Antithrombin Effect of Jidabokuippo and Identification of Active Compounds

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Abstract
Static blood (Oketsu) is a blood disorder of Kampo medicine. Many natural medicines have been used to cure static blood, and these drugs are called blood stasis-resolving formula. Some of these were reported to inhibit blood coagulation. Jidabokuippo (JDI), one of the blood stasis-resolving formulas, has been used to treat bruises and sprains. In this study, we evaluated the antithrombin activity of JDI and its active compounds using thrombin and an artificial substrate. Bioassay-guided isolation was performed for JDI, and the active compounds were identified using spectral data (NMR spectroscopy and mass spectrometry) by comparison with reported data. JDI depressed thrombin activity in a dose-dependent manner, ranging from 0.125 to 1 mg/mL. Chrysophanol-1-O-β-D-glucoside (1) and chrysophanol-8-O-β-D-glucoside (2) were obtained from JDI and showed thrombin inhibitory activity. This study provided the first report of the antithrombin effect of JDI and the isolation from it of two active compounds (1 and 2). These findings might contribute to the understanding of the mechanism of static blood and blood stasis-resolving formula.

Keywords
quinones, blood disorder, static blood, Oketsu, Kampo, traditional medicine, antithrombin, jidabokuippo

Introduction
Static blood (Oketsu) is a pathological and physiological concept in oriental traditional medicine and recognized as blood stagnation and/or stasis syndrome.¹ Shadows, skin and mucosa purpura, swelling of the sublingual artery, and menopausal discomfort are typical characteristics of static blood. These are used to diagnose static blood. Kampo is traditional medicine in Japan. Kampo prescriptions, used to treat static blood, are called blood stasis-resolving formulas. Tokishakuyakusan, keishibukuryogan, and kamishoyosan are representative blood stasis-resolving formulas that have been reported to be biologically active, including inhibition of activated partial thromboplastin time and prothrombin time,¹ ³ anticoagulation in vitro, and improvement of blood viscosity in vitro.¹ ⁴ Although the previous reports suggest that static blood may be related to the blood coagulation–fibrinolysis system, a few studies of the mechanism of static blood exist.

Jidabokuippo (JDI), a blood stasis-resolving formula used to treat bruises and sprains,² consists of seven crude drugs, quercus bark, cinnamon bark, cnicium rhizome, nuphar rhizome, glycyrrhiza, rhubarb, and clove (Table 1). Several clinical studies on JDI have been reported recently. Komasawa et al⁵ reported that JDI effectively decreased the severity of postoperative pain after tooth extraction with mandibular bone removal. Nakae et al⁶ reported that JDI could shorten the treatment time in patients with rib fractures compared with NSAIDs. Yoshinaga et al⁷ reported that JDI could treat ankle sprains with long-term swelling and pain; however, scientific studies on JDI are limited.

We hypothesized that blood stasis-resolving formulas might inhibit thrombin because it plays an important role in blood coagulation. In primary hemostasis, thrombin works as an activator of platelets, whereas thrombin works as an activator of some coagulation factors and alters fibrinogen to fibrin in secondary hemostasis. Therefore, in our continuing scientific study of static blood and blood stasis-resolving formulas, we investigated the antithrombin effect of JDI and its active substances.

Here, we describe the evaluation of the antithrombin activity of JDI, bioassay-guided isolation, and identification of the active compounds of JDI.

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Results and Discussion

Effect of JDI on thrombin

The effect of JDI on thrombin is shown in Figure 1. JDI inhibited thrombin activity dose-dependently, and the inhibition rate at 1 mg/mL was 71.1% ± 4.5%. Furthermore, the inhibitory effect of the Et2O-soluble and water-soluble fractions of JDI on thrombin was evaluated (Figure 2). Although both fractions inhibited thrombin activity at 1 mg/mL, the Et2O-soluble fraction showed stronger activity than the water-soluble fraction.

Effect of fractions E1 to E5 and E2-1 to E2-4 on thrombin

The inhibitory effect of fractions E1 to E5 (each 1 mg/mL) on thrombin is shown in Table 2. Among them, E2 and E4 represented extremely strong inhibition of thrombin. Fraction E2 was further fractioned into four fractions (E2-1-E2-4), and their inhibitory activity was evaluated. Inhibitory rates of fractions E2-2 to E2-4 at 1 mg/mL were over 95%.

Isolation and identification of compounds 1 and 2

The JDI decoction was separated using bioassay-guided fractionation. The Et2O-soluble fraction was repeatedly separated using silica gel and octadecylsilane (ODS) columns to generate four fractions (Fr. E2-1-E2-4). Fractions E2-2 to E2-4 showed significant antithrombin activity. Finally, compounds 1 and 2 were isolated as the desired compounds by preparative HPLC. Both compounds 1 and 2 were obtained as amorphous yellow powders. The molecular formulas of 1 and 2 were determined as C_{21}H_{20}O_{9} from the high-resolution electrospray ionization (ESI)-MS data \{1, m/z 439.1005 [M + Na]^+ (calculated for C_{21}H_{20}O_{9}Na: 439.1005); 2, m/z 439.1006 [M + Na]^+ (calculated for C_{21}H_{20}O_{9}Na: 439.1005)\}. The 1H and 13C NMR data of 1 and 2 showed characteristic signals of anthraquinone glycosides. Finally, both compounds were identified by...
comparing the spectral data with those in previous literature (Figure 3).9,10

**Effect of chrysophanol-1-O-β-D-glucoside (1) and chrysophanol-8-O-β-D-glucoside (2) on thrombin**

Compounds 1 and 2 are well-known constituents of rhubarb. Because of the shortage of these two compounds obtained from JDI extract for thrombin assay, we obtained sufficient amounts from rhubarb. The effect of compounds 1 and 2 on thrombin activity is shown in Figure 4. Both significantly inhibited thrombin activity, with inhibition rates at 1 mg/mL of 83% and 63%, respectively.

In this study, we found that JDI extract inhibited thrombin activity in a dose-dependent manner (Figure 1). In one case study, JDI was reported to improve venous thrombosis of a patient by combination therapy with other clinical drugs.11 However, to our knowledge, this is the first report of the antithrombin activity of JDI. Our results indicated that the improvement in venous thrombosis by JDI could be due to its antithrombin activity. Furthermore, we identified chrysophanol-1-O-β-D-glucoside (1) and chrysophanol-8-O-β-D-glucoside (2) as active substances of JDI. Compounds 1 and 2 significantly suppressed thrombin activity at 1 mg/mL. Glycosides are generally considered to be absorbed after hydrolysis by intestinal bacteria. However, several anthraquinone glycosides, including chrysophanol-8-O-β-D-glucoside, were reported to be absorbed by orally administrated rats, not only in hydrolyzed forms, but also intact forms.12 Thus, compounds 1 and 2 might show antithrombin activity in vivo. Seo et al13 reported that chrysophanol-8-O-β-D-glucoside (2) from rhubarb inhibited platelet aggregation induced by collagen, thrombin, and arachidonic acid in vitro. Although their study suggested that 2 may inhibit primary hemostasis via antiplatelet activity, anticoagulant activity of anthraquinones in secondary hemostasis was unclear. On the other hand, our experimental system focused on their direct effects against protease activity of thrombin related to secondary hemostasis. This study revealed that JDI extract could inhibit thrombin directly, and compounds 1 and 2 may be active components. Our results and the previous report suggest that JDI could be used in the treatment of blood stasis by inhibiting the blood coagulation system in primary and secondary hemostasis.11 However, this study clarified only two active compounds in JDI, though several fractions also inhibited thrombin. We examined the other active fractions from the Et2O-soluble materials of JDI, but active compounds were inseparable because of the limitation of amount, and complexity of constituents. Thus, further investigations of the water-soluble fractions are ongoing, aimed at finding active components.

**Conclusion**

In conclusion, this study provided the first report of the antithrombin effect of JDI and two active compounds. JDI inhibited thrombin activity dose-dependently. Chrysophanol-1-O-β-D-glucoside (1) and chrysophanol-8-O-β-D-glucoside (2) were identified as active compounds in JDI. These findings might contribute to the understanding of the mechanism of static blood and blood stasis-resolving formula.

**Experimental**

**Materials**

All crude drugs used for the preparation of JDI were purchased from Uchida Waikanyu Co. Ltd (Tokyo, Japan). Thrombin was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA), Boc-Val-Pro-Arg-MCA from Peptide Institute, Inc. (Osaka, Japan), Tris (hydroxymethyl) aminomethane from Nakalai Tesque, Inc. (Kyoto, Japan), and Argatroban from FujiFilm Wako Pure Chemical Co. (Osaka, Japan).
General Experimental Procedures

$^1H$ and $^{13}C$ NMR spectra were measured on a JEOL JNM-LA-500 (500 MHz for $^1H$, 125 MHz for $^{13}C$) spectrometer. The 2.49 ppm resonance of residual DMSO-$d_6$ in DMSO-$d_6$ was used as an internal reference for $^1H$ NMR spectra and the 39.5 ppm resonance of DMSO-$d_6$ for the $^{13}C$ NMR spectra. The MS were recorded on a JMS-T100LP AccuTOF LC-plus4G (JEOL, Japan). Column chromatography was performed using silica gel (Sylicycle Inc., Canada), Chromatorex ODS (Fuji Silysia Chemical Ltd, Japan) and Diaion HP-20 (Mitsubishi Chemical, Japan). HPLC was performed using silica gel (Sylicycle Inc., Canada), Mightysil RP-18 GP (φ20 × 250 mm, Kanto Chemical Co., Inc.).

Thrombin Assay

The substrate solution consisting of 200 µL of 0.1 M NaCl/0.05 M Tris-HCl buffer (pH 8) and 3 µL of 2.5 mM Boc-Val-Pro-Arg-MCA was prepared and added to each well of a 96-well flat microplate. All samples, dissolved in either dimethyl sulfoxide or Tris-HCl buffer 6 µL, were added to sample wells (A) and sample blank wells (D) at different concentrations (final concentrations ranged from 0.01 to 1 mg/mL). Conversely, 6 µL of Tris-HCl buffer was added to blank wells (B) and control wells (C) instead of sample solutions. Eighty µL of the stop solution (water:MeOH:ν-BuOH = 7:6:7) was added to blank and sample blank wells. Incubation was continued at 37°C for 5 min. After incubation, 10 µL of 0.1 U/mL thrombin was added and then the plate was incubated at 37°C for 1 h. The reaction was stopped by adding 80 µL of the stop solution. The fluorescence intensity (excitation wavelength at 380 nm; emission wavelength at 470 nm) of the supernatants was measured on an Infinite M200 (TECAN). The inhibition rate (%) of thrombin activity was calculated as follows: % inhibition = [(C−B)−(A−D)]/(C−B) × 100. Argatroban, a clinically used antithrombin drug, was used as a positive control.

Extraction and Isolation

The JDI extract was prepared from the composition of crude drugs as shown in Table 1. They were mixed and decocted at 100°C with 600 mL of water until the volume of water decreased to 300 mL. The decoction was filtered through filter paper, and the filtrate evaporated in vacuo. The extract was suspended in water (325 mL) and extracted with Et$_2$O (2.4 L) to give Et$_2$O-soluble (489 mg) and water-soluble fractions (12.3 g). The Et$_2$O-soluble fraction was subjected to silica gel column chromatography (eluent, CHCl$_3$:MeOH:Water = 8:2:0.5) to give five fractions (E1–E5). Fraction E2 (101.4 mg) was chromatographed on an ODS column (eluent, 40% MeOH aq.) to yield four fractions (E2-1–E2-4). Fraction E2-3 (17.7 mg) and Fr. E2-4 (28.0 mg) decreased to 300 mL. The decoction was suspended in water (325 mL) and extracted with Et$_2$O (2.4 L) to give Et$_2$O-soluble (489 mg) and water-soluble fractions (12.3 g). The Et$_2$O-soluble fraction was subjected to silica gel column chromatography (eluent, CHCl$_3$:MeOH:Water = 8:2:0.5) to give five fractions (E1–E5). Fraction E2 (101.4 mg) was chromatographed on an ODS column (eluent, 40% MeOH aq.) to yield four fractions (E2-1–E2-4). Fraction E2-3 (17.7 mg) and Fr. E2-4 (28.0 mg) depressed thrombin activity. TLC analysis [silica gel 60 F$_{254}$ (Merck); solvent, CHCl$_3$:MeOH = 85:15; UV detection at 365 nm] of E2-2 to E2-4 revealed that E2-3 and E2-4 had two major spots (R$_f$ values: 0.25 and 0.43, respectively) colored orange by UV irradiation (365 nm). Further purification of Fr. 2-3 and Fr. 2-4 by C$_{18}$ HPLC [Mightysil RP-18 GP, φ20 × 250 mm; eluent, 30% CH$_3$CN aq.; flow rate 5 mL/min, UV detection at 254 nm] was carried out to obtain chrysophanol-1-O-β-D-glucoside (1, 0.26 mg) and chrysophanol-8-O-β-D-glucoside (2, 0.17 mg). Both compounds were identified by comparing their spectral data with those in the literature.9,10

Statistical Analysis

Results are expressed as mean ± SD of at least three times experiments.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical Approval

Not applicable, because this article does not contain any studies with human or animal subjects.

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Informed Consent

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Trial Registration

Not applicable, because this article does not contain any clinical trials.

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