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Antiinflammatory Activity of Isoflavonoids from *Pueraria* Radix and Biochanin A Derivatives

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For comparing with flavones/flavonols, isoflavonoids isolated from *Pueraria* radix and chemically synthesized from biochanin A were evaluated for the antiinflammatory activity using mouse ear edema test. Isoflavonoids such as daidzein and puerarin showed the significant antiinflammatory activity at a dose of 2 mg/mouse, although their activity was generally less than that of flavones/flavonols. 7-O-Substitution of biochanin A was not favorable for the antiinflammatory activity.

Key words: Antiinflammation, Isoflavonoid, *Pueraria* radix, Biochanin A

INTRODUCTION

Flavonoids have been shown to possess the various biological activities including antibacterial, antiviral, anticancer, anti-inflammatory and immunoregulatory activities (Havsteen, 1983). Recently, research interests are focusing on the antiinflammatory activity of flavonoids because flavonoids are believed to show less side-effects compared to nonsteroidal acidic drugs (NSAIDs) and steroidal antiinflammatory drugs (SAIDs). Many investigators demonstrated that flavonoid derivatives including quercetin, luteoline and kaempferol showed the anti-inflammatory activity *in vivo* (Gábor, 1986; Lewis, 1989). And the structure-activity relationships of flavonoids commercially available and isolated from plants were established by us (Kim *et al.*, 1993; Lee *et al.*, 1993). Mainly flavones/flavonols were found to possess the antiinflammatory activity *in vivo* and a C-2,3-double bond might be important. However, the antiinflammatory activity of isoflavonoids remains to be elucidated. Gábor (1986) found that sophoricoside (isoflavone glycoside) possessed the antiinflammatory activity against carrageenan-induced rat paw edema. Huh *et al.* (1987) reported that daidzein showed the anti-inflammatory activity. Kim and Chung (1990) demonstrated the inhibitory effects of daidzein on type I-IV hypersensitivities in experimental animals. Previously, we found that biochanin A (isoflavone) showed the comparable antiinflammatory activity with quercetin (Kim *et al.*, 1993). Although all of these previous results indicated that the certain isoflavonoids may show the antiinflammatory activity, the structure-activity relationship of isoflavonoids was not established yet. Therefore, it is worthy of studying the antiinflammatory activity of isoflavonoids, especially, isoflavones having a C-2,3-double bond. In this study, the several isoflavonoids were isolated from *Pueraria* radix, and the 7-O-substituted biochanin A derivatives were chemically synthesized. These derivatives were evaluated for the antiinflammatory activity using mouse ear edema test and the structure-activity relationship was discussed.

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MATERIALS AND METHODS

Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. ^{1}H-NMR and ^{13}C-NMR spectra were obtained on a Varian 200 MHz NMR using TMS as an internal standard. The purity of the compounds was checked using TLC (Kieselgel 60 F254 glass plate, Merck). Quercetin and biochanin A were obtained from Aldrich Chem. Co. (USA). Arachidonic acid (AA), croton-oil, hydrocortisone and indomethacin were products of Sigma Chem. Co. (USA). The other reagents used were the highest purity chemicals available.

Isolation of Isoflavonoids from *Pueraria* Radix

Pueraria radix purchased from a local market was refluxed with methanol for 3 hrs three times. The dried methanol extract was partitioned between ether and

water. The dried ether fraction was subjected to SiO_2 column chromatography using chloroform : methanol (95 : 5) as a mobile phase and afforded daidzein. The combined eluents except the fractions containing daidzein were dried in vacuo, subjected to SiO_2 colomn chromatography again and eluted using hexane : ethyl acetate (7 : 3) to give formononetin, genistein and puerarol. The water fraction was further extracted with n-butanol. From the n-butanol fraction, SiO_2 colomn chromatography using chloroform : methanol : water (40 : 16 : 3) afforded three isoflavonoid glycosides ; daidzin, puerarin and PG-3.

Daidzein: Crystallized from chloroform as pale yellow platelets, m.p. >300℃, ^{1}H-NMR (DMSO-d_6) δ 8.28 (s, 1H, C-2), 7.95 (d, 1H, C-5), 7.38 (d, 2H, C-2′ and 6′), 6.92 (dd, 1H, C-6), 6.84 (d, 1H, C-8), 6.79 (d, 2H, C-3′ and 5′); ^{13}C-NMR: see Table I.

Genistein: Crystallized from chloroform as white amorphous powder, m.p. =282-3℃, ^{1}H-NMR (DMSO-d_6) δ 8.33 (s, 1H, C-2), 7.39 (d, 2H, C-2′ and 6′), 6.83 (dd, 2H, C-3′ and 5′), 6.37, 6.21 (2d, 2H, C-6 and 8).

Formononetin: Identified by direct comparison with an authentic sample kindly provided by Prof. Sam Sik Kang (Natural Products Institute, Seoul National Univ.).

Puerarol: Crystallized from chloroform as pale yellow platelets, m.p. =236-7℃, ^{1}H-NMR (DMSO-d_6) δ 9.90 (s, 1H, -OH), 9.29 (s, 1H, -OH), 7.77 (d, 1H, C-2′), 7.62 (s, 1H, C-5′), 6.99 (s, 1H, C-5), 6.93 (dd, 1H, C-3′), 5.93 (m, 1H, allyl), 5.14 (m, 1H, allyl), 3.38 (d, 2H, benzyl-CH_2), 2.11 (m, 4H, 2 allyl-CH_2), 1.72, 1.69, 1.61 (3s, 9H, 3-allyl-CH_3).

Puerarin: Crystallized from acetone as yellow needles, m.p. =193-4℃, ^{1}H-NMR (DMSO-d_6) δ 8.34 (s, 1H, C-2), 7.92 (d, 1H, C-5), 7.40 (dd, 2H, C-2′ and 6′), 6.95 (d, 1H, C-6), 6.81 (dd, 2H, C-3′ and 5′), 4.79 (d, 1H, Glc-C-1); ^{13}C-NMR: see Table I.

Daidzin: Crystallized from acetone as colorless needles, m.p. =205-6℃, ^{1}H-NMR (DMSO-d_6) δ 8.38 (s, 1H, C-2), 8.04 (d, 1H, C-5), 7.39 (dd, 2H, C-2′ and 6′), 7.22 (s, 1H, C-8), 7.13 (dd, 1H, C-6), 6.80 (d, 2H, C-3′ and 5′), 5.09 (d, 1H, Glc-C-1); ^{13}C-NMR: see Table I.

PG-3: Crystallized from acetone as colorless needles, m.p. =215-6℃, ^{1}H-NMR (DMSO-d_6) δ 8.41 (s, 1H, C-2), 7.94 (d, 1H, C-5), 7.19 (s, 1H, C-2′), 7.05 (d, 1H, C-6′), 6.98 (d, 1H, C-6), 6.83 (d, 1H, C-5′), 4.82 (d, 1H, Glc-C-1), 3.82 (s, 3H, -OCH_3); ^{13}C-NMR: see Table I.

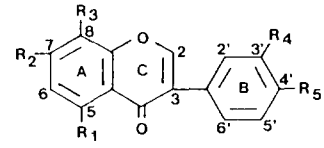
The chemical structures of the isoflavonoids used in this study were shown in Fig. 1.

Synthesis of Biochanin A Derivatives

Table I. ^{13}C-NMR spectral data of isoflavonoids from *Pueraria* radix (200 MHz, DMSO-d_6)

		Daidzein	Puerarin	Daidzin	PG-3
C	2	153.9	152.6 (152.8)	153.3 (153.2)	152.9 (153.0)
	3	124.6	122.9 (123.0)	123.9 (123.9)	122.8 (123.0)
	4	175.9	174.9 (175.0)	174.1 (174.0)	174.8 (175.0)
	5	128.4	126.1 (126.5)	126.9 (127.0)	126.2 (126.5)
	6	116.3	114.8 (115.2)	114.7 (115.0)	115.0 (115.2)
	7	163.8	161.2 (161.0)	161.4 (161.5)	161.0 (161.0)
	8	103.2	112.5 (112.8)	99.8 (100.0)	112.5 (112.8)
	9	158.6	156.1 (156.9)	157.3 (157.3)	156.0 (156.0)
	10	117.6	116.6 (117.0)	118.4 (118.5)	116.5 (117.0)
	1′	123.6	122.4 (122.5)	122.1 (122.3)	122.9 (123.1)
	2′	131.2	130.0 (130.0)	130.0 (130.1)	112.8 (113.0)
	3′	116.0	114.8 (115.1)	114.9 (115.5)	146.3 (146.8)
	4′	158.3	157.1 (157.0)	157.1 (157.0)	147.1 (147.2)
	5′	116.0	114.8 (115.1)	114.9 (115.5)	112.8 (113.0)
	6′	131.2	130.0 (130.0)	130.0 (130.2)	121.4 (121.8)
Glucose	1		78.6 (78.9)	103.3 (103.5)	78.6 (79.0)
	2		73.2 (73.5)	73.1 (73.2)	73.2 (73.8)
	3		70.5 (70.5)	76.3 (76.5)	70.5 (70.9)
	4		70.5 (70.5)	69.4 (69.6)	70.5 (70.9)
	5		81.7 (82.5)	77.1 (77.3)	81.8 (82.0)
	6		61.1 (61.5)	60.6 (60.7)	61.3 (61.9)
3′-OMe					55.4 (56.0)

Data in parenthesis are the results of Ohshima *et al.* (1988).



	R_1	R_2	R_3	R_4	R_5
Daidzein	H	OH	H	H	OH
Formononetin	H	OH	H	H	OMe
Puerarol*					
Genistein	OH	OH	H	H	OH
Puerarin	H	OH	-Glc	H	OH
Daidzin	H	O-Glc	H	H	OH
PG-3	H	OH	-Glc	OMe	OH
Biochanin A	OH	OH	H	H	OMe
Methylbiochanin A	OH	OMe	H	H	OMe
Dimethylbiochanin A	OMe	OMe	H	H	OMe
Ethylbiochanin A	OH	OEt	H	H	OMe
Isopropylbiochanin A	OH	O-iso-Pro	H	H	OMe
Isobutylbiochanin A	OH	O-iso-But	H	H	OMe
Allylbiochanin A	OH	O-Allyl	H	H	OMe

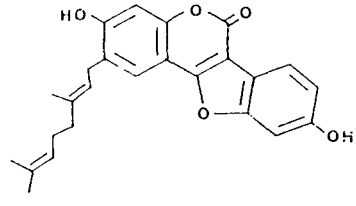


Fig. 1. Chemical structures of isoflavonoids used in this study

The 7-O-substituted biochanin A derivatives were synthesized from biochanin A and alkylbromide/DMF in the presence of K_2CO_3 as a catalyst, except 7-O-methyl and 5,7-O-dimethylbiochanin A. For 7-O-methylbiochanin A, methyl iodide was reacted with biochanin A on a 1 : 1 molar base. Excess methyl iodide treatment gave 5,7-O-dimethylbiochanin A.

7-O-Methylbiochanin A: 55% yield, m.p. =141℃, ^{1}H-NMR ($CDCl_3$) δ 12.87 (s, 1H, 5-OH), 7.87 (s, 1H, C-2), 7.46, 6.98 (2d, 4H, 3-aryl), 6.38-6.41 (m, 2H, C-6 and 8), 3.87, 3.85 (2s, 6H, 2-OCH_3).

7-O-Ethylbiochanin A: 70%, m.p. =161℃, ^{1}H-NMR ($CDCl_3$) δ 12.84 (s, 1H, 5-OH), 7.86 (s, 1H, C-2), 7.46, 6.98 (2d, 4H, 3-aryl), 6.38-6.39 (m, 2H, C-6 and 8), 4.11 (q, 2H, -$\underline{CH_2}$-CH_3), 3.84 (s, 3H, 4'-OCH_3), 1.53 (t, 3H, -CH_2-$\underline{CH_3}$).

7-O-Isopropylbiochanin A: 79%, m.p. =130-1℃, ^{1}H-NMR ($CDCl_3$) δ 12.83 (s, 1H, 5-OH), 7.85 (s, 1H, C-2), 7.46, 6.98 (2d, 4H, 3-aryl), 6.35-6.38 (m, 2H, C-6 and 8), 4.63 (q, 1H, -$\underline{CH}$=(CH_3)$_2$), 3.85 (s, 3H, 4'-OCH_3), 1.39 (d, 6H, -CH=($\underline{CH_3}$)$_2$).

7-O-Isobutylbiochanin A: 52%, m.p. =138-9℃, ^{1}H-NMR ($CDCl_3$) δ 12.83 (s, 1H, 5-OH), 7.86 (s, 1H, C-2), 7.47, 7.13 (2d, 4H, 3-aryl), 6.37-6.39 (m, 2H, C-6 and 8), 3.88 (s, 3H, 4'-OCH_3), 3.83 (d, 2H, -$\underline{CH_2}$-CH=), 2.10 (m, 1H, -CH=(CH_3)$_2$), 1.64 (d, 6H, -CH=($\underline{CH_3}$)$_2$).

7-O-Allylbiochanin A: 80%, m.p. =166-7℃, ^{1}H-NMR ($CDCl_3$) δ 12.85 (s, 1H, 5-OH), 7.87 (s, 1H, C-2), 7.46, 6.98 (2d, 4H, 3-aryl), 6.40-6.42 (m, 2H, C-6 and 8), 5.30-6.20 (m, 3H, -CH=CH_2), 4.61 (m, 2H, -O-CH_2-).

5,7-O-Dimethylbiochanin A: 85%, m.p. =161-162℃, ^{1}H-NMR ($CDCl_3$) δ 7.77 (s, 1H, C-2), 7.48, 6.93 (2d, 4H, 3-aryl), 6.38-6.44 (m, 2H, C-6 and 8), 3.94, 3.89, 3.83 (3s, 9H, 3 -O-CH_3).

Mouse Ear Edema Test

ICR mice (18-22 g) were maintained in our animal facility under the conditions of 22± 1℃, 12 hr/12 hr (L/D) cycle. Mice were feeding with pellet lab. chow and tap water *ad libitum*. The whole procedures of arachidonic acid (AA) and croton-oil induced ear edema tests were same as previously described by Kim *et al.* (1993). The test compounds were administered to each mouse 1 hr (oral) or 0.5 hr (topical to ear) prior to AA/croton-oil application. Student-t-test was used for comparing the statistical significance.

RESULTS AND DISCUSSION

The antiinflammatory activity of isoflavonoids was evaluated comparing with flavones/flavonols. Isoflavonoids used in this study were isolated from *Pueraria* radix and chemically synthesized from biochanin A. The chemical structures of the isolated isoflavonoids from *Pueraria* radix were chemically and analytically identified based on the previously described results of Shibata *et al.* (1959) and Ohshima *et al.* (1988). The ^{13}C-NMR spectra in Table I were in agreement with the previous results of Ohshima *et al.* (1988).

Table II. Croton-oil induced ear edema inhibition

Group	Thickness increased (mm)	% inhibition
Experiment 1		
Control	0.22± 0.02	–
Biochanin A	0.16± 0.01*	26
Daidzein	0.19± 0.01	13
Formononetin	0.21± 0.02	4
Puerarol	0.20± 0.01	9
Experiment 2		
Control	0.22± 0.01	–
Biochanin A	0.17± 0.01*	23
Daidzin	0.21± 0.02	4
Puerarin	0.19± 0.01*	14
PG-3	0.22± 0.01	–
Experiment 3		
Control	0.24± 0.01	–
Hydrocortisone	0.06± 0.02**	75
Quercetin	0.18± 0.02*	25
Biochanin A	0.18± 0.01*	25
Methylbiochanin A	0.22± 0.02	8
Dimethylbiochanin A	0.22± 0.03	8
Ethylbiochanin A	0.20± 0.02	16
Isopropylbiochanin A	0.19± 0.02*	20
Isobutylbiochanin A	0.26± 0.03	–
Allylbiochanin A	0.23± 0.03	4

All compounds in Experiment 1 and 3 were topically applied to ears of mice (2 mg/ear, n=8). All compounds in Experiment 2 were orally administered to mice (2 mg/mouse, n= 10). *: $P<0.01$, **: $P<0.001$, significantly different from control.

Table II represented the antiinflammatory activity of isoflavonoids from *Pueraria* radix and the synthesized biochanin A derivatives against croton-oil induced ear edema. At a dose of 2 mg/mouse, only puerarin showed a significant inhibition via oral administration among the isoflavones from *Pueraria* radix. For the synthesized biochanin A derivatives, only 7-O-isopropylbiochanin A showed a significant inhibition when topically administered. Biochanin A was found to be the most active isoflavonoid. No one among isoflavonoids tested was revealed to be more active than biochanin A. Table III demonstrated the antiinflammatory activity of isoflavonoids in AA-induced mouse ear edema test at a dose of 2 mg/mouse. Daidzein and puerarin were significantly active. Among the biochanin A derivatives, only 7-O-ethyl and 7-O-isopropylbiochanin A showed the comparable antiinflammatory activity with biochanin A. Comparing with the antiinflammatory activity

Table III. Arachidonic acid induced ear edema inhibition

Group	Thickness increased (mm)	% inhibition
Experiment 1		
Control	0.15± 0.02	–
Biochanin A	0.08± 0.01**	41
Daidzein	0.11± 0.03*	30
Formononetin	0.12± 0.03	20
Puerarol	0.15± 0.01	–
Experiment 2		
Control	0.15± 0.02	–
Biochanin A	0.11± 0.02*	27
Daidzin	0.13± 0.02	13
Puerarin	0.11± 0.02*	27
PG-3	0.13± 0.02	13
Experiment 3		
Control	0.16± 0.02	–
Indomethacin	0.04± 0.01**	76
Quercetin	0.09± 0.01**	45
Biochanin A	0.08± 0.02**	51
Methylbiochanin A	0.12± 0.03	26
Dimethylbiochanin A	0.13± 0.03	20
Ethylbiochanin A	0.08± 0.02**	52
Isopropylbiochanin A	0.07± 0.02**	54
Isobutylbiochanin A	0.15± 0.02	6
Allylbiochanin A	0.11± 0.06	29

All compounds in Experiment 1 and 3 were topically applied to ears of mice (2 mg/ear, n=8). All compounds in Experiment 2 were orally administered to mice (2 mg/mouse, n= 10). *: $P<0.01$, **: $P<0.001$, significantly different from control.

of flavones/flavonols (Kim *et al.*, 1993), isoflavonoids were found to be generally less active. It was also found that isoflavonoids tested showed the higher activity against AA-induced ear edema than that against croton-oil induced ear edema when applied topically. Formononetin was not active in croton-oil or AA-induced ear edema, while 5-OH formononetin (biochanin A) was active. These results were well correlated with the previous results of Huh *et al.* (1987) demonstrating that daidzein was active in carrageenan-induced rat paw edema, but formononetin was not.

It is known that NSAIDs show the potent activity against AA-induced ear edema via cyclooxygenase inhibition and SAIDs show the higher activity against croton-oil ear edema assay (Arner *et al.*, 1985; Bouclier *et al.*, 1990). Flavonoids, mainly flavones/flavonols, showed the higher activity against AA-induced ear edema than that against croton-oil induced ear edema when applied topically (Kim *et al.*, 1993), which suggested that flavones/flavonols may affect cyclooxygenase (CO)/lipoxygenase (LO) activity in vivo as reported by the various researchers (Welton *et al.*, 1988; Landolfi *et al.*, 1991). Isoflavonoids also showed the higher activity against AA-induced ear edema, however, the activity was less than that of flavones/flavonols. For the 7-O-substituted biochanin A derivatives, only 7-O-isopropylbiochanin A in croton-oil edema, and 7-O-ethyl and 7-O-isopropylbiochanin A in AA-induced edema showed similar potency of the antiinflammatory activity to biochanin A. Therefore, it is suggested that a certain size limitation of 7-position substituents would be existed, and 7-O-substitution of isoflavonoids may not be favorable for the antiinflammatory activity of isoflavonoids.

In conclusion, the certain isoflavonoids such as daidzein and puerarin showed the antiinflammatory activity in mice although they were generally less active than flavones/flavonols. And this effect may participate to the known biological actions of antipyresis and spasmolysis by *Pueraria* radix.

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