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Title page

Isolation and identification of urinary metabolites of kakkalide in rats

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Running title page

Running title: Metabolites of kakkalide, in rat urine

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ABBREVIATIONS: NMR, nuclear magnetic resonance; HPLC, high performance liquid chromatography; TOF-MS, time-of-flight tandem mass spectrometry; ESI-MS, electrospray ionization-mass spectrometry; UV, ultraviolet; IR, infrared; TMS, tetramethylsilane; DMSO, dimethyl sulfoxide; CC, column chromatography.

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Abstract

Kakkalide is a major isoflavonoid of 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 rat. Thirteen metabolites were isolated using solvent extraction and repeated chromatographic methods, and identified using spectroscopic methods including UV, IR, MS, NMR and CD experiments. Four new compounds were identified as irisolidone-7-*O*-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 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 human.

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Introduction

Puerariae Flos has been used to relieve some symptoms such as drunkenness, headache and red face and ameliorate liver injure caused by excessive drinking of alcohol in China, Japan and some Asian countries (Song et al., 2001; Niiho et al., 1989). It is botanically from the flowers of *Pueraria lobata* (Willd.) Ohwi of which kakkalide is a predominant isoflavone, and *P. thomsonii* Benth. of which tectoridin is a major isoflavone (Zhang et al., 2009). Pharmacologically, kakkalide, irisolidone-7-*O*-*β*-D-xylopyranosyl-(1→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 SW and Kim DH, 2007), and antioxidation (Kang et al., 2008), etc. The major metabolite in the blood of rats orally given kakkalide was its aglycone irisolidone, but not kakkalide itself. Han et al isolated irisolidone and kakkalidone (irisolidone-7-*O*-*β*-D-glucopyranoside) from an anaerobic medium containing kakkalide and human fecal bacteria, and found that kakkalide exerts the protective activity against ethanol-induced mortality and hepatic injury in mice only by oral route (Han et al., 2003). On the other hand, the activity of irisolidone was stronger than that of kakkalide no matter it was given orally or intraperitoneally (Shin et al., 2006; Yamaki et al., 2002, Min SW and Kim DH, 2007). Also 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.

Recently, phytochemicals containing Puerariae Flos have become the good selling herbal medicines for the diseases of alcohol intoxication and liver injure in Chine and Japan. According to our previous study (Zhang et al., 2009), the contents of kakkalide in the flower of *P. lobata* and in its water extracts were more than 2% and 10%, respectively. Thus, peoples consuming phytochemicals

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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). It is thus necessary to understand the metabolic fate of kakkalide.

LC/MS^n 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 et al., 2008; Zhang et al., 2009). In these studies, the glucuronide or sulfate-conjugated position of several phase II metabolites cannot be definitely determined only by means of LC/MS^n method. Isolation of metabolites and their further structural confirmation on the basis of UV, IR, NMR and MS data are valuable as well.

In the present study, we conducted the systematic isolation of urinary metabolites of orally administrated kakkalide in rat, and the structural determination using chemical and spectroscopic experiments. On the basis of the metabolite profile, the possible metabolic pathway of kakkalide was proposed.

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Materials and Methods

Materials and chemicals. Kakkalide and irisolidone were isolated from the flowers of *P. lobata*, and tectorigenin and tectoridin from the flowers of *P. thomsonii*, following the previously reported methods (Yuan et al., 2009; Chang et al., 2009). The identity of these compounds were confirmed by melting point, UV, IR, ^{1}H- and ^{13}C-NMR and MS. The purity of kakkalide evaluated with HPLC-UV was 98.6%, and those of others were more than 95%. *β*-glucuronidase and sulphatase were purchased from Sigma (USA), macroporous resin D101 from Fushun Xintai Fine Chemical Factory (Fushun, China), Sephadex LH-20 from GE Healthcare (Sweden), ODS from YMC Co., Ltd. (Japan) and Silica gel GF_{254} for thin-layer chromatography and silica gel CC from Qindao Ocean Chemical Co. (China). Other chemical reagents were of analysis or HPLC grades. Double distilled water was used in this study.

Animals. Male Wistar rats (200±20 g body weight) 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, so was normal water at all times.

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

Isolation of metabolites. The cumulative urinary samples (approximately 5 L in total) were thawed

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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-H_2O (10:90-100:0). The fraction eluted with MeOH-H_2O (35:65) was further subjected to a Sephadex LH-20 column eluting with MeOH-H_2O (50:50) to give M-1, so were the fraction eluted with MeOH-H_2O (40:60) to give M-2 and M-3 and the fraction eluted with MeOH-H_2O (50:50) to give M-4 and M-7.

The 70% EtOH fraction was further separated by silica gel CC eluting with a $CHCl_3$-MeOH gradient solvent system. M-6 was given from the fraction eluted with $CHCl_3$-MeOH (120:1). The fraction eluted with $CHCl_3$-MeOH (100:1) was further subjected to a Sephadex LH-20 column eluting with $CHCl_3$-MeOH (1:1) to give M-9, so were the fraction eluted with $CHCl_3$-MeOH (70:1) to a Sephadex LH-20 column eluting with MeOH to give M-11, and the fraction eluted with $CHCl_3$-MeOH (50:1) to a Sephadex LH-20 column eluting with MeOH to give M-12.

The 95% EtOH fraction was further separated by silica gel CC eluting with a $CHCl_3$-MeOH gradient solvent system. M-5 was obtained from the fraction eluted with $CHCl_3$-MeOH (500:1). The fraction eluted with $CHCl_3$-MeOH (300:1) was further subjected to a Sephadex LH-20 column to give M-8, eluting with $CHCl_3$-MeOH (3:1), so were the fraction eluted with $CHCl_3$-MeOH (200:1) to a Sephadex LH-20 column eluting with $CHCl_3$-MeOH (1:1) to yield M-10, and the fraction eluted with $CHCl_3$-MeOH (150:1) to a Sephadex LH-20 column eluting with $CHCl_3$-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 μl, 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 μl,

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1000 units, type H-1) were 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 methanol for HPLC-UV analysis.

Spectroscopic methods. ESI-single quadrupole MS (Shimadzu QP8000α, Shimadzu co. Ltd., 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 50-800, a heating capillary temperature at 250 °C, and dry air at a flow rate of 4.5 $L \cdot min^{-1}$.

TOF-MS (Bruker MicroTOF-Q 125, Bruker, Newark, DE, USA) 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 using full scan negative ion mode with spectra acquired over a mass range from m/z 50–1000. The ESI source was set to the following conditions: drying gas (N_2) 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 TMS. All compounds were dissolved in DMSO-d_6. UV spectra were obtained using a Shimadzu UV-2201 spectrophotometer. CD spectra were recorded on a JASCO CD-2095 plus spectrophotometer. IR spectra were obtained on a Bruker IFS-55 infrared spectrometer.

HPLC condition Wates HPLC system (Waters Co., Milford, MA, US) consisting of model 510 pump, automated gradient controler, 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 μm, Tianjin Scientific Instruments CO. Ltd., China). The on-line UV spectra were recorded in

the range of 200 ~ 400 nm. The injection volume was 20 μl. The mobile phase consisted of a gradient system of solution A, water containing 0.05% TFA, and solution B, acetonitrile containing 0.05% TFA at a flow rate of 0.8 ml/min. The gradient program were as following: 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.

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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, 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, thirteen metabolites were isolated from the rat urinary 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) and M-13 (2.4 mg) and M-7 (2.0mg).

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

Metabolite M-1 (irisolidone-7-*O*-*β*-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/z 489.1024 (calc. 489.1027) in the ESI-TOF-MS. The $[M-H]^-$ 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.3-A). M-1 was hydrolyzed with *β*-glucuronidase to give the aglycone irisolidone, which identity was confirmed through co-chromatography with irisolidone standard by HPLC coupled to ultraviolet photodiode array

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detection based on the same retention time and UV spectral. In the ^{1}H 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'. Also 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 the literature values of the ^{1}H and ^{13}C 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 $C_{16}H_{12}O_9S$ from the $[M-H]^-$ quasimolecular ion peak at m/z 379.0117 (calc. 379.0118) in the ESI-TOF-MS. The negative ESI-MS displayed a $[M-H]^-$ ion peak at *m/z* 379 and a $[M-SO_3H]^-$ ion peak at *m/z* 299, further indicating the presence of a sulfate group (Fig.3-B). M-2 was hydrolyzed by sulfatase to give the aglycone tectorigennin, which identity was also confirmed through co-chromatography with tectorigennin standard using HPLC-UV. Moreover, the resonances for protons and carbons of the aglycone moiety in the ^{1}H- and ^{13}C-NMR spectrum had close resemblance to those of the known

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tectorigenin, and they are assigned according to the literature values of the ^{1}H and ^{13}C 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 (cald. 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/z* 299 were clearly shown in ESI-MS (Fig.3-C). The enzymatic hydrolysis of M-3 with sulfatase gave tectorigenin, which identity was also confirmed through co-chromatography with tectorigenin standard using HPLC-UV. The resonances for protons and carbons of the aglycone moiety in the ^{1}H- and ^{13}C-NMR spectrum also had close resemblance to 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 -5' (+ 4.9 ppm) relative to the corresponding signals of tectorigennin (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 yellowish 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 $C_{16}H_{12}O_9S$ from the $[M-H]^-$ quasimolecular ion peak at m/z 379.0117 (cald. 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

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were also clearly shown in ESI-MS (Fig.3-D). The resonances for protons and carbons of the aglycone moiety of M-4 in the ^{1}H- and ^{13}C-NMR spectrum had close resemblance to those of the known 6-hydroxybiochanin A, and they are assigned according to the literature values of the ^{1}H and ^{13}C 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 ~ M-13 were identified as irisolidone, tectorigenin, tectoridin, 5,7-dihydroxy-8,4'-dimethoxyisoflavone, isotectorigenin, biochanin A, genistein, daidzein, equol, respectively, by comparing their UV, NMR, MS or CD data (Tables 2) with the reported values (Yin et al., 2006, Kang et al., 2008, Bashir et al., 1991, Moriyasu et al., 2007, Talukdar et al., 2000, Yasuda et al., 1994, Yasuda and Ohsawa., 1998). M-6 ~ M-13 were first isolated as the metabolites of kakkalide.

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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 ones (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 was consistent with the previous literatures (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 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 undergo 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 urinary sample is more than that of three conjugates M-1 ~ M-3. In addition, we also isolated some minor metabolites, including seven phase I metabolites (M-7~M-13) and a phase II metabolites (M-4). It is worth noting that the 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

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decrease or eliminate their biological activity (Liska, 1998). The wide existence of phase I metabolites of 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 is demethoxylation. The formations of M-10 and M-11 that are the minor metabolites in our study involved in demethoxylation of M-5 and M-6 at C-6 position. Simons et al reported that glycitein was transformed into daidzein by human gut microflora, which undergoes the demethoxylation at 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 occured *in vivo* to form chrysophanol (Sun and Chen, 1986). Above results indicate that demethoxylation is a potential but minor pathway for the metabolism of isoflavone or flavonone *in vivo*. The second 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 co-incubation of icarrin (3,5,7-trihydroxyl-4'-methoxyl-8-isopentenylflavone-3-*O*-α-L-rhamnopyranosyl-7-*O*-β-D-glucopyran oside) with gut microflora (Liu et al., 2000), which produced an unusual metabolite, 3,5,7-trihydroxyl-4'-methoxyl-6-isopentenyl-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 is dehydroxylation. M-12 was isolated in trace amount from rat urine, which was structurally from the dehydroxylation of M-11 at C-5 position. Yasuda et al. reported that genistein and daizein were transformed into equol *via* hydrogenation, reduction and dehydroxylation *in vivo* (Yasuda et al., 2001), 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

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of irisolidone involved in 6-*O*-demethylation to give 6-OH-biochanin A, and instant sulfate-conjugation at C-6.

Tectoridin was a structural analogue of kakkalide. It showed similar hepatoprotective, anti-inflammatory and estrogenic effects to those of kakkalide (Lee et al., 2005b; Park et al., 2007; Shin et al., 2006). 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 di- hydroxylations at B-ring. (Chen et al., 2008; zhang et al., 2008). Although M-3 (tectorigenin-4'-*O*-sulfate) was reported to be identified in rat urine after orally administration of tectoridin by LC–MS^n techniques (Chen et al., 2008), the proposed MS or MS^n data could not provided sufficient evidence to confirm the sulfate-conjugated position is at C-4' or at C-7. In our study, conjugated position of four phase II metabolites were clearly elucidated using NMR as well as MS data. Additionally, different from 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; Yasuda et al., 2001; Bursztyka et al., 2008). Further study on the biological activity of these metabolites will be the next topic of our research.

In summary, thirteen 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 would appear to be the formations of irisolidone through the microbial hydrolysis in the gastrointestinal tract and tectorigennin 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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Fig. 1. HPLC-UV chromatograms of blank urine (A), a rat urine sample during 0-24 h after oral administration of kakkalide at a dose of 200 mg/kg (B); metabolite standards (C).

Fig. 2. Proposed metabolic pathways of kakkalide

〔〕 refers to possible intermediates that were not obtained in the present study.

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).

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Tables

Table 1. NMR of kakkalide metabolites M-1~M-4

position	M-1		M-2		M-3		M-4	
	δ_C [a]	δ_H [a] (J in Hz)	δ_C [a]	δ_H [a] (J in Hz)	δ_C [a]	δ_H [a] (J in Hz)	δ_C [a]	δ_H [a] (J 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-d_6.

1" ~ 6" refer to the numbering of glucuronic acid moiety.

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Table 2. ^{1}H-NMR date of kakkalide metabolites M-5~ M-13

position	δ_H [a], (*J* in Hz)								
	M5	**M6**	**M7**	**M8**	**M9**	**M10**	**M11**	**M12**	**M13**
2	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)
3									2.96-3.04, m
4									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'	7.00, d (8.6)	6.83, d (8.4)	6.82, d (8.7)	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)
Glc-1			5.08, d (7.4)						
2			3.30, m						
3			3.30, m						
4			3.16, m						
5			3.44, m						
6			3.46, m α 3.70, m β						
6-OMe	3.79, s	3.75, s	3.76, s						
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.

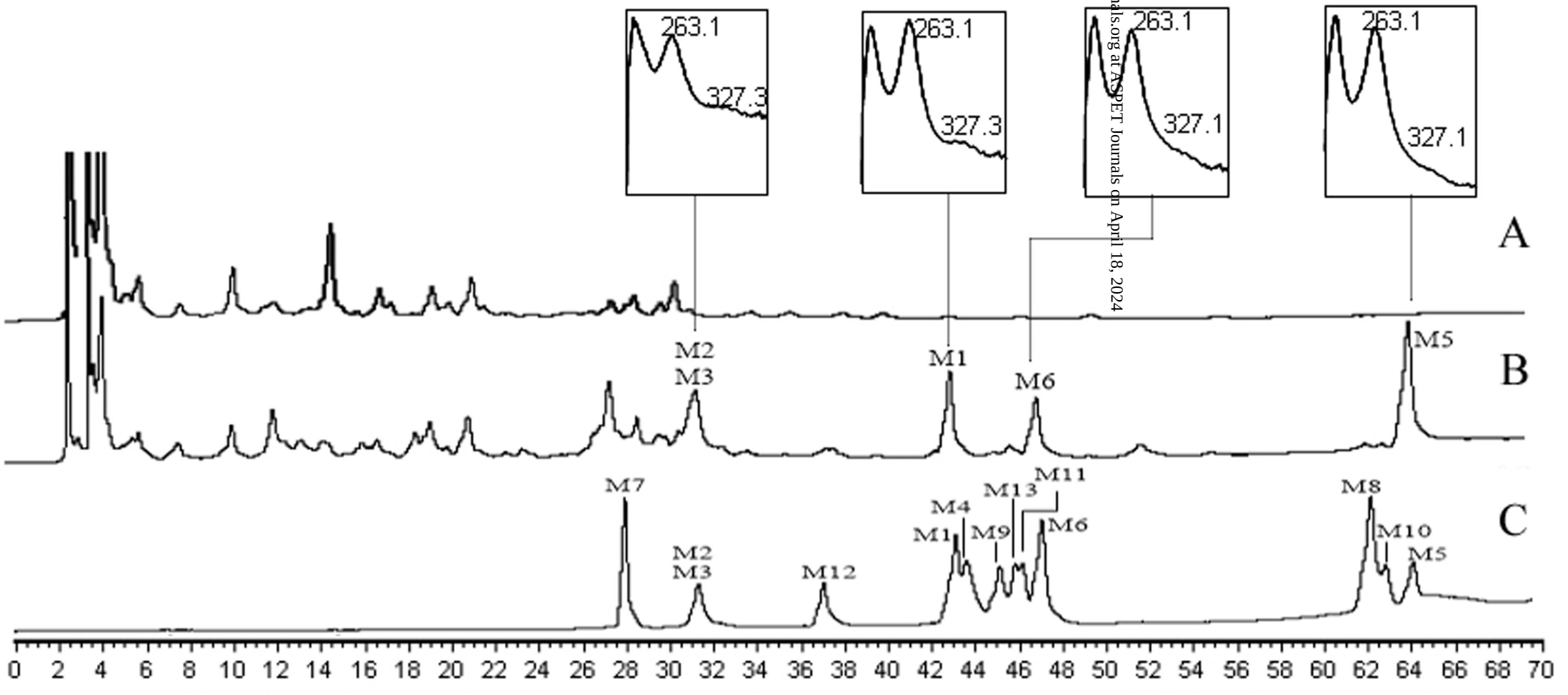


Fig.1

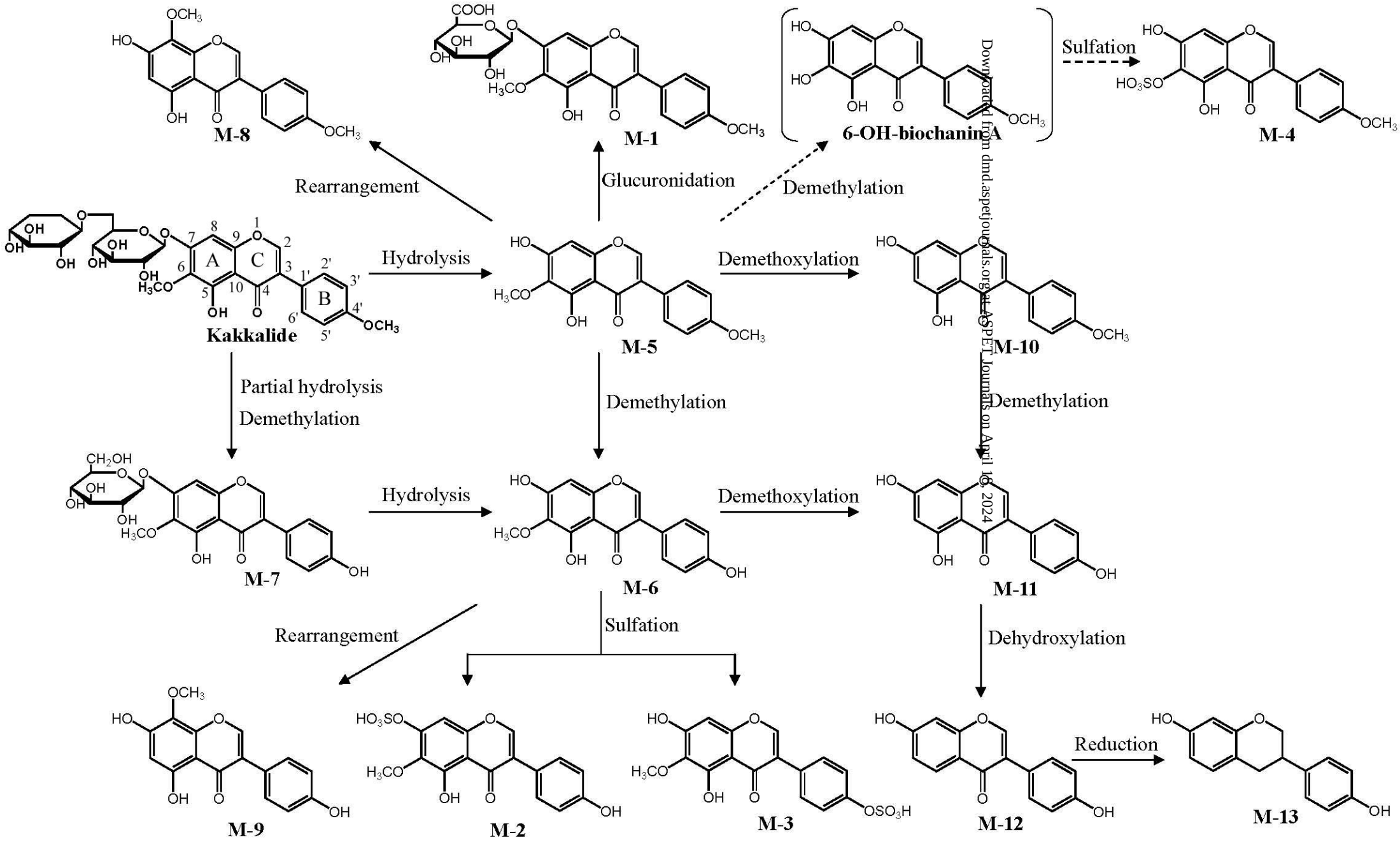
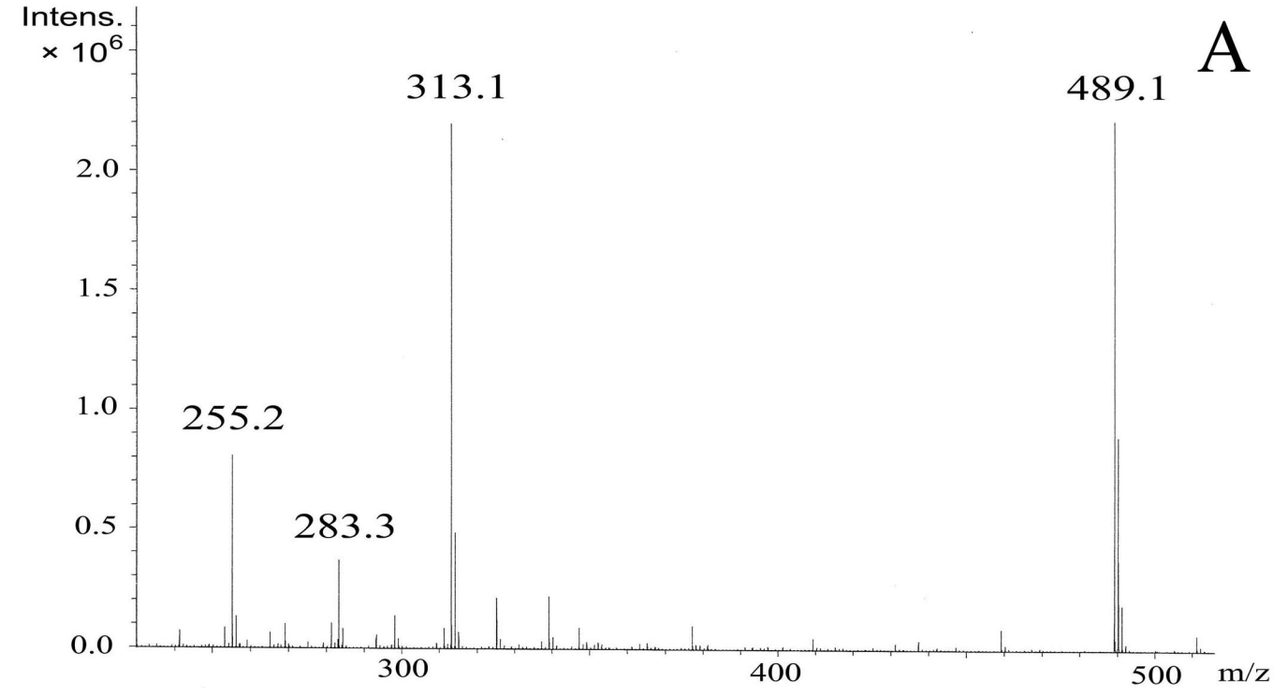
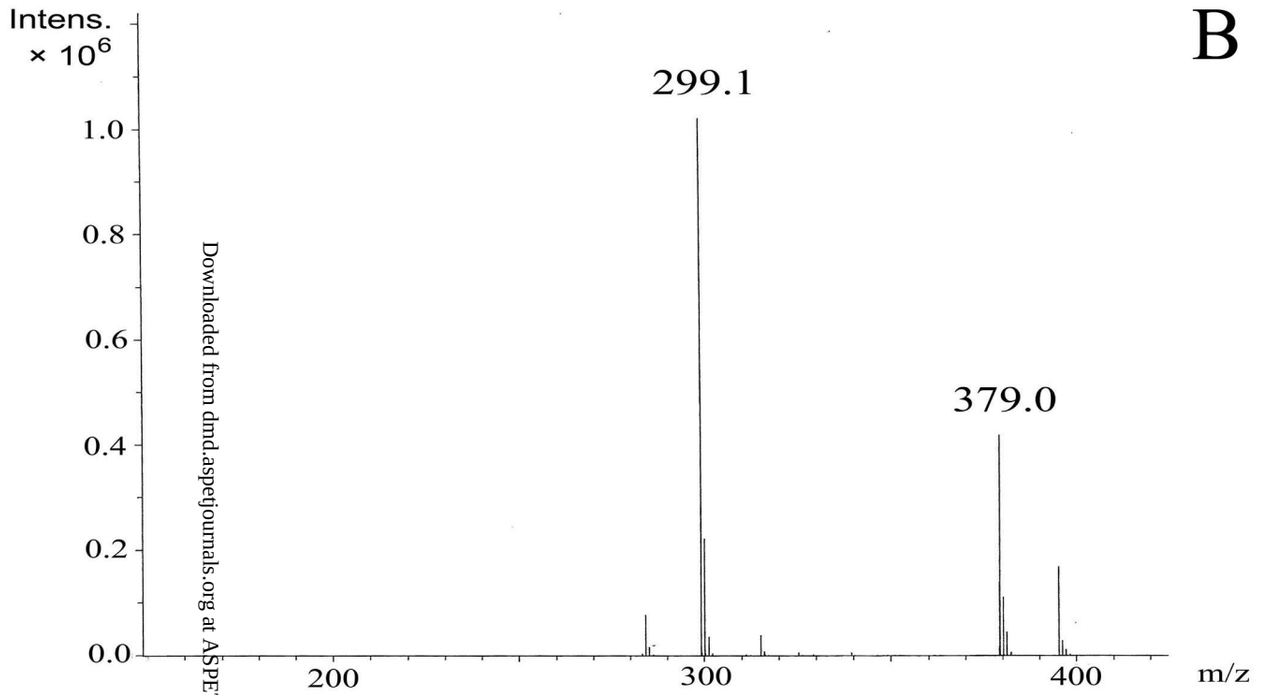
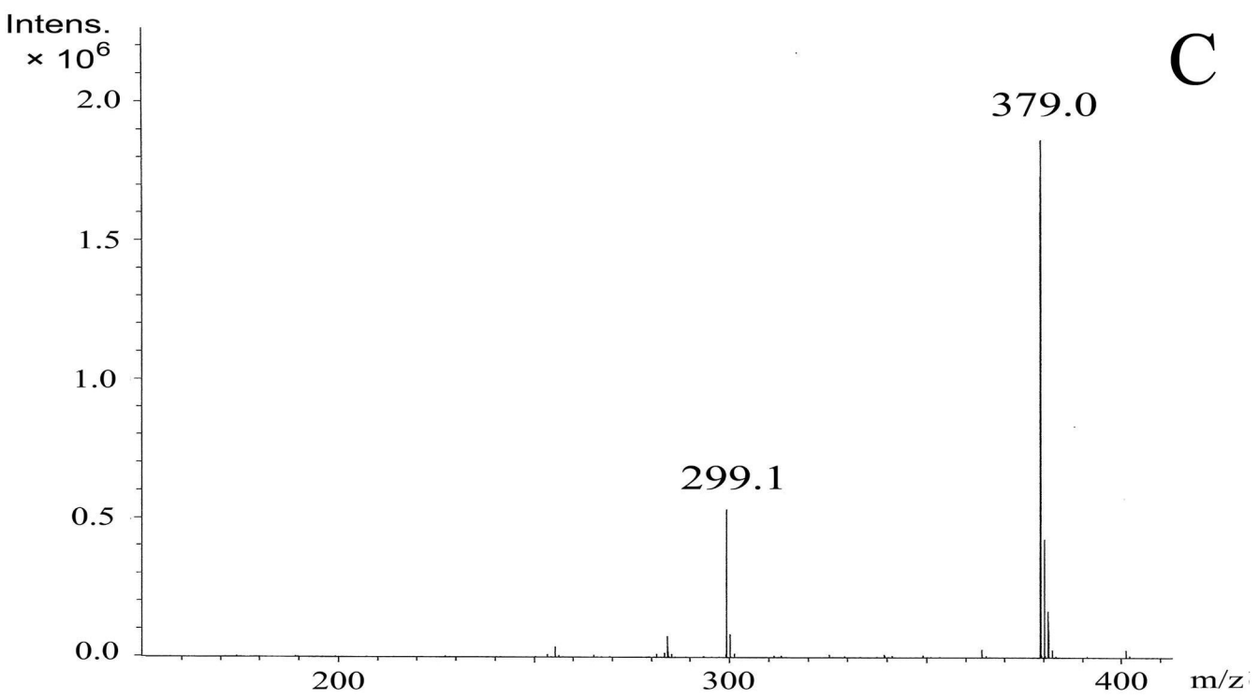


Fig.2







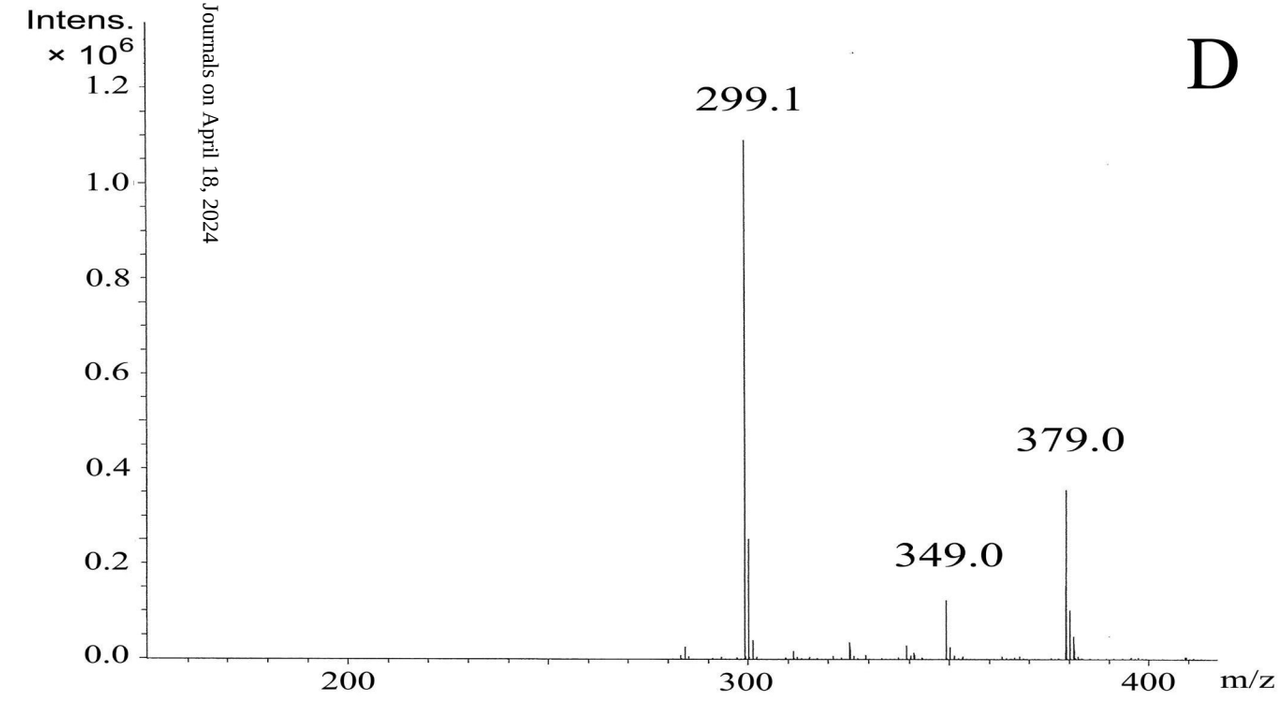


Fig.3