The Anti-thrombotic Active Constituents from *Centella asiatica*

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Received December 9, 2006; accepted February 21, 2007; published online March 6, 2007

The *in vitro* effects of a methanol extract from the aerial parts of *Centella asiatica* on shear-induced platelet activation and coagulation were assessed after oral administration to rats, by subjecting non-anticoagulated blood to haemostatometry. 3,5-Di-*O*-caffeoyl quinic acid, 1,5-di-*O*-caffeoyl quinic acid, 3,4-di-*O*-caffeoyl quinic acid, 4,5-di-*O*-caffeoyl quinic acid, and chlorogenic acid, together with asiaticoside, kaempferol, quercetine, kaempferol-3-*O*-*β*-D-glucoside and quercetin-3-*O*-*β*-D-glucoside were all isolated from the methanol extract. Amongst these, only 3,5-di-*O*-caffeoylquinic acid showed significant inhibition of shear-induced platelet activation and dynamic coagulation. The reactive curve of the inhibitory effect on the platelet reaction and the dynamic coagulation showed a bell-shape.

Key words *Centella asiatica*; anti-thrombotic activity; 3,5-di-*O*-caffeoylquinic acid; shear stress; non-anticoagulated blood; haemostatometry

In the course of our work to find natural products capable of preventing lifestyle-related diseases, we have investigated the ability of constituents of *Centella asiatica* (L.) Urban (Japanese name: Tsubokusa) to inhibit thrombosis. *C. asiatica* (Umbelliferae) is distributed in sunny, moist grounds in all parts of the world (South east Asia, Australia, Africa, Brazil, Europe. *etc.*). In India, this plant has long been used in the Ayurvedic system of medicine to treat a variety of ailments and even now this plant is used in the treatment of dermatitis, diabetes, cough, cataract and other eye troubles, and for improving memory.[1,2] An infusion of the aerial part of *C. asiatica* is also used in the treatment of wounds and ulcers in Europe,[3] and in the treatment of dermatitis, the purification of the blood, the amelioration of hypertension and in the improvement of the memory in Indonesia[4,5] and Brazil.[6] In Malays, this plant is used in treatments of bronchitis, asthma, gastric catarrh, dysentery, kidney trouble, urethritis, and dropsy. An infusion prepared from this plant is commonly sold in the towns of Malaya as a tonic and a cold drink, which are used to treat liver complaints, tuberculosis and to treat the passing of blood in the urine.[7] In Japan, this plant was used as a detoxicant and diuretic but is not used in recent times.[8]

From the usage of this plant in Indonesia, Brazil and India, we expected that it contained some compounds that had anti-thrombotic activity (that is, promoting blood circulation to remove blood stasis). However, despite the many reports on the saponins (contain asiaticoside) isolated from *C. asiatica*,[9—18] no anti-thrombotic compounds or anti-thrombotic effects have been reported with regard to this plant.

In the meantime, it is well known that platelets are activated by shear forces as well as by various agonists, resulting in the formation of a platelet-rich thrombus. A variety of *in vitro* test have been used for the assessment of platelet activation *in vivo*. One of these is the measurement of shear-induced platelet-rich haemostatic plug formation and coagulation in flowing blood by haemostatometry.[19—21]

Thus, we studied the inhibitory effect of constituents isolated from *C. asiatica* on thrombosis, using this haemostatometry. By this we found a significantly active compound, 3,5-di-*O*-caffeoyl quinic acid (**2**),[22—24] together with chlorogenic acid (**1**),[22—24] 1,5-di-*O*-caffeoylquinic acid (**3**),[25] 3,4-di-*O*-caffeoylquinic acid (**4**),[22—24] 4,5-di-*O*-caffeoylquinic acid (**5**),[22—24] and also the flavonoids, kaempferol (**7**), quercetin (**8**), kaempferol 3-*O*-*β*-D-glucuronide (**9**),[26] quercetin- 3-*O*-*β*-D-glucuronide (**10**),[26,27] and the saponin, asiaticoside (**6**).[10,18] In this paper, we report an active anti-thrombotic compound, and the isolation and characterization of ten constituents isolated from this plant.

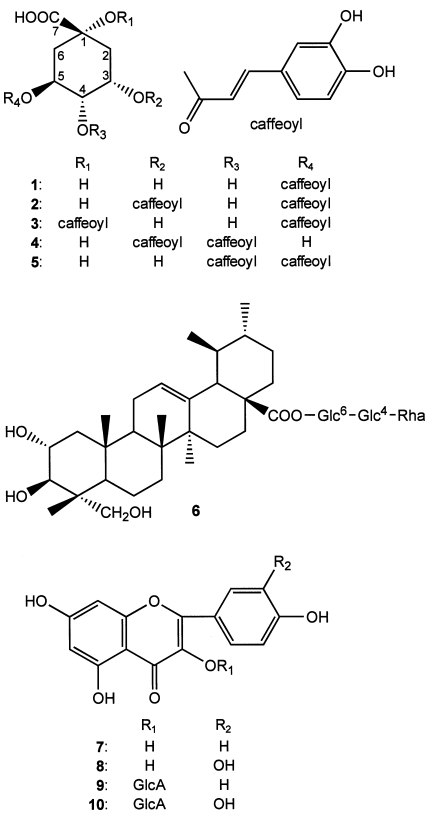

Fig. 1. Chemical Structures of Compounds **1**—**10**

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Table 1. ^{1}H-NMR Spectral Data for Quinic Acid, **1**, **2**, **3**, **4** and **5** in CD_3OD

	Quinic acid	**1**	**2**	**3**	**4**	**5**
2	2.04—2.06 (m)	α 1.95 (ddd, 14.5, 2.9, 2.8)	2.21—2.34 (m)	α 2.49 (ddd, 14.4, 4.6, 1.6)	α 2.15 (ddd, 14.9, 4.3, 2.3)	α 2.11 (ddd, 14.4, 5.2, 1.6)
		β 2.17 (dd, 14.5, 3.1)		β 2.42 (dd, 14.4, 4.0)	β 2.37 (dd, 14.9, 3.7)	β 2.30 (dd, 14.4, 3.1)
3	4.10 (q, 3.2)	4.13 (ddd, 3.2, 3.1, 2.9)	5.47 (m)	4.29 (ddd, 4.6, 4.0, 3.4)	5.65 (ddd, 4.3, 3.7, 3.4)	4.37 (dt, 5.2, 3.1)
4	3.40 (dd, 9.1, 3.2)	3.69 (dd, 10.0, 3.2)	4.01 (dd, 8.1, 3.1)	3.78 (dd, 8.1, 3.4)	5.01 (dd, 9.0, 3.4)	5.12 (dd, 9.0, 3.1)
5	4.01 (ddd, 10.9, 9.1, 4.7)	5.39 (ddd, 11.4, 10.0, 5.1)	5.44 (m)	5.39 (ddd, 9.0, 8.1, 4.0)	4.39 (ddd, 10.3, 9.0, 4.3)	5.63 (ddd, 9.6, 9.0, 5.1)
6	α 2.14 (dd, 13.2, 4.7)	α 2.10 (ddd, 12.8, 5.1, 2.8)	2.22—2.28 (m)	α 2.58 (ddd, 13.7, 4.0, 1.6)	α 2.25 (ddd, 11.9, 4.3, 2.3)	α 2.29 (ddd, 13.2, 5.1, 1.6)
	β 1.86 (dd, 13.2, 10.9)	β 2.02 (dd, 12.8, 11.4)		β 2.07 (dd, 13.7, 9.0)	β 2.11 (dd, 11.9, 10.3)	β 2.23 (dd, 13.2, 9.6)
2′		7.06 (d, 2.1)	7.07 (d, 2.0)	7.07 (d, 2.0)	7.05 (d, 2.0)	7.00 (d, 2.0)
5′		6.79 (d, 8.2)	6.79 (d, 8.1)	6.79 (d, 8.1)	6.78 (d, 8.3)	6.74 (d, 8.1)
6′		6.93 (dd, 8.2, 2.1)	6.96 (dd, 8.1, 2.0)	6.97 (dd, 8.1, 2.0)	6.93 (dd, 8.3, 2.0)	6.90 (dd, 8.1, 2.0)
7′		7.57 (d, 15.9)	7.62 (d, 15.9)	7.58 (d, 15.9)	7.58 (d, 15.9)	7.52 (d, 15.9)
8′		6.29 (d, 15.9)	6.34 (d, 15.9)	6.30 (d, 15.9)	6.30 (d, 15.9)	6.19 (d, 15.9)
2″			7.07 (d, 2.0)	7.06 (d, 2.0)	7.03 (d, 2.0)	7.02 (d, 2.0)
5″			6.79 (d, 8.1)	6.79 (d, 8.1)	6.74 (d, 8.3)	6.76 (d, 8.1)
6″			6.94 (dd, 8.1, 2.0)	6.96 (dd, 8.1, 2.0)	6.87 (dd, 8.3, 2.0)	6.92 (dd, 8.1, 2.0)
7″			7.60 (d, 15.9)	7.58 (d, 15.9)	7.55 (d, 15.9)	7.59 (d, 15.9)
8″			6.30 (d, 15.9)	6.27 (d, 15.9)	6.27 (d, 15.9)	6.28 (d, 15.9)

All assignments are based on the HH-COSY, HMQC and HMBC spectral data. Coupling patterns and constants (*J*) in Hz are given in parentheses.

Table 2. ^{13}C-NMR Spectral Data for Quinic Acid, **1**, **2**, **3**, **4** and **5** in CD_3OD

	Q	**1**	**2**	**3**	**4**	**5**
1	76.85	77.74	74.77	81.06	76.53	76.07
2	38.37	39.17	35.97	35.67	36.96	38.42
3	71.91	73.08	72.56	69.46	70.14	69.37
4	77.04	75.20	70.81	72.86	75.19	75.76
5	67.98	72.50	71.91	71.55	65.77	68.99
6	42.35	40.71	37.73	36.93	41.91	39.52
7	177.61	181.07	177.42	174.94	177.93	176.80
1′		127.74	127.82	127.83	127.75	127.68
2′		115.17	115.25	115.26	115.24	115.19
3′		146.83	146.51	146.76	146.73	146.72
4′		149.66	149.34	149.58	149.53	149.65
5′		116.57	116.43	116.50	116.50	116.49
6′		122.93	122.97	123.07	123.09	123.18
7′		146.91	147.00	147.43	147.37	147.57
8′		115.49	115.46	115.33	114.99	114.72
9′		169.30	168.87	168.02	168.55	168.23
1″			127.70	127.80	127.69	127.73
2″			115.14	115.17	115.15	115.21
3″			146.51	146.76	146.70	146.75
4″			149.43	149.57	149.53	149.67
5″			116.43	116.49	116.46	116.49
6″			123.04	123.01	123.21	123.23
7″			147.19	147.27	147.31	147.70
8″			115.01	115.14	114.91	114.77
9″			168.42	168.67	168.63	168.56

All assignments are based on the HH-COSY, HMQC and HMBC spectral data. Q: quinic acid.

MATERIALS AND METHODS

Plant Material *Centella asiatica* was cultivated in the botanical garden of Faculty of Pharmaceutical Science, Kobe Gakuin University and harvested at July in 2001. A voucher specimen has been retained in our laboratory.

Materials Male Wistar ST rats aged 10—11 weeks (280—340 g, Japan SLC, Hamamatsu, Japan), Nembutal (sodium pentobarbital, Dainippon Pharmaceutical Co., Ltd.) as an anesthetic.

General Procedures Optical rotations were measured using a Jasco DIP-1000 digital polarimeter. HR-FAB-MS were performed with a JEOL JMS-BU 20 spectrometer. IR and UV spectra were measured on a Shimadzu FT-IR 8300 infrared spectrometer and a Hitachi U-3000 spectrometer, respectively. The NMR spectra were recorded in CD_3OD, C_5D_5N and DMSO-d_6 on a Bruker DPX-400 instrument. TLC was performed on Merck precoated TLC plates (Kieselgel 60F_{254}, Rp-18F_{254}). Column chromatography was conducted with Kieselgel 60 (70—230 mesh, Merck) and Sephadex LH-20 (Pharmacia). Medium pressure liquid chromatography (MPLC, micro pump KP-7, Kusano Scientific Co., Tokyo, Japan) was carried out on a CIG column [ODS (C-18)].

Extraction and Isolation The air-dried aerial parts of *C. asiatica* (790 g) were extracted 6 times with methanol (4 l) at 60 °C for 6 h. The combined MeOH was evaporated under reduced pressure to give the MeOH extract (460.2 g), which was suspended in H_2O : MeOH (3 : 1) and then was sequentially partitioned with $CHCl_3$, EtOAc and *n*-BuOH. Each solvent was removed under reduced pressure to yield the $CHCl_3$ soluble phase (91.4 g), the EtOAc soluble phase (14.1 g), the *n*-BuOH soluble phase (91.5 g) and H_2O soluble phase (258.2 g). The *n*-BuOH soluble phase was subjected to column chromatography on Sephadex LH-20 (MeOH) to give fractions 1—5. From fraction 2, compound **6** (1.50 g) was isolated. Fraction 3 was subjected to further column chromatography on Sephadex LH-20 (H_2O : CH_3CN = 1 : 1) followed by MPLC [Rp-18 (H_2O)] to furnish compound **1** (95 mg).

Fraction 4 was further purified by column chromatography on Sephadex LH-20 (H_2O : CH_3CN = 1 : 1) followed by MPLC [Rp-18 (H_2O : MeOH = 1 : 1) to give compounds **9** (130 mg) and **10** (120 mg). Fraction 5 was subjected to column chromatography on Sephadex LH-20 (H_2O : MeOH = 1 : 1) followed by MPLC [Rp-18 (H_2O : MeOH = 5 : 2)] to yield compounds **2** (900 mg), **3** (470 mg), **4** (15 mg), and **5** (98 mg).

The EtOAc soluble phase underwent column chromatography on Sephadex LH-20 (MeOH) to give fractions 1—4. Fraction 3 was subjected to MPLC [Rp-18 (H_2O : MeOH = 5 : 2)] to furnish compounds **7** (60 mg) and **8** (175 mg). Fraction 2 was purified by MPLC [Rp-18 (H_2O : MeOH = 5 : 2) to

afford compounds **2** (550 mg) and **3** (400 mg). Fraction 1 was further separated by chromatography on ODS [Rp-8 (H_2O : MeOH = 5 : 2)] and Sephadex LH-20 (H_2O : MeOH = 1 : 1) to purify **2** (900 mg), **3** (800 mg), **4** (45 mg), and **5** (25 mg).

The structures of these compounds were elucidated on the basis of following data and comparison with authentic samples.

Compound 1 (Chlorogenic Acid) Colorless needles from water and methanol, $[\alpha]_D^{26}$ −28° (c=0.8, H_2O); IR ν_{max} (KBr) cm^{-1}: 3350 (OH), 1685 (C=O), 1652, 978 (*trans* C=C), 1600, 1516, 1445 (aromatic ring); UV λ_{max} (MeOH) nm (log ε): 332 (4.60), 328 (4.55), 301 (4.51); ^{1}H-NMR (400 MHz, CD_3OD): Table 1; ^{13}C-NMR (100 MHz, CD_3OD): Table 2.

Acid Hydrolysis of Compound 1 Compound **1** (15 mg) was hydrolyzed with 3% HCl (5 ml) at 90 °C for 3 h under stirring. The reaction mixture, after cooling, was neutralized with Ag_2CO_3, and the insoluble material was filtered off. The filtrate was evaporated to dryness and the residue was subjected to chromatography on Sephadex LH-20 (MeOH) to give L-quinic acid, $[\alpha]_D^{26}$ −38° (c=0.3, MeOH).

Compound 2 (3,5-Di-*O*-caffeoylquinic Acid) Colorless amorphous powder, $[\alpha]_D^{26}$ −175.8° (c=0.84, MeOH); negative-FAB-MS *m/z*: 515 $[M-H]^-$; IR ν_{max} (KBr) cm^{-1}: 3400 (OH), 1683 (C=O), 1652, 978 (*trans* C=C), 1602, 1516, 1447 (aromatic ring); UV λ_{max} (MeOH) nm (log ε): 332 (4.60), 297 (4.40), 245 (4.28), 219 (4.44); ^{1}H-NMR (400 MHz, CD_3OD): Table1; ^{13}C-NMR (100 MHz, CD_3OD): Table 2.

Compound 3 (1,5-Di-*O*-caffeoylquinic Acid) Colorless amorphous powder, $[\alpha]_D^{26}$ +204° (c=0.68, MeOH); positive-FAB-MS *m/z*: 517 $[M+H]^+$; IR ν_{max} (KBr) cm^{-1}: 3400 (OH), 1692 (C=O), 1650, 979 (*trans* C=C), 1602, 1522, 1447 (aromatic ring); UV λ_{max} (MeOH) nm (log ε): 329 (4.60), 302 (4.42), 245 (4.28), 219 (4.44); ^{1}H-NMR (400 MHz, CD_3OD): Table 1; ^{13}C-NMR (100 MHz, CD_3OD): Table 2.

Compound 4 (3,4-Di-*O*-caffeoylquinic Acid) Colorless amorphous powder, $[\alpha]_D^{26}$ −134.7° (c=0.63, MeOH); positive-FAB-MS *m/z*: 517 $[M+H]^+$; IR ν_{max} (KBr) cm^{-1}: 3370 (OH), 1693 (C=O), 1640, 978 (*trans* C=C), 1605, 1522, 1447 (aromatic ring); UV λ_{max} (MeOH) nm (log ε): 328 (4.50), 245 (4.28), 218 (4.42); ^{1}H-NMR (400 MHz, CD_3OD): Table 1; ^{13}C-NMR (100 MHz, CD_3OD): Table 2.

Compound 5 (4,5-Di-*O*-caffeoylquinic Acid) Colorless amorphous powder, $[\alpha]_D^{26}$ −232.4° (c=0.43, MeOH); positive-FAB-MS *m/z*: 517 $[M+H]^+$, IR ν_{max} (KBr) cm^{-1}: 3265 (OH), 1695 (C=O), 1640, 978 (*trans* C=C), 1601, 1516, 1447 (aromatic ring); UV λ_{max} (MeOH) nm (log ε): 329 (4.51), 245 (4.27), 218 (4.40); ^{1}H-NMR (400 MHz, CD_3OD): Table 1; ^{13}C-NMR (100 MHz, CD_3OD): Table 2.

Compound 6 (Asiaticoside) Colorless needles from MeOH, mp 230—232 °C (decomp.), $[\alpha]_D^{26}$ −16° (c=0.52, MeOH); negative-FAB-MS *m/z*: 957 $[M-H]^-$; IR ν_{max} (KBr) cm^{-1}: 3246 (OH), 2878 (CH_3, CH_2), 1734 (C=O), 1636 (C=C); ^{13}C-NMR (δ, C_5D_5N): 14.4 (C-24), 17.3 (C-29), 17.6 (C-25), 17.8 (C-26), 18.4 (C-6‴), 18.5 (C-6), 21.3 (C-30), 23.7 (C-27), 23.8 (C-11), 24.5 (C-16), 28.7 (C-15), 30.8 (C-21), 33.1 (C-7), 36.8 (C-22), 38.3 (C-10), 39.1 (C-19), 39.3 (C-20), 40.2 (C-8), 42.5 (C-14), 43.6 (C-4), 47.9 (C-5), 48.1 (C-9), 48.1 (C-1), 48.4 (C-17), 53.2 (C-18), 61.3 (C-6″), 66.5 (C-23), 68.9 (C-2), 69.3 (C-6′), 70.3 (C-5‴), 71.0 (C-4′), 72.5 (C-2‴), 72.7 (C-3‴), 73.7 (C-2′), 73.9 (C-4‴), 75.2 (C-2″), 76.4 (C-3″), 77.1 (C-5″), 77.8 (C-5′), 78.2 (C-3), 78.2 (C-4″), 78.7 (C-3′), 95.6 (C-1′), 102.6 (C-1‴), 104.8 (C-1″), 126.0 (C-12), 138.5 (C-13), 176.3 (C-28).

Compound 7 (Kaempferol) Yellow amorphous powder; IR ν_{max} (KBr) cm^{-1}: 3385 (OH), 1660 (C=O), 1602, 1503, 1450 (aromatic ring); UV λ_{max} (MeOH) nm (log ε): 256 (4.14), 371 (4.15); ^{1}H-NMR (δ, DMSO-d_6): 6.18 (1H, d, J=2.1 Hz, H-6), 6.38 (1H, d, J=2.1 Hz, H-8), 8.06 (2H, d, J=9.1 Hz, H-2′, H-6′), 6.90 (2H, d, J=9.1 Hz, H-3′, H-5′), ^{13}C-NMR (δ, DMSO-d_6): 148.03 (C-2), 137.06 (C-3), 177.30 (C-4), 162.42 (C-5), 99.27 (C-6), 165.49 (C-7), 94.49 (C-8), 158.20 (C-9), 104.52 (C-10), 123.71 (C-1′), 130.65 (C-2′, C-6′), 116.29 (C-3′, C-5′).

Compound 8 (Quercetin) Yellow amorphous powder; IR ν_{max} (KBr) cm^{-1}: 3380 (OH), 1652 (C=O), 1613, 1512, 1450 (aromatic ring); UV λ_{max} (MeOH) nm (log ε): 255 (4.36), 373 (4.38); ^{1}H-NMR (δ, DMSO-d_6): 6.20 (1H, d, J=2.1 Hz, H-6), 6.42 (1H, d, J=2.1 Hz, H-8), 7.69 (1H, d, J=2.2 Hz, H-2′), 6.90 (1H, d, J=8.5Hz, H-5′), 7.55 (1H, dd, J=8.5, 2.2 Hz, H-6′); ^{13}C-NMR (δ, DMSO-d_6): 146.79 (C-2), 135.69 (C-3), 175.69 (C-4), 160.71 (C-5), 98.16 (C-6), 163.85 (C-7), 93.33 (C-8), 156.13 (C-9), 103.00 (C-10), 121.95 (C-1′), 115,05 (C-2′), 145.01 (C-3′), 147.65 (C-4′), 115,58 (C-5′), 119.97 (C-6′).

Compound 9 (Kaempferol 3-*O*-*β*-D-Glucuronide) Yellow amorphous powder, IR ν_{max} (KBr) cm^{-1}: 3380 (OH), 1652 (C=O), 1613, 1512, 1450 (aromatic ring); ^{1}H-NMR (δ, DMSO-d_6): 6.10 (1H, d, J=2.0 Hz, H-6), 6.31 (1H, d, J=2.0 Hz, H-8), 8.02 (2H, d, J=8.9 Hz, H-2′, H-6′), 6.85 (2H, d, J=8.9 Hz, H-3′, H-5′), 5.52 (1H, d, J=7.5 Hz, H-1″), 3.26—3.29 (2H, m, H-2″, H-3″), 3.22 (1H, m, H-4″), 3.40 (1H, d, J=9.4 Hz, H-5″); ^{13}C-NMR (δ, DMSO-d_6): 156.24 (C-2), 133.48 (C-3), 177.38 (C-4), 160.14 (C-5), 98.81 (C-6), 164.96 (C-7), 93.86 (C-8), 156.37 (C-9), 103.60 (C-10), 120.85 (C-1′), 130.93 (C-2′, C-6′), 115.16 (C-3′, C-5′), 160.98 (C-4′), 101.29 (C-1″), 74.48 (C-2″), 76.40 (C-3″), 71.94 (C-4″), 74.06 (C-5″), 172.74 (C-6″).

Compound 10 (Quercetin 3-*O*-*β*-D-Glucuronide) Yellow amorphous powder, IR ν_{max} (KBr) cm^{-1}: 3380 (OH), 1652 (C=O), 1613, 1512, 1450 (aromatic ring); ^{1}H-NMR (δ, DMSO-d_6): 6.17 (1H, d, J=2.0 Hz, H-6), 6.36 (1H, d, J=2.0 Hz, H-8), 8.47 (1H, d, J=2.2 Hz, H-2′), 6.82 (1H, d, J=8.4 Hz, H-5′), 7.29 (1H, dd, J=8.4, 2.2 Hz, H-6′), 5.23 (1H, d, J=7.3 Hz, H-1″), 3.24—3.27 (2H, m, H-2″, H-3″), 3.20 (1H, dd, J=9.7, 9.7 Hz, H-4″), 3.39 (1H, d, J=9.7 Hz, H-5″); ^{13}C-NMR (δ, DMSO-d_6): 157.55 (C-2), 133.97 (C-3), 177.50 (C-4), 160.95 (C-5), 99.00 (C-6), 165.14 (C-7), 93.85 (C-8), 156.56 (C-9), 103.51 (C-10), 120.61 (C-1′), 115.44 (C-2′), 144.80 (C-3′), 148.38 (C-4′), 115.44 (C-5′), 119.97 (C-6′), 102.94 (C-1″), 74.31 (C-2″), 76.73 (C-3″), 71.85 (C-4″), 74.00 (C-5″), 172.20 (C-6″).

Acid Hydrolysis of 9 and 10 for Analysis of Sugar Moiety Refluxing of each compound **9** and **10** (10 mg) in 5% HCl for 4 h gave quercetin and kaempferol as aglycon moiety, respectively and D-glucuronic acid as sugar moiety (identified by comparison with authentic sample).

Oral-Administration of Samples To evaluate the antithrombotic effect on rat blood, the MeOH extract, EtOAc sol-

uble phase, *n*-BuOH soluble phase, H_2O soluble phase, and compounds (**2**, **3**, **6**, **7**, **8**) which were dissolved in distilled water (4 ml/kg body weight, *p.o.*), were administrated orally through a gastric tube to rats at a dosage of 4 ml/kg (body weight, *p.o.*) twice a day at intervals of 6 h for two weeks (each $n=6$).

Preparation of Samples Group MeOH: MeOH extract (45 mg/kg body weight, *p.o.*) was dissolved in distillated water (4 ml/kg body weight, *p.o.*). Group EtOAc, *n*-BuOH and H_2O: EtOAc, *n*-BuOH and H_2O soluble phases (each 14 mg/kg body weight, *p.o.*) were dissolved in distilled water of 4 ml/kg (body weight, *p.o.*).

Evaluation of Anti-thrombotic Effect Using Haemostatometry The haemostatometer was invented by Gorog and Kovacs.[28—30] On the basis of their published data, a three-channel haemostatometer was constructed by Yamamoto, *et al.*, at the Faculty of Nutrition of Kobe Gakuin University for research purposes.

The principle of haemostatometry is shown in Figs. 2 and 3. The haemostatometer is composed of three parts A, B and C as shown Fig. 2. The blood, which was withdrawn from the abdominal aorta of the rat at 40 min after Nembutal anesthesia (60 mg/kg, intramuscularly), was dispensed in 1.0 ml of syringes without anti-coagulant. The syringe was inserted vertically into a syringe holder at 37 °C (Fig. 2A). Paraffin liquid was infused into the blood sample at the constant flow rate of 0.057 ml/min and at a pressure of 60 mmHg, and non-anticoagulated blood was displaced by the paraffin liquid, resulting in blood flow in the other polyethylene tubing (o.d., 1.00 ± 0.02 mm; i.d., 0.50 ± 0.01 mm). While blood was flowing in it, the tubing was pierced with a fine needle at 150s after blood withdrawal, which resulted in 'bleeding' from the pieced tube into surrounding warm saline (initial pressure decrease, Fig. 2B). The initial shear stress was 375 dynes/cm^2. Eventually 'bleeding' stopped due to the formation of platelet-rich haemostatic plugs in the holes. Subsequently, the flow in the main tube stopped, and the pressure in C decreased, indicating coagulation. (second pressure decrease, Fig. 2C). The recorded pressure changes reflected both the haemostatic and coagulation processes.

The pressure changes were analyzed by computer as shown in Fig. 3. The pressure recovery curve was used to assess platelet activation. The areas until 30% (H1) and 90% (H2) pressure recovery from the maximal pressure drop after the piercing-induced initial pressure drop reflected the initial haemostatic reaction.

After initial haemostasis was achieved, the non-anticoagulated blood clotted resulting in a second decrease of the perfusion pressure. The time until a 10 mmHg decrease of pressure from the original level (60 mmHg) was defined as clot-

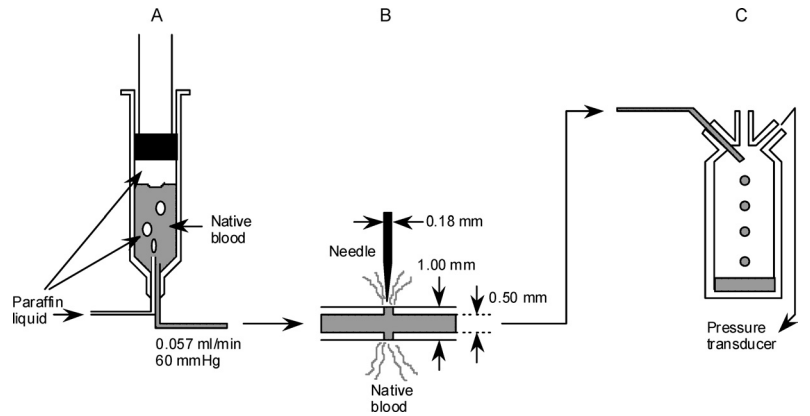

Fig. 2. Principle of Haemostatometry

Haemostatometer is composed of three parts, A, B and C. Non-anticoagulated blood in a syringe is kept vertically in a holder at 37 °C (A). Non-anticoagulated blood is displaced by paraffin liquid at 0.057 ml/min, resulting in blood flow in the other polyethylene tubing (o.d., 1.00 ± 0.02 mm; i.d., 0.50 ± 0.01 mm). Bleeding in surrounding saline at 37 °C is caused by punching with a needle, 0.18 mm in diameter, followed by haemostasis, and shear-induced platelet-rich plug formation (B). Polyethylene tubing is connected to a blood waste reservoir under 60 mmHg and changes in pressure are recorded (C).

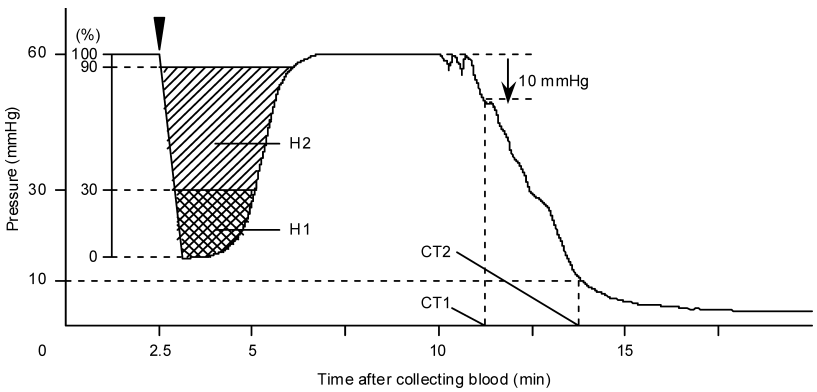

Fig. 3. A Typical Haemostatograph

▼, punching. H1, the area until 30% pressure recovery; H2, the area until 90% pressure recovery; CT1, the time until a pressure drop of 10 mmHg from baseline, 60 mmHg, by clotting; CT2, the time to reach 10 mmHg keeping below 10 mmHg for 1 min.

ting time 1 (CT1, min), while the time until the pressure declined to 10 mmHg was defined as clotting time 2 (CT2, min).

The effect of samples on platelet reactivity and coagulation was assessed by the changes in (H1, H2) and (CT1, CT2), respectively. Numerical increases or decreases in H1 and H2 reflected inhibition or enhancement of platelet reactivity, respectively. Prolongation of CT1 and CT2 indicated inhibition, while shortened times indicated accelerated dynamic coagulation.

Statistical Analysis All data were analyzed by ANOVA, followed by Fisher's *post hoc* test.

RESULTS

The ability of the MeOH extract and EtOAc, *n*-BuOH, and H_2O soluble phases to inhibit platelet reactivity (H2) and dynamic coagulation (CT2) *ex vivo* was measured by haemostatometry. As there was a significant positive correlation between H1 and H2 data, as well as between CT1 and CT2 data, only H2 and CT2 data were presented in Fig. 4. As shown in Fig. 4, the MeOH extract exhibited inhibition of platelet reactivity but did not affect dynamic coagulation. The EtOAc soluble phase inhibited both platelet reactivity and dynamic coagulation, whereas *n*-BuOH soluble phase exhibited inhibition of platelet reactivity but did not affect dynamic coagulation. From the highly active EtOAc soluble phase, 3,5-di-*O*-caffeoylquinic acid (**2**, 1.45 g) and 1,5-di-*O*-caffeoylquinic acid (**3**, 1.20 g) were isolated as major components, together with 3,4-di-*O*-caffeoylquinic acid (**4**, 45 mg), 4,5-di-*O*-caffeoylquinic acid (**5**, 70 mg), kaempferol (**7**, 60 mg), quercetin (**8**, 175 mg) as minor components. From the *n*-BuOH soluble phase, chlorogenic acid (**1**, 98 mg), **2** (900 mg), **3** (470 mg), **4** (15 mg) and **5** (98 mg) were isolated together with kaempferol 3-*O*-β-D-glucuronide (**9**, 130 mg), quercetin 3-*O*-β-D-glucuronide (**10**, 120 mg) and asiaticoside (**6**, 1.50 g). Compounds **2**, **3**, **6**, **7**, and **8** were yielded in comparatively large quantities, and so their individual inhibitory effects on platelet reactivity and dynamic coagulation were studied by haemostatometry. Blood samples were withdrawn from the abdominal aorta of rats after oral administration of each compound twice a day at intervals of 6 h for a period of 14 d (each 4 mg compounds/4 ml distilled water/kg/d). A significant inhibitory effect on platelet reactivity was observed after oral treatment of compound **2**. This effect was even observed on dilution ($\times$10, $\times$20). However, compound **3**, which is a 1,5-disubstituted isomer of **2**, as well as flavonoids (**8**, **9**) had no effect on platelet reactivity or dynamic coagulation. Furthermore, asiaticoside (**6**), which is the main saponin constituent of this plant, did not inhibit platelet reactivity and dynamic coagulation.

DISCUSSION

Centella asiatica has long been used in the Ayurvedic system of medicine in India to treat a variety of diseases and has been used all over the world to heal many kinds of ailments. There have been many investigations on the bioactivity of this plant, but most of these publications are of studies of the well-known asiaticoside (**6**), the main saponin.[9—18] We were unable to find any papers referring to anti-thrombotic effects of *C. asiatica*. It is known that platelet-rich thrombi play a pivotal role in myocardial infarction and stroke, having formed at sites of damage or stenosis in arteries.[31,32] Platelets are activated by shear forces as well as by various agonists, resulting in the formation of platelet-rich thrombi. This present study is the first to demonstrate the inhibitory effects of the MeOH extract of *C. asiatica* and its compo-

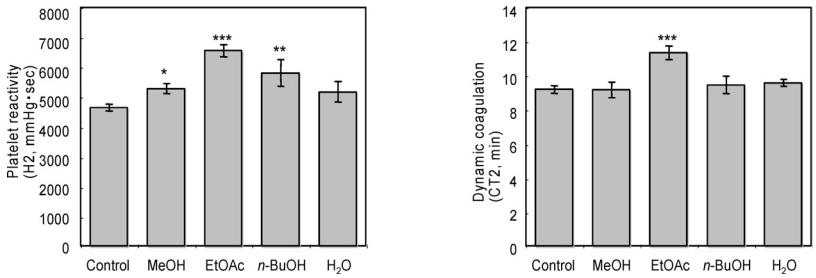

Fig. 4. Inhibitory Effect of Orally Administrated MeOH Extract (45 mg/kg) and EtOAc, *n*-BuOH and H_2O Soluble Phases (Each, 14 mg/kg) Twice a Daily for a Period of 14 d on Platelet Reactivity (H2) and Dynamic Coagulation (CT2)

$*p<0.05$, $**p<0.01$, $***p<0.001$ by ANOVA with subsequent Fisher's *post hoc* test compared with distilled water control.

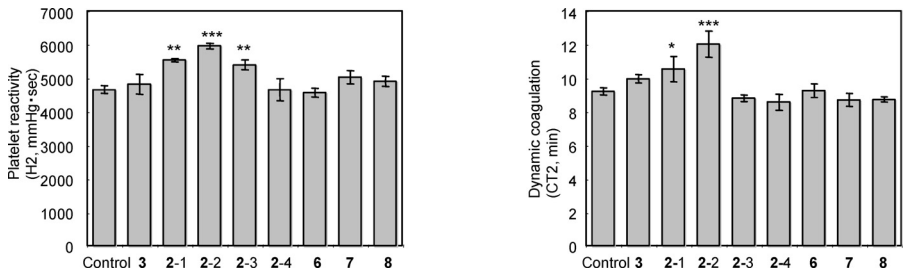

Fig. 5. Inhibitory Effect of Orally Administrated **3** (4 mg/kg), **2**-1 (4 mg/kg), **2**-2 (0.4 mg/kg), **2**-3 (0.2 mg/kg), **2**-4 (0.04 mg/kg), **6** (4 mg/kg), **7** (4 mg/kg) and **8** (4 mg/kg) Twice a Daily for a Period of 14 d on Platelet Reactivity (H2) and Dynamic Coagulation (CT2)

$*p<0.05$, $**p<0.01$, $***p<0.001$ by ANOVA with subsequent Fisher's *post hoc* test compared with distilled water control.

nent, on shear-induced platelet activation and coagulation in non-anticoagulated blood after oral administration to rats. In the system presented here, the effect of chronic administration (twice a day for 14 d) on the inhibition of the platelet reactivity and the dynamic coagulation were assessed. The results showed that the EtOAc soluble phase from the MeOH extract exhibited the strongest inhibitory activity. From the EtOAc soluble phase, 3,5-di-*O*-caffeoylquinic acid (**2**) and 1,5-di-*O*-caffeoylquinic acid (**3**) were isolated as major constituents together with minor quantities of 3,4-di-*O*-caffeoylquinic acid (**4**), 4,5-di-*O*-caffeoyl-quinic acid (**5**), and flavonoids (**7**, **8**). In the biological experiment, only the five compounds (**2**, **3**, **6**, **7**, **8**) obtained in high yield were examined, as a large amount of test samples were required for oral administration twice a day for a period of two weeks. Among these compounds, only 3,5-di-*O*-caffeoylquinic acid (**2**) exhibited an anti-thrombotic effect and inhibitory effect on dynamic coagulation. As shown in Fig. 5, the reactive curve of the inhibitory effect in regard to the platelet reaction and the dynamic coagulation, showed a bell-shape with maximum activity at a concentration of 0.4 mg/kg, which decreased at 4 mg/kg and 0.2 mg/kg.

As about 0.23 mg of compound **2** was calculated to be contained in 45 mg of the MeOH extract, it was expected that it might show a considerable inhibitory effect (** $p<0.01$) on the platelet activation. However, the MeOH extract inhibited to a weak level only (* $p<0.05$, Fig. 4). This finding indicated that some compounds which promoted thrombi, might be included in the MeOH extract, but these compounds have not yet been confirmed. The 900 mg of **2** isolated from the *n*-BuOH soluble phase (91.5 g) corresponded to 0.14 mg of **2** in the *n*-BuOH soluble phase (14 mg) on calculation, however this phase inhibited relatively strongly the platelet activation (** $p<0.01$) while not inhibiting dynamic coagulation. Accordingly, another minor constituent (ex. compounds **1**, **4**, **5**) in the *n*-BuOH soluble phase might also have an anti-thrombotic effect. However as only compounds **2** and **3** have been investigated so far, the other ester derivatives of a quinic acid and caffeic acids need to be studied with regard to anti-thrombotic effects.

Furthermore, 3,5-di-*O*-caffeoylquinic acid (**2**), 3,4-di-*O*-caffeoylquinic acid (**4**) and 3,4,5-tri-*O*-caffeoylquinic acid have been reported to have anti-hypertensive effects by unknown mechanisms.[33] So, the observation that **2** has an anti-thrombotic effect supports the fact that 3,5-di-*O*-caffeoylquinic acid (**2**) has the anti-hypertensive effect. Compounds such as ester derivatives of quinic acid and caffeic acids are not particularly unusual as natural compounds, but they are normally found only in small quantities.

Thus, *C. asiatica* was shown to promote blood circulation to remove blood stasis.

Consequently, *C. asiatica* is a useful medicinal plant for the prevention of lifestyle-related diseases such as hypertension, cardiopathy and cerebral apoplexy caused by arteriosclerosis.

Acknowledgments We are grateful to Dr. K. Hori, Akita Research Institute of Food and Brewing, for measurements of HR-FAB-MS, and Miss C. Yamasita and S. Umehara, Kobe Gakuin University, for their cooperation in experiments.

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