Isolation and Identification of Urinary Metabolites of Kakkalide in Rats

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ABSTRACT:

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Kakkalide is a major isoflavonoid from the flowers of Pueraria lobata (Willd.) Ohwi, possessing the protective effect against ethanol-induced intoxication and hepatic injury. The metabolism of kakkalide was investigated in rats. Thirteen metabolites were isolated by using solvent extraction and repeated chromatographic methods and identified by using spectroscopic methods including UV, IR, mass spectrometry, NMR, and circular dichroism experiments. Four new compounds were identified as irisolidone-7-0-

In China, Japan, and other Asian countries, Puerariae Flos has been used to relieve some symptoms such as drunkenness, headache, and red face and ameliorate liver injury caused by excessive drinking of alcohol (Niiho et al., 1989; Song et al., 2001). Puerariae Flos is botanically from the flowers of Pueraria lobata (Willd.) Ohwi, where kakkalide is a predominant isoflavone, and Pueraria thomsonii Benth, where tectoridin is a major isoflavone (Zhang et al., 2009). Pharmacologically, kakkalide, irisolidone-7-O- β -D-xylopyranosyl-(1 \rightarrow 6)- β -D-glucopyranoside, shows a wide spectrum of bioactivities such as hepatoprotection (Yamazaki et al., 1997; Han et al., 2003; Lee et al., 2005a), estrogenic effect (Shin et al., 2006), anti-inflammation (Park et al., 2007), antihyperlipidemia (Min and Kim, 2007), antioxidation (Kang et al., 2008), and others. The major metabolite in the blood of rats orally given kakkalide was its aglycone irisolidone, but not kakkalide itself. Han et al. (2003) isolated irisolidone and kakkalidone (irisolidone-7-O-B-D-glucopyranoside) from an anaerobic medium containing kakkalide and human fecal bacteria, and they found that kakkalide exerts the protective activity against ethanol-induced mortality and hepatic injury in mice only by oral route. On the other hand, the activity of irisolidone was stronger than that of kakkalide whether it was given orally or intraperitoneally (Yamaki et al., 2002; Min and Kim, 2007; Shin et al., 2006). In addition, some isoflavone aglycone metabolites such as tectorigenin, glycitein, and genistein showed more potent activity than their glycoside precursors (Yamaki et al., 2002). These results indicate that kakkalide is in essence a prodrug.

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glucuronide (M-1), tectorigenin-7-O-sulfate (M-2), tectorigenin-4'-O-sulfate (M-3), and biochanin A-6-O-sulfate (M-4) together with nine known compounds identified as irisolidone (M-5), tectorigenin (M-6), tectoridin (M-7), 5,7-dihydroxy-8,4'-dimethoxyisoflavone (M-8), isotectorigenin (M-9), biochanin A (M-10), genistein (M-11), daidzein (M-12), and equol (M-13). The metabolic pathway of kakkalide was proposed, which is important to understand its metabolic fate and disposition in humans.

More recently, in China and Japan, phytochemicals containing Puerariae Flos have become one of the better selling herbal medicines for treatment of diseases such as alcohol intoxication and liver injury. According to our previous study (Zhang et al., 2009), the contents of kakkalide in the P. lobata flower and in its water extracts were more than 2 and 10%, respectively. Thus, peoples consuming phytochemicals containing Puerariae Flos may be exposed to high levels of kakkalide. As a phytoestrogen, the metabolic forms of an isoflavone would be responsible for its activity and side effects (Shin et al., 2006). Thus, it is necessary to understand the metabolic fate of kakkalide.

The liquid chromatography-tandem mass spectrometry (LC-MSⁿ) technique was applied to characterize the urinary and biliary metabolites of tectoridin and tectorigenin, the major isoflavones of P. thomsonii (Chen et al., 2008; Zhang, 2008; Zhang et al., 2009). In these studies, the glucuronide or sulfate-conjugated position of several phase II metabolites cannot be definitively determined means of the LC-MSⁿ method alone. Isolation of metabolites and their further structural confirmation on the basis of UV, IR, NMR, and mass spectrometry (MS) data are valuable as well.

In the present study, we conducted the systematic isolation of urinary metabolites in rats given kakkalide orally and determined the structure by using chemical and spectroscopic experiments. On the basis of the metabolite profile, the possible metabolic pathway of kakkalide was proposed.

Materials and Methods

Materials and Chemicals. Kakkalide and irisolidone were isolated from the flowers of P. lobata, and tectorigenin and tectoridin were isolated from the flowers of P. thomsonii, following the previously reported methods of Chang et al. (2009) and Yuan et al. (2009). The identity of these compounds was confirmed by melting point, UV, IR, ¹H and ¹³C NMR, and MS. The purity

ABBREVIATIONS: LC-MSⁿ, liquid chromatography-tandem mass spectrometry; MS, mass spectrometry; HPLC, high-performance liquid chromatography; CC, column chromatography; CD, circular dichroism; ESI, electrospray ionization; TOF-MS, time-of-flight tandem mass spectrometry.

of kakkalide evaluated with high-performance liquid chromatography (HPLC)-UV was 98.6%, and those of others were more than 95%. β -Glucuronidase and sulfatase were purchased from Sigma-Aldrich (St. Louis, MO); macroporous resin D101 was obtained from Fushun Xintai Fine Chemical Factory (Fushun, China); Sephadex LH-20 was from GE Healthcare (Uppsala, Sweden); ODS was obtained from YMC Co., Ltd. (Kyoto, Japan); and Silica gel GF254 for thin-layer chromatography and silica gel column chromatography (CC) were from Qindao Ocean Chemical Co. (Qingdao, China). Other chemical reagents were of analytical or HPLC grade. Double-distilled water was used in this study.

Animals. Male Wistar rats $(200 \pm 20 \text{ g b.wt.})$ were purchased from the Animal Center of Shenyang Pharmaceutical University (Shenyang, China). The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Shenyang Pharmaceutical University. They were kept in a breeding room to be acclimated for 4 days before use. Normal foods were available before experiments, and normal water was available at all times.

Urine Collection. Male Wistar rats were fasted 12 h before experiments. Kakkalide (9 g) was given orally to 48 rats at a dose of 200 mg/kg and administered reiteratively in an interval of 7 days (2 days for administration and 5 days for recovery) for the collection of urine samples. The urine samples were collected from 0 to 36 h. During the collection, water and sugar were available freely. Phosphoric acid was added to urine samples to adjust pH to 5.0, and samples were subsequently placed in the refrigerator at -10° C.

Isolation of Metabolites. The cumulative urine samples (approximately 5 liters in total) were thawed at room temperature and successively passed through a macroporous absorption resin D101 column eluting with a gradient of EtOH- H_2O (H_2O , 30% EtOH, 70% EtOH, and 95% EtOH elutions) to yield four major fractions.

The 30% EtOH fraction was further separated through an ODS open column eluting with a gradient of MeOH-H₂O (10:90-100:0). The fraction eluted with MeOH-H₂O (35:65) was further subjected to a Sephadex LH-20 column eluting with MeOH-H₂O (50:50) to give M-1, so were the fractions eluted with MeOH-H₂O (40:60) to yield M-2 and M-3 and the fractions eluted with MeOH-H₂O (50:50) to yield M-4 and M-7.

The 70% EtOH fraction was further separated by silica gel CC eluting with a CHCl₃-MeOH gradient solvent system. M-6 was given from the fraction eluted with CHCl₃-MeOH (120:1). The fraction eluted with CHCl₃-MeOH (100:1) was further subjected to a Sephadex LH-20 column eluting with CHCl₃-MeOH (1:1) to give M-9, so were the fractions eluted with CHCl₃-MeOH (70:1) to a Sephadex LH-20 column eluting with MeOH to yield M-11, and the fractions eluted with CHCl₃-MeOH (50:1) to a Sephadex LH-20 column eluting with MeOH to yield M-12.

The 95% EtOH fraction was further separated by silica gel CC eluting with a CHCl₃-MeOH gradient solvent system. M-5 was obtained from the fraction eluted with CHCl₃-MeOH (500:1). The fraction eluted with CHCl₃-MeOH (300:1) was further subjected to a Sephadex LH-20 column to give M-8, eluting with CHCl₃-MeOH (3:1), so were the fractions eluted with CHCl₃-MeOH (200:1) to a Sephadex LH-20 column eluting with CHCl₃-MeOH (1:1) to yield M-10, and the fraction eluted with CHCl₃-MeOH (1:1) to yield M-13.

Enzymatic Hydrolysis of Metabolites M-1, M-2, and M-3. M-1 (0.1 mg) was incubated with β -glucuronidase (20 μ l, 2000 units, type B-1) in 0.05 M ammonium dihydrogen phosphate buffer (1.0 ml, pH 5.0) for 2 h at 37°C. The hydrolysis of M-2 and M-3 (each 0.1 mg) with sulfatase (20 μ l, 1000 units, type H-1) was conducted following the hydrolytic method of M-1 as described above. Each reaction mixture was extracted with ethyl acetate, and the organic layer was evaporated to dryness in vacuo to give the aglycone powder, which dissolved in 0.2 ml of methanol for HPLC-UV analysis.

Spectroscopic Methods. Electrospray ionization (ESI)-single quadrupole MS (Shimadzu QP8000 α ; Shimadzu Co. Ltd., Kyoto, Japan) was used in the beginning to scan each fraction for novel metabolites. The mass spectrometer was operated under the following conditions: direct infusion of sample, ESI in positive and negative mode, an electrospray voltage of 4.0 kV, a mass scan range at m/z 50 to 800, a heating capillary temperature at 250°C, and dry air at a flow rate of 4.5 l \cdot min⁻¹.

Time-of-flight tandem mass spectrometry (TOF-MS) (Bruker MicroTOF-Q 125; Bruker, Newark, DE) was used to acquire both the exact molecular weight and the product ion spectra of any novel compounds detected by the single-

quadrupole mass spectrometer. Parameters for analysis were set by using full-scan negative ion mode with spectra acquired over a mass range from m/z 50 to 1000. The ESI source was set to the following conditions: drying gas (N₂) flow rate, 4.0 l/min; drying gas temperature, 190°C; nebulizer, 0.4 bar; capillary voltage, 3.2 kV.

NMR spectra were measured on a Bruker ARX-600 spectrometer, and chemical shifts are given in ppm downfield relative to tetramethylsilane. All compounds were dissolved in dimethyl sulfoxide-*d*₆. UV spectra were obtained using a Shimadzu UV-2201 spectrophotometer. Circular dichroism (CD) spectra were recorded on a JASCO CD-2095 plus spectrophotometer (Jasco, Tokyo, Japan). IR spectra were obtained on a Bruker IFS-55 IR spectrometer.

HPLC Condition. Waters HPLC system (Waters, Milford, MA) consisting of model 510 pump, automated gradient controller, model 2996 photodiode array detector, and Millennium32 PDA software was used. HPLC analyses were carried out at 35°C on a Kromasil C18 column (4.6 × 250 mm, 5 μ m; Tianjin Scientific Instruments Co. Ltd., Tianjin, China). The on-line UV spectra were recorded in the range of 200 ~ 400 nm. The injection volume was 20 μ l. The mobile phase consisted of a gradient system of solution A, water containing 0.05% trifluoroacetic acid, and solution B, acetonitrile containing 0.05% trifluoroacetic acid at a flow rate of 0.8 ml/min. The gradient program was as follows: linear gradient from solution A-B (90:10, v/v) to solution A-B (60:40, v/v) in 48 min, followed by linear gradient to solution A-B (30:70, v/v) in 32 min.

Results

Isolation and Structural Elucidation of Kakkalide Metabolites. Representative HPLC profiles showing the rat urinary metabolites are given in Fig. 1. Kakkalide and its metabolites were selectively detected at 265 nm due to their characteristic benzoyl group. Five major metabolites, M-1, M-2/-3, M-5, and M-6, were clearly observed in rat urine (Fig. 1). By means of repeated chromatographic methods on the columns of silica gel, Sephadex LH-20, or reverse-phase ODS, 13 metabolites were isolated from the rat urine sample, including four new compounds, a glucuronide conjugate, M-1 (2.5 mg), and three sulfate ones, M-2 (3.5 mg), M-3 (3.0 mg), and M-4 (2.5 mg), together with nine known isoflavones, M-5 (15 mg), M-6 (10 mg), M-8 (1.4 mg), M-9 (1.1 mg), M-10 (1.1 mg), M-11 (1.4 mg), M-12 (1.2 mg), M-13 (2.4 mg), and M-7 (2.0 mg).

The maximal absorption at 263 to 265 nm in UV spectra and the absorption bands at 1649 to 1659 cm⁻¹ due to conjugated carbonyl and at 1457 to 1616 cm⁻¹ due to aromatic functions in IR spectra indicate that M-1 \sim M-12 have an isoflavone skeleton. The structures of M-1 \sim M-13, as shown in Fig. 2, were elucidated using UV, IR, ¹H and ¹³C NMR, MS, or CD techniques.

Metabolite M-1 (irisolidone-7-O-B-D-glucuronide) was isolated as a white amorphous powder. The molecular formula was determined to be $C_{23}H_{21}O_{12}$ from the $[M - H]^-$ quasimolecular ion peak at m/z489.1024 (calculated 489.1027) in the ESI-TOF-MS. The $[M - H]^{-1}$ ion at m/z 489 and an important fragment ion at m/z 313 originating from the eliminating 176 mass units (glucuronic acid) from [M - H]ion indicated that M-1 should be a glucuronide conjugate (Fig. 3A). M-1 was hydrolyzed with β -glucuronidase to give the aglycone irisolidone, the identity of which was confirmed through cochromatography with irisolidone standard by HPLC coupled to UV photodiode array detection based on the same retention time and UV spectral. In the ¹H NMR spectrum, the characteristic isoflavone signal for H-2 was observed at δ 8.49 (1H, s). A singlet at δ 6.91 due to an aromatic proton suggested that three substituents were linked to A-ring. The protons due to an AA'BB' aromatic system appeared at δ 7.53 (d, J = 8.4 Hz, H-2', 6') and 7.01 (d, J = 8.4 Hz, H-3', 5'), indicating that B-ring was substituted at C-4'. In addition, two singlets at δ 3.80 and 3.78 due to two methoxyl groups were observed. The resonances for carbons and protons of the aglycone moiety had a close resemblance to those of the known irisolidone, and they are assigned according to



0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50 52 54 56 58 60 62 64 66 68 70 Fig. 1. A, HPLC-UV chromatograms of blank urine; B, a rat urine sample during 0 to 24 h after oral administration of kakkalide at a dose of 200 mg/kg; C, metabolite standards.



Fig. 2. Proposed metabolic pathways of kakkalide. [] refers to possible intermediates that were not obtained in the present study.

the literature values of the ¹H and ¹³C NMR data for irisolidone (Yin et al., 2006). The signals of an anomeric proton (δ 5.08, 1H, d, J = 6.6 Hz) and a carboxylic group at C-6' at δc 171.0 indicated the presence of a β -D-glucuronic acid moiety (Yasuda et al., 1994). The β -D-glucuronic acid moiety could be attached to C-7 position according to the upfield shift of C-7 (-0.8 ppm) and the downfield shifts of C-6 (+1.1 ppm) and C-8 (+0.2 ppm) relative to the corresponding signals

of irisolidone (Yasuda et al., 1994; Yin et al., 2006). The full assignments of carbon and proton signals are summarized in Table 1. Thus, M-1 was determined to be irisolidone 7-O- β -D-glucuronide.

Metabolite M-2 (tectorigenin-7-O-sulfate) was isolated as a yellowish amorphous powder. IR spectrum showed an absorption band at 1038 cm⁻¹ due to a sulfate group, suggesting the sulfate-conjugated structure of M-2. The molecular formula was determined to be





FIG. 3. ESI-TOF-MS spectra of metabolite M-1, irisolidone-7-*O*-glucuronide (A); metabolite M-2, tectorigenin-7-*O*-sulfate (B); metabolite M-3, tectorigenin-4'-*O*-sulfate (C); and metabolite M-4, biochanin A-6-*O*-sulfate (D).

				TABLE 1				
			NMR og	f kakkalide metabolite.	s M-1~M-4			
Position	M-1		M-2		M-3		M-4	
	$\delta_{C}{}^{a}$	$\delta_{\text{H}}{}^{a} (J \text{ in Hz})$	$\delta_{C}^{\ a}$	$\delta_{\mathrm{H}}{}^{a}(J \text{ in Hz})$	$\delta_{C}{}^{a}$	$\delta_{\text{H}}{}^{a}(J \text{ in Hz})$	$\delta_{C}{}^{a}$	$\delta_{\text{H}}{}^{a}(J \text{ in Hz})$
2	155.1	8.49, s	154.9	8.44, s	154.6	8.39, s	154.7	8.4, s
3	122.9		122.0		125.4		120.3	
4	180.8		181.0		180.4		180.2	
5	153.0		153.2		153.2		154.1	
6	132.6		133.7		131.5		125.3	
7	156.8		153.0		157.5		157.6	
8	94.2	6.91, s	98.0	7.26, s	93.9	6.50, s	95.0	6.55, s
9	152.5		151.7		152.7		154.1	
10	106.5		107.0		104.8		105.0	
1'	121.8		121.2		121.6		121.6	
2',6'	130.3	7.53, d (8.4)	130.3	7.40, d (8.4)	129.5	7.46, d (8.4)	130.3	7.51, d (9.0)
3',5'	113.8	7.01, d (8.4)	115.2	6.83, d (8.4)	120.1	7.22, d (8.4)	113.8	7.00, d (9.0)
4'	159.3		157.5		153.5		159.2	
1″	100.0	5.08, d (6.6)						
2″	73.1							
3″	76.7							
4″	71.9							
5″	73.9							
6"	171.0							
6-OMe	60.4	3.78, s	60.3	3.77, s	59.9	3.75, s		
4'-Ome	55.3	3.80, s					55.3	3.79, s

^a All spectra were recorded on a Bruker ARX-600 spectrometer, in DMSO-d₆.

 $1'' \sim 6''$ refer to the numbering of glucuronic acid moiety.

 $C_{16}H_{12}O_{9}S$ from the $[M - H]^{-}$ quasimolecular ion peak at m/z 379.0117 (calculated 379.0118) in the ESI-TOF-MS. The negative ESI-MS displayed a $[M - H]^{-}$ ion peak at m/z 379 and a $[M-SO_{3}H]^{-}$ ion peak at m/z 299, further indicating the presence of a sulfate group (Fig. 3B). M-2 was hydrolyzed by sulfatase to give the aglycone tectorigenin, the identity of which was also confirmed through co-chromatography with tectorigenin standard using HPLC-UV. Moreover, the resonances for protons and carbons of the aglycone moiety

in the ¹H and ¹³C NMR spectrum closely resembled those of the known tectorigenin, and they are assigned according to the literature values of the ¹H and ¹³C NMR data for tectorigenin (Kang et al., 2008). The sulfate moiety could be attached to C-7 position according to a upfield shift of C-7 (-4.5 ppm) and the downfield shifts of C-6 (+2.2 ppm), C-8 (+4.0 ppm) together with H-8 (+0.76 ppm), and C-10 (+2.1 ppm) relative to the corresponding signals of tectorigenin (Yasuda et al., 1994; Kang et al., 2008). The

full assignments of carbon and proton signals are summarized in Table 1. Thus, M-2 was determined to be tectorigenin-7-O-sulfate.

Metabolite M-3 (tectorigenin-4'-O-sulfate) was isolated as a yellowish amorphous powder. IR spectrum also showed an absorption band at 1038 cm^{-1} due to a sulfate group. The molecular formula was determined to be $C_{16}H_{12}O_9S$ from the $[M - H]^-$ quasimolecular ion peak at m/z 379.0117 (calculated 379.0118) in the ESI-TOF-MS. The $[M - H]^-$ ion peak at m/z 379 and a $[M - SO_3H]^-$ ion peak at m/z299 were clearly shown in ESI-MS (Fig. 3C). The enzymatic hydrolysis of M-3 with sulfatase gave tectorigenin, the identity of which was also confirmed through cochromatography with tectorigenin standard using HPLC-UV. The resonances for protons and carbons of the aglycone moiety in the ¹H and ¹³C NMR spectrum also closely resembled those of tectorigenin (Kang et al., 2008). The sulfate moiety could be attached to C-4' position according to an upfield shift of C-4' (-4.2 ppm) and the downfield shifts of C-3' and C-5' (+4.9 ppm) relative to the corresponding signals of tectorigenin (Yasuda et al., 1994; Kang et al., 2008). The full assignments of carbon and proton signals are summarized in Table 1. Thus, M-3 was determined to be tectorigenin-7-O-sulfate.

Metabolite M-4 (biochanin A-6-O-sulfate) was isolated as a vellowish amorphous powder. The IR spectrum showed an absorption band at 1045 cm⁻¹ due to a sulfate group. The molecular formula was determined to be $C_{16}H_{12}O_{9}S$ from the $[M - H]^{-}$ quasimolecular ion peak at m/z 379.0117 (calculated 379.0118) in the ESI-TOF-MS. The $[M - H]^-$ ion peak at m/z 379 and $[M - SO_3H]^-$ ion peak at m/z 299 were also clearly shown in ESI-MS (Fig. 3D). The resonances for protons and carbons of the aglycone moiety of M-4 in the ¹H and ¹³C NMR spectrum closely resembled those of the known 6-hydroxybiochanin A, and they are assigned according to the literature values of the ¹H and ¹³C NMR data for 6-hydroxybiochanin A (Horie et al., 1996). The sulfate moiety could be attached to C-6 according to the upfield shift of C-6 (-4.1 ppm) and downfield shifts of C-7 (+4.1 ppm) and C-5 (+6.8 ppm) relative to the corresponding signals of 6-hydroxybiochanin A (Yasuda et al., 1994; Horie et al., 1996). The full assignments of carbon and proton signals are summarized in Table 1. Thus, M-4 was determined to be biochanin A-6-O-sulfate.

Other Metabolites. M-5 \sim M-13 were identified as irisolidone, tectorigenin, tectoridin, 5,7-dihydroxy-8,4'-dimethoxyisoflavone, isotectorigenin, biochanin A, genistein, daidzein, and equol, respectively,

by comparing their UV, NMR, MS, or CD data (Tables 2) with the reported values (Bashir et al., 1991; Yasuda et al., 1994; Yasuda and Ohsawa, 1998; Talukdar et al., 2000; Yin et al., 2006; Moriyasu et al., 2007; Kang et al., 2008). M-6 \sim M-13 were first isolated as the metabolites of kakkalide.

Discussion

The metabolic pathways of soy isoflavones in vivo are related to many reactions such as hydrolysis, *O*-methylation, glucuronidation, sulfation, hydroxylation, decyclization, and reduction (Yasuda and Ohsawa, 1998; Yasuda et al., 2001; Bursztyka et al., 2008). In the present study, 13 metabolites were isolated from the urine of rats given kakkalide orally and structurally confirmed on the basis of UV, IR, NMR, MS, and CD data. According to the metabolite profile, the possible metabolic pathways of kakkalide in rats are proposed as shown in Fig. 2.

Two phase I metabolites (M-5 and M-6) and three phase II metabolites (M-1, M-2, and M-3) were clearly identified in the HPLC-UV profile of rat urine (Fig. 1). Irisolidone (M-5) is a major metabolite that is believed to be formed through the microbial hydrolysis of kakkalide in the gastrointestinal tract, which is consistent with the previous literature (Han et al., 2003). Moreover, M-5 is also a reactive metabolite that can be successively converted to a glucuronide-conjugate (M1) via further biotransformation at C-7 position catalyzed by UDP-glucuronosyltransferases. Most importantly, we found that M-6 is another key metabolite derived from the O-demethylation of M-5 at C-4' position catalyzed by cytochrome P450 (Meyer et al., 2009), which has not been reported in the previous studies on the metabolism of kakkalide. It also subsequently undergoes sulfate-conjugation at the C-4' and C-7 positions (Fig. 3) catalyzed by sulfotransferases, forming M-2 and M-3. The total amount of M-5 and M-6 in urine samples is more than that of three conjugates $(M-1 \sim M-3)$. In addition, we also isolated some minor metabolites, including seven phase I metabolites (M-7 \sim M-13) and a phase II metabolites (M-4). It is worth noting that the purity of the dosed kakkalide was found to be 98.6%, and that these minor metabolites were not impurities in the dosed kakkalide according to HPLC analysis. In general, the role of phase II metabolism in vivo is drug detoxification by means of conjugation of phase I metabolites with endogenous substances to increase their water solubility and decrease or eliminate their biological activity (Liska, 1998). The wide existence of phase I metabolites of

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TABLE 2									
¹ H NMR	date	of	kakkalide	metabolites	M-5~M-13				

Position	$\delta_{\rm H}^{\ a}(J \text{ in Hz})$										
	M5	M6	M7	M8	M9	M10	M11	M12	M13		
2 3 4	8.38, s	8.33, s	8.44, s	8.45, s	8.37, s	8.36, s	8.31, s	8.28, s	3.89, t α (10.5) 4.15, dd β (10.5, 1.8) 2.96–3.04, m 2.78–2.88, m		
5								7.95, d (9.0)	6.86, d (8.4)		
6				6.31, s	6.26, s	6.22, d (2.4)	6.22, d (2.0)	6.92, dd (9.0, 2.4)	6.29, dd (8.4, 2.1)		
8	6.51, s	6.50, s	6.88, s			6.39, d (2.4)	6.42, d (2.0)	6.84, d (2.4)	6.19, d (2.1)		
2',6'	7.50, d (8.6)	7.38, d (8.4)	7.39, d (8.7)	7.50, d (9.0)	7.36, d (8.4)	7.50, d (8.4)	7.40, d (8.5)	7.37, d (8.4)	7.10, d (8.4)		
3',5' Glc-1 2 3 4 5 6 6-OMe	7.00, d (8.6) 3.79, s	6.83, d (8.4) 3.75, s	6.82, d (8.7) 5.08, d (7.4) 3.30, m 3.16, m 3.44, m 3.46, m α 3.70, m β 3.76, s	7.01, d (9.0)	6.80, d (8.4)	7.00, d (8.4)	6.83, d (8.5)	6.79, d (8.4)	6.72, d (8.4)		
8-OMe				3.77, s	3.74, s						
4'-OMe	3.76, s			3.77, s		3.79, s					

 a All spectra were recorded on a Bruker ARX-600 spectrometer, in DMSO- d_{6}

kakkalide should be ascribed to high polarity of their multi-O-substituted isoflavone skeleton.

Some of the proposed metabolic pathways or reactions were relatively rare. The first rare pathway is demethoxylation. The formations of M-10 and M-11 that are the minor metabolites in our study were involved in demethoxylation of M-5 and M-6 at C-6 position, respectively. Simons et al. (2005) reported that glycitein was transformed into daidzein by human gut microflora, which undergoes the demethoxylation at the C-6 position of isoflavone skeleton. However, they suggested that such a demethoxylation reaction may not be a major pathway of metabolism in humans and hamsters (Simons et al., 2005). The demethoxylation of physcion at C-6 position also occurred in vivo to form chrysophanol (Sun and Chen, 1986). The results above indicate that demethoxylation is a potential but minor pathway for the metabolism of isoflavone or flavonone in vivo. The second rare pathway is methoxyl group migration intermediated by decyclization reaction. M-8 and M-9, the isomeric metabolites of M-5 and M-6, are both minor urinary metabolites possessing 8-methoxyl group. A pathway related to isopentenyl group migration was found in the metabolites of coincubation of icariin (3,5,7-trihydroxyl-4'methoxyl-8-isopentenylflavone-3-O- α -L-rhamnopyranosyl-7-O- β -D-glucopyranoside) with gut microflora (Liu et al., 2000), which produced an unusual metabolite, 3,5,7-trihydroxyl-4'-methoxyl-6isopentenyl-flavone. On the basis of the above-mentioned study, we postulated that these metabolites may be formed by rearrangement of irisolidone or tectorigenin. The third rare pathway is dehydroxylation. M-12 was isolated in trace amounts from rat urine, which was structurally from the dehydroxylation of M-11 at C-5 position. Yasuda et al. (1998, 2001) reported that genistein and daidzein were transformed into equol via hydrogenation, reduction, and dehydroxylation in vivo, which suggest that dehydroxylation reaction may occur in the metabolism of isoflavone in vivo. Lastly, M-4 is a minor phase II metabolite. It should be the product of subsequent metabolism of irisolidone involved in 6-O-demethylation to give 6-OH-biochanin A and instant sulfate-conjugation at C-6.

Tectoridin was a structural analog of kakkalide. It showed similar hepatoprotective, anti-inflammatory, and estrogenic effects to those of kakkalide (Lee et al., 2005b; Shin et al., 2006; Park et al., 2007). The metabolic fates of tectoridin and tectorigenin were associated with hydrolysis of 7-O-glucosyl, O-methylation, sulfation, disulfation, and glucuronidation of hydroxyl groups and mono- and dihydroxylations at B-ring (Chen et al., 2008; Zhang, 2008). Although M-3 (tectorigenin-4'-O-sulfate) was reported to be identified in rat urine after oral administration of tectoridin by LC-MSⁿ techniques (Chen et al., 2008), the proposed MS or MSⁿ data could not provide sufficient evidence to confirm the sulfate-conjugated position at C-4' or at C-7. In our study, the conjugated position of four phase II metabolites was clearly elucidated by using NMR as well as MS data. In addition, and in contrast to other studies of metabolism, we also pay attention to the systematic isolation and identification of phase I metabolites because many reports showed that isoflavone aglycones may exert more potent biological activities or side effects than their glycosides Yasuda et al., 1998, 2001; Bursztyka et al., 2008). Further study on the biological activity of these metabolites will be the next topic of our research.

In summary, 13 metabolites were isolated from the urine of rats given kakkalide orally and structurally confirmed by UV, IR, NMR, MS, and CD techniques. The initial metabolic pathways appear to be the formations of irisolidone through the microbial hydrolysis in the gastrointestinal tract and tectorigenin through the *O*-demethylation of irisolidone at C-4'. Both metabolites were excreted either free or conjugated. The phase II metabolism of tectorigenin and irisolidone forms a glucuronide conjugate and three sulfate conjugates that are isolated and identified for the first time.

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