Antioxidant activity of prenylated flavonoids from the West African medicinal plant *Dorstenia mannii*

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Abstract

The antioxidant activities of three prenylated flavonoids from *Dorstenia mannii* (6,8-diprenyleriodictyol, dorsmanin C and dorsmanin F) were compared to the common, non-prenylated flavonoid, quercetin. The prenylated flavonoids were found to be potent scavengers of the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH), and are more potent than butylated hydroxy toluene (BHT), a common antioxidant used as a food additive. The prenylated flavonoids also inhibited Cu^{2+}-mediated oxidation of human low density lipoprotein (LDL). Dose–response studies indicated that the prenylated flavonoids were effective inhibitors of lipoprotein oxidation with IC_{50} values <1 μM and had similar inhibitory potency compared to quercetin, but was not directly related to Cu binding. Unlike quercetin, they did not show any pro-oxidant activity at high doses in the Cu^{2+}-mediated lipoprotein oxidation system. The medicinal action of *Dorstenia mannii* may be related to the high concentration of potent antioxidant prenylated flavonoids in this species.

Keywords: Low density lipoprotein; Flavonoids; Quercetin; Antioxidant

1. Introduction

The oxidation of lipoproteins, in particular low density lipoprotein (LDL), is thought to play a critical role in the development of atherosclerosis (Witztum and Steinberg, 1991). Oxidative damage to LDL produces particles containing lipid oxidation products and damaged proteins, which enhance the atherogenic effects of these particles. Oxidised lipoproteins contribute to all stages of the atherosclerotic process, including activation of inflammatory events, endothelial damage, recruitment of macrophages and unregulated uptake of oxidised lipid particles by these cells to form foam cells, the hallmark of early atherosclerotic lesions (Berliner and Heinecke, 1996). Since oxidative damage plays a key role in cardiovascular and other degenerative diseases, there has been considerable interest in the role of antioxidants to inhibit these processes (Diaz et al., 1997). While endogenous lipophilic antioxidants such as α-tocopherol (Vitamin E) no doubt play a role in protecting cell membranes and lipoproteins from oxidation, intervention studies using Vitamin E supplements have not shown consistent results to improve cardiovascular disease (Halliwell, 2000). We have been particularly interested in plant-derived antioxidant phenolic compounds which may act as antioxidants or co-antioxidants to protect LDL from oxidative damage (Morton et al., 2000). The flavonoids, a group of secondary plant metabolites occurring widely throughout the plant kingdom, have been shown to possess a range of biological activities, some of which, such as inhibition of xanthine oxidase, may be related to their well recognised antioxidant activity (Cotelle et al., 1996; Morton et al., 2000; Takahama, 1985). While the structure–activity relationships for the antioxidant properties of flavonoids has been elucidated (Rice-Evans et al., 1996; Chen et al., 1996; Cao et al., 1997), little is known about the effects of prenylation on the antioxidant activity of the flavonoids.

Many species of the genus *Dorstenia* (Moraceae) are used for medicinal purposes (Africa, Middle East, Central and South America). To date, only the African *Dorstenia* has yielded a variety of mono-, di-, and triprenylated and also mono- and digeranylated flavonoids (Abegaz et al., 2000). *Dorstenia mannii* Hook.f., a perennial herb growing in the tropical rain forest of Central Africa, has its twigs and leaves sold in local markets for the treatment of various diseases,

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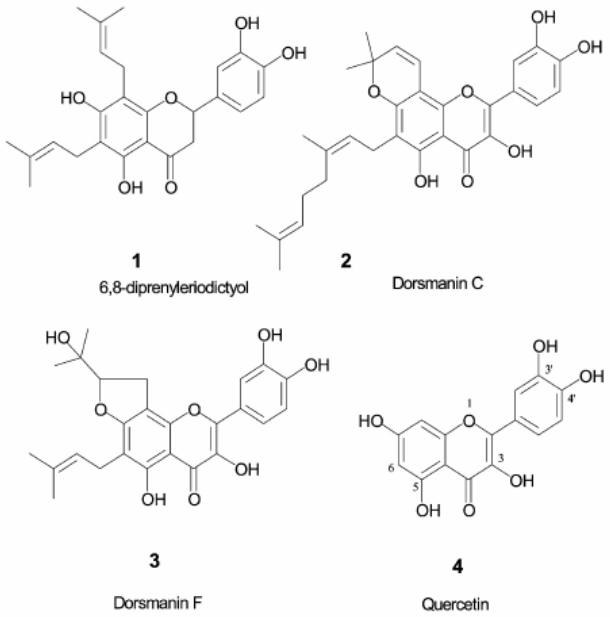

Fig. 1. Structure of prenylated flavonoids isolated from *Dorstenia mannii* and the non-prenylated flavonoid quercetin for comparison.

such as rheumatism and stomach disorders (Bouquet, 1969). No pharmacological studies have been made of this plant, but a number of flavonoids showing varying degrees of prenylation of the A ring have been isolated (Ngadjui et al., 1998, 1999). Examples of some of these compounds are shown in Fig. 1 together with the structure of the wide-spread flavonoid, quercetin, for comparison. Since the introduction of prenyl groups is likely to increase lipophilicity of these compounds compared to unsubstituted flavonoids, we examined their antioxidant activities against lipoprotein oxidation. An understanding of the antioxidant activity of these compounds may assist in rationalising the claimed use of this medicinal plant.

2. Methods

2.1. Compounds

Plant samples were collected from Nkoljobe mountain, Central Province of Cameroon and a voucher specimen (No. 2135) was deposited at the National Herbarium, Cameroon. Prenylated flavonoid compounds were isolated from the twig and leaf samples as previously described (Ngadjui et al., 1998, 1999): 6,8-diprenyleriodictyol (compound **1**, Fig. 1) as yellow crystals, melting point (m.p.) 141–142 °C; dorsmanin C (compound **2**) as yellow crystals, m.p. 213–215 °C; compound **3** as an epimeric mixture of diasteromers, beige crystals, m.p. 164–165 °C (Ngadjui et al., 2000). Quercetin (compound **4**), butylated hydroxy toluene (BHT) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma Chemicals, St. Louis (USA).

2.2. LDL purification

Blood was collected by venipuncture into EDTA (1 g/l) and centrifuged immediately at 1000 × *g* for 10 min at 4 °C. LDL was isolated by density gradient ultracentrifugation as previously described (Croft et al., 1991). Briefly, plasma density was increased to 1.07 by addition of NaCl. A 4-step gradient was then constructed over the plasma, using the following densities (kg/l): 0.5 ml of 1.063 NaCl, 0.5 ml of 1.04 NaCl, 0.5 ml of 1.02 NaCl and 0.9 ml of double distilled water. To protect the LDL against oxidative modification during ultracentrifugation, each density solution contained 100 mg/l NaEDTA. Samples were ultracentrifuged at 205,000 × *g* (average) for 20 h at 4 °C using a L-80 Ultracentrifuge (Beckman, USA). The LDL band was collected by aspiration and passed through a Pharmacia PD10 Sephadex column to remove the excess salt and the majority of the EDTA. The isolated LDL was stored in the dark at 4 °C.

2.3. LDL oxidation studies

The LDL oxidation procedure was similar to that previously described (Croft et al., 1992). Briefly, freshly isolated

LDL was passed through a second Pharmacia PD10 Sephadex column to remove any remaining EDTA. The cholesterol concentration of the LDL was measured using a standard enzymatic method (Monotest, Boehringer Mannheim, Germany), and the protein content was measured using the Lowery procedure with bovine serum albumin (Sigma) as the standard. The LDL was diluted with 0.15 M phosphate buffer saline (PBS), pre-treated with chelex chelating resin (Sigma), to a standard concentration of 0.1 mg/ml. Oxidation reactions were initiated by the addition of freshly prepared $CuSO_4$ (final concentration 5 μmol/l).

For dose–response studies test compounds were dissolved in ethanol and added to the LDL to give a final concentration of 0.1–10 μM. The total volume of ethanol in the buffer solution was 0.2%, and the same volume of ethanol was added to control oxidations. The extent of lipoprotein oxidation was determined by measuring the formation of lipid hydroperoxides at various time-points, up to 150 min. Lipid hydroperoxides were measured by the ferrous oxidation-xylenol orange (FOX) assay (Nourooz-Zadeh et al., 1994) as previously described (Proudfoot et al., 1997). The percent inhibition of lipid hydroperoxide formation is expressed as the change in lag time compared to control oxidations. For dose–response and time course studies, oxidation kinetics were determined by monitoring lipid hydroperoxides every 15 min over a 125-min period.

2.4. Free radical scavenging capacity

The free radical scavenging activity of each compound was analysed by the DPPH assay. Test compounds at concentrations ranging from 1 to 100 μM were mixed with 3 ml of 0.1 mmol DPPH/l (in ethanol) in a cuvette. The time course of the change in absorbance at 517 nm was monitored over 20 min.

2.5. Copper interaction studies

Stock solutions of each flavonoid (1 mM) were prepared in methanol. The absorption spectra of each compound between 200 and 800 nm was recorded after dilution of each compound to 25 μM with PBS (10 mM, pH 7.4). Scans with 12.5, 25, 37.5, 50 μM $CuSO_4$ were taken after 10 s and compared with the flavonoid alone.

3. Results

3.1. Activity of prenylated flavonoids as radical quenching antioxidants

The radical scavenging activity of the prenylated flavonoids (compounds **1–3**) was estimated by reactivity with DPPH. The absorbance of DPPH did not change over the period of the experiments (20 min) in the absence of test compounds. The addition of each compound to DPPH caused a rapid decrease in absorbance at 517 nm, reaching a plateau at 15 min (Fig. 2). The inset to Fig. 2 shows the dose–response for DPPH radical scavenging for a range of concentration for each test compound (1–100 μM) compared to the BHT at the same dose range. It can be seen that the potency of free radical scavenging activity has the following range: compound *2* > *1* > *3* ≫ BHT.

3.2. Inhibition of low density lipoprotein oxidation

To test the ability of prenylated flavonoids to act as antioxidants in a physiologically relevant setting we examined the effect of each compound over a wide range of doses on lipid hydroperoxide formation during Cu^{2+}-catalysed oxidation of LDL. The kinetics of LDL oxidation were

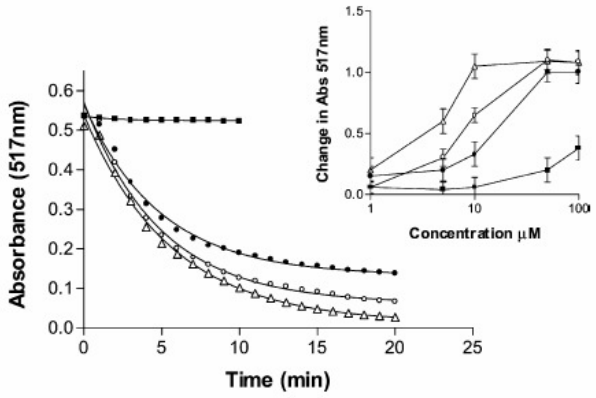

Fig. 2. Radical scavenging of DPPH by 10 μM of the prenylated flavonoids, compound **1** (○), compound **2** (△) and compound 3 (●) compared to control (■) as indicated by decrease in absorbance at 517 nm. Insert represents dose–response for these compounds compared to BHT (■). Values are the mean and standard error of three experiments.

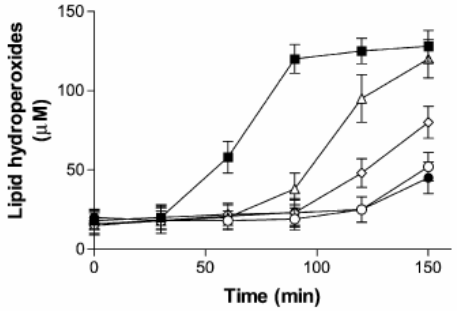

Fig. 3. Kinetics of LDL oxidation measure by the formation of lipid hydroperoxides in the presence of 0.5 μM of 6,8-diprenyleriodictyol, **1** (○), dorsmanin C, **2** (△), dorsmanin F, 3 (◇) and quercetin, **4** (●) compared to control oxidation (■). Values are the mean and standard error of three separate experiments.

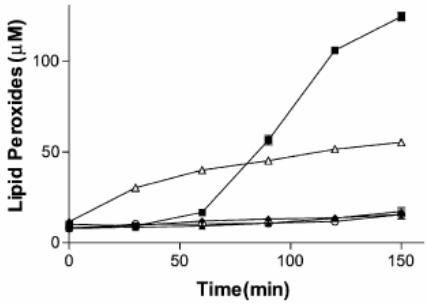

Fig. 5. The effect of different doses of compound **1** and quercetin on the kinetics of LDL oxidation. 6,8-Diprenyleriodictyol, **1** at 1 μM (▲) and 100 μM (◆), quercetin at 1 μM (○) and 100 μM (△) compared to control oxidation (■). Results are the mean and standard error of three experiments.

examined over a period of 150 min. Fig. 3 shows the kinetics of LDL oxidation for control and with the addition of 0.5 μM of each compound. All compounds showed significant prolongation of the lag time to oxidation (indicated by the rapid formation of lipid hydroperoxides). 6,8-Diprenyleriodictyol (**1**) and quercetin (**4**) showed the greatest activity in this assay. Fig. 4 shows the dose–response curve for the prenylated flavonoids expressed as percent inhibition compared to control oxidation. It can be seen that all compounds have an IC_{50} of <1 μM under these assay conditions. When comparing quercetin under the same conditions, it was noted that at high doses this compound actually exhibited a pro-oxidant effect. Fig. 5 shows the LDL oxidation kinetics for 6,8-diprenyleriodictyol (**1**) and quercetin (**4**) at 1 and 100 μM. At a higher dose quercetin stimulates lipid hydroperoxide formation and there is no apparent lag time for this oxidation to occur.

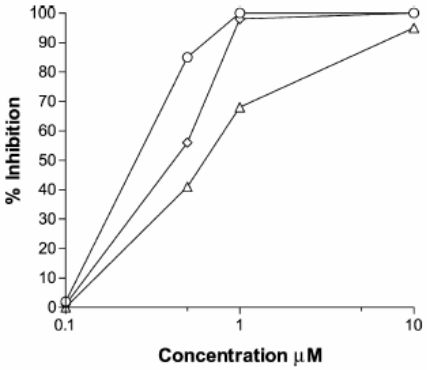

Fig. 4. Dose–response curve for the inhibition of Cu-mediated LDL oxidation all compounds tested in the range of 0.1–10 μM. 6,8-Diprenyleriodictyol, **1** (○), dorsmanin C, **2** (△), dorsmanin F, 3 (◇). Values are the average of duplicate experiments.

Table 1
Spectral shift of dorsmanin C (**2**) and quercetin (**4**)[a]

Flavonoid	Peak position (nm)	
	Control	+Cu (25 μM)
Compound **2**	242	255
	288	304
	347	279
	405	443
Quercetin	260	275
	375	450

[a] The concentration of flavonoids was 25 μM in each case with the addition of equimolar amount of Cu^{2+}. Data are representative of three experiments.

3.3. Copper interaction studies

The direct interaction of the flavonoids with Cu^{2+} ions at pH 7.4 were assessed by UV-Vis spectroscopy. Only dorsmanin C (**2**) showed any interaction with Cu^{2+} ions indicated by significant bothochromic shift in major absorbance bands upon addition of equimolar concentrations of Cu^{2+} ions. Table 1 shows the absorption shift for 6,8-diprenyleriodictyol (**1**) compared to that seen with quercetin (**4**). Only compounds with the hydroxyl in the 3 position of the flavone ring C show chelating properties with Cu^{2+}. This shift is reversible as shown with the addition of EDTA (Brown et al., 1998).

4. Discussion and conclusions

This study shows that the prenylated flavonoids isolated from the medicinal plant *Dorstenia mannii* have antioxidant activity against LDL oxidation. All the compounds tested

were efficient free radical scavengers, although only dorsmanin C (**2**) showed significant binding to copper ions. The antioxidant activity of the prenylated flavonoids was similar to that of the non-prenylated flavonoid quercetin (**4**), however, unlike quercetin they show no pro-oxidant activity at high concentrations under conditions of copper-mediated LDL oxidation.

There has been a considerable amount of work to examine the structure–antioxidant activity relationships for flavonoids (Rice-Evans et al., 1996; Chen et al., 1996; Cao et al., 1997), but very little information is available on the effect of prenylation on their antioxidant activity. Ko et al. (1998) examined the radical scavenging and antioxidant properties of several prenylflavones isolated from *Artocarpus heterophyllus* Lam. (Moraceae), a medicinal plant from South-east Asia. Several of the compounds tested inhibited iron-induced lipid peroxidation and scavenged DPPH, particularly those with free hydroxyl groups on the A and B ring. Since no comparison was made with non-prenylated flavonoids, no conclusions can be drawn on the effect of prenylation on antioxidant activity. In the present study, all compounds showed very strong antioxidant action against copper-induced LDL oxidation and were of similar activity to the non-prenylated compound quercetin, which has similar distribution of hydroxyl groups. These results indicate that in this particular model of lipoprotein oxidation, modification of the flavonoid structure by prenylation has little influence on antioxidant activity.

Since the ability of flavonoids to bind or chelate Cu^{2+} ions may be related to their activity in protecting LDL against Cu^{2+} ion-induced oxidation (Brown et al., 1998), the interaction of the prenylated flavonoids was examined with Cu^{2+} ions using UV spectroscopy. The results indicate that only the compound with the hydroxyl group on the C ring (dorsmanin C, **2**) showed any significant interaction with Cu^{2+} ions. Interestingly, compound **2** had the lowest antioxidant activity against Cu^{2+} ion-induced LDL oxidation (Fig. 4), from which it was concluded that Cu^{2+} binding plays a minor role in the activity of these compounds. This is perhaps not surprising given that all compounds showed activity at doses between 0.5 and 1 μM and that Cu^{2+} ions were present at 5 μM.

At low doses (<10 μM) all compounds showed antioxidant activity against Cu^{2+} ion-induced LDL oxidation. At a dose of 100 μM quercetin (**4**) actually showed pro-oxidant activity causing the formation of lipid hydroperoxides from early time-points in the oxidation (Fig. 5). By comparison, 6,8-diprenyleriodictyol (**1**) showed only inhibitory activity even at the highest dose. The reason for this difference is not clear; pro-oxidant activity of some flavonoids and phenolic acids has been noted previously (Cao et al., 1997; Yamanaka et al., 1997). Such pro-oxidant activity may be related to the ability of these redox active compounds to reduce metal ions and hence enable the formation of free radical chain propagating species. It can be seen from Fig. 5 that high dose quercetin increases the early propagation phase of lipid peroxidation but has an overall effect of inhibition on the total production of lipid hydroperoxides. The lack of pro-oxidant activity for the prenylated compounds could be beneficial in terms of overall biological activity.

Epidemiological studies have demonstrated that intake of flavonoids is inversely related to the mortality from diseases such as coronary heart disease (Hertog et al., 1993). The flavonoid content of fruits and vegetables may also explain why such dietary components have a protective effect against chronic diseases such as cancer and cardiovascular disease. The common link between these effects is mediated by the antioxidant activity of these compounds. In addition, prenylated flavonoids have also been shown to inhibit platelet aggregation (Ko et al., 1999) and influence cyclooxygenase and lipoxygenase activity (Kimura et al., 1986; Chi et al., 2001). This latter activity may have relevance to anti-inflammatory action of medicinal plants containing such compounds. We have shown that the prenylated flavonoids from *Dorstenia mannii* have potent antioxidant activity while not sharing some of the pro-oxidant action of some of the non-prenylated flavonoids. The effect of prenylation on lipid solubility may also make these compounds more readily absorbed or associated with lipoprotein particles in the blood. This activity may correlate with the ethnopharmacological use of this plant for treatment of inflammatory conditions such as rheumatism, which involves oxidative stress. Further conclusions on the actual absorption and bioavailability of these compounds must await detailed in vivo studies.

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