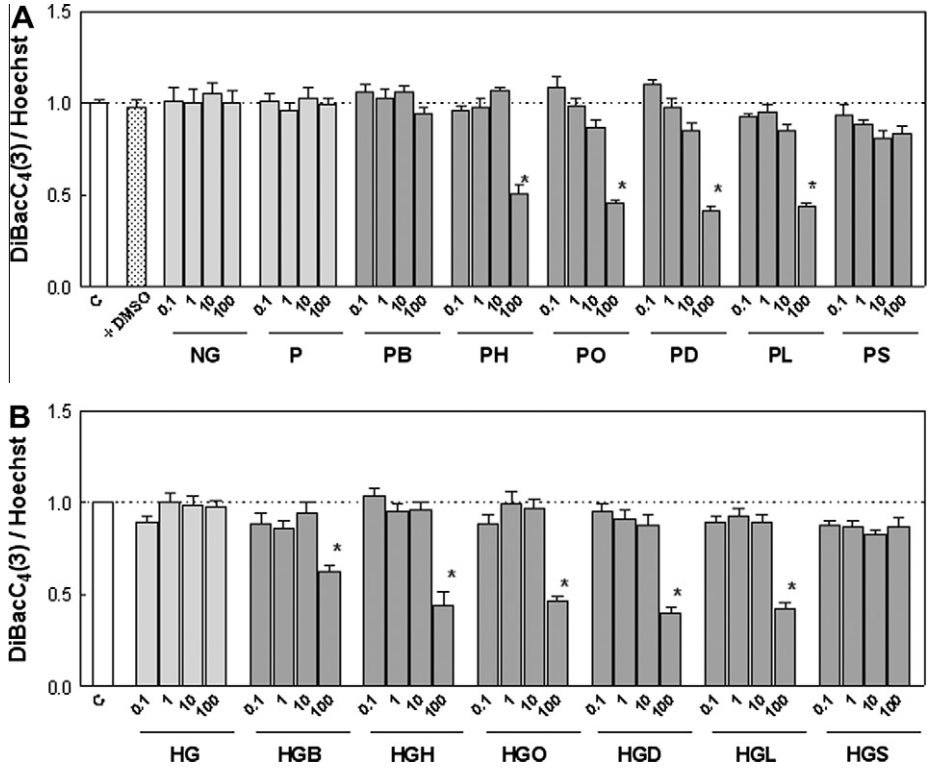

Fig. 5. Flavanones-alkyl esters cause cell hyperpolarization. Jurkat cells were incubated for 1 h at 37 °C in the presence of 0.1–100 μM of (A) NG, P or P-alkyl esters; (B) HG or HG-alkyl esters. After incubation, cell transmembrane potential was evaluated from the uptake of the fluorescent probe $DiBacC_4(3)$ as indicated in Section 2.6. Results were corrected by DNA content measured from nuclear staining with the probe Hoechst 32258, and are shown as the mean ± SEM of four independent experiments. * Significantly different from the value measured in control (C) cells ($P < 0.05$, ANOVA).

compounds. Experimental evidences indicate that as a consequence of flavonoids interactions with different components of the biological membranes, their biophysical properties may be altered, ultimately affecting certain membrane-associated processes such as the activity of enzymes, ligand–receptor interactions, metabolites uptake, and cell signaling, among others (Hendrich, 2006; Morris and Zhang, 2006; Verstraeten et al., 2009). In addition, flavonoids diminish either the initiation and/or the propagation of the oxidative damage to membrane lipids and the disruption of the lipid bilayer by external agents (Verstraeten et al., 2003; Lopez-Revuelta et al., 2006).

The nature and extent of the interaction of flavonoids with membranes rely on the chemical features of these molecules such as their molecular size, tridimensional structure and their solubility in hydrophilic or hydrophobic milieu. As an example, highly hydrophilic flavonoids such as the procyanidins mainly interact with the polar headgroup of membrane phospholipids and locate at the water–lipid interface (Verstraeten et al., 2003, 2008; Erlejman et al., 2004). In contrast, more hydrophobic flavonoids like certain flavonols and flavanones, penetrate membranes according to their hydrophobicity and locate in the region that contains the alkyl chains of phospholipids (for a review see Verstraeten et al., 2009). This is the case of P- and HG-alkyl esters. These compounds are amphiphilic, bearing alkyl chains with variable length (C_4–C_{18}) that will determine a differential partition in water and lipid environments. Supporting that, a recent study demonstrates that the glucosyl flavanone P partitions equally into water and n-octanol, whereas P-alkyl esters increase the solubility in n-octanol with the length of the bound alkyl chain (Céliz and Daz, 2011). Therefore, it is expected that, when in contact with biological membranes, alkyl chains will determine the spontaneous incorporation of these compounds into the lipid bilayer situating the glucosyl flavanone moiety at the water–lipid interface. Based on that, we hypothesized that the incorporation of P- and HG-alkyl esters into cell plasma membrane might have differential effects on membrane-associated events.

First, we investigated if these compounds may affect cell viability, by means of two different approaches. The first one involves the metabolization of the dye MTT. In living cells, MTT is reduced to formazan by the mitochondrial succinate dehydrogenase complex (E.C. 1.3.99.1) that participates in the electron transfer chain. Thus, this method mainly evaluates the amount of cells with preserved mitochondrial functionality. We observed that almost every compound assessed decreased cell capacity to metabolize MTT and that this effect depended on the concentration of the compound in the media. Since P and HG did not affect MTT metabolization *per se*, we investigated if this effect was caused by the presence of the alkyl chains in the compounds. Individual alkyl acids did not affect MTT metabolization, except LA that decreased MTT reduction in a concentration-dependent manner. Therefore, it is possible to conclude that the effects observed for P- and HG-alkyl esters were caused by the entire molecule and cannot be ascribed separately to the hydrophilic or hydrophobic portions. Interestingly, these alkyl compounds have a maximal effect on MTT metabolization in a range of chain lengths of C_8–C_{10} for P series and C_6–C_{12} for HG series. At the highest alkyl chain length assessed (C_{18}) no toxic effects were evidenced. C_{18} acyl chains are the second most abundant species present in the phospholipids from Jurkat cells (Stulnig et al., 1998). Therefore, and in opposition to the shorter acyl chains that do not match the average length of phospholipids, the insertion of C_{18} alkyl ester into the bilayer will introduce minimal membrane perturbations.

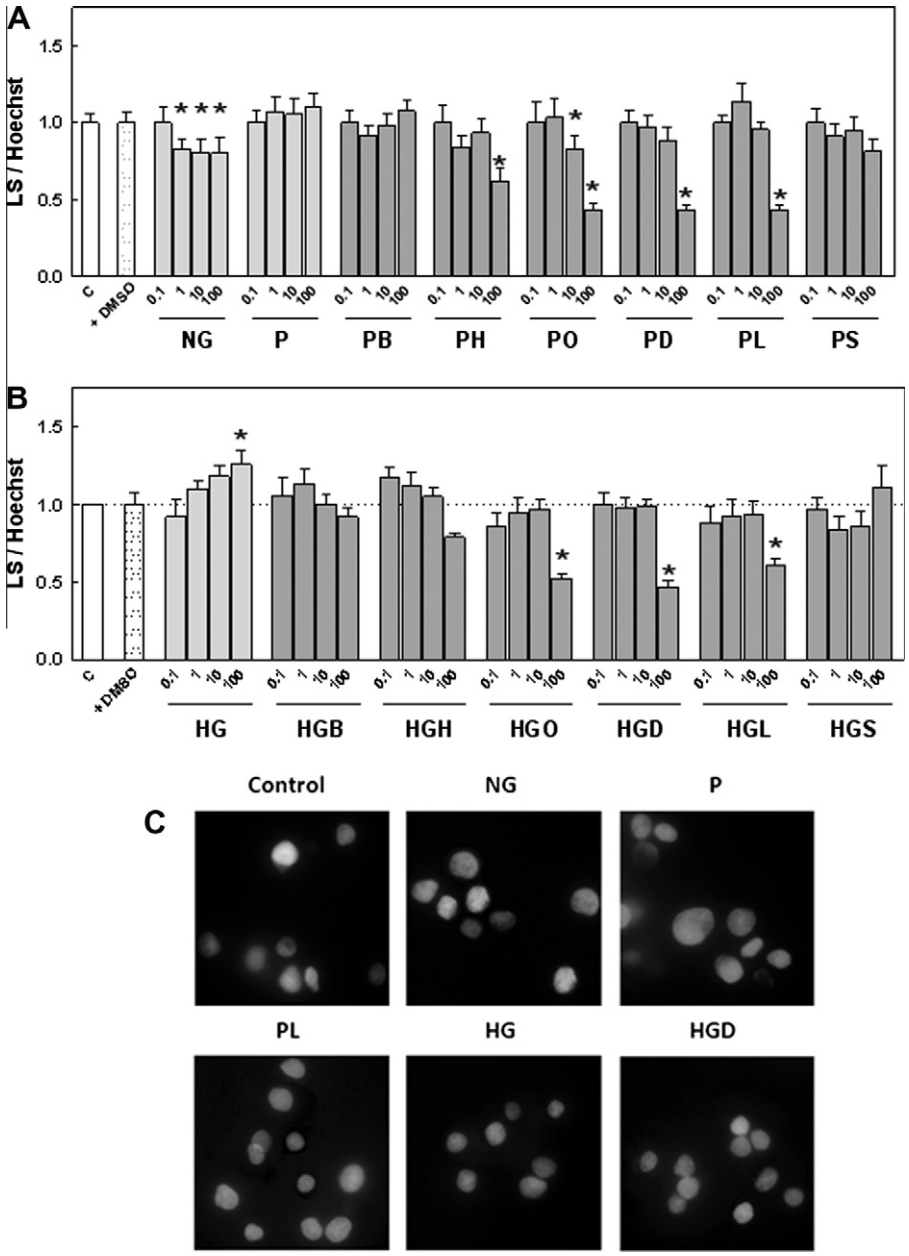

Fig. 6. Flavanones-alkyl esters cause cell shrinkage. Jurkat cells were incubated for 1 h at 37 °C in the presence of 0.1–100 μM of (A) NG, P or P-alkyl esters; (B) HG or HG-alkyl esters. After incubation, changes in cell size were evaluated in the samples from the variations in scattered light (LS) as indicated in Section 2.7. Results were corrected by DNA content measured from nuclear staining with the probe Hoechst 32258, and are shown as the mean ± SEM of four independent experiments. * Significantly different from the value measured in control (C) cells ($P < 0.05$, ANOVA). (C) Cells were incubated for 1 h at 37 °C in the presence of 100 μM NG, P, LP, HG or HGD and nuclei were stained with the probe Hoechst 32258 and observed by fluorescence microscopy (magnification 600×).

Since mitochondrial functionality strongly depends on the integrity of the plasma membrane, we evaluated the extent of the damage from the reaction between DNA and PI, a compound that only can reach cell nucleus upon plasma membrane damage. According to the observed in MTT reduction experiments, free alkyl acids did not affect the integrity of cell plasma membrane, exception made by LA that promoted cell lysis when present at 100 μM concentration. P- and HG-alkyl esters only displayed mild effects on plasma membrane integrity. Maximal cell lysis was achieved with 100 μM HGD and corresponded only to 21% decrease in the amount of intact cells. Therefore, cell lysis does not account for the major decrease in mitochondrial functionality observed in the current experimental system, and further experiments are required to determine the causes of such mitochondrial impairment. Considering that the exposure time of cells to these compounds was relatively short (1 h), it seems unlikely that they were able to reach mitochondria and cause a direct effect on the functionality of the electron transfer chain. Rather, it is plausible that once inserted in the plasma membrane they trigger certain membrane-associated signals that impact onto mitochondria (Nicholls, 2004).

The maintenance of plasma membrane potential is a key event in cellular homeostasis. In non-excitable cells such as Jurkat cells, plasma membrane hyperpolarization provides an electrochemical gradient that constitutes the driving force for Ca^{2+} influx from the extracellular space (Lewis, 2001). Sustained increase of Ca^{2+}

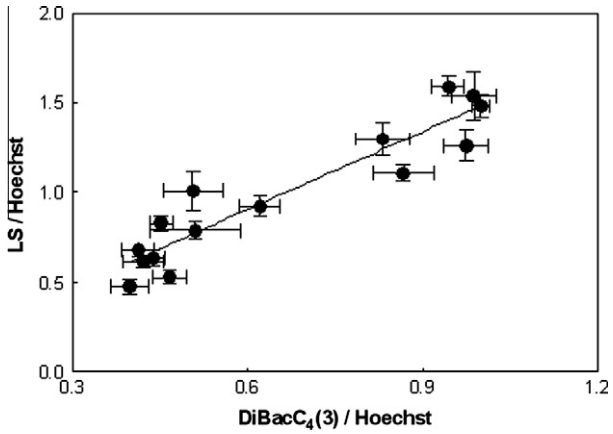

Fig. 7. Correlation between flavanones-mediated cell hyperpolarization and shrinkage. Data taken from Figs. 5 and 6 and corresponding to the value measured at 100 μM flavanones concentration.

concentration in cytoplasm is in turn a signal that triggers cell apoptosis (Orrenius et al., 2003). To evaluate if in the current experimental model P- and/or HG-alkyl esters modified the potential of the plasma membrane, the uptake of the fluorescent probe $DiBacC_4(3)$ was analyzed. This probe is water-soluble and crosses cell plasma membrane according to their transmembrane potential, with low intracellular fluorescence indicating a hyperpolarized state of the cell (Yamada et al., 2001). When cells were treated with NG, P or HG no significant changes in $DiBacC_4(3)$ uptake were evidenced, indicating that these compounds do not alter the potential of the plasma membrane. Conversely, the esters decreased the uptake of the probe, suggesting cell hyperpolarization. This effect of the esters was observed only for the highest concentration assessed. Even though cell lysis will result in lower probe uptake, $DiBacC_4(3)$ uptake decreased ~50% respect to control values, largely exceeding the maximal 20% cell lysis attained with these compounds.

As mentioned before, plasma membrane hyperpolarization determines Ca^{2+} influx. Among other effects, an increase of intracellular Ca^{2+} concentration stimulates Ca^{2+}-activated K^+ channels with the consequent net efflux of K^+. In order to maintain the intracellular potential, the efflux of Cl^- and water increases resulting in a contraction of cell volume (Reetz and Reiser, 1996). The flavanones assessed in this study showed differential effects on cell size. NG caused significant cell shrinkage but the glucosyl flavanones either had no effects on cell size (P) or even enlarged cells (HG). On the other hand, at a 100 μM concentration, all the esterified compounds reduced cell volume regardless their polar headgroup. A significant correlation between plasma membrane potential and cell size was observed. Although the mechanisms that mediate cell shrinkage in this model were not investigated, based on the observation that cells exposed to those alkyl compounds that caused the highest cell shrinkage maintained normal nuclear morphology, it is possible to discard that at that time cells were apoptotic. However, an alteration in the capacity of cells to regulate their volume can be suspected, involving an imbalance of Ca^{2+} and K^+ fluxes resulting in an overall efflux of water which ultimately may lead to apoptosis. This hypothesis deserves further investigations.

Working with artificial membranes, Arora et al. demonstrated that NG promotes membrane rigidification, an effect that was minimal at the bilayer surface (probe 6-AS) and progressively increased towards the innermost region of the bilayer (probes 12-AS and 16-AP) (Arora et al., 2000). This possibility was evaluated

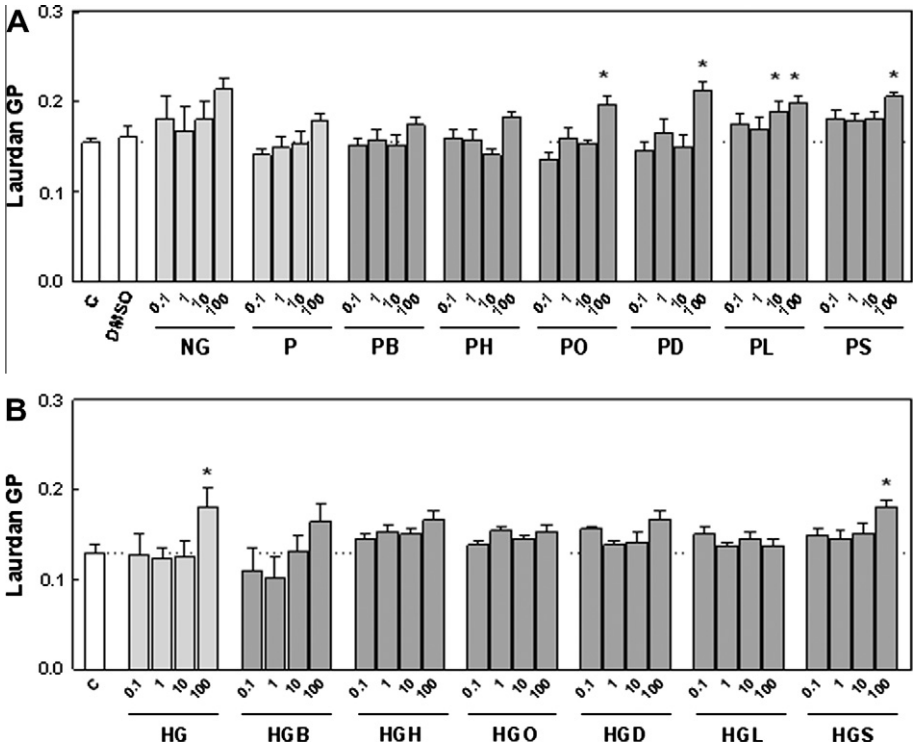

Fig. 8. Flavanones-alkyl esters cause slight cell plasma membrane dehydration. Jurkat cells were incubated for 1 h at 37 °C in the presence of 0.1–100 μM of (A) NG, P or P-alkyl esters; (B) HG or HG-alkyl esters. After incubation, cell plasma membrane hydration was evaluated from the changes in Laurdan generalized polarization (GP) as indicated in Section 2.9. Results are shown as the mean ± SEM of four independent experiments. * Significantly different from the value measured in control (C) cells ($P < 0.05$, ANOVA).

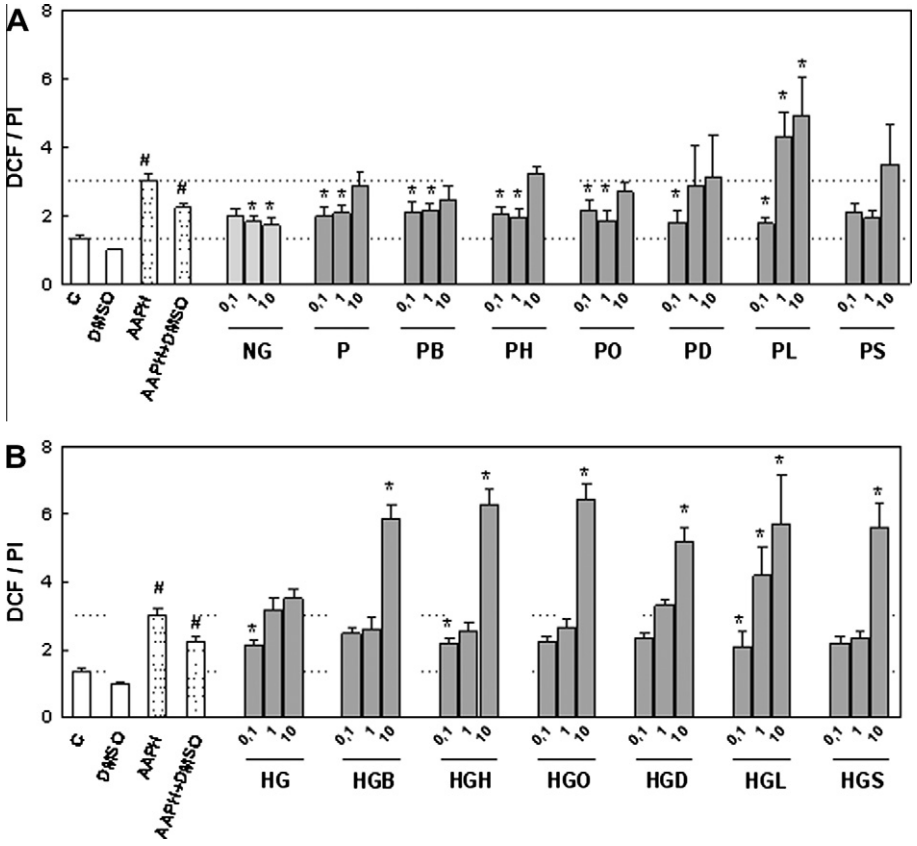

Fig. 9. Flavanones-alkyl esters enhance AAPH-mediated oxidative damage to cells. Jurkat cells were incubated for 1 h at 37 °C in the presence of 0.1–100 μM of (A) NG, P or P-alkyl esters; (B) HG or HG-alkyl esters. After incubation, AAPH-mediated oxidative damage was evaluated from increase in the fluorescence emission of the probe DCF as indicated in Section 2.10. Results were corrected by DNA content measured from nuclear staining with the probe propidium iodide (PI), and are shown as the mean ± SEM of five independent experiments. # Significantly different from the value measured in control (C) cells ($P < 0.005$, ANOVA). * Significantly different from the value measured in AAPH-treated cells ($P < 0.05$, ANOVA).

in our experimental model. In opposition to the previous findings in liposomes, NG did not alter cell membrane fluidity, regardless the depth of the bilayer investigated. Similarly, P, HG, and their corresponding alkyl esters did not affect plasma membrane fluidity. In spite of this, a slight although not significant dehydration of the plasma membrane was observed for the esters, as evidenced with the probe Laurdan. This probe senses the penetration of water molecules into its immediate surroundings (Parasassi and Gratton, 1995), a phenomenon that is regulated by the packing of lipids in the bilayer. In this sense, membrane dehydration implies a tighter packing of lipids and hence, a decrease in membrane fluidity. Alternatively, flavonoid moieties may reside at the water–lipid interface and limit the access of water molecules into the hydrophobic core of the bilayer. The lower fluidity evidenced with Laurdan seems to be in conflict with the results obtained with 6-AS, 12-AS and 16-AP; however, in our experience Laurdan is a highly sensitive probe and is able to detect minor changes in membrane fluidity that are undetectable with other probes (Buffone et al., 2009).

In the last decades flavonoids gained recognition as natural antioxidant compounds, with the capacity to prevent and/or ameliorate the oxidative damage to biological molecules in vitro and *in vivo* (for recent reviews see Galleano et al., 2010; Gulcin, 2012). On the basis of their chemical structure, Bors et al. (1990) defined three criteria to suspect a maximal antioxidant capacity in a given flavonoid, that involves the presence of (1) a catechol group (3′ and 4′ hydroxyl groups) in ring B, (2) 2–3 double bond in conjugation with a 4-keto function in ring C, and (3) 3- and 5-hydroxyl groups in rings A and C. As the studied compounds belong to the flavanone family, they only partially met these criteria because (1) they possess a phenolic group in ring B, (2) there is a single bond between positions 2 and 3, and (3) they lack the hydroxyl group in position 3 (see Fig. 1A). Therefore, high antioxidant capacities were not expected *a priori* for the studied compounds. On the other hand, it has been proposed that, under certain experimental conditions, flavonoids may act as pro-oxidants (Halliwell, 2008). The mechanism underlying this effect of flavonoids is still not completely elucidated but involves their interaction with molecular oxygen, rendering superoxide anion which can oxidize a second flavonoid moiety and generate H_2O_2. However, the first reaction is not thermodynamically favorable and thus the relevance of this mechanism in biological conditions may be limited (for review see Galleano et al., 2010).

In order to characterize the antioxidant effects of the flavonones and their alkyl esters in Jurkat cells, their behavior against two different oxidant molecules were analyzed. The first oxidant investigated was the azo-compound AAPH. This is a water-soluble compound that upon thermal decomposition generates a constant flux of alkylperoxyl radicals ($ROO^{\cdot}$) (Rojas Wahl et al., 1998). In turn, $ROO^{\cdot}$ react with biological molecules, p. e. lipids, proteins and nucleic acids, rendering oxidized derivatives that might lose their functions and/or acquire new ones. It has been demonstrated that during the first 5 h of incubation at 40 °C (pH 7.2) the concentration of AAPH only decreases in 15% (Rojas Wahl et al., 1998), and therefore, its concentration can be considered as constant during the incubation period used for the current experiments.

Experiments were performed in the flavanones range of concentration 0.1–10 μM where the compounds did not affect cell integ-

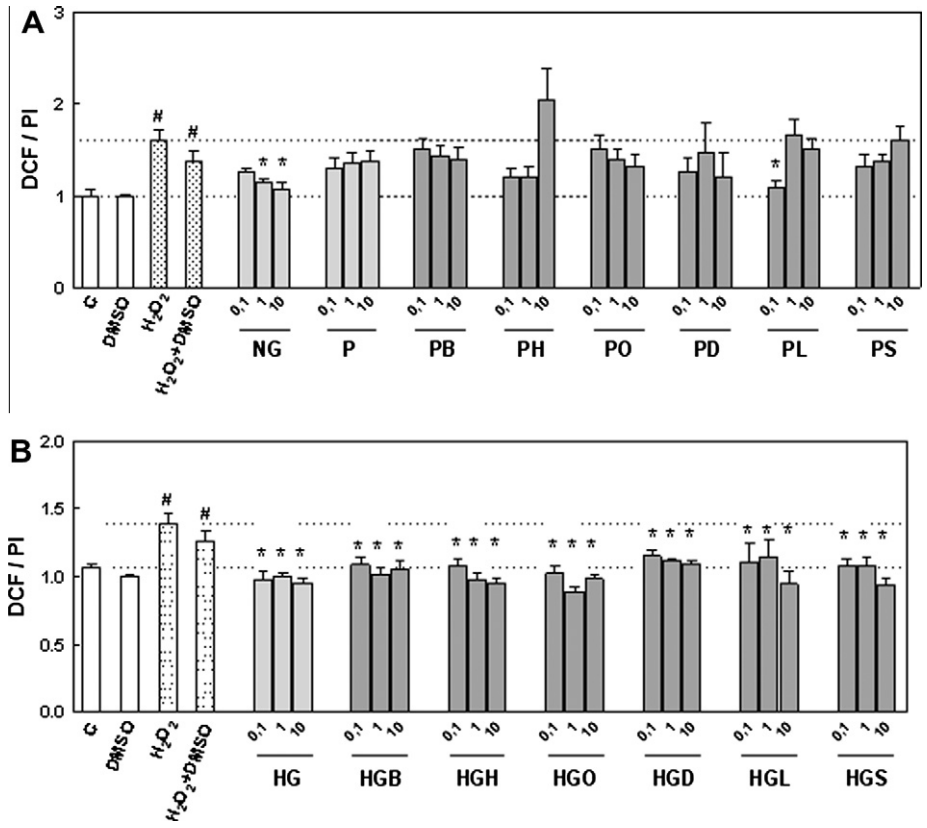

Fig. 10. Flavanones-alkyl esters prevent H_2O_2-mediated oxidative damage to cells. Jurkat cells were incubated for 1 h at 37 °C in the presence of 0.1–100 μM of (A) NG, P or P-alkyl esters; (B) HG or HG-alkyl esters. After incubation, H_2O_2-mediated oxidative damage was evaluated from increase in the fluorescence emission of the probe DCF as indicated in Section 2.10. Results were corrected by DNA content measured from nuclear staining with the probe propidium iodide (PI), and are shown as the mean ± SEM of five independent experiments. # Significantly different from the value measured in control (C) cells ($P < 0.005$, ANOVA). * Significantly different from the value measured in H_2O_2-treated cells ($P < 0.05$, ANOVA).

rity. As expected from the analysis of their chemical structure, the flavanones NG and P have a similar behavior in protecting cells from AAPH-mediated oxidative damage. However, when assessed at 10 μM concentration, P lost its capacity to act as an antioxidant, suggesting a biphasic behavior based on its concentration. This feature was conserved along the P-series with PL having the maximal pro-oxidant effect. As found for P, HG effects inversely related to its concentration in the media, a characteristic that was conserved along the HG family. To notice, at 10 μM concentration, all HG derivatives assessed acted as strong pro-oxidants, causing a ~2-fold enhancement of AAPH effect on the content of cytoplasmic oxidants. On the other hand, being AAPH a water-soluble compound and used at a relatively low concentration, the impact on lipid peroxidation was minimal in this system. In addition, none of the compounds displayed a pro-oxidant effect.

The second oxidant evaluated was H_2O_2. This is a small, diffusible molecule that passively enters cells and that is unable to oxidize other macromolecules *per se*, requiring the presence of a peroxidase or a peroxidase-like molecule to exert its oxidant actions. In this model, H_2O_2 did not promote C_{11}-BODIPY but DCDCDHF oxidation, an effect that was partially (P series) or fully (HG series) prevented by all the compounds assessed. The different pattern of effects found upon cell treatment with AAPH or H_2O_2 may be related to the capacity of the hydroxyl group in position 5 in ring A to be oxidized. The oxidation of this particular phenolic group by peroxidases and/or heme-containing enzymes renders a phenoxyl radical that can co-oxidize other macromolecules (Galati et al., 2002). Working with the chromophore ABTS (2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid)), which upon oxidation renders the radical $ABTS^{\cdot+}$, Chan et al. (2003) demonstrated that NG acts both as a pro-oxidant and antioxidant depending on its concentration in the media. This pro-oxidant capacity of NG was ascribed to the generation of a phenoxyl radical in position 5 of ring A capable to enhance ABTS oxidation (Chan et al., 2003). If this mechanism is operative in our experimental model, flavanones-alkyl esters might differentially respond to a given oxidative insult, based not only on the nature of the oxidant but also on the potential chemical modifications that these compounds may undergo.

5. Conclusions

The alkyl esters of the glucosyl flavanones P and HG are hydrophobic compounds that are able to interact with biological membranes. After a short-term (1 h) exposure and within the concentration range 0.1–10 μM, these compounds seem to be innocuous for cultured Jurkat cells, but at higher concentrations certain evidences of cell toxicity were found. Even when the insertion of the esters into cell plasma membrane did not affect their biophysical properties, they caused cell hyperpolarization through still non-elucidated mechanisms. In addition, these compounds may act either as antioxidants or pro-oxidants, depending on the source of the oxidative insult. Further experiments are required to establish the long-term harmlessness of these compounds to humans.

Conflict of interest

The authors declare no conflict of interest.

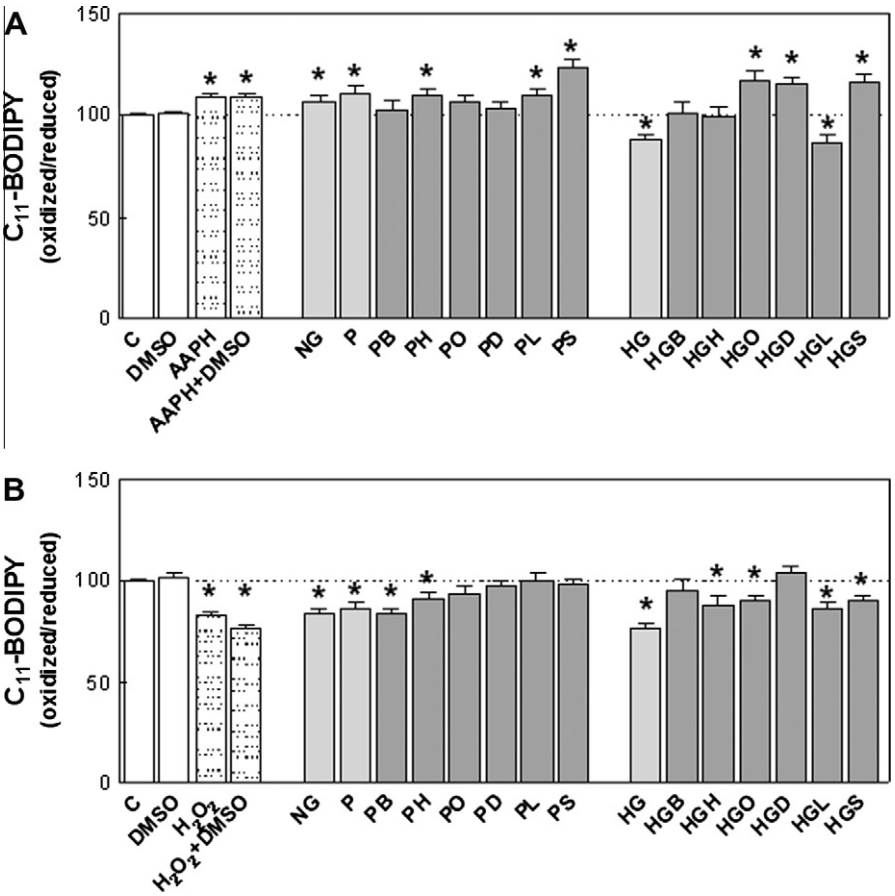

Fig. 11. Effects of flavanones-alkyl esters on H_2O_2- or AAPH-mediated lipid oxidation. Jurkat cells were labeled with 2 μM C_{11}-BODIPY and further incubated for 1 h at 37 °C in the presence of 10 μM of NG, P or P-alkyl esters, HG or HG-alkyl esters. After incubation, (A) AAPH- and (B) H_2O_2- mediated oxidative damage was evaluated from the increase in the oxidized to reduced C_{11}-BODIPY fluorescence ratio as indicated in Section 2.10.2. Results are shown as the mean ± SEM of five independent experiments. * Significantly different from the value measured in control cells ($P < 0.01$, ANOVA).

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fct.2013.01.011.

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