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Antioxidant properties of gallocatechin and prodelphinidins from pomegranate peel

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Galocatechins and a range of prodelphinidins were purified by high performance liquid chromatography from pomegranate peel. Gallocatechin, gallocatechin-(4-8)-catechin, gallocatechin-(4-8)-gallocatechin and catechin-(4-8)-gallocatechin were all identified, purified and quantified by LC-DAD-MS and MS-MS. The antioxidant properties of these compounds were assessed using two methods: (i) inhibition of ascorbate/iron-induced peroxidation of phosphatidylcholine liposomes; and (ii) scavenging of the radical cation of 2,2-azinobis (3-ethyl-benzothiazoline-6-sulphonate, ABTS) relative to the water-soluble vitamin E analogue Trolox C (expressed as Trolox C equivalent antioxidant capacity, TEAC). The prodelphinidin dimers were potent antioxidants in the aqueous phase, being much more effective than the gallocatechin monomer. However, in the lipid phase, only one of the dimers (gallocatechin-(4-8)-catechin) was significantly more effective than the monomer in the inhibition of lipid peroxidation of phosphatidylcholine vesicles. This study represents the first report on the antioxidant properties of prodelphinidins.

INTRODUCTION

The association between a diet rich in fruit and vegetables and a decreased risk of cardiovascular disease and certain forms of cancer is supported by a considerable amount of epidemiological evidence.^{1,2} Recent work has highlighted that, in addition to the antioxidant nutrients, the polyphenolic components of higher plants (such as flavonoids) may also contribute to the beneficial health effects of fruit and vegetables.^{3,4} Flavonoids are a class of compounds distributed widely in the plant kingdom,⁵ individual compounds of which have demonstrated high antioxidant activity,⁶ antimutagenic activity,⁷ and the ability to act as vasodilators.⁸ The antioxidant action of individual flavonoid compounds includes both the ability to scavenge free radicals^{9,10} and to induce phase II enzymes such as quinone reductase.¹¹

Prodelphinidins are a subclass of the pro-anthocyanidins or condensed tannins within the flavonoid family. They are termed prodelphinidins, since they liberate delphinidin upon acid hydrolysis. Pro-anthocyanidins have demonstrated several biological activities in both epidemiological and *in vitro* studies. They are able to complex proteins and metallic ions, and to act as antioxidants and radical scavengers.^{12–16} Their presence in the diet has also been associated with a decreased risk of some chronic diseases.^{17–19} Pro-anthocyanidins in wine have been associated with a cardiovascular protective effect, the so-called 'French paradox'.²⁰ Through interactions with anthocyanins, pro-anthocyanidins contribute to the colour in red wine²¹ and the astringency of wine and other plant foods.²²

In wine, oligomeric procyanidins have been isolated, characterized, and studied thoroughly.^{23,24} Prodelphinidins, on the other hand, although known to be present in grape skins, have only recently been found in wine²⁵ and subsequently identified and characterized.²⁶ Three novel delphinidins were identified: gallocatechin-(4-8)-catechin, gallocatechin-(4-8)-gallocatechin and catechin-(4-8)-gallocatechin. In this paper, we report the antioxidant properties of these compounds in comparison to the monomer gallocatechin.

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MATERIALS AND METHODS

Chemicals

All chemicals were of the highest grade available supplied by Sigma Chemical Company (Poole, UK). Gallicocatechin and prodelphinidins were obtained from pomegranate peel as described below.

Preparation of gallicocatechin and prodelphinidins

Frozen samples of pomegranate peel were ground and extracted with cold methanol (-20°C). The mixtures were sonicated for 15 min and then centrifuged at 4000 g at -10°C . The supernatant was collected and the residue processed a further four times as described above. Water was added and the methanol eliminated in a vacuum. The aqueous extract was washed with *n*-hexane and the gallicocatechin and prodelphinidins extracted with ethyl acetate and subsequently lyophilised. Further fractionation was performed using a Sephadex LH-20 column with 96% ethanol; the collection of fractions was monitored at 280 nm (Lambda 3B, Perkin Elmer) and by performing thin layer chromatography (TLC) on silica gel plates with toluene:acetone:formic acid (3:6:1, v:v:v) as eluents and a DMACA solution (1% *p*-dimethylaminocinnamaldehyde in 1.5 M H_2SO_4 in methanol) as developing reagent. Pure compounds were isolated from the fractions by high performance liquid chromatography (HPLC) using a gradient pump (Waters 600), a radial compression cartridge (Prep Nova-Pak C_{18} , 6 μm , 25 x 100 mm, Waters) and a UV detector (Waters 486) set at 270 nm. Solvent A was water, solvent B was 2.5% acetic acid and solvent C was methanol. Elution commenced with a linear gradient from 100% solvent A to 100% solvent B in 5 min, from 100% solvent B to 1% solvent C in 5 min, continuing with 1% solvent C isocratically for 5 min, a linear gradient from 1–2% solvent C in 5 min, 2% solvent C isocratically for 5 min, linear gradient from 2–5% solvent C in 5 min and 5% solvent C isocratically for 5 min followed by washing and reconditioning of the column with 100% solvent A for 15 min. The flow was 12 ml/min throughout.

Identification of compounds

Partial acid cleavage was performed to identify the terminal subunits of the prodelphinidins.²⁷ Acid hydrolysis, in the presence of phloroglucinol and phenylmethanethiol,²⁸ with subsequent desulphuration of the thioethers,²⁹ was carried out for the identification of the upper subunits.

Pure compounds obtained were also characterised by LC-MS (LCQ, Finnigan) using electrospray ionisation (ESI), the capillary temperature was 200°C and the capil-

lary voltage 31 V. The HPLC was connected to the probe of the mass spectrometer via the UV cell outlet, using PEEK tubing. Both the auxiliary and the sheath gas were a mixture of nitrogen and helium. Spectra were recorded in the positive mode and the mass spectrometer was programmed to do a series of three scans: a full mass, a zoom scan of the most abundant ion in the first scan, and a MS-MS also of the most abundant ion using a relative collision energy of 20.

For the HPLC analyses, an HP1100 pump (Hewlett Packard) was used with a Spherisorb S3 ODS-2 C_{18} , 4.6 x 150 mm, column (Waters) and a diode array detector (HP 1100). The solvents were 2.5% acetic acid (A), acetonitrile/2.5% acetic acid (1/9, v/v) (B) and pure acetonitrile (C). The gradient programme was from 100% A to 100% B in 5 min, from 100% B to 15% C in 25 min and from 15–50% C in 5 min at a flow rate of 0.5 ml/min.

Lipid phase antioxidant activity

Phosphatidylcholine (final concentration 1 mg/ml) was added to 150 mM KCl containing 0.2 mM FeCl_3 and test compound at a range of concentrations. Peroxidation was started as described previously³⁰ with ascorbate (final concentration 0.05 mM), in a final volume of 0.4 ml. Samples were incubated at 37°C for 40 min and the reaction terminated by the addition of 0.8 ml of 20% (w/v) trichloroacetic acid/0.4% (w/v) thiobarbituric acid/0.25 N HCl and 0.01 ml of butylated hydroxytoluene in ethanol. The production of thiobarbituric acid-reactive substances (TBARS) was measured after boiling for 15 min, cooling and centrifuging. Results are expressed as percentage of inhibition of peroxidation, where 100% inhibition is defined as baseline peroxidation of phosphatidylcholine without added iron/ascorbate, and 0% inhibition with added iron/ascorbate. Prodelphinidins and gallicocatechin were tested at a range of concentrations (5–100 μM)

Aqueous phase antioxidant activity

The Trolox equivalent antioxidant capacity (TEAC) was measured as described previously³¹ using Trolox C as the standard. The assay is based on the relative ability of antioxidants to scavenge the radical cation of 2,2'-azino-bis(3-ethyl-benzothiazoline-6-sulphonate, ABTS). Since the radical is generated by interaction with activated metmyoglobin and H_2O_2 , the assay is also influenced by how well the test compound inhibits formation of the radical. The extent of quenching of the ABTS radical was measured spectrophotometrically at 734 nm and compared to standard amounts of Trolox C. Prodelphinidins and gallicocatechin were tested as 0.5 mM solutions in sodium phosphate buffer at pH 7.4.

RESULTS AND DISCUSSION

The TEAC values for gallocatechin and the three prodelphinidins (structures shown in Fig. 1) are listed in Table 1. Gallocatechin is a good antioxidant in this assay ($\text{TEAC} = 2.2 \pm 0.08$), more than twice as effective as the vitamin E analogue ($\text{TEAC} = 1$). One might have expected this value to be higher, since addition of a third hydroxyl group to the B ring of epicatechin ($\text{TEAC} = 2.23 \pm 0.02$) to form epigallocatechin ($\text{TEAC} = 3.69 \pm 0.02$) significantly increases antioxidant efficacy. The antioxidant potency of catechin, which has a TEAC of 2.47 ± 0.02 , is not enhanced by the addition of a third hydroxyl in the B ring, as in gallocatechin. Clearly, the availability of the B ring hydroxyls to donate hydrogen is hindered with epimerisation from epigallocatechin to gallocatechin.

For maximum effectiveness as an antioxidant in the TEAC assay, there is a requirement for an *ortho*-dihydroxy structure in the B ring, a free $-\text{OH}$ group at the 3-position

attached to the 2,3 double bond and adjacent to the carbonyl moiety in the C ring, as in quercetin.⁶ Removal of the 2,3 double bond and 4-oxo group (as in catechin) drastically reduces antioxidant activity in this assay. The altered bonding in the C ring of catechin does not allow the delocalisation of electrons between the A and B rings, stabilizing the resultant radical after hydrogen donation. The TEAC values of the prodelphinidin dimers are all in the range 3.36–3.56 which is significantly less than those obtained previously for the equivalent procyanidin dimers (in the range 4.39–4.73) isolated from grape seed, apple skin and almond.¹⁴ Once again, the third hydroxyl group in the B ring reduces the ability of the B ring hydroxyls to donate hydrogen. Therefore, the 3'4' *ortho*-dihydroxy configuration in the B ring is an important determinant for antioxidant action in this assay.

The effect of gallocatechin and the prodelphinidins on the inhibition of lipid peroxidation of phosphatidylcholine is shown in Figure 2. The degree of inhibition

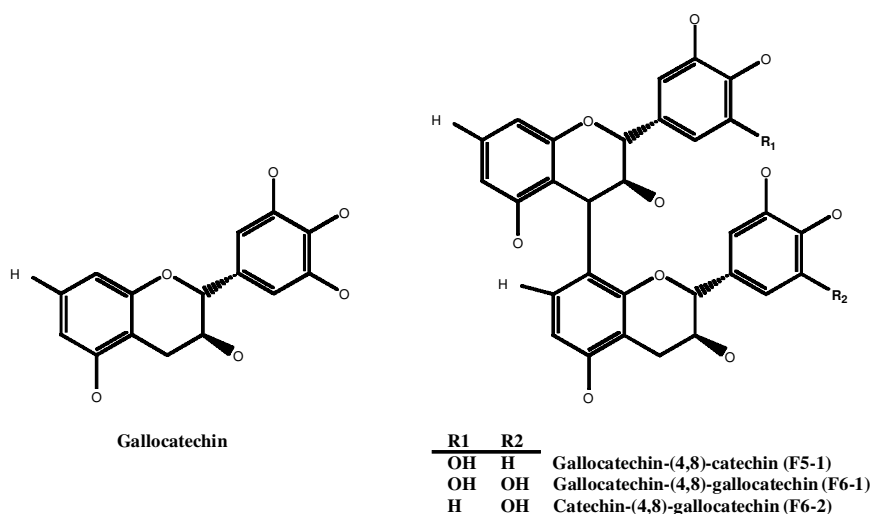


Fig. 1. Structures of gallocatechin and the three prodelphinidins.

Table 1. TEAC values and IC_{50} concentrations from lipid peroxidation assay for gallocatechin and prodelphinidins from pomegranate peel together with catechin, epicatechin, epigallocatechin and quercetin for comparative purposes

Compound	Trolox equivalent antioxidant capacity (TEAC)	Inhibition of lipid peroxidation IC_{50} (μM)
Gallocatechin	2.20 ± 0.08	38.4 ± 1.1
Gallocatechin-(4-8)-catechin	3.56 ± 0.11	26.2 ± 0.9
Catechin-(4-8)-gallocatechin	3.50 ± 0.04	36.4 ± 1.2
Gallocatechin-(4-8)-gallocatechin	3.36 ± 0.03	48.4 ± 1.6
Catechin	2.47 ± 0.02	3.4 ± 0.5
Epicatechin	2.23 ± 0.02	5.3 ± 0.3
Epigallocatechin	3.69 ± 0.02	48.7 ± 3.4
Quercetin	4.70 ± 0.10	7.7 ± 0.3

The values shown for the antioxidant assays are the mean and SD of at least three determinations. The TEAC values for catechin, epicatechin, epigallocatechin and quercetin are consistent with previous studies.⁶

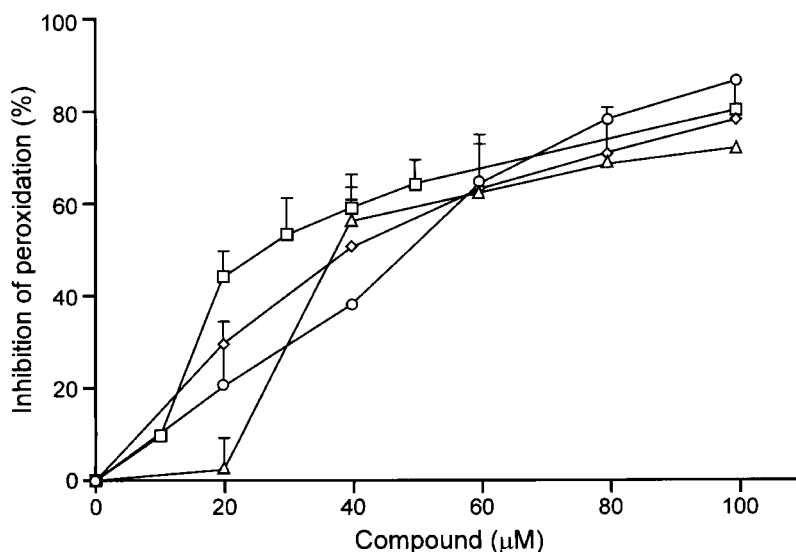


Fig. 2. Effect of gallicocatechin and the three prodelphinidins on the inhibition of iron/ascorbate-induced lipid peroxidation of phosphatidylcholine vesicles. The peroxidation was performed in the presence of (diamonds) gallicocatechin, (squares) gallicocatechin-(4-8)-catechin, (triangles) catechin-(4-8)-gallicocatechin and (circles) gallicocatechin-(4-8)-gallicocatechin. Values represent the mean and SD of three determinations.

was measured by estimating the IC_{50} values (concentration of test compound which inhibits peroxidation by 50%) which are listed in Table 1. As a comparison to the flavonols under study, the IC_{50} values for butylated hydroxytoluene and Trolox were $5.0 \pm 0.2 \mu M$ and $12.6 \pm 1.4 \mu M$, respectively. Gallicocatechin is much less effective at inhibiting lipid peroxidation compared to catechin. The specific mode of antioxidant action by the flavonoids in this assay is difficult to determine. It may be a function or combination of: (i) scavenging peroxy and lipid alkoxyl radicals; (ii) chelating iron ions; or (iii) acting as a chain-breaking antioxidant, donating hydrogen to a lipid radical. These factors will also be influenced by the partition coefficient of the molecule, *i.e.* the likelihood that the molecule will be in the aqueous phase or in the lipid phase to exert its action. Gallicocatechin is less effective than catechin in the lipid peroxidation assay for two reasons. First, it is more likely to be localized near the membrane surface; second, gallicocatechin does not possess the preferred 3'4' dihydroxy motif in the B ring for hydrogen donation. A third hydroxyl group in the B ring does not enhance antioxidant action in either the aqueous or lipid phases.

Figure 2 shows that none of the prodelphinidin dimers analysed was a potent inhibitor of lipid peroxidation in this system, possessing IC_{50} values in the range 26–49 μM . It is interesting to note that the antioxidant effectiveness in this assay is not increased by 'adding' two catechins together – the IC_{50} for gallicocatechin and dimers being in the same range. The prodelphinidin with the highest IC_{50} (*i.e.* the worst antioxidant) was the dimer with two gallicocatechins linked

together. A previous study¹⁴ demonstrated that procyanidin dimers were highly effective at protecting phosphatidylcholine vesicles against oxidation ($IC_{50} = 3\text{--}5 \mu M$). The lower values observed for the prodelphinidins generally, and the gallicocatechin-(4-8)-gallicocatechin specifically, can be explained by their decreased hydrogen-donating ability and their decreased tendency to partition into the membrane.

The TEAC and lipid peroxidation assays are invaluable in providing an indication of the potential of an antioxidant in exerting these same effects *in vivo* in the aqueous and lipid phases, respectively. These, and other similar assays (FRAP, DPPH, *etc.*) are an initial screening stage in the identification of important dietary antioxidants. Further studies, such as additional *in vitro* and *in vivo* assays, studies on metabolism and absorption, *etc.*, can then be employed to characterise fully the antioxidant and its mechanism of action. One problem with the lipid peroxidation assay is that added metal ions, H_2O_2 , antioxidants and chelating agents can influence the peroxidation in the incubation media in addition to the peroxide decomposition during the assay.³² However, despite its limitations, the assay provides a good first stage screening to identify antioxidant compounds for further, more detailed analysis.

In previous studies, a variety of advantageous antioxidant properties have been found using extracts from pomegranate. Fermented juice and oil seed demonstrated higher antioxidant activity compared to BHA, red wine and green tea when measuring the coupled oxidation of carotene and linoleic acid.³³ In addition, consumption of pomegranate juice by human subjects resulted in a marked decrease in AAPH-induced LDL oxidation³⁴ in plasma. Finally,

pomegranate extracts showed higher antioxidant activities compared to red wine in a range of assays (ABTS, DPPH, FRAP).³⁵ Although the main contribution to the antioxidant properties in this study was found to be the hydrolysable tannins, the anthocyanins had a significant contribution to the total antioxidant activity. Clearly, the anti-atherogenic capabilities of pomegranate juice are due to a host of phytochemicals present in the fruit, of which the compounds presented herein make a small, but significant, contribution. Studies isolating individual classes and/or components and more detailed characterization will further our understanding of the compounds which are the most bioactive and the mechanism of their action. This will yield information for dietary advice, projects for plant breeding and identify materials for natural food preservatives and therapeutic agents.

CONCLUSIONS

Pro-anthocyanidins are found widely in the plant kingdom and there are large differences in both the amounts and the nature of the species present in plants. Procyanidins consisting of catechin and epicatechin units have previously been shown to be highly potent antioxidants, particularly in the lipid phase.¹⁴ In this communication, we have shown that the prodelphinidins, although less potent than the procyanidins, still make a significant contribution to the antioxidant capacity of the total polyphenolics. A knowledge of these differences is important when assessing the antioxidant activity of pro-anthocyanidin containing foods because of the reduced potency of the gallo catechin-containing dimers compared to the catechin-containing dimers.

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