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Free radical scavenging and cytoprotective activities of phenolic antioxidants

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The free radical scavenging activities of three flavonoids (quercetin, rutin and catechin) and four hydroxycinnamic acids (caffeic, ferulic, sinapic, and chlorogenic acids) were evaluated using both oxygen radical absorbance capacity (ORAC) and lipid peroxidation inhibition capacity (LPIC) assays. The cytoprotective effects of these compounds were also measured by the degree of protection against H_2O_2-induced damage of human Jurkat cells. All compounds exhibited protection against H_2O_2-mediated cytotoxicity in a dose-dependent manner. The concentrations required to result in a 50% reduction in cell death (EC_{50} value) were calculated from their dose-response curves. These ranged from 0.15 – 2.65 μM. Overall, the four hydroxycinnamic acids tested were less effective than the three flavonoids, and of all compounds tested, quercetin offered the strongest protection against H_2O_2-induced cell death. A comparison of the results showed that the ability to inhibit peroxidation of lipids in a liposomal system (LPIC) correlated well with the cytoprotective activities (EC_{50}), but not with the ability to protect an aqueous fluorescent substrate in the ORAC assays. The results suggest that the behavior of antioxidants in a liposomal membrane is to some extent similar to the mechanism involved in the protection of living cells from oxidative damage.

Keywords: Antioxidant / Flavonoids / Hydroxycinnamic acids / Lipid peroxidation inhibition capacity / Lipophilicity

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1 Introduction

Flavonoids and hydroxycinnamic acids are ubiquitous phenolic secondary metabolites synthesized by plants for defensive purposes [1]. When consumed as food these diet-derived compounds have been recognized to act as antioxidants and are thought to be contributors to the health benefits of fruit and vegetables [2, 3]. The antioxidant properties of plant-derived phenolic compounds have been extensively studied by using *in vitro* chemical systems [4 – 7]. These systems have the advantage of being relatively simple and inexpensive to carry out. However, such *in vitro* assays can only rank antioxidant activity for their particular reaction system and their relevance to *in vivo* health-protective activities is uncertain. It is therefore considered prudent to use more than one antioxidant assay system to measure antioxidant activities, as there may be distinct mechanisms involved, resulting in different outcomes, depending on the method of test [8].

The oxygen radical absorbance capacity (ORAC) assay was devised by Cao *et al.* [5, 9] to provide a means of measuring the total antioxidant activity in a way that was independent of reaction kinetics. It is an aqueous based system and has been frequently used to measure the antioxidant capacity of various biological samples from purified phytochemicals to complex plant extracts [10]. However, many phenolic antioxidants such as flavonoids and phenolic acids have a high lipophilicity and therefore will partition into a hydrophobic phase more than into water. The lipid peroxidation inhibition capacity (LPIC) assay [11] uses a fluorescent 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid (C_{11}-BOD-

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Abbreviations: AAPH, 2,2′-azobis(2-amidinopropane)hydrochloride; **C_{11}-BODIPY**, 4, 4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid; **CDI**, cell death index; **DOPC**, 1,2-dioleoyl-sn-glycero-3-phosphocholine; **EC_{50}**, concentration required to result in a 50% reduction in cell death; **LPIC**, lipid peroxidation inhibition capacity; **ORAC**, oxygen radical absorbance capacity; **PI**, propidium iodide

IPY) probe dissolved into the membrane phase of the liposome. This assay simulates the ORAC assay but in a liposomal form that is sensitive to the membrane partitioning behavior of antioxidants. LPIC and ORAC assays therefore address different aspects of antioxidant properties.

The mechanisms by which antioxidant compounds protect cells are still not clear and thus it is difficult to extrapolate from *in vitro* antioxidant data to human health effects. The antioxidant capacity of compounds alone does not predict the ability of antioxidants to protect cells exposed to oxidative stress [12]. *In vivo* mechanisms of action of antioxidants in disease prevention and health promotion go beyond the antioxidant activity of scavenging free radicals [12, 13]. However, animal models and human studies are expensive and not suited to screening the biological activity of diverse phytochemical antioxidants. Cell culture models can be used to investigate some of the biological effects of antioxidants but there is a need to understand their relationship to both *in vitro* antioxidant assay results and *in vivo* animal or human studies.

Attempts to mimic a biological environment for experimental assay purposes can be achieved by means of membrane models, which are typically liposomes made up of lipid bilayers. These are comparable to cellular membrane systems. *In vivo* lipophilic antioxidants can penetrate cell membranes and may prevent peroxidative damage to cells through membrane-related partitioning effects that supplement their chemical activities [14].

Exposure of cells to oxidative stress induces a range of cellular events that can result in apoptosis or necrosis [15]. Apoptotic cells can be evaluated based on measurement of the loss of plasma membrane asymmetry [16]. Under normal physiological conditions, a cell maintains a strictly asymmetric distribution of phospholipids in the two leaflets of the cellular membranes with phosphatidylserine (PS) facing the cytosolic side [17]. However, during early apoptosis this membrane asymmetry is rapidly lost without concomitant loss of membrane integrity [16]. Cell surface exposure of phosphatidylserine (PS), which precedes the loss of membrane integrity, can be detected by FITC-labeled annexin V, a reagent that has high affinity for PS residues in the presence of millimolar concentrations of calcium (Ca) [18]. By simultaneous probing of membrane integrity by means of exclusion of the nuclear dye propidium iodide (PI), apoptotic cells can be discriminated from necrotic cells [19].

In the present study, the antioxidant activities of three flavonoids (quercetin, rutin and catechin) and four hydroxycinnamic acids (caffeic, ferulic, sinapic, and chlorogenic acids) were evaluated using both ORAC and LPIC assays. The cytoprotective effects of these compounds were also measured by the degree of protection against H_2O_2-induced cellular damage using both apoptosis and necrosis endpoints. These cytoprotective effects were then compared with the measured LPIC and ORAC activities to determine which chemical antioxidant testing system could better predict their ability to protect cells exposed to oxidative stress.

2 Materials and methods

2.1 Chemicals

The fatty acid fluorescent probe C_{11}-BODIPY was obtained from Molecular Probes (Eugene, OR). The 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC), β-phycoerythrin (PE) (lot number 109A4039), 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH), PI, quercetin, rutin, catechin, sinapic acid and chlorogenic acid were purchased from Sigma (St. Louis, MO). Caffeic acid was purchased from Acros Organics (Milwaukee, WI). Ferulic acid and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Aldrich Chemicals (Milwaukee, WI). Hydrogen peroxide was obtained from BDH Chemicals (Poole, UK). All other solvents were of HPLC grade. Water used in experiments was deionized (MilliQ).

2.2 The LPIC assay

The antioxidants were either incorporated into liposomes ($LPIC_{Inco}$) or mixed with preformed DOPC-unilamellar vesicles ($LPIC_{Mixed}$) and the LPIC assay was performed as previously described [11]. Briefly, in the final assay mixtures (2 mL of total volume), the fluorescent probe C_{11}-BODIPY (2.4 μM) was used as a target of free radical damage, AAPH (40 mM) as a peroxyl radical generator and Trolox as a control standard. Antioxidants were either incorporated into or mixed with preformed DOPC-C_{11}-BODIPY vesicles, based on their solubility. The fluorescence was measured every minute on a spectrophotometer (Perkin Elmer LS50B, Wellesley, MA) at excitation and emission wavelengths of 540 and 600 nm, respectively. Final results were calculated using the differences in areas under the fluorescence decay curves of C_{11}-BODIPY between a blank and a sample and were expressed as Trolox equivalents (in μmol Trolox per μmol antioxidant).

2.3 The ORAC assay

The ORAC assay procedure was based on the method of Cao *et al*. [5]. Briefly, in the final assay mixture (2 mL total volume), β-phycoerythrin (68 mg/L) was used as a target of free radical damage and AAPH (40 mM) as a peroxyl radical generator. Trolox was used as the control standard. After

the addition of AAPH, the zero point was immediately recorded using a fluorescence spectrophotometer (Perkin Elmer LS50B) at excitation and emission wavelengths of 540 and 565 nm, respectively. Final results were calculated using the differences of areas under the quenching curves of β-phycoerythrin between a blank and a sample and compared with a standard curve prepared with different concentrations of Trolox (0–100 μM). The results were expressed as Trolox equivalents (in μmol Trolox per μmol antioxidant).

2.4 Cytoprotection assay

2.4.1 Culture and treatment of human Jurkat T cells

Jurkat cells (clone E6-1), a human T cell leukemic cell line was obtained from the ATCC (American Type Culture Collection; Manassas, VA). Cells were cultured in RPMI-1640 medium with 10% heat-inactivated fetal calf serum and 2 mM glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin (Invitrogen-Life Technologies, Carlsbad, CA) at 37°C in humidified air with 5% CO_2. The experimental design consisted of measuring the extent of cytotoxicity and cytoprotection that antioxidants provided against hydrogen peroxide-induced cell death. Cells in RPMI-1640 medium were seeded at a density of 5×10^5 cells/mL and then different concentrations of phenolic compounds were added. Hydrogen peroxide (50 μM) was then added to one set of duplicated wells simultaneously with phenolic compounds. After 18-h incubation, treated and untreated cells were harvested by centrifugation (1200 rpm, 5 min) and washed twice with PBS.

2.4.2 Annexin V staining and flow cytometric analysis

Annexin V coupled with FITC is typically used in conjunction with a vital dye such as PI to identify different stages of apoptotic and necrotic cells using flow cytometry. This assay was performed according to the method described by Vermes *et al*. [20] with slight modifications. Briefly, the washed cells were resuspended in 100 μL of binding buffer containing Annexin V-FITC (BD Biosciences, San Diego, CA) and incubated in the dark for 20 min. Then, another 400 μL binding buffer containing PI was added and incubated for a further 10 min. Flow cytometric analysis was performed within 1 h using a Cytomics FC500 MPL (Beckman Coulter, Miami, FL). The total cell count was set to 35 000 cells/sample.

2.4.3 Calculation of cell death index

Apoptosis or cell death is characterized by a variety of morphological changes including alterations to the plasma membrane. Annexin V staining precedes the loss of membrane integrity, which accompanies the later stages of cell death resulting from apoptosis. Annexin V in conjunction with PI is used to identify early apoptotic cells. PI is used to evaluate the increased membrane permeability of apoptotic cells. The percentages of viable, early, late apoptotic and necrotic cells were determined as illustrated in the cytogram (Fig. 1). The viable cells are located in the lower left corner (negative in both annexin V-FITC and PI, A3). Early apoptotic cells are in the lower right corner (annexin V-FITC positive only, A4). Late apoptotic cells that show progressive cellular membrane and nuclear damage are in the upper right corner (both annexin V-FITC and PI positive, A2). Necrotic cells are located in the upper left corner (PI positive only, A1). The total percentage of damaged cells (both apoptotic and necrotic) was considered as (A1+A2+A4). The cell death index (CDI) was calculated based on the cytogram by the following equation:

$$\mathrm{CDI} = \frac{(\mathrm{A1} + \mathrm{A2} + \mathrm{A4})}{\mathrm{A3}} \times 100 \quad (1)$$

The CDI is the ratio of total damaged cells to viable cells and is used to remove inter-experiment variations in cell density. The net cell damage (ΔCDI) is derived by subtracting the CDI of incubated control cells (Fig. 1B) from that of treated cells (Fig. 1C):

$$\Delta\mathrm{CDI} = \mathrm{CDI}_{\text{Treated cells}} - \mathrm{CDI}_{\text{Incubated control cells}} \quad (2)$$

2.4.4 Calculation of 50% reduction in cell death (EC_{50})

The cytoprotective effects of antioxidants were measured by the inhibition of total cell death induced by 50 μM H_2O_2 (approximately caused 50% total cell death). The percentage of inhibition of cell death was calculated by:

$$\%\ \text{Inhibition of cell death} = \frac{\Delta\mathrm{CDI}_{\mathrm{HP}} - \Delta\mathrm{CDI}_{\mathrm{Sample}}}{\Delta\mathrm{CDI}_{\mathrm{HP}}} \times 100 \quad (3)$$

where $\Delta\mathrm{CDI}_{\mathrm{HP}}$ and $\Delta\mathrm{CDI}_{\mathrm{Sample}}$ are the net cell damage caused by 50 μM H_2O_2 and test sample, respectively. The EC_{50} values were calculated from the dose-response relationship between the concentrations of antioxidant and % inhibition of cell death.

2.5 Data analysis

The mean values were calculated from data taken from at least two separate experiments with each analysis. The analysis of flow cytometric data was carried using CXP data analysis software (Beckman Coulter, Miami, FL). The

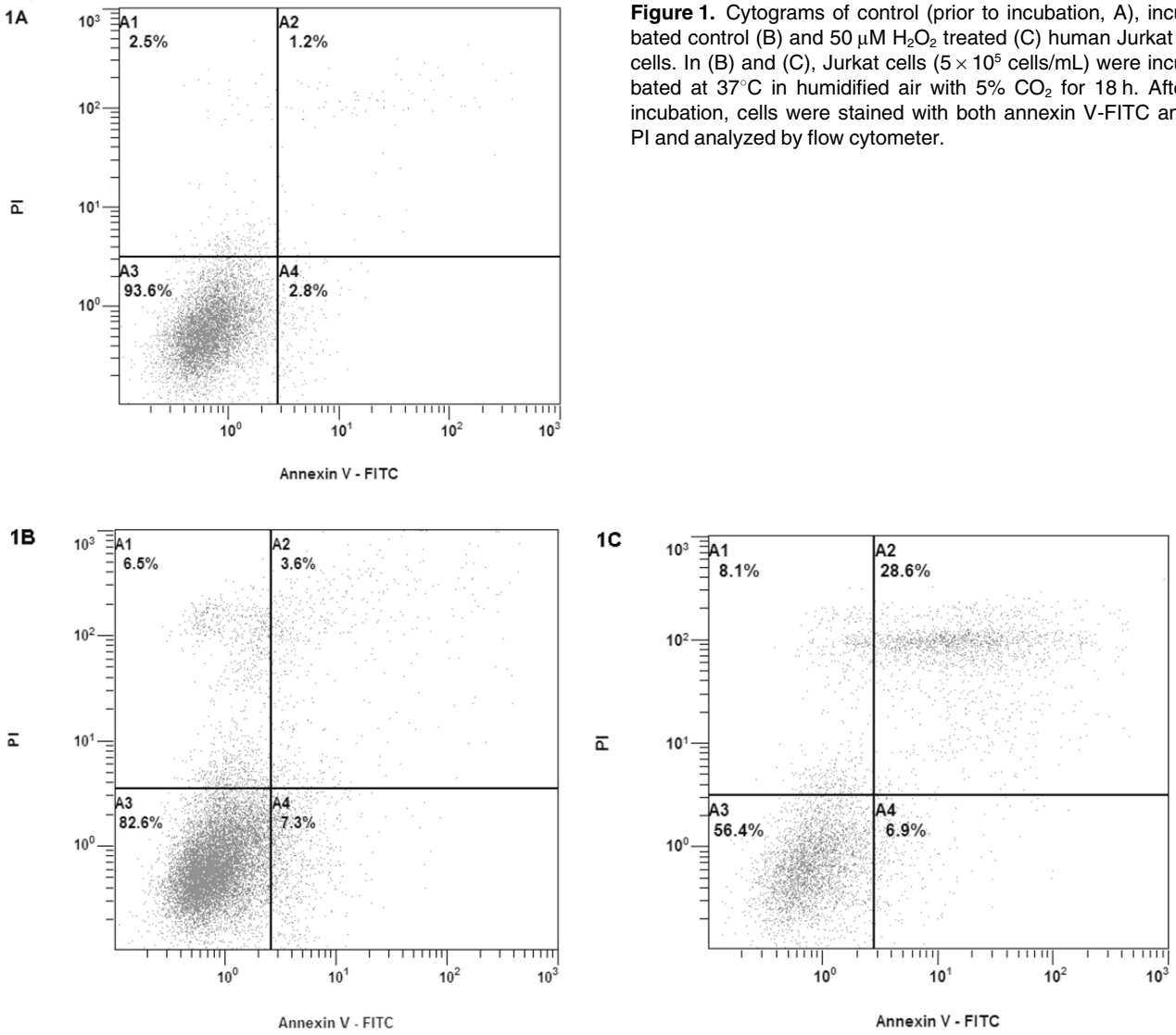

Figure 1. Cytograms of control (prior to incubation, A), incubated control (B) and 50 μM H_2O_2 treated (C) human Jurkat T cells. In (B) and (C), Jurkat cells (5×10^5 cells/mL) were incubated at 37°C in humidified air with 5% CO_2 for 18 h. After incubation, cells were stained with both annexin V-FITC and PI and analyzed by flow cytometer.

EC_{50} was calculated using the Sigmoidal dose-response function in GraphPad Prism 4.0 software (http://www.graphpad.com/prism/Prism.htm).

3 Results and discussion

3.1 The relationship between antioxidant capacity measured by the ORAC and the LPIC assays

The LPIC and ORAC values are shown in Table 1. The lipid peroxidation inhibition capacity of the flavonoids and hydroxycinnamic acids was tested by both direct addition to preformed unilamellar vesicles ($LPIC_{Mixed}$) and by incorporation into the unilamellar vesicles during the manufacturing process ($LPIC_{Inco}$). There was effectively no correlation ($r^2 = 0.18$) between the $LPIC_{Mixed}$ and the $LPIC_{Inco}$. However, the $LPIC_{Mixed}$ values were approximately double the response compared to the $LPIC_{Inco}$ values when Trolox was used as the standard [11]. This may be due to higher incorporation of antioxidants during the making of the vesicles, and therefore more activity, being achieved by the $LPIC_{Inco}$ method.

There was only a weak linear correlation between the measured ORAC and $LPIC_{Mixed}$ values ($r^2 = 0.45$). The $LPIC_{Mixed}$ values tended to increase with increasing ORAC values (Fig. 2A). There was no relationship between the measured ORAC and $LPIC_{Inco}$ values ($r^2 = 0.006$) (Fig. 2B). The lack of a relationship between the methods is probably an inherent effect of the different assay mechanisms. In the LPIC

Table 1. LPIC, ORAC and EC_{50} values of flavonoids and hydroxycinnamic acids[a)]

Compound	ORAC (μmol)	$LPIC_{Inco}$ (μmol)	$LPIC_{Mixed}$ (μmol)	EC_{50} (μM)	$1/EC_{50}$ $(\mu M)^{-1}$
Quercetin	3.56 ± 0.08	4.34 ± 0.07	8.64 ± 0.05	0.151 ± 0.01	6.61
Caffeic acid	2.24 ± 0.18	3.92 ± 0.04	5.59 ± 0.09	0.394 ± 0.01	2.54
Rutin	0.78 ± 0.01	3.62 ± 0.03	4.68 ± 0.04	0.254 ± 0.03	3.94
Catechin	2.35 ± 0.04	3.11 ± 0.02	6.48 ± 0.07	0.296 ± 0.03	3.38
Chlorogenic acid	3.06 ± 0.05	2.57 ± 0.02	8.26 ± 0.10	0.403 ± 0.09	2.48
Sinapic acid	2.28 ± 0.07	2.31 ± 0.08	3.66 ± 0.10	2.648 ± 0.53	0.38
Ferulic acid	1.26 ± 0.04	2.23 ± 0.01	4.28 ± 0.01	1.305 ± 0.33	0.77

a) Data are the mean ± SD of two separate determinations.

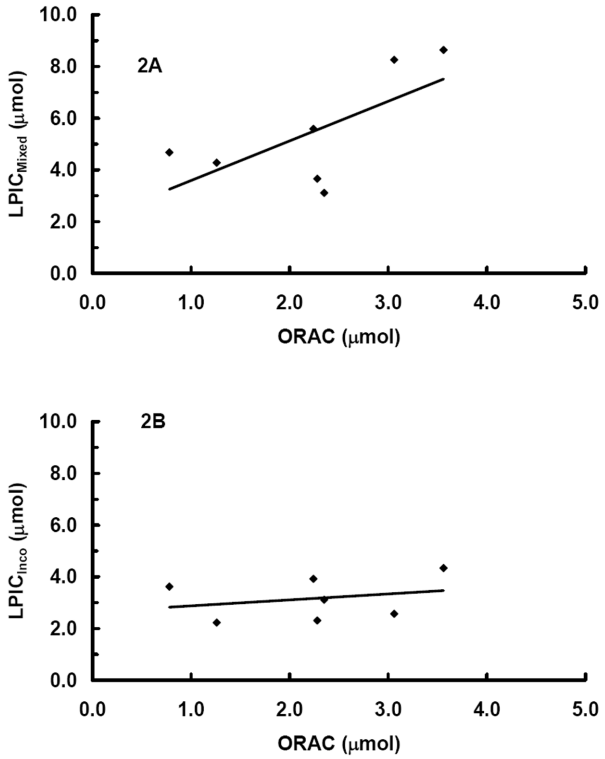

Figure 2. Correlation between LPIC and ORAC values of three flavonoids and four hydroxycinnamic acids. (A) Shows the data for $LPIC_{Mixed}$ v. ORAC values (r^2 = 0.45). (B) Shows the data for $LPIC_{Inco}$ v. ORAC values (r^2 = 0.006).

assay system, and particularly with the $LPIC_{Inco}$ assay, antioxidants were incorporated into unilamellar vesicles along with the lipid-soluble fluorescent probe when they were made. The fluorescent end of the probe sits on the membrane surface [21]. The AAPH radicals also have an affinity to the surface of lipid bilayers [22]. Therefore, this makes the affinity of antioxidants for the membrane an important co-determinate of their antioxidant activity. The LPIC assay thus measures the ability of antioxidants to protect a substrate embedded in a lipid membrane. Hence, the LPIC assay is suitable to study the effectiveness of antioxidants against membrane lipid peroxidation, which is an important mechanism in causing oxidative stress to the cells [11].

The results obtained in the ORAC assay did not show the same order of effectiveness shown in either the $LPIC_{Inco}$ or $LPIC_{Mixed}$ assays. Several factors influence the reactivity of antioxidants and these factors are different when assays are carried out in homogeneous solutions compared with liposomal membranes. The activity measured by either the $LPIC_{Inco}$ or the $LPIC_{Mixed}$ assay is governed, in part, by the capacity of phenolic antioxidants to bind to, and to penetrate, the lipid bilayers. Antioxidants with moderate lipophilicity, such as quercetin, are found both at the interface of the lipid bilayers and inside the bilayers [23, 24]. Therefore, they can be effective both in inhibition of attack by free radicals in the aqueous phase and effective in scavenging lipophilic free radicals inside lipid bilayers. In contrast, the ORAC assay and its variants [5, 9] are capable of measuring the activity of both lipophilic and hydrophilic antioxidants by employing a non-polar fluorophore. However, the simple effect of oxidation of a hydrophilic fluorescent protein does not necessarily reflect the extent of antioxidant protection against oxidative damage in biological systems. There is no partitioning lipid phase or surface reactivity in the ORAC assay system and thus a potentially important mechanisms for comparing antioxidant activity is not accounted for.

3.2 Cytoprotection assay

3.2.1 Cytotoxic effects of H_2O_2

Hydrogen peroxide is known to be able to induce both apoptosis and necrosis in cells [25 – 27], with the required concentrations and exposure time dependent on the cell type being investigated. The response of human Jurkat T cells to H_2O_2, in terms of both concentration and exposure time, was determined to calculate the dosage required to kill approximately half the cells. The CDI increased with increasing concentrations of H_2O_2 (Fig. 3) giving a CDI value of 52.2 ± 5.4 (mean of ten separate determinations) at a dose of 50 μM H_2O_2.

A time course showed a rapid increase of total cell death in the first 2 h after cells were treated with 50 μM H_2O_2 and then a slower increase up to 18 h (Fig. 4). Therefore, a concentration of 50 μM H_2O_2 and an 18-h incubation time was

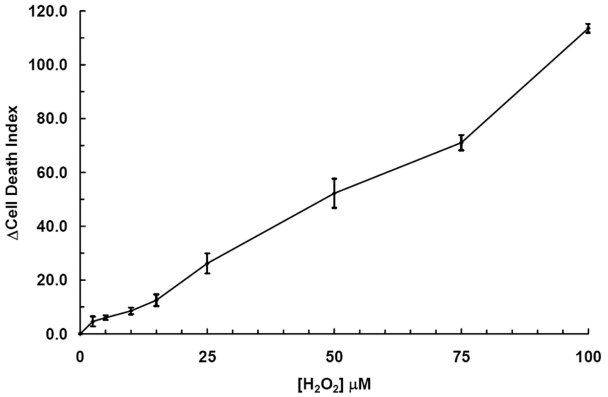

Figure 3. Cell death responses of Jurkat cells exposed to increasing concentration of H_2O_2. Jurkat cells (5×10^5 cells/mL) were exposed to different concentrations of hydrogen peroxide and incubated at 37°C in humidified air with 5% CO_2 for 18 h. After incubation, cells were stained with both annexin V-FITC and PI and analyzed by flow cytometer. The total cell death was calculated using Eq. (2). Bars indicate SD from the mean of two separate determinations.

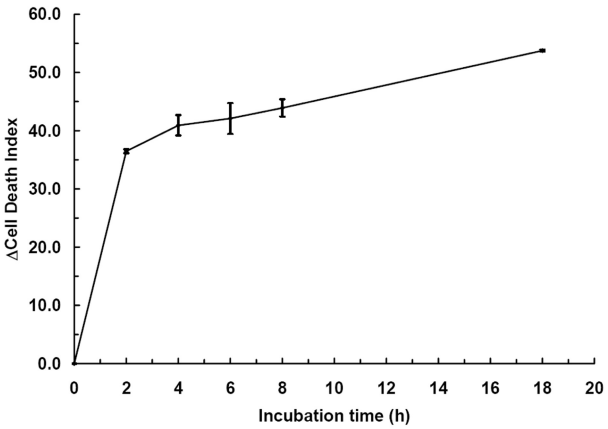

Figure 4. Effect of 50 μM hydrogen peroxide on the time course of cell death in Jurkat cells treated with H_2O_2. Jurkat cells (5×10^5 cells/mL) were incubated at 37°C in humidified air with 5% CO_2 for 18 h. After incubation, cells were stained with both annexin V-FITC and PI and analyzed by flow cytometer. The total cell death was calculated using Eq. (2). Bars indicate SD from the mean of two separate determinations.

used in this study to investigate the protection effects of phenolic compounds against H_2O_2-induced total cell death.

3.2.2 Cytotoxicity of phenolic compounds

There are a number of studies showing that dietary flavonoids can induce apoptosis in Jurkat T cells [28]. Dose-response relationships were determined, in duplicate experiments, for the intrinsic toxicity of quercetin, catechin, rutin, caffeic acid, chlorogenic acid, ferulic acid and sinapic acid (Figs. 5A and B). Quercetin, rutin, chlorogenic acid,

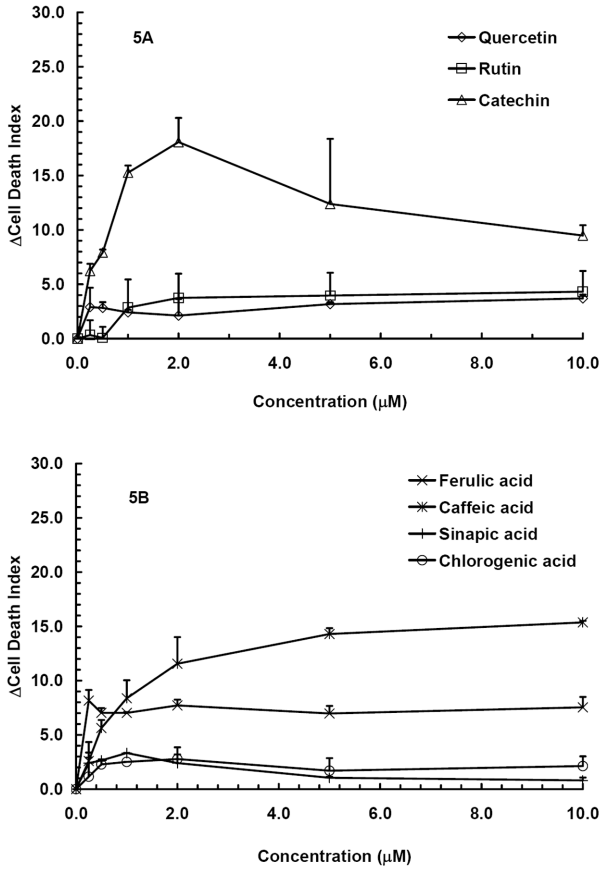

Figure 5. Dose-response curves for toxicity to Jurkat cells of phenolic compound tested. Jurkat cells (5×10^5 cells/mL) were incubated at 37°C in humidified air with 5% CO_2 for 18 h. After incubation, cells were stained with both annexin V-FITC and PI and analyzed by flow cytometer. The total cell death was calculated using Eq. (2). Bars indicate SD from the mean of two separate determinations. (A) shows the effect of three flavonoids and (B) shows the effect of four hydroxycinnamic acids.

ferulic acid and sinapic acid showed little or no cytotoxicity (CDI < 10). Catechin induced cell death at lower concentrations; the CDI reached a value of 18 at 2 μM but then decreased at higher concentrations. This high cytotoxicity of catechin may be due to its ability to produce H_2O_2 in cell culture systems. The resulting increase in H_2O_2 levels could trigger Fe(II)-dependent formation of highly toxic hydroxyl radicals, which in turn could induce cell death [29, 30]. This observation showed the pro-oxidant effect of catechin [31]. It has been reported that resveratrol can trigger apoptosis in human leukemia cells through eliciting pro-oxidant properties at lower concentrations [32, 33]. However, the cytotoxicity seen with catechin was lower at higher concentrations (>2 μM) presumably because of the antioxidant action of catechin, which would then start to depress the toxic hydroxyl radicals generated at lower concentrations. Similarly, caffeic acid induced cell death in a dose-depen-

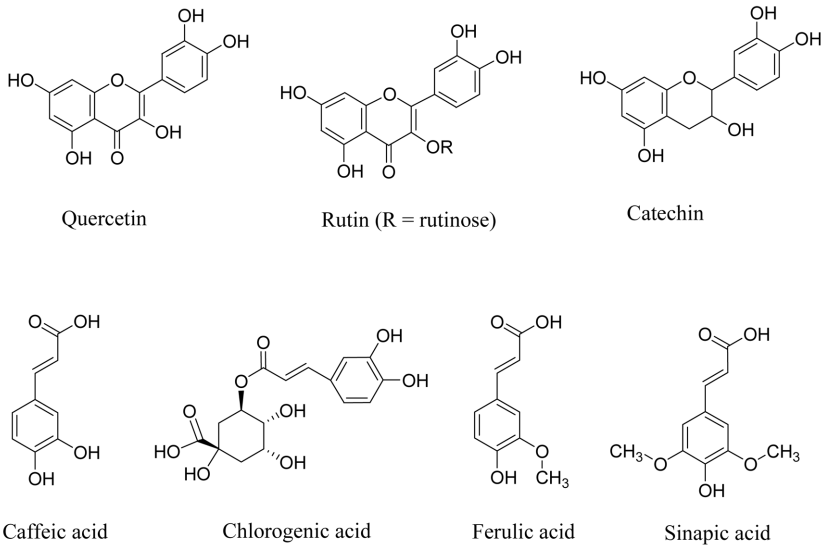

Figure 6. Chemical structure of flavonoids and hydroxycinnamic acids.

dent manner, with the CDI reaching a plateau (CDI ≈ 14) at 5 μM with little increase up to 10 μM.

3.2.3 The influence of flavonoids and hydroxycinnamic acids on cytotoxic effects of H_2O_2

Measurement of the potential health effects of dietary derived phenolic compounds needs to be undertaken at concentration ranges that are relevant to levels that might be achieved *in vivo*. Therefore, the polyphenol test concentrations used should be of the same order as the maximum plasma concentrations attained after a polyphenol-rich meal. These are thought to be in the range of 0.1 – 10 μM [34]. Hence, the three flavonoids and four hydroxycinnamic acids (Fig. 6) were added to Jurkat cells in the doses of 0.25, 0.5, 1.0, 2.0, and 10.0 μM immediately prior to the addition of the H_2O_2.

All compounds tested exhibited protection against H_2O_2-mediated cytotoxicity in a dose-dependent manner. The EC_{50} value (Table 1) of each compound tested was calculated from their dose-response curves. Of all compounds tested, quercetin offered the strongest protection against H_2O_2-induced cell death (Fig. 7). Catechin has the same number and location of hydroxyl groups as quercetin, but catechin lacks the 2,3-double bond and the 4-keto group, so there is no electron delocalization between the A-ring and B-ring because of the saturation of the heterocyclic C-ring. This caused a nearly 40% reduction in its cytoprotective activity compared to quercetin. Replacement of the hydroxyl group at the C-3 position of quercetin by rutinose in rutin reduced its cytoprotective activity but rutin was still more active than catechin.

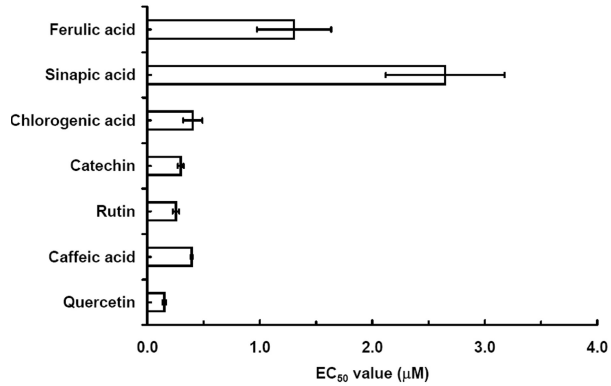

Figure 7. EC_{50} values of three flavonoids and four hydroxycinnamic acids. Jurkat cells (5×10^5 cells/mL) were incubated at 37°C in humidified air with 5% CO_2 for 18 h. After incubation, cells were stained with both annexin V-FITC and PI and analyzed by flow cytometer. The total cell death was calculated using Eq. (2) and EC_{50} values were calculated through a dose-response curve plotted by the concentrations of antioxidant versus % inhibition of cell death as calculated using Eq. (3). Bars indicate SD from the mean of two separate determinations.

Overall, the four hydroxycinnamic acids tested were less effective than the three flavonoids. Chlorogenic acid, which is the ester of caffeic acid and quinic acid, is slightly less effective than caffeic acid. Substitution of the 3-hydroxyl group of caffeic acid by a methoxy group (ferulic acid) considerably reduced its effectiveness. The effectiveness of sinapic acid, which has an additional methoxy group, was almost 50% less than that of ferulic acid. Hydroxycinnamic acids such as caffeic and chlorogenic acids are mainly in their ionized form at pH 7.4 and therefore, largely remain in the extracellular medium or associated with cell mem-

branes. On the other hand, more hydrophobic molecules, such as quercetin, rutin and catechin, would be expected to interact more strongly with the lipid membranes resulting in higher local concentrations and greater ability to protect cell membranes. H_2O_2 has been reported to generate DNA damage through oxygen-radical mechanisms and induce chromosomal aberrations, gene mutations and DNA single strand breaks [35]. H_2O_2 may be involved, via a Fenton-type reaction, in the formation of hydroxyl radicals, which are highly reactive and result in direct DNA damage [30, 36]. The results presented here relate to previously reported studies on protective properties of polyphenols in human Jurkat T cells challenged with H_2O_2 [37 – 40] in human myelogenous leukemia K562 cells [41] and in H_2O_2-treated murine leukemia L1210 cells [42]. However, it is considered unlikely that only the free radical scavenging activity of the flavonoids and hydroxycinnamic acids protect cells against the action of reactive oxygen species (ROS) [43]. Other biological activities of these phenolic compounds, for example, modulation of cell signaling, may also contribute to their cellular protective properties [13]. It has been reported that polyphenols can also protect human normal peripheral blood mononuclear cells from H_2O_2-induced apoptosis [44].

There is a growing number of reports showing the metabolism of common flavonoids in cultured cells [45 – 47]. An initial study in our laboratory indicated that quercetin showed approximately 25% conversion to a slightly more polar, but unconjugated derivative, possibly an oxidized quinone form after 18-h incubation with Jurkat T cells (Deng *et al*. *FEBS Letters*, accepted for publication). The ability of flavonoids to act as effective antioxidants *in vivo* is dependent on the extent of their conjugation and biotransformation on absorption [48]. It has been reported that the oxidative metabolism of quercetin in fibroblasts, occurs in such a physiological intracellular environment, via enzymic processes or the non-enzymic oxidation of quercetin [49, 50]. It has been confirmed that the most effective flavonoid as an antioxidant *in vitro*, are those that are metabolized to the greatest extent in *vivo* [51]. This may explain why quercetin exhibited the strongest cytoprotective effect in our testing system. Further studies are in progress to investigate the metabolism of polyphenols during incubation with cells in the presence or absence of H_2O_2.

3.3 The relationship between antioxidant capacity and protection against H_2O_2-mediated cytotoxicity

In order to understand how chemical antioxidant assays and a cell-based assay compared for their ability to measure biological activities of polyphenols, the measured ORAC and LPIC values of the three flavonoids and four hydroxycinnamic acids were compared with the $1/EC_{50}$ values derived

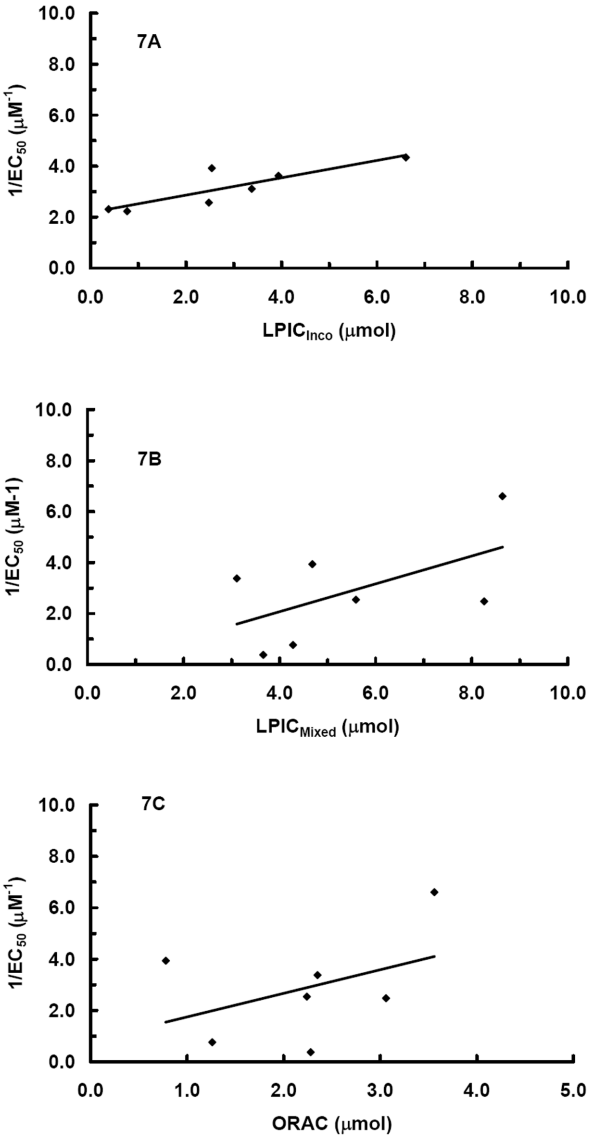

Figure 8. Correlation between measured antioxidant activities and $1/EC_{50}$ values of three flavonoids and four hydroxycinnamic acids. (A) shows the data for $LPIC_{Inco}$ v. $1/EC_{50}$ (r^2 = 0.72), (B) shows the data for $LPIC_{Mixed}$ v. $1/EC_{50}$ (r^2 = 0.59) and (C) shows the data for ORAC v. $1/EC_{50}$ (r^2 = 0.04).

from the protection of H_2O_2-mediated cytotoxicity of human Jurkat T cells. As can be seen from Table 1, the order of efficacy of $LPIC_{Inco}$ is almost the same as that of EC_{50} values except for caffeic acid and catechin, which are in reversed order. There was a linear correlation between $LPIC_{Inco}$ and $1/EC_{50}$ values of tested antioxidants as shown in Fig. 8A (r^2 = 0.72). Similarly, there was also a linear correlation between $LPIC_{Mixed}$ and $1/EC_{50}$ values as shown in Fig. 8B (r^2 = 0.59). In contrast, there was almost no correlation between the ORAC and $1/IC_{50}$ values as shown in Fig. 8C (r^2 = 0.04). In principle, *in vitro* antioxidant activity

assays do not necessarily reflect the cellular physiological conditions and do not consider bioavailability and metabolism issues. However, the strong relationship between LPIC activity and the protection against H_2O_2-induced cell death indicates that there are some degrees of similarity between liposomal membranes and living cell environments. With the ORAC method it is assumed that the oxidative deterioration and, in turn, the antioxidative mechanism and protection of the fluorescence protein β-phycoerythrin can mimic critical biological substrates. The simple effect of oxidation of the photoreceptor portion of β-phycoerythrin on fluorescence measurements does not necessarily reflect the extent of antioxidant protection afforded against oxidative damage of the protein itself. There are no lipid substrates in the ORAC reaction system. Results from a purely hydrophilic media may not be directly comparable with antioxidant effectiveness in lipid-rich foods and biological systems.

4 Concluding remarks

Antioxidant activity methodologies must be validated and compared in order to identify the most appropriate methods for determining antioxidant activity that will mostly closely relate to, and predict, the effectiveness of antioxidants in providing health benefits to the human body *in vivo*. The antioxidant activity measured in the LPIC assay, particularly the $LPIC_{Inco}$ variation, correlates well to cytoprotection activity measured in an oxidatively stressed cell model. The LPIC assay is therefore an improved chemical model to screen and compare the antioxidant activity of diverse phytochemical antioxidants before further testing by *in vitro* cell culture or animal and human *in vivo* bioassay systems.

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