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Effects of naturally occurring prenylated flavonoids on enzymes metabolizing arachidonic acid: Cyclooxygenases and lipoxygenases

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Abstract

Prenylated flavonoids are chemical entities having an isoprenyl, a geranyl, a 1,1-dimethylallyl, and/or a lavandulyl moiety as part of their flavonoid backbone structure. In this study, the effects of 19 naturally occurring prenylated flavonoids, isolated from medicinal plants, on cyclooxygenase (COX)-1 and COX-2 and on 5-lipoxygenase (5-LOX) and 12-LOX were investigated using [^{14}C]arachidonic acid as a substrate. The homogenates of bovine platelets and polymorphonuclear leukocytes were used as COX-1, 12-LOX, and 5-LOX enzyme sources; the homogenate of aspirin-pretreated lipopolysaccharide-induced RAW 264.7 cells was used for the COX-2 enzyme source. Among the 19 prenylated flavonoids, morusin, kuwanon C, sanggenon B, sanggenon D and kazinol B inhibited COX-2 activity (IC_{50} = 73–100 μM), but the potencies were far less than that of NS-398 (IC_{50} = 2.9 μM). In contrast, many prenylated flavonoids, such as kuraridin, kuwanon C and sophoraisoflavanone A, inhibited COX-1 activity. Of the COX-1 inhibiting prenylated flavonoids, kuraridin, kurarinone, and sophoraflavanone G, all having a C-8 lavandulyl moiety, showed potent activity (IC_{50} = 0.1 to 1 μM) comparable to that of indomethacin (IC_{50} = 0.7 μM). Most of the prenylated flavonoids tested inhibited 5-LOX activity with IC_{50} values ranging from 0.09 to 100 μM. Of these, only kuwanon C, papyriflavonol A and sophoraflavanone G showed inhibitory activity against 12-LOX at low concentration ranges (IC_{50} = 19–69 μM) comparable to that of NDGA (IC_{50} = 2.6 μM). Our results suggest that the position and the nature of the prenyl substitution greatly influence *in vitro* biological activities of these molecules.

Keywords: Flavonoid; Prenylated flavonoid; Sophoraflavanone; Sanggenon; Cyclooxygenases; Lipoxygenases; Anti-inflammatory activity

1. Introduction

Flavonoids, naturally occurring plant products, when consumed medicinally have mild if any side-effects and are, therefore, referred to as "tender drugs." They demonstrate various pharmacological activities *in vitro* and *in vivo* including anticancer, anti-inflammatory, and antiallergic activities. Among these actions, the anti-inflammatory activity of flavonoids may be mediated by the inhibition of the AA-metabolizing enzymes, COX/LOX, as well as by their antioxidative properties.

Various flavonoids modulate the activities of these eicosanoid-generating enzymes. It was demonstrated previously that several flavonoids including luteolin, galangin and epicatechin at relatively high concentrations inhibit COX and LOX [1]. Some chalcones having a 3,4-dihydroxyl substitution inhibit COX and 12-LOX from mouse epidermis at concentrations of 0.1 to 10 μM [2]. And several flavones including chrysin, 3-hydroxyflavone and galangin have been reported to be COX inhibitors as determined by measuring TXB_2 formation from A-23187-stimulated leukocytes [3].

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Abbreviations: AA, arachidonic acid; COX, cyclooxygenase; LOX, lipoxygenase; PG, prostaglandin; TX, thromboxane; HETE, hydroxyeicosatetraenoic acid; NDGA, nordihydroguaiaretic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; LPS, lipopolysaccharide; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; and PMN, polymorphonuclear leukocyte.

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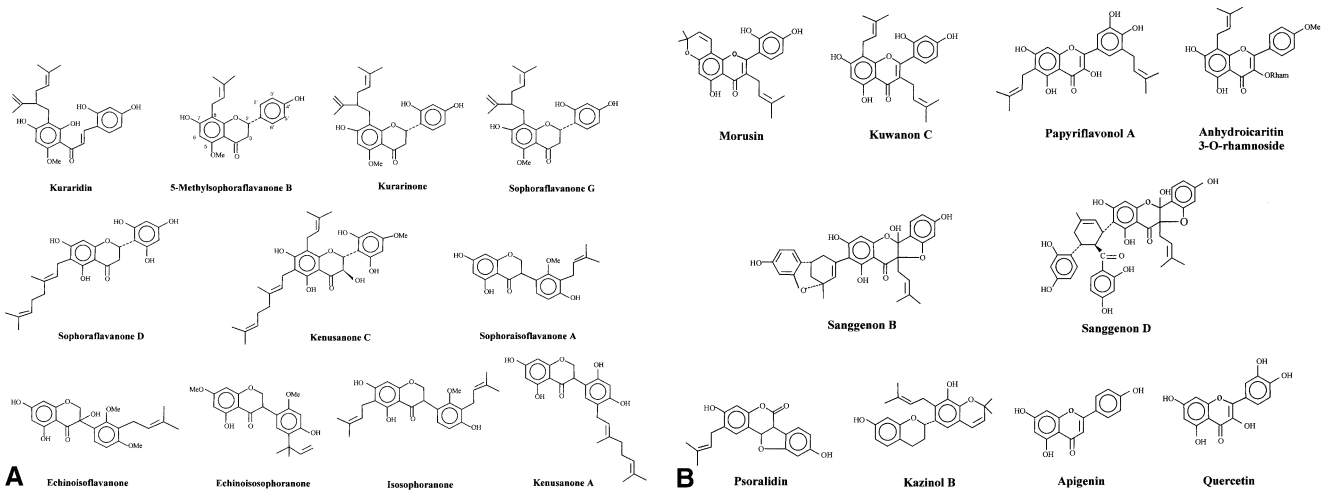

Fig. 1. Chemical structures of the flavonoids and prenylated flavonoids used in this study.

Recently, amentoflavone (biflavone) was found to be a selective inhibitor of COX-1 from guinea pig epidermis, comparable to indomethacin [4]. Many flavonoids have been found to be LOX inhibitors. For instance, flavonols such as kaempferol, fisetin, morin, and quercetin are 5-LOX inhibitors [5]. It has also been shown that in terms of their IC_{50} values most flavonol derivatives were stronger inhibitors of 12-LOX than of COX-1 as measured in sheep platelet homogenates [6]. All of these previous investigations suggest that flavones are relatively selective inhibitors of COX-1, while flavonols are stronger inhibitors of LOX. There have been only a few reports studying the effects of flavonoids on COX-2, an inducible isoform of COX. It was demonstrated recently that flavan-3-ols such as (+)-catechin, 4′-*O*-methyl-ent-gallocatechin and mearnsitrin weakly inhibit COX-2 at pharmacologically unobtainable concentrations (>100 μM); no selectivity over COX-1 was observed [7,8].

Prenylated flavonoids have limited distribution in the plant kingdom, i.e. the Moraceae family. Most of these plants have been used as anti-inflammatory agents in Chinese medicine, and their major constituents are various types of prenylated flavonoids [9]. These molecules are chemical entities having an isoprenyl (3,3-dimethylallyl), a geranyl (E-3,7-dimethyl-2,6-octadienyl), a 1,1-dimethylallyl, and/or a lavandulyl (5-methyl-2-isoprophenyl-hex-4-enyl) moiety as part of their flavonoid backbone structure. Hence, they usually are more hydrophobic than the conventional flavonoids, suggesting easy penetration through the cell membrane and skin barrier when used topically [10]. Thus, they may have advantages for topical use as anti-inflammatory agents if the prenylated ones have inhibitory activities on AA-metabolizing enzymes. It was reported previously that several prenylated flavonoids such as morusin, kuwanon C, kuwanon G, kuwanon H and sanggenon C inhibit COX-1 at a relatively high concentration [11,12]. Sanggenon D was found to inhibit both COX-1 and 12-LOX. It was also demonstrated that morusin and artonin E are potent inhibitors of purified 5-, 12-, and 15-LOXs at micromolar concentrations, having the greatest potency on 5-LOX [13]. However, these previous studies did not extensively investigate the various chemically different groups of prenylated flavonoids, i.e. sophoraflavanones, kenusanones, and echinoisoflavanones. Moreover, there has been no report describing the effects of prenylated flavonoids on COX-2 activity. Therefore, in the present investigation, the effects of 19 chemically different groups of prenylated flavonoids on COX-1, COX-2, 5-LOX, and 12-LOX activities were studied and compared with the conventional flavonoids.

2. Materials and methods

2.1. Chemicals

[1-^{14}C]AA (54.6 mCi/mmol) was obtained from NEN. NS-398 (*N*-[2-cyclohexyloxy-4-nitrophenyl]methane sulfonamide) was obtained from Biomol. MTT, LPS (*Escherichia coli* 0127:B8), aspirin, indomethacin, and NDGA were purchased from the Sigma Chemical Co. DMEM and other cell culture reagents including FBS were products of Gibco BRL. TLC plates (20 × 20 cm) were products of the Merck Co. PGE_2, $PGF_{2\alpha}$, TXB_2, and 5-, 12-, and 15-HETE were the products of Cayman Chem. Apigenin and quercetin were purchased from the Aldrich Chemical Co.

2.2. Isolation of prenylated flavonoids from medicinal plants

Kuraridin, kurarinone, 5-methylsophoraflavanone B, and sophoraflavanone G (Fig. 1) were isolated from the roots of *Sophora flavescens* Ait. (Fabaceae) and structurally identified as previously described [14]. Kenusanone A and C, isosophoranone, sophoraflavanone D, sophoraisoflavanone A, echinoisoflavanone and echinoisosophoranone were isolated from the roots of *Echinosophora koreensis* Nakai

(Fabaceae) and identified according to previously published articles [15–18]. Psoralidin and β-anhydroicaritin 3-*O*-rhamnoside were isolated from the seeds of *Psoralea corylifolia* L. (Fabaceae) and the roots of *Epimedium koreanum* Nakai (Berberidaceae), respectively [19,20]. Morusin and kuwanon C were isolated from the root barks of *Morus alba* L. (Moraceae), and sanggenon B and D were obtained from the root barks of a commercial crude drug of *Morus mongolica* Schneider (Moraceae). Kazinol B and papyriflavonol A were isolated from the root barks of *Broussnetia papyriferra* (L.) Vent. (Moraceae) [21,22]. The purity of the isolated compounds was > 95% (w/w).

2.3. Preparation of platelets and PMN homogenates from bovine blood

Bovine blood was obtained from a local slaughterhouse; it was withdrawn with 3.8% sodium citrate (10%, v/v) and centrifuged at 400 *g* for 10 min at room temperature to obtain platelet-rich plasma (PRP). PRP was centrifuged again at 1000 *g* for 10 min at room temperature, and the precipitated platelets were washed twice with 25 mM Tris–HCl buffer containing 1 mM EDTA (pH 7.4). The platelet homogenate was obtained by sonication for 3 sec three times on ice. From bovine blood, PMNs were separated by a double-density gradient method using Histopaque (1077 and 1119, Sigma) according to the recommendation of the manufacturer. After determination of protein concentration with a Bio-Rad protein assay kit, platelet homogenates were used without further purification as the source of COX-1/12-LOX. PMN homogenates were used as the source of 5-LOX.

2.4. COX-1 assay

The experimental procedure for measuring COX-1 activity was essentially the same as that described previously [4]. In brief, the incubation mixture consisted of 0.01 μCi [^{14}C]AA and 100 μg protein of platelet homogenate in 100 mM Tris–HCl buffer, pH 8.0, with 5 mM EDTA, 2 mM reduced glutathione, 50 mM l-tryptophan, and 2 μM hemoglobin with or without the test compounds. Reference compounds or flavonoid derivatives were dissolved in DMSO and diluted to appropriate concentrations with the above buffer solution on the day of the experiment. The same amount of DMSO was added in the control tube. The total incubation mixture was 100 μL/tube, in which the final concentration of DMSO was always 0.1% (v/v). The mixture was incubated at 37° for 20 min, and the reaction was terminated with ice-cold 0.15 N HCl (50 μL). Chloroform: methanol (2:1; 900 μL) was added immediately, and the products were extracted by vigorous vortexing. The organic layer obtained was evaporated with N_2. COX products were separated twice with TLC using ethyl acetate:acetic acid (99:1) as the mobile phase, and the TLC plate was autoradiographed for 7 days. The radioactive spots that comigrated with authentic standard TXB_2 were scraped out and counted in LSC (Pharmacia 1209). In several experiments, the extracted and dried metabolites obtained from the COX reaction were analyzed by HPLC (Shimadzu LC-10AT). The radioactive peaks were detected with an on-line radioactivity flow detector (Packard 150TR) as previously described [23]. HPLC was performed in an ODS-II reverse phase column (15 cm, Shinwa Chem.) at a flow rate of 1 mL/min using gradient elution (A:B, 94:6 → 0:100) for 50 min (solvent A: 26% acetonitrile, 10% methanol, and 0.02% acetic acid in water; solvent B: 0.05% acetic acid in acetonitrile).

2.5. COX-2 assay

RAW 264.7 cells obtained from the American Type Culture Collection were cultured in DMEM supplemented with 10% FBS and 1% antibiotics under 5% CO_2 at 37° [24]. Briefly, cells were plated in a 150-mm tissue culture dish. To inactivate COX-1, cells were pretreated with aspirin (250 μM) for 1.5 hr according to a previously described procedure [23]. After washing the cells three times with serum-free DMEM, LPS (1 μg/mL) was added to induce COX-2. Twenty hours later, the cells were scraped and homogenized in 20 mM Tris–HCl buffer, pH 7.5, containing 2 mM EDTA, 0.5 mM EGTA, 330 mM sucrose, and 2 mM phenylmethylsulfonyl fluoride. After centrifugation at 15,000 *g* for 30 min at 4°, the supernatant obtained was used for the COX-2 enzyme source. The procedure for measuring COX-2 activity was the same as the procedure for the COX-1 assay described above, except that 20 μg protein/tube was used. Under this assay condition, PGE_2 and PGD_2 were the major metabolites, with the amount of PGE_2 being greater than that of PGD_2. Thus, the percent inhibition of COX-2 activity was calculated based on the PGE_2 concentration, and the HPLC separation procedure was the same as that described for the COX-1 assay.

2.6. 5- and 12-LOX assays

For measuring 12-LOX activity, the standard assay system consisted of platelet homogenate (20 μg protein/tube) and 0.01 μCi [^{14}C]AA in 100 mM Tris–HCl buffer, pH 7.4, containing 1 mM EDTA and 2 mM reduced glutathione with or without the test compound [4]. For the 5-LOX assay, PMN homogenate (40 μg protein/tube) in 100 mM Tris–HCl buffer, pH 7.4, with 2 mM ATP and 2 mM $CaCl_2$ was used as described previously [25]. The incubation and extraction procedures were essentially the same as used for the COX-1 assay, except for an incubation time of 30 min. LOX products were separated by TLC using petroleum ether:diethyl ether:acetic acid (50:50:1) as a mobile phase. After autoradiography, the spots corresponding to 12-HETE or 5-HETE were scraped out, and radioactivity was counted.

Table 1
IC_{50} values of the prenylated flavonoids on COXs and LOXs

Compounds	IC_{50} (μM)			
	COX-1	COX-2	5-LOX	12-LOX
Chalcone				
Kuraridin	0.6–1[a]	-[b]	5.4–6.9[a]	>100[c]
Flavanones				
5-Methylsophoraflavanone B	>100	-	>100	>100
Kurarinone	0.6–1[a]	-	22	>100
Sophoraflavanone G	0.1–0.6[a]	-	0.09–0.25[a]	20
Sophoraflavanone D	-	-	>100	>100
Kenusanone C	>100	-	-	100
Isoflavanones				
Sophoraisoflavanone A	5–7.6[a]	-	-	-
Echinoisoflavanone	>100	-	-	-
Echinoisosophoranone	-	-	19	-
Isosophoranone	-	-	>100	-
Kenusanone A	-	-	0.5–0.9[a]	>100
Flavones and flavonols				
Morusin	>100	100	>100	>100
Kuwanon C	62	>100	12	19
Papyriflavonol A	-	-	7	69
β-Anhydroicaritin 3-*O*-rhamnoside	>100	-	>100	-
Miscellaneous				
Sanggenon B	42	>100	6	-
Sanggenon D	59	73	4	100
Psoralidin	23	-	3.6–8.8[a]	>100
Kazinol B	>100	>100	71	-
References				
Apigenin (flavone)	-	-	-	-
Quercetin (flavonol)	8	76	0.8	12
Indomethacin	0.4–1.3[d]	1.5–5.0[d]	[e]	
NS-398	-	1.9–5.6[d]		
NDGA			0.6–0.9[d]	2.6

[a] Ranges of IC_{50} values from three separate experiments of a concentration-dependent study.
[b] No or less than 20% inhibition up to 100 μM.
[c] Less than 40% inhibition was observed at the tested concentration of 100 μM.
[d] Ranges of IC_{50} values from ten separate experiments of a concentration-dependent study.
[e] Blank space indicates that no experiment was performed.

3. Results

For testing the effects of prenylated flavonoids on COX-1 and on 5- and 12-LOX, the homogenates of bovine platelets and PMNs were used as the enzyme sources. For the COX-2 source, the cell homogenate of aspirin-pretreated LPS-induced RAW 264.7 macrophages was used. Under each assay condition, indomethacin (a COX-1/COX-2 inhibitor) potently inhibited the formation of TXB_2 and PGE_2, while NDGA (an LOX inhibitor) inhibited the formation of 12-HETE and 5-HETE, its effect being more potent on 5-HETE formation (Table 1). NS-398 (COX-2 inhibitor) potently inhibited COX-2 without affecting COX-1 up to 100 μM. To establish the inhibitory activities of flavonoids, each flavonoid was initially tested at 10 and 100 μM. The flavonoids showing more than 40% inhibition at 100 μM were tested again at 0.1 to 100 μM to obtain their IC_{50} values. The potency of inhibition against COX-1 was as follows: sophoraflavanone G > indomethacin > kuraridin = kurarinone > sophoraisoflavanone A > quercetin (Table 1 and Fig. 2a). Against COX-2, only morusin, kuwanon C, sanggenon B, sanggenon D and kazinol B showed inhibition, although it was not potent. The order of inhibition was NS-398 $\gg$ sanggenon D > quercetin > morusin > kuwanon C = sanggenon B = kazinol B (Table 1 and Fig. 2b). The inhibitory activities of sophoraflavanone G and sanggenon D against COX-1 and COX-2, respectively, were also confirmed by HPLC analysis (Fig. 3). For the 5-LOX reaction, most prenylated flavonoids were found to be active. The order of inhibition was: sophoraflavanone G > kenusanone A = NDGA > quercetin > psoralidin > sanggenon D (Table 1 and Fig. 2c). The prenylated flavonoids tested were generally less active inhibitors of 12-LOX than of 5-LOX. None exceeded the inhibitory potency of NDGA. And the order of NDGA $\gg$ quercetin > kuwanon C > sophoraflavanone G > papyriflavonol A > kenusanone C = sanggenon D was found (Table 1 and Fig. 2d). From all of the above results, it was revealed that sophoraisoflavanone A was a selective inhibitor of COX-1, whereas papyriflavonol A and kenusanone A were selective inhibitors of 5-LOX and 12-LOX without affecting COX-1/COX-2 activity up to 100 μM. Echinoisosophoranone and isosophoranone were selective inhibitors of 5-LOX. In contrast, morusin, kuwanon C, and sanggenon D were inhibitors of COX-1, COX-2, 5-LOX, and 12-LOX with varying degrees of potency, although they were far less active than the reference compounds (indomethacin, NS-398, and NDGA). It is interesting to note that kuraridin and kurarinone were potent and selective inhibitors against COX-1 and 5-LOX. Sophoraflavanone G was found to be the most potent inhibitor against COX-1 and 5-LOX among the flavonoids tested in this study. The potency of sophoraflavanone G was similar or even higher than those of the reference compounds, indomethacin and NDGA. Quercetin, used as a reference flavonoid, was found to be an inhibitor against COXs and LOXs, being more active on LOXs, while apigenin did not inhibit these enzymes significantly up to 100 μM.

4. Discussion

Many researchers have studied the effects of flavonoids on COXs/LOXs in the hope of finding an active ingredient in plant-derived drugs and finding lead compounds for pharmacological use. In this study, 19 prenylated flavonoid derivatives were isolated from several plant extracts and evaluated for their inhibitory activities on AA-metabolizing enzymes, including COX-2.

The prenylated flavonoids tested showed varying degrees of inhibition on COXs and LOXs depending upon their chemical structures. The structure–activity relationships

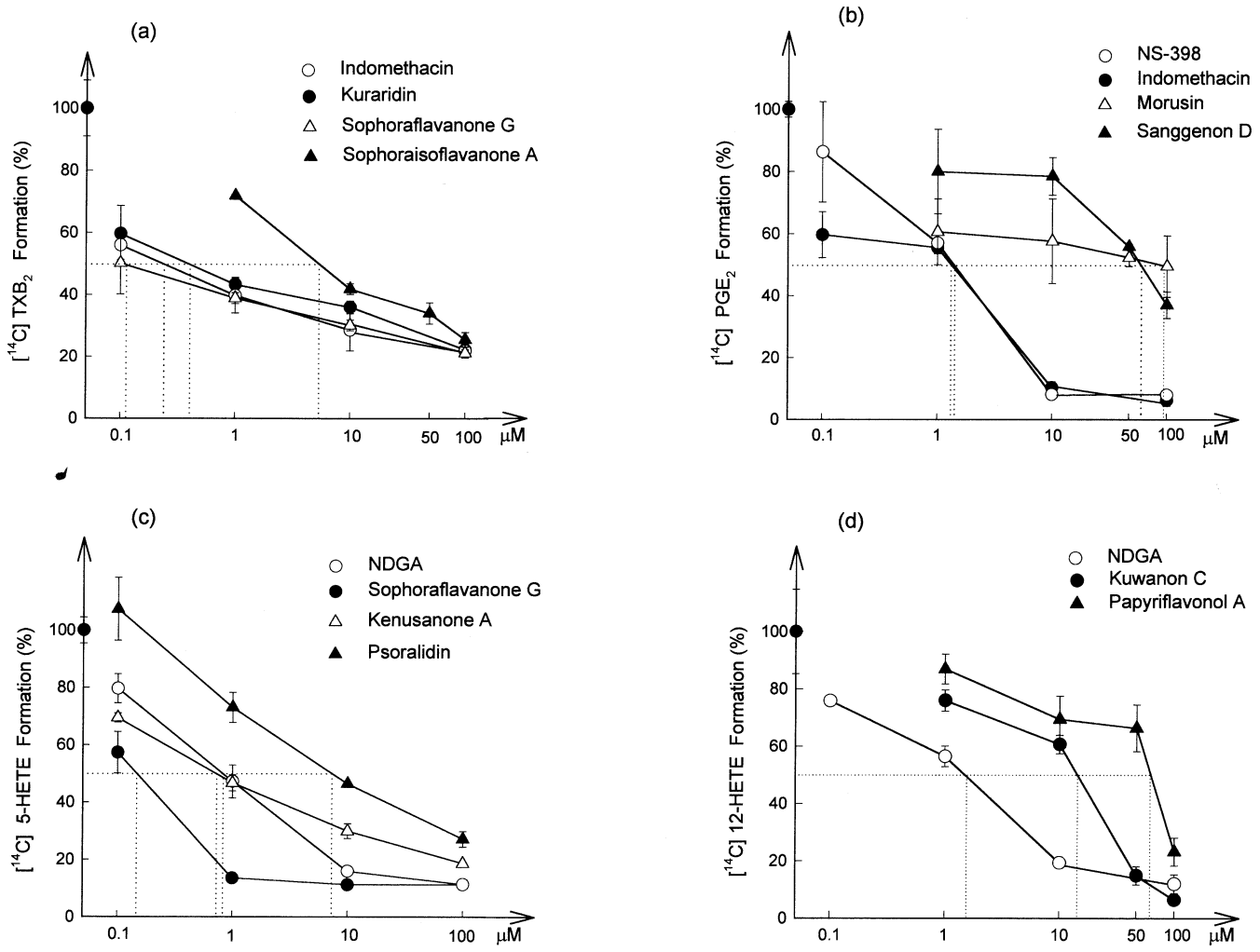

Fig. 2. Inhibition of eicosanoid-metabolizing enzyme activities by several selected prenylated flavonoids. COX-1 and -2, and 5- and 12-LOX reactions were carried out as described in "Materials and methods." These results represent one of several sets of experimental data (N = 3, means ± SD). (a) COX-1 reaction (867 ± 176 cpm of TXB_2/tube without inhibitor), (b) COX-2 reaction (1178 ± 48 cpm of PGE_2/tube without inhibitor), (c) 5-LOX reaction (1009 ± 46 cpm of 5-HETE without inhibitor), and (d) 12-LOX reaction (1423 ± 235 cpm of 12-HETE without inhibitor).

found were as follows. Many prenylated flavonoids, such as sophoraflavonoids and sanggenons, showed promising inhibitory activity on COX-1. The prenylated flavonoids having a C-8 substitution with a lavandulyl group at the A-ring were potent COX-1 inhibitors (kuraridin, kurarinone, and sophoraflavanone G), suggesting the importance of a lavandulyl residue at this position. The flavonoids having a C-8 isoprenyl (3,3-dimethylallyl) residue also inhibited COX-1, but were less active (5-methylsophoraflavanone B, morusin, kuwanon C, β-anhydroicaritin 3-*O*-rhamnoside). It was previously claimed that, in addition to the presence of an isoprenyl group at C-3, a 2′,4′-dihydroxyl group in the B-ring or a 2,4-dihydroxybenzoyl moiety might be essential for inhibiting COX-1 among the prenylated flavonoids tested (morusin, kuwanon C, G, H, and M, and sanggenon C and D) [10,11]. In accordance with these previous findings, the prenylated flavonoids having potent COX-1 inhibitory activity also possessed a 2′,4′-dihydroxyl group in the B-ring (kuraridin, kurarinone, and sophoraflavanone G). In contrast, most of the prenylated flavonoids did not inhibit COX-2. Among the 19 derivatives tested, morusin, kuwanon C, sanggenon B, sanggenon D, and kazinol B were found to be weak COX-2 inhibitors. The common structural moiety of the prenylated flavonoids inhibiting COX-2 is an isoprenyl (3,3-dimethylallyl) group attached to the C-3 position of the flavonoid C-ring, except for kazinol B. However, any selectivity for COX-2 over COX-1 was not observed. Only sanggenon D inhibited COX-1/COX-2 with similar potencies. With respect to the weak inhibitory activity toward COX-2, it is suggested that COX-2 inhibition may not be a major anti-inflammatory mechanism of prenylated flavonoids. In general, the inhibitory activity of prenylated flavonoids against 5-LOX was much stronger than against 12-LOX. A methoxyl group at the C-5 position seemed to reduce the inhibitory activity of 5-LOX (kuraridin and kurarinone vs sophoraflavanone G). Moreover, the introduction of an isoprenyl or a geranyl residue on the C-6 position of the flavonoid A ring completely abolished or

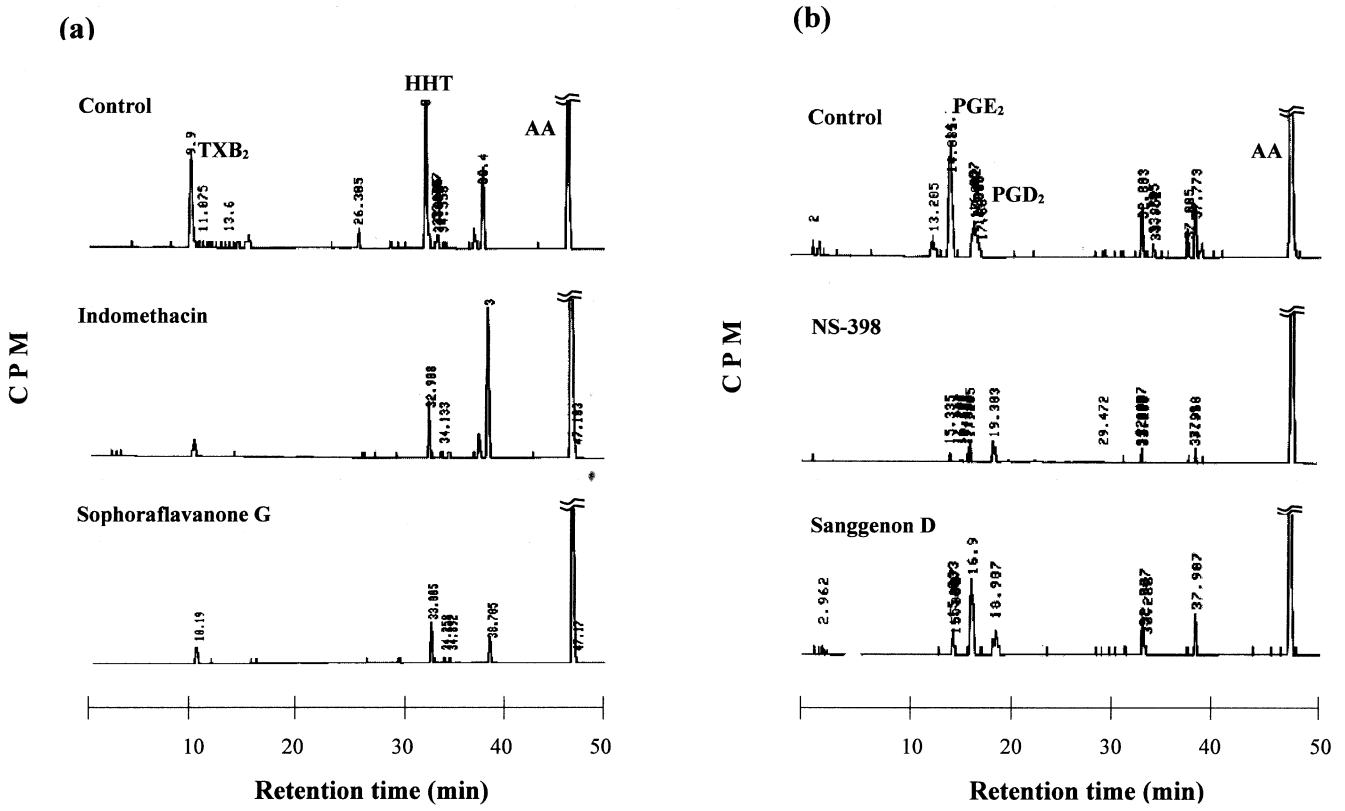

Fig. 3. HPLC analysis of the metabolites from COX-1 and COX-2 reactions. (a) Metabolite separation from COX-1 reaction. Selective inhibition of TXB_2 and HHT formation by indomethacin (10 μM) was noted, while sophoraflavanone G (10 μM) potently inhibited TXB_2 and HHT formation, and moderately inhibited 12-HETE formation at the tested concentration. (b) Metabolite separation from the COX-2 reaction. NS-398 (10 μM) potently inhibited the formation of PGE_2, whereas sanggenon D (10 μM) weakly inhibited the formation of PGE_2. In these reactions, a 10-fold amount of [^{14}C]AA and the enzyme homogenates were added to obtain the proper detection of radioactive metabolites.

greatly reduced COX inhibitory activity, but were usually LOX inhibitors (sophoraflavanone D, kenusanone C, isosophoranone, and papyriflavonol A). Indeed, introduction of an isoprenyl group at C-6 of sophoraisoflavanone A (isosophoranone) caused a loss of COX-1 inhibitory activity, and 5′,6-diisoprenyl quercetin (papyriflavonol A) did not inhibit COX-1. Similar findings that a methoxyl or an alkyl group substitution at C-6 or C-8 increased the inhibitory activity of LOX have been demonstrated previously [26]. There were some discrepancies between the experimental results of the present investigation and the findings of others. Morusin was reported previously to inhibit purified COX-1 and 5- and 12-LOX from porcine leukocytes at the 0.1 to 10 μM range [13], but morusin showed IC_{50} values of over 100 μM against COXs and LOXs in our study. And the IC_{50} value of quercetin against COX-1 from bovine platelet homogenate was 8 μM in this study, while the IC_{50} value of the same compound was 50 μM from the homogenate of guinea pig epidermis [4]. These discrepancies in experimental results may be explained, at least in part, by the differences in animal species and the purity of the enzymes used.

In summary, certain prenylated flavonoids such as morusin, papyriflavonol A, kuraridin and sophoraflavanones inhibited COX-1, COX-2, 5-LOX, and/or 12-LOX depending upon the position and the nature of the prenyl substitution. For the first time, morusin, kuwanon C, sanggenon B and D, and kazinol B were found to be weak COX-2 inhibitors. A C-8 isoprenyl or a lavandulyl group favors COX-1 inhibition, whereas a C-3 isoprenyl group favors a COX-2 reaction. Generally, the inhibitory activity of prenylated flavonoids against 5-LOX was much stronger than against 12-LOX. The present study revealed the importance of a prenyl group(s) attached to the flavonoid molecule on the inhibitory activities against COXs and LOXs. These inhibitory activities may contribute to the anti-inflammatory effect of medicinal plants having prenylated flavonoids as major constituents.

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