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Determination of hydrophobic constituents of the root of *Polygonum multiflorum* using LC-MS/MS and their anti-inflammatory effect in rat hepatocytes

Saki Shirako[1]; Siti Mariyah Ulfa[2, 3]; Takanari Hattori[4]; Seiji Horie[4]; Jun Watanabe[4]; Faith Hays[5]; Mikio Nishizawa[1]

[1]Ritsumeikan University, Kusatsu, Japan; [2]Brawijaya University, Malang, Indonesia; [3]Ritsumeikan University, Ibaraki, Japan; [4]Shimadzu Corporation, Kyoto, Japan; [5]Shimadzu Scientific Instruments, Inc., Columbia, MD

1. Overview

The root of *Polygonum multiflorum* Thunberg (Polygonaceae) is a crude drug known as *Kashu* and thought to improve *blood* deficiency as a Kampo concept. This crude drug has been included in Kampo formulas, such as *Tokiinshi.* In this study, we isolated six constituents from *Polygonum* roots by extraction and fractionation based on hydrophobicity. The content of each constituent was determined using liquid chromatography-triple quadrupole mass spectrometer (LC-MS/MS).To assess anti-inflammatory effect of the constituents, the interleukin (IL)-1β-induced production of nitric oxide (NO), an inflammatory mediator, in rat hepatocytes was monitored. The EtOAc-soluble fraction significantly suppressed IL-1β-induced NO production without showing cytotoxicity, whereas the other fractions did not. Among six constituents emodin and emodin-8-*O*-β-D-glucopyranoside significantly suppressed NO production in hepatocytes, whereas the others showed less potency in suppressing NO production. These results suggest that hydrophobic constituents in the EtOAc-soluble fraction may be responsible for the anti-inflammatory effect of *Polygonum* roots.

2. Introduction

The root of *Polygonum multiflorum* Thunberg (Polygonaceae) is a crude drug known as *Kashu* and thought to improve *blood* deficiency as a Kampo concept. This crude drug has been included in Kampo formulas, such as *Tokiinshi* to treat eczema and dermatitis with itchiness. There are some reports on the constituents in *Polygonum* roots with anti-inflammatory. However, comparison of anti-inflammatory potencies of these constituents is hardly reported. Here, we isolated constituents by extraction from *Polygonum* roots and fractionation based on hydrophobicity and examined their anti-inflammatory effect using primary cultured rat hepatocytes. The content of each constituent was determined using liquid chromatography-triple quadrupole mass spectrometer (LC-MS/MS).

3. Materials and Methods

3-1. Preparation of sample .

Dried roots of *Polygonum multiflorum* Thunberg (collected from Sichuan Province, China) were pulverized and extracted with methanol under reflux. The methanol extract was resuspended in water and partitioned based on hydrophobicity into ethyl acetate (EtOAc)-soluble fraction (fraction A), *n*-butanol-soluble fraction (fraction B), and water-soluble fraction (fraction C). From fraction A of a *Polygonum* root extract, constituents were purified by silica gel chromatography, identified by NMR spectrum analyses, and numbered as compounds **1**-**6**.

3-2. Assessment of anti-inflammatory effect

A liver of Wistar rat was perfused with collagenase, and the dispersed cells were centrifuged, resuspended, and seeded at 1.2 × 10^6 cells per 35 mm diameter dish. Interleukin (IL)-1β-induced production of nitric oxide (NO), an inflammatory mediator, was monitored using the primary cultured rat hepatocytes to assess anti-inflammatory activity.

3-3. Analytical Conditions

LC-MS/MS analysis was performed using a Nexera X3 system coupled with a LCMS-8060 (Shimadzu Corporation, Japan). Separation was achieved on an Inertsil ODS-HL column (GL Sciences, Japan.) under gradient elution.

HPLC (Nexera X3 system)

Mobile phase: 0.1% formic acid in water (solvent A)
0.1% formic acid in acetonitrile (solvent B)
Flow rate 0–5 min, 10–100% B; 5–7.5 min, 100% B;
7.5–7.51 min, 100–10% B; and 7.51–10 min, 10% B.
Injection vol.: 1 μL

MS (LCMS-8060)

Ionization: ESI (Negative mode)
Mode: MRM (Table. 1)

Figure 1 Nexera X3 + LCMS-8060

Table 1 MRM condition

No	Compounds	MRM (Precursor ion > Product ion)
1	Physcion	283.7 > 269.05, 225.11, 241.1
2	Emodin (Em)	269.05 > 225.15, 241.1, 197.1
3	Physcion-8-*O*-β-D-glucopyranoside	445.1 > 283.05, 240.1, 325.05
4	Catechin	289.1 > 245.15, 203.15, 109.1
5	Emodin-8-*O*-β-D-glucopyranoside (Em-Glc)	430.9 > 269.1, 225.1, 240.15
6	2,3,5,4'-tetrahydroxystilbene-2-O-β-D-glucoside (THSG)	405.1 > 243.05, 173.15, 137.05

4. Result

4-1. Fractionation of methanol extract of *Polygonum* roots

The constituents of Fraction A of a *Polygonum* root extract were purified by silica gel chromatography and identified by NMR spectrum analyses (Figure 2). The constituents isolated were physcion (Compound **1**), emodin (**2**), physcion-8-*O*-β-D-glucopyranoside (**3**), emodinnd-8-*O*-β-D-glucopyranoside (**5**), and catechin (**4**), and THSG (**6**).

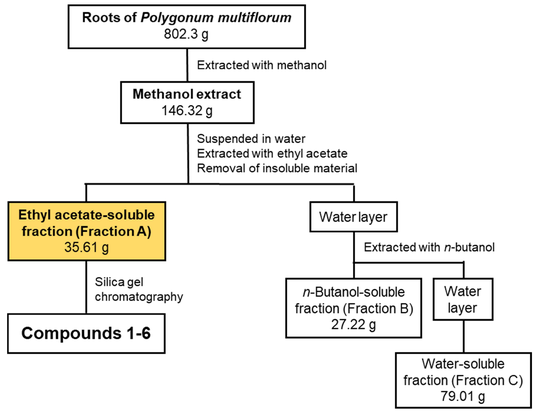

Figure 2 Fractionation of methanol extract of *Polygonum* roots

Among the constituents detected by LC-MS/MS, the content of THSG was the highest (83.9%) in fraction A (Figure 3). Emodin and physcion were 5.41% and 3.61%, respectively, whereas the content of their glycosides was around 1%. Given that the content of THSG was the highest in fraction A and the yield of fraction A was 24.3%, the content of THSG is calculated as 20.4% in the *Polygonum* root extract.

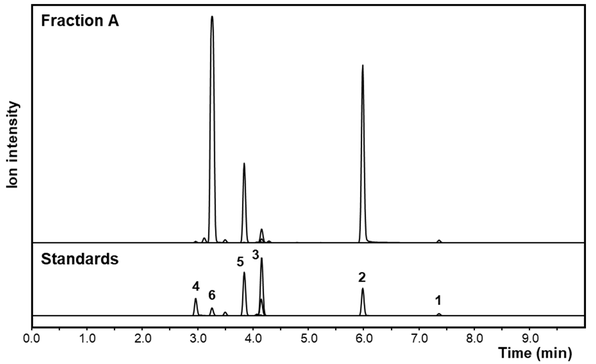

Figure 3 LC-MS/MS analysis of constituents present in Fraction A of a *Polygonum* root extract.

4-2. Anti-inflammatory effect of fractions and constituents

NO production was induced by IL-1β in primary cultured rat hepatocytes, and the effects of a *Polygonum* root extract and it fractions were assessed using the hepatocytes. NO production increased in the presence of IL-1β, whereas NO production was not observed in the absence of IL-1β. When a *Polygonum* root extract was added into the medium, it decreased IL-1β-induced NO production without showing cytotoxicity. When fraction A was added, it decreased NO production in a concentration-dependent manner, without showing cytotoxicity at the concentrations applied. Fraction B also decreased NO production, whereas fraction C did not cause a significant change.

Then, the potencies of the six compounds in the suppression of NO production in the hepatocytes were estimated. Among the compounds, THSG, did not affect NO production at a concentration up to 800 μM, and an IC_{50} value could not be calculated. Emodin and emodin-8-*O*-β-D-glucopyranoside efficiently suppressed NO production, without showing cytotoxicity (Figure 4). In contrast, catechin did not significantly inhibit NO production.

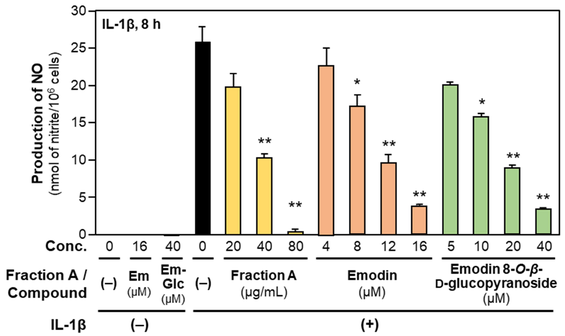

Figure 4 Comparison of anti-inflammatory effect of Fraction A, emodin, and emodin-8-*O*-β-D-glucopyranoside

5. Conclusions

✓ **Our results suggest that hydrophobic constituents in the EtOAc-soluble fraction may possess the anti-inflammatory effect by *Polygonum* roots.**