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Anti-inflammatory effects of anthraquinones of *Polygonum multiflorum* roots

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ABSTRACT

Background: The roots of *Polygonum multiflorum* Thunberg (Polygonaceae) are a crude drug known as *Kashu* and are thought to improve *blood* deficiency as a Kampo concept. Kashu is added to Kampo formulas, including *Tokiinshi,* which is used to treat eczema and dermatitis with itchiness by facilitating blood circulation in the skin. Although anti-inflammatory effects were expected, the reports on anti-inflammatory constituents of *Polygonum* roots are very few.

Objective: To purify pharmacologically active constituents from *Polygonum* roots and compare their anti-inflammatory potency using hepatocytes.

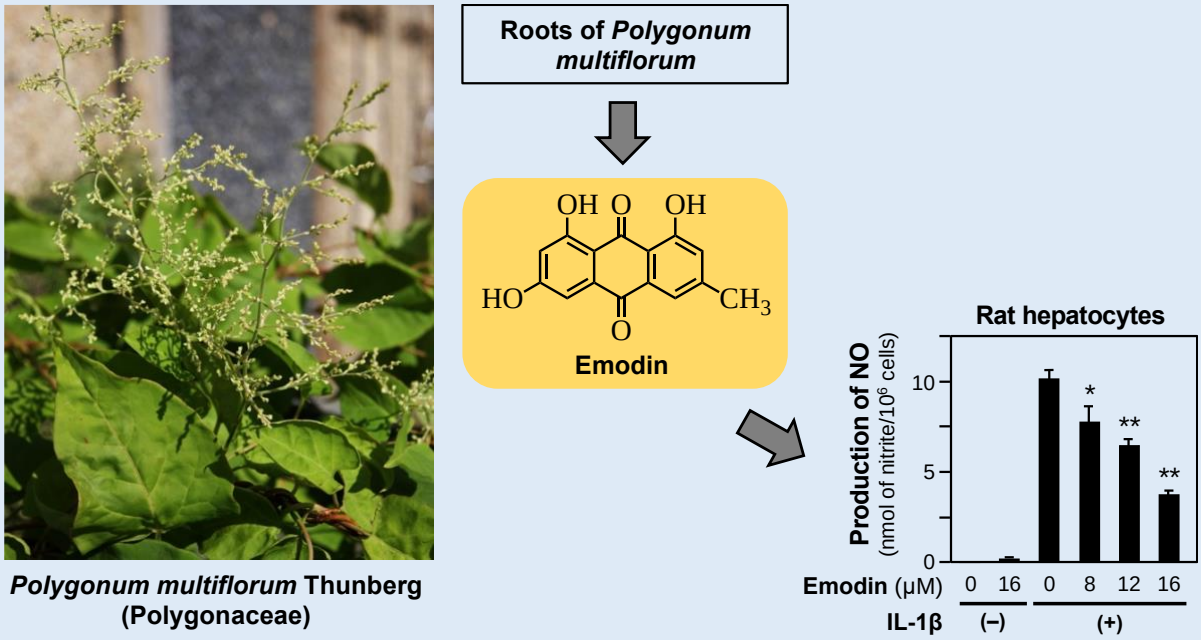
Methods: *Polygonum* roots were extracted and fractionated based on hydrophobicity to three fractions, the ethyl acetate (EtOAc)-soluble, *n*-butanol-soluble, and water-soluble fractions. Constituents were purified from the EtOAc-soluble fraction, and its contents were measured using liquid chromatography-triple quadrupole mass spectrometer. The interleukin (IL)-1β-induced production of nitric oxide (NO), a proinflammatory mediator, in rat hepatocytes was monitored to assess anti-inflammatory activity.

Results: The EtOAc-soluble fraction inhibited IL-1β-induced NO production, whereas the other fractions did not. Six

constituents were isolated from the EtOAc-soluble fraction and identified as (*E*)-2,3,5,4'-tetrahydroxystilbene-2-*O*-β-D-glucoside, emodin, emodin-8-*O*-β-D-glucopyranoside, physcion, physcion-8-*O*-β-D-glucopyranoside, and catechin. Emodin and emodin-8-*O*-β-D-glucopyranoside significantly inhibited NO production in hepatocytes, and the others showed less potency in suppressing NO production. These two anthraquinones decreased the protein and mRNA levels of inducible NO synthase, as well as the mRNA levels of tumor necrosis factor α. In a search of the literature to date, the comparison of pharmacological activity of the constituents of *Polygonum* roots was not reported.

Conclusion: These results suggest that hydrophobic constituents in the EtOAc-soluble fraction, especially anthraquinones, may exert the anti-inflammatory effects by treatment with *Polygonum* roots.

Keywords: anthraquinone, nitric oxide, inflammation, Kampo medicine, LC–MS/MS.



***Polygonum multiflorum* Thunberg (Polygonaceae)**

INTRODUCTION

Polygonum multiflorum Thunberg (Polygonaceae) is a perennial vine-like plant that grows in China. Its roots (called *Kashu*) have been utilized in traditional Japanese medicine, *i.e.*, Kampo medicine. *Polygonum* roots are considered to improve a deficiency of *blood*, which is a physiological concept based on Kampo medicine and defined as the red fluid that flows through the body, carries nutrients, and maintains life [1]. *Blood* deficiency in the skin causes insufficient blood microcirculation, resulting in dryness and itching. This crude drug has been included in several Kampo formulas, for example, *Tokiinshi,* which is used to treat eczema and dermatitis with itchiness. Therefore, *Polygonum* roots are expected to possess anti-inflammatory effects.

A major constituent of *Polygonum* roots is 2,3,5,4'-tetrahydroxystilbene-2-*O*-β-D-glucoside (THSG), a stilbene glycoside [2], and the anti-inflammatory effects of THSG have been examined *in vitro* and *in vivo*. When THSG and bacterial endotoxin (*i.e.*, lipopolysaccharide, LPS) were added together to the culture medium of a mouse macrophage line, RAW264.7, THSG inhibited the LPS-induced production of prostaglandin E_2 (PGE_2), which is a proinflammatory mediator that is synthesized by cyclooxygenase 2 (COX-2) [3]. Another proinflammatory mediator, nitric oxide (NO), is synthesized by inducible nitric oxide synthase (iNOS) in macrophages and hepatocytes [4]. PGE_2 and NO play crucial roles in

physiological and pathophysiological conditions [5–6]. THSG inhibits the expression of the iNOS protein induced in response to interleukin (IL)-1β in rat chondrocytes [7]. In addition, THSG has been shown to attenuate carrageenan-induced paw edema and dimethylbenzene-induced ear edema and improve mono-iodoacetate-induced osteoarthritis in experimental rat models [7]. Although there are other constituents, such as anthraquinones (*i.e.*, emodin and physcion) [8] and flavonoids (*e.g.*, catechin), in *Polygonum* roots, comparisons of the anti-inflammatory potencies of these constituents are rarely reported.

When foreign pathogens induce inflammation, NO plays crucial roles in physiological and pathophysiological conditions [6]. LPS stimulates the resident hepatic macrophages (Kupffer cells) to produce PGE_2 and IL-1β, which stimulates hepatocytes to express pro-inflammatory genes, including the *iNOS* gene [4]. IL-1β-treated rat hepatocytes mimic liver injury, and the inhibition of NO production by a constituent is correlated with the anti-inflammatory effect of that constituent. Therefore, NO levels are monitored to evaluate the anti-inflammatory effect of the constituents of crude drugs, *e.g.*, Cnidii Rhizoma (rhizomes of *Cnidium officinale*) [9] and Glycyrrhizae Radix (roots and stolons of *Glycyrrhiza uralensis*) [10].

In the current study, the anti-inflammatory effects of *Polygonum* roots were examined by isolating the constituents from a root extract. Then, the potencies of the constituents in the inhibition of IL-1β-induced NO production and the induction of proinflammatory gene expression in the rat hepatocytes were estimated.

MATERIALS AND METHODS

General experimental procedures: Using a JNM-ECS400 NMR spectrometer (JEOL Ltd., Akishima, Tokyo, Japan) operated at 400 MHz (^{1}H) and 100 MHz (^{13}C), NMR spectra were recorded. An HPLC system using an LC-20AD pump equipped with an SPD-20A UV/VIS detector (Shimadzu Corporation, Kyoto, Japan) was used. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was carried out with an LCMS-8060 (Shimadzu Corporation) equipped with an Inertsil ODS-HL column [2.1 mm internal diameter (i.d.) × 100 mm; particle size, 3 µm; GL Science, Tokyo, Japan] and LabSolutions software (Shimadzu Corporation). A JMS-700 MStation mass spectrometer (JEOL Ltd.) was used to obtain FAB-MS spectra. Optical rotations of compounds were measured on a DIP-1000 polarimeter (JASCO Corporation, Hachioji, Tokyo, Japan). Silica gel column chromatography was run with Wakogel C-300 HG (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) or Silica Gel 60 (Nacalai Tesque Inc., Kyoto, Japan). Tetramethylsilane (internal standard of NMR spectrometry), deuterated chloroform ($CDCl_3$), and deuterated dimethyl sulfoxide (DMSO-d_6) were purchased from Euriso-Top (Saint-Aubin, France). Cosmonice Filter S (pore size, 0.45 µm; 4 mm filter diameter) was purchased from Nacalai Tesque Inc. The *n*-octanol/water partition coefficient (Log P) was predicted using the ALOGPS 2.1 program [11].

Plant material: Roots of *Polygonum multiflorum* Thunberg collected from Sichuan Province, China, were purchased from Tochimoto Tenkaido Co., Ltd. (Osaka, Japan). Dr. Yutaka Yamamoto (Tochimoto Tenkaido Co., Ltd.) authenticated as Kashu. The voucher sample was deposited in the Ritsumeikan Herbarium of Pharmacognosy, Ritsumeikan University (code number RIN-PM-30).

Purification of constituents from the roots of Polygonum multiflorum: Dried roots of *Polygonum multiflorum* (802.3 g) were pulverized and subjected to methanol (MeOH; 8 L) extraction under reflux three times. Evaporation of the filtrate under vacuum yielded 146.32 g (18.24%) of black gum. The MeOH extract was resuspended in water and partitioned with ethyl acetate

(EtOAc), as previously described [12]. After the removal of insoluble materials, the fraction was concentrated to yield 35.61 g of EtOAc-soluble fraction (Fraction A). Fraction A, which showed the highest suppressive effect against NO production in hepatocytes, was subjected to purification by Silica Gel 60 column chromatography (20–230 mesh; 7.0 cm i.d. × 20 cm; Nacalai Tesque, Inc.) with stepwise elution using $CHCl_3$:MeOH (100:0 to 0:100) to produce nine subfractions (A1–A9). Subfraction A1 (460 mg) was purified by silica gel column chromatography with stepwise elution using $CHCl_3$:*n*-hexane (70:30 → 100:0) to yield compound **1**, a yellow powder (156 mg). Subfraction A3 was obtained as an orange powder and confirmed to be compound **2** (217 mg) after elution by silica gel column chromatography using $CHCl_3$:MeOH (100:0 → 0:100). Subfraction A5 (662 mg) was recrystallized from MeOH to produce the yellow precipitate A5.1 (137 mg), and the filtrate was evaporated to produce the orange powder A5.2 (369 mg). Purification of A5.1 by preparative HPLC using a Cosmosil 5C18-AR-II (10 mm i.d. × 250 mm) column with the isocratic solvent 0.1% acetic acid/MeOH (10:90) produced A.5.1.2, a yellow powder (25.15 mg) identified as compound **3**. Purification of A.5.2 (369 mg) by silica gel column chromatography with the gradient eluent $CHCl_3$/MeOH (90:10 → 0:100) followed by crystallization over MeOH produced Subfraction A.5.2.7.4a, a brown powder (20 mg) that was identified as compound **4**. Recrystallization of Subfraction A6 (268 mg) using MeOH produced the precipitate A6.2, a yellow powder (63 mg) as compound **5**. Subfraction A8 (6.168 mg) was collected as light-brown crystals as compound 6.

Quantification of the content of each compound by LC–MS/MS analysis: Each purified compound was dissolved in 60% acetonitrile and clarified by passing through a Cosmonice Filter S. Each filtrate was injected into an LCMS-8060 (Shimadzu Corporation) equipped with an Inertsil ODS-HL column. The multiple reaction monitoring (MRM) condition was optimized using LabSolutions (Shimadzu Corporation). A binary linear gradient was used with 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The gradient program was as follows: 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. The column was maintained at 40 ℃. The content of each compound in Fraction A of a *Polygonum* root extract was quantified via LC–MS/MS in the MRM mode. The calibration equations and correlation coefficients of the standard compounds were as follows: physcion, y = 580384x—1932.7 (R^2 = 0.9999); emodin, y = 50000000x + 48427 (R^2 = 0.9999); physcion-8-*O*-β-D-glucopyranoside, y = 30000000x + 16347 (R^2 = 0.9999); emodin-8-*O*-β-D-glucopyranoside, y = 80000000x + 17846 (R^2 = 0.9999); (*E*)-THSG, y = 10000000x—5138.4 (R^2 = 0.9999); and catechin, y = 2000000x—6548.1 (R^2 = 1.0000). The peak areas of each compound in the sample were fit to the calibration curves. The content was calculated as each compound (mg) divided by Fraction A of a *Polygonum* root extract (mg).

Animals: All animal care and experimental procedures were carried out in accordance with the laws and guidelines of the Japanese government and were approved by the Animal Care Committee of Ritsumeikan University, Biwako-Kusatsu Campus (BKC2017-052 and BKC2020-045). Specific pathogen-free Wistar rats (male, 5–6 weeks old; Charles River Laboratories Japan Inc., Yokohama, Japan) were housed at 21–23 ℃ under a 12-h light/dark cycle. The animals were fed a γ-ray-irradiated CRF-1 diet (Charles River Laboratories Japan) with water available *ad libitum* and acclimatized to the housing for at least seven days.

Primary cultured rat hepatocytes: Hepatocytes were isolated from the liver of Wistar rats, according to the previously published method [13]. The liver was perfused

with collagenase (FUJIFILM Wako Pure Chemical Corporation). The dispersed cells were centrifuged and resuspended. The purity of the hepatocytes was more than 99% by microscopic observation (data not shown). The hepatocytes were seeded at 1.2×10^6 cells per 35-mm-diameter dish and incubated at 37 ℃ for 2 h. After the replacement of a fresh medium, the cells were further incubated at 37 ℃ overnight.

NO production and lactate dehydrogenase (LDH) activity: On Day 1, each fraction or compound was added to culture medium, and hepatocytes were incubated for 8 h. The concentration of nitrite, which is a stable metabolite of NO, in the medium was measured using the Griess method [14–15]. The NO levels measured in the absence and presence of IL-1β were set as 0% and 100%, respectively. The half-maximal inhibitory concentration (IC_{50}) value for nitrite was determined in triplicate for at least three different concentrations of an extract, a fraction, or a compound [14]. As a positive control, loxoprofen sodium (Kolon Life Science, Inchon, South Korea) was included [14]. Using an LDH Cytotoxicity Detection Kit (Takara Bio Inc., Kusatsu, Japan), LDH activity in culture medium was estimated to monitor cytotoxicity. The IC_{50} value was calculated to assess the potency of a fraction or compound in inhibiting NO production when it did not show cytotoxicity.

Direct NO quenching activity: Each compound was added to culture medium containing 25 μM $NaNO_2$ and incubated at 37 ℃ for 1.5 h, as previously described [10]. The final concentration of each compound was 10 μM (emodin) or 15 μM (emodin-8-*O*-β-D-glucopyranoside). The medium was mixed with Griess reagent [14–15] and incubated at 23 ℃ for 5 min. To determine a decrease of nitrite by the compound, the absorbance at 540 nm was measured.

Western blot analysis: Hepatocytes were incubated with 1 nM IL-1β and a fraction or compound for 8 h and lysed. The lysates were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a Sequi-Blot membrane (Bio–Rad, Hercules, CA, USA) followed by blocking with 5% Difco skim milk (BD Biosciences, San Jose, CA, USA) [9]. For immunostaining, primary antibodies against iNOS (Clone 54; BD Biosciences) and β-tubulin (Cell Signaling Technology Inc., Danvers, MA, USA) and an anti-immunoglobulin Fc antibody conjugated with horseradish peroxidase were used. The protein was visualized with ECL Western Blotting Detection Reagents (Cytiva, Tokyo, Japan) and detected using an Amersham Imager 600 (Cytiva).

Quantitative reverse transcription-polymerase chain reaction (RT–PCR): Total RNA was extracted from rat hepatocytes using Sepasol I Super G solution (Nacalai Tesque, Inc.) and treated with a TURBO DNA-free Kit (Applied Biosystems, Waltham, MA, USA) [16]. The isolated total RNA was reverse transcribed into cDNA, which was then amplified by PCR with previously described primers [17–18]. Real-time PCR using SYBR Green I and a Thermal Cycler Dice Real Time System (Takara Bio Inc.) was performed to measure mRNA levels in triplicate. The dissociation curve of the PCR-amplified product for each mRNA transcript was checked. When a single-peak melting temperature was observed, their relative mRNA level was calculated from obtained the threshold cycle (Ct) value according to the ΔΔCt method. Ct values were normalized to those for the elongation factor 1α (EF) mRNA transcript [17], which is a housekeeping gene and was used as an internal control. The normalized mRNA levels in total RNA from

hepatocytes in the presence of IL-1β alone were set as 100%.

Statistical analysis: Data are representative of at least three independent experiments with similar findings. Values are expressed as the mean ± standard deviation (SD). Differences between values were analyzed using Student's *t*-test followed by the Bonferroni correction. $P < 0.05$ and $P < 0.01$ were considered to indicate statistical significance.

RESULTS

Suppression of NO production by crude fractions from a Polygonum root extract: The roots of *Polygonum multiflorum* were extracted with MeOH, as previously described [12]. As shown in Figure 1, the resultant MeOH extract was successively partitioned based on hydrophobicity into Fraction A (EtOAc-soluble fraction), Fraction B (*n*-butanol-soluble fraction), and Fraction C (water-soluble fraction).

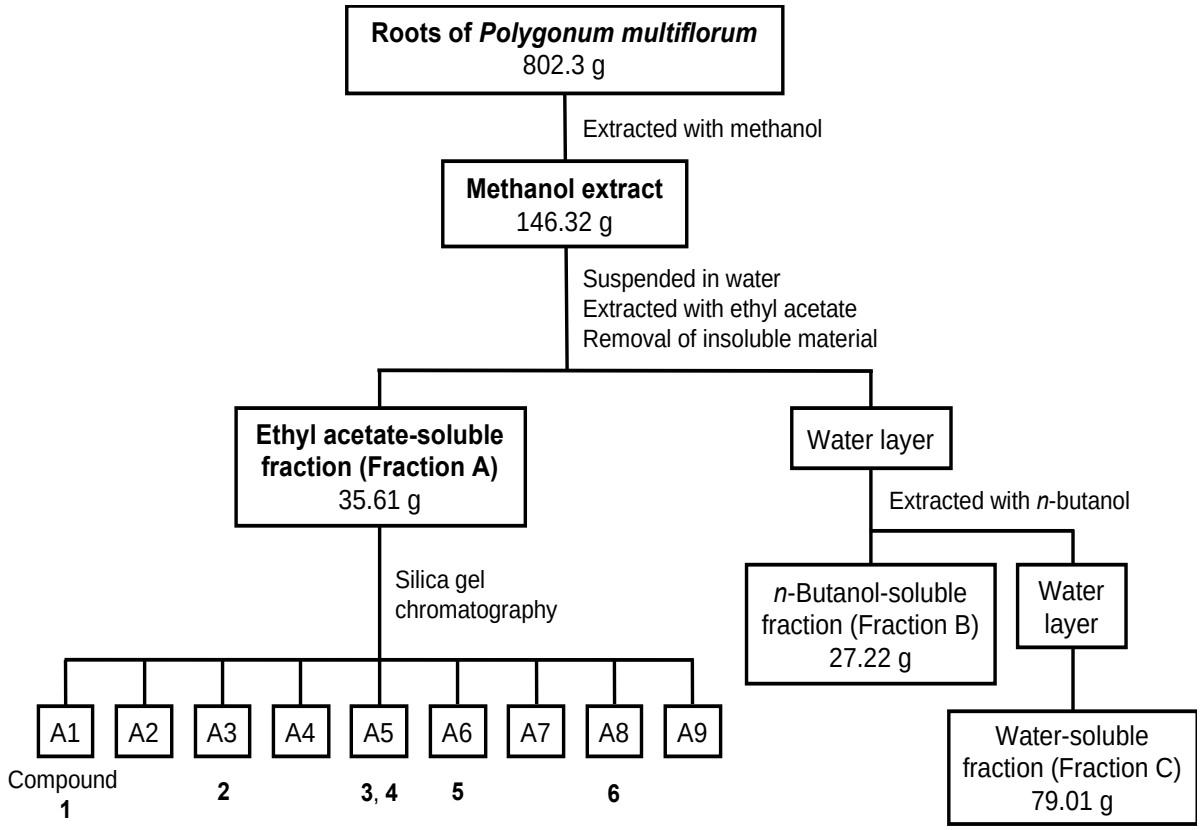


Figure 1. Purification of constituents from the roots of *Polygonum multiflorum*. Flowchart showing the procedures used to fractionate constituents from the roots of *Polygonum multiflorum*. The plant material was extracted with MeOH, and the dried extract was dissolved in water and successively fractionated based on hydrophobicity with EtOAc, *n*-butanol, and water to obtain Fractions A, B, and C, respectively. Constituents isolated in this study are depicted under the relevant subfraction.

The IC_{50} values of the *Polygonum* root extract and its crude fractions were calculated and are summarized in Table 1. Fractions A and B showed lower IC_{50} values than the *Polygonum* root extract for NO production in IL-1β-treated hepatocytes. Because it had the highest potency among the fractions, Fraction A (EtOAc-soluble fraction) was speculated to include hydrophobic constituents that suppress IL-1β-induced NO production.

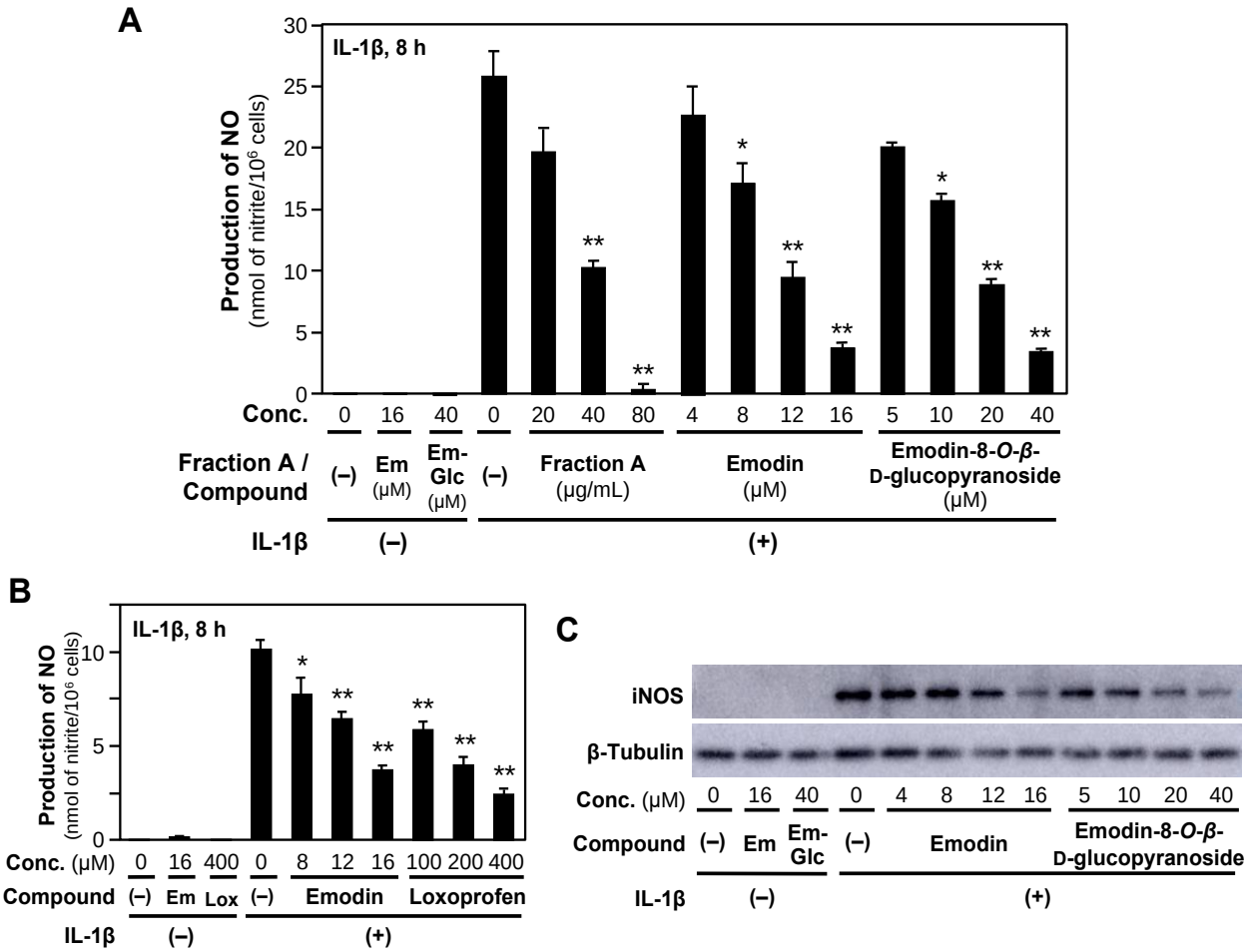


Figure 2. Fraction A and constituents in a *Polygonum* root extract suppress *iNOS* gene expression in hepatocytes. **(A)** The effects of Fraction A, emodin (Em), and emodin-8-*O*-β-D-glucopyranoside (Em-Glc) on NO production. Hepatocytes were treated with 1 nM IL-1β in the presence or absence of Fraction A or a constituent for 8 h. The levels of nitrite (a major metabolite of NO) in the medium were measured in triplicate. The values are shown as the mean ± SD (n = 3). * $P < 0.05$ and ** $P < 0.01$ *versus* IL-1β alone. (**B**) The effects of emodin and loxoprofen on NO production. Hepatocytes were treated with 1 nM IL-1β in the presence or absence of emodin or loxoprofen (Lox; positive control) for 8 h. The levels of nitrite were measured and shown similarly to (A). (**C**) Effects of emodin and emodin-8-*O*-β-D-glucopyranoside on iNOS expression. Cell extracts were prepared from the hepatocytes treated in (A). Each extract (20 μg per lane) was analyzed by Western blotting to detect iNOS (130 kDa) and β-tubulin (55 kDa; internal control).

Table 1. Fractionation of a *Polygonum multiflorum* root extract and the effects on NO production.

Fraction	Yield [%][a]	IC_{50} [μg/mL][b]
Methanol extract	100	105 ± 8.9
A (ethyl acetate soluble)	24.3	24.5 ± 6.0
B (*n*-butanol soluble)	18.6	NA
C (water soluble)	54.0	NA

[a]The percentage was calculated as the weight of each fraction divided by the sum of three fractions. [b] The half-maximal inhibitory concentration for NO production in hepatocytes in the presence of IL-1β (mean ± SD). NA, not applied due to low activity.

Purification of constituents from Fraction A of a Polygonum root extract: The constituents of Fraction A of a *Polygonum* root extract were purified by silica gel chromatography and identified by NMR spectrum analyses, as described in the Materials and Methods. The constituents isolated were (*E*)-THSG (compound **6**) and anthraquinones including physcion (**1**), emodin (**2**), physcion-8-*O*-β-D-glucopyranoside (**3**), emodin-8-*O*-β-D-glucopyranoside (**5**), and catechin (**4**). The spectral characteristics used to determine chemical structures are

described in the Supplementary Data.

Compound **1** was identified as physcion based on the 2D heteronuclear correlation of the ^{1}H NMR and ^{13}C NMR spectra, which were in agreement with published data [19]. Correlation analysis of the ^{1}H NMR and ^{13}C NMR spectra using heteronuclear single quantum correlation (HSQC) and heteronuclear multiple bond connectivity (HMBC) matched published data [19–20], suggesting that compound **2** was identified as emodin. Compound **3** was determined to be physcion-8-*O*-β-D-glucopyranoside based on the ^{1}H NMR and ^{13}C NMR analyses and comparison with published data [19]. Similarly, compound **4** was identified as catechin based on a comparison with published data [21–22]. Compounds **5** and **6** were identified as emodin-8-*O*-β-D-glucopyranoside and (*E*)-2,3,5,4'-tetrahydroxystillbene-2-*O*-β-D-glucopyranoside (THSG), respectively, which were supported by previously reported data [19,23].

The content of the constituents from Fraction A of a Polygonum root extract: Next, the content of each constituent in Fraction A of a *Polygonum* root extract was measured. Using the above-mentioned constituents as standards, LC-MS/MS analysis was performed (Figure 3). Among the constituents detected by LC-MS/MS, THSG accounted for the most content (83.9%) in Fraction A (Table 2). Emodin and physcion had content levels of 5.41% and 3.61%, respectively, whereas the content of their glycosides was approximately 1%.

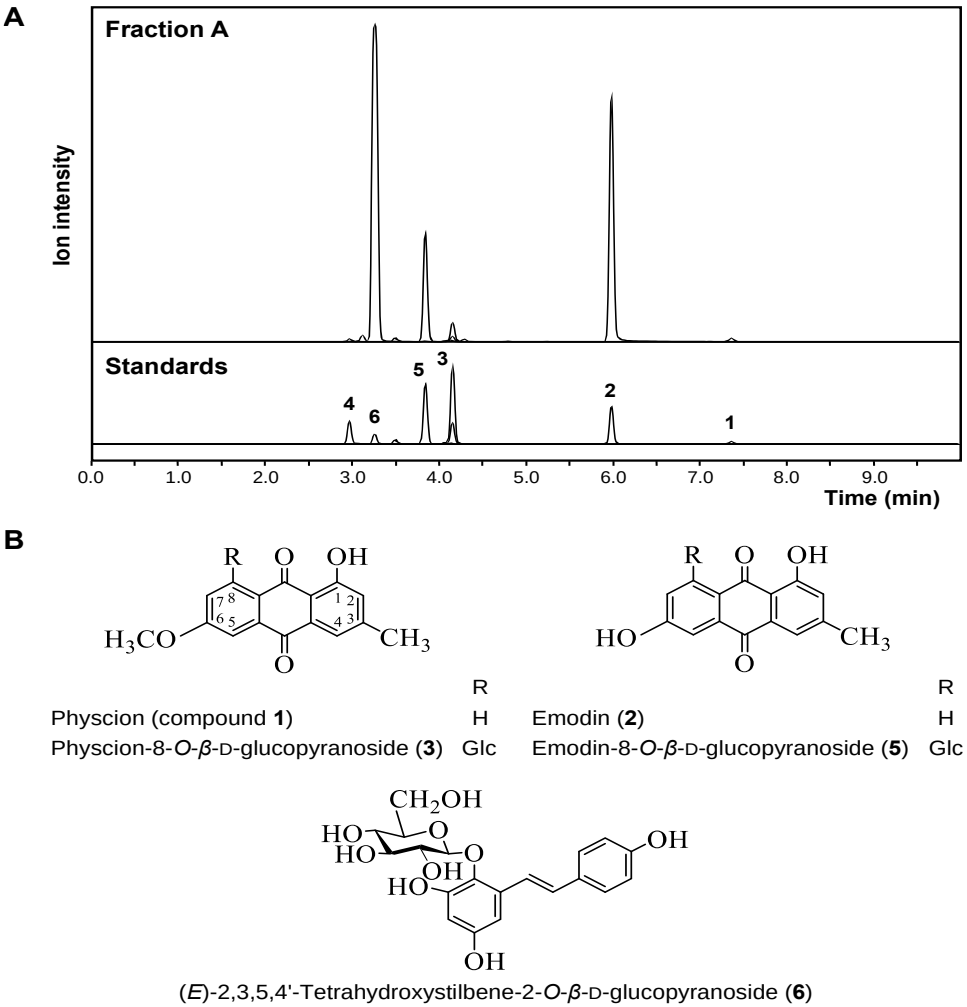


Figure 3. Constituents present in Fraction A of a *Polygonum* root extract. (Top) Total ion chromatograms of constituents in Fraction A of a *Polygonum multiflorum* root extract detected by LC-MS/MS in the multiple reaction monitoring mode, as described in the Materials and Methods. The constituents identified (compounds **1** to **5**) were used as standards. (Bottom) The chemical structures of the constituents present in Fraction A of a *Polygonum* root extract are depicted under the MS chromatogram. The number of each peak in the chromatograms corresponds to the compound number. Glc, glucose.

Table 2. The effects of ethyl acetate-soluble fraction (Fraction A) on NO production.

Constituent (Compound)	Content [%][a]	IC_{50} [μM][b]	Log P[c]
Fraction A	100	——	
(*E*)-2,3,5,4'-Tetrahydroxystilbene-2-*O*-β-D-glucoside (6)	83.9	NA	0.40
Emodin (2)	5.41	7.33 ± 1.53	2.66
Physcion (1)	3.61	ND	2.95
Emodin-8-*O*-β-D-glucopyranoside (5)	1.27	12.2 ± 1.59	0.71
Physcion-8-*O*-β-D-glucopyranoside (3)	0.17	ND	0.67
Catechin (4)	0.85	NA	1.02

[a] The content of each constituent was measured by LC–MS/MS and is shown as the percentage of the dry weight of Fraction A. [b] The half-maximal inhibitory concentration for NO production in IL-1β-treated hepatocytes (mean ± SD). [c] *n*-Octanol/water partition coefficient as predicted using the ALOGPS 2.1 program [11]. NA, not applied due to low activity; ND, not determined due to very low solubility in the medium.

***Inhibition of NO production in hepatocytes by the constituents of Fraction A*:** We examined whether the constituents in Fraction A of a *Polygonum* root extract could inhibit NO induction in IL-1β-treated hepatocytes. The major constituent, THSG, did not affect NO production at a concentration up to 800 μM, and the IC_{50} value could not be calculated (data not shown). Emodin and emodin-8-*O*-β-D-glucopyranoside efficiently suppressed NO production, but neither constituent alone induced NO production (Figure 2A). LDH assays showed that these constituents were not cytotoxic to hepatocytes (data not shown). To compare the effect of NO production inhibition, the non-steroidal anti-inflammatory drug loxoprofen was used as a positive control because it inhibited NO production in hepatocytes [14]. Emodin more efficiently suppressed NO production than loxoprofen (Figure 2B). Physcion and physcion-8-*O*-β-D-glucopyranoside were highly hydrophobic and almost insoluble in water or medium (data not shown). Thus, they could not be analyzed in the NO assays. In contrast, catechin did not significantly inhibit NO production, as previously reported [24].

Emodin and emodin-8-*O*-β-D-glucopyranoside were further investigated. When the NO quenching effect of these two constituents was estimated, there were no significant changes in the NO level between the medium containing these constituents and the medium including $NaNO_2$ alone (data not shown). These data imply that these two anthraquinones did not possess direct NO quenching activity. Therefore, emodin and emodin-8-*O*-β-D-glucopyranoside were used in subsequent experiments as constituents characteristic of the *Polygonum* root extract.

Inhibition of the expression of pro-inflammatory genes by the constituents in a Polygonum root extract: The effects of emodin and emodin-8-*O*-β-D-glucopyranoside on *iNOS* gene expression were further evaluated. When emodin or emodin-8-*O*-β-D-glucopyranoside was added to culture medium, the iNOS protein level in IL-1β-treated hepatocytes was reduced (Figure 2C). In the absence of IL-1β, neither constituent alone affected the induction of iNOS protein expression.

Quantitative RT–PCR analysis of total RNA from treated hepatocytes was performed using EF mRNA as an internal control, and the Ct values were normalized to those of EF. As shown in Figure 4A, the mRNA expression of iNOS was increased in the presence of IL-1β alone (a positive control). When emodin or emodin-8-*O*-β-D-glucopyranoside was added to the medium with IL-1β, the mRNA levels of iNOS decreased in a dose-dependent manner. The iNOS mRNA levels were extremely low in the

absence of IL-1β, and no amplification was observed when emodin or emodin-8-*O*-β-D-glucopyranoside alone was added to the medium (data not shown). These results imply that emodin and emodin-8-*O*-β-D-glucopyranoside inhibit the induction of *iNOS* gene expression by reducing *iNOS* mRNA levels. In addition, primary cultured rat hepatocytes included more than 99% hepatocytes and the expression of neither endothelial NOS (eNOS) nor neuronal NOS (nNOS) mRNA was detected in these hepatocytes (unpublished data).

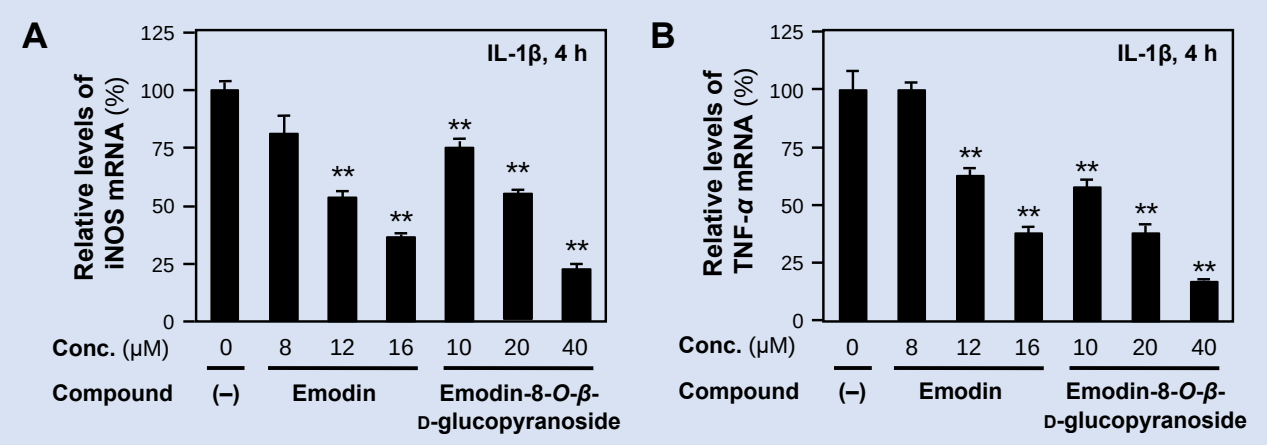


Figure 4. Emodin and emodin-8-*O*-β-D-glucopyranoside reduce the expression of proinflammatory genes. (**A**) The effects of emodin and emodin-8-*O*-β-D-glucopyranoside on the mRNA expression of iNOS. Each constituent and 1 nM IL-1β were added to the hepatocyte culture medium. After a 4-hr incubation, total RNA was prepared and analyzed by quantitative RT–PCR. mRNA levels were measured using EF as an internal control. The mRNA expression levels of iNOS were measured and normalized to the EF mRNA level. Relative mRNA levels are presented as the mean ± SD (n = 3) of the resulting percentage; the mRNA level measured in the presence of IL-1β alone was set as 100%. Amplification was not observed when IL-1β was not added or when each constituent alone was added to the culture medium (data not shown). (**B**) The effects of emodin and emodin-8-*O*-β-D-glucopyranoside on the mRNA expression of tumor necrosis factor (TNF) α. Similar to (A), the expression levels of TNF-α mRNA were measured and normalized to the EF mRNA level. Relative mRNA levels are presented as the mean ± SD (n = 3) of the resulting percentage; the mRNA level measured in the presence of IL-1β alone was set as 100%. Amplification was not observed when IL-1β was not added or when each constituent alone was added to the culture medium (data not shown). $P < 0.01$ *versus* IL-1β alone.

Finally, we examined whether emodin or emodin-8-*O*-β-D-glucopyranoside inhibits the expression of genes encoding proinflammatory cytokines, *e.g.*, TNF-α. Quantitative RT-PCR analysis indicated that TNF-α mRNA levels were increased by IL-1β alone (Figure 4B). When emodin or emodin-8-*O*-β-D-glucopyranoside was added to the medium, the mRNA expression of TNF-α in IL-1β-treated hepatocytes was reduced. No amplification was observed when cells were individually treated with emodin or emodin-8-*O*-β-D-glucopyranoside (data not shown).

DISCUSSION

In this study, we purified the constituents, including anthraquinones, of the roots of *Polygonum multiflorum* and determined their chemical identities. LC-MS/MS analysis demonstrated the content of each constituent in an extract of *Polygonum* roots (Figure 2, Table 2). The content of THSG was the highest (83.9%) in Fraction A; the yield of Fraction A was 24.3% (Table 1). Therefore, the content of THSG was calculated to be 20.4% of the *Polygonum* root extract. When we systematically analyzed several constituents in this crude drug, LC-MS/MS analysis facilitated quantitative estimation and provided reproducible results for the content. This analysis is advantageous compared to conventional HPLC analysis, which generally requires a much longer time.

We compared the anti-inflammatory activities of the six compounds, specifically the ability to suppress NO production in rat hepatocytes in the presence of IL-1β to

evaluate the anti-inflammatory activities of the constituents of the crude drug [16]. Our comparison indicated that emodin and emodin-8-*O*-β-D-glucopyranoside efficiently inhibited NO production, whereas THSG showed a lower potency (Table 2). In a search of the literature to date, the comparison of anti-inflammatory potency of the constituents of *Polygonum* roots was not reported.

The IC_{50} values of emodin (7.33 μM) and emodin-8-*O*-β-D-glucopyranoside (12.2 μM) were compared with those of other constituents in crude drugs: 8.25 μM (atractylodin from the rhizomes of *Atractylodes chinensis*) [25], 12.0 μM (berberine from the rhizomes of *Coptis chinensis*) [16], and 12.3 μM [(*Z*)-ligustilide from the rhizomes of *Cnidium officinale*] [9]. These results imply that emodin and emodin-8-*O*-β-D-glucopyranoside possess potency comparable to that of those constituents.

Two anthraquinones, *i.e.*, emodin and emodin-8-*O*-β-D-glucopyranoside, decreased *iNOS* mRNA levels to inhibit *iNOS* gene expression in hepatocytes in the presence of IL-1β (Figures 3, 4). Previous studies suggest that down-regulation of the *iNOS* gene correlates with the suppression of the expression of proinflammatory genes [for example, 9,25]. Indeed, these anthraquinones also inhibited the mRNA expression of TNF-α (Figure 4). Nuclear factor κB (NF-κB), a key transcription factor, plays an important role in inflammation to upregulate both *iNOS* and *TNF-α* genes [27–28]. The molecular mechanisms underlying the anti-inflammatory effects of the anthraquinones may be involved in the pathways mediated by NF-κB, as in our previous studies [14,16,26]. It is necessary to be examined whether NF-κB is indeed involved in the reduced expression of the *iNOS* and *TNF-α* genes induced by emodin or other constituents of *Polygonum* roots.

Emodin is an anthraquinone, which has a para-quinone structure. Fourier transform infrared (FTIR) spectroscopy and UV absorption spectra analysis suggested that emodin may interact with double-stranded DNA by intercalation and external binding [29]. Therefore, it is likely that emodin inhibits transcription by interacting with DNA. The structure-activity relationship of anthraquinones should be investigated using emodin-8-*O*-β-D-glucopyranoside and other anthraquinone derivatives.

In contrast, several pharmacological effects of THSG have been reported [30]; for example, THSG induces the iNOS protein in IL-1β-treated chondrocytes [7]. Our results using rat hepatocytes, however, indicated that the potency of THSG in suppressing NO production in hepatocytes was very low (Table 2). Further detailed investigation of the constituents in *Polygonum multiflorum* roots and their pharmacological activities using animals is in progress.

CONCLUSION

We first compared potency in suppressing NO production by pharmacologically active constituents in the EtOAc-soluble fraction of *Polygonum multiflorum* roots. The results imply that anthraquinones may be responsible for the anti-inflammatory effects of *Polygonum* roots.

List of Abbreviations: THSG: 2,3,5,4'-tetrahydroxy-stilbene-2-*O*-β-D-glucoside, NO: nitric oxide, iNOS: inducible nitric oxide synthase, IL-1β: interleukin 1β, LC-MS/MS: liquid chromatography-tandem mass spectrometry, i.d.: internal diameter, MeOH, methanol, EtOAc; ethyl acetate, IC_{50}: half-maximal inhibitory concentration, LDH: lactate dehydrogenase, EF: elongation factor 1α, SD: standard deviation.

Authors' Contributions: MN and YI designed the research; SMU, SS, MS, DRD, TO, SH, and JW performed the experiments and analyzed the data; MN, SMU, SS, and YI prepared the manuscript; and all authors were involved in the performance of the experiments and preparation of the manuscript. SMU and SS equally contributed to this work.

Competing Interests: SH and JW are employees of Shimadzu Corporation. MS performed this study as an undergraduate student at the College of Life Sciences,

Ritsumeikan University. The other authors declare that they have no conflicts of interest.

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