Methylated polyphenols are poor “chemical” antioxidants but can still effectively protect cells from hydrogen peroxide-induced cytotoxicity

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Keywords: Polyphenolic; Antioxidant mechanism; Hydrogen peroxide; Apoptosis

1. Introduction

Polyphenolic compounds, such as flavonoids (e.g., quercitin) and phenolic acids (e.g., caffeic acid) are effective antioxidants in vitro, mainly because of their ability to scavenge damaging free radicals [1]. This activity has long been thought to be equally important in vivo [1]. The real in vitro direct antioxidant effect of dietary polyphenolics, however, is increasingly being questioned and may actually be quantitatively insignificant [2,3]. The bioavailability of most polyphenolics is low, in the range of 0.1–10 µM plasma concentration [4] and they can act as pro-oxidants in some circumstances [1,5].

Nobiletin, a natural poly-methylated citrus flavonoid, has been shown in Caco-2 cell-based absorption studies to cross the cell monolayer almost twice as fast as un-methylated luteolin and to accumulate in the cells, whereas luteolin did not [6].

Abstract Several polyphenolic compounds, including flavonoids and phenolic acids, were compared with their per-methylated forms in both chemical and cell-based assays for antioxidant capacity. Methylation largely eliminated “chemical” antioxidant capacity, according to ferric reducing antioxidant power and oxygen radical absorbance capacity assays. Methylation, however, only moderately reduced protection of human Jurkat cells in culture, from hydrogen peroxide-mediated cytotoxicity, at physiologically relevant concentrations. Neither methylated nor un-methylated compounds were detectably metabolized by the cells. It appears that the protective mechanism of polyphenolic antioxidants against high concentrations of hydrogen peroxide in human cells may be largely unrelated to chemical antioxidant capacity.

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2. Materials and methods

2.1. Materials

Quercetin, phloridzin, phloretin, catechin, caffeic acid, ferulic acid, 3,4-dimethoxy cinnamic acid (caffeic acid-Me) and cinnamic acid were obtained from Sigma Chemicals (St Louis, MO), Acros Organics (NJ, USA), or Lancaster Synthesis (Lancs., UK).

2.2. Methylation of flavonoids

Quercetin, catechin and phloridzin were methylated with dimethyl sulphate to give partially or fully methylated derivatives. For example, a mixture of phloridzin (30 mg), dimethyl sulphate (1 ml) and potassium carbonate (200 mg) in acetic acid (10 ml) was stirred at 45–50 °C for 24 h. The solvent was removed under nitrogen flow. The residue was suspended in water (3 ml) and ammonia solution (25% w/v; 0.5 ml) and extracted with ethyl acetate (3 × 3 ml). The combined ethyl acetate extracts were dried and purified by silica gel chromatography to afford phloridzin-Me (28 mg). Phloridzin-Me (15 mg) was deglycosylated in aqueous hydrochloric acid (0.5 M, 2 ml), stirred at room temperature overnight and extracted with ethyl acetate (3 × 1 ml). The combined ethyl acetate extracts were dried to give phloridzin-Me quantitatively. Structure determination was by liquid chromatography mass spectrometry (LCMS) and 1H and 13C NMR, with confirmation of assignment of NMR resonances by HMBC (heteronuclear multiple bond correlation).

2.3. LCMS characterization of methylated polyphenolics

Phenolic compounds and methylated derivatives were identified and quantified by LCMS using a LCQ Deca ion trap mass spectrometer fitted with an electrospray ionisation (ESI) interface (ThermoQuest, Finnigan, San Jose, CA, USA) and coupled to a Surveyor™ high performance liquid chromatography (HPLC) and diode-array detector.

The HPLC column used was a 150 × 2 mm Aqua, 3 µm particle size, 125 Å pore size, C-18, analytical column (Phenomenex, Auckland, NZ) maintained at 35 °C. A 0.2 µm in-line filter (Alltech, Deerfield, IL, USA) was installed before the column. Solvents were (A) water + 0.1% formic acid and (B) acetonitrile + 0.1% formic acid and the flow rate was 200 µl/min. The mobile phase composition was 100% A from 0 to 10 min, ramped to 10% B at 25 min, 30% at 35 min, 50% at 40 min, 95% between 44 and 47 min and back to 0% between 49 and 65 min. Samples were dissolved in solvent A (100 µl). Sample injection volume was 10 µl. UV–Visible detection was by absorbance at 200–600 nm. Solvent flow to the MS was
2.4. Ferric reducing antioxidant power (FRAP) assay
FRAP of phenolic compounds was compared, in duplicate, with that of the reference antioxidant Trolox (R)-(+)-6-hydroxy-2,5,7,8-tetramethyldihydroxirrovan-2-carboxylic acid, Sigma) using a published procedure [7]. Results were calculated as μmol Trolox/mmol of test compound.

2.5. Oxygen radical absorbance capacity (ORAC) assay
ORAC of phenolic compounds was compared, in triplicate, with Trolox using a published procedure [8], a modification of the fully aqueous method [9] in which the sample solutions are made up in methanol to improve solubility of hydrophobic compounds. Results were calculated as μmol Trolox/mmol of test compound.

2.6. Culture of Jurkat cells and measurement of hydrogen peroxide mediated cytotoxicity
Jurkat cells (clone E6-1) were cultured and incubated as described previously [10], under four different conditions, i.e., in the presence of: 50 μM H2O2, test phenolic compound (concentrations from 0.25 to 10 μM), both, or neither, in duplicate, for 18 h. Cells were classified as normal, apoptotic or necrotic using fluorescent staining and flow cytometry [10]. Results were expressed as cell death index (CDI), defined as: ((apoptotic + necrotic)/healthy cells) × 100. The percentage of inhibition of H2O2-induced cell death by test compounds was calculated by the following equation:

$$\text{%Inhibition} = \frac{\Delta CDI_{HP} - \Delta CDI_{Sample}}{\Delta CDI_{HP}} \times 100$$

where ΔCDIHP and ΔCDISample are the CDIs resulting from 50 μM H2O2 without and with test compound, respectively. The “effective concentration” at which a test compound halved the CDI (EC50) values were calculated through dose–response curves (not shown) of the concentration of test compound (μM) against percent inhibition.

2.7. LCMS analysis of test compounds after incubation with Jurkat cells
Test compounds (10 μM) were incubated with cells as described in Section 2.6 and the whole incubation mixtures frozen and stored at −80 °C until extracted. Recovery standards of test compounds were prepared by adding compounds to cell incubations and freezing immediately. Some incubations were separated into cell and growth medium fractions by centrifugation and some cell pellets were washed with fresh growth medium. Samples were prepared for analysis by freeze-drying and then sonication with acetonitrile/water (1:1, 1 ml) at room temperature. After centrifugation at 13000 rpm, the supernatants were vacuum dried (Centriprep centrifugal concentrator, Labconco, Kansas City, MO). Samples were stored at −80 °C until analysed by LCMS (see Section 2.3). Identification and quantification of test compounds and correction for incomplete extraction or degradation during analysis was achieved by comparison of UV and MS-molecular ion peak areas with the un-incubated recovery standards. Ionisation of permethylated compounds (although observed with pure samples, see above) was apparently suppressed in the relatively dilute and impure cell extracts (with the exception of phloridzin-Me) so only UV quantification was used.

3. Results

3.1. Methyl derivative synthesis
Quercetin and catechin were successfully per-methylated (quercetin-Me, catechin-Me). Methylation of phloridzin was accomplished selectively on the phenolic hydroxyl groups of the flavonoid, leaving the glucosyl residue un-methylated (phloridzin-Me). Phloretin-Me was prepared, with one free hydroxyl group, by de-glycosylation of phloridzin-Me (Fig. 1). NMR signal integration indicated a >95% extent of methylation.

3.2. FRAP and ORAC values of methylated vs. un-methylated compounds
Methylation reduced FRAP values much more than ORAC values, for example, quercetin had a FRAP 650 times higher than quercetin-Me, and an ORAC 15 times higher (Table 1). Corresponding ratios were 81 and 29 for catechin and 427 and 30 for caffeic acid. Mono-methylated caffeic acid (i.e., ferulic acid) behaved similarly to caffeic acid in both assays.

![Fig. 1. Structures of synthesised methylated flavonoids showing percentage purities from HPLC, 1H and 13C NMR methyl resonances (ppm; in parentheses) predicted by Chemdraw Ultra® (CambridgeSoft Corp.) and those found. All compounds showed the expected NMR resonances for the flavonoid nucleus and the expected molecular ions by LCMS (not shown).](image-url)
acid-Me and cinnamic acid (effectively caffeic acid without its hydroxyl groups) had very little activity in either assay. Methylation of phloridzin almost eliminated both activities. It appears that free hydroxyl groups are essential for reduction of ferric ions, but less so for radical scavenging.

3.3. Protective effects of phenolic compounds on hydrogen peroxide-mediated cytotoxicity

The quantitative reduction in EC$_{50}$ values of polyphenolics, resulting from methylation, was mostly considerably lower than the reduction in ORAC and FRAP values (Table 1 and Fig. 2). EC$_{50}$ values decreased 5–6-fold for quercetin, phloridzin and phloretin, 43-fold for catechin and 10-fold for caffeic acid. Removal of hydroxyl groups, as in cinnamic acid, essentially eliminated the protective effect. Most compounds had EC$_{50}$ values well within the range of plasma concentration (0.1–10 $\mu$M) typically observed for dietary polyphenolics [4].

The very low cytotoxicity of quercetin and phloridzin was increased several fold by methylation, but the increased toxicity was only moderately greater than that of phloretin or catechin. Methylation of phloretin did not change cytotoxicity significantly and that of catechin was reduced by methylation. The relatively high toxicity of caffeic acid was reduced by mono- or di-methylation but increased by removal of its OH groups (cinnamic acid).

3.4. LCMS analysis of test compounds after incubation with Jurkat cells

This analysis was carried out to compare the extent of any degradation, or derivatisation, of methylated and un-methylated test compounds during the incubation period. The presence of hydrogen peroxide did not significantly decrease recoveries. Recoveries of methylated compounds were quantitative, within the limits of error. Catechin and phloridzin showed recoveries of 85 $\pm$ 5%, phloretin and ferulic acid, 50 $\pm$ 5% and caffeic acid 30 $\pm$ 10%. Quercetin was apparently converted into $\sim$50% of a slightly more polar derivative, which could not be identified, but had an identical UV spectrum and a trace of a less polar derivative, possibly methylated. Overall recovery was 30 $\pm$ 10%. There was no detectable deglycosylation of phloridzin or phloridzin-Me. Apart from quercetin, 

### Table 1
Comparison of FRAP and ORAC values with protective effects from hydrogen peroxide on cultured Jurkat cells, of methylated and un-methylated polyphenolics

<table>
<thead>
<tr>
<th>Compound</th>
<th>FRAP (μmol Trolox/mmol)</th>
<th>ORAC (μmol Trolox/mmol)</th>
<th>EC$_{50}$ ($\mu$M)</th>
<th>Cytotoxicity at 10 $\mu$M$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quercetin</td>
<td>2628 ± 88</td>
<td>10900 ± 260</td>
<td>0.15 ± 0.01</td>
<td>3.7 ± 0.3</td>
</tr>
<tr>
<td>Quercetin-Me</td>
<td>4 ± 0.1</td>
<td>700 ± 30</td>
<td>0.90 ± 0.17</td>
<td>14.8 ± 0.7</td>
</tr>
<tr>
<td>Phloridzin</td>
<td>76 ± 1.6</td>
<td>5180 ± 110</td>
<td>0.15 ± 0.02</td>
<td>2.1 ± 1.2</td>
</tr>
<tr>
<td>Phloridzin-Me</td>
<td>0 ± 0.1</td>
<td>8 ± 1</td>
<td>0.78 ± 0.23</td>
<td>16.9 ± 1.3</td>
</tr>
<tr>
<td>Phloretin</td>
<td>636 ± 12</td>
<td>4440 ± 270</td>
<td>0.14 ± 0.01</td>
<td>12.7 ± 1.8</td>
</tr>
<tr>
<td>Phloretin-Me</td>
<td>0 ± 0.3</td>
<td>700 ± 50</td>
<td>0.70 ± 0.03</td>
<td>12.6 ± 2.3</td>
</tr>
<tr>
<td>Catechin</td>
<td>976 ± 36</td>
<td>14560 ± 280</td>
<td>0.30 ± 0.03</td>
<td>9.5 ± 1.0</td>
</tr>
<tr>
<td>Catechin-Me</td>
<td>12 ± 0.8</td>
<td>500 ± 40</td>
<td>13.07 ± 1.52</td>
<td>5.8 ± 0.8</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>1708 ± 32</td>
<td>3970 ± 90</td>
<td>0.39 ± 0.01</td>
<td>15.4 ± 0.2</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>1520 ± 3</td>
<td>3640 ± 290</td>
<td>1.30 ± 0.33</td>
<td>7.5 ± 1.0</td>
</tr>
<tr>
<td>Caffeic acid-Me</td>
<td>4 ± 0.4</td>
<td>130 ± 6</td>
<td>4.15 ± 0.16</td>
<td>7.3 ± 1.0</td>
</tr>
<tr>
<td>Cinnamic acid</td>
<td>4 ± 0.4</td>
<td>120 ± 9</td>
<td>18.10 ± 3.58</td>
<td>18.4 ± 4.9</td>
</tr>
</tbody>
</table>

$^a$Defined as the concentration required to provide a 50% reduction in 50 $\mu$M hydrogen peroxide-induced cell death.

$^b$Defined as the cell death index (CDI) produced by the test compound at 10 $\mu$M, in the absence of hydrogen peroxide. The CDI of the H$_2$O$_2$ control was 52 ± 2.5.
no derivative of any compound was detected. The maximum effect of the degradation of the least stable compounds, on biological activity, would be approximately a 3-fold increase in apparent EC₅₀ for protection of Jurkat cells, resulting from decreased concentrations by the end of the experiment.

In experiments in which cells and growth medium were separated by centrifugation and analysed separately for level of test compound, observed percentages of total test compound in the unwashed cell fraction were: quercetin (27%), quercetin-Me (9%), phloridzin (26%) and phloridzin-Me (18%). Washing of the cells did not significantly change the level of quercetin, but removed ~85% of quercetin-Me and all detectable phloridzin and phloridzin-Me. Methylated compounds appear to be somewhat less available to the cells and of the compounds tested, only quercetin appears to be significantly bound to, or absorbed by the cells.

4. Discussion

It has long been assumed that polyphenolics function in the body similarly to their well-established ability to protect foods from oxidation by scavenging reactive oxygen species and for example, inhibiting oxidation of PUFAs [1]. The results from this study have demonstrated that the polyphenolics studied, at least in cultured Jurkat cells, could provide strong protection from hydrogen peroxide-mediated cytotoxicity, at concentrations likely to be achieved in vivo, even after their redox activity was largely eliminated by chemical derivatisation. Contrary to expectations, the hydrophobic methyl derivatives of quercetin and phloridzin appeared to be less bioavailable to the cells than the parent, un-methylated compounds. Unmethylated compounds were less stable during incubation with Jurkat cells, but this could account for, at most, a threefold increase in apparent EC₅₀ compared with methylated analogues. It appears, therefore, that the methylated derivatives may be, if anything, more potent in protecting Jurkat cells than their measured EC₅₀ values indicate. Washing of the centrifuged cells had no effect on quercetin, but largely removed the other three compounds tested. This indicates that absorption by the cells may not necessarily be required for phenolic compounds to protect them from hydrogen peroxide.

The significance of polyphenolics’ in vivo “chemical” antioxidant activity has recently been called into question as the quantity of polyphenolics in the body, even after relatively high consumption, is only a few percent of total plasma redundant activity has been demonstrated previously. These include direct binding to DNA and RNA [12], inhibition of gene transcription [13] and binding to regulating cellular proteins [14]. It appears that the benefits of polyphenolics may arise from multiple interactions, of which “chemical” antioxidant effect is just one.

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References