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Tissue Factor Inhibitory Flavonoids from the Fruits of *Chaenomeles sinensis*

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Tissue factor (TF, tissue thromboplastin or coagulation factor III) accelerates the blood clotting, activating both the intrinsic and the extrinsic pathways to serve as a cofactor. In order to isolate TF inhibitors from the fruits of *Chaenomeles sinensis*, an activity-guided purification utilizing a bio-assay method of prothrombin time prolongation, was carried out to yield five active flavoniods such as hovetrichoside C (**1**) (IC_{50} = 14.0 μg), luteolin-7-*O*-β-D-glucuronide (**3**) (IC_{50} = 31.9 μg), hyperin (**4**) (IC_{50} = 20.8 μg), avicularin (**6**) (IC_{50} = 54.8 μg) and quercitrin (**10**) (IC_{50} = 135.7 μg), along with other inactive compounds such as (±)-(2*E*,4*E*)-*O*-β-D-glucopyranosyl-4'-hydroxy-β-ionylideneacetic acid ester (**2**), genistein-7-*O*-β-D-glucopyranoside (**5**), luteolin-3'-methoxy-4'-*O*-β-D-glucopyranoside (**7**), luteolin-7-*O*-β-D-glucuronide methyl ester (**8**), tricetin-3'-methoxy-4'-*O*-β-D-glucopyranoside (selagin-4'-*O*-β-D-glucopyranoside) (**9**), (−)-epicatechin (**11**), luteolin-4'-*O*-β-D-glucopyranoside (**12**) and apigenin-7-*O*-β-D-glucuronide methyl ester (**13**). The structures of the isolated compounds were elucidated through spectral analysis. Among them, compounds **1** to **9**, **12** and **13** were isolated for the first time from the fruits of this plant and the compound **9** is a new flavonoid.

Key words: *Chaenomeles sinensis* (Rosaceae), Tricetin-3'-methoxy-4'-O-β-D-glucopyranoside, Tissue factor inhibitors

INTRODUCTION

TF is a cell surface receptor of FVII (coagulation factor VII) and is the principal initiator of the vertebrate coagulation cascade (Nemerson, 1988). Vascular damage exposes blood to cells expressing TF, which participates to form a TF/FVII (a)/phospholipid/Ca^{2+} complex. The complex mediates the activation of both the intrinsic and the extrinsic pathways (Osterud and Rapaport, 1977). This critical position of the TF/FVII (a) complex within the blood coagulation cascade makes it an attractive target for anti-coagulant drug discovery (Harker *et al.*, 1995), so the researches of TF inhibitors from natural products may have a profound significance. We have screened various plants belonging to Rosaceae for examining their inhibition on TF by measuring the prolongation of prothrombin time after addition to TF in rat plasma, and the fruits of *C. sinensis* were turned out to have the strong inhibitory activity on TF.

The fruits of *Chaenomeles sinensis* (Thunb.) Koehne (Rosaceae), Chinese quince, have been used in a traditional medicine for the treatment of cough, common cold, pain and diarrhea (Lee, 1966; Namba, 1992) in Korea, Japan and China. It is known that the fruits contain some organic acids, ursolic acid-3-*O*-behenate, 3-acetyl ursolic acid, 3-acetyl pomolic acid, betulinic acid (Sun and Hong, 2000), oleanolic acid, ursolic acid (Im and Roh, 1991), chaenoside A and B (Im and Roh, 1994), maslinic acid, tormentic acid, euscaphic acid (Roh *et al.*, 1995), 2-hydroxy-naringenin-7-*O*-β-D-glucoside (Kim *et al.*, 2000), 5-*O*-*p*-coumaroylquinic acid, (−)-epicatechin (Osawa *et al.*, 2001), α-terpineol, isozedoarondiol, isofuranogermacrene, quercetin, rutin, quercitrin (TradiMed, 1996), proanthocyanidin (Mattic and Porter, 1984) and tannin (Matsuo and Ito, 1981). The methanolic extract of *C. sinensis* fruits is known to show anti-influenza A virus activity (Kim *et al.*, 2000). This report describes the isolation and characterization of TF inhibitors from the fruits of this plant.

MATERIALS AND METHODS

Plant materials

The dried pieces of the fruits of *C. sinensis* were pur-

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chased from a Kyungdong market in Seoul, Korea and verified by Emeritus Prof. H. J. Chi, Seoul National University Korea. A voucher specimen (NPRI 010201) was deposited at the Herbarium of Natural Products Research Institute, Seoul National University, Korea.

General experimental procedures

Melting point was determined on a Büchi B-540 apparatus and was uncorrected. UV spectra were recorded on a Hitachi U-3210 UV-VIS spectrophotometer. IR spectra were obtained on a Jasco FT/IR-5300 spectrophotometer. FAB-MS was measured on a Jeol Mstation JMS-700 mass spectrometer and EI-MS on a Hewlett Packard model 5989B GC/MS spectrometer. NMR spectra were recorded on a Varian Gemini-2000 spectrometer or on a Bruker Advance 400 spectrometer operating at 300 or 400 MHz for ^{1}H and 75 or 100 MHz for ^{13}C-NMR. Optical rotations were determined on a Jasco P-1020 polarimeter. Centrifuges were taken at Sorvall RT 6000 centrifuge and Sorvall OTD 65B ultracentrifuge, rotor T 865. Silica gel (63-200 μm, Merck KGaA), Sephadex LH-20 (25-100 μm, Sigma Fluka) and LiChroprep RP-18 (40-63 μm, Merck KGaA) were used for open chromatography. TLC was performed on silica gel 60 F_{254} (Merck) and RP-18 F_{254s} (Merck).

Preparation of tissue factor

The microsomal fraction of rat lung tissues was used as a tissue factor source (Han and Rhee, 1998). The lung tissue of Sprague-Dawley rats was taken and one g of the tissue was homogenized with 4 ml of 0.15 M NaCl solution for 2 min with a homogenizer in an ice bath. The homogenate was centrifuged at 2,000 rpm for 20 min and an aliquot (2.5 ml) of the supernatant was centrifuged at 31,000 rpm (105,000×g), 4 °C for 1 hr to give the microsoma fraction. The fraction was suspended in 2.5 ml of 0.15 M NaCl to give a suspension, which was used as a TF stock solution. The dilution of the stock by 100-200 times was used for assay, which gave the 1 to 10 units of TF.

Determination of the tissue factor activity

Prothrombin time was measured to determine the TF activity using citrated plasma from rats (Han and Rhee, 1998). Blood was taken from heart using syringe containing 3.13% sodium citrate, of which final concentration after taking blood was 10%. Plasma was prepared by centrifugation at 2,500 rpm for 15 min. In a plastic tube prewarmed at a 37 °C water bath, 100 μl of plasma, 100 μl of TF solution diluted to the proper concentration with saline and 100 μl of 25 mM $CaCl_2$ were taken, and prothrombin time was measured after the addition of $CaCl_2$. It was executed in duplicate and the average was taken.

A standard curve was made from the measurements of the prothrombin time assayed on the lung tissues from 25 rats over several times against the pooled up plasma from 9 rats. It was considered as 100% TF activity when the prothrombin time (PT) with TF was 18 sec, and the plasma recalcification time (RT) without TF as 0% TF activity. Clotting acceleration percentage was calculated according to Eq. 1.

$$\textit{Plasma clotting acceleration}\ (\%)=\frac{RT-PT}{RT-18}\times 100 \qquad \text{(Eq. 1)}$$

The standard curve was drawn from the acceleration (%) on the Y-axis and the unit of TF in log scale on the X-axis. The amount of TF that accelerated 50% the prothrombin time was arbitrarily defined as one unit of TF.

The inhibition percentage was calculated by decrease extent of TF activity in unit as Eq. 2.

$$\textit{Inhibition}\ (\%)=\frac{Bu-Au}{Bu}\times 100 \qquad \text{(Eq. 2)}$$

Au, unit of TF with TF and sample; Bu, unit of TF with TF and without sample. The IC_{50} value of TF inhibitor was obtained from a logit-log graph on relationship between the inhibition percentage and the concentration of inhibitor in log scale (Fig. 1).

Extraction and isolation

The dried pieces of the fruits of *C. sinensis* (20 kg) were percolated with MeOH. The methanolic extract (4.6 kg) was successively partitioned between *n*-hexane, EtOAc, *n*-BuOH and H_2O to afford 140, 350, 973 and 2960 g of residues, respectively. Repetitive chromatography of the EtOAc fraction (350 g) over Sephadex LH-20 eluting with MeOH-H_2O (1 : 1) afforded two active fractions A (19 g) and B (38 g). The fraction A was subjected to chromatography on a silica gel column eluting with a gradient of $CHCl_3$-MeOH-H_2O to give an active sub-fractions A_1 (1 g) and an inactive sub-fractions A_2 (2 g). Further chromatography of the sub-fraction A_1 on silica gel (a gradient of EtOAc-MeOH-H_2O) and RP-18 (MeOH-H_2O, 2 : 1) columns yielded compound **1** (100 mg); that of the sub-fraction A_2 on silica gel (a gradient of $CHCl_3$-MeOH-H_2O), RP-18 (MeOH-H_2O, 1 : 1) and silica gel (EtOAc-MeOH-H_2O, 500 : 13.5 : 10) columns yielded compound **2** (60 mg). The fraction B was divided into two parts according to its solubility in MeOH-H_2O (1 : 1). The part of precipitate (285 mg) was chromatographed on a silica gel column ($CHCl_3$-MeOH-H_2O, 70 : 30 : 4) yielded compounds **3** (50 mg) and **4** (50 mg). The soluble part was chromatographed on a silica gel column eluting with a gradient of $CHCl_3$-MeOH-H_2O to give three sub-fractions B_1 (5 g), B_2 (3 g) and B_3 (318 mg). Further chromatography of the sub-fraction B_1 on a silica gel column (a gradient of EtOAc-

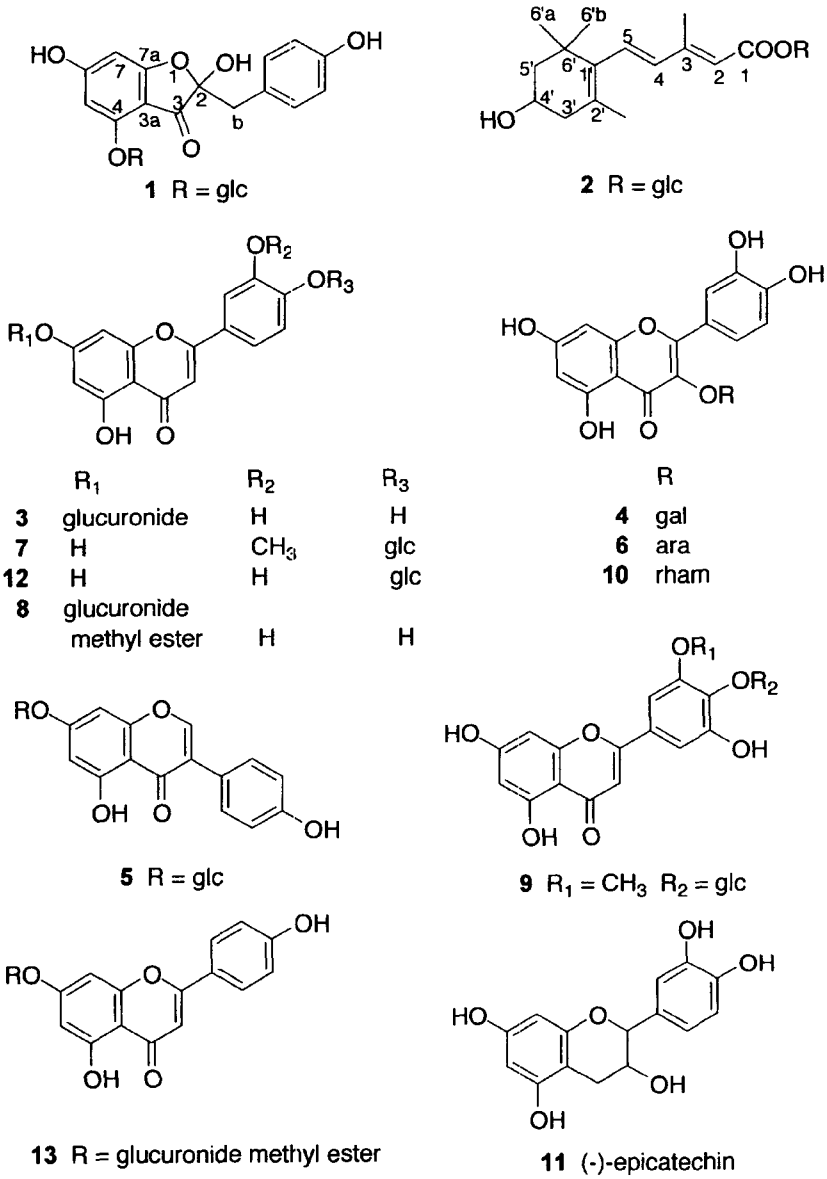

Fig. 1. The structures of compounds **1** to **13** isolated from the fruits of *Chaenomeles sinensis*

MeOH-H_2O) yielded compounds **5** (25 mg), **6** (60 mg), **7** (20 mg), **8** (50 mg) and **9** (2.5 mg). The sub-fraction B_2 was chromatographed on a silica gel column (a gradient of EtOAc-MeOH-H_2O) to give fractions B_{21}, B_{22} and B_{23}. The fraction B_{21} was on RP-18 (MeOH-H_2O, 1 : 1) and silica gel (EtOAc-MeOH-H_2O, 300 : 13.5 : 10) columns to yield compound **10** (300 mg); the fraction B_{22} was on silica gel ($CHCl_3$-MeOH-H_2O, 3 : 1 : 0.1), RP-18 (MeOH-H_2O, 1 : 1) and Sephadex LH-20 (MeOH-H_2O, 1 : 1) columns to yield compound **11** (150 mg); the fraction B_{23} was on a RP-18 (MeOH-H_2O, 1 : 1) column to yield compound **12** (25 mg). Finally, the sub-fraction B_3 was chromatographed on a silica gel column ($CHCl_3$-MeOH, 6 : 1) to give compound **13** (35 mg).

Compound 1 (hovetrichosides C); mp: 179-181C; $[\alpha]_D^{13}$-17.5 (*c* 0.1, MeOH); UV λ_{max} (MeOH) nm (log ε): 210 (4.3), 228 (4.2), 291 (4.2); IR ν_{max} (KBr) cm^{-1}: 3405 (OH), 1688 (C = O), 1620, 1516 (aromatic C = C); FAB-MS *m/z* (rel. int.): 451 ($[M+1]^+$, 6.8), 289 ($[M\text{-glc}+H]^+$, 36.8), 154 (100), 137 (98.5); ^1H and ^{13}C-NMR: see Table II.

Compound 2 ((±)-(2*E*,4*E*)-*O*-β-D-glucopyranosyl-4'-hydroxy-β-ionylideneacetic ester; mp: 105-107°C; $[\alpha]_D^{13}$-32.5 (*c* 0.1, MeOH); UV λ_{max} (MeOH) nm (log ε): 301 (4.1, β-ionone), 258 (4.1); IR ν_{max} (KBr) cm^{-1}: 3432 (OH), 1721 (C = O), 1608 (C = C), 1073; FAB-MS *m/z*: 413 $[M+1]^+$; ^1H-NMR (300 MHz, pyridine-d_5) $_H$ (ppm): 5.99 (1H, *brs*, H-2), 6.63 (1H, *d*, *J* = 16.2 Hz, H-4), 6.18 (1H, *d*, *J* = 16.2

Hz, H-5), 2.35 (1H, *dd*, *J* = 17.1, 8.4 Hz, H-3'*ax*.), 2.59 (1H, *dd*, *J* = 17.1, 5.1 Hz, H-3'*eq*.), 4.32 (1H, *m*, H-4'), 1.78 (1H, *dd*, *J* = 12.0, 8.3 Hz, H-5'*ax*.), 2.04 (1H, *dd*, *J* = 12.0, 1.5 Hz, H-5'*eq*.), 2.41 (3H, *s*, 3-CH_3), 1.73 (*s*, 2'-CH_3), 1.09 (3H, *s*, 6'a-CH_3), 1.11 (3H, *s*, β-form, 6'b-CH_3), 6.44 (1H, *d*, *J* = 7.8 Hz, glu H-1), 4.08-4.51 (5H, *m*, glu H-2, 3, 4, 5, 6); ^{13}C-NMR (75 MHz, pyridine-d_5) δ_C (ppm): 165.9 (C-1), 118.4 (C-2), 154.6 (C-3), 134.2 (C-4), 136.7 (C-5), 137.0 (C-1'), 129.6 (C-2'), 43.5 (C-3'), 63.7 (C-4'), 49.2 (C-5'), 37.0 (C-6'), 13.8 (3-CH_3), 21.7 (2'-CH_3), 30.3 (6'a-CH_3), 28.7 (6'b-CH_3), 95.5 (glu C-1), 74.2 (glu C-2), 78.5 (glu C-3), 71.1 (glu C-4), 79.4 (glu C-5), 62.3 (glu C-6).

Compound 3 (luteolin-7-*O*-β-D-glucuronide) (Schulz *et al*., 1985) ^{1}H-NMR (300 MHz, DMSO-d_6) δ_H (ppm): 6.71 (1H, *s*, H-3), 12.97 (1H, *s*, OH-5), 6.40 (1H, *d*, *J* = 2.1 Hz, H-6), 6.75 (1H, *d*, *J* = 2.1 Hz, H-8), 7.42 (1H, *d*, *J* = 2.4 Hz, H-2'), 6.86 (1H, *d*, *J* = 9.0 Hz, H-5'), 7.40 (1H, *dd*, *J* = 9.0, 2.4 Hz, H-6'), 5.05 (1H, *d*, *J* = 7.2 Hz, glu H-1), 3.12-3.58 (4H, *m*, glu H-2, 3, 4, 5); ^{13}C-NMR (75 MHz, DMSO-d_6) δ_C ppm): 164.6 (C-2), 103.1 (C-3), 182.0 (C-4), 161.2 (C-5), 99.8 (C-6), 163.2 (C-7), 94.7 (C-8), 157.1 (C-9), 105.4 (C-10), 121.2 (C-1'), 113.5 (C-2'), 146.1 (C-3'), 150.4 (C-4'), 116.2 (C-5'), 119.3 (C-6'), 99.8 (glu C-1), 73.1 (glu C-2), 76.7 (glu C-3), 72.1 (glu C-4), 73.9 (glu C-5), 171.9 (glu COOH-6).

Compound 4 (hyperin, hyperoside, quercetin-3-*O*-β-D-galactopyranoside) (Lu and Yeap, 1997) ^{1}H-NMR (300 MHz, DMSO-d_6) δ_H (ppm): 12.62 (1H, *s*, OH-5), 6.19 (1H, *d*, *J* = 2.1 Hz, H-6), 6.40 (1H, *d*, *J* = 2.1 Hz, H-8), 7.51 (1H, *d*, *J* = 2.1 Hz, H-2'), 6.80 (1H, *d*, *J* = 8.7 Hz, H-5'), 7.66 (1H, *dd*, *J* = 8.7, 2.1 Hz, H-6'), 5.37 (1H, *d*, *J* = 7.8 Hz, gal H-1), 3.15-3.65 (5H, *m*, gal H-2, 3, 4, 5, 6); ^{13}C-NMR (75 MHz, DMSO-d_6) δ_C (ppm): 156.5 (C-2), 133.6 (C-3), 177.7 (C-4), 161.4 (C-5), 98.8 (C-6), 164.3 (C-7), 93.7 (C-8), 156.5 (C-9), 104.1 (C-10), 121.3 (C-1'), 115.3 (C-2'), 145.0 (C-3'), 148.6 (C-4'), 116.1 (C-5'), 122.1 (C-6'), 102.0 (gal C-1), 71.4 (gal C-2), 73.4 (gal C-3), 68.1 (gal C-4), 76.0 (gal C-5), 60.3 (gal C-6).

Compound 5 (genistein-7-*O*-β-D-glucopyranoside) (Mazurek *et al*., 2000) ^{1}H-NMR (300 MHz, DMSO-d_6) δ_H (ppm): 8.42 (1H, *s*, H-2), 12.94 (1H, *s*, OH-5), 6.46 (1H, *d*, *J* = 2.4 Hz, H-6), 6.71 (1H, *d*, *J* = 2.1 Hz, H-8), 7.38 (2H, *d*, *J* = 8.7 Hz, H-2', 6'), 6.82 (2H, *d*, *J* = 8.7 Hz, H-3', 5'), 5.06 (1H, *d*, *J* = 7.2 Hz, glu H-1), 3.14-3.72 (5H, *m*, glu H-2, 3, 4, 5, 6); ^{13}C-NMR (75 MHz, DMSO-d_6) δ_C (ppm): 154.8 (C-2), 121.2 (C-3), 180.7 (C-4), 161.8 (C-5), 99.8 (C-6), 163.2 (C-7), 94.7 (C-8), 157.7 (C-9), 106.3 (C-10), 122.7 (C-1'), 130.4 (C-2', 6'), 115.3 (C-3', 5'), 157.4 (C-4'), 100.0 (glu C-1), 73.3 (glu C-2), 76.6 (glu C-3), 69.8 (glu C-4), 77.4 (glu C-5), 60.8 (glu C-6).

Compound 6 (avicularin, quercetin-3-O-α-L-arabinofuranoside) (Lu and Yeap, 1997) ^{1}H-NMR (300 MHz, DMSO-d_6) δ_H (ppm): 12.64 (1H, *s*, OH-5), 6.19 (1H, *d*, *J* = 2.1 Hz, H-6), 6.40 (1H, *d*, *J* = 2.1 Hz, H-8), 7.47 (1H, *d*, *J* = 2.1 Hz, H-2'), 6.84 (1H, *d*, *J* = 8.4 Hz, H-5'), 7.55 (1H, *dd*, *J* = 8.4, 2.1 Hz, H-6'), 5.58 (1H, *d*, *J* = 1.2 Hz, ara H-1), 3.21-3.73 (4H, *m*, ara H-2, 3, 4, 5), 4.14 (1H, *t* like, ara H-5); ^{13}C-NMR (75 MHz, DMSO-d_6) δ_C (ppm): 157.1 (C-2), 133.6 (C-3), 177.9 (C-4), 161.4 (C-5), 98.9 (C-6), 164.4 (C-7), 93.7 (C-8), 156.5 (C-9), 104.2 (C-10), 121.1 (C-1'), 115.7 (C-2'), 145.3 (C-3'), 148.7 (C-4'), 115.7 (C-5'), 121.9 (C-6'), 108.0 (ara C-1), 82.3 (ara C-2), 77.1 (ara C-3), 86.0 (ara C-4), 60.8 (ara C-5).

Compound 7 (luteolin-3'-methoxy-4'-*O*-β-D-glucopyranoside) (Bennini *et al*., 1992; Nørbæk *et al*., 1999) ^{1}H-NMR (300 MHz, DMSO-d_6) δ_H (ppm): 6.99 (1H, *s*, H-3), 12.90 (1H, *s*, OH-5), 6.20 (1H, *d*, *J* = 2.1 Hz, H-6), 6.54 (1H, *d*, *J* = 2.1 Hz, H-8), 7.60 (1H, *d*, *J* = 2.4 Hz, H-2'), 7.23 (1H, *d*, *J* = 8.7 Hz, H-5'), 7.64 (1H, *dd*, *J* = 8.7, 2.1 Hz, H-6'), 5.07 (1H, m, overlap with OH peak glu H-1), 3.16-3.70 (5H, *m*, glu H-2, 3, 4, 5, 6), 3.88 (3H, *s*, OCH_3): ^{13}C-NMR (75 MHz, DMSO-d_6) δ_C (ppm): 163.3 (C-2), 104.0 (C-3), 182.1 (C-4), 161.6 (C-5), 99.1 (C-6), 164.5 (C-7), 94.4 (C-8), 157.6 (C-9), 104.3 (C-10), 124.2 (C-1'), 110.4 (C-2'), 149.4 (C-3'), 149.9 (C-4'), 115.2 (C-5'), 120.0 (C-6'), 99.7 (glu C-1), 73.3 (glu C-2), 77.3 (glu C-3), 69.8 (glu C-4), 77.0 (glu C-5), 60.8 (glu C-6), 56.2 (OCH_3).

Compound 8 (luteolin-7-*O*-β-D-glucuronide methyl ester) (Schulz *et al*., 1985) ^{1}H-NMR (300 MHz, DMSO-d_6) δ_H (ppm): 6.75 (1H, *s*, H-3), 13.00 (1H, *s*, OH-5), 6.46 (1H, *d*, *J* = 2.1 Hz, H-6), 6.81 (1H, *d*, *J* = 2.1 Hz, H-8), 7.41 (1H, *d*, *J* = 2.1 Hz, H-2'), 6.89 (1H, *d*, *J* = 8.1 Hz, H-5'), 7.44 (1H, *dd*, *J* = 8.1, 2.1 Hz, H-6'), 5.32 (1H, *d*, *J* = 7.2 Hz, glu H-1), 3.24-3.42 (3H, *m*, glu H-2, 3, 4), 4.20 (1H, *d*, *J* = 9.3 Hz, glu H-5), 3.66 (3H, *s*, OCH_3); ^{13}C-NMR (75 MHz, DMSO-d_6) δ_C (ppm): 164.8 (C-2), 103.6 (C-3), 182.2 (C-4), 161.2 (C-5), 99.6 (C-6), 162.7 (C-7), 95.0 (C-8), 157.3 (C-9), 105.8 (C-10), 121.7 (C-1'), 113.8 (C-2'), 145.9 (C-3'), 150.0 (C-4'), 116.3 (C-5'), 119.6 (C-6'), 99.4 (glu C-1), 72.9 (glu C-2), 75.4 (glu C-3, 5), 71.5 (glu C-4), 169.5 (glu C-6), 52.4 (OCH_3).

Compound 9 (tricetin-3'-methoxy-4'-*O*-β-D-glucopyranoside); mp: 243-245 °C; UV λ_{max} (MeOH) nm (log ε): 270 (4.3), 290sh (4.0), 327 (4.3), λ_{max} (NaOMe) nm (log ε): 276sh (4.5), 290, 364 (4.3), λ_{max} (NaOAc) nm (log ε): 276 (4.4), 290, 364 (4.1), λ_{max} (NaOAc/H_3BO_3) nm (log ε): 270 (4.3), 327 (4.3), λ_{max} ($AlCl_3$) nm (log ε): 256sh (4.1), 278 (4.2), 296 (4.1), 343 (4.2), 385, λ_{max} ($AlCl_3$-HCl) nm (log ε): 256sh (4.1), 280 (4.2), 296 (4.1), 338 (4.2), 385; IR ν_{max} (KBr) cm^{-1}: 3437 (OH), 1637 (br. C = O), 1508 (C = C);

FAB-MS *m/z*: 501.46 $[M+Na]^+$; ^{1}H-NMR (300 MHz, DMSO-d_6) δ_H (ppm): 6.94 (1H, *s*, H-3), 12.85 (1H, *s*, OH-5), 6.20 (1H, *d*, *J* = 2.1 Hz, H-6), 6.50 (1H, *d*, *J* = 2.1 Hz, H-8), 7.17 (2H, *s*, H-2', 6'), 4.82 (1H, *d*, *J* = 7.5 Hz, glu H-1), 3.15-3.64 (5H, *m*, glu H-2,3,4,5,6), 3.86 (3H, *s*, OCH_3-3'); ^{13}C-NMR (75 MHz, DMSO-d_6): see Table III.

Compound 10 (quercitrin, quercetin-3-O-α-L-rhamnopyranoside) (Lu and Yeap, 1997; TradiMed., 1996) ^{1}H-NMR (300 MHz, DMSO-d_6) δ_H (ppm): 12.65 (1H, *s*, OH-5), 6.19 (1H, *d*, *J* = 2.1 Hz, H-6), 6.38 (1H, *d*, *J* = 2.1 Hz, H-8), 7.29 (1H, *d*, *J* = 2.1 Hz, H-2'), 6.85 (1H, *d*, *J* = 8.4 Hz, H-5'), 7.24 (1H, *dd*, *J* = 8.4, 2.1 Hz, H-6'), 5.24 (1H, *d*, *J* = 1.8 Hz, rha H-1), 3.96 (1H, *s*, rha H-2), 3.10-3.49 (3H, *m*, rha H-3,4,5), 0.80 (3H, *d*, *J* = 5.7 Hz, rha CH_3); ^{13}C-NMR (75 MHz, DMSO-d_6) δ_C (ppm): 157.5 (C-2), 134.4 (C-3), 178.0 (C-4), 161.5 (C-5), 98.9 (C-6), 164.4 (C-7), 93.9 (C-8), 156.7 (C-9), 104.3 (C-10), 120.9 (C-1'), 115.7 (C-2'), 145.4 (C-3'), 148.7 (C-4'), 115.9 (C-5'), 121.3 (C-6'), 102.0 (rha C-1), 70.8 (rha C-2), 70.6 (rha C-3), 71.4 (rha C-4), 70.3 (rha C-5), 17.7 (CH_3).

Compound 11 ((–)-epicatechin) (Osawa *et al.*, 2001; Mattice and Porter, 1984) ^{1}H-NMR (300 MHz, Aceton-d_6) δ_H (ppm): 4.87 (1H, *brs*, H-2), 4.19 (1H, *m*, H-3), 2.72 (1H, *dd*, *J* = 16.5, 3.3 Hz, H-4a), 2.83 (1H, *dd*, *J* = 16.5, 4.5 Hz, H-4b), 5.91 (1H, *d*, *J* = 2.4 Hz, H-6), 6.01 (1H, *d*, *J* = 2.4 Hz, H-8), 7.04 (1H, *d*, *J* = 1.8 Hz, H-2'), 6.77 (1H, *d*, *J* = 8.4 Hz, H-5'), 6.83 (1H, *dd*, *J* = 8.4, 1.8 Hz, H-6'); ^{13}C-NMR (75 MHz, Aceton-d_6) δ_C (ppm): 79.3 (C-2), 66.9 (C-3), 28.9 (C-4), 157.5 (C-5, 7), 96.1 (C-6), 95.6 (C-8), 157.0 (C-9), 99.7 (C-10), 132.2 (C-1'), 115.4 (C-2'), 145.2 (C-3'), 145.3 (C-4'), 115.2 (C-5'), 119.3 (C-6').

Compound 12 (luteolin-4'-O-β-D-glucopyranoside) (Schulz *et al.*, 1985) ^{1}H-NMR (300 MHz, DMSO-d_6) δ_H (ppm): 6.80 (1H, *s*, H-3), 12.89 (1H, *s*, OH-5), 6.17 (1H, *d*, *J* = 2.1 Hz, H-6), 6.47 (1H, *d*, *J* = 2.1 Hz, H-8), 7.48 (1H, *d*, *J* = 2.1 Hz, H-2'), 7.23 (1H, *d*, *J* = 8.7 Hz, H-5'), 7.50 (1H, *dd*, *J* = 8.1, 2.1 Hz, H-6'), 4.87 (1H, *d*, *J* = 7.5 Hz, glu H-1), 3.16-3.73 (5H, *m*, glu H-2, 3, 4, 5, 6); ^{13}C-NMR (75 MHz, DMSO-d_6) δ_C (ppm): 163.3 (C-2), 103.9 (C-3), 181.9 (C-4), 161.6 (C-5), 99.2 (C-6), 164.9 (C-7), 94.3 (C-8), 157.6 (C-9), 104.2 (C-10), 124.9 (C-1'), 113.8 (C-2'), 147.2 (C-3'), 148.7 (C-4'), 116.2 (C-5'), 118.7 (C-6'), 101.4 (glu C-1), 73.4 (glu C-2), 76.1 (glu C-3), 70.0 (glu C-4), 77.5 (glu C-5), 60.9 (glu C-6).

Compound 13 (apigenin-7-O-β-D-glucuronide methyl ester) (Allais *et al.*, 1991) ^{1}H-NMR (300 MHz, DMSO-d_6) δ_H (ppm): 6.86 (1H, *s*, H-3), 12.97 (1H, *s*, OH-5), 6.46 (1H, *d*, *J* = 2.4 Hz, H-6), 6.85 (1H, *d*, *J* = 2.4 Hz, H-8), 7.95 (2H, *d*, *J* = 8.7 Hz, H-2', 6'), 6.93 (2H, *d*, *J* = 8.7 Hz, H-3', 5'), 5.31 (1H, *d*, *J* = 6.3, glu H-1), 3.15-3.44 (3H, *m*, glu H-2, 3, 4), 4.19 (1H, *d*, *J* = 9.0, glu H-5), 3.66 (3H, *s*, OCH_3); ^{13}C-NMR (75 MHz, DMSO-d_6) δ_C (ppm): 164.5 (C-2), 103.3 (C-3), 182.2 (C-4), 161.5 (C-5), 99.5 (C-6), 161.4 (C-7), 94.8 (C-8), 157.1 (C-9), 105.7 (C-10), 121.2 (C-1'), 128.8 (C-2', 6'), 116.2 (C-3', 5'), 162.6 (C-4'), 99.2 (glu C-1), 72.9 (glu C-2), 75.6 (glu C-3), 71.5 (glu C-4), 75.3 (glu C-5), 169.4 (glu C-6), 52.1 (OCH_3).

RESULTS AND DISCUSSION

Chemical structure of compounds

The solvent fractionation of the MeOH extract of the fruits of *C. sinensis* revealed that the EtOAc soluble fraction had the strongest inhibitory activity on TF with the highest specific activity as shown in Table I. Activity-guided isolation of the EtOAc fraction yielded compounds **1, 3, 4, 6** and **10** as TF inhibitors and compounds **2, 5, 7, 8, 9, 11, 12** and **13** were inactive in the assay system used.

Compound 1 was amorphous powder. The molecular formula $C_{21}H_{22}O_{11}$ was deduced by FAB-MS giving a quasimolecular ion $[M+1]^+$ peak at *m/z* 451. The ^{1}H- and ^{13}C-NMR spectra of **1** displayed two sets of signals due to a measopsin and a hexosyl group (Table II). The ratio of the intensity of each pair of signals was 3 : 2, which existed as enantiomeric pairs due to the reversible nature of the hemiketal at C-2. The attachment of a glucosyl moiety in **1** produced a pair of diastereoisomers, thus giving rise to a double set of signals. In the ^{1}H-NMR spectrum of **1**, one anomeric proton signals appeared at 4.88 (0.6 H, *d*, *J* = 7.2 Hz) and 4.93 (0.4 H, *d*, *J* = 7.2 Hz), disclosing the glucose having β-configuration. Further, a HMBC spectrum showed connectivities between the major H-1 (δ 4.88) of glucose and major C-4 (δ 156.9) of the aglycone. In a comparison of the ^{1}H- and ^{13}C-NMR spectra of **1** with those of hovetrichoside C, maesopsin-4-O-glucoside isolated from *Hovenia trichocarea* (Yoshikawa *et al.*, 1998), the proton and carbon signals of the two compounds were in good agreement. Compound **1** was isolated for the first time from the fruits of *C. sinensis*.

Table I. Tissue factor inhibitory activities of various fractions

Fractions	Amount (g)	IC_{50}/TF unit* (g/unit)	Total inhibitory activity (10^7 units**)	Specific inhibitory activity (10^5 units/g)
n-Hexane	140	10.0	1.4	1.0
EtOAc	350	0.6	58.3	16.7
n-BuOH	973	5.3	18.4	1.9
H_2O	2960	2.3	128.7	4.3

*The amount of TF that accelerated 50% the prothrombin time was defined as one unit of TF.

**The amount of sample that inhibited 50% TF activity was defined as one unit of TF inhibitor.

Table II ^{1}H- and ^{13}C-NMR of compound 1 (hovetrichoside C) in DMSO-d_6, 400 MHz and 100 MHz

Carbon No.	major		minor	
	^{13}C	^{1}H (multiplicity, J in Hz)	^{13}C	^{1}H (multiplicity, J in Hz)
2	105.6		105.6	
3	192.4		192.7	
3a	101.3		101.5	
4	156.9		156.9	
5	95.9	5.96 (*s*)	96.5	5.99 (*s*)
6	169.8		169.8	
7	91.8	5.89 (*s*)	92.0	5.89 (*s*)
7a	172.1		172.0	
1′	124.3		124.3	
2′, 6′	131.4	6.93 (*d*, 8.4)	131.4	6.95 (*d*, 8.4)
3′, 5′	114.8	6.55 (*d*, 8.7)	114.7	6.57 (*d*, 8.7)
4′	155.9		155.9	
b	40.1	2.87 (*d*, 14.1)	40.1	2.85 (*d*, 14.1)
		2.95 (*d*, 14.1)		2.92 (*d*, 14.1)
Glc-1	99.6	4.88 (*d*, 7.2)	99.5	4.93 (*d*, 7.2)
2	72.9	3.17-3.43 (*m*)	72.9	3.17-3.43 (*m*)
3	76.7	3.17-3.43 (*m*)	76.7	3.17-3.43 (*m*)
4	69.3	3.17-3.43 (*m*)	69.3	3.17-3.43 (*m*)
5	77.2	3.17-3.43 (*m*)	77.2	3.17-3.43 (*m*)
6	60.3	3.62 (*dd*, 11.1, 5.1)	60.4	3.46 (*dd*, 11.1, 5.1)
		3.65 (*dd*, 11.1, 3.0)		3.49 (*dd*, 11.1, 3.0)

Compound 2 was obtained as white powder. The molecular formula $C_{21}H_{32}O_8$ was deduced by FAB-MS giving a qausimolecular ion $[M+1]^+$ peak at *m/z* 413. Its skeleton could be deduced as β-ionylideneacetic acid from the ^{1}H- and ^{13}C-NMR data (Winterhalter *et al.*, 1991; Oritani and Yamashita, 1979). The UV absorption maximum at 301 nm indicated compound **2** was β-ionone (Oritani and Yamashita, 1972). The presence of an ester glucoside was confirmed by the observation of an carboxyl carbon at 165.9, an anomeric carbon at δ 95.5 and an anomeric proton at δ 6.44 (*d*, *J* = 7.8 Hz). The IR spectrum showed absorption band at 1721 cm^{-1} for carboxylic ester and strong absorption bands at 3432 and 1073 cm^{-1}, supporting the glycosidic structure. The comparison of ^{1}H-NMR with literature data (Oritani and Yamashita, 1979) revealed that the 2.41 (3 H, *s*, 3-CH_3) peak was for 2*E* configuration as well as the 6.18 (1 H, *d*, *J* = 16.2 Hz, H-5) and 6.63 (1 H, *d*, *J* = 16.2 Hz, H-4) peaks for 4*E* configuration. In a H-H COSY experiment, the signals at 1.78 (1 H, *dd*, *J* = 12.0, 8.3 Hz, H-5′*ax*.), 2.04 (1 H, *dd*, *J* = 12.0, 1.5 Hz, H-5′*eq*.), 2.35 (1 H, *dd*, *J* = 17.1, 8.3 Hz, H-3′*ax*.) and 2.59 (1 H, *dd*, *J* = 17.1, 5.1 Hz, H-3′*eq*.) gave across peaks with 4.28 (1 H, *ddd*, *J* = 8.3, 5.1, 1.5 Hz, H-4′), suggesting the presence of a hydroxyl group substituted at 4′ position. These evidence allowed us to formulating the structure of compound **2** as (±)-(2*E*,4*E*)-glucopyranosyl-4′-hydroxy-β-ionylideneacetic acid ester. Compound **2** was isolated for the first time from the fruits of *C. sinensis*.

Compound 9 was obtained as amorphous yellow powder. The molecular formula $C_{22}H_{22}O_{12}$ was deduced by FAB-MS giving a qausimolecular ion $[M+Na]^+$ peak at *m/z* 501.46. The ^{1}H-NMR spectrum of **9** showed several signals due to a flavone glycoside having one methoxyl group (δ 3.86, 3 H, s) as follows; The 5,7-disubstitution pattern of A-ring was indicated by the two doublets at δ 6.20 (H-6) and 6.50 (H-8) with a meta coupling constant of 2.1 Hz and the singlet at δ 6.94 (H-3) integrating for one proton supported the flavone skeleton, while the singlet at δ 7.17 (2 H) accounted for H-2′ and H-6′ that gave this signal instead of a doublet as was also observed in 3′,4′-dimethoxy tricetin (Ricardo and Alicia, 1985). An anomeric proton doublet was observed at 4.82 (*d*, 7.5 Hz) that is characteristic for a β-linked glucose, which was conformed by ^{13}C-NMR data (Table III) (Sharaf *et al.*, 1999). The presence of a free hydroxyl at 5-position was confirmed by a signal at 12.85. The UV spectrum of **9** showed absorption maxima at 270, 290 and 327 nm, which was coincident with those of 3′,4′-dimethoxy tricetin (Ricardo and Alicia, 1985), indicating the absence of 3-hydroxyl group. The presence of the chelated hydroxyl group at the 5-position was also supported by a bathochromic shift of 58 nm in UV on treatment with aluminum chloride-hydrochloric acid. The shift with sodium acetate (△λ 6 nm) indicated the presence of a free hydroxyl at the 7-position. No shift in wave length on treatment of **9** with sodium acetate-boric acid ruled out the presence of a 3′,4′-dihydroxyl group in the B-ring. The shift with sodium methoxide (△λ 37 nm) also indicated the absence of a free hydroxyl group at the 4′-position, since the flavone having 4′-hydroxyl group such as tricin and 3′-methoxy tricetin showed a shift higher than 50 nm (Ricardo and Alicia, 1985). Moreover the ^{13}C-NMR shifts of the aglycone moiety of **9** corresponded well to those of tricetin (Sharaf *et al.*, 1999), the only differences being downfield shifts of the signal assigned to C-1′ by approximately 5.8 ppm (126.7-120.9) and of the ortho-related carbons of C-4′ (Table III). The downfield shifts of C-3′, C-5′ and C-1′ was caused by 4′-glucosyl glycosylation (Sharaf *et al.*, 1999; Nørbæk *et al.*, 1999). From the above data compound **9** was characterized as tricetin-3′-methoxy-4′-*O*-β-D-glucopyranoside, a new flavonoid.

Except the three compounds established above, the structures of other compounds were easily identified from ^{1}H- and ^{13}C-NMR data described in a text book (Markham and Geiger, 1994). Flavone glycosides having luteolin as an aglycone were compounds **3** (luteolin-7-O-β-D-glu-

Table III.. ^{13}C-NMR of tricetin (I) and compound **7** and **9** in DMSO-d_6, 75 MHz

Position	9	I[a]	7
Aglycone 2	163.2	164.2	163.3
3	104.0	103.2	104.0
4	182.0	181.6	182.1
5	161.6	161.6	161.6
6	99.1	99.0	99.1
7	164.6	164.2	164.5
8	94.3	93.9	94.4
9	157.6	157.9	157.6
10	104.2	104.0	104.3
1'	126.7	120.9	124.2
2'	108.0	106.0	110.4
3'	153.4	146.5	149.4
4'	137.0	137.9	149.4
5'	150.9	146.5	115.2
6'	105.1	106.0	120.0
Glucose 1	103.0		99.7
2	74.0		73.3
3	77.5		77.3
4	69.8		69.8
5	76.4		77.0
6	60.8		60.8
-OCH_3	56.8		56.2

[a] ^{13}C-NMR of compounds I were cited from a literature of Sharaf (1999). G, glucose; R, rhamnose.

curonide), **7** (luteolin-3'-methoxy-4'-*O*-β-D-glucopyranoside), **8** (luteolin-7-*O*-β-D-glucuronide methyl ester) and **12** (luteolin-4'-*O*-β-D-glucopyranoside), and flavone glycoside having apigenin as an aglycone was compound **13** (apigenin-7-*O*-β-D-glucuronide methyl ester).

Flavonol glycosides with quercetin as a genin were compounds **4** (quercetin-3-*O*-β-D-galactopyranoside), **6** (quercetin-3-*O*-α-L-arabinofuranoside) and **10** (quercetin-3-*O*-α-L-rhamnopuranoside, quercitrin).

Isoflavone glycoside having genistein as a genin was compound **5** (genistein-7-*O*-β-D-glucuronide). Compound **11** was proved to be (-)-epicatechin.

Copounds **10** and **11** were already isolated together with quercetin and rutin from the fruits of *C. sinensis* (Mattice and Porter, 1984; Osawa *et al.*, 2001; TradiMed, 1996). Compounds **3** to **8**, **12** and **13** occur in other plants, but were isolated for the first time from the fruits of *C. sinensis* by us.

TF inhibitory activity and SAR analysis

The TF inhibitory profiles of compounds **1**, **3**, **4**, **6** and **10** were exhibited in Fig. 2, and the inhibitory activities of

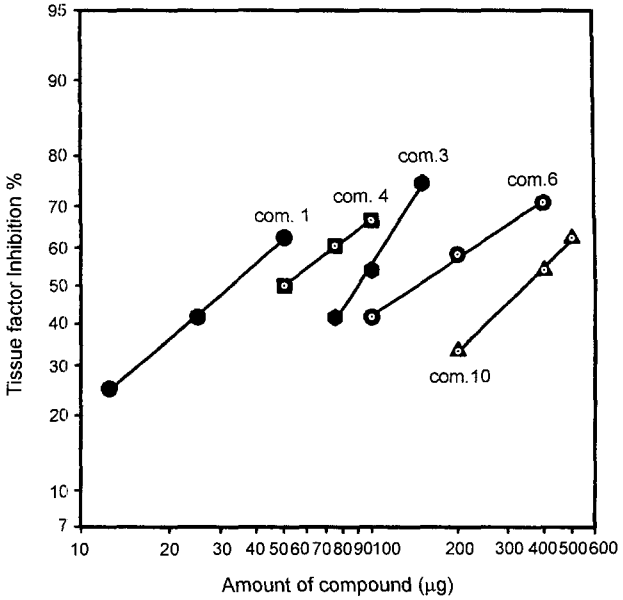

Fig. 2. Inhibition profiles of compounds **1**, **3**, **4**, **6** and **10** on tissue factor. Each reaction mixture contained the 2.4 units of TF.

Table IV. The IC_{50} values of the compounds isolated from *C. sinensis* and flavonoids frequently found in plants

Compounds	IC_{50} (μg/unit of TF)
1 (maesopsin[a])	14.0 ± 1.4[b]
Maesopsin	ND[c]
2 (4'-hydroxy-β-ionylideneacetic acid)	NA[d]
4'-hydroxy-β-ionylideneacetic acid	NA
3 (luteolin)	31.9 ± 5.0
Luteolin	NA
4 (quercetin)	20.8 ± 3.6
Quercetin	>150
5 (genistein)	NA
Genistein	NA
6 (quercetin)	54.8 ± 4.9
7 (luteolin)	NA
8 (luteolin)	NA
9 (tricetin)	NA
Tricetin	NA
10 (quercetin)	135.7 ± 8.4
11	NA
12 (luteolin)	NA
13 (apigenin)	NA
Apigenin	NA
Acacetin	NA
Diosmetin	NA
Kaempferol	NA
Rutin (quercetin)	NA

[a] Each compound in parenthesis is the aglycone of the corresponding compound. [b] Mean ± S. D. (n = 3). [c] Not determined. [d] Non-active

them and flavonoids frequently found in plants were shown in Table IV. The TF inhibitory activities were found in some flavonoid glycosides, but not in aglycones. Compound **1** is the C-ring pentacyclic flavonol with the IC_{50} value of 14.0 μg. Compounds **3** and **13** are the C-ring hexacyclic flavones having glucuronic acid and its methyl ester, respectively, but the former compound showed the TF inhibitory activity with the IC_{50} value of 31.9 μg, while the latter did not, indicating that free carboxyl group could be an important determinant of the activity. Quercetin and its glycosides such as rutin, compounds **4**, **6** and **10** are the C-ring hexacyclic flavonols with the IC_{50} values of about 150, 0, 20.8, 54.8 and 135.7 μg, respectively, indicating that carbon 3-oxygen of quercetin moiety and one sugar moiety attached to the C_3-OH play pivotal role in exhibiting the TF inhibitory activity of the flavonol glycosides

The results showed that the TF inhibitory activity of the C-ring pentacyclic flavonol was evidently stronger than that of C-ring hexacyclic flavonol. This discovery could provide a possibility to find new drug candidates from natural product resources for regulating the blood coagulation cascade. It may be possible in the near future after further studies for designing and synthesizing more effective TF inhibitors based on the compounds isolated from *C. sinensis*.

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