Antioxidant and Prooxidant Actions of Prenylated and Nonprenylated Chalcones and Flavanones in Vitro

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Prenylated flavonoids found in hops and beer, i.e., prenylchalcones and prenylflavanones, were examined for their ability to inhibit in vitro oxidation of human low-density lipoprotein (LDL). The oxidation of LDL was assessed by the formation of conjugated dienes and thiobarbituric acid-reactive substances (TBARS) and the loss of tryptophan fluorescence. At concentrations of 5 and 25 μM, all of the prenylchalcones tested inhibited the oxidation of LDL (50 μg protein/ml) induced by 2 μM copper sulfate. The prenylflavanones showed less antioxidant activity than the prenylchalcones, both at 5 and 25 μM. At 25 μM, the nonprenylated chalcone, chalconaringenin (CN), and the nonprenylated flavanone, naringenin (NG), exerted prooxidant effects on LDL oxidation, based on TBARS formation. Xanthohumol (XN), the major prenylchalcone in hops and beer, showed high antioxidant activity in inhibiting LDL oxidation, higher than α-tocopherol and the isoflavone genistein but lower than the flavonol quercetin. When combined, XN and α-tocopherol completely inhibited copper-mediated LDL oxidation. These findings suggest that prenylchalcones and prenylflavanones found in hops and beer protect human LDL from oxidation and that prenylation antagonizes the prooxidant effects of the chalcone, CN, and the flavanone, NG.

Keywords: *Antioxidants; flavonoids; prenylated chalcones; prenylated flavanones; low-density lipoprotein*

Oxidation of low-density lipoprotein (LDL) is thought to play a central role in atherosclerosis (Steinberg et al., 1989; Diaz et al., 1997). Oxidation of LDL can be initiated by catalytic transition metal ions such as copper. During the oxidation of LDL, the polyunsaturated fatty acids in the lipoprotein are rapidly converted to lipid hydroperoxides and aldehydic breakdown products (Esterbauer et al., 1992). LDL oxidation also results in the oxidative modification of the apoprotein, which plays a role in macrophage uptake and atherogenesis (Steinberg et al., 1989; Diaz et al., 1997; Esterbauer et al., 1992). The mechanism by which Cu^{2+} oxidizes LDL in vitro involves the oxidation of LDL-associated α-tocopherol to the α-tocopheroxyl radical, which subsequently initiates lipid peroxidation (Bowry and Stocker, 1993). In addition, preformed lipid hydroperoxides, if present in LDL, may play a role via the Cu^{2+}-mediated formation of lipid peroxyl and alkoxyl radicals (Esterbauer et al., 1992; Frei and Gaziano, 1993).

Numerous flavonoids have been shown to protect LDL from Cu^{2+}-catalyzed oxidation (Viana et al., 1996; Teissedre et al., 1996; Cheng et al., 1998). Flavonoids, including chalcones (with an open C-ring), are a group of phenolic compounds with wide distribution in the plant kingdom. The antioxidant activities of flavonoids are related to their ability to chelate metal ions and scavenge singlet oxygen, superoxide radicals, peroxyl radicals, hydroxyl radicals, and peroxynitrite (Briviba and Sies, 1994; Bors et al., 1997). These reactive oxygen and nitrogen species can react with critical cellular components such as DNA, lipids, and proteins leading to tissue injury and contributing to chronic disease. A high intake of flavonoids has been associated with a lower incidence of cardiovascular diseases (Hertog et al., 1993). The low mortality rate from cardiovascular diseases in populations consuming a high fat diet along with frequent drinking of red wine (the "French paradox") may be accounted for, in part, by the high content of polyphenolic compounds in red wine (Renaud et al., 1992; Constant, 1997).

Recently, several flavonoids such as chalcones and flavanones with prenyl or geranyl side chains have been identified in hops and beer (Stevens et al., 1997, 1998, 1999b). As phenolic compounds, these flavonoids may be responsible for the antioxidant activity of lager beer, which is higher than that of green tea, red wine, or grape juice (Vinson et al., 1999). Isoxanthohumol, xanthohumol, and 6-prenylnaringenin were the major prenylflavonoids found in beer, accompanied by minor amounts of 8-prenylnaringenin and 6-geranylnaringenin (Stevens et al., 1999b). To assess the antioxidant activity of the prenylated flavonoids, we evaluated their capacity to inhibit Cu^{2+}-mediated oxidation of LDL by measuring the formation of conjugated dienes and thiobarbituric acid-reactive substances (TBARS) and the oxidation of tryptophan residues. The antioxidant properties of the prenylflavonoids were compared to those of quercetin (a flavonol), genistein (an isoflavone), chalconaringenin

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(a nonprenylated chalcone), naringenin (a nonprenylated flavanone), and α-tocopherol. The possible interaction of xanthohumol, the major prenylchalcone in beer, with α-tocopherol to inhibit Cu^{2+}-mediated LDL oxidation was also examined.

MATERIALS AND METHODS

Materials. The chalcones XN (xanthohumol), XG (xanthogalenol), PX (5′- prenylxanthohumol), TP (desmethylxanthohumol or 2′,4′,6′,4-tetrahydroxy-3′-*C*-prenylchalcone), TG (2′,4′,6′,4-tetrahydroxy-3′-*C*-geranylchalcone), DX (dehydrocycloxanthohumol), DH (dehydrocycloxanthohumol hydrate), MXN (4′-*O*-methylxanthohumol), and MCN (4′,6′-dimethoxy-2′,4-dihydroxychalcone) and the flavanone IX (isoxanthohumol) were isolated from hops as described by Stevens et al. (1997, 2000). The prenylated flavanones 6PN (6-prenylnaringenin), 8PN (8-prenylnaringenin), DPN (6,8-diprenylnaringenin), 6GN (6-geranylnaringenin), and 8GN (8-geranylnaringenin) were prepared from naringenin and 2-hydroxy-2-methylbut-3-ene or linalool (Stevens et al., 1999a,b). The remaining chalcones CN (chalconaringenin), TX (tetrahydroxanthohumol), and DPX (4′-*O*-5′-C-diprenylxanthohumol) were prepared as described below. Naringenin (NG), genistein (GS), quercetin (QC), and α-tocopherol (TOC) were purchased from Sigma Chemical Co. (St. Louis, MO). The chemical structures of the individual flavonoids are shown in Figure 1.

CN was prepared by treatment of NG with 5% NaOH in MeOH (50 mL) under reflux conditions for 1 h. The reaction mixture was poured into 200 mL of 2 N HCl, and the aqueous layer was extracted with ethyl acetate (1 × 150 mL, 2 × 100 mL). The combined ethyl acetate layers were washed with water (3 × 100 mL), dried over anhydrous Na_2SO_4, and evaporated in vacuo. CN was separated from unconverted naringenin by preparative HPLC, yielding a yellow powder after lyophilization. UV [λ_{max} (MeOH)]: 365 nm. APCI-MS [*m*/*z* (rel int)]: 273 $[MH]^+$ (100%). NMR [δ_H (DMSO-d_6, 600 MHz)]: 12.52 (s, OH-2′/6′), 10.38 (s, OH-4′), 10.05 (s, OH-4), 7.96 (d, J = 15.6 Hz, H-α), 7.65 (d, J = 15.6 Hz, H-*β*), 7.52 (d, J = 8.5 Hz, H-2/6), 6.83 (d, J = 8.5 Hz, H-3/5), 5.84 (s, H-3′/5′).

TX was obtained from XN by hydrogenation in the presence of Pd/C. Lyophilization of the TX peak fraction yielded a white solid. APCI-MS [*m*/*z* (rel int)]: 359 $[MH]^+$ (100%), consistent with $C_{21}H_{26}O_5$. APCI-MS-MS [*m*/*z* (rel int)]: 341 $[MH - H_2O]^+$ (19%), 253 [C_2 − A ring, $C_{14}H_{21}O_4$]$^+$ (95%), 211 $[253 - C_2H_2O]^+$ (60%), 149 [C_3 − B ring, $C_9H_9O_2$]$^+$ (39%), 107 $[149 - C_2H_2O]^+$ (100%). NMR [δ_H (DMSO-d_6, 600 MHz)]: 14.19 (s, OH-2′), 10.43 (s, OH-4′), 9.13 (s, OH-4), 7.01 (d, J = 8.3 Hz, H-2/6), 6.66 d, J = 8.3 Hz, H-3/5), 6.05 (s, H-5′), 3.78 (s, 6′-OMe), 3.16 (t, J = 8 Hz, H-α), 2.76 (t, J = 8 Hz, H-*β*), 2.43 (t, J = 8 Hz, H-1″), 1.52−1.46 (m, H-3″), 1.29−1.26 (m, H-2″), 0.89 and 0.88 (2 × s, Me-4″ and Me-5″).

DPX was prepared from XN and 2-hydroxy-2-methylbut-3-ene by following a procedure described by Jain et al. (1978). The reaction mixture, containing XN, PX, DPX, and other prenylated xanthohumols, was submitted to preparative HPLC. DPX was obtained as a yellow-orange solid, M_r 490, by APCI-MS, consistent with $C_{31}H_{38}O_5$. APCI-MS-MS [*m*/*z* (rel int)]: 491 $[MH]^+$ (100%), 435 $[MH - C_4H_8]^+$ (36%), 371 $[A]^+$ (21%), 315 $[371 - C_4H_8]^+$ (20%), 247 $[315 - C_5H_8]^+$ (8%), 191 $[247 - C_4H_8]^+$ (24%). Because a *C*-prenyl function gives rise to loss of 56 mass units (C_4H_8) and an *O*-prenyl to loss of 68 mass units (C_5H_8), it was determined from the fragmentation pattern that the compound carries two *C*-prenyls (one C_4H_8 released from MH^+ and two C_4H_8 neutrals from the retro Diels−Alder A-ring fragment with *m*/*z* 371) and one *O*-prenyl substituent. Loss of 120 mass units from the molecular ion, $[MH]^+$, and the absence of a fragment with $[MH - 68]^+$, indicated that the *O*-prenyl substituent was located at one of the two free A-ring hydroxyls. Because the ^1H NMR spectrum showed a hydrogen-bonded OH signal at δ_H 13.89 (OH-2′) and one other OH signal (OH-4, δ_H 10.10), the position of the *O*-prenyl substituent was determined to be at C-4′. Other resonances were assigned to the bridge protons (H-α and H-*β*, δ_H 7.78 and 7.73, both doublets with J = 15.5 Hz), the B-ring protons (H-2′/6 and H-3′/5′, δ_H 7.59 and 6.85, both doublets with J = 8.5 Hz), and the methyl protons of the three prenyl substituents (δ_H 1.75, 1.70, 1.64, 1.58, 1.41, and 1.15, all singlets).

LDL Isolation. Heparinized blood, collected from healthy, normolipidemic volunteers, was centrifuged at 1200*g* for 20 min at 4 °C to obtain plasma. LDL was isolated from plasma using the method of Chung et al. (1986) as modified by Sattler et al. (1994). The LDL solution obtained from the single spin procedure was adjusted to a density of 1.067 g/mL with NaBr and centrifuged at 540 000*g* for 14 h at 5 °C. The second centrifugation step was included to concentrate the LDL and to ensure that the preparation was free from albumin. Isolated LDL was desalted by gel filtration [PD-10 column equilibrated with PBS (10 mM phosphate, 150 mM NaCl, pH 7.4)] and stored at 4 °C under nitrogen until use.

Assessment of Cu^{2+}-Catalyzed LDL Oxidation. The individual flavonoids (5, 12.5, or 25 μM, in ethanol) or α-tocopherol (12.5 or 25 μM, in ethanol) were added to LDL (diluted with PBS to a final protein concentration of 50 μg/mL) in a total volume of 200 μL/well of a 96-well plate. Ethanol (1 μL/well) was added to LDL in control wells. The 96-well plate was placed inside the temperature-controlled (37 °C) chamber of a microplate reader (SpectraMax 250, Molecular Devices, Sunnyvale, CA), and the light absorbance at 250 nm was recorded as zero time values. Copper sulfate (2 μM) was then added to the individual wells. Negative control wells contained LDL without Cu^{2+}. To determine if the flavonoids themselves act as prooxidants, LDL was incubated with these compounds in the absence of Cu^{2+}. The plate was returned to the plate reader set at 37 °C. Conjugated diene formation in each well was measured at 30-min intervals for 5 h by monitoring the increase in absorbance at 250 nm (Camejo et al., 1998). Although conjugated diene formation is usually followed in conventional spectrophotometers using the peak maximum at 234 nm, measurement of the absorbance at 250 nm with a microtiter spectrophotometer is also an accepted method (Camejo et al., 1998).

After 5 h of incubation, the amount of TBARS formed in the sample was measured as described by Buege and Aust (1978) with some modifications. The content of each well was transferred to a 1.5-ml microcentrifuge tube containing 20 μL of ice-cold 50% trichloroacetic acid. The tubes were vortexed, and then 100 μL of 1% thiobarbituric acid in 0.28% NaOH was added to each tube. The samples were acidified by the addition of 20 μL of 1 N HCl. The tubes were heated at 90 °C for 20 min and centrifuged at 16 000*g* for 5 min. An aliquot (200 μL) of the supernatant was transferred to a 96-well plate, and the absorbance at 535 nm was measured using a microplate reader (SpectraMax 250).

To assess the effects of the flavonoids on Cu^{2+}-mediated oxidation of tryptophan residues in LDL, the fluorescence of the LDL samples was read at excitation and emission wavelengths of 280 and 331 nm, respectively, in a Hitachi F-2500 spectrofluorometer (Keaney and Frei, 1994).

Chelation of Copper by Flavonoids. Copper chelation by flavonoids was determined by the spectral shifts produced when copper ions were incubated with the test compounds. The reaction mixtures consisted of 0.5 mL of phosphate buffer (0.2 M, pH 7.4), 0.5 mL of acetonitrile, 50 μL of flavonoid solution (2 mM in DMSO), 50 μL of $CuSO_4$ solution (1 mM in water), and He-degassed water to a total volume of 10 mL. The Cu^{2+} and flavonoid concentrations in these mixtures were 5 and 10 μM, respectively. The flavonoids tested were CN, XN, XG, TP, IX, 8PN, and NG. Spectra (200−600 nm) were recorded immediately after preparation of the mixtures and again after 10 min, in the presence and absence of copper ions.

Statistical Analysis. Multiple mean comparisons were performed by one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) test using the SAS computer software. Statistical significance was accepted if the null hypothesis was rejected at the $p < 0.05$ level.

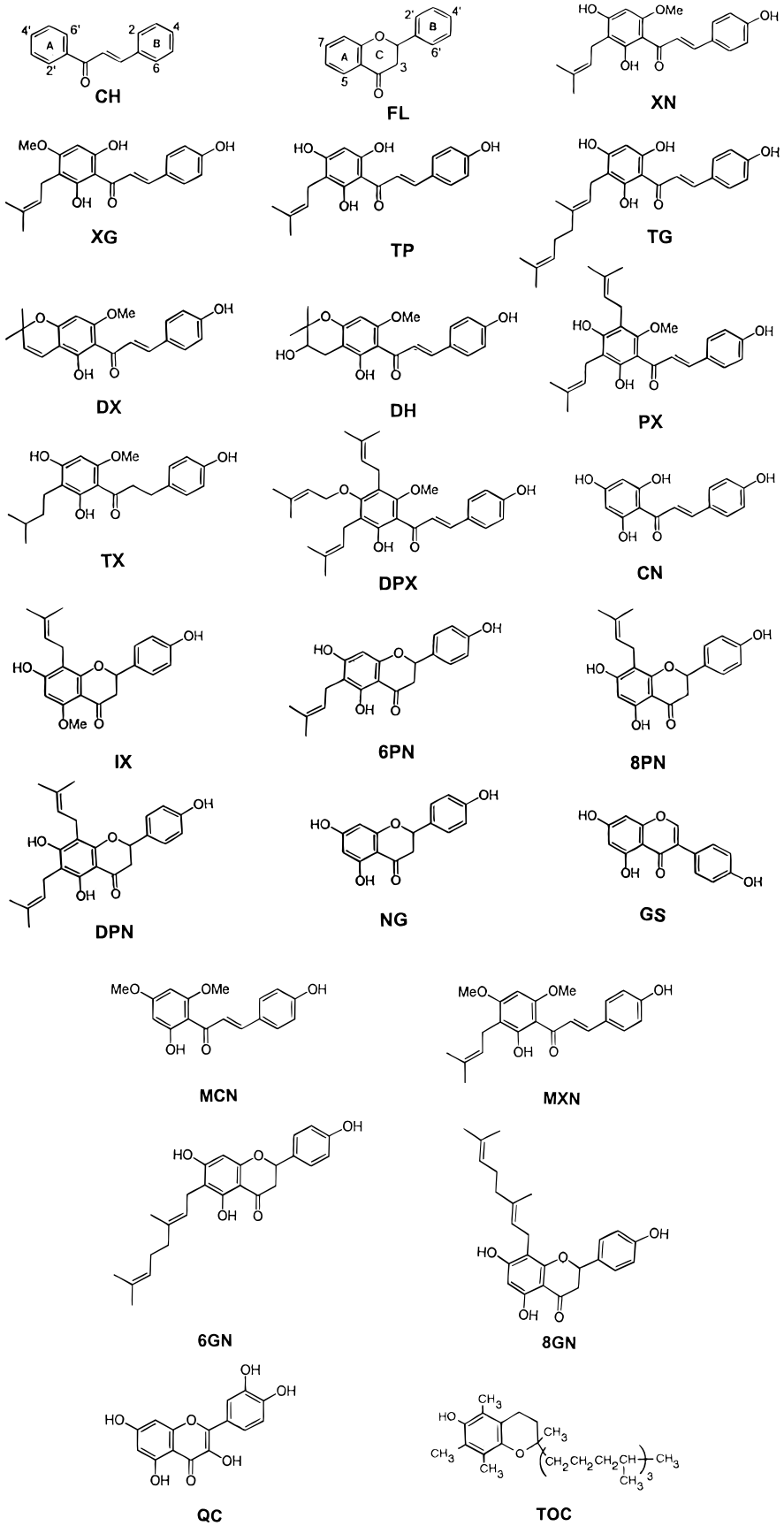

Figure 1. Chemical structures of flavonoids and α-tocopherol: CH, chalcone; FL, flavanone; XN, xanthohumol; XG, xanthogalenol; TP, 2′,4′,6′,4-tetrahydroxy-3′-*C*-prenylchalcone (desmethylxanthohumol); TG, 2′,4′,6′,4-tetrahydroxy-3′-*C*-geranylchalcone); DX, dehydrocycloxanthohumol; DH, dehydrocycloxanthohumol hydrate; PX, 5′-prenylxanthohumol; TX, tetrahydroxanthohumol; DPX, 4′-*O*-5′-*C*-diprenylxanthohumol; CN, chalconaringenin; IX, isoxanthohumol; 6PN, 6-prenylnaringenin; 8PN, 8-prenylnaringenin; DPN, 6,8-diprenylnaringenin; NG, naringenin; GS, genistein; MCN, 4′,6′-dimethoxy-2′,4-dihydroxychalcone; MXN, 4′-*O*-methylxanthohumol; 6GN, 6-geranylnaringenin, 8GN, 8-geranylnaringenin; QC, quercetin; TOC, α-tocopherol.

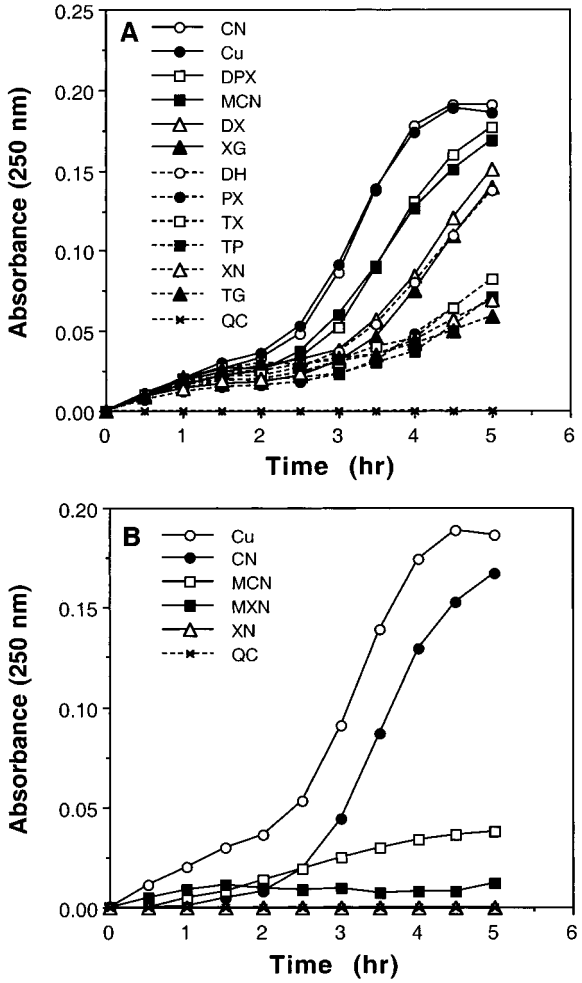

Figure 2. Conjugated diene formation in human LDL (50 μg protein/mL) incubated with 2 μM Cu^{2+} in the presence or absence of prenylated or nonprenylated chalcones at a concentration of 5 μM (A) or 25 μM (B). Results represent the mean of three determinations.

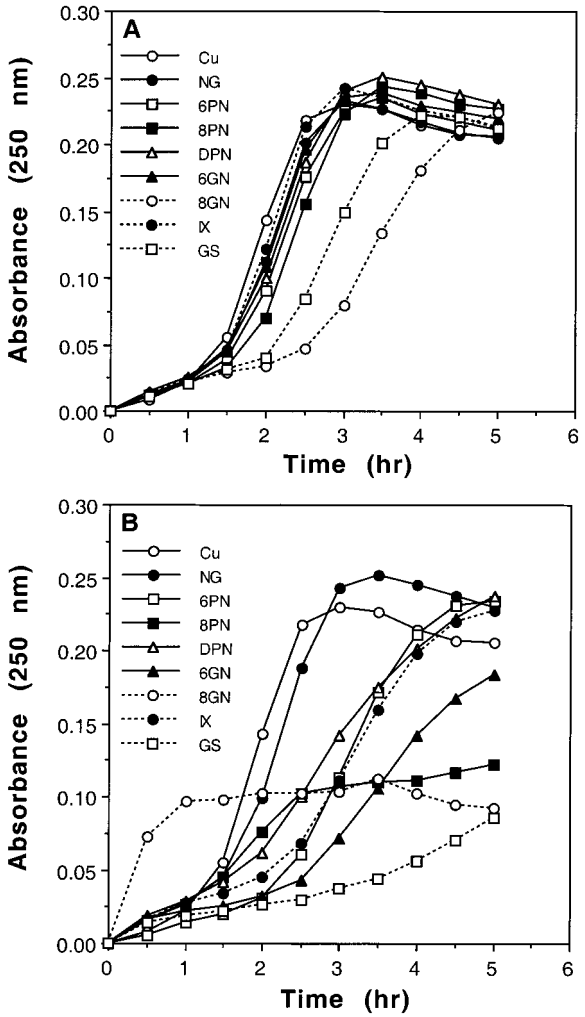

Figure 3. Conjugated diene formation in human LDL (50 μg protein/mL) incubated with 2 μM Cu^{2+} in the presence or absence of prenylated or nonprenylated flavanones and isoflavone (GS) at a concentration of 5 μM (A) or 25 μM (B). Results represent the mean of three determinations.

RESULTS

Effects of Prenylated Chalcones on Conjugated Diene Formation in LDL. When freshly isolated human LDL (50 μg protein/mL) was incubated with 2 μM Cu^{2+} at 37 °C, the typical time course of conjugated diene formation was observed (Esterbauer et al., 1992), consisting of a lag phase, a propagation phase, and a decomposition phase (Figure 2). Under these incubation conditions, the lag time of LDL oxidation in the absence of flavonoids was approximately 2.0 h, and the maximum absorbance was about 0.19 absorbance units, reached after 4.5 h of incubation (Figure 2). Addition of prenylchalcones (5 μM) increased the lag time and decreased the rate of conjugated diene formation during the propagation phase (Figure 2A). The three most effective prenylchalcones were TG, XN, and TP, which decreased conjugated diene formation by more than 70% after 5 h of incubation compared to the vehicle control (LDL and Cu^{2+}). The nonprenylated chalcone, CN, had no effect on LDL oxidation whereas QC (positive control) completely prevented the formation of conjugated dienes in LDL incubated for up to 5 h with Cu^{2+} (Figure 2A). Furthermore, when LDL was incubated with the flavonoids for 5 h in the absence of Cu^{2+}, no increase in conjugated diene formation was observed (data not shown).

At a concentration of 25 μM, quercetin, XN (Figure 2B), and several other prenylchalcones (data not shown) completely inhibited the Cu^{2+}-mediated formation of conjugated dienes during the 5-h incubation period. MXN, which contains two methoxy groups, was almost as inhibitory as XN, which contains only one methoxy group (Figure 2B). However, the nonprenylated chalcone, CN, at 25 μM showed only a slight inhibitory activity toward conjugated diene formation, whereas the nonprenylated chalcone, MCN, which contains two methoxy groups, strongly inhibited (Figure 2B).

Effects of Prenylated Flavanones on Conjugated Diene Formation in LDL. In contrast to the prenylchalcones (Figure 2A), the prenylflavanones at a concentration of 5 μM had very little effect on Cu^{2+}-mediated LDL oxidation, except for 8GN (Figure 3A). At a concentration of 25 μM, the prenylflavanones were more effective at inhibiting LDL oxidation (Figure 3B). The nonprenylated flavanone, NG, at 25 μM also slightly increased the lag time of conjugated diene formation but was considerably less effective than the prenylflavanones. Interestingly, NG and the prenylflavanones investigated, except for 6PN, appeared to exert a slight prooxidant effect during the initial 30 min of incubation of LDL with Cu^{2+}. In fact, 25 μM 8GN exerted a strong

Table 1. Effect of Prenylated and Nonprenylated Flavonoids on Cu^{2+}-Dependent Lipid Peroxidation in Human Low-Density Lipoprotein (LDL)[a]

addition to LDL	TBARS (A_{535}) 5 μM flavonoid	25 μM flavonoid
none	0.030 ± 0.002	0.020 ± 0.001
Cu^{2+} (control)	0.081 ± 0.001[a] (100)	0.075 ± 0.001[a] (100)
Cu^{2+} + chalcone		
CN	0.121 ± 0.003[b] (140)	0.112 ± 0.001[b] (149)
MCN	0.080 ± 0.002[a] (98.8)	0.044 ± 0.004[c] (58.7)
TP	0.097 ± 0.003[c] (120)	0.030 ± 0.001[d] (40.0)
XG	0.083 ± 0.003[a] (102)	0.022 ± 0.001[e] (29.3)
XN	0.055 ± 0.002[d] (67.9)	0.020 ± 0.001[e,f] (26.6)
PX	0.026 ± 0.001[e] (32.1)	0.005 ± 0.001[g,h] (6.7)
MXN	0.040 ± 0.001[f] (49.4)	0.019 ± 0.001[e,f] (25.3)
TG	0.042 ± 0.001[f] (51.9)	0.012 ± 0.001[i] (16.0)
Cu^{2+} + flavanone		
NG	0.062 ± 0.002[g] (76.5)	0.120 ± 0.003[j] (160)
8PN	0.080 ± 0.001[a] (98.8)	0.032 ± 0.001[d] (42.7)
DPN	0.076 ± 0.001[h] (93.8)	0.056 ± 0.004[k] (74.7)
8GN	0.062 ± 0.002[g] (76.5)	0.009 ± 0.001[h,i] (12.0)
IX	0.068 ± 0.001[i] (83.9)	0.048 ± 0.001[c] (64.0)
Cu^{2+} + isoflavone		
GS	0.050 ± 0.001[j] (61.7)	0.069 ± 0.001[l] (92.0)
Cu^{2+} + flavonol		
QC	0.019 ± 0.003[k] (23.4)	0.014 ± 0.001[f,i] (18.7)

[a] Results are expressed as mean values ± SE for four determinations. TBARS analysis was performed on human LDL (50 μg protein/mL) exposed to 2 μM Cu^{2+} and 5 or 25 μM flavonoid for 24 h at 25 °C. Values in the same column followed by the same letter are not significantly different at $p < 0.05$. Values in parentheses indicate percent of control (LDL plus Cu^{2+}).

prooxidant effect, with no observable lag phase of diene conjugation (Figure 3B). Unlike the prenylchalcones, none of the prenylflavanones was able to completely prevent the oxidation of LDL catalyzed by Cu^{2+} during the 5-h incubation period.

Effects of Prenylated Chalcones and Flavanones on TBARS Formation in LDL. The effects of prenylchalcones on Cu^{2+}-mediated LDL oxidation were further evaluated by measuring TBARS levels after incubation for 5 h at 37 °C. At a concentration of 5 μM, the prenylchalcones and QC were ranked as follows (using ANOVA) in terms of their ability to inhibit TBARS formation: QC > TG > PX, XN, TP, TX > DH, MXN, DX, XG, DPX. These data are in full agreement with those on conjugated diene formation (see Figure 2A). The nonprenylated chalcone, CN, had no inhibitory effect at 5 μM and at 25 μM significantly increased TBARS formation by 6%. In contrast to the prenylchalcones, the prenylflavanones at a concentration of 5 μM had no inhibitory effect, except for 8GN and DPN. At a concentration of 25 μM, the prenylflavanones and GS significantly inhibited TBARS formation and were ranked as follows: 8GN > GS > DPN, 6GN, 8PN > IX, 6PN. These data are consistent with those on diene conjugation (see Figure 3). The nonprenylated flavanone, NG, at 25 μM, significantly increased TBARS levels by 13%.

The effects of the flavonoids on TBARS formation were also assessed following incubation of LDL with 2 μM Cu^{2+} at 25 °C for 24 h (Table 1). At 5 μM, the prenylchalcones PX, MXN, TG, and XN and the prenylflavanones 8GN, IX, and DPN significantly inhibited LDL oxidation. XG and 8PN had no effect, whereas CN and TP stimulated TBARS formation. At 25 μM, CN and NG acted as strong prooxidants and significantly increased TBARS levels by 49 and 60%, respectively, while MCN acted as an antioxidant (Table 1). The

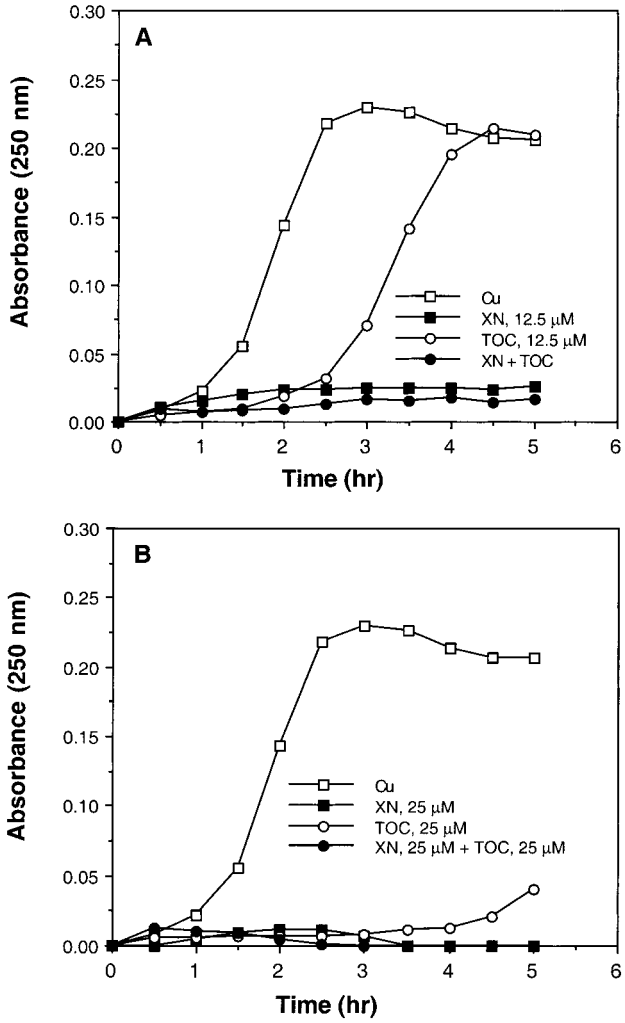

Figure 4. Conjugated diene formation in human LDL (50 μg protein/mL) incubated with 2 μM Cu^{2+} in the presence or absence of xanthohumol or α-tocopherol, alone or in combination, at a concentration of 12.5 μM (A) or 25 μM (B). Results represent the mean of three determinations.

chalcones with prenyl (PX, MXN, XN, XG, and TP) or geranyl (TG) side chains significantly inhibited Cu^{2+}-induced formation of TBARS, as did the flavanones with prenyl (8PN, IX, and DPN) or geranyl (8GN) side chains. At 25 μM, GS was less effective than the prenylchalcones in inhibiting TBARS formation, whereas QC was as inhibitory as XN, MXN, TG, and 8GN (Table 1).

Effects of α-Tocopherol, Alone or in Combination with XN, on LDL Oxidation. α-Tocopherol (TOC) is known to protect LDL from Cu^{2+}-mediated oxidation, if the Cu^{2+}:LDL ratio is relatively high (Bowry and Stocker, 1993; Keaney and Frei, 1994). It was of interest to compare the relative antioxidant activity of TOC and XN, the major chalcone in hops and in some beers. TOC was used at concentrations of 12.5 and 25 μM, which are within the physiological range in human plasma. At 12.5 μM, TOC extended the lag time of conjugated diene formation from 1.2 to 2.5 h (Figure 4A). In contrast, 12.5 μM XN extended the lag time over 5 h. Equimolar concentrations (12.5 μM) of TOC and XN inhibited conjugated diene formation to a similar extent as XN alone throughout the 5-h incubation period (Figure 4A). At 25 μM, TOC alone or XN alone almost completely inhibited the formation of conjugated dienes (Figure 4B). When TOC and XN were combined to-

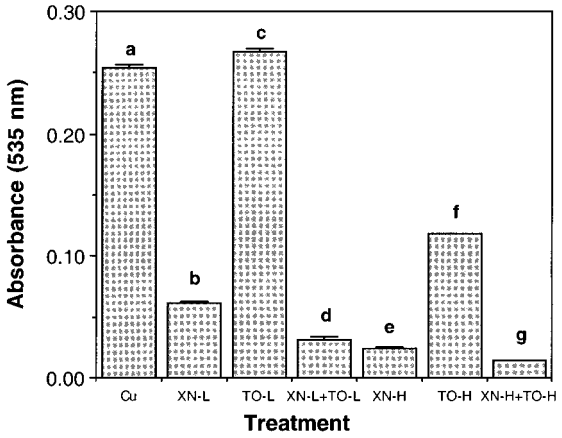

Figure 5. Effects of xanthohumol (XN) and α-tocopherol (TO), alone or in combination, on Cu^{2+}-mediated LDL oxidation as assessed by the formation of thiobarbituric acid-reactive substances. XN and TO were used at low (12.5 μM each, XN−L and TO−L) or high (25 μM each, XN−H and TO−H) concentrations in reaction mixtures containing LDL (50 μg protein/mL) and 2 μM Cu^{2+}. Results represent the mean ± SE of three determinations. Bars labeled with different letters are significantly different from each other, $p < 0.05$.

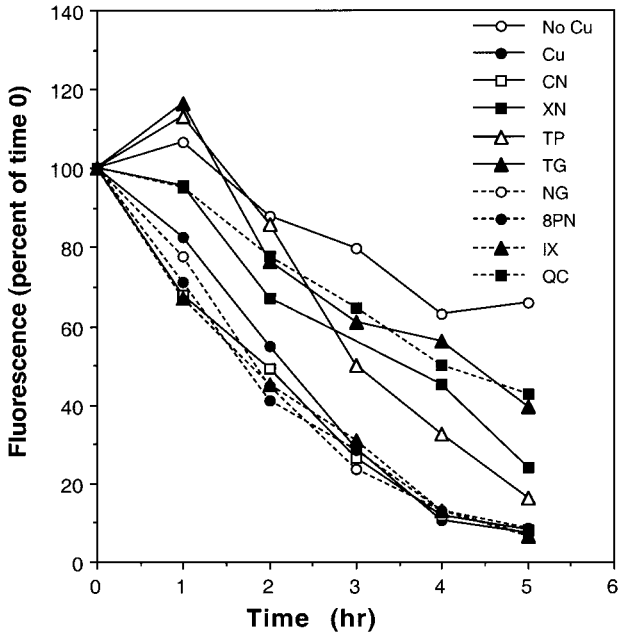

Figure 6. Tryptophan fluorescence (excitation = 280 nm, emission = 331 nm) in human LDL (50 μg protein/mL) incubated with 2 μM Cu^{2+} in the presence or absence of 25 μM flavonoids.

gether, there was complete inhibition of conjugated diene formation (Figure 4B).

The effects of XN, alone or in combination with TOC, on TBARS formation following 5 h of incubation of LDL with 2 μM Cu^{2+} are shown in Figure 5. At 12.5 μM, TOC appeared to slightly increase TBARS levels, whereas XN strongly inhibited. TBARS formation was significantly lower in the presence of both TOC and XN compared to each compound alone. Similar results were obtained at the higher concentration (25 μM) of XN and TOC (Figure 5).

Tryptophan Oxidation in LDL. The reduction in tryptophan fluorescence (excitation/emission = 280/331 nm) was used to evaluate the protective effects of flavonoids on Cu^{2+}-mediated protein oxidation in LDL during incubation at 37 °C. Copper causes rapid oxidation of LDL tryptophan residues which can be monitored fluorometrically (Giessauf et al., 1995). As shown in Figure 6, copper (2 μM) caused an almost linear decrease in LDL tryptophan fluorescence with increasing time of incubation. The flavonoids XN, TP, TG, and QC inhibited tryptophan oxidation, in contrast to CN, NG, 8PN, and IX.

Chelation of Cu^{2+} by Flavonoids. Flavonoids display characteristic spectra that undergo changes upon interaction with metal ions. For example, the chalcone butein exhibits maxima at 260 and 395 nm, which shift to 286 and 454 nm in the presence of Cu^{2+} ions (Cheng et al., 1998). In the present study, however, little or no change of the spectra was observed for XN, IX, 8PN, MXN, PX, and DPX (data not shown). The 2′,6′-dihydroxychalcones CN, XG, and TP developed new maxima around 290 nm over a 10-min period, which was attributed to conversion of the chalcones to their isomeric flavanones rather than chelation of copper ions.

DISCUSSION

The results of the present study show that prenylated chalcones are capable of inhibiting Cu^{2+}-mediated oxidation of LDL. The prenylchalcones prolonged the lag phase of conjugated diene formation and reduced the rate of LDL lipid peroxidation during the propagation phase. Furthermore, the prenylchalcones inhibited the formation of TBARS and the oxidation of tryptophan residues in LDL, providing additional evidence for antioxidant activity of these compounds. The prenylflavanones also displayed antioxidant activity but were not as effective as the prenylchalcones. These findings suggest that prenylchalcones and, to a lesser extent, prenylflavanones provide protection against oxidative modification of LDL.

Prenylated chalcones and flavanones are found in hops and beer. Vinson et al. (1995, 1999) compared several beverages, including beer, for antioxidant activity toward Cu^{2+}-induced LDL oxidation. On the basis of the formation of TBARS, the antioxidant activity of the beverages was ranked as follows: black tea > coffee > prune juice = beer > green tea > orange juice > red wine > tangerine juice > red grape juice > white grape juice > grapefruit juice (Vinson et al., 1999). The antioxidant activity of the fruit juices and tea could be explained by their high content of polyphenolic flavonoids. However, the specific compounds responsible for the antioxidant activity in beer have not been identified. The prenylated chalcone XN and the prenylated flavanones IX, 6PN, 8PN, and 6GN, are polyphenolic compounds recently identified in beer (Stevens et al., 1999b). The concentrations of these prenylflavonoids vary with the type of beer (Stevens et al., 1998). For example, XN and IX, a flavanone isomer of XN, have been reported to be present in beer at concentrations of 0.69 mg/L (1.95 μM) and 3.44 mg/L (9.7 μM), respectively, and the total prenylflavonoid content of beer could be as high as 4 mg/L, or approximately 11 μM (based on the molecular weight of XN and IX of 354 Da). The prenylflavanone constituents of beer do not originate directly from hops but from the isomerization of the prenylchalcones in hops during the brewing process (Stevens et al., 1999b). These prenylflavonoids and other phenolic compounds, such as glycosides of kaempferol and quercetin and catechins (Vinson et al., 1995; Sä-

gesser et al., 1996), may all contribute to the antioxidant activity of beer.

Studies on structure−activity relationships have shown that the presence of hydroxyl groups and methoxy groups in the A and B rings appear to be important in antioxidant and free radical scavenging activities of chalcone compounds (Anto et al., 1995). Butein, a chalcone with a catechol in the B ring and two hydroxyl groups in the A ring, is a potent inhibitor of Cu^{2+}-mediated oxidation of LDL (Cheng et al., 1998). Another chalcone, isoliquiritigenin, with two hydroxyl groups in the A ring and one hydroxyl group in the B ring (phenol B ring), also is a strong inhibitor of 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH)-induced oxidation of LDL (Vaya et al., 1997). The prenylated chalcone isolated from licorice roots, isoprenylchalcone, likewise inhibited LDL oxidation induced by AAPH (Vaya et al., 1997).

In the present study, we have shown that prenyl groups may have a marked influence on the antioxidant activities of the different prenylflavonoids containing phenol B rings. Specifically, prenylation may endow hydroxylated chalcones with enhanced antioxidant activity. This is exemplified by TP and TG that showed very high antioxidant activity as opposed to CN, a nonprenylated hydroxylated chalcone. CN even acted as a prooxidant by increasing TBARS formation during incubation of LDL with Cu^{2+}. The addition of a methoxy group also confers antioxidant activity to the chalcones, since 25 μM MCN (a chalcone with two methoxy groups), unlike 25 μM CN, effectively inhibited formation of conjugated dienes and TBARS. The prenyl and geranyl groups also appeared to be important in the antioxidant activity of flavanones at high concentration (25 μM) because the nonprenylated flavanone, NG, acted as a prooxidant, whereas 8PN, DPN, and 8GN inhibited LDL oxidation.

The mechanisms by which prenyl and methoxy groups enhance the antioxidant capacity of chalcones and flavanones remain to be established. It is conceivable that *C*-prenylation and *O*-methylation add lipophilicity to the flavonoids, NG and CN, which could result in an increase of the flavonoid concentration in the lipophilic environment within the LDL particle due to "lipid−lipid" partitioning or noncovalent interactions. Model experiments with XN and IX have shown that the "solubility" of both flavonoids is much higher in wort (27 and 122 μM, respectively) than in water (3.7 and 14 μM), which indicates that a significant proportion of these flavonoids is complexed in wort with macromolecules, mainly carbohydrates and proteins (Stevens et al., 1999a). It is in the lipophilic environment of the LDL particles where peroxidation of unsaturated fatty acids takes place, and hence, protection by the prenylated flavonoids against lipid peroxidation is most effective.

Copper chelation has been proposed as a possible mechanism for the antioxidant activities of quercetin (Afanas'ev et al., 1989) and butein (Cheng et al., 1998). Though representatives of different flavonoid classes, quercetin and butein have a B ring catechol moiety in common, which is known to be important for Cu^{2+}-chelate formation (Brown et al., 1998). None of the flavonoids investigated in this study, however, carry 3′,4′-dihydroxy substituents on the B ring, which may explain why only small spectral shifts or no shifts were observed in the presence of copper ions. Copper chelation is therefore considered to play a minor role in the antioxidant activities of substituted naringenins and chalconaringenins.

The antioxidant activity of the prenylflavonoids may also involve the scavenging of lipid peroxyl radicals, resulting in the inhibition of the propagation of lipid peroxidation in LDL. Like other flavonoids, the prenylated chalcones and flavanones contain phenolic hydroxyl groups that likely act as hydrogen donors in the scavenging of free radicals (Neuzil et al., 1997). Evidence for the ability of prenylchalcones to scavenge peroxyl radicals is provided by the inhibition of lipid peroxidation in rat liver microsomes induced by the radical generator AAPH (Rodriguez et al., 1999). In addition, prenylflavonoids may neutralize preformed lipid hydroperoxides; e.g., the peroxides may be reduced by hydrogens donated from phenolic hydroxy groups.

α-Tocopherol, the most abundant lipid-soluble antioxidant in human plasma and lipoproteins, exerts antioxidant and prooxidant activity in isolated LDL depending on the incubation conditions (Bowry and Stocker, 1993; Neuzil et al., 1997; Kontush et al., 1996). Under strong oxidative conditions in the presence of high amounts of Cu^{2+}, TOC supplementation shows antioxidant activity. Under conditions of mild oxidation, TOC acts as an antioxidant in the presence of coantioxidants, such as ubiquinol-10 and ascorbate, but as a prooxidant in their absence (Bowry and Stocker, 1993; Neuzil et al., 1997). Ascorbate is known to regenerate TOC from the potentially prooxidant α-tocopheroxyl radical. In the present study, we found evidence for an interaction between TOC and a prenylchalcone, XN, in the protection against Cu^{2+}-mediated LDL oxidation. When combined, TOC and XN inhibited the formation of conjugated dienes and TBARS to a greater extent than either TOC or XN alone. Further studies are required to determine whether this represents a synergistic interaction or an additive effect and whether XN can regenerate TOC from the α-tocopheroxyl radical.

Like α-tocopherol, flavonoids may behave as prooxidants under certain conditions. The flavonols quercetin and myricetin accelerate the generation of hydroxyl radicals in reaction mixtures containing hydrogen peroxide and Fe^{3+}−EDTA, as measured by deoxyribose degradation (Laughton et al., 1989). Depending upon the number of hydroxyl groups in their backbone structure, flavones, isoflavones, or flavanones may exert prooxidant activities in the presence of Cu^{2+} ions (Cao et al., 1997). Catechins found in green tea, such as (−)-epicatechin and (−)-epigallocatechin, accelerate Cu^{2+}-mediated LDL oxidation during the propagation phase (Yamanaka et al., 1997). Other flavonoids also accelerate Cu^{2+}-mediated LDL oxidation when added during the propagation phase of lipid peroxidation (Otero et al., 1997). Upon activation by peroxidases, the flavanone NG and the flavone apigenin become prooxidants and cause lipid peroxidation and extensive oxidation of glutathione (Galati et al., 1999) and NADH (Chan et al., 1999) at physiological pH. In the presence of a peroxidase, flavonoid compounds with phenol B rings may form prooxidant phenoxyl radicals, which during redox cycling can generate superoxide radicals and hydrogen peroxide. Naringenin also induced a concentration-dependent peroxidation of membrane lipids concurrent with DNA strand breaks in isolated rat liver nuclei (Sahu and Gray, 1997). In the present study, NG and CN, with phenol B rings, showed a tendency to act

as prooxidants at 25 μM by increasing TBARS production during exposure of LDL to Cu^{2+}. This prooxidant activity was not observed in chalcones and flavanones with prenyl or geranyl groups, suggesting that these substituents influence the redox potential of the phenoxyl radicals potentially formed during Cu^{2+}-mediated oxidation of LDL. The mechanistic role of prenyl and geranyl groups in antagonizing the prooxidant action of flavanones and other flavonoids with phenol B rings merits further investigation.

The potential impact of the prenylated flavonoids found in beer and hops, with respect to their antioxidant properties, on human health is not known. It remains to be investigated whether the prenylchalcones and prenylflavanones are absorbed from the intestines and are antioxidatively active in vivo or are metabolized to products with different antioxidant properties than their parent compounds. In vivo studies are needed to establish the potential health benefits of prenylflavonoids, alone or in combination, found in alcoholic and nonalcoholic beverages or in dietary supplements.

ABBREVIATIONS USED

CN, chalconaringenin; DH, dehydrocycloxanthohumol hydrate; DPN, 6,8-diprenylnaringenin; DPX, 4′-*O*-5′-*C*-diprenylxanthohumol; DX, dehydrocycloxanthohumol; 6GN, 6-geranylnaringenin; 8GN, 8-geranylnaringenin; GS, genistein; IX, isoxanthohumol; MCN, 4′,6′-dimethoxy-2′,4-dihydroxychalcone; MXN, 4′-*O*-methylxanthohumol; NG, naringenin; 6PN, 6-prenylnaringenin; 8PN, 8-prenylnaringenin; PX, 5′- prenylxanthohumol; QC, quercetin; TG, 2′,4′,6′,4-tetrahydroxy-3′-*C*-geranylchalcone; TOC, α-tocopherol; TP, 2′,4′,6′,4-tetrahydroxy-3′-*C*-prenylchalcone (desmethylxanthohumol); TX, tetrahydroxanthohumol; XG, xanthogalenol; XN, xanthohumol.

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