Metabolism of Liriodendrin and Syringin by Human Intestinal Bacteria and their Relation to *in Vitro* Cytotoxicity

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When liriodendrin or syringin was incubated for 24 h with human intestinal bacteria, two metabolites, (+)-syringaresinol-β-D-glucopyranoside and (+)-syringaresinol, from liriodendrin and one metabolite, synapyl alcohol, from syringin were produced. The metabolic time course of liriodendrin was as follows: at early time, liriodendrin was converted to (+)-syringaresinol-β-D-glucopyranoside, and then (+)-syringaresinol. The *in vitro* cytotoxicities of these metabolites, (+)-syringaresinol and synapyl alcohol, were superior to those of liriodendrin and syringin.

Key words : Liriodendrin, Syringin, Intestinal bacteria, Cytotoxicity

INTRODUCTION

Most herbal medicines are administered orally as decoctions. The components of these herbal medicines are therefore inevitably brought into contact with intestinal microflora in the alimentary tract. Most components of herbal medicines can be transformed by the intestinal bacteria before absorption from the gastrointestinal tract. Studies on the metabolism of the components by human intestinal microflora are of a great importance to an understanding of their biological effects (Kim, 1995; Kobashi and Akao, 1997). In Korea, the stem bark of *Kalopanax pictus* belonging to the Araliaceae family has been used in tonic, analgesic and antidiabetic preparations. From these plants, a number of constituents, such as hederagenin glycosides (kalopanaxsaponin A-G), hederagenin and 22α-hydroxyhederagenin have been isolated (Shao *et al.*, 1989a; Shao *et al.*, 1989b; Sano *et al.*, 1991; Porzel *et al.*, 1992). Similar constituents have been isolated from *K. pictum* var. *typicum*, *K. pictum* var. *magnificum* and *K. pictum* var. *chinese* (Cho and Hahn, 1991; Lee and Hahn, 1991; Park and Hahn, 1991). The biological activities of this plant have not been studied except the antihepatotoxic and analgesic activity of liriodendrin and the antidiabetic activity of kalopanaxsaponins (Lee *et al.*, 1995; Park *et al.*, 1998). We could not obtain any information about the metabolism of liriodendrin and syringin, although the metabolic pathway of kalopanaxsaponins was studied (Kim *et al.*, 1998). Furthermore, these intact glycosides are not easily absorbed from the gastrointestinal tract. In order to understand the nature of the metabolites of liriodendrin and syringin produced by human intestinal bacteria and how their biological activity changes, we investigated the metabolism of liriodendrin and syringin by human intestinal bacteria and their *in vitro* cytotoxicity to tumor cell lines.

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MATERIALS AND METHODS

Materials

General anaerobic medium (GAM) and glucose blood liver medium (BL) were purchased from Nissui Pharmaceutical Co., Ltd. The other media were purchased from Difco Co. (U.S.A.). Tumor cell lines were purchased from the Korean Cell Line Bank (Seoul National University, Korea). Liriodendrin and syringin were isolated from *Kalopanax pictus* according to our previous report (Park *et al.*, 1998).

Instruments

Melting points were determined on an electrothermal digital melting point apparatus (Jasco DIP 360). ^{1}H- and ^{13}C-NMR spectra were taken on a Brucker-AMX 500 spectrometer with tetramethylsilane (TMS) as an internal standard. Electron impact-mass spectra (EI-MS) was taken on high resolution mass spectra (VG-VESQ, VG ANALYTICAL. UK. mass spectrophotometer).

Metabolites

To isolate the metabolites of liriodendrin (**I**) or syringin

(**IV**) by human intestinal bacteria, a reaction mixture containing 0.4 mM liriodendrin (or syringin) and 0.5 g fresh human fecal microflora (healthy man, twenties) in a final volume of 50 ml of an anaerobic dilution medium (Mitsuoka, 1980) was incubated at 37°C for 20 h. The reaction mixture was extracted three times with ethylacetate. The ethylacetate extract was applied to silica gel column chromatography (1.5× 20 cm) with $CHCl_3$:MeOH (9:1). Two metabolites, **II** and **III**, isolated from liriodendrin were crystallized with MeOH. One metabolite, **V**, isolated from syringin was crystallized with MeOH.

Compound II ((+)-syringaresinol-β-D-glucopyranoside): White powder (from MeOH). IR (KBr) cm^{-1}: 3424 (OH), 1596, 1516 (aromatic ring). EI-MS *m/z*: 580 (M^+). ^{1}H-NMR (500 MHz, CD_3OD) δ: 3.66 (12H, s, OCH_3x4). 6.60, 6.65 (2H each, s, aromatic H). ^{13}C-NMR (125 MHz, CD_3OD)δ: see Table I.

Compound III ((+)-syringaresinol): Colorless needles (from MeOH), mp 182~183°C, $[\alpha]_D^{20}$ +22.7 (c=7.7, $CHCl_3$). IR (KBr) cm^{-1}: 3428 (OH), 1614, 1520 (aromatic ring). EI-MS *m/z*: 418.3 (M^+). ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 3.20 (2H, m, H-1, 5), 3.91 (12H, s, OCH_3x4) 4.30~4.40 (4H, m, H-4, 8), 4.73 (2H, d, *J*=4.2 Hz, H-2, 6), 6.58 (4H, s, aromatic H). ^{13}C-NMR (125 MHz, $CDCl_3$) δ: see Table I.

Table I. ^{13}C-NMR spectral data of metabolites of liriodendrin and siringin by human intestinal bacteria

Carbon number	Chemical shift (ppm)		
	I	II	III
1	53.6	53.6	54.3
5	53.6	53.6	54.3
4	71.4	71.2	71.7
8	71.4	71.2	71.7
2	85.0	85.3	86.0
6	85.0	85.1	86.0
1'	134.0	134.1	132.3
1''	134.0	134.4	132.3
2'	104.5	104.4	102.7
2''	104.5	104.0	102.7
3'	152.6	152.6	147.1
3''	152.6	148.0	147.1
4'	137.1	137.2	134.3
4''	137.1	135.1	134.3
5'	152.6	152.6	147.1
5''	152.6	148.0	147.1
6'	104.5	104.4	102.7
6''	104.5	104.0	102.7
Glc-1	102.8	102.9	
2	74.2	74.2	
3	76.5	76.5	
4	70.1	70.1	
5	77.1	77.1	
6	61.0	61.1	
OCH_3	56.2	56.2	56.3
OCH_3	56.2	56.2	56.3

Thin layer chromatography (TLC)

TLC for liriodendrin (**I**), syringin (**IV**), **II**, **III** and **V** were performed on silica gel plates (Merck, silica gel 60F-254) as follows: developing solvents system, $CHCl_3$: methanol (4:1). The quantity of these compounds were assayed with a TLC scanner (Shimadzu CS-920).

Time courses of metabolism of liriodendrin and syringin by intestinal bacteria

Liriodendrin- and syringin-metabolizing activities were measured as follows. First, the assay mixture contained 0.5 mM liriodendrin (or 1.0 mM syrigin) and 0.5 g fresh human fecal microflora in a final volume of 20 ml of an anaerobic dilution medium. The mixture was incubated at 37°C for one day and an aliquot (2 ml) of the reaction mixture was periodically extracted twice with 10 ml of ethylacetate. The ethylacetate fraction was analyzed by TLC.

Second, 5 ml of the 10-fold diluted fresh human fecal suspension or the isolated bacteria which was previously cultured was inoculated into 45 ml of GAM broth containing 0.5 mM liriodendrin (or 1.0 mM syrigin) and was then incubated at 37°C for 1 day, and an aliquot (2 ml) of the reaction mixture was periodically extracted twice with 5 ml of ethylacetate. The ethylacetate fraction was analyzed by TLC.

Isolation of intestinal bacteria metabolizing liriodendrin

A suspension of the fresh feces of a Korean man was diluted 10^5 to 10^8-fold with the GAM medium. An aliquot (200 μl) of the 10^7-diluted human feces was inoculated into a BL agar plate, and was then anaerobically incubated at 37°C for 4 days. Each colony was incubated in 5 ml of GAM broth containing 0.2 mg liriodendrin. The cultured media were extracted with 5 ml of ethylacetate. After evaporation, each ethylacetate fraction was analyzed for liriodendrin, (+)-syringaresinol-β-D-glucopyranoside and (+)-syringaresinol by TLC. Although most of intestinal bacteria isolated from human feces metabolized liriodendrin to (+)-syringaresinol, K-111 had the potent liriodendrin metabolizing activity. Identification of these isolated bacteria was performed according to Bergey's mannual (Kreig, 1984). K-111, which was a gram-positive, β-glucosidase-positive, α-rhamnosidase-negative, methyl red test-positive and anaerobic rod, was identified as *Bifidobacterium breve*.

In vitro cytotoxicity assay

The *in vitro* cytotoxicity assay was performed according to the method of Carmichael *et al.* (1987).

RESULTS AND DISCUSSION

Metabolites of liriodendrin and syringin by human intestinal bacteria

To investigate the metabolites of liriodendrin by human intestinal bacteria, liriodendrin was anaerobically incubated for 12 h with a bacterial mixture from human feces. Then, the metabolites were extracted with ethylacetate, separated by silica gel column chromatography, and analyzed by TLC, ^{13}C- and ^{1}H-NMR and EI-MS.

Two metabolites (**II** and **III**) were observed by TLC. These metabolites exhibited absorption due to hydroxyl (3420~3250 cm^{-1}) and aromatic function (1614~1516 cm^{-1}) in the IR spectra. The Rf of metabolite **II** was 0.45 on TLC (developing solvent system in MATERIALS AND METHODS). EI-MS of **II** showed a molecular ion peak at *m/z* 580 (M^+). TLC chromatogram and ^{1}H- and ^{13}C-NMR spectra of **II** showed that the lignan skeleton of liriodendrin was intact but one glucosyl moiety was missing among two glycosyl moiety. By these results, **II** was (+)-syringaresinol-β-D-glucopyranoside, which was previously isolated from *Cistanchis herba* (Kobayashi *et al.*, 1985). The Rf of metabolite **III** was 0.70 on TLC (developing solvent system in MATERIALS AND METHODS). TLC chromatogram and ^{1}H- and ^{13}C-NMR spectra of **III** showed that the lignan skeleton of liriodendrin was intact but all two glycosyl moeities were missing. EI-MS of **III** showed a molecular ion peak at *m/z* 418.3 (M^+). By these results, **III** was identified as (+)-syringaresinol.

To compare the metabolites of syringin with those of liriodendrin, syringin was also anaerobically incubated for 12 h with a bacterial mixture of fresh human feces. One metabolite, **V**, was separated by means of silica gel column chromatography. The Rf of metabolite **V** was 0.74 on TLC (developing solvent system in MATERIALS AND METHODS). Comparing with an authentic compound, **V** was identified as synapyl alcohol.

The time course of metabolism of liriodnedrin and syringin

The time course of transformation of liriodendrin and syringin by human intestinal bacteria was shown in Fig. 1. Liriodendrin started to be converted to **II** and it was quickly transformed to **III** during incubation time. The major metabolite was **III** for 6 h incubation with human intestinal bacteria. When syringin was incubated with human intestinal bacteria, syringin was quickly converted to synapyl alcohol. The other metabolites could not be identified. When liriodendrin was anaerobically incubated with fresh human fecal suspension in GAM broth, it was transformed to **II** during the growth of human intestinal bacteria and then **II** was quickly transformed to **III**. The biotransformtion of syringin to **V** proceeded more quickly than that of liriodendrin to **III**. The metabolic pathways of liriodendrin and syringin by K-111, which was *Bifidobacterium*

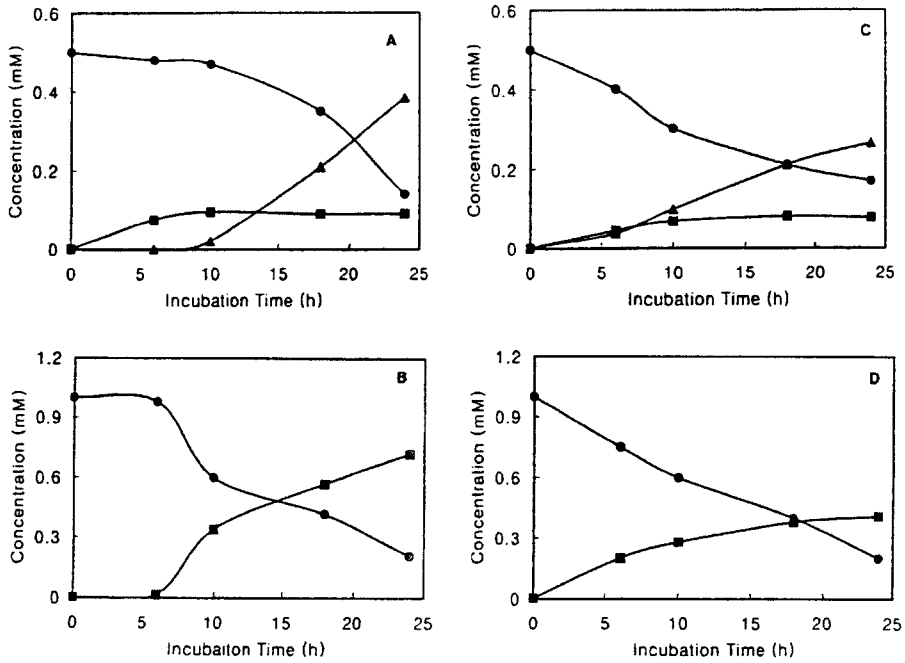
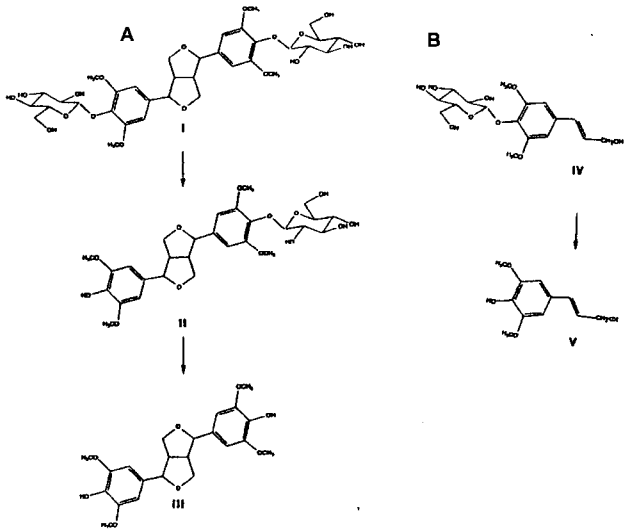


Fig. 1. Time course of metabolism of liriodendrin (A, C) and syringin (B, D): A and B, metabolism by human intestinal bacteria; C and D, metabolism by *Bifidobacterium breve* K-111. Symbols of A and C are indicated as follows: ●, liriodendrin; ■, (+)-syringaresinol-β-D-glucopyranoside; ▲, (+)-syringaresinol. Symbols of B and D are indicated as follows: ●, syringin; ■, synapylalcohol.



Scheme 1. Proposed metabolic pathways of liriodendrin (A) and syringin (B) by human intestinal bacteria: **I**, liriodendrin; **II**, (+)-syringaresinol-β-D-glucopyranoside; **III**, (+)-syringaresinol; **IV**, syringin; **V**, synapyl alcohol.

breve isolated from human intestinal bacteria, were similar to those of liriodendrin and syringin by human intestinal bacteria, respectively. The method of chemical hydrolysis, such as HCl or NaOH, is not suitable due to the formation of the isomers of (+)-syringaresinol by the chemical method to transform liriodendrin to (+)-syringaresinol. However, this enzymatic method did not make its isomers. Therefore, it was thought that β-glucosidase of *B. breve* K-111 could be useful to transform liriodendrin to (+)-syringaresinol.

It is likely that liriodendrin and syringin can be easily transformed to **III** and **V**, respectively in the intestine by human intestinal bacteria (Scheme 1), and these metabolic processes may be important in the pharmacological effect of these compounds.

In vitro cytotoxicity of metabolites against tumor cell lines

The *in vitro* cytotoxicity of liriodendrin, syringin and their metabolites by human intestinal bacteria on tumor cell lines was assayed (Table II). The metabolites, **III** and **V**, showed more potent cytotoxicities against tumor cell lines than liriodendrin and syringin. The ED_{50} values of **III** and **V** were 34~56 and 0.8~1.4 mM, respectively. We found that the cytotoxicity was increased when the glycosides of liriodendrin and syringin were metabolized to their aglycones by human intestinal bacteria. These results suggest that natural glycosides are prodrugs which can be transformed to active compounds by intestinal bacteria.

Table II. *In vitro* cytotoxicity of liriodendrin, syringin and their metabolites against tumor cell lines

Compound	ED_{50} (mM)		
	P388[a]	L-1210[b]	U-937[c]
Liriodendrin (I)	84	68	75
III	56	34	47
Siringin (IV)	>100	>100	>100
V	0.8	1.0	11.4

[a]P-388, mouse lymphoid neoplasma cell line.
[b]L-1210, mouse lymphocytic leukemia cell line.
[c]U-937, human promyelocytic leukemia cell line. III, (+)-syringaresinol. V, synapyl alcohol.

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