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Note

Inhibition of Xanthine Oxidase by Flavonoids

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Various dietary flavonoids were evaluated *in vitro* for their inhibitory effect on xanthine oxidase, which has been implicated in oxidative injury to tissue by ischemia-reperfusion. Xanthine oxidase activity was determined by directly measuring uric acid formation by HPLC. The structure-activity relationship revealed that the planar flavones and flavonols with a 7-hydroxyl group such as chrysin, luteolin, kaempferol, quercetin, myricetin, and isorhamnetin inhibited xanthine oxidase activity at low concentrations (IC_{50} values from 0.40 to 5.02 μM) in a mixed-type mode, while the nonplanar flavonoids, isoflavones and anthocyanidins were less inhibitory. These results suggest that certain flavonoids might suppress *in vivo* the formation of active oxygen species and urate by xanthine oxidase.

Key words: xanthine oxidase; flavonoid; inhibition; oxidative injury; gout

Flavonoids are widely distributed in various foods and beverages of plant origin.[1)] Some flavonoids are well known to act *in vitro* as strong antioxidants by their radical-scavenging activity and metal ion chelation.[2–6)] In addition to their antioxidative activity, flavonoids have been reported to inhibit various enzymes such as cyclooxygenase and lipoxygenase related to inflammation.[7)] Moreover, epidemiological studies have shown an inverse relationship between the intake of dietary flavonoids and mortality from coronary heart disease.[8)] Thus, dietary flavonoids have attracted strong attention for potential beneficial effects on human health. Several flavonoids have been found to show an inhibitory effect on xanthine oxidase (XO) which produces hydrogen peroxide and superoxide anion during the oxidation of hypoxanthine to xanthine and then to uric acid. These active oxygen species produced by XO have been proposed to cause oxidative injury to tissues by ischemia-reperfusion,[9–11)] although this hypothesis is still controversial. Allopurinol, one of the XO inhibitors, was found to suppress oxidative injury by ischemia-reperfusion, and is also clinically used for gout treatment to prevent urate from accumulating in the joints. Therefore, flavonoids with an inhibitory effect on XO might suppress oxidative lesions by ischemia-reperfusion and could be used for preventing gout. In this study, a wide range of flavonoids was evaluated for their inhibitory effect on XO, and certain ones were found to inhibit XO at a low concentration of 10^{-7} M.

Xanthine oxidase activity was determined by quantifying the amount of uric acid produced from xanthine in the reaction mixture by HPLC. The reaction medium contained 50 μM xanthine, 12.5 mM potassium phosphate buffer, pH 7.8, 10 μl of ethanol containing a flavonoid and 0.1 mU XO in a total volume of 0.2 ml. The final concentration of the flavonoid in the reaction medium was less than 100 μM. The reaction was started by adding XO after preincubating at 37°C for 5 min and was conducted at 37°C for 10 min, while control incubation was done without XO. The reaction was stopped by adding 25 μl of 3.2% perchloric acid, and then 25 μl of 0.4 M potassium phosphate buffer, pH 7.8 and 250 μl of acetonitrile were added. The mixture (100 μl) was subjected to an HPLC analysis which was performed with a Hewlett-Packard 1100 chromatographic system equipped with a G1311A quaternary pump, a G1314A variable-wavelength detector, and a G1329A autosampler (Yokogawa Analytical Systems, Tokyo, Japan). Uric acid was separated on a Lichrosphere 100 NH_2 normal-phase column of 4×250 mm, which was attached to a guard column of 4×4 mm (E. Merck, Darmstadt, Germany), with acetonitrile/20 mM KH_2PO_4 (1:1, v/v) as the mobile phase at a flow rate of 1.0 ml/min and monitored at 290 nm. Uric acid eluted at 3.9 min was quantified from its peak area by comparing with that of an external standard. The detection limit for uric acid was 8 pmol of uric acid/200 μl of reaction mixture (S/N=5). Under the standard assay conditions, urate formation was proportional to both the incubation time (up to 15 min) and amount of the enzyme (up to 0.15 mU/200 μl of the reaction medium).

XO activity has usually been determined by following the rate of urate formation from xanthine spectrophotometrically at 295 nm. Xanthine itself has UV absorption at around 260 nm, and some flavonoids also have strong absorption from the UV to visible region. Such absorption not only enhances the background absorption but also sometimes disturbs an accurate spectrophotometric determination of urate formation. We determined XO activity by analyzing the amount of uric acid separating on a HPLC column. This method could eliminate the disturbance by other UV-absorbing com-

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Abbreviation: XO, xanthine oxidase

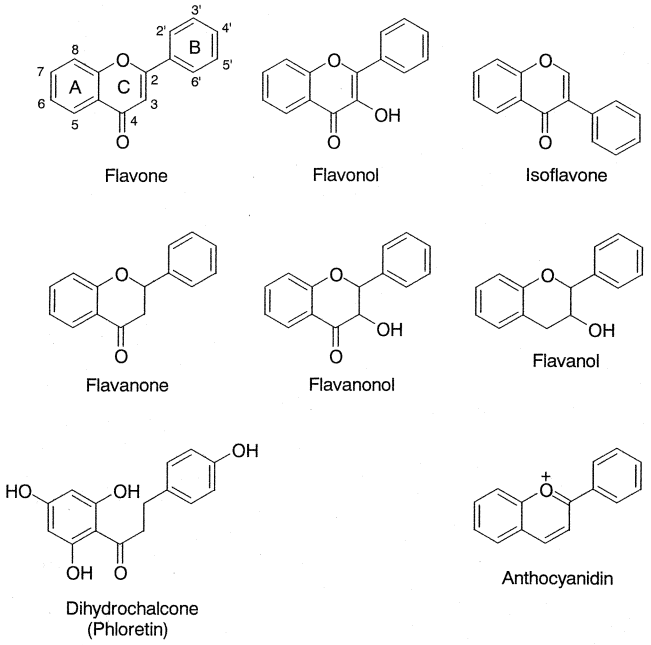

Fig. 1. Basic Structure of Flavonoids Evaluated for Their Inhibition of Xanthine Oxidase.

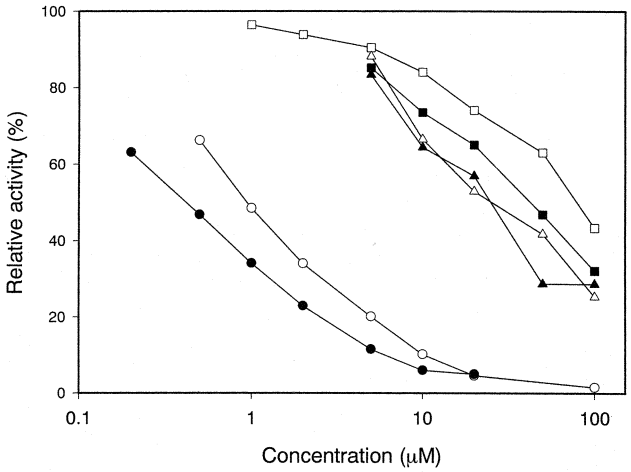

Fig. 2. Effect of Flavonoid Concentration on Xanthine Oxidase Activity.

● quercetin; ○ luteolin; ▲ hesperetin; △ cyanidin; ■ (−)-epigallocatechin gallate; □ genistein.

pounds present in the reaction medium. This method is therefore useful to evaluate the inhibitory effect on XO activity of various compounds and extracts from biological tissues.

Some flavonoids inhibited XO in a concentration-dependent manner as shown in Figure 2, and the concentration of flavonoid required to give 50% inhibition (IC_{50}) was evaluated (Table 1). Chrysin and luteolin with hydroxyl groups at C-5 and C-7 of the flavone skeleton strongly inhibited XO activity, but not flavone itself. Quercetin, a 3-hydroxy derivative of luteolin, was as inhibitory as luteolin. These results indicate that a hydroxyl group at C-5 and/or C-7 was essential for the inhibition of XO activity, but not a hydroxyl group at C-3 of

Table 1. Inhibition of Xanthine Oxidase by Various Flavonoids

Class	Compound	Substituent	IC_{50} value (μM)	K_i value (μM)
Flavone	Chrysin	5,7-OH	5.02	
	Luteolin	5,7,3′,4′-OH	0.96	0.31
	Flavone		No inhibition	
Flavonol	Kaempferol	3,5,7,4′-OH	0.67	0.23
	Quercetin	3,5,7,3′,4′-OH	0.44	0.28
	Myricetin	3,5,7,3′,4′,5′-OH	1.27	0.75
	Rhamnetin	3,5,3′,4′-OH; 7-OMe	>50	
	Isorhamnetin	3,5,7,3′-OH; 4′-OMe	0.40	0.17
	Tangeretin	3,5-OH; 6,7,8-OMe	>100	
	Rutin	5,7,3′,4′-OH; 3-*O*-rutinosyl	46.8	
Isoflavone	Genistein	5,7,4′-OH	83.0	
	Daidzein	7,4′-OH	>100	
Flavanone	Hesperetin	5,7,3′-OH; 4′-OMe	27.4	
Flavanonol	Taxifolin	3,5,7,3′,4′-OH	>100	
Flavanol	(+)-Catechin	3,5,7,3′,4′-OH	No inhibition	
	(−)-Epicatechin	3,5,7,3′,4′-OH	No inhibition	
	(−)-Epigallocatechin	3,5,7,3′,4′,5′-OH	>100	
	(−)-Epicatechin gallate	5,7,3′,4′-OH; 3-*O*-galloyl	48.5	
	(−)-Epigallocatechin gallate	5,7,3′,4′,5′-OH; 3-*O*-galloyl	44.7	
Dihydrochalcone	Phloretin	*	0.66	0.24
Anthocyanidin	Apigenidin	5,7,4′-OH	29.1	
	Pelargonidin	3,5,7,4′-OH	21.9	
	Cyanidin	3,5,7,3′,4′-OH	27.8	
	Peonidin	3,5,7,4′-OH; 3′-OMe	26.0	
	Delphinidin	3,5,7,3′,4′,5′-OH	52.4	

* The substitents of phloretin are shown in Figure 1.

the flavone structure. Rhamnetin, a 7-methoxy derivative of quercetin, showed much lower inhibitory effect than quercetin. Therefore, the C-7 position in flavones and flavonols was evidently involved in the inhibitory effect on XO activity, while the dependence of inhibition on the C-5 position was not clear from the data of the flavonoids that were evaluated. These results are consistent with the previous finding that 7-hydroxy-flavones among 24 hydroxy-flavones tested could significantly inhibit XO activity.[12] Similarly, baicalein (5,6,7-trihydroxyflavone) was found to be as inhibitory as quercetin.[13] The effects of substituents in the B ring of the flavone structure on the inhibitory activity against XO are not clear. Luteolin, a 3′,4′-dihydroxy derivatives of chrysin, showed five-fold lower IC_{50} value than chrysin. On the other hand, quercetin, with hydroxyl groups at C-3′ and C-4′ of the B ring, showed slightly lower IC_{50} value than kaempferol (3′-OH) or myricetin (3′,4′,5′-OH). Isorhamnetin, a 4′-methoxy derivative of quercetin, was as inhibitory as quercetin. Therefore, the catechol structure of the B ring, which gives antioxidative potential to flavonoids, was not related to XO inhibition.

Genistein and daidzein with an isoflavone structure were less inhibitory, although they had a hydroxyl group at C-7. Taxifolin and hesperetin, which are 2,3-dihydro derivatives of quercetin and isorhamnetin, respectively, were far less inhibitory. These results indicate that a planar flavone structure rather than a nonplanar flavanone structure was essential to the inhibition of XO activity. This is also confirmed by the facts that such flavanols as (−)-catechin and (−)-epicatechin did not inhibit XO activity. A planar structure due to conjugation over three rings in the flavone skeleton might be required for strong inhibition of XO activity. However, nonplanar phloretin without ring C inhibited XO at a low concentration ($IC_{50}=0.66\,\mu M$). Phloretin might have a similar conformation to the structure of kaempferol when binding to XO. These different inhibitory effects between planar and nonplanar flavonoids have also been observed in cyclooxygenase inhibition.[14] (−)-Epicatechin gallate and (−)-epigallocatechin gallate, which have a galloyl group, were moderately inhibitory. The anthocyanidins tested moderately inhibited XO activity, although they have a 7-hydroxyl group and planar structure. The flavylium cation might have reduced the inhibitory effect.

Like the results shown in this study, Chang *et al.* also found no inhibitory effect of (−)-catechin. On the other hand, Aucamp *et al.*[15] have reported K_i values of 304 and 21 μM for (−)-catechin and (−)-epicatechin, respectively, under their assay conditions for XO activity, where catechins and XO were preincubated before the enzyme reaction. They also found much lower K_i values of 11, 2.9, and 0.76 μM for (−)-epigallocatechin, (−)-epicatechin gallate and (−)-epigallocatechin gallate, respectively, than those obtained in this study (Table 1). In our works as well as in Chang's report, the enzyme reaction was started by adding XO. Therefore, catechins might inactivate XO in a time-dependent manner during preincubation.

The K_i values for the flavonoids with strong inhibitory effects ranged from 0.17 to 0.75 μM as shown in Table 1, with kaempferol and luteolin, as well as the other compounds tested, showing mixed-type inhibition (Figure 3). Different types of inhibition by flavonoids have been reported in the previous studies. Chang *et al.*[13] reported uncompetitive inhibition by baicalein ($K_i=2.48\,\mu M$) and competitive inhibition by quercetin ($K_i=0.13\,\mu M$). Cotelle *et al.*[12] reported noncompetitive inhibition by 3′,4′,5′-trihydroxyflavone, and competitive inhibition by luteolin and two 7-hydroxyflavones, and suggested that 7-OH of flavones took the place of 2 or 6-OH of xanthine in the active site of XO. However, Aucamp *et al.*[15] reported mixed-type inhibition by quercetin ($K_i=0.25\,\mu M$) and kaempferol ($K_i=0.33\,\mu M$) which is consistent with our results. The results suggest that these flavonoids inhibit XO activity not only by competitive mode, but also by interaction with the enzyme at a site other than the active center. Further detailed work is needed to clarify inhibition mechanism by flavonoids.

Significant *in vitro* inhibition of XO by the flavonoids at 10^{-7} M suggests that they may suppress *in vivo* the

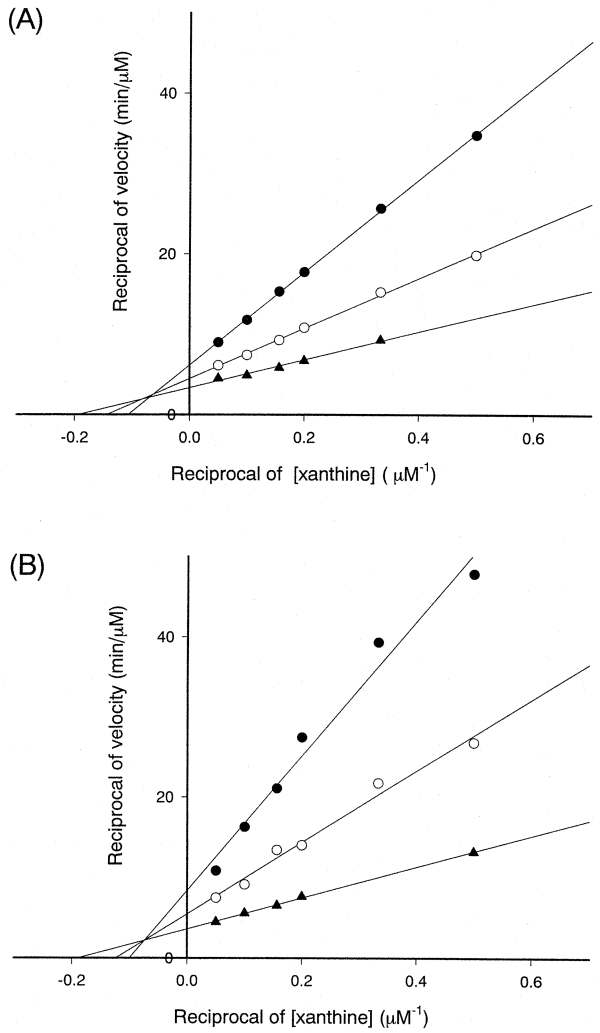

Fig. 3. Lineweaver-Burk Plots for the Inhibition of Xanthine Oxidase by Kaempferol and Luteolin.

(A) Inhibition by quercetin: ▲ control; ○ 0.2 μM; ● 0.5 μM (B) Inhibition by luteolin: ▲ control; ○ 0.4 μM; ● 1.0 μM.

production of active oxygen species under the conditions, at which XO works. Moreover, their IC_{50} values being comparable to that of allopurinol (0.77 μM), a therapeutic drug for treating gout, indicate a possible function of dietary flavonoids in preventing gout. However, recent studies have shown that most quercetin present in human plasma was found in the form of metabolites.[16,17] Therefore, the data obtained in this study cannot be directly used to evaluate the *in vivo* effect of dietary flavonoids on XO, and further determination of the inhibitory effects by individual flavonoid metabolites will be required.

In this study, a wide range of dietary flavonoids was evaluated for the inhibition of XO activity. The planar flavones and flavonols with a 7-hydroxyl group had a highly inhibitory effect on xanthine oxidase. The detailed mechanism for inhibition and the *in vivo* effect of these flavonoids on oxidative injury and urate accumulation by XO remain to be studied to clarify the roles of dietary flavonoids in human health.

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