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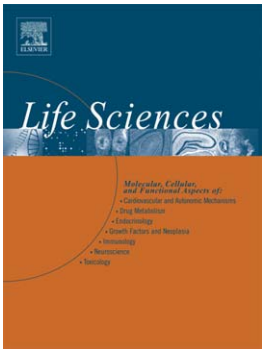

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Phenolic compounds protect HepG2 cells from oxidative damage: Relevance of glutathione levels

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Abstract

In the present work, the potential hepatoprotective effects of five phenolic compounds against oxidative damages induced by *tert*-butyl hydroperoxide (*t*-BHP) were evaluated in HepG2 cells in order to relate in vitro antioxidant activity with cytoprotective effects. *t*-BHP induced considerable cell damage in HepG2 cells as shown by significant LDH leakage, increased lipid peroxidation, DNA damage as well as decreased levels of reduced glutathione (GSH). All tested phenolic compounds significantly decreased cell death induced by *t*-BHP (when in co-incubation). If the effects of quercetin are given the reference value 1, the compounds rank in the following order according to inhibition of cell death: luteolin (4.0) > quercetin (1.0) > rosmarinic acid (0.34) > luteolin-7-glucoside (0.30) > caffeic acid (0.21). The results underscore the importance of the compound's lipophilicity in addition to its antioxidant potential for its biological

activity. All tested phenolic compounds were found to significantly decrease lipid peroxidation and prevent GSH depletion induced by *t*-BHP, but only luteolin and quercetin significantly decreased DNA damage. Therefore, the lipophilicity of the natural antioxidants tested appeared to be of even greater importance for DNA protection than for cell survival. The protective potential against cell death was probably achieved mainly by preventing intracellular GSH depletion. The phenolic compounds studied here showed protective potential against oxidative damage induced in HepG2 cells. This could be beneficial against liver diseases where it is known that oxidative stress plays a crucial role.

Keywords: Phenolic compounds; Liver; Oxidative stress; HepG2 cells; *tert*-butyl hydroperoxide; Antioxidants

Introduction

An overall increase in cellular levels of reactive oxygen species (ROS) above the cells' defenses results in oxidative stress that can ultimately cause cell death. Oxidative stress has been recognized to be involved in the etiology of several age-related and chronic diseases such as cancer, diabetes, neurodegenerative and cardiovascular diseases (Tiwari, 2004; Cui et al., 2004; Ceriello and Motz, 2004; Klaunig and Kamendulis, 2004; Willcox et al., 2004; Ballinger, 2005; Gibson and Huang, 2005). In particular with respect to liver diseases such as hepatocellular carcinoma, viral and alcoholic hepatitis and non-alcoholic steatosis, it is known that ROS and reactive nitrogen species play a crucial role in disease induction and progression (Adachi and Ishii, 2002; Loguercio and Federico, 2003; Vitaglione et al., 2004). The liver is particularly susceptible to toxicants since the portal vein brings blood to this organ after intestinal absorption. The absorbed drugs and xenobiotics in a concentrated form can cause ROS- and free radical-mediated damage that may result in inflammatory and fibrotic processes (Jaeschke et al., 2002).

Because oxidative stress plays a central role in liver diseases pathology, dietary antioxidants have been proposed as therapeutic agents to counteract liver damage (Vitaglione et al., 2004). This same idea has also been suggested for other oxidative stress-based chronic diseases (Tiwari, 2004; Willcox et al., 2004). In fact, several epidemiological studies have shown that diets rich in fruit and vegetables and other plant foods (including tea and wine) are associated with a decreased risk of premature death and mortality from chronic diseases, such as cardiovascular diseases and some types of cancer (Stanner et al., 2004; Scalbert et al., 2005). Phenolic compounds (PhC), and in particular polyphenols, are believed to be, at least in part, responsible for such effects. Results from some human clinical trials support the role of these compounds in prevention of some

chronic diseases (Ren et al., 2003; Spencer et al., 2004; Tiwari, 2004; Willcox et al., 2004; Scalbert et al., 2005).

Today much is known about the chemistry and antioxidant potential of PhC as a result of in vitro chemical and sub-cellular studies (Rice-Evans et al., 1997; Croft, 1998). However, besides their strong free radical scavenging activity, PhC can also act as antioxidants by chelating metal ions, preventing radical formation, and indirectly by modulating enzyme activities and altering the expression levels of important proteins, such as antioxidant and detoxifying enzymes (Ferguson, 2001; Ross and Kasum, 2002; Ferguson et al., 2004). Few studies, however, address the biological effects of PhC, and the ones performed using cellular and in vivo models indicate a poor correlation between the antioxidant potency of PhC measured in vitro and the compound's biological activity. The biological effect of PhC and their in vivo circulating metabolites will ultimately depend on their cellular uptake and/or the extent to which they associate with cell membranes (Spencer et al., 2004).

HepG2 cells, a human hepatoma cell line, are considered a good model to study in vitro xenobiotic metabolism and toxicity to the liver, since they retain many of the specialized functions which characterize normal human hepatocytes (Knasmuller et al., 1998). In particular, HepG2 cells retain the activity of many phase I, phase II and antioxidant enzymes ensuring that they constitute a good tool to study cytoprotective, genotoxic and antigenotoxic effects of compounds (Knasmuller et al., 2004; Mersch-Sundermann et al., 2004). Recently, studies of cytoprotection by natural antioxidants in HepG2 cells have increasingly been using *tert*-butyl hydroperoxide (*t*-BHP), an organic hydroperoxide, as the toxic agent (Thabrew et al., 1997; Kinjo et al., 2003; Mersch-Sundermann et al., 2004; Lee et al., 2005a, 2005b; Alia et al., 2006). *t*-BHP can be metabolized in the hepatocyte by glutathione peroxidase, generating oxidized glutathione (GSSG) (Sies and Summer, 1975;

87 Rush et al., 1985). GSSG is converted back to reduced glutathione (GSH) at the expense of
88 NADPH by glutathione reductase (GR). Depletion of GSH and NADPH oxidation are
89 associated with altered calcium homeostasis, leading to loss of cell viability (Bellomo et al.,
90 1982; Martin et al., 2001). Alternatively, *t*-BHP can be converted into its peroxyl and
91 alkoxyl free radicals by cytochrome P450 enzymes and by free iron-dependent reactions.
92 These free radicals can subsequently initiate lipid peroxidation, form covalent bonds with
93 cellular molecules (such as DNA and proteins) and further decrease GSH levels. The latter
94 effect, in addition to altering calcium homeostasis, affects mitochondrial membrane
95 potential, eventually causing cell death (Rush et al., 1985; Nicotera et al., 1988; Masaki et
96 al., 1989; Davies, 1989; Buc-Calderon et al., 1991; Kass et al., 1992; VanderZee et al.,
97 1996; Hix et al., 2000).
98 In this study we evaluate hepatoprotective effects of PhC against *t*-BHP-induced oxidative
99 damage in HepG2 cells, in order to relate in vitro antioxidant activity with cytoprotective
100 effects. Two phenolic acids, caffeic acid and rosmarinic acid (an ester of caffeic acid and
101 3,4-dihydroxyphenyllactic acid), and three flavonoids, luteolin (flavone), luteolin-7-
102 glucoside (flavone glycoside) and quercetin (flavonol), were used (Fig. 1). Firstly, the
103 concentrations of PhC that protected by 50% (IC_{50}) against *t*-BHP-induced cell death were
104 determined. Based on the IC_{50} values for each compound, biological activity was related to
105 both antiradical efficiency and hydrophobicity. Subsequently, IC_{80} values, a concentration
106 that effectively protects 80% of the cells against *t*-BHP-induced cell death, were used to
107 evaluate the effects of each compound on several markers of oxidative damage, such as
108 intracellular glutathione, lipid peroxidation, glutathione-related enzyme such as
109 glutathione-S-transferase (GST), GR and glutathione peroxidase (GPox), as well as on
110 DNA damage. The relative importance of effects of PhC on these parameters to protection
111 against *t*-BHP-induced cell death is discussed.

Materials and methods

Chemicals

Minimum Essential Medium Eagle (MEM), *tert*-butyl hydroperoxide, quercetin, rosmarinic acid, caffeic acid and Bradford reagent were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was obtained from Biochrom KG (Germany). Luteolin and luteolin-7-*O*-glucoside were purchase from Extrasynthese (Genay, France). All other reagents were of analytical grade.

Cell culture

HepG2 cells (hepatocellular carcinoma cell line), obtained from the American Type Culture Collection (ATCC), were maintained in culture in 75 cm^2 polystyrene flasks (Falcon) with MEM containing 10% FBS, 1% antibiotic-antimycotic solution, 1 mM sodium pyruvate and 1.5 g/l sodium bicarbonate under an atmosphere of 5% CO_2 at 37ºC.

Assay for t-BHP cytotoxicity and protection by phenolic compounds

HepG2 cells were plated in 24-multiwell culture plates at 2.5×10^5 cells per well. To study *t*-BHP cytotoxicity, forty hours after plating, the medium was discarded and fresh medium containing *t*-BHP at various concentrations was added. At different time points, cellular viability was determined by the MTT assay (Mosmann, 1983) and by lactate dehydrogenase (LDH) leakage assay (Lima et al., 2005). In order to determine the concentration of PhC that protects 50% of the cells from damage induced by the toxicant (IC_{50}), cells were incubated with 2 mM of *t*-BHP for 5 h to induce significant cell death. The prevention of LDH leakage (cell death) was measured in co-incubations with PhC dissolved in DMSO (1% v/v final concentration, controls with DMSO only) at several

concentrations. The IC_{50} and the Hill slope – the slope of the PhC concentrations (in logarithm) plotted versus cell death protection relative to the control (2 mM *t*-BHP, 5 h) – were calculated graphically using a computer program (GraphPad Prism, version 4.00, GraphPad Software Inc.). Based on the dose–response curves of cell death protection by PhC against the *t*-BHP-induced oxidative damage in HepG2 cells, the IC_{80} concentrations were estimated and used in the following experiments to evaluate the protective potential of the compounds on several cellular parameters.

Evaluation of the effects of t-BHP and PhC at the IC_{80} concentration on lipid peroxidation, glutathione levels and glutathione-related enzyme activities in HepG2 cells

HepG2 cells were plated in 6-multiwell culture plates at 7.5×10^5 cells per well. Forty hours after plating, the medium was discarded and fresh medium containing 2 mM *t*-BHP and/or the IC_{80} concentration of each PhC was added. Five hours later, cell culture medium and cell scrapings were harvested and kept at -80ºC for following quantification of several parameters. Cell scrapings were harvested in lysis buffer (25 mM KH_2PO_4, 2 mM $MgCl_2$, 5 mM KCl, 1 mM EDTA, 1 mM EGTA, 100 µM PMSF, pH 7.5) after rinsing the cells with PBS (137 mM NaCl, 2.7 mM KCl, 8.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4).

Evaluation of the effects of t-BHP and PhC at the IC_{80} concentration on DNA damage in HepG2 cells

HepG2 cells were plated in 6-multiwell culture plates at 5×10^5 cells per well. To study *t*-BHP-induced DNA damage, 16 h after plating, the medium was discarded and fresh medium containing *t*-BHP at various concentrations was added. After 1 hour of incubation, cells were rinsed in warm PBS and then incubated for 5 min with 0.125% (w/v) trypsin in PBS. The cells were then harvested in PBS to be used in the alkaline version of the comet

assay for evaluation of DNA damage. To study the protective potential of PhC at IC_{80} concentration on *t*-BHP-induced DNA damage, cells were incubated with 200 µM *t*-BHP for 1 h to induce significant DNA damage. For that, sixteen hours after plating, the medium was discarded and fresh medium containing 200 µM *t*-BHP and/or the IC_{80} concentration of each PhC was added to the cells. After 1 h incubation, cells were treated as above to carry out the comet assay.

Comet assay

The single cell gel electrophoresis (comet) assay was performed based on previous descriptions (Klaude et al., 1996; Uhl et al., 1999, 2000) with slight modifications. Briefly, 40,000 cells in PBS were centrifuged (80 ×*g*, 2 min), the pellet was mixed with 100 µl of low melting agarose 0.5% (w/v) in PBS, at 37ºC and spread on agarose coated slides. The agarose was allowed to set at 4ºC for 10 min, and then the slides were immersed in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10 with NaOH, triton X-100 1% v/v added fresh) at 4ºC for 2 h. After being rinsed with distilled water, the slides were immersed in a horizontal electrophoresis tank with electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH >13) at 4ºC and exposed for 40 min to allow alkaline unwinding. Afterwards, electrophoresis was carried out under alkaline conditions for 20 min, 300 mA, at 0.8 V/cm in a cold room (4ºC). Finally, the slides were neutralized by washing three times for 5 min each with 0.4 M Tris, pH 7.5, at 4ºC, fixed with methanol and kept at 4ºC until evaluation. For analysis of the comet images, the DNA was stained with ethidium bromide and scored under a fluorescent microscope using a computer assisted image analysis system and/or a visual scoring method avoiding analyzing cells at the edges of the gel. The computer image analyses were done using a public domain image-analysis program – NIH image (Helma and Uhl, 2000), and the results expressed in terms of tail

length, tail moment and % DNA in tail of 50 cells in 4 independent experiments. In the semiquantitative method of visual scoring, the comet images were classified in five classes according to the intensity of fluorescence in the comet tail, attributing a value of 0, 1, 2, 3 or 4 from undamaged to maximal damage. In this way, the total score for 100 images can range from 0 (all undamaged) to 400 (all maximally damaged, giving the overall DNA damage of the cell population expressed in arbitrary units (Duthie and Dobson, 1999; Duthie, 2003).

Biochemical analyses

Lipid peroxidation

The extent of lipid peroxidation was estimated by the levels of malondialdehyde measured using the thiobarbituric acid reactive substances (TBARS) assay at 535 nm following a methodology previously described (Lima et al., 2005). The results are expressed as nmol/mg of protein using a molar extinction coefficient of 1.56×10^5 $M^{-1}cm^{-1}$.

Glutathione levels

The glutathione levels from the cell cultures were determined by the DTNB-GSSG reductase recycling assay as previously described (Anderson, 1985), with some modifications (Lima et al., 2004). The results are expressed as nmol GSH/mg of protein.

Glutathione-related enzyme activities

For measurement of the glutathione-related enzyme activities, the cell scraping homogenates were centrifuged at 10,000 ×*g* for 10 min at 4ºC and the supernatant collected.

GST and GR activities were measured spectrophotometrically at 30ºC as previously described (Lima et al., 2005) and the results expressed in nmol/min/mg protein (mU/mg).

The selenium-dependent and -independent GPox activity was assayed as previously described (Martin-Aragon et al., 2001) with some modifications. Briefly, GPox activity was measured at 30ºC following NADPH oxidation at 340 nm on a plate reader spectrophotometer (Spectra Max 340pc, Molecular Devices, Sunnyvale, CA, USA) in the presence of 1 mM GSH, 0.18 mM NADPH, 1 mM EDTA, 0.5 U/ml GR and 0.7 mM *t*-BHP in 50 mM imidazole (pH 7.4). The activity was expressed as nmol of substrate oxidized per minute per mg of protein (mU/mg).

Protein

Protein content was measured with the Bradford Reagent purchased from Sigma using bovine serum albumin as a standard.

Antiradical activity

The free radical scavenging (antiradical) activity of PhC was studied against two radicals: the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH?) and the superoxide radical. For DPPH scavenging activity, after addition of different concentrations of PhC to DPPH (90 µM), the percentage of remaining DPPH was determined at different times from the absorbance at 515 nm using a plate reader spectrophotometer. As suggested by Sanchez-Moreno and collaborators (Sanchez-Moreno et al., 1998), the amount of antioxidant necessary to decrease by 50% the initial DPPH concentration (IC_{50}) was expressed in terms of initial concentration of DPPH to make the results easier comparable with other published results. However, we put the results of the PhC in terms of moles instead of grams to better relate the results with the chemical structures (Fig. 1) of the PhC studied. We also calculated the parameter antiradical efficiency (AE) (Sanchez-Moreno et al., 1998) using the estimated T_{IC50} – time needed to reach the steady state at the corresponding IC_{50} concentration, where $AE = 1/(IC_{50} \times T_{IC50})$. Finally, a new parameter is also shown –

the Hill slope, the graphically calculated slope from the plotted PhC concentration (in logarithm) versus the remaining DPPH concentration (GraphPad Prism). The higher this value, the narrower the concentration range from 0 to 100% of antiradical activity. This graph was also used to calculate the IC_{50} of each compound.

The superoxide radical scavenging activity was determined using the phenazine methosulphate-NADH nonenzymatic assay as previously described (Valentao et al., 2001). As for DPPH assay, we also show the Hill slope from the graphics used to calculate the IC_{50} (GraphPad Prism).

Measurement of the partition coefficients

The degree of hydrophobicity of the PhC was examined by measuring the partition coefficients taken in logarithm using an n-octanol/HEPES system (K_{ow}) as previously described (Areias et al., 2001), at ambient temperature (~25ºC).

Statistical analysis

Data are expressed as means ± SEM. Statistical significances were determined using a one-way ANOVA followed by the Student-Newman-Keuls post-hoc test. *P* values = 0.05 were considered statistically significant.

Results

t-BHP cytotoxicity

The cytotoxicity of *t*-BHP to liver cells has been extensively studied although its mechanisms of action have not been totally established (Sies and Summer, 1975; Cadenas and Sies, 1982; Bellomo et al., 1982; Rush et al., 1985; Jewell et al., 1986; Nicotera et al., 1988; Masaki et al., 1989; Davies, 1989; Buc-Calderon et al., 1991; Kass et al., 1992; Hix

et al., 2000; Martin et al., 2001). Recently, HepG2 cells have been used to study the hepatotoxicity of *t*-BHP (Kim et al., 1998, 2000; Piret et al., 2002, 2004; Alia et al., 2005), and this model suggested to evaluate the protective properties of natural compounds and plant extracts against oxidative damages (Thabrew et al., 1997; Kinjo et al., 2003; Lee et al., 2005a, 2005b; Alia et al., 2006). However, because the cell's response to *t*-BHP depends on culture conditions, we first studied HepG2 cells' response to *t*-BHP dose (1 mM and 2 mM) and incubation time (1-16 h) by measuring LDH leakage and by the MTT assay (Fig. 2). All our experiments with HepG2 cells were done with a culture medium containing 10% (v/v) FBS. LDH leakage and MTT assay gave similar results for effects on cell viability in response to *t*-BHP at both studied concentrations (Fig. 2). In the subsequent studies, cell incubations were performed with 2 mM of *t*-BHP for 5 h to induce 40-50% of cell death (Fig. 2) and used to evaluate the hepatoprotective potential of PhC against this oxidant insult.

Potential hepatoprotective effects of the PhC against t-BHP-induced toxicity in HepG2 cells

The potential hepatoprotective effects of the five PhC against the *t*-BHP-induced toxicity (2 mM, 5 h) was evaluated by determining protection of cell viability, as measured by LDH leakage (Fig. 3) in HepG2 cells. From the graphically computed values (Fig. 3), IC_{50} and Hill slope values for each compound were obtained (Table 1). As shown in Table 1, of the tested compounds, luteolin had the highest protective activity against *t*-BHP-induced toxicity. The glycosylation of the hydroxyl group at position 7, present in luteolin-7-glucoside, significantly decreased both IC_{50} and Hill slope (Table 1). Quercetin, the flavonol of luteolin, in addition to a higher IC_{50} also had a lower Hill slope, indicating a lower hepatoprotective potential when compared to the flavone. Rosmarinic and caffeic

286 acids had lower protective potentials against the oxidant insult to HepG2 cells when
287 compared to the flavonoids – higher IC_{50} values and lower Hill slopes (Table 1).
288 Comparing the phenolic acids (Table 1), the polyphenol rosmarinic acid had higher
289 hepatoprotective potential than caffeic acid, which correlates well with the presence of one
290 more ortho dihydroxy phenolic structure (Fig. 1).
291 Based on the dose–response curves of protection from cell death, the PhC IC_{80}
292 concentrations were extrapolated (Table 1) and used to evaluate the effects of each
293 compound against *t*-BHP-induced oxidative injuries in HepG2 cells in terms of lipid
294 peroxidation, glutathione levels, glutathione-related enzyme activities and DNA damage.
295 The level of protection of cell viability obtained for each compound was correlated with
296 the effect on each of the several parameters outlined above.
297
298 *Effects of the t-BHP and PhC at the IC_{80} concentration on lipid peroxidation, glutathione*
299 *levels and glutathione-related enzyme activities in HepG2 cells*
300 The incubation of HepG2 cells with 2 mM *t*-BHP for 5 h decreased cell viability by 40-
301 50% (Fig. 2), along with a significant increase in lipid peroxidation and GSSG levels
302 (Table 2), as well as a decrease in GSH levels (Fig. 4). The toxicant also significantly
303 decreased the GR and GPox activities and had no significant effect on GST activity (Table
304 3).
305 All the PhC tested at IC_{80} concentration decreased significantly the *t*-BHP-induced
306 increase in lipid peroxidation (Table 2), caffeic acid being the most powerful with a 35%
307 reduction and the weakest being luteolin-7-glucoside with a 25% reduction. None of the
308 PhC significantly changed lipid peroxidation and GSSG levels in cells incubated alone
309 (without *t*-BHP) for 5 hours. As shown in Table 2, all the compounds reduced the *t*-BHP-
310 induced increases in GSSG levels, but the effect was significant only for rosmarinic acid.

311 The decrease in the GSH levels induced by *t*-BHP was significantly attenuated by all of the
312 PhC (Fig. 4). Luteolin-7-glucoside showed the best protective effect (81%) against the *t*-
313 BHP-induced decrease in GSH levels, followed by luteolin (53%), quercetin (40%), caffeic
314 acid (36%) and rosmarinic acid (34%). When HepG2 cells were incubated alone with the
315 PhC for 5 h, rosmarinic acid and the three tested flavonoids slightly decreased basal GSH
316 levels, although not significantly (Fig. 4). When this effect is taken into consideration,
317 luteolin-7-glucoside almost completely prevented the decrease of GSH induced by the
318 toxicant.
319 When incubated alone with HepG2 cells, luteolin-7-glucoside decreased significantly the
320 GST activity by 17% (Table 3). As observed in Table 3, the *t*-BHP-induced decreases in
321 GR and GPox activities were only slightly attenuated by the PhC, and only quercetin
322 showed a significant protective effect (19%) on GPox activity.
323
324 *t-BHP-induced DNA damage in HepG2 cells*
325 The extent of DNA damage produced by 1-hour incubations with increasing concentrations
326 of *t*-BHP were determined by the comet assay and the images analyzed both by computer
327 assisted program and visual scoring. This model of *t*-BHP-induced DNA damage in
328 HepG2 cells has been used by other authors (Woods et al., 1999, 2001). As stated
329 previously, due to effects of culture conditions a dose–response to *t*-BHP on DNA damage
330 was studied. As shown in Figure 5, *t*-BHP concentrations of 200 µM and higher result in
331 significant DNA damage as visualized by the comet assay. The semiquantitative method of
332 visual scoring used has been extensively validated by comparison with computerized
333 image analysis systems and correlates well with more quantitative measures, such as %
334 DNA in the tail and tail moment (Duthie, 2003). Our results also showed good correlations
335 between the semiquantitative method and the parameters given by computer analysis

system (Fig. 5E). To evaluate the effect of the PhC at IC_{80} concentration on *t*-BHP-induced DNA damage, HepG2 cells were co-incubated for 1 h with the different PhC plus 200 µM *t*-BHP, and the DNA damage was assessed using the alkaline version of the comet assay (results scored using the semiquantitative method). Incubation conditions of 1 h with 200 µM *t*-BHP were chosen to test the protective effects of PhC because intermediate damage to the DNA was produced (~200 AU).

Effects of PhC at the IC_{80} concentration on t-BHP-induced DNA damage in HepG2 cells

Of the PhC tested, quercetin and luteolin conferred the best protection against *t*-BHP-induced DNA damage (Fig. 6). Even if the IC_{80} concentration for luteolin is 4 times lower than that for quercetin (Table 1), luteolin gave better protection than the flavonol (76% and 58%, respectively) (Fig. 6). Both quercetin (Fig. 7) and luteolin (data not shown) showed a concentration-dependent DNA protection. As shown in Figure 7B, the protective effect of quercetin was visually clear in the comet assay images. Rosmarinic acid (14%) and luteolin-7-glucoside (18%) also protected significantly from DNA damages, although to a much lower extent. At IC_{80} concentration, caffeic acid did not show protection of the DNA. None of the PhC tested induced DNA damage when incubated alone for 1 h at IC_{80} concentration (Fig. 6).

Antiradical activity

The antiradical activity of the PhC used in this study was evaluated by the DPPH and superoxide radical scavenging assays. Figure 8 shows graphically the results from the DPPH scavenging assay of caffeic acid as an example, which was used to calculate the IC_{50} and the Hill slope for the compound. Rosmarinic acid had the best IC_{50} values both against DPPH and superoxide radicals (Tables 4 and 5). The IC_{50} values in both antiradical

activity assays for caffeic acid were, as expected, significantly higher than those for rosmarinic acid, but both compounds showed similar Hill slopes. Quercetin presented lower IC_{50} values than the other flavonoids against both radicals (Tables 4 and 5). On the other hand, quercetin had the lowest AE (Table 4). Comparing luteolin with its glucoside, the aglycone had a slightly lower IC_{50} and a higher Hill slope against both radicals. In the case of the DPPH scavenging activity, the higher AE value with a similar IC_{50} means that for luteolin-7-glucoside the time needed for it to reach the steady state at the corresponding IC_{50} concentration was shorter than for luteolin.

The antiradical activity of some of these PhC has been extensively studied by many authors (Sanchez-Moreno et al., 1998; Moridani et al., 2003; Butkovic et al., 2004; Parejo et al., 2004; Kosar et al., 2004), and our results are, in general, in agreement with theirs.

Partition coefficients

The degree of hydrophobicity of the PhC was examined by measuring the partition coefficients using an n-octanol/HEPES system. Flavonoids are much more hydrophobic than phenolic acids (Table 6). As expected, the glycosylation of the hydroxyl group at position 7 of luteolin decreased considerably the degree of hydrophobicity of this compound. Luteolin had a slightly higher PC than that of quercetin (Table 6). The experimentally determined hydrophobicity of these two flavonoids has often been referred in the literature, but the results are controversial. Some authors describe luteolin as more hydrophobic than quercetin (Brown et al., 1998; Areias et al., 2001; Murata et al., 2004) whereas others hold the opposite to be true (Moridani et al., 2003). The computer program that can be accessed at http://www.esc.syrres.com, the KowWin (LogKow) software, gives a lower degree of hydrophobicity for quercetin than for luteolin, 1.48 and 2.36, respectively. This program uses fragmental analysis of the compound's structure for the

prediction and the computed values show usually a high correlation with quoted experimental values ($r^2 = 0.95$).

Discussion

The present work demonstrates that all the tested PhC possess protective effects against *t*-BHP-induced cell death in HepG2 cells. Conferred protection decreased in the following order: luteolin > quercetin > rosmarinic acid > luteolin-7-glucoside > caffeic acid as shown by IC_{50} values. Considering the compounds' hydrophobicity (luteolin > quercetin > luteolin-7-glucoside > rosmarinic acid > caffeic acid) and the antiradical activity evaluated both for DPPH (rosmarinic acid > quercetin > caffeic acid > luteolin > luteolin-7-glucoside) and superoxide radical (rosmarinic acid > quercetin > luteolin > luteolin-7-glucoside > caffeic acid) scavenging activities, the results show that the hepatoprotective potential of these PhC correlates primarily with their degree of hydrophobicity and only secondarily with their antiradical capacity. In fact, Rice-Evans et al. (1996) and Spencer et al. (2004) suggested that the antioxidant biological activity of PhC will depend more heavily on the extent to which they associate, interact and permeate cell membranes than on its antiradical activity alone. In agreement with this, it was only for compounds with comparable hydrophobicities, such as the two tested phenolic acids, that a direct correlation between biological activity and antiradical activity was obtained.

The importance of the compound's lipophilicity in addition to the antiradical capacity is corroborated by comparisons between structurally related compounds. When luteolin is glycosylated at position 7 in the A ring to become luteolin-7-glucoside, the compound's hydrophobicity decreases dramatically. As a result, although the antiradical activity of luteolin-7-glucoside was only slightly affected (5% to 11%), its biological activity decreased dramatically (about 13 times lower) when compared with that for luteolin. The

411 results observed for quercetin and luteolin also implicate hydrophobicity as an important
412 factor for this cytoprotective antioxidant effect of compounds. The absence of the hydroxyl
413 group at position 3 (C ring) decreases the antiradical (hydrogen-donating) activity of
414 luteolin while increasing its hydrophobicity relative to quercetin. In agreement with the
415 previously stated, in co-incubations with *t*-BHP, luteolin showed the best protection with
416 an IC_{50} four times lower than that for quercetin. Also, in certain types of non cellular
417 lipophilic oxidation systems, luteolin showed higher antioxidant effects than those of
418 quercetin (Brown et al., 1998; Filipe et al., 2001; Hirano et al., 2001).

419 The importance of the compounds' hydrophobicity is also shown by comparing the results
420 between rosmarinic acid and luteolin-7-glucoside. Although rosmarinic acid had higher
421 antiradical scavenging activity, because the degree of hydrophobicity of luteolin-7-
422 glucoside was higher than rosmarinic acid, both compounds showed similar biological
423 effect (similar IC_{50} values).

424 Because our model of cytoprotection tests the PhC in co-incubations with the toxicant,
425 their antioxidant effects may reflect mainly their direct actions on mediators of *t*-BHP
426 toxicity. These direct effects include, besides the antiradical scavenging or hydrogen-
427 donating activity measured in this study, the compounds' ability to chelate metal ions
428 (Rice-Evans et al., 1996). Iron chelation could indeed be important for the protection
429 against *t*-BHP toxicity, which is known to be mediated by intracellular iron ions (Hix et al.,
430 2000). PhC may also indirectly act as antioxidants in cells by modulating the activity of
431 antioxidant, detoxifying and repairing enzymes as well as enzymes involved in the
432 bioactivation of xenobiotics (Ferguson, 2001; Ross and Kasum, 2002; Ferguson et al.,
433 2004). In the present study, where short term simultaneous incubations were used, PhC
434 protection through increased activity of glutathione-related enzymes seems not to be
435 relevant. In fact, the activity of GST, an important phase II detoxifying enzyme (Ferguson,

2001; Ferguson et al., 2004), was decreased rather than increased in controls exposed to luteolin-7-glucoside, the only compound that had a significant effect on glutathione-related enzymes. Longer term pre-incubations would provide the opportunity for induction of proteins and enzymes, such as antioxidant enzymes, by interaction with antioxidant response elements (Ferguson et al., 2004).

t-BHP-induced cell death was accompanied by increased lipid peroxidation and GSSG levels, and DNA damage as well as decreased GSH levels and glutathione-related enzyme activity. The increase in GSSG levels was not in the same range as the decrease in GSH levels. This indicates that *t*-BHP reduced GSH levels mainly through formation of GSH conjugates rather than oxidation to GSSG. These effects are in accordance with previous studies in liver cells (Sies and Summer, 1975; Bellomo et al., 1982; Rush et al., 1985; Jewell et al., 1986; Nic otera et al., 1988; Masaki et al., 1989; Buc-Calderon et al., 1991; Kass et al., 1992; Thabrew et al., 1997; Martin et al., 2001; Kinjo et al., 2003; Alia et al., 2005, 2006; Lee et al., 2005a, 2005b). However, particularly in HepG2 cells, *t*-BHP exposure conditions are different among different studies published so far (Thabrew et al., 1997; Kim et al., 1998, 2000; Piret et al., 2002, 2004; Kinjo et al., 2003; Alia et al., 2005, 2006; Lee et al., 2005a, 2005b). Previous reports indeed alert to the fact that different origins of HepG2 clones, culture medium composition and cultivation time (age of cells) may affect the experimental outcome through differences in sensitivity towards drugs (Knasmuller et al., 2004; Mersch-Sundermann et al., 2004). It therefore becomes imperative to characterize the cells' response to the toxicant as well as the experimental conditions used for the detection of protective effects of test compounds.

In an attempt to explain the observed cytoprotective effects of the tested PhC, we looked at their effects at IC_{80} concentration on several markers of cellular oxidative stress, such as lipid peroxidation, glutathione levels and DNA damage.

461 *t*-BHP-induced lipid peroxidation in HepG2 cells was attenuated by all tested PhC at IC_{80}
462 concentrations to a similar extent, of about 30% (25% to 35%). A good correlation seems
463 to exist between hepatoprotective effects and the prevention of lipid peroxidation. The
464 ability of PhC to chelate metal ions and/or to act as chain breaking antioxidants by
465 scavenging (as hydrogen donors) lipid alkoxyl and peroxyl radicals (Rice-Evans et al.,
466 1996; Brown et al., 1998) could provide an explanation for the observed reduction in lipid
467 peroxidation. Nevertheless, the extent of this reduction was relatively small, only about
468 30%. This indicates that it is most likely not only through reduction of lipid peroxidation
469 that PhC protect HepG2 cells against death. In agreement with this, previous reports
470 indicated that *t*-BHP-induced toxicity was not mediated by lipid peroxidation (Rush et al.,
471 1985; Jewell et al., 1986; Buc-Calderon et al., 1991; Martin et al., 2001). Moreover, our
472 own observations (data not shown) and a previous work (Rush et al., 1985) reported that
473 incubations of liver cells with the oxidant pair ascorbate/iron induced massive cell lipid
474 peroxidation without significantly affecting cell viability. Preservation of cell viability
475 seems therefore to depend also on effects at other levels.

476 All tested PhC also significantly attenuate the decrease of GSH levels induced by *t*-BHP at
477 their IC_{80} concentrations. GSH plays an important role in hepatocyte defence against ROS,
478 free radicals and electrophilic metabolites (Kedderis, 1996; Castell et al., 1997). A severe
479 GSH depletion leaves cells more vulnerable to oxidative damage by radicals and increases
480 protein thiolation or oxidation of SH groups that may lead to alterations in cellular calcium
481 homeostasis (Castell et al., 1997). A sustained increase in cytosolic calcium levels results
482 in activation of enzymes (phospholipases, non-lysomal proteases, endonucleases) and
483 cytoskeletal damage which ultimately causes cell death (Castell et al., 1997). The decrease
484 of GSH levels has indeed been suggested as one of the primary mechanisms of *t*-BHP-
485 induced toxicity in liver cells (Jewell et al., 1986; Buc-Calderon et al., 1991; Martin et al.,

2001) that is generally followed by an increase in the intracellular levels of calcium (Bellomo et al., 1982; Nicotera et al., 1988; Buc-Calderon et al., 1991; Kass et al., 1992). Thus, the potential of PhC to maintain GSH at reasonably high levels could be of great importance against *t*-BHP-induced toxicity. Therefore, the ability of the tested PhC in preventing against *t*-BHP-induced GSH depletion by about 40% was probably a major contribution to their cytoprotective effects. In the case of luteolin-7-glucoside, there was a higher protection (~80%) of GSH levels that did not reflect higher cytoprotection (all compounds were tested at their IC_{80} concentration). This may have been due to the observed inhibitory effect of luteolin-7-glucoside on GST having a sparing effect on GSH. Because protection by PhC against increases of GSSG levels induced by *t*-BHP was weak, it seems that PhC protect against the decrease of GSH levels mainly by preventing the formation of GSH conjugates rather than oxidation to GSSG.

In spite of this general protection of GSH, when incubated alone, PhC decreased GSH levels by 5% in the case of rosmarinic acid and between 10% and 14% for the tested flavonoids. Although not statistically significant, this effect seems to indicate some pro-oxidant activity of these compounds. Previous studies also found a decrease in GSH induced by flavonoids (Duthie et al., 1997; Galati et al., 2002). For flavonoids with a 3',4'-dihydroxyl group on the B ring (catechol B ring), as is the case here, the decrease of GSH levels was found to be through formation of GSH conjugates instead of oxidation to GSSG (Galati et al., 2002).

Incubations of HepG2 cells with *t*-BHP induced DNA damage in a concentration-dependent manner, as visualized by the comet assay. Exposure to 200 µM *t*-BHP induced significant DNA damage without inducing cell mortality (data not shown). This seems to indicate that *t*-BHP-induced DNA damage was not implicated in the cell death induced by this organic peroxide in HepG2 cells. In fact, caffeic acid, at IC_{80} concentration,

511 significantly decreased *t*-BHP- induced cell death without protecting DNA from damage.
512 Also, previous reports showed a dissociation between the oxidative DNA damage induced
513 by *t*-BHP from the killing of hepatocytes (Coleman et al., 1989; Latour et al., 1995).
514 Latour and collaborators (1995) ruled out both the formation of oxidized DNA bases and
515 the activation of a calcium-dependent endonuclease as mechanisms by which *t*-BHP
516 induces DNA single strand breaks. They showed that *t*-BHP causes DNA single strand
517 breaks most likely by covalent binding of free radicals to DNA by mechanisms dependent
518 on iron ions (Latour et al., 1995). Iron-dependent reactions have been proposed as the key
519 factor to the DNA damage induced by *t*-BHP since it can be prevented by iron chelators
520 but not by free radical scavengers, such as butylated hydroxytoluene and trolox (Coleman
521 et al., 1989; Latour et al., 1995; Guidarelli et al., 1997; Sestili et al., 1998, 2002). Recently,
522 another study using a different model showed the importance of iron chelation on DNA
523 protection over free radical scavenger activity (Melidou et al., 2005). In our study, where
524 the compounds were tested at their IC_{80} concentration (concentration that protected 80%
525 against cell death), only luteolin and quercetin conferred a very clear protection against
526 DNA damage. An ortho dihydroxy phenolic structure is one of the requirements for PhC
527 ability to chelate transition metal ions such as copper and iron (Rice-Evans et al., 1996;
528 Williams et al., 2004). All the compounds used in this study possess this element, but only
529 luteolin and quercetin conferred noticeable protection against DNA damage. It seems
530 therefore, that even more than in the case of preserved cell viability, the degree of
531 hydrophobicity of the compound is an important factor for protecting from DNA damage,
532 since this could explain the higher effects obtained for luteolin and quercetin. Also in
533 accordance with this are the results obtained from the comparison between quercetin and
534 luteolin themselves. Metal ion chelation ability of flavonoids appears to be not only
535 dependent on the presence of the catechol B ring but also an oxo group at position 4 in C

ring in combination with hydroxyl group either at position 5 or 3 (Mira et al., 2002; Williams et al., 2004). Therefore, quercetin probably has higher metal ion chelation ability than luteolin, which lacks the OH group at position 3. In fact, previous results showed higher capacity of quercetin to chelate iron and copper than luteolin (Mira et al., 2002). Our results show that luteolin, although at a concentration 4 times lower, protected DNA against damage better than quercetin, which emphasizes the importance of the compounds' lipophilicity. Also others have already drawn attention to the fact that the biological effects of a compound would be a direct function of its lipophilicity, which is expected to increase the cellular uptake of these agents, as well as their subcellular localization in lipid compartments (Sestili et al., 2002; Spencer et al., 2004). Studies using other models and/or different cell types showed that luteolin had higher potential to decrease DNA damage than quercetin (Noroozi et al., 1998; Romanova et al., 2001; Horvathova et al., 2004, 2005), or the opposite—quercetin having higher ability to reduce DNA damage than luteolin (Horvathova et al., 2003; Melidou et al., 2005). As well, higher cytoprotective effects of luteolin over quercetin were found by some authors (Kaneko and Baba, 1999; Sasaki et al., 2003), although others reported the opposite (Ishige et al., 2001). It seems, therefore, that the protective potential of luteolin and quercetin is cell type specific and/or dependent on the agent used to induce DNA damage. Nevertheless, although DNA damage induced by *t*-BHP in HepG2 cells seems not to be a crucial event for cell death, this experimental model can be of use to extensively study the protective potential of PhC against DNA damage. It would be, for example, a good model for structure-activity relationships between several classes of flavonoids.

In conclusion, the PhC studied here showed protective effects against oxidative damages induced in HepG2 cells that could be of use against liver diseases where it is known that oxidative stress plays a crucial role. Moreover, their protective potential seems to be

561 dependent on the compound's lipophilicity in conjunction with its antioxidant activity.
562 Their effects on protection against *t*-BHP-induced GSH depletion seem to be an important
563 factor for preserving cell viability.

564

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Results (tables)

Table 1 – Potential hepatoprotective effects of the tested PhC against *t*-BHP-induced toxicity in HepG2 cells.

TABLE

Hepatoprotective effects of PhC were tested in co-incubations with 2 mM of *t*-BHP (5 h) in HepG2 cells. IC_{50} and the Hill slope were taken from the plotted dose–response curve (Fig. 3). IC_{80} concentration was estimated from the same dose–response curve. Values are mean ± SEM of at least 4 independent experiments.

Table 2 – Effects of *t*-BHP and PhC at IC_{80} concentration on lipid peroxidation and oxidized glutathione levels in HepG2 cells.

TABLE

HepG2 cells were incubated with *t*-BHP 2 mM (5 h) and/or with individual PhC at IC_{80} concentration and lipid peroxidation (as estimated by TBARS assay) and GSSG levels measured. Values are mean ± SEM, n = 5 (TBARS), n = 4 (GSSG). *** P=0.001 when compared with the negative control. $^{\#}$ P=0.05 and $^{\#\#\#}$ P=0.001 when compared with the *t*-BHP control.

Table 3 – Effects of *t*-BHP and PhC at IC_{80} concentration on glutathione-related enzyme activities in HepG2 cells.

TABLE

HepG2 cells were incubated with *t*-BHP 2 mM (5 h) and/or with individual PhC at IC_{80} concentration and the activities of GST, GR and GPox measured. Values are mean ± SEM, n = 5. * P=0.05, ** P=0.01 and *** P=0.001 when compared with the negative control. $^{\#}$ P=0.05 when compared with the *t*-BHP control.

Table 4 – DPPH scavenging activity of the tested PhC.

TABLE

Different concentrations of each PhC were added to the ethanolic solution of DPPH and the discoloration measured spectrophotometrically at 515 nm. From the results expressed as the percentage of the remaining DPPH obtained for each PhC concentration (Fig. 8), the IC_{50} and Hill slope were taken. From the results, the AE was also calculated for each PhC. Values represent mean ± SD of 5 replicates.

Table 5 – Superoxide radical scavenging activity of the tested PhC.

TABLE

Using the phenazine methosulphate-NADH nonenzymatic assay, superoxide radicals were produced continuously and measured spectrophotometrically at 560 nm. In co-incubations with individual PhC at several concentrations, the scavenging of superoxide radical was measured and from the plotted results the IC_{50} and the Hill slope were taken.

Values represent mean ± SD of 3 independent experiments with 3 replicates each.

Table 6 – Experimental partition coefficients values obtained for each tested PhC.

TABLE

Partition coefficient values in logarithm (K_{ow}) were measured in an n-octanol/HEPES (20 mM, pH 7.4) system. Values are mean ± SD of 3 independent experiments.

Results (figures)

Figure 1 – Chemical structures of the phenolic compounds used in this study.

Figure 2 – *t*-BHP-induced toxicity in HepG2 cells. HepG2 cells were incubated with *t*-BHP 1 mM and 2 mM for different time periods and cell viability measured by LDH leakage (% of LDH in the extracelular medium) (**A**) and MTT assay (**B**). Time scale was logarithmized in order to obtain sigmoidal response curves. Values represent mean ± SEM, n = 4. In **A**: * P=0.05 and *** P=0.001 when compared to the same time point in the control situation.

Figure 3 – Dose–response effect of the tested PhC against *t*-BHP-induced toxicity in HepG2 cells. After incubating HepG2 cells with 2 mM of *t*-BHP and individual PhC for 5 h, protection against cell death (as measured by LDH leakage) versus PhC concentration (in logarithm) were plotted in order to take the IC_{50} and Hill slope of each compound (Table 1). Values are mean ± SEM of at least 4 independent experiments.

Figure 4 – Effects of *t*-BHP and PhC at the IC_{80} concentration on reduced glutathione levels in HepG2 cells. HepG2 cells were incubated with *t*-BHP 2 mM (5 h) and/or with individual PhC (CA – caffeic acid; RA – rosmarinic acid; L-7-G – luteolin-7-glucoside;

L – luteolin; Q – quercetin) at IC_{80} concentration and GSH levels measured. Values are mean ± SEM, n = 5. *** P=0.001 when compared with the negative control. # P=0.05, ## P=0.01 and ### P=0.001 when compared with the *t*-BHP control.

Figure 5 – *t*-BHP-induced DNA damage in HepG2 cells. HepG2 cells were incubated with different concentrations *t*-BHP for 1 h and DNA damage assessed by the comet assay. Comet images were examined by computer assisted image analysis system (**A** – tail length; **B** – tail moment; **C** – % DNA in the tail) and by a semiquantitative method of visual scoring (**D**). The correlation coefficients between the semiquantitative method and the computer assisted parameters are given in graph **E**. Values are mean ± SEM, n=4. * P=0.05, ** P=0.01 and *** P=0.001 when compared with the control.

Figure 6 – Effects of *t*-BHP and PhC at IC_{80} concentration on DNA damage in HepG2 cells. HepG2 cells were incubated with *t*-BHP 200 µM (1 h) and/or with individual PhC at IC_{80} concentration and DNA damage evaluated by the comet assay. DNA damage was assessed by the semiquantitative method of visual scoring. Values are mean ± SEM, n = 4. *** P=0.001 when compared with the negative control. ### P=0.001 when compared with the *t*-BHP control.

Figure 7 – Dose-dependent protection of *t*-BHP-induced DNA damage in HepG2 cells by quercetin (**A**). HepG2 cells were incubated with *t*-BHP 200 µM (1 h) and/or with quercetin at different concentrations and DNA damage evaluated by the comet assay.

DNA damage was assessed by the semiquantitative method of visual scoring. Values are mean ± SEM, n = 4. *** P =0.001 when compared with the negative control. ### P =0.001 when compared with the *t*-BHP control. **B** – Representative pictures of the comet assay results.

Figure 8 – Dose-dependent DPPH scavenging activity of caffeic acid. Different concentrations of caffeic acid were added to the ethanolic solution of DPPH and the discoloration measured spectrophotometrically at 515 nm. At the time point where all tested concentrations had reached the steady state (9 min), the percentages of the remaining DPPH were plotted against the corresponding caffeic acid concentrations (in logarithm). From this graph, the IC_{50} and Hill slope were taken (Table 4). Values represent mean ± SD of 5 replicates.

Compound	IC_{50} (μM)	Hill slope	IC_{80} (μM)
Caffeic acid	114.1 ± 11.5	1.17 ± 0.16	370
Rosmarinic acid	69.2 ± 5.3	1.48 ± 0.16	180
Luteolin-7-O-glucoside	78.0 ± 7.6	1.47 ± 0.22	200
Luteolin	5.9 ± 0.5	2.46 ± 0.44	11
Quercetin	23.5 ± 1.4	2.12 ± 0.27	45

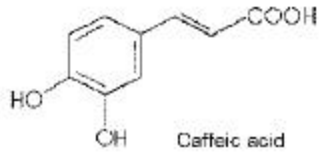
Phenolic compound	*t*-BHP 2 mM, ~5 h	Parameter	
		TBARS (nmol/mg)	GSSG (nmol GSH equiv/mg)
–	–	0.20 ± 0.05	1.2 ± 0.3
	+	2.25 ± 0.13 ***	5.0 ± 0.4 ***
Caffeic acid	–	0.19 ± 0.10	1.2 ± 0.4
	+	1.54 ± 0.10 ###	4.2 ± 0.1
Rosmarinic acid	–	0.15 ± 0.05	1.2 ± 0.3
	+	1.71 ± 0.08 ###	3.5 ± 0.3 #
Luteolin-7-glucoside	–	0.15 ± 0.02	1.3 ± 0.2
	+	1.74 ± 0.08 ###	3.8 ± 0.5
Luteolin	–	0.20 ± 0.07	1.6 ± 0.4
	+	1.66 ± 0.12 ###	4.4 ± 0.4
Quercetin	–	0.15 ± 0.06	1.5 ± 0.3
	+	1.64 ± 0.12 ###	4.6 ± 0.2

Phenolic compound	*t*-BHP 2 mM, ~5 h	Enzyme activity (mU/mg)		
		GST	GR	GPox
–	–	24.7 ± 1.0	25.9 ± 0.8	18.2 ± 0.5
	+	23.5 ± 0.5	21.8 ± 0.9 *	6.3 ± 0.6 ***
Caffeic acid	–	24.4 ± 0.8	25.6 ± 1.3	17.8 ± 0.3
	+	23.1 ± 0.9	23.2 ± 0.8	7.5 ± 0.8
Rosmarinic acid	–	23.1 ± 0.4	23.6 ± 0.4	16.1 ± 0.5
	+	24.5 ± 0.3	21.3 ± 0.9	5.5 ± 0.5
Luteolin-7-glucoside	–	20.6 ± 0.6 **	23.6 ± 0.3	16.0 ± 0.5
	+	23.7 ± 0.6	22.7 ± 0.8	5.7 ± 0.5
Luteolin	–	22.6 ± 0.6	24.6 ± 0.3	16.1 ± 0.7
	+	26.1 ± 0.8	23.2 ± 0.8	5.6 ± 0.7
Quercetin	–	24.0 ± 1.0	25.9 ± 1.0	17.2 ± 0.4
	+	22.6 ± 0.8	22.9 ± 0.6	8.6 ± 1.1 #

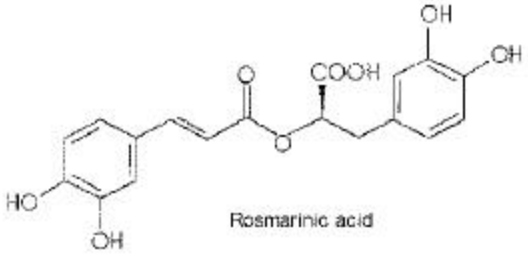
Compound	IC_{50} (mmol/mol DPPH)	Hill slope	AE ($\times 10^{-3}$)
Caffeic acid	179.6 ± 4.1	2.03 ± 0.06	0.81
Rosmarinic acid	102.6 ± 2.2	2.07 ± 0.09	0.53
Luteolin-7-O-glucoside	277.3 ± 14.9	1.48 ± 0.06	1.21
Luteolin	263.9 ± 11.0	1.66 ± 0.03	0.70
Quercetin	126.0 ± 2.4	1.66 ± 0.05	0.36

Compound	IC_{50} (μM)	Hill slope
Caffeic acid	99.1 ± 5.3	1.02 ± 0.06
Rosmarinic acid	21.0 ± 0.9	0.95 ± 0.04
Luteolin-7-O-glucoside	50.4 ± 2.4	0.93 ± 0.05
Luteolin	45.3 ± 3.0	1.70 ± 0.19
Quercetin	35.1 ± 3.3	1.69 ± 0.25

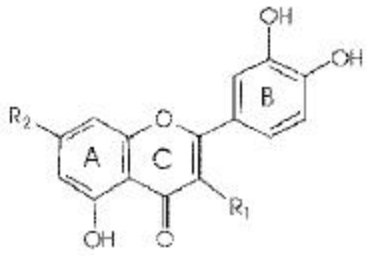
Phenolic compound	K_{ow}
Caffeic acid	-0.89 ± 0.10
Rosmarinic acid	-0.44 ± 0.13
Luteolin-7-glucoside	1.22 ± 0.01
Luteolin	2.68 ± 0.05
Quercetin	2.60 ± 0.09



Caffeic acid



Rosmarinic acid



Compound	R_1	R_2
Luteolin-7-O-glucoside	H	O-Glucose
Luteolin	H	OH
Quercetin	OH	OH