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Antioxidant Activity of Phytoestrogenic Isoflavones

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The aim of this work was to determine the antioxidant activities of a range of phytoestrogenic isoflavones. The antioxidant activity in the aqueous phase was determined by means of the $ABTS^{\bullet+}$ total antioxidant activity assay. The results show that the order of reactivity in scavenging the radical in the aqueous phase is genistein > daidzein = genistin ≈ biochanin A = daidzin > formononetin ≈ ononin, the latter displaying no antioxidant activity. The importance of the single 4'-hydroxyl group in the reactivity of the isoflavones, as scavengers of aqueous phase radicals, as well as the 5,7-dihydroxy structure is demonstrated. Examination of their abilities to enhance the resistance of low density lipoproteins to oxidation supports the observation that genistein is the most potent antioxidant among this family of compounds studied, both in the aqueous and in the lipophilic phases.

INTRODUCTION

Phytoestrogens, which are the major components of soybean, have been shown to be protective against several diseases, particularly cancer.[1–5] One of the two main classes of oestrogenic substances found in plants is the isoflavones, which constitute a large group of compounds containing a number of phenolic hydroxyl groups attached to ring structures (Fig. 1). The naturally occurring isoflavones with oestrogenic activity, measured by the uterine enlargement of immature female mice,[6] are genistein, daidzein, their glucosides genistin and daidzin, and their 4-methyl esters, biochanin A and formononetin, respectively. These compounds are particularly abundant in soya milk, a 12 ounce portion of which has been reported to contain 13.7 mg of genistein/genistin and 12.4 mg of daidzein/daidzin.[7]

Their biological potencies vary with the chemical structure, genistein being the most potent.[6] It was reported that isoflavones possessing a free 4'-hydroxyl group (genistein, daidzein) are more potent uterotropic agents than their 4-methyl esters (biochanin A and formononetin, respectively).[8] Most of the isoflavones occur in the intact plant as glycosides, but are transformed into the aglycone form during their bacterial metabolism in the intestine.[9] Thus, formononetin is converted by demethylation into daidzein and

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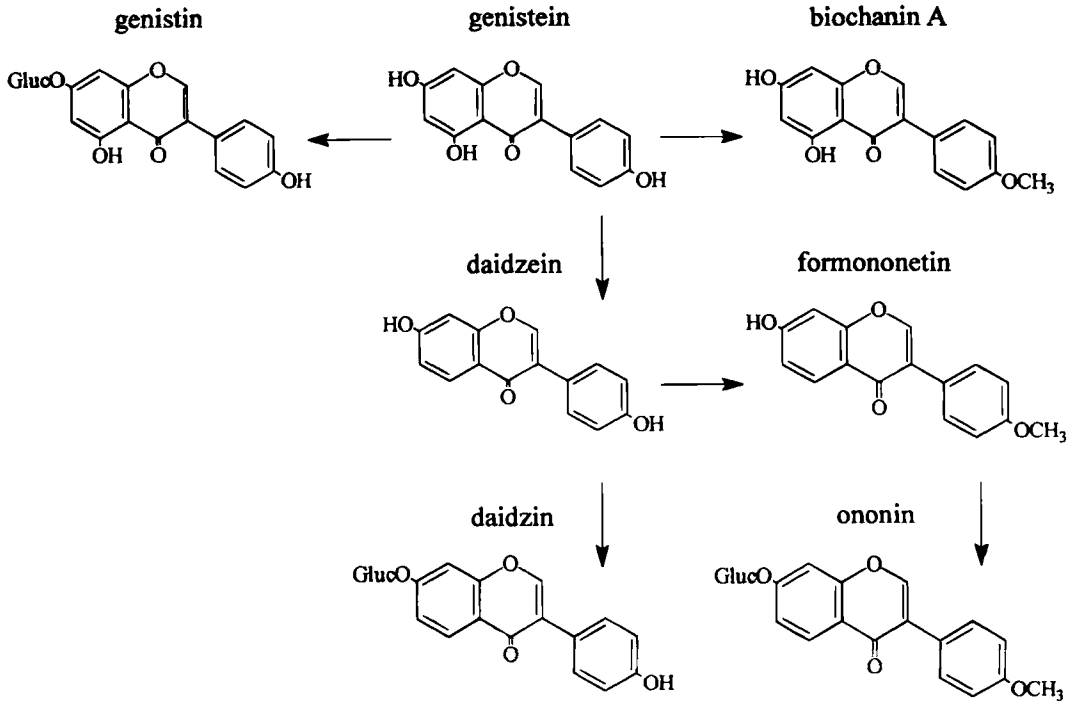

FIGURE 1 Structures of the isoflavone family of phytoestrogens.

to a higher degree to the isoflavan equol, an active oestrogenic metabolite.[9] Biochanin A is metabolized to genistein and subsequently to the non-oestrogenic p-ethylphenol. Daidzein, equol and genistein are the major phytoestrogens detected in the urine of humans and animals. These and other isoflavones have been shown to reduce the proliferation of cells, including breast cancer lines, and other tumors.[10–12]

There is increasing evidence that dietary intake of carotenoid-rich fruit and vegetables contribute to the protection from certain cancers.[13–24] Fruit and vegetables contain antioxidants such as vitamin C and carotenoids, flavonoids, hydroxycinnamates as well as many other constituents which may contribute to these protective effects through a range of possible anticarcinogenic mechanisms.

The aim of this work was to determine the antioxidant activity of the isoflavones, a class of phytoestrogens with varying oestrogenic activity. The antioxidant activity in the aqueous phase was determined by means of the $ABTS^{+\bullet}$ total antioxidant activity assay.[25,26] The ability of four structurally related isoflavones, genistein, genistin, daidzein and biochanin A to enhance the resistance of LDL to oxidation was also examined in order to assess the antioxidant potency of isoflavones in a lipophilic system. The results show that genistein is the most potent antioxidant in the aqueous phase and in lipophilic systems. The importance of the single 4′-hydroxyl group in the reactivity of the isoflavones as scavengers of radicals generated in the aqueous phase is demonstrated, in contrast to the case with flavone structures.

MATERIALS AND METHODS

Genistein, genistin, daidzein, daidzin, biochanin A, formononetin and ononin were obtained from

Extrasynthese (B.P. 62-69730 Genay Cedex, France) and dissolved for analysis in HPLC grade ethanol (Rathburn Chemicals Ltd., Caberston Road, Walkerburn, Scotland EH43 6AU). Trolox (®Hoffman-La Roche) and ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt) were obtained from Aldrich Chemical Co. (The Old Brickyard, Gillingham, Dorset SP8 4JL) while equine metmyoglobin was obtained from Sigma-Aldrich Co. Ltd. (Fancy Road, Poole, Dorset BH12 4QH). All other chemicals used were purchased from Merck Ltd (Hunter Boulevard, Lutterworth, Leics LE17 4XN) and were of the highest grade available.

The Trolox Equivalent Antioxidant Capacity (TEAC) of the isoflavonoids was estimated by measuring the total antioxidant activity of solutions of these compounds, relative to that of standard solutions of Trolox.[26] This spectrophotometric method is based on the reduction of the blue-green $ABTS^{\bullet+}$ radical by hydrogen-donating or electron-donating antioxidants. The reaction occurs in the aqueous phase and is measured by the suppression of the characteristic long wave absorption spectrum of $ABTS^{\bullet+}$ at 734 nm. $ABTS^{\bullet+}$ was generated by the interaction of ABTS during the formation of the ferrylmyoglobin radical species, using metmyoglobin (2.5 μM), H_2O_2 (75 μM) and ABTS (150 μM) (final concentrations). Absorbance readings were taken using a Cobas Fara centrifugal analyser and data reduction was carried out using a logit/log 4 curve fit. Stock solutions prepared by dissolving the isoflavonoids in ethanol at a concentration of 5 mmol/L. Three different dilutions were then selected which gave values on analysis that corresponded to between 40–80% inhibition of the blank, equivalent to an initial concentration of 1.0–2.0 mM Trolox). The three different dilutions of each compound were freshly prepared daily and analysed in triplicate (i.e. 9 determinations) on separate days (n = 3). The TEAC was calculated for each dilution and the mean value (± the standard deviation) of all the results derived: the TEAC is defined as the millimolar concentration of a Trolox solution having an antioxidant capacity equivalent to a 1.0 mM solution of the substance under investigation.

For estimation of the inhibition of LDL oxidation by the isoflavonoids, fresh whole human blood was obtained by venipuncture from healthy volunteers and was immediately transferred into acid citrate dextrose (ACD) buffer as an anticoagulant. LDL was isolated from the plasma as previously described,[27,28] was stored at 4°C in 100 μM EDTA and used within 4 days. LDL was dialysed immediately prior to use. Protein concentrations were estimated by the method of Markwell *et al.*[29] using bovine serum albumin as standard. LDL (62.5 μg LDL protein/ml final concentration in 5 mM phosphate buffered saline) was oxidised with 1.66 μM Cu^{2+} (final concentration) in the presence of varying concentrations of the isoflavonoids and the formation of conjugated dienes was monitored by continuous scanning from 220 to 300 nm at 10 minute intervals for 6 hours at 37°C using a Beckman DU7500 diode array spectrophotometer.[30] Control LDL aliquots were also incubated with the appropriate volumes of ethanol. The increased absorbance at 234 nm from each spectrum was plotted against time and the lag phase estimated.[31] The percentage increase in lag phase with each concentration of added isoflavonoid was calculated. The ability of the isoflavonoids to chelate copper was assessed spectrophotometrically using a Hewlett-Packard series 8 diode array spectrophotometer to scan solutions of the compounds after copper addition.

RESULTS AND DISCUSSION

Previous studies on polyphenolic flavonoid structures have shown that it is the phenolic hydroxyl groups and their structural arrangements that confer the antioxidant activity,[32] through their H-donating properties. Measurement of the total antioxidant activity of the isoflavone genistein (4',5,7-trihydroxyisoflavone) gives a TEAC value

of 2.90 ± 0.08 (Table I), nearly three times the antioxidant activity of Trolox or vitamin C.[33] Manipulation of the 5,7-dihydroxy structure in the A ring to a monophenolic group dramatically affects the antioxidant activity. The removal of the 5-hydroxyl group on the A ring of the structure (as in daidzein) is associated with a decrease of 60% of the antioxidant activity (TEAC = 1.25 ± 0.02). Glycosylation of genistein in the 7-position of the A ring (as in genistin) attenuates the antioxidant activity to a similar extent (TEAC = 1.24 ± 0.02). Methoxylation of the 4'-hydroxyl group of the B ring of genistein (as in biochanin A) also has a similar effect on the antioxidant activity (TEAC = 1.16).

Daidzin, the 7-glycoside of daizein has a similar TEAC value (1.15 ± 0.01), demonstrating the significance of the metadihydroxy structural feature in the A ring. In contrast, methylation of the 4'-hydroxyl group in the B ring of diadzein (as in formononetin) has a strongly suppressive effect on the antioxidant activity, emphasising the lack of contribution to the antioxidant activity from a lone hydroxyl group in the A ring. The basic ononin structure has no free hydroxyl groups and displays no antioxidant activity as expected.

These results indicate that there is a major contribution to the total antioxidant activity from the single hydroxyl group at position 4' of the B ring of the isoflavones in the presence of either one or two hydroxyl groups in the A-ring of the isoflavone molecule. It can also be deduced from the results that when the A ring lacks the 5,7-dihydroxy arrangement and only contains one hydroxyl substituent, the lone phenolic group on the A ring is not an effective hydrogen donor in this chemical arrangement, thus demonstrating the strong influence of this diphenolic conformation in the A ring on the antioxidant activity.

TABLE I Total antioxidant activity of isoflavones

Isoflavone	TEAC mM
Genistin	1.24 ± 0.02
Genistein	2.90 ± 0.10
Biochanin A	1.16 ± 0.02
Daidzein	1.25 ± 0.02
Formononetin	0.11 ± 0.02
Daidzin	1.15 ± 0.01
Ononin	0.05 ± 0.04

±SD, n = 5–6 determinations

Determination of the enhancement of the resistance of LDL to oxidation was applied to investigate the relative efficacies of the isoflavones as chain-breaking antioxidants. Oxidation of LDL was promoted by copper ions through the catalysis of the sequential reductive and oxidative decomposition of lipid hydroperoxides, generating alkoxyl and peroxyl radicals which propagate the peroxidation. The extent of oxidation of LDL (final concentration 62.5 μg/ml) promoted by 1.66 μM Cu^{2+} (final concentration) was measured by increased formation of conjugated dienes at 37°C. In the absence of phytoestrogens, the time-course of LDL oxidation showed an iinitial lag phase of approximately 60 min followed by a propagation phase over the next 1 hour. The addition of a range of concentrations of phytoestrogens, prior to the prooxidant, and delayed the onset of the propagation step, i.e. prolonged the lag phase to oxidation, to different extents (Fig. 2). The compounds are compared on the basis of the percent enhancement of the lag phase to oxidation as a function of concentration. Fig. 3 illustrates that the sequence of efficacies as antioxidants is genistein > genistin > biochanin A > daidzein. The concentration required to enhance the lag phase to oxidation of LDL by 50% are 13 μM, 29 μM, 44 μM and 56 μM, respectively for genistein, genistin, biochanin A and diadzein.

In order to investigate whether copper chelation to the 5 hydroxy, 4-keto groups (as defined by Thompson *et al.*[34]) was playing a role in the inhibition of the oxidation, spectroscopic studies were undertaken of the formation of copper complexes with the relevant isoflavones at 30 μM and 50 μM concentrations. The results show no spectral modifications of the phytoestrogen by addition of copper, indicating no chelation with

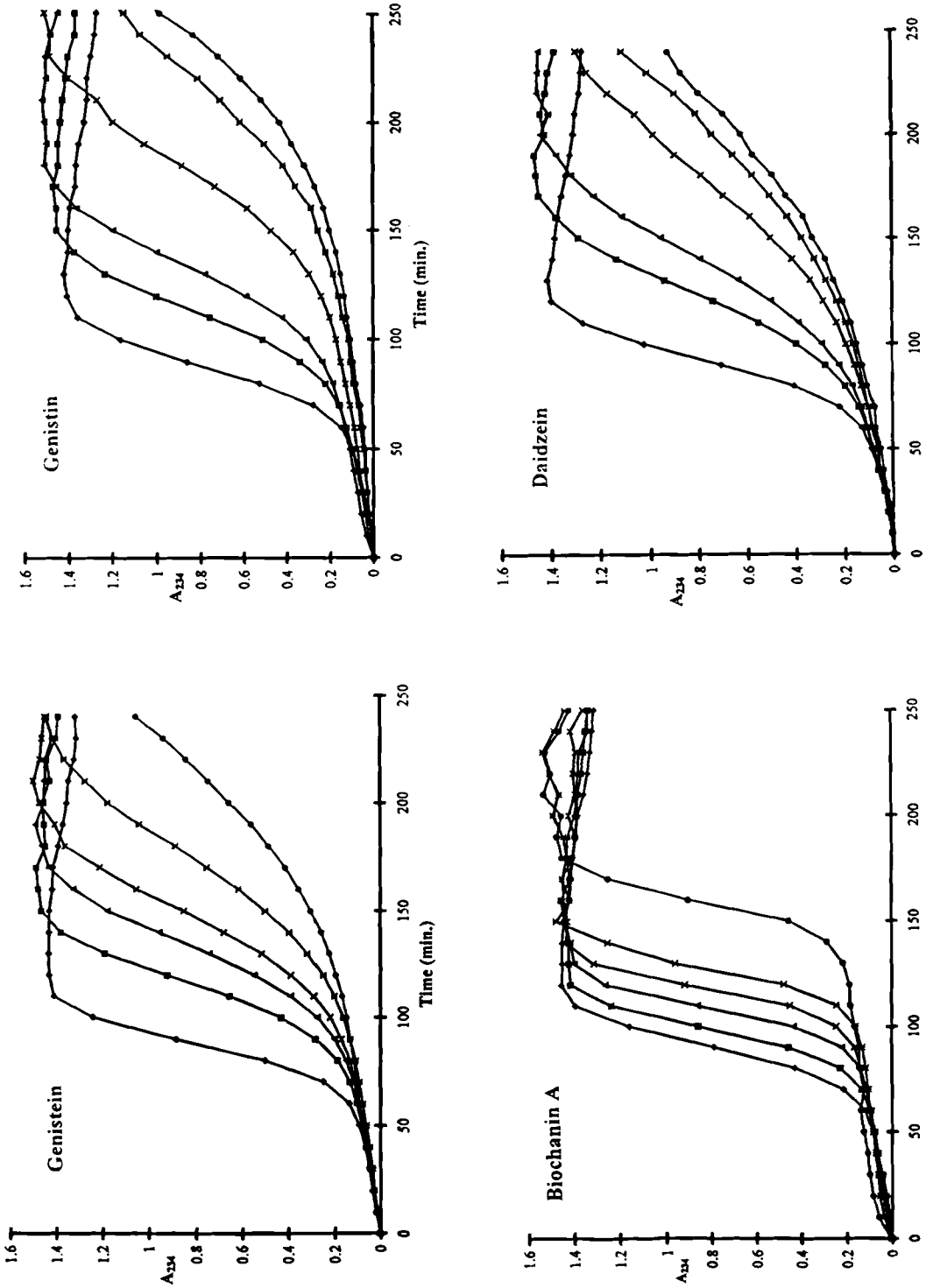

FIGURE 2 The effects of isoflavones on the lag phase to oxidation of LDL (62.5 μg LDL protein/ml final concentration) promoted by Cu^{2+} (1.66 μM final concentration). genistein, concentrations 0, 5, 10, 15, 20, 30 μM; genistin, concentrations 0, 10, 20, 40, 60, 80 μM; biochanin A, concentrations 0, 10, 20, 40, 60, 100 μM; daidzein, concentrations 0, 10, 20, 40, 60, 100 μM.

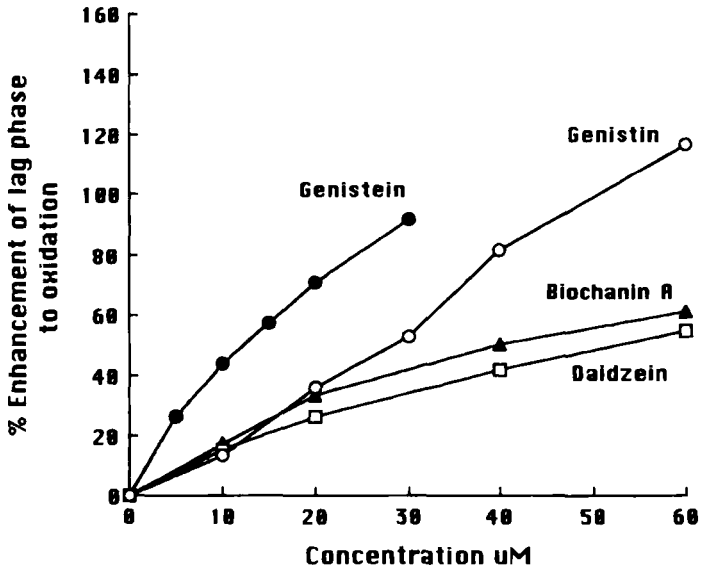

FIGURE 3 The percent enhancement of the lag phase to oxidation of LDL 62.5 μg LDL protein/ml (final concentration) promoted by 1.66 μM Cu^{2+} (final concentration) as a function of concentrations of genistein, genistin, biochanin A and daidzein.

copper. The order of effectiveness for phytoestrogens against LDL-oxidation does not correlate with their electron-donating abilities to the $ABTS^{\bullet+}$ radical cation (genistein > daidzein = genistin ≈ biochanin A) suggesting that the compounds have differential rates of reaction with the peroxyl radical and $ABTS^{\bullet+}$ radical cation, but the accessibility of the isoflavones to the radicals within the LDL and their partitioning characteristics will also be significant in the former case.

Our recent studies have demonstrated the features underlying the structure/antioxidant activity relationships of the flavonoids.[32] The importance of specific structural criteria defining the free radical scavenging activities of flavonoids have been characterised by Bors *et al.*[35] These include: the 2,3-double bond with the 4-oxo group and the 3-hydroxyl group in the C ring, the 5,7-dihydroxyl structure in the A ring and the orthodihydroxy structure in the B ring. The studies on the isoflavonoids described here also underline the contribution from the 5,7-dihydroxy groups in the A ring—giving an approximately similar contribution to the total antioxidant activity as in the flavonoids. However, the positioning of the B ring in the 3 position adjacent to the 4-keto group in the isoflavonoids, compared to the 2-position in the flavonoids, allows the 4-hydroxyl on the B ring to contribute more significantly to the antioxidant activity, in terms of hydrogen donation and subsequent electron delocalization. However, a single hydroxyl group in the 4′ position of the B ring in the flavonoids does not contribute to the antioxidant activity.

Other workers have shown that the antioxidant activity of isoflavones against hydrogen peroxide production by HL-60 cells is in the order of genistein > diadzein >>> biochanin A, with no effect from the latter.[36] Their interpretation of the importance of the hydroxyl group at the 4′-position of the B ring being crucial to the antioxidant activity is consistent with our findings here for direct radical scavenging of $ABTS^{\bullet+}$ by these compounds.

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