Plant-derived phenolic compounds prevent the DNA single-strand breakage and cytotoxicity induced by *tert*-butylhydroperoxide via an iron-chelating mechanism

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The protective effects of selected members from a series of caffeic acid esters and flavonoids were tested in various toxicity paradigms using U937 cells, previously shown to be sensitive to either iron chelators or *bona fide* radical scavengers or to both classes of compounds. It was found that all the protective polyphenols were active at very low concentrations and that their effects were observed only under those conditions in which iron chelators also afforded protection. Consistently, active polyphenolic compounds, unlike the inactive ones, effectively chelated iron in an *in vitro* system. It follows that, at least under the experimental conditions utilized in the present study, the most prominent activity of these polyphenolic compounds resides in their ability to chelate iron. Further studies revealed that the protective effects afforded by the caffeic acid esters and flavonoids were largely mediated by the catechol moiety and that the relative biological potency of these compounds was a direct function of their lipophilicity.

Key words: caffeic acid ester, flavonoid, polyphenol.

INTRODUCTION

There is currently a wealth of experimental evidence available suggesting that naturally occurring polyphenol compounds display potent antioxidant activities [1–19]. An important source of these substances is represented by the propolis from the honeybee hives which contains as prominent constituents two distinct series of polyphenols, i.e. flavonoids and caffeic acid esters [20]. In particular, the latter group of compounds is present in very large amounts accounting for up to 20% (w/w) of the propolis [20].

While the ability of members of the flavonoid family [3,16,18] and some caffeic acid esters [6,8,9,12,15] to counteract the deleterious effects mediated by different sources of reactive oxygen species is well documented, very few studies have investigated the molecular basis for these effects. In principle, the polyphenol structure allows both the scavenging of free radicals [10,11,19] and the chelation of transition metals, including iron [4,10,14,19]. It is generally believed that the 3′,4′-catechol structure of the B-ring is an important structural determinant for the antioxidant potential of flavonoids [12,21–23]; the catechol group, however, not only provides a reducing potential but also allows metal chelation. Hydroxy groups in different position may allow scavenging of reactive oxygen species but at the same time markedly decrease the lipophilicity and therefore the ability of the polyphenols to penetrate into the cells. As a consequence some of these compounds, while effective *in vitro*, cannot display cytoprotective properties for the simple reason that they are not efficiently taken up by the cells. A good example is given by catechin, a flavanol lacking the 4-oxo group in the C-ring, but otherwise identical to quercetin, which is unable to prevent (unlike quercetin) the deleterious effects mediated by hydrogen peroxide in PC12 cells [21]. Thus, it appears obvious that, while the structure–activity relationships of polyphenols are quite well understood in *in vitro* systems, various difficulties arise in the interpretation of results obtained in *in vivo* studies. For the same and other reasons it is difficult to establish the relative contribution of the radical scavenging versus the iron-chelating properties of the polyphenols to the antioxidant activity. Indeed, to our best knowledge, there are no detailed studies that have specifically addressed this issue using *in vivo* systems.

We have recently developed an experimental approach which, in a relevant biological setting, discriminates free radical scavenging versus iron-chelating mechanisms and shows that the most prominent activity of quercetin resides in its ability to chelate iron [24]. In the present study, the same approach was used to analyse a series of selected flavonoids and caffeic acid esters.

Our results indicate that cytoprotection afforded by these compounds in oxidatively injured cells is mediated by an iron-chelating mechanism which appears to be entirely dependent on the catechol moiety. The biological potency of these compounds was a direct function of their lipophilicity.

MATERIALS AND METHODS

Cell culture and treatments

U937 cells were grown in RPMI 1640 culture medium (HyClone Laboratories, Logan, UT, U.S.A.) supplemented with 10% (v/v) Fetal clone III (HyClone), penicillin (50 units/ml), and

Abbreviations used: BC, benzyl caffeate; BHT, butylated hydroxytoluene; CAPE, phenylethyl caffeate; DF, 3′,4′-dihydroxyflavone; ClogP, n-octanol/water partition coefficient; DPPD, *N,N′*-diphenyl-1,4-phenylene-diamine; MBC, 3-methyl-2-butenyl caffeate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; P3HC, phenylethyl-3-hydroxycinnamate; P4HC, phenylethyl-4-hydroxycinnamate; PDH, phenylethyl-3,4-dihydroxyhydrocinnamate; RNSF, relative nuclear spreading factor; *t*B-OOH, *tert*-butylhydroperoxide; mp, melting point.

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streptomycin (50 μg/ml) at 37 °C in T-75 tissue culture flasks (Corning, Corning, NY, U.S.A.) gassed with an atmosphere of air/CO_2 (19:1). Reagent-grade chemicals, *tert*-butylhydroperoxide (*t*B-OOH), Trolox, *o*-phenanthroline, butylated hydroxytoluene (BHT), *N*,*N*′-diphenyl-1,4-phenylene-diamine (DPPD), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), quercetin, morin and chrysine were obtained from Sigma-Aldrich (Milan, Italy). 3′,4′-Dihydroxyflavone (DF) was purchased from Lancaster (Milan, Italy). Stock solutions of *t*B-OOH, or H_2O_2, were freshly prepared in saline A (8.182 g/l NaCl, 0.372 g/l KCl, 0.336 g/l $NaHCO_3$ and 0.9 g/l glucose). Trolox was dissolved in 1 M $NaHCO_3$. Phenolic compounds and *o*-phenanthroline were dissolved in DMSO. BHT and DPPD were dissolved in 95 % (v/v) ethanol. At the treatment stage the final DMSO/ethanol concentration was never higher than 0.05 %. Under these conditions ethanol and DMSO were neither toxic nor DNA-damaging, nor did they affect the cyto-genotoxic properties of *t*B-OOH or H_2O_2.

Cells (2.5×10^5/ml) were treated with *t*B-OOH (30 min) or H_2O_2 (60 min) in saline A (2 ml), washed and either analysed immediately for DNA damage or post-incubated for 6 h in complete medium and then analysed for cell viability. Antioxidants, *o*-phenanthroline and phenolic compounds were added to the cultures 5 min prior to the addition of *t*B-OOH or H_2O_2.

Phenolic compounds synthesis

The structure and purity of all synthesized products were supported by analytical and spectroscopic data. The synthesis of derivatives 3-methyl-2-butenyl caffeate (MBC) and benzyl caffeate (BC) was achieved as follows: 2 mmol of 1,3-dicyclohexylcarbodiimide and 2 mmol of the opportune alcohol were added to a solution of 2 mmol of caffeic acid in 6 ml of dry tetrahydrofuran at 0 °C. The mixture was stirred at room temperature for 17 h and filtered; the solvent was then evaporated and the residue purified by flash chromatography (cyclohexane/ethyl acetate mixture, 1:1, v/v) and by crystallization. MBC: melting point (mp) 126 °C (diethyl ether/hexane); mp 123–125 °C [22]. BC: mp 151 °C (diethyl ether/hexane); mp 151–153 °C [23].

Phenylethyl caffeate (CAPE), phenylethyl-3,4-dihydroxyhydrocinnamate (PDH), phenylethyl-4-hydroxycinnamate (P4HC), phenylethyl-3-hydroxycinnamate (P3HC) were prepared according to the following general method: 100 mmol of phenylethyl alcohol, 6.75 mmol of the opportune acid and 50 mg of *p*-toluenesulphonic acid were suspended/dissolved in 25 ml of toluene. The mixture was stirred for 24 h at reflux with a Dean-Stark trap. Solvent and excess of alcohol were removed by distillation and the residue was purified by flash chromatography (cyclohexane/ethyl acetate mixture, 1:1, v/v) and by crystallization. CAPE: mp 128 °C (diethyl ether/hexane); mp 124.5–126 °C [25]. PDH mp 71 °C (toluene); mp 72.5–73.5 °C [26]. P4HC: mp 89 °C (diethyl ether/hexane) [27]. P3HC: mp 99 °C (diethyl ether/hexane). ^{1}H NMR (200 MHz, [^{2}H]chloroform): δ 3.03 (t, 2H, $J = 7.3$ Hz); 4.43 (t, 2H, $J = 7.3$ Hz); 5.24 (brs, 1H); 6.39 (d, 1H, $J = 15.7$ Hz); 6.84–7.38 (m, 9H); 7.61 (d, 1H, $J = 15.7$ Hz). MS (EI-70 eV): m/z 268, 164, 147, 119, 104.

Cytotoxicity assays

After the treatments, the cells were washed with saline A and suspended in pre-warmed RPMI 1640 medium before being plated into 35-mm tissue culture dishes and incubated at 37 °C for 6 h. Cytotoxicity was determined using the Trypan Blue exclusion assay. Briefly, an aliquot of the cell suspension was diluted 1:1 (v/v) with 0.4 % Trypan Blue and the cells were counted using a haemocytometer. Results are expressed as the percentage of dead cells (ratio of stained cells to the total number of cells).

In some experiments, cytotoxicity was determined by the MTT assay. Cells were treated as described above and, after 5 h of post-challenge incubation, MTT was added (50 μg/ml) to each well and the plate was incubated for a further 1 h at 37 °C. Cells were then washed, centrifuged and dissolved in 1 ml of DMSO. Formation of blue formazan was measured spectrophotometrically at 570 nm. Results are expressed as the percentage of MTT reducing activity (absorbance in treated versus control cells).

Measurement of DNA single-strand breakage by the alkaline halo assay

DNA single-strand breakage was determined using the alkaline halo assay described in [28] with minor modifications. After the treatments, the cells (2.5×10^5/ml) were resuspended at 2.0×10^4 cells/100 μl in 1.5 % (w/v) low-melting-point agarose in phosphate-buffered saline (8 g/l NaCl, 1.15 g/l Na_2HPO_4, 0.2 g/l KH_2PO_4, 0.2 g/l KCl) containing 5 mM EDTA and immediately sandwiched between an agarose-coated slide and a coverslip. After complete gelling, the coverslips were removed and the slides were immersed in an alkaline buffer (0.1 M NaOH/1 mM EDTA, pH 13, for 20 min), washed and stained for 5 min with 10 μg/ml ethidium bromide.

The ethidium bromide-labelled DNA was visualized using a Bio Rad DVC 250 confocal laser microscope (Bio Rad, Richmond, CA, U.S.A.) and the resulting images were taken and processed with a Hamamatsu chilled CCD 5985 camera (Hamamatsu Italy S.p.a., Milan, Italy) coupled with an Apple Macintosh computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

The level of DNA single-strand breakage was quantified by calculating the nuclear spreading factor values, which represent the ratio between the area of the halo (obtained by subtracting the area of the nucleus from the total area, nucleus + halo) and that of the nucleus, from 50 to 75 randomly selected cells/experiment/treatment condition. Data are expressed as relative nuclear spreading factor (RNSF) values calculated by subtracting the nuclear spreading factor values of control cells from those of treated cells.

Spectrophotometric determination of iron chelation

The ability of polyphenols to chelate iron was determined spectrophotometrically [29,30]. UV-visible absorption spectra (200–500 nm) were measured using a Beckman DU640 UV-visible spectrophotometer. Polyphenols were dissolved in PBS (50 mM, pH 7.4) and the spectral changes were measured in the absence or presence of 50 μM $FeSO_4$.

Determination of n-octanol/water partition coefficient (ClogP) values

ClogP was calculated with CS ChemDraw Ultra 5.0 (CambridgeSoft, Cambridge, MA, U.S.A.).

RESULTS AND DISCUSSION

The aim of this work was to determine the antioxidant activity of a series of polyphenolic compounds and gather information on the mechanism(s) involved in these responses as well as on the structure–activity relationships.

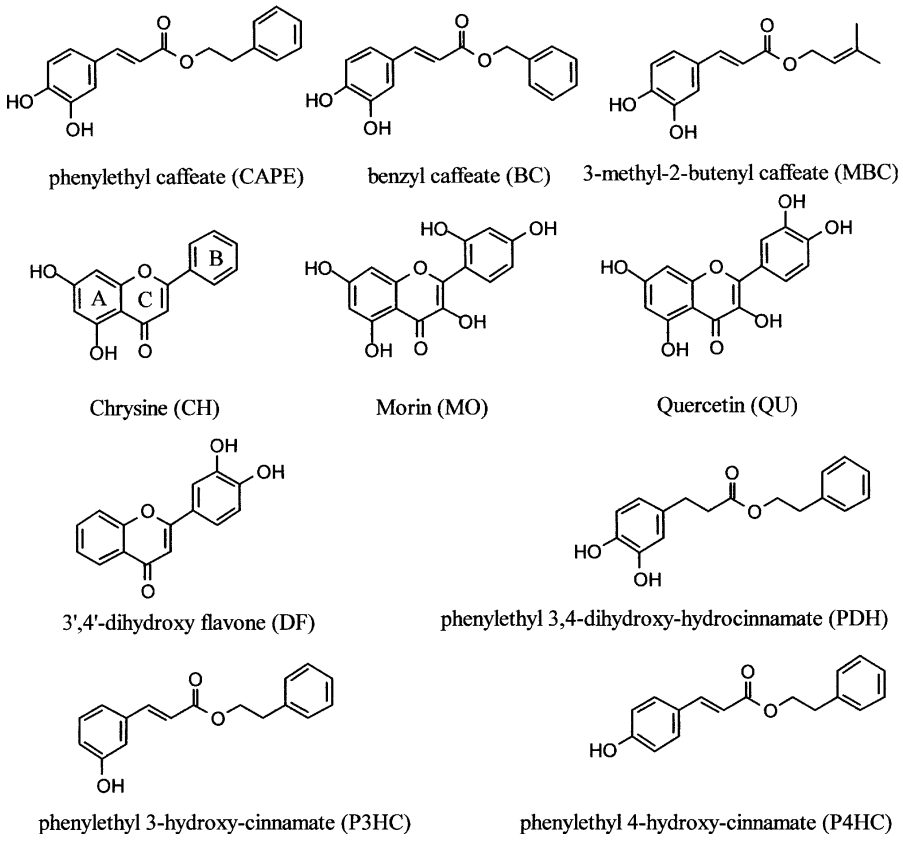

Figure 1 Structures of the phenolic compounds

Table 1 Effect of *bona fide* antioxidants and of an iron chelator on the DNA damage and toxicity caused by *t*B-OOH in U937 cells

In cytotoxicity experiments the cells were exposed for 30 min to 3 mM *t*B-OOH in saline A in the absence or presence of the indicated compounds. The relative number of dead cells was measured after 6 h of post-challenge growth in fresh culture medium using the Trypan Blue exclusion assay. Results are expressed as the percentage of dead cells in each sample. In DNA single-strand breakage experiments cells were treated for 30 min with 200 μM *t*B-OOH in saline A in the absence or presence of the indicated compounds and immediately assayed for DNA damage with the alkaline halo assay, as detailed in the Materials and methods section. Results are expressed as RNSF values measured in cells treated with *t*B-OOH in the absence or presence of the indicated compounds. Results are the mean $\pm$ S.E.M. for at least three separate experiments, each performed in duplicate.

Compound	Cytotoxicity (% dead cells)	DNA single-strand breakage (RNSF values)
–	35.6 $\pm$ 2.12	5.25 $\pm$ 0.49
BHT (200 μM)	4.04 $\pm$ 0.41	5.04 $\pm$ 0.41
DPPD (10 μM)	3.62 $\pm$ 0.33	5.62 $\pm$ 0.51
Trolox (1 mM)	3.22 $\pm$ 0.21	5.19 $\pm$ 0.43
o-Phenanthroline (25 μM)	2.37 $\pm$ 0.15	0.21 $\pm$ 0.015

Figure 1 shows the structures of the ten polyphenols tested. It can be seen that CAPE, BC and MBC are caffeic acid esters with different alcoholic residues. PDH is similar to CAPE except that the double bond in the α position was removed. P3HC and P4HC are CAPE analogues in which the catechol moiety was replaced with a *m*-hydroxylphenyl (P3HC) or *p*-hydroxylphenyl (P4HC). Chrysine, morin, quercetin and DF belong to the flavonoid family. The structure of morin is the same as that of quercetin except for the relative positions of the hydroxy groups in the B ring. Similarly, the structure of DF is the same as that of a quercetin lacking the hydroxy groups in the A and C rings.

The effect of these compounds was tested in a toxicity paradigm in which U937 cells were exposed for 30 min to 3 mM *t*B-OOH and then post-incubated in fresh culture medium for 6 h. Under these conditions, H_2O_2 caused a 35.6 $\pm$ 2.12% cell death that could be prevented (Table 1) by each of three different *bona fide* antioxidants, BHT (200 μM), DPPD (10 μM) and Trolox (1 mM), or by an intracellular iron chelator, *o*-phenanthroline (25 μM). These results are consistent with the findings presented in published work previously from this [24,31,32] and other [33,34] laboratories, and confirm the notion that the toxicity mediated by H_2O_2 requires a source of iron and the formation of lipid peroxidation products.

Using an identical toxicity paradigm, non-toxic levels of CAPE, BC, MBC, PDH, quercetin and DF mitigated the cytotoxic response evoked by *t*B-OOH in a dose-dependent manner (Figure 2). The calculated IC_{50} values (Table 2) emphasize the notion that each of the above compounds is extremely effective in reducing H_2O_2-induced cell death. In particular, the IC_{50} values of CAPE, BC, MBC and PDH were all below 50 nM. Other compounds tested at concentrations up to 100 μM, namely chrysine, morin, P3HC and P4HC, showed no effects in this toxicity paradigm (Table 2).

Finally, experiments were performed in which the cells were treated as detailed above but analysed for cytotoxicity using the MTT assay. Under these conditions BHT (200 μM), DPPD (10 μM), Trolox (1 mM) and *o*-phenanthroline (25 μM) inhibited

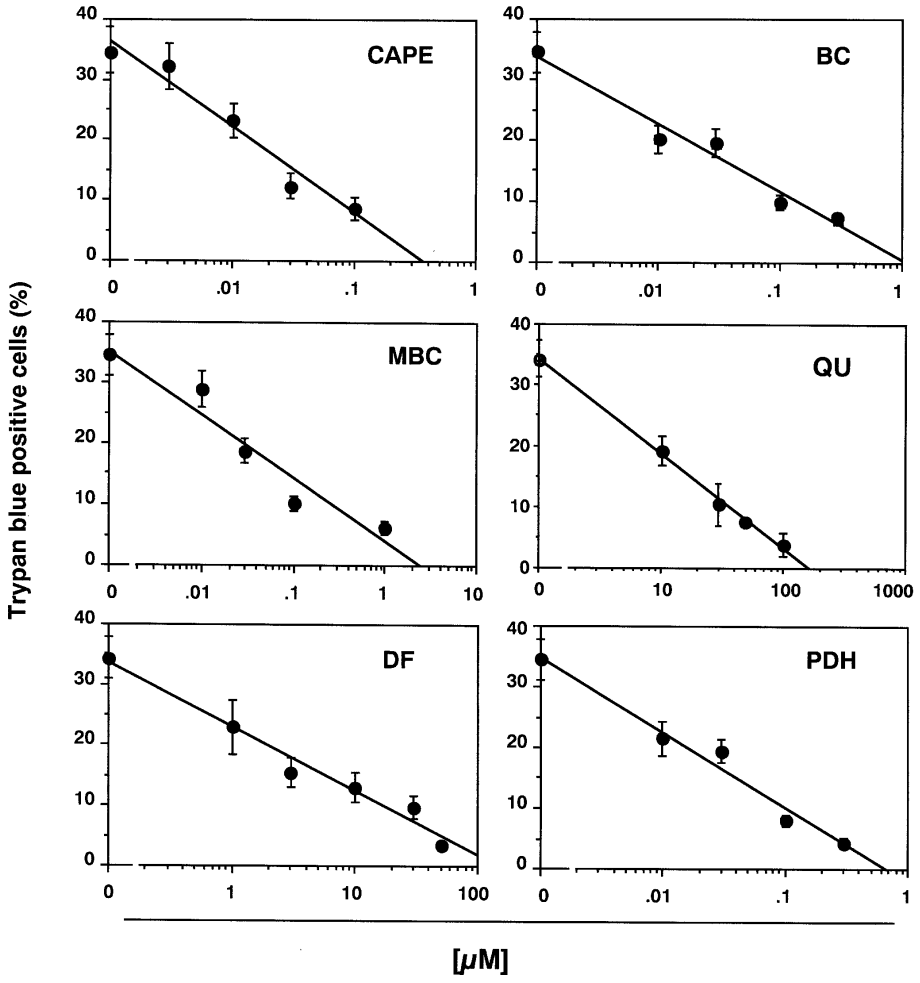

Figure 2 Effect of phenolic compounds on *t*B-OOH-induced U937 cell death

The cells were plated at a density of 2.5×10^5 cells/ml in saline A and exposed for 30 min to 3 mM *t*B-OOH, in the absence or presence of increasing concentrations of the indicated compounds. The relative number of dead cells was measured after 6 h of post-challenge growth in fresh culture medium using the Trypan Blue exclusion assay. Results are the mean ± S.E.M. for at least three separate experiments, each performed in duplicate. QU, quercetin.

Table 2 Effect of selected polyphenols on the DNA damage and toxicity caused by *t*B-OOH in U937 cells

ClogP values were calculated as detailed in the Materials and methods section. Iron chelation was determined spectrophotometrically, as detailed in the Materials and methods section. Results are the mean ± S.E.M. for at least three separate experiments, each performed in duplicate.

Compound	IC_{50}* (μM)	IC_{50}† (μM)	ClogP	Iron chelation
CAPE	0.0203 ± 0.0017	0.0062 ± 0.0005	3.43	+
BC	0.0305 ± 0.0021	0.013 ± 0.001	3.15	+
MBC	0.0502 ± 0.0038	0.015 ± 0.001	2.65	+
Chrysine	Inactive‡	Inactive	Not tested	−
Morin	Inactive	Inactive	Not tested	−
Quercetin	12.67 ± 0.86	2.73 ± 0.29	0.35	+
DF	4.05 ± 0.38	0.59 ± 0.05	2.29	+
PDH	0.0240 ± 0.0019	0.0098 ± 0.0009	3.32	+
P3HC	Inactive	Inactive	Not tested	−
P4HC	Inactive	Inactive	Not tested	−

* Concentration of the compound promoting a 50% reduction of the *t*B-OOH (3 mM)-induced cytotoxicity.
† Concentration of the compound promoting a 50% reduction of the *t*B-OOH (200 μM)-induced DNA single-strand breakage.
‡ Inactive at concentrations up to 100 μM.

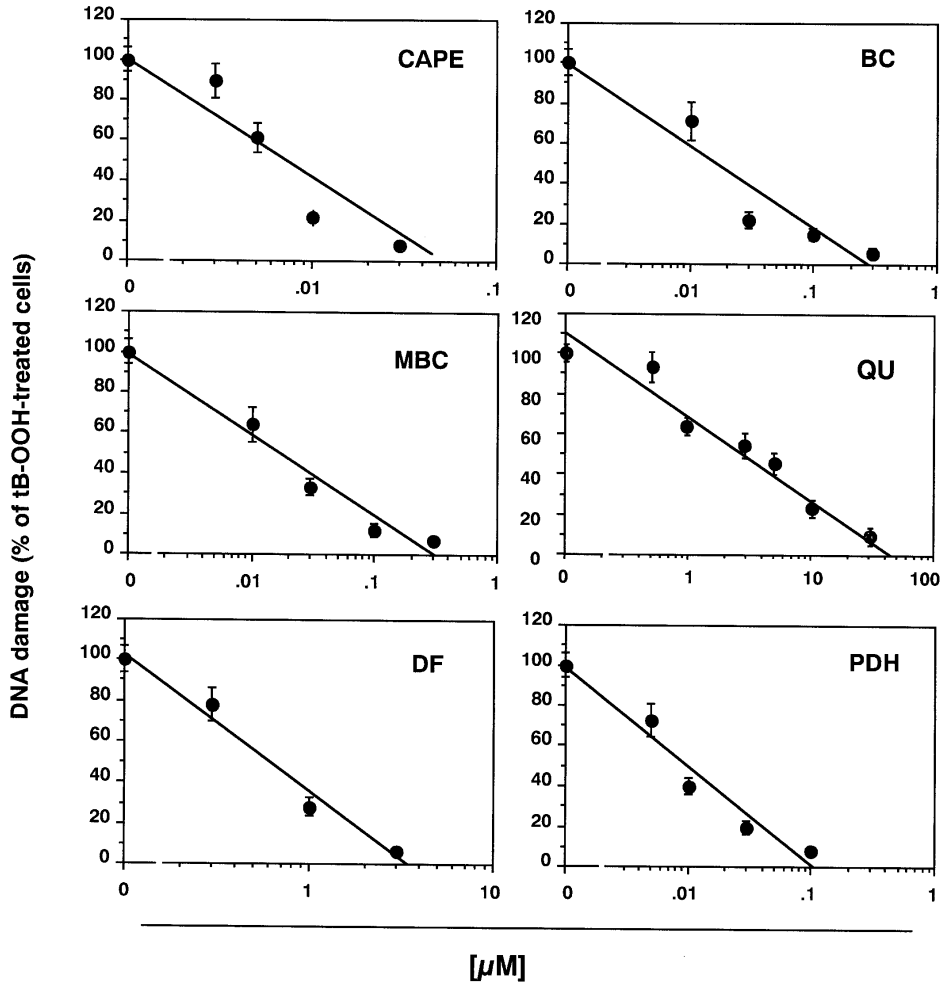

Figure 3 Effect of phenolic compounds on *t*B-OOH-induced DNA single-strand breakage

The cells were treated for 30 min with 200 μM *t*B-OOH in saline A, in the absence or presence of increasing concentrations of the indicated compounds, and immediately assayed for DNA damage with the alkaline halo assay, as detailed in the Materials and methods section. Under these conditions, *t*B-OOH caused an RNSF of 5.25 ± 0.49. Results are expressed as the percent ratio between the RNSF values measured in cells treated with *t*B-OOH in the presence of the polyphenols and that of cells treated with *t*B-OOH alone. Data are the mean $\pm$ S.E.M. for at least three separate experiments, each performed in duplicate. QU, quercetin.

the lethal response evoked by H_2O_2 (Table 3). All the polyphenolic compounds effectively reduced toxicity (Table 3) when used at concentrations affording an almost complete protection in experiments in which cytotoxicity was assayed using the Trypan Blue exclusion assay (Figure 2). Furthermore, all the inactive compounds listed in Table 2 did not prevent the loss of MTT-metabolizing activity induced by H_2O_2 (Table 3).

Taken together the above results, while allowing the identification of a number of polyphenolic derivatives which display remarkable cytoprotection in oxidatively-injured cells, do not allow us to conclude whether these effects are mediated by a radical-scavenging or iron-chelation mechanism, or by a combination of these two activities.

With the aim of gathering information in this direction, we utilized an approach exploited in another study previously [24] that is based on the notion that the DNA single-strand breakage induced by *t*B-OOH is prevented by iron chelators but insensitive to *bona fide* antioxidants [24,31,33,34]. Using this approach we provided experimental evidence suggesting that the most prominent activity of the flavonoid quercetin resides in its ability to chelate iron [24], a finding recently confirmed by another laboratory [35].

From the above premise it follows that agents which prevent cell death induced by *t*B-OOH are more likely to act via an iron-chelating mechanism when capable of affording a parallel protection against the DNA cleavage induced by H_2O_2. On the contrary, the inability of preventing the latter effect associated with cytoprotection would be taken as a strong indication that the tested molecule acts as a scavenger.

We therefore performed experiments in which the cells were exposed for 30 min to 200 μM *t*B-OOH, in the absence or presence of different compounds, and the level of DNA single-strand breakage was measured immediately after the treatments using the alkaline halo assay [28]. Under these conditions *t*B-OOH caused a RNSF of 5.25 ± 0.49. As expected, this DNA-damaging response was both insensitive to BHT (200 μM), DPPD (10 μM) and Trolox (1 mM), and abolished by *o*-phenanthroline (25 μM; Table 1).

The results illustrated in Figure 3 and Table 2 indicate that each of the polyphenolic compounds affording protection in

Table 3 Effect of selected polyphenols on the loss of MTT-reducing activity caused by *t*B-OOH in U937 cells

Cells were exposed for 30 min to 3 mM *t*B-OOH in saline A in the absence or presence of the indicated compounds. Cytotoxicity was measured after 6 h of post-challenge growth in fresh culture medium using the MTT assay. Results are expressed as the percent ratio between the MTT-reducing activity in treated versus control cells, and are the mean ± S.E.M. for at least three separate experiments, each performed in duplicate.

Compound	*t*B-OOH (% ratio)
–	55.4 ± 3.21
CAPE (0.1 μM)	90.2 ± 5.07
BC (0.3 μM)	91.7 ± 7.31
MBC (1 μM)	93.4 ± 6.45
Chrysine (100 μM)	55.3 ± 4.11
Morin (100 μM)	57.1 ± 4.23
Quercetin (100 μM)	86.5 ± 8.61
DF (50 μM)	85.7 ± 4.38
PDH (100 μM)	105 ± 11.92
P3HC (100 μM)	57.1 ± 5.25
P4HC (100 μM)	55.8 ± 6.16
BHT (200 μM)	85.04 ± 7.41
DPPD (10 μM)	88.62 ± 7.74
Trolox (1 mM)	86.22 ± 8.11
o-Phenanthroline (25 μM)	91.37 ± 6.25

Table 4 Effect of selected polyphenols on the toxicity induced by H_2O_2 in U937 cells

Cells were exposed for 1 h to 3 mM H_2O_2 in saline A in the absence or presence of the indicated compounds. The relative number of dead cells was measured after 6 h of post-challenge growth in fresh culture medium using the Trypan Blue exclusion assay. Results are expressed as the percentage of dead cells in each sample and are the mean ± S.E.M. for at least three separate experiments, each performed in duplicate.

Compound	H_2O_2 (% dead cells)
–	34.06 ± 2.41
CAPE (0.1 μM)	5.55 ± 0.53
BC (0.3 μM)	6.26 ± 0.61
MBC (1 μM)	5.02 ± 0.45
Chrysine (100 μM)	32.96 ± 3.11
Morin (100 μM)	34.61 ± 3.23
Quercetin (100 μM)	6.91 ± 0.71
DF (50 μM)	5.55 ± 0.38
PDH (100 μM)	7.85 ± 0.8
P3HC (100 μM)	40.8 ± 6.15
P4HC (100 μM)	38.8 ± 2.36
BHT (200 μM)	37.17 ± 3.12
DPPD (10 μM)	34.43 ± 2.87
Trolox (1 mM)	35.55 ± 3.01
o-Phenanthroline (25 μM)	5.89 ± 0.44

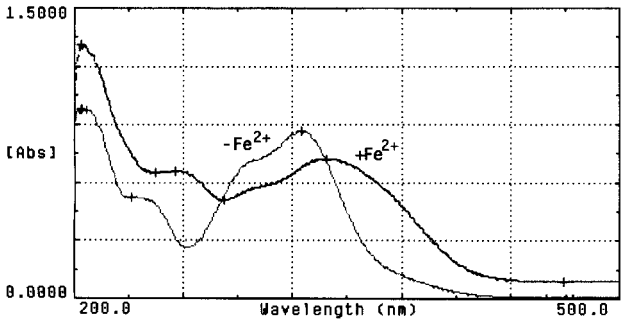

Figure 4 UV-visible absorption spectra of CAPE in the absence or presence of Fe^{2+} ions

CAPE was dissolved at 50 μM in phosphate buffer (50 mM, pH 7.4) in the absence or presence of 50 μM $FeSO_4$ and spectra (200 to 500 nm) were immediately recorded.

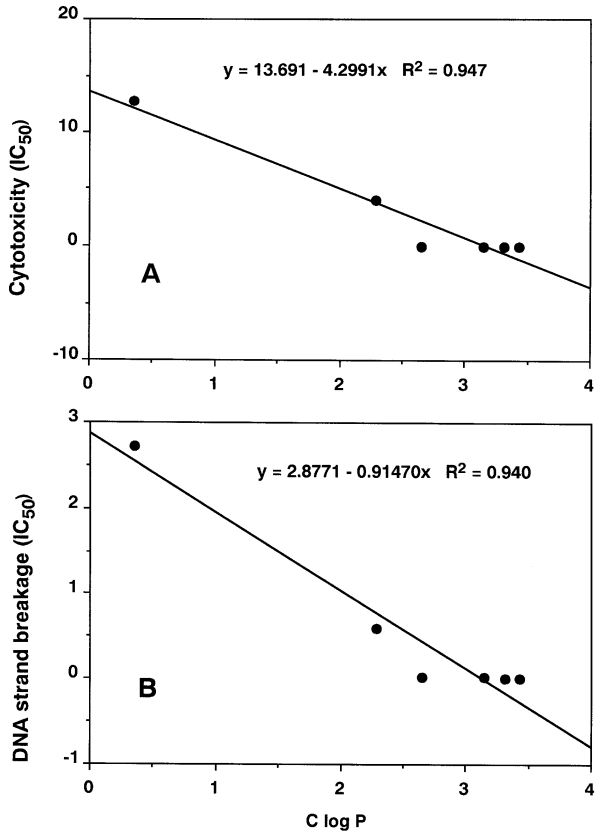

Figure 5 Correlation analysis for lipophilicity and the protective effects of phenolic compounds on *t*B-OOH-induced cytotoxicity and genotoxicity

The IC_{50} values (Table 2) of the active polyphenols in cytotoxicity (**A**) and genotoxicity (**B**) studies were plotted versus their lipophilicity, expressed as ClogP values calculated as detailed in the Materials and methods section.

cytotoxicity studies (Figure 2 and Table 2) also reduced the DNA strand scission caused by H_2O_2. Interestingly, a comparison of the IC_{50} values for both effects clearly indicates that, on a molar basis, each of these compounds is at least 4-fold more effective in preventing the DNA cleavage than cell death mediated by *t*B-OOH exposure. Not surprisingly the same compounds which did not reduce cytotoxicity, also failed to mitigate the extent of the DNA strand scission induced by *t*B-OOH.

Thus, under the experimental conditions utilized in this study, the most prominent activity of CAPE, BC, MBC, quercetin, DF or PDH appears to be iron chelation. In order to provide additional experimental support for this notion we have taken advantage of our recent observation that exposure of U937 cells to high concentrations of H_2O_2 promotes a lethal response that is insensitive to *bona fide* antioxidants but prevented by iron chelators (L. Palomba and O. Cantoni, unpublished work). We reasoned that agents displaying cytoprotective properties in this toxicity paradigm were likely to act via an iron-chelating mechanism. We therefore performed experiments in which the cells

were exposed for 60 min to 3 mM H_2O_2, in the absence or presence of different compounds, and cytotoxicity was measured after 6 h of post-treatment incubation in fresh culture medium. Under these conditions $34.06 \pm 2.41\%$ of the cells lost their ability to exclude Trypan Blue (Table 4). As expected, this lethal response was both insensitive to BHT (200 μM), or DPPD (10 μM), and abolished by *o*-phenanthroline (25 μM) (Table 4).

The results illustrated in Table 4 indicate that each of the polyphenolic compounds affording protection in cytotoxicity studies illustrated in Figure 2 and Table 2 also reduced the toxicity caused by H_2O_2. In addition, all the inactive compounds listed in Table 2 failed to mitigate the toxicity induced by H_2O_2.

Thus, these results are consistent with the above inference that the most prominent activity of the polyphenols employed in the present study is iron chelation.

The experimental results illustrated in Figure 4 and Table 2 are consistent with this notion. Indeed, measurement of the UV-visible absorption spectrum for CAPE showed, upon addition of Fe^{2+} ions, a characteristic absorbance band-shift indicative of the formation of iron chelates. No absorbance band-shift was detected using P4HC (results not shown). The remaining compounds active in cytotoxicity and genotoxicity studies, unlike the inactive ones, produced typical UV-visible absorbance shifts indicative of the formation of iron chelates (Table 2).

It is important to note that all the compounds displaying cytoprotective and iron-chelating activities bear a catechol group in one of the aromatic rings whereas this group is absent in the inactive compounds, which do, however, show strict structural analogies with some of the active derivatives. This would suggest that the presence of two hydroxy groups in the *o*-position is essential for the chelation of iron. An example is given by chrysine, morin and quercetin. Removal (chrysine) or replacement of the catechol group of quercetin with two hydroxy groups in the *m*-position (morin) abolished the protective and iron-chelating effects of the flavonoid (Table 2). Similarly, the strong activities of CAPE were lost when one of the hydroxy groups of the catechol ring was removed (P3HC and P4HC; Table 2). Removal of the double bond in the α-position of CAPE generated a derivative (PDH) with similar biological activity; a finding consistent with the notion that in both circumstances the availability of electrons in the catechol moiety allows efficient iron chelation.

Our results are therefore in apparent conflict with the general view that the non-catecholic hydroxy groups in flavonoids or caffeic acid esters may themselves act as scavengers by abstracting an electron from radicals [36] in a biologically relevant setting. Two additional lines of evidence support our conclusion. Firstly, chrysine and morin, quercetin analogues lacking the catechol group in the B ring with two hydroxy groups in the *m*-position in the A ring, were not active. Secondly, quercetin was less potent than DF, an *o*-diphenolic analogue lacking the hydroxy groups on the A and C rings. Thus, the antioxidant activity of these compounds would appear to be negatively related to the number of the non-catecholic hydroxy groups which are expected to reduce their lipophilicity and cellular uptake.

In order to assess the relationships between the protective effects and the lipophilicity, the ClogP values (Table 2) of these compounds were plotted against the IC_{50} values of the caffeic acid esters and flavonoids obtained in cytotoxicity and DNA damage experiments. An excellent direct correlation was observed in both analyses: the higher the level of lipophilicity, the higher the extent of cytoprotection (Figure 5A, $r^2 = 0.947$) or the ability to prevent DNA strand scission (Figure 5B, $r^2 = 0.940$).

In conclusion, the results presented in this study strongly suggest that, in a specific toxicity paradigm, the antioxidant activity of a series of caffeic acid esters and flavonoids is largely mediated by an iron-chelating mechanism. In this response a critical role of the catechol ring was identified. In the absence of this structure, the presence of hydroxy groups did not promote any antioxidant activity. Hydroxy groups in different positions of the molecules bearing the catechol groups caused a decrease in biological activity, possibly because these modifications reduce the lipophilicity of the polyphenols. Their biological potencies were a direct function of the lipophilicity which is expected to increase the cellular uptake of these agents, as well as their subcellular localization in lipid compartments. As a final note, it is important to emphasize that our conclusions are based on experimental results obtained using a specific toxicity paradigm. Thus, although the iron-chelating mechanism has the potential of being biologically relevant, in that it was found to operate at very low levels of the polyphenols, the possibility exists that in different systems (different cells and different sources of reactive oxygen species) the caffeic acid esters and flavonoids may also exhibit protective effects via direct free radical scavenging.

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