HPLC Profile Analysis of Hepatoprotective Oleanene-Glucuronides in Puerariae Flos[1)]

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In order to confirm the constitution of hepatoprotective oleanene glucuronide (OG), HPLC profile analyses of the total OG fractions of both Puerariae Thomsonii Flos (the flowers of *Pueraria thomsonii*) and Puerariae Lobatae Flos (the flowers of *P. lobata*) were performed. No remarkable difference in the HPLC profiles with respect to OGs in the flowers was shown, in contrast to those of the roots. By repeated chromatography of the total OG fraction of Puerariae Thomsonii Flos, soyasaponin I (1), kaikasaponin III (2) and kakkasaponin I (3), which had been already isolated from Puerariae Lobatae Flos, were obtained. The hepatoprotective activity of 2 towards immunologically induced liver injury was significantly more effective than that of 1. This information supported previously obtained structure–hepatoprotective relationship data which was measured on another model. The structure–activity relationship information which suggested that the hydroxymethyl group of the galactosyl unit would enhance the hepatoprotective activity was also substantiated.

Key words *Pueraria thomsonii*; *Pueraria lobata*; triterpenoidal saponin; oleanene glucuronide; HPLC profile analysis; hepatoprotective activity

Puerariae Lobatae Flos, the flowers of *Pueraria* (*P.*) *lobata*, is a crude drug used to counteract the overconsumption of alcohol in Japan and China.[2)] During the course of our studies on this crude drug, we have reported a series of olean-12-ene type triterpenoidal glucuronides (oleanene glucuronide, OG).[3)] Furthermore, we confirmed that the total OG fraction was effective in treating an experimental *in vivo* liver injury model.[4)] On the other hand, we have isolated eleven new OGs, together with two known ones,[5)] from Puerariae Lobatae Radix, the roots of *P. lobata*. Although we have obtained five OGs from Puerariae Thomsonii Radix, which is the other Puerariae Radix (the roots of *P. thomsonii*),[6)] the structures of OGs obtained from both crude drugs were different. Moreover, we ascertained that the HPLC profile of the OG fraction which originated from Puerariae Thomsonii Radix differed from that of Puerariae Lobatae Radix.[7)] In addition to differences in the HPLC profiles, the preventive effects of the total OG fraction of both crude drugs against the immunological *in vitro* liver injury model[8a)] were also different.[7)]

Herein we describe the HPLC profile analyses of the total OG fractions for both Puerariae Flos (the flowers of *P. lobata* and *P. thomsonii*) and the hepatoprotective activities of isolated OGs, discussing the structure–activity relationships.

Results and Discussion

A methanolic extract of Puerariae Thomsonii Flos (the flowers of *P. thomsonii*) was separated by Sephadex LH-20 column chromatography to afford the total OG fraction. Similarly, the total OG fraction in Puerariae Lobatae Flos (the flowers of *P. lobata*) was prepared. In order to confirm the constitution of OGs, HPLC profile analyses of the total OG fractions of both Puerariae Flos were performed (Fig. 1). Although the HPLC profile for the total OG fraction in the roots of *P. lobata* differed from that of *P. thomsonii*,[7)] no great difference in the HPLC profiles with respect to OGs in the flowers was readily apparent.

After repeated silica gel chromatography of the total OG fraction in Puerariae Thomsonii Flos, compounds **1**—**3** were obtained. They were identified as soyasaponin I,[3,9)] kaikasaponin III,[3,10)] and kakkasaponin I,[3)] respectively. Since they had already been isolated from Puerariae Lobatae Flos,[3)] the constitution of OGs in both crude drugs was concluded to be almost identical.

Next, we compared the hepatoprotective actions of these compounds (**1**—**3**). In a previous paper,[8a)] we reported that the activity of alanine aminotransferase (ALT) in the medium was in good agreement with the extent of hepatocyte damage induced by immunological liver injury. Therefore, cell damage was evaluated by means of ALT activity. The hepatoprotective activity is summarized in Table 1. All tested compounds exhibited protective activity. However, since the levels of activity differed, it may be speculated that hepatoprotective activity depends on some structural features.

The tested OGs were classified into two groups, namely, soyasapogenol B glycoside (**1**) and sophoradiol glycosides (**2** and **3**). These OGs were also divided into two groups which have different sugar moieties linked at C-3. These were α-L-rhamnopyranosyl-(1→2)-β-D-galactopyranosyl-(1→2)-β-D-glucuronopyranosyl derivatives (S_1) (**1** and **2**) and an α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl-(1→2)-β-D-glucuronopyranosyl derivative (S_2) (**3**). When the actions of **1** and **2** were compared, compound **2** was significantly effective at a concentration less than 100 μM, although **1** was only effective at the highest dose (500 μM). This indicates that the hydroxy group at C-24 would reduce the hepatoprotective activity. This information supported previously obtained structure–hepatoprotective relationship data which was measured on another model.[11)] Since we reported a similar effect for the hydroxyl group at C-23,[12)] the hydroxymethyl group at C-4 seems to reduce the hepatoprotective action, regardless of configuration. From a comparative study in the S_1 and S_2

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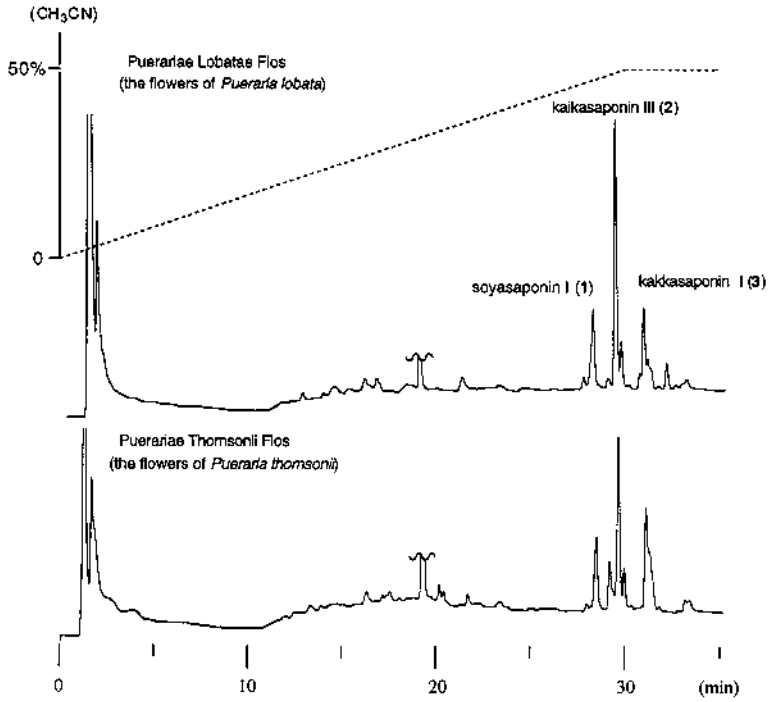
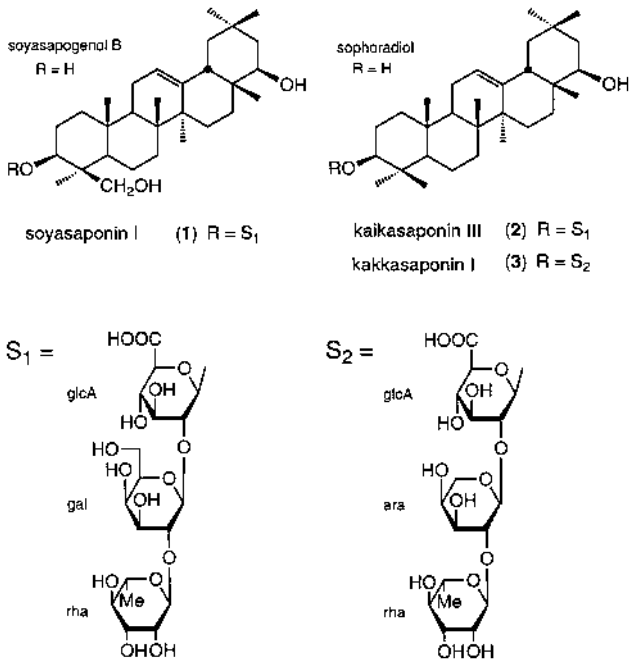


Fig. 1. HPLC Profile for OGs Originated from the Flowers of *Pueraria lobata* and *P. thomsonii*



group, the OG having a galactosyl unit (**2**) in the central sugar moiety shows greater action at any dose than that of the arabinosyl unit (**3**). Hence, the hydroxymethyl group of the galactosyl unit would enhance the hepatoprotective activity. This information also substantiated previously obtained structure–activity relationship data.[8c)]

Table 1. Hepatoprotective Activity of Compounds **1**—**3**

Substance	Dose (μM)	ALT	
		IU/l	Protection (%)
Control	—	11.92±1.0	—
Soyasaponin I (**1**)	0 (ref.)	31.50±1.9	—
	10	36.25±2.5	−24
	30	36.00±2.2	−23
	90	33.25±1.0	−9
	200	29.75±2.2	9
	500	20.50±1.7[a)]	56
Kaikasaponin III (**2**)	0 (ref.)	34.75±0.5	—
	10	37.00±1.4	−10
	30	36.50±3.0	−8
	90	28.75±0.5[a)]	26
	200	26.25±1.0[b)]	37
	500	17.50±2.1[b)]	76
Kakkasaponin I (**3**)	0 (ref.)	39.00±2.7	—
	10	42.25±2.4	−12
	30	40.50±1.0	−6
	90	37.50±1.7	6
	200	31.00±1.4[a)]	30
	500	21.00±3.8[b)]	66

Hepatoprotective actions of compounds **1**—**3** toward *in vitro* immunological liver injury in primary cultured rat hepatocytes. Control is the value of hepatocytes which were not treated with the antiserum. Reference (ref.) is the value of hepatocytes which were treated with the antiserum and not treated with the tested samples. The percent of protection is calculated as [1−(substance−control)/(ref.−control)]×100. Significantly different from ref., effective *a*) $p<0.05$, *b*) $p<0.001$.

Experimental

The instruments and reagents used in this study were the same as those described in the previous papers.[8)]

Extraction and Isolation Half-dried flowers (360 g) of *P. lobata* collected in Kumamoto Prefecture were extracted with MeOH, and the extract (100 g) was partitioned between EtOAc and 40% MeOH. The 40% MeOH layer (80 g) was separated by Sephadex LH-20 column chromatography using MeOH to give the total OG fraction (7 g). Similarly, dried flowers (500 g) of *P. thomsonii* produced in Taiwan gave the total OG fraction (18 g). Subsequently, silica gel column chromatography of a part of the latter OG fraction using $CHCl_3$: MeOH : H_2O=7 : 3 : 0.5→6 : 4 : 1 and 1-BuOH : AcOH : H_2O=4 : 1 : 2 provided compounds **1** (0.002%), **2** (0.014%) and **3** (0.0016%), which were identical with authentic samples.[3)]

Apparatus and Conditions for HPLC Profile Analysis HPLC was carried out on a system which included a pump: CCPM (Tosoh), a UV detector: UV-970 (JASCO), a column heater: U-620 (Sugai) and a column:

Nova-Pak C_{18} (4 μm, 8×100 mm) with a Radial-Pak RCM 8×10 module. The mobile phases were solvent A, H_2O: trifluoroacetic acid (TFA)= 100 : 0.05 (v/v) and solvent B, $CH_3CN : H_2O : TFA = 60 : 40 : 0.05$ (v/v). Analysis was carried out on an elution in a program: 0→83% solvent B (30 min), 83% solvent B (5 min), 83→100% solvent B (5 min), an 100% solvent B (5 min). The flow rate was 1 ml/min, column temperature was 40 °C and the detection was done by UV at 205 nm.

Assay for Hepatoprotective Activity The procedure was described in the preceding paper.[8e] The control is the value of hepatocytes which were not administered antiserum. The control value was 11.92±1.0 (IU/l). The percent of protection is calculated as [1−(sample−control)/(reference−control)]×100. The protection percentage for glycyrrhizin (positive control) was 35% at 500 μM.

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References and Notes

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