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Preparative purification of plasmin activity stimulating phenolic derivatives from *Gastrodia elata* using centrifugal partition chromatography

Je-Seung Jeon, Jeeyoung Kim, Soyoung Park, Chongsuk Ryou* and Chul Young Kim*

ABSTRACT: *Gastrodia* rhizome, a dried and steamed tuber of *Gastrodia elata* Blume (Orchidaceae), has been traditionally used in Korea, China and Japan for the treatment of neurological and nervous disorders such as headaches, dizziness, vertigo and convulsive illnesses. The ethyl acetate and water extracts of *G. elata* stimulated plasmin activity. The active ethyl acetate fraction was subjected to centrifugal partition chromatography (CPC) with a two-phase solvent system, composed of *n*-hexane–ethyl acetate–methanol–water (3:7:4:6, v/v) followed by semi-preparative HPLC purification to separate active compounds and the water fraction was purified by Diaion HP-20 resin and semi-preparative HPLC. In ethyl acetate extract, 4-hydroxybenzyl alcohol (1), 4-hydroxybenzoic acid (2), 4-hydroxybenzaldehyde (3), 4-ethoxymethylphenol (4), 4,4′-oxy*bis*(methylene)diphenol (5) and 4,4′-methylenediphenol (6) were obtained with high purities. Parishin (7) and parishin B (8) were isolated from water extract. Among isolated compounds, 4-hydroxybenzyl alcohol (1), 4-hydroxybenzaldehyde (3) and 4-ethoxymethylphenol (4) significantly stimulated plasmin activity. Copyright © 2015 John Wiley & Sons, Ltd.

Keywords: centrifugal partition chromatography; *Gastrodia elata* Rhizome; plasmin activity; phenolic derivatives

Introduction

Gastrodia rhizome, a dried and steamed tuber of *Gastrodia elata* Blume (Orchidaceae), has been traditionally used in Oriental countries for the treatment of neurological and nervous disorders like headaches, migraine, dizziness, rheumatism, paralysis and epilepsy, or as an anti-convulsant and a sedative (Jang *et al*., 2010; Lee *et al*., 2012). The extracts and isolated compounds of *G. elata* exert diverse biological activities such as neuroprotective, anti-inflammatory, antidepressant, anti-osteoporotic, anti-thrombic and anti-platelet effects (Ahn *et al*., 2007; Chen and Sheen, 2011; Pyo *et al*., 2000, 2004; Seo *et al*., 2010; Tsai *et al*., 2011; Zhou *et al*., 2006). Previous phytochemical study on these rhizomes resulted in the isolation of more than 30 phenolic compounds, such as 4-hydroxybenzyl alcohol (gastrodigenin), 4-hydroxybenzaldehyde, gastrodin, gastrol, parishin and *bis*(4-hydroxybenzyl) sulfoxide, and polysaccharides (Baek *et al*., 1999; Hayashi *et al*., 2002; Jung *et al*., 2006, Lee *et al*., 2006).

Plasmin is a serine protease that degrades fibrin clots in blood (Castellino and Ploplis, 2005). In addition to fibrinolysis, plasmin is also involved in many physiological events such as processing of proenzymes (Deryugina and Quigley, 2012), ovulation (Mondéjar *et al*., 2012), wound healing (Li *et al*., 2003) and liver homeostasis (Bezerra *et al*., 1999). Furthermore, activation of plasminogen to plasmin via plasminogen activators is proposed to play a key role in a number of pathological conditions such as Alzheimer's disease (Dotti *et al*., 2003) and cerebral thrombosis, leading to ischemic stroke (Murray *et al*., 2010), of which clinical features include headaches, migraine, dizziness and paralysis. Induction of plasmin levels by plasminogen activators could dissolve the clots, as shown in tissue-type plasminogen activator treatment for stroke patients, although adverse side effects are uncontrollable (Marder *et al*., 2010). In Alzheimer's conditions, the decreased plasmin level results in an increase of neurotoxic amyloid β (Tucker *et al*., 2000). Thus, stimulation of plasmin activity without involving plasminogen activator would be an alternative approach to ameliorate the disease conditions.

Isolation and purification of the bioactive compounds from the crude extract has been assumed to be an essential step for a further biological activity test. However, classical methods of separation need time-consuming chromatographic steps and consume too much organic solvent (Wang *et al*., 2008; Yan *et al*., 2004). Centrifugal partition chromatography (CPC) is a solid matrix-free liquid–liquid partition technique, which contributes many advantages such as a higher sample loading capacity, the absence of sample loss and irreversible sample adsorption to the solid column. With these merits, CPC is an excellent tool for isolation bioactive natural compounds using activity-guided purification methods (Dai *et al*., 2013; Jin *et al*., 2013).

In this study, plasmin activity stimulating phenolic compounds were purified by CPC and subsequently preparative HPLC. In addi-

* Correspondence to: C. Y. Kim and C. Ryou, College of Pharmacy and Institute of Pharmaceutical Science and Technology, Hanyang University, Ansan, Gyeonggi-do 15588, Republic of Korea. Email: chulykim@hanyang.ac.kr, cryou2@hanyang.ac.kr

College of Pharmacy and Institute of Pharmaceutical Science and Technology, Hanyang University, Ansan, Gyeonggi-do 15588, Republic of Korea

tion, it is the first report of *G. elata* extract and purified compounds enhancing plasmin activity.

Experimental

Apparatus and materials

The *Gastrodia* rhizomes were purchased from a Kyungdong Oriental Herb Market, Seoul, Korea. A voucher specimen was deposited in the Herbarium of the College of Pharmacy, Hanyang University (HYU-GE-001). Preparative CPC was carried out on a SCPC 1000 (Armen Instruments, France) apparatus with a spot prep II HPLC instrument (an automated HPLC system comprising a pump, an injector, a UV–Vis detector, a fraction collector and a digital screen; Armen instrument, France). Armen Glider CPC software was used to control the SCPC1000 and Spot prep II system. The analytical Agilent 1260 HPLC system (Agilent Technologies, Waldbronn, Germany) consisted of a G1312C binary pump, a G1329B autosampler, a G1315D DAD detector, a G1316A column oven and ChemStation software. All HPLC and analytical-grade solvents were obtained from Daejung chemical (Gyonggi-do, Korea). Diaion HP-20 resin used in this experiment was purchased from Mitsubishi (Tokyo, Japan)

Preparation of crude extract of *Gastrodia* rhizome

Gastrodia rhizomes (1.8 kg) were ground into powder and extracted three times with methanol (3 L) for 90 min using an ultrasonic apparatus and subsequently filtered *in vacuo* through a defatted cotton filter. The filtered solute was then concentrated to dryness (101 g) by rotary evaporation under reduced pressure at 40°C. Next, 96 g of methanolic extract was suspended in 1000 mL water and successively partitioned with *n*-hexane, ethyl acetate and *n*-butanol (1 L × 3 times) to obtain 2.07 g of *n*-hexane, 2.7 g of ethyl acetate and 9.42 g of *n*-butanol extracts; it was stored in a refrigerator (4°C).

In vitro plasmin activity assay

Human plasmin was purchased from Hematologic Technologies (Essex Junction, VT, USA). Plasmin activity was measured using a SensoLyte AFC (7-amido-4-trifluormethylcoumarin) plasmin activity assay kit (AnaSpec, Inc., Fremont, CA, USA), which is based on the quantification of AFC fluorophore released from a fluorogenic substrate upon plasmin activity. The assay was performed according to a provided protocol. Briefly, crude extracts or isolated compounds from *G. elata* were added as indicated concentration into 96-well flat black plastic plates. Next, 50 nM plasmin and 15 nM AFC plasmin substrate were added into each cell. Then, the fluorescence was detected at 380 nm excitation and 500 nm emission using a Tecan Infinite M200 PRO plate reader (Tecan, Maennedorf, Switzerland) and recorded data every 5 min for 60 min. Acetylsalicylic acid (aspirin; Hanmi Pharmaceutical, Seoul, Korea), previously reported to stimulate plasmin activity (Milwidsky *et al.*, 1991), was assayed in parallel as a positive control to evaluate the activity level of the tested compounds.

Selection of the solvent system

Several variations of the two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water were selected on the bases of the partition coefficient (*K*-value) of the target compounds. The *K*-values were determined by HPLC. Briefly, about 15 mg of the ethyl acetate extract was weighted in 2 mL test tubes to which 900 μL of the pre-equilibrated upper and lower phase solvent was added. These test tubes were forcefully shaken and then allowed to settle. Aliquots of 200 μL of equilibrated upper and lower solutes were made up to 500 μL with methanol for analysis. The *K*-value was expressed as the peak area of each compound in the upper phase divided by that in the lower phase.

CPC and preparative HPLC separation of ethyl acetate extract

The two-phase solvent system selected for the separation was of *n*-hexane–ethyl acetate–methanol–water (3:7:4:6, v/v). This was prepared by adding the solvent to a separation funnel according to the volume ratios and thoroughly equilibrated at room temperature. The two phases were divided and degassed by sonication for 20 min before use. The sample solution was prepared by dissolving 2.6 g of the ethyl acetate extract in 30 mL of mixed upper and lower phases of the solvent system. In CPC separation, the upper organic phase, serving as the stationary phase, was pumped into the CPC column at a flow rate 40 mL/min at 500 rpm in ascending mode. After the column was entirely filled with the stationary phase, the lower aqueous phase, dedicated as a mobile phase, was conveyed via the stationary phase at a flow rate of 10 mL/min at 1200 rpm in descending mode. When CPC column reached hydrostatic equilibrium, evidenced by the mobile phase eluting clearly from the outlet (460 mL of stationary upper phase was eluted, 45 bar), 30 mL of the sample solution was injected. The effluent for the tail end of the column was continuously monitored at the wavelength of 254 nm and scan mode (230–400 nm). Every peak fraction was collected at 20 mL per tube and separated according to the elution profile to yield five CPC peak fractions **I–V**. The fractions were dried and analyzed by HPLC. For the further purification of the CPC peak fractions **I–V**, after HPLC analysis, an Armen Spot prep II HPLC instrument with an INNO Column C_{18} (20.0 × 250mm, 5 μm, YoungJin Biochrom, Korea) was utilized. After preparative HPLC, compounds **1–6** were obtained with high purities: compound **1** (18.8 mg) from CPC peak fraction **I**; compound **2** (5.8 mg) from CPC peak fraction **II**; compound **3** (20 mg) from CPC peak fraction **III**; compounds **4** (32.3 mg) and **5** (133.0 mg) from CPC peak fraction **IV**; and compound 6 (13.5 mg) from CPC peak fraction **V**.

Purification of water extract using Diaion HP-20 resin and preparative HPLC

Water fraction was dissolved in deionized water and subjected to column chromatography on Diaion HP-20 resin (150 g) using a stepwise gradient of 30, 40, 50 and 100% methanol.

Parishin (**7**) was isolated from 40% methanol eluent by preparative HPLC with methanol gradient (0–50 min, 25–30% of methanol, 10 mL/min), and parishin B (**8**) was further purified from 50% methanol eluent by preparative HPLC with 15% methanol elution (10 mL/min).

HPLC analysis

An Agilent 1260 HPLC system with an Inno column C_{18} (4.6 × 250mm, YoungJin Biochrom, Korea) was employed for the analysis of the ethyl acetate extract and respective peak fractions obtained from CPC and prep-HPLC. A linear gradient system of acetonitrile with 1% formic acid (solvent A) and water with 1% formic acid (solvent B) was used as follows: 0–25 min, 10–60% of A; 25–28 min, 60–100% A; 28–32 min, 100% A. The flow rate was 1 mL/min and an injection volume was 10 μL. The chromatographic profile of the effluents was recorded at 210 and 254 nm ranging from 210 to 400 nm.

Identification of purified compounds by ESI-MS and NMR

Structural elucidation of the purified compounds was carried out by ESI-MS and NMR analysis. Each compound was dissolved in methanol and directly injected into Advion expression CMS mass spectrometry (Advion, Ithaca, NY, USA) through a Rheodyne injector (2 μL loop). The ESI-MS spectra conditions were as follows: positive or negative ion mode; mass range, *m/z* 100–1200; capillary temperature, 200°C; capillary voltage, 150 V; source voltage offset, 100; source voltage span, 50; source gas temperature, 150°C; ESI voltage, 3.5 kV. In addition, purified compounds (5–15 mg) were dissolved in 600 μL of methanol-d_4 for the NMR experiment. The ^{1}H, ^{13}C (400 and 100 MHz, respectively) together with 2D including ^{1}H–^{1}H COSY, HSQC and HMBC were executed on a Bruker model digital AVANCE III 400 NMR and processed using MestReNova software (Mestrelab Research, Santiago de Compostela, Spain).

Statistical analysis

All data are reported as means ± SE. Statistically significant differences were assessed by Student's *t*-test. A probability value of less than 0.05 was considered significant.

Results and discussion

Stimulation of plasmin activity of *G. elata* extract

Using *in vitro* plasmin activity assay system, crude extracts (*n*-hexane, ethyl acetate, *n*-butanol and water extracts) of *G. elata* were evaluated. The steamed and dried rhizomes of the *G. elata* (1.8 kg) were extracted using methanol and dried completely *in vacuo*. *n*-Hexane, ethyl acetate, *n*-butanol and water fractions were yielded through successive partitioning from the *Gastrodia* crude methanolic extract. Chromatograms of crude extract and sub-fractions are represented in Fig. 1(A). When the extracts were applied to our plasmin activity assay, ethyl acetate and water extracts enhanced plasmin activity, while *n*-hexane and *n*-butanol fractions inhibited plasmin activity (Fig. 1B). These two active extracts were evaluated again at different concentrations (5, 50, 500 and 5000 μg/mL). The ethyl acetate extract enhanced plasmin activity but did not show a dose-dependent response (Fig. 1C), and the water extract increased plasmin activity in a dose-dependent manner (Fig. 1D). These data suggested that ethyl acetate and water extracts included active substances enhancing plasmin activity. Thus these two extracts were further purified to isolate active molecules with stimulation of plasmin activity.

Isolation of phenolic compounds from active ethyl acetate fraction by CPC

Successful purification of CPC depends on a two-phase solvent system. To optimize CPC solvent conditions, the *K*-values of the target compounds in a solvent system consisting of *n*-hexane–ethyl acetate–methanol–water in different ratios were examined (Table 1).

The two-phase solvent system 3:7:4:6 showed suitable *K*-values and separation factors amongst the compounds even if its separation factor between compounds **3** and **4** was only about 1.2, which was a bigger separation factor than that of solvent system 3:4:5:5 or 4:6:4:6, resulting in fine separation in the present CPC study. Two-phase solvents systems 3:4:5:5 and 4:6:4:6 had too small separation factors between compounds **3** and **4** (0.93 and 1.1,

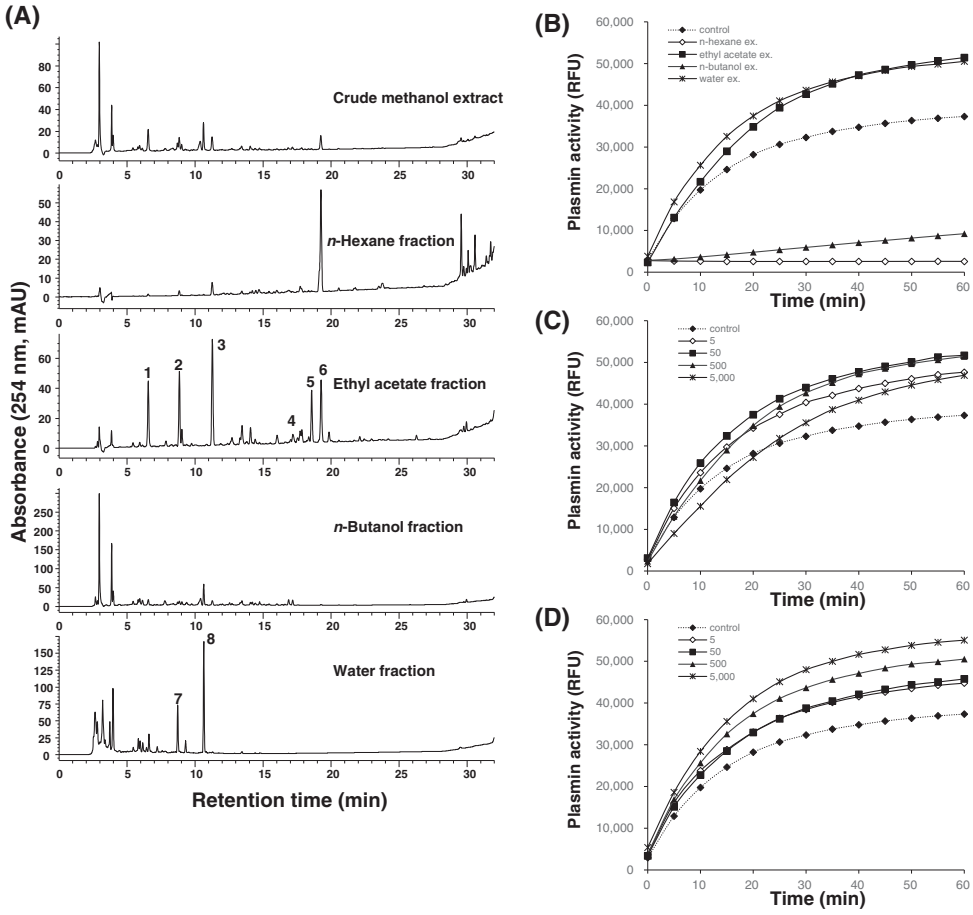

Figure 1. HPLC analysis of crude extract and stimulation of plasmin activity by extract and sub-fractions. (A) HPLC chromatograms of *G. elata* extract and sub-fractions; HPLC conditions are described in Materials and Methods section. Peaks: 4-hydroxybenzyl alcohol (**1**), 4-hydroxybenzoic acid (**2**), 4-hydroxybenzaldehyde (**3**), 4-ethoxymethyl phenol (**4**), 4,4′-oxy*bis*(methylene)diphenol (**5**), 4,4′-methylenediphenol (**6**), parashin (**7**) and parishin B (**8**). (B) Enhancement of plasmin activity by crude methanol extract, *n*-hexane, ethyl acetate, *n*-butanol and water fractions; (C) stimulation of plasmin activity by ethyl acetate fraction; (D) stimulation of plasmin activity by water fraction. The arbitrary fluorescence levels achieved in the presence of *G. elata* extracts were presented as a function of time.

Table 1. The *K*-values of target compounds of ethyl acetate extract of *Gastrodia elata* in different solvent systems

n-Hexane–ethyl acetate–methanol–water (v/v)	*K*-values				
	4-Hydroxybenzyl alcohol (**1**)	4-Hydroxybenzoic acid (**2**)	4-Hydroxybenzaldehyde (**3**)	4,4′-Oxy*bis*(methylene)diphenol (**5**)	4,4′-Methylenediphenol (**6**)
3:4:5:5	0.23	0.36	0.88	0.82	1.55
4:6:4:6	0.23	0.47	1.26	1.38	2.74
3:7:4:6	0.36	0.62	1.47	1.75	2.85
3:7:3:7	0.47	1.03	2.25	2.8	3.66

respectively). In addition, solvent 3:7:3:7 had quite a large *K*-value for compound **6** (3.66), which was anticipated to have excessive band broadening.

A preparative CPC was carried out with the solvent system of 3:7:4:6 at a constant flow rate of 10 mL/min, 1200 rpm in descending mode. From 2.6 g of the ethyl acetate extract, five CPC peak fractions **I–V** were separated and their analytical HPLC chromatogram is shown in Fig. 2. The CPC peak fractions **I–V** were further subjected to a preparative HPLC using a methanol–water gradient or an isocratic. Consequently, compounds **1–3** were purified from corresponding CPC peak fractions **I**, **II** and **III**, respectively, while compounds **4** and **5** came from the CPC peak fraction **IV**. Additionally, compound **6** was refined from CPC peak fraction **V**. The purities of isolated compounds **1–6** were >90% based on HPLC peak area. Purification of the bioactive compounds from the crude extract has been assumed to be an essential step for further biological activity tests. However, classical methods using silica gel, Sephadex LH-20 or C_{18} open column chromatography require repeated time-consuming chromatographic steps, and some polyphenolic compounds were not eluted in the column and were irreversibly adsorbed to the solid material. Furthermore, separation yields of our research are higher than in a previous study (Pyo *et al.*, 2000). Therefore, CPC is a good choice for the separation of active molecules from natural products.

Isolation of compounds from water fraction using Diaion HP-20 resin and preparative HPLC

The water fraction was dissolved in 30% methanol and subjected to column chromatography on Diaion HP-20 resin (150 g) using a stepwise gradient of 40, 50 and 100% methanol. Parishin was further purified by preparative HPLC from 50% methanol eluent and parishin B was isolated by preparative HPLC from 40% methanol eluent fraction.

Structural elucidation of isolated compounds

Structural elucidation of the purified compounds was carried out by ESI-MS and NMR analysis. After comparison of their spectroscopic date with those reported in the literature (Cho *et al.*, 1998; Lee *et al.*, 2006; Lin *et al.*, 1996; Sun *et al.*, 2001; Taguchi *et al.*, 1981), the purified compounds were identified as 4-hydroxybenzyl alcohol (**1**), 4-hydroxybenzoic acid (**2**), 4-hydroxybenzaldehyde (**3**), 4-ethoxymethyl phenol (**4**), 4,4′-oxy*bis*(methylene)diphenol (**5**), 4,4′-methylenediphenol (**6**), parishin (**7**) and parishin B (**8**).

4-Hydroxybenzyl alcohol (1). Pale yellow crystal; UV λ_{max} (nm): 224.3, 274.5; ^{1}H NMR (400 MHz, methanol-d_4) δ 7.17 (2H, d, J = 8.4 Hz, H-2, 6), 6.75 (2H, d, J = 8.4 Hz, H-3, 5), 4.48 (1H, s, H-7); ^{13}C NMR (100 MHz, methanol-d_4) δ 158.0 (C, C-4), 133.4 (C, C-1), 129.9 (CH, C-2, 6), 116.1 (CH, C-3, 5), 65.2 (CH_2, C-7); ESIMS *m/z* 147 $[M + Na]^+$.

4-Hydroxybenzoic acid (2). Brown amorphous solid; UV λ_{max} (nm): 254.6; ^{1}H NMR (400 MHz, methanol-d_4) δ 7.87 (2H, d, J = 8.3 Hz, H-2, 6), 6.82 (2H, d, J = 8.3 Hz, H-3, 5); ^{13}C NMR (100 MHz, methanol-d_4) δ 169.5 (C, C-7), 163.4 (C, C-4), 132.8 (CH, C-2, 6), 116.2 (CH, C-3, 5); ESIMS *m/z* 137 $[M - H]^-$.

4-Hydroxybenzaldehyde (3). Pale brown needle; UV λ_{max} (nm): 222.5, 284.9; ^{1}H NMR (400 MHz, methanol-d_4) δ 9.75 (1H, s, H-7), 7.77 (2H, d, J = 8.5, H-2, 6), 6.91 (2H, d, J = 8.5, H-3, 5); ^{13}C NMR (100 MHz, methanol-d_4) δ 192.8 (C, C-7), 165.2 (C, C-4), 130.2 (C, C-1), 133.4 (CH, C-2, 6), 116.8 (CH, C-3, 5): ESIMS *m/z* 145 $[M + Na]^+$, 121 $[M - H]^-$.

4-Ethoxymethyl phenol (4). Colorless oil; UV λ_{max} (nm): 227.1,273.8; ^{1}H NMR (400 MHz, methanol-d_4) δ 7.15 (2H, d, J = 8.4, H-2, 6), 6.76 (2H, d, J = 8.4, H-3, 5), 4.37 (2H, s, H-7), 3.50 (2H, q, J = 7.0, H-8), 1.19 (3H, t, J = 7.0, H-9); ^{13}C NMR (100 MHz, methanol-d_4) δ 158.4 (C, C-1), 130.8 (CH, C-2, 6), 130.3 (C, C-4), 116.1 (CH, C-3, 5), 73.6 (CH_2, C-7), 66.4 (CH_2, C-8), 15.4 (CH_3, C-9); ESIMS *m/z* 150.9 $[M - H]^-$.

4,4′-Oxy*bis*(methylene)diphenol (5). Brown amorphous solid; UV λ_{max} (nm): 230.1, 275.3 ; ^{1}H NMR (400 MHz, methanol-d_4) δ 7.15 (4H, d, J = 8.4, H-2, 6, 2′, 6′), 6.76 (4H, d, J = 8.4, H-3, 5, 3′, 5′), 4.38 (4H, s, H-7, 7′); ^{13}C NMR (100 MHz, methanol-d_4) δ 158.2 (C, C-1, 1′), 130.9 (CH, C-2, 6, 2′, 6′), 130.2 (C, C-4, 4′), 116.1 (CH, C-3, 5, 3′, 5′), 72.6 (CH_2, C-7, 7′); ESIMS *m/z* 229 $[M - H]^-$.

4,4′-Methylenediphenol (6). Colorless needle; UV λ_{max} (nm): 232.1, 278.0; ^{1}H NMR (400 MHz, methanol-d_4) δ 6.96 (4H, d, J = 8.4, H-2, 6, 2′, 6′), 6.68 (4H, d, J = 8.4, H-3, 5, 3′, 5′), 3.74 (2H, s, H-7); ^{13}C NMR (100 MHz, methanol-d_4) δ 156.4 (C, C-1, 1′), 134.4 (C, C-4, 4′), 130.7 (CH, C-2, 6, 2′, 6′), 116.1 (CH, C-3, 5, 3′, 5′), 41.1 (CH_2, C-7); ESIMS *m/z* 199 $[M - H]^-$.

Parishin (7). Amorphous solid; UV λ_{max} (nm): 228.4, 268.9; ^{1}H NMR (400 MHz, methanol-d_4) δ 7.26 (4H, dd, J = 8.8, H-2, 2″, 6, 6′′), 7.16 (2H, d, J = 8.7, H-2′, 6′), 7.07 (4H, dd, J = 8.7, H-3, 3″, 5, 5″), 7.04 (2H, d, J = 8.7, H-3′, 5′), 4.98 (5H, m, H-7′, 8, 8′, 8″), 4.90 (4H, m, H-7, 7″), 3.88 (3H, dd, J = 12.1, 1.7, H-13a, 13′a, 13″a), 3.69 (3H, dd, J = 12.0, 5.1, H-13b, 13′b, 13″b), 3.52–3.35 (12H, D-glucopyranosyl moiety), 2.95 (2H, d, J = 15.3, H-15a, 15″a), 2.78 (2H, d, J = 15.3, H-15b, 15″b); ^{13}C NMR (100 MHz, methanol-d_4) δ 174.3 (C, C-14′), 171.0 (C, C-14, 14″), 159.1 (C, C-4, 4′, 4″), 131.0 (CH, C-2, 2′, 2″, 6, 6′, 6″), 130.6 (C, C-1, 1′, 1″), 117.7 (CH, C-3, 3′, 3″, 5, 5′, 5″), 102.2 (CH, C-8, 8′, 8″), 78.0 (CH, C-10, 10′, 10″), 77.8 (CH, C-12, 12′, 12″), 74.8 (CH, C-9, 9′, 9″), 74.7 (C, C-15′), 71.2 (CH, C-11′, 11″, 11‴), 68.2 (CH_2, C-7′), 67.3 (CH_2, C-7, 7″), 62.4 (CH_2, C-13, 13′, 13‴), 44.7 (CH_2, C-15, 15″); ESIMS *m/z* 995 $[M - H]^-$.

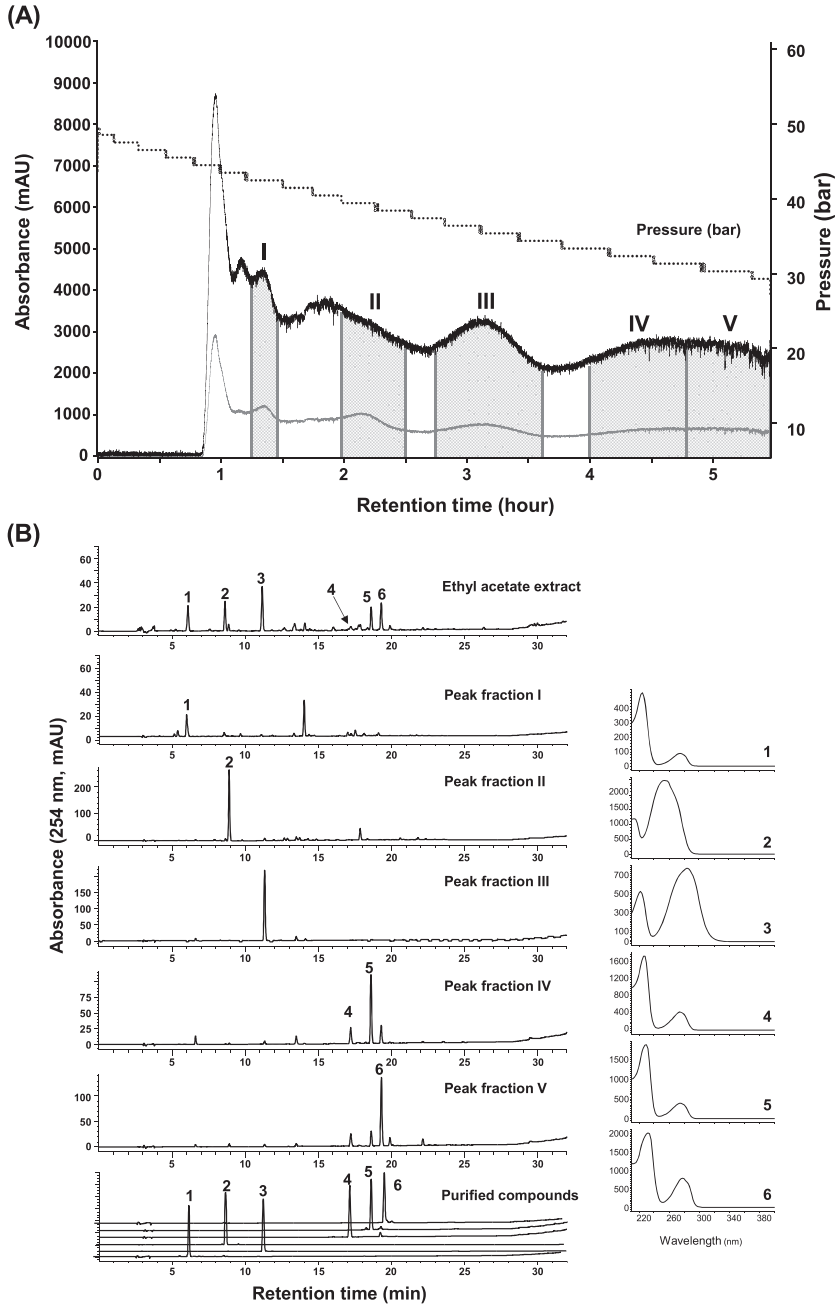

Figure 2. CPC chromatogram of the *Gastrodia* rhizome ethyl fraction (A) and HPLC analysis of CPC peak fractions I–V and purified compounds (B). CPC conditions: two-phase solvent system, *n*-hexane–ethyl acetate–methanol–water (3:7:4:6, v/v); descending mode, mobile phase – lower aqueous phase; flow-rate, 10 mL/min; rotation speed, 1200 rpm; monitored at 254 nm and scan mode (230–400 nm). HPLC conditions are same as described above.

Parishin B (8). Amorphous solid; UV λ_{max} (nm): 224.0, 268.2; ^{1}H NMR (400 MHz, methanol-d_4) δ 7.29–7.22 (4H, m, H-2, 2′, 6, 6′), 7.10–7.04 (4H, m, H-3, 3′, 5, 5′), 5.02 (2H, s, H-7), 4.99 (2H, s, H-7′), 4.90 (2H, m, H-8, 8′), 3.88 (2H, dt, J = 12.1, 1.7, H-13a, 13′a), 3.7 (2H, dt, J = 12.1, 5.2, H-13b, 13′b), 3.50–3.35 (8H, glucopyranosyl moiety), 2.95 (1H, d, J = 15.2, H-15″a), 2.89 (1H, d, J = 15.7, H-15a), 2.80 (1H, d, J = 15.2, H-15″b), 2.73 (1H, d, J = 15.7, H-15b); ^{13}C NMR (100 MHz, methanol-d_4) δ 174.6 (C, C-14′), 173.5 (C, C-14″), 171.1 (C, C-14), 159.1 (C, C-4, 4′), 131.0 (CH, C-2, 2′, 6, 6′), 130.8 (C, C-1, 1′), 117.7 (CH, C-3, 3′, 5, 5′), 102.2 (CH, C-8, 8′), 78.1 (CH, C-10, 10′), 77.9 (CH, C-12, 12′), 74.9 (CH, C-9, 9′), 74.6 (C, C-15′), 71.3 (CH, C-11, 11′), 68.2 (CH_2, C-7′), 67.3 (CH_2, C-7), 62.5 (CH_2, C-13, 13′), 44.3 (CH_2, C-15, 15″); ESIMS *m/z* 727 $[M - H]^-$.

Enhancement of plasmin activity by purified compounds from *G. elata* extract

Plasmin activity was measured at the end of linear velocity period (t = 60 min) after incubation with purified compounds (Fig. 3A). Ability of stimulation of plasmin activity by purified compounds was evaluated at a concentration of 1 μM. Compared with control (plasmin 50 nM), 4-hydroxybenzyl alcohol (**1**), 4-hydroxybenzaldehyde

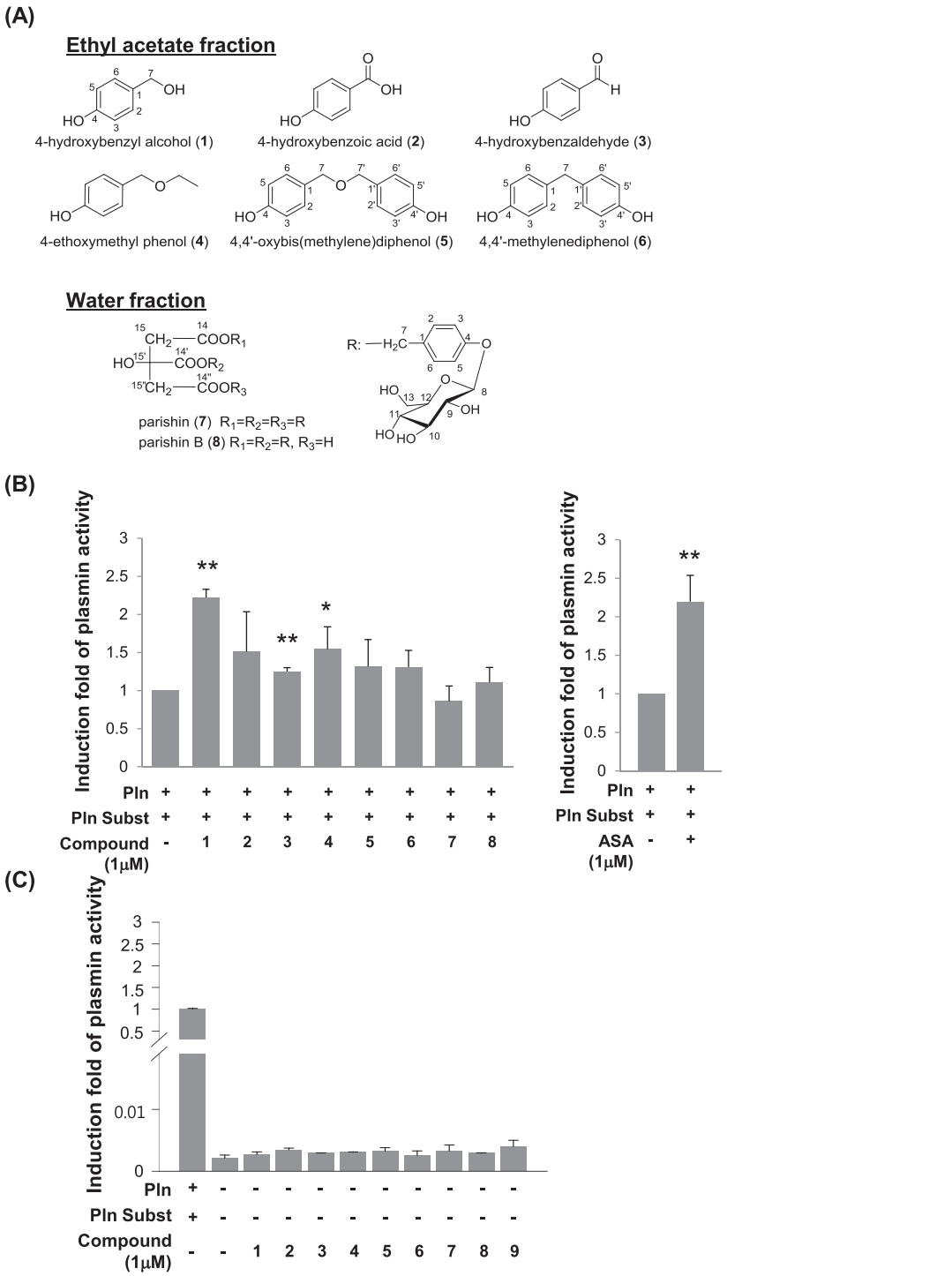

Figure 3. Chemical structures of isolated compounds (A), stimulation of plasmin activity by purified compounds **1–8** and acetylsalicylic acid (B) and their intrinsic fluorescence (C). Plasmin activity was measured at the end point ($t = 60$ min) after incubation with 1 μM. Average and standard deviation of triplicate assay results were presented. * $p < 0.05$; ** $p < 0.01$; Pln, plasmin; Pln Subst, plasmin substrate; ASA, acetylsalicylic acid.

(**3**) and 4-ethoxymethylphenol (**4**) significantly ($p < 0.05$) stimulated plasmin activity (Fig. 3B). Among these compounds, 4-hydroxybenzyl alcohol (**1**) showed the most potent stimulating activity, more than 2-fold compared with the control value. This was comparable with the results obtained with acetylsalicylic acid. The fluorescence readouts indicating induction of plasmin activity by the tested compounds and acetylsalicylic acid were not due to the intrinsic fluorescence of the compounds, as demonstrated in Fig. 3(C), in which the fluorescence levels of the compounds were very low.

Conclusions

In this study, we successfully identified plasmin activity stimulators from *G. elata* extract using CPC or Diaion HP-20 macroporous resin

and subsequent preparative HPLC method. This is the first report of *G. elata* and purified compounds enhancing plasmin activity. In particular, plasmin activity enhanced compounds were first isolated from natural resources. In addition, CPC methods for large amounts of active molecules were developed. Also, this large-scale isolation and purification method for natural compounds can be applied to future studies aimed at discovering new functional foods or ingredients.

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