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Antithrombotic Phenolics from the Stems of *Parthenocissus tricuspidata* Possess Anti-inflammatory Effect

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In the course of our program to search for antithrombotic and anti-inflammatory agents from plants, twelve phenolics (**1–12**) were isolated from the stems of *Parthenocissus tricuspidata*. Their structures were elucidated on the basis of spectroscopic (1D and 2D NMR, and MS) data analyses, and comparison with published data. At the concentration of 100 μg/mL, compounds **2**, **4**, **6** and **10** possessed potential effects on anti-blood coagulation, with inhibitory percentage of 216, 174, 148 and 225%, respectively; while aspirin used as positive control showed 181% inhibition at the same concentration. Furthermore, the anti-inflammatory activity of isolated compounds (**1–12**) was investigated on lipopolysaccharide (LPS)-induced murine macrophage cells (RAW264.7). Compounds **2**, **4** and **6** also potential inhibited the production of nitric oxide, with IC_{50} values of 11.9 ± 0.3, 2.9 ± 0.2 and 29.0 ± 0.6 μM, respectively. Celastrol, the positive control used, gave an IC_{50} value of 1.0 ± 0.1 μM.

Key Words : *Parthenocissus tricuspidata*, Anticoagulants, Thrombin inhibitors, NO production inhibitors, Anti-inflammatory activity

Introduction

Inflammation is a process that involves multiple factors that act in concert. The ingress of leukocytes into sites of inflammation is an important aspect of the pathogenesis of inflammatory conditions.[1] For example, macrophages are recruited to inflammatory sites, and are activated by various signals that stimulate many intracellular cascades of cytokines and chemokines.[2] In macrophages, lipopolysaccharide (LPS), a well-known endotoxin, induces the productions of inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-1β, and inflammatory mediators, such as nitric oxide (NO) and prostaglandin E2 (PGE2), which are synthesized by inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2), respectively.[3,4] The iNOS generates high levels of NO that modulate inflammations through multiple pathways, and plays an important role in the regulation of immune reactions.[5] Low concentrations of NO produced by iNOS possess beneficial roles in the host defense mechanism against pathogens; while excessive amounts of NO can cause various inflammatory diseases, such as septic shock, tissue damage following inflammation, and rheumatoid arthritis.[6] Therefore, NO production induced by LPS through iNOS can reflect the degree of inflammation; and a change in NO level, through inhibition of iNOS enzyme activity or iNOS induction, provides a means of assessing the effect of agents on the inflammatory process.

Arterial thrombosis is the most common cause of myocardial infarction and ischemic stroke; whereas, deep vein thrombosis can lead to pulmonary embolism. In the USA, pulmonary embolism causes almost 300,000 deaths per annum.[7] Great advances have been made in understanding the molecular and cellular basis of thrombus formation in the past few decades, with anticoagulants remaining the cornerstone for the prevention and treatment of thromboembolic disorders.[8] Inhibition of thrombin generation, activation, or both is therefore a logical target in the treatment of TD. Heparin has been used clinically as the drug of choice in the prevention and treatment of thromboembolic diseases.[9] However, its use is accompanied by some side effects, frequently requiring monitoring of partially activated thromboplastin time, and resulting in other hemorrhagic complications.[10] Although it is well established that aspirin still provides an effective secondary prevention of ischemic cardiovascular disorders, this drug can produce hemorrhagic events and upper gastrointestinal bleeding as major drawbacks.[11] Thus, the search for alternative anticoagulants with reduced side effects is still needed, and urgent.

In our continuing program to search for anti-inflammatory and antithrombotic agents from plants, we found that an EtOAc-soluble extract of the stems of *P. tricuspidata* exhibited significant activities on both *in vitro* LPS-induced NO production in RAW264.7 cells, and thrombin time (TT) assay. *P. tricuspidata* (Vitaceae) is a woody vine that typically grows 30–50 feet or more. The leaves have been used as folk medicine in South Asia, for treating arthritis, jaundice, insect bites, and neuralgia.[12] Previous phytochemical studies on this plant had revealed that it is a rich source of phenolic compounds.[13-16] In this study, we reported the bioassay-guided isolation, chemical structure elucidation, and biological investigation of the isolated phenolics (**1–12**) from the stems of *P. tricuspidata*, on LPS-induced NO pro-

duction and blood coagulation inhibition.

Experimental Section

General Procedures. The optical rotations were determined on a Rudolph Autopol AP 589 polarimeter using a 100 mm glass microcell. The IR spectra were recorded on a Nicolet 6700 FT-IR (Thermo electron Corp.). UV spectra were recorded in MeOH using a Shimadzu spectrometer. The NMR spectra were recorded in methanol-d_4 (CD_3OD), pyridine-d_5 (C_5D_5N) on Varian OXFORD-AS 400 MHz instrument (PaloAlto, CA, USA) with TMS as the internal standard at the Department of Pharmacy, Catholic University of Daegu, Korea. All mass experiments were performed on a Micromass QTOF2 (Micromass, Wythenshawe, UK) mass spectrometer. Silica Gel (Merck, 63-200 μm particle size) and RP-18 (Merck, 150 μm particle size) were used for column chromatography. For thin-layer chromatography, pre-coated TLC was carried out on Silica Gel 60 F_{254} and RP-18 F_{254} plates from Merck. HPLC runs were carried out using a Gilson system with a UV detector and an Optima Pak C18 column (10 × 250 mm, 10 μm particle size, RS Tech Corp., Korea).

Plant Material. The stems of *P. tricuspidata* were collected in August 2002 from the Palgong mountain of Kyungbuk, Korea, and dried at room temperature for 2 weeks. The plant was verified by Professor Byung Sun Min, College of Pharmacy, Catholic University of Daegu, Korea. A voucher specimen (CUDP 2002-02) was deposited at the College of Pharmacy, Catholic University of Daegu, Korea.

Extraction and Isolation. Stems of *Parthenocissus tricuspidata* (10 kg) were dried at room temperature, cut into small pieces, and extracted with methanol (MeOH) at refluxing temperature, to yield about 1.2 kg of MeOH extract. This extract was suspended in distilled H_2O, and successively partitioned with dichloromethane (CH_2Cl_2), ethyl acetate (EtOAC), and *n*-butanol (*n*-BuOH), to yield each of the three fractions. The CH_2Cl_2, EtOAc, *n*-BuOH, and H_2O-soluble layers were tested on both thrombin time (TT) assay, and NO production inhibition assay. Among these, the EtOAc fraction showed strongest activities. Thus, this fraction (108.6 g) was subjected to a silica gel column chromatography (15 × 60 cm; 63–200 μm particle size), using gradient solvents of CH_2Cl_2:MeOH (100:1 → 0:1), to yield ten combined fractions (F.1 to F.10), according to their TLC profiles. These fractions were assayed for blood coagulants and NO production inhibition assay. Strong active fractions 4, 6 and 8 were continuously chromatographed, for activity-guided isolation. Fraction 8 was further purified by semi-preparative Gilson HPLC, using an isocratic solvent system of 35% MeOH in H_2O over 40 min [RS Tech Optima Pak C18 column (10 × 250 mm, 5 μm particle size); mobile phase MeOH/H_2O containing 0.1% formic acid (0–40 min: 35% MeOH, 40–45 min: 35–100% MeOH, 45–60 min: 100% MeOH); UV detections at 205 and 254 nm], resulting in the isolation of compounds **11** (117.1 mg, t_R = 24.9 min), **12** (23.7 mg, t_R = 29.1 min), **1** (15.6 mg, t_R = 33.8 min), **2** (10.0 mg, t_R = 36.8 min) and **3** (22.5 mg, t_R = 39.5 min). Compound **10** (51.6 mg) was purified from fraction 4, by using an open RP-C18 column (3.5 × 20 cm), eluting with a gradient solvent of 45% MeOH in H_2O. Fraction 6 was also purified by semi-preparative Gilson HPLC, using an isocratic solvent system of 40% MeOH in H_2O, over 70 min [RS Tech Optima Pak C18 column (10 × 250 mm, 5 μm particle size); mobile phase MeOH/H_2O containing 0.1% formic acid (0–70 min: 40% MeOH, 70–75 min: 40–100% MeOH, 75–85 min: 100% MeOH); UV detections at 205 and 254 nm], resulting in the isolation of compounds **7** (7.9 mg, t_R = 31.5 min), **8** (5.6 mg, t_R = 36.5 min), **5** (4.9 mg, t_R = 47.5 min), **9** (18.9 mg, t_R = 53.3 min), **4** (6.8 mg, t_R = 60.1 min) and **6** (58.5 mg, t_R = 65.7 min), respectively.

Protocatechuic Acid (1): Brown powder; ^{1}H-NMR (Methanol-d_4, 400 MHz) δ 7.37 (1H, d, *J* = 2.0 Hz, H-2), 7.36 (1H, dd, *J* = 2.0, 8.4 Hz, H-6), 6.73 (1H, d, *J* = 8.4 Hz, H-5); ^{13}C-NMR (Methanol-d_4, 100 MHz) δ 169.0 (-COO-), 151.9 (C-4), 146.3 (C-3), 123.8 (C-6), 122.7 (C-1), 117.5 (C-2), 116.0 (C-5).

Benzoic Acid (2): White powder; ^{1}H-NMR (Methanol-d_4, 400 MHz) δ 7.43 (1H, d, *J* = 2.0 Hz, H-2), 7.42 (1H, d, *J* = 2.0, 8.4 Hz, H-6), 6.80 (1H, d, *J* = 8.4 Hz, H-5), 3.84 (3H, s, -OCH_3); ^{13}C-NMR (Methanol-d_4, 100 MHz) δ 172.1 (-COO-), 151.9 (C-4), 146.3 (C-3), 123.8 (C-6), 122.7 (C-1), 117.5 (C-2), 116.0 (C-5), 52.4 (-OCH_3).

Caffeic Acid (3): White powder; ^{1}H-NMR (Methanol-d_4, 400 MHz) δ 7.60 (1H, d, *J* = 16.0 Hz, H-7) , 7.05 (1H, d, *J* = 2.0 Hz, H-2), 6.95 (1H, dd, 2.0, 8.0 Hz, H-6), 6.78 (1H, d, *J* = 8.0 Hz, H-5), 6.31 (1H, d, *J* = 16.0 Hz, H-8); ^{13}C-NMR (Methanol-d_4, 100 MHz) δ 171.0 (-COO-), 152.1 (C-4), 147.9 (C-3), 124.4 (C-6), 117.2 (C-2), 115.8 (C-5).

Methyl 3,4-Dihydroxycinnamate (4): White powder; ^{1}H-NMR (Methanol-d_4, 400 MHz) δ 7.54 (1H, d, *J* = 16.0 Hz, H-7), 7.03 (1H, d, *J* = 2.0 Hz, H-2), 6.94 (1H, d, *J* = 2.0, 8.0 Hz, H-6), 6.78 (1H, d, *J* = 8.4 Hz, H-5), 6.26 (1H, d, *J* = 16.0 Hz, H-8), 3.76 (3H, s, -OCH_3); ^{13}C-NMR (Methanol-d_4, 100 MHz) δ 170.1 (-COO-), 149.9 (C-4), 148.3 (C-3), 128.9 (C-6), 122.4 (C-1), 116.9 (C-2), 115.6 (C-5), 52.1 (-OCH_3).

Caffeoylglycolic Acid (5): White amorphous powder; ^{1}H-NMR (Methanol-d_4, 400 MHz) δ 7.61 (1H, d, *J* = 16.0 Hz, H-7), 7.06 (1H, d, *J* = 2.0 Hz, H-2), 6.95 (1H, dd, *J* = 2.0, 8.0 Hz, H-6), 6.78 (1H, d, *J* = 8.0 Hz, H-5), 6.33 (1H, d, *J* = 16.0 Hz, H-8), 4.66 (2H, s, H-10); ^{13}C-NMR (Methanol-d_4, 100 MHz) δ 172.7 (11-COOH), 168.7 (9-COO-), 149.8 (C-4), 147.8 (C-7), 146.9 (C-3), 127.8 (C-1), 123.2 (C-6), 116.7 (C-5), 115.3 (C-2), 114.6 (C-8), 62.2 (C-10).

Caffeoylglycolic Acid Methyl Ester (6): White amorphous powder; ^{1}H-NMR (Methanol-d_4, 400 MHz) δ 7.61 (1H, d, *J* = 16.0 Hz, H-7), 7.06 (1H, d, *J* = 2.0 Hz, H-2), 6.95 (1H, dd, *J* = 2.0, 8.0 Hz, H-6), 6.78 (1H, d, *J* = 8.0 Hz, H-5), 6.32 (1H, d, *J* = 16.0 Hz, H-8), 4.72 (2H, s, H-10), 3.75 (-OCH_3); ^{13}C-NMR (Methanol-d_4, 100 MHz) δ 170.6 (11-COO-), 168.5 (9-COO-), 150.0 (C-4), 148.2 (C-7), 147.0 (C-3), 127.7 (C-1), 123.3 (C-6), 116.7 (C-5), 115.4 (C-2), 114.1 (C-8), 61.7 (C-10), 52.8 (-OCH_3).

3,4',5-Trihydroxybenzophenone (7): Amorphous powder;

^{1}H-NMR (400 MHz, methanol-d_4) δ 7.72 (2H, d, *J* = 8.8 Hz, H-2'/H-6'), 6.87 (2H, d, *J* = 8.8, H-3'/H-5'), 6.59 (2H, d, *J* = 2.4 Hz, H-2/H-6), 6.48 (1H, t, *J* = 2.4, H-4); ^{13}C NMR (100 MHz, methanol-d_4) δ 198.1 (C=O), 163.8 (C-4'), 159.8 (C-3/C-5), 141.8 (C-1), 134.1 (C-2'/C-6'), 130.1 (C-1'), 116.2 (C-3'/C-5'), 109.2 (C-2/C-6), 107.3 (C-4).

5-(4-Hydroxybenzyl)benzene-1,3-diol (8): Amorphous powder; ^{1}H-NMR (400 MHz, methanol-d_4) δ 7.05 (1H, d, *J* = 8.8 Hz, H-2'/H-6'), 6.93 (2H, d, *J* = 8.8 Hz, H-2/H-6), 6.72 (2H, d, *J* = 8.8, H-3'/H-5'), 6.47 (1H, t, *J* = 2.4, H-4), 4.10 (2H, s, H-7); ^{13}C NMR (100 MHz, methanol-d_4) δ 156.2 (C-4'), 158.8 (C-3/C-5), 138.9 (C-1), 130.3 (C-2'/C-6'), 129.2 (C-1'), 115.2 (C-3'/C-5'), 106.8 (C-2/C-6), 107.2 (C-4), 44.3 (C-7).

2*R*,3*R*-3,5,6,7,4′-Pentahydroxy-flavanonol (9): Yellow amorphous powder; ^{1}H-NMR (methanol-d_4, 400 MHz) δ 7.35 (2H, d, *J* = 8.4 Hz, H-2'/H-6'), 6.83 (2H, d, *J* = 8.4 Hz, H-3'/H-5'), 5.86 (1H, s, H-8), 4.97 (1H, d, *J* = 11.6 Hz, H-2), 4.57 (1H, d, *J* = 11.6 Hz, H-3); ^{13}C-NMR (Methanol-d_4, 100 MHz) δ 198.7 (C-4), 168.8 (C-7), 164.7 (C-5), 164.7 (C-9), 159.4 (C-4'), 147.0 (C-6), 130.5 (C-2'/C-6'), 129.4 (C-1'), 116.3 (C-3'/C-5'), 102.0 (C-10), 97.5 (C-8), 85.1 (C-2), 73.8 (C-3).

Acacetin (10): Yellow amorphous powder; ^{1}H-NMR (Methanol-d_4, 400 MHz) δ 7.83 (2H, d, *J* = 8.4 Hz, H-2'/H-6'), 6.96 (2H, d, *J* = 8.4 Hz, H-3'/H-5'), 6.59 (1H, d, *J* = 2.4 Hz, H-6), 6.57 (1H, s, H-3), 6.45 (1H, d, *J* = 2.4 Hz, H-8), 3.94 (3H, 4'-OCH_3); ^{13}C-NMR (Methanol-d_4, 100 MHz) δ 180.3 (C-4), 164.9 (C-7), 163.6 (C-2), 162.3 (C-5), 162.1 (C-4'), 161.2 (C-9), 129.0 (C-2'/C-6'), 123.2 (C-1'), 116.9 (C-3'/C-5'), 108.2 (C-10), 106.5 (C-3), 56.5 (4'-OCH_3).

(+)-Catechin (11): White amorphous powder; ^{1}H-NMR (Methanol-d_4, 400 MHz) δ 6.84 (1H, d, *J* = 1.6 Hz, H-6'), 6.77 (1H, d, *J* = 8.0 Hz, H-3'), 6.72 (1H, dd, *J* = 2.0, 8.0 Hz, H-2'), 5.94 (1H, d, *J* = 2.4 Hz, H-8), 5.86 (1H, d, *J* = 2.4 Hz, H-6), 4.57 (1H, d, *J* = 7.2 Hz, H-2), 3.99 (1H, m, H-3), 2.86 (1H, dd, *J* = 5.6, 16.0 Hz, H-4a), 2.51 (1H, dd, *J* = 8.0, 16.0 Hz, H-3b); ^{13}C NMR (Methanol-d_4, 100 MHz) δ 156.6 (C-5), 156.4 (C-7), 155.7 (C-9), 145.1 (C-4'), 145.0 (C-5'), 131.0 (C-2'), 118.9 (C-6'), 114.9 (C-3'), 114.1 (C-1'), 99.6 (C-10), 195.1 (C-6), 94.3 (C-8), 81.7 (C-2), 67.6 (C-3), 27.3 (C-4).

(–)-Catechin (12): Brownish amorphous powder; ^{1}H-NMR (Methanol-d_4, 400 MHz) δ 6.84 (1H, d, *J* = 2.0 Hz, H-6'), 6.76 (1H, d, *J* = 8.0 Hz, H-3'), 6.71 (1H, dd, *J* = 2.0, 8.0 Hz, H-2'), 5.92 (1H, d, *J* = 2.4 Hz, H-8), 5.85 (1H, d, *J* = 2.4 Hz, H-6), 4.56 (1H, d, *J* = 7.6 Hz, H-2), 3.97 (1H, m, H-3), 2.84 (1H, dd, *J* = 5.2, 16.0 Hz, H-4a), 2.50 (1H, dd, *J* = 8.0, 16.0 Hz, H-3b).

Determination of NO Production and the Cell Viability Assay. The level of NO production was determined by measuring the amount of nitrite from the cell culture supernatants as described previously. Briefly, the RAW264.7 cells (1×10^5 cells/well) were stimulated with or without 1 μg/mL of LPS (Sigma Chemical Co., St. Louis, MO) for 24 h in the presence or absence of the test compounds (5–50 μM). The cell culture supernatant (100 μL) was then reacted with 100 μL of Griess reagent. The remaining cells after the Griess assay were used to test their viability using a MTT (Sigma Chemical Co., St. Louis, MO)-based colorimetric assay as previously described.[17]

Thrombin Time (TT) Assay. To assess anticoagulant action of sample, the effect of sample on thrombin time (TT) was determined using an Auto Blood Coagulation Analyzer (Sysmex CA-540, Japan), according to the manufacturer's instructions.[18] Briefly, 50 μL of human thrombin (Sigma, St. Louis, MO, USA) was preincubated for 10 min at 37 °C with 10 μL of individual samples dissolved in DMSO before mixing with 50 μL of 20 mM $CaCl_2$ and 100 μL of standard human plasma (Siemens, Marburg, Germany). DMSO and aspirin dissolved in DMSO were used as negative and positive controls, respectively. The time period required for coagulation of the mixture was measured by the Auto Analyzer.

Statistical Analysis. All data in the present study were obtained as average of experiments that were performed in triplicate and are expressed as mean ± S.D. Statistical significance was determined using the software SPSS 19.0.

Results and Discussion

Phytochemical study on the EtOAc-soluble extract of *P. tricuspidata* using *in vitro* thrombin time assay and repeated column chromatographic separation yielded twelve phenolics **1–12** as active principles (Fig. 1). The ^{1}H and ^{13}C NMR

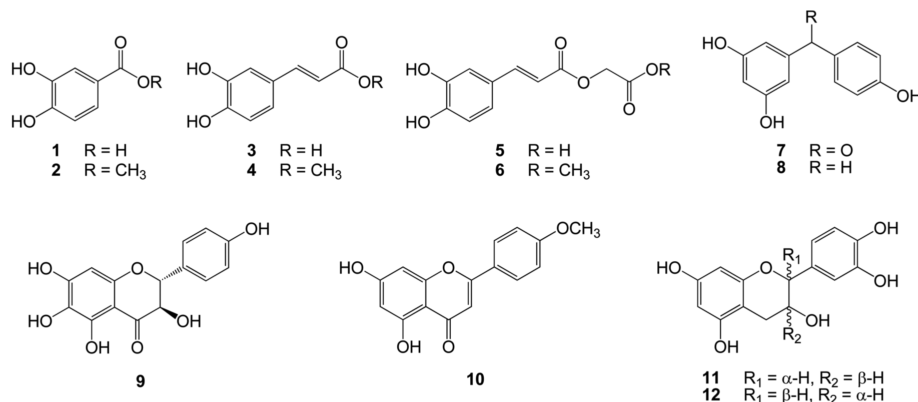

Figure 1. Chemical structures of the isolated compounds (**1**–**12**) from the stems of *Parthenocissus tricuspidata.*

spectra of compounds **1–4** revealed that they were caffeic acid derivatives with an ABX-spin system (δ_H 7.37–7.63), two *trans*-olefinic protons (δ_H 7.60–7.54 and 6.31–6.26, each 1H, d, J = 16.0 Hz), and a carboxylic group (δ_C 169.0–172.1). Compounds **5** and **6** were isolated as white amorphous powders. Their ^{1}H and ^{13}C NMR spectra also gave an ABX-spin system at δ_H 7.06–6.32, two *trans*-olefinic protons at δ_H 7.61 and 6.33–6.31 (each 1H, d, J = 16.0 Hz), and a carboxylic group (δ_C 168.7–168.5). In addition, the signals at δ_H 4.66–4.72 (2H, s) were attributed to oxygenated methylene of glycolic acid moiety (**5**), and a methoxyl group of methyl ester in **6** (3.75, 3H, s). In the ^{13}C NMR spectra, the carbonyl carbon at δ_C 168.7–168.5 (C-9) of caffeoyl moiety and δ_C 170.6–172.7 (C-11) of glycolic acid moiety further supported the above assignments. Compounds **5** and **6** were thus elucidated as caffeoylglycolic acid and its methyl ester, respectively.[16,18] Compound **7** was identified as 3,4',5-trihydroxybenzophenone, which was first reported as a synthesized product having inhibitory effects on several cancer cells, such as MCF-7, pancreas BXPC-3, lung NCI-H460, and colon KM20L2.[19] 3,4',5-trihydroxybenzophenone was first isolated, and reported as a natural product by Ito *et al.*[20] However, both compounds **7** and **8** [5-(4-hydroxybenzyl)-benzene-1,3-diol] were isolated from this plant for the first time.

Compound **9** was isolated as yellow amorphous powder, and its ESI mass spectrum gave an ion peak [M-H]$^-$ at *m/z* 303. From the mass and ^{13}C NMR data, the molecular formula $C_{15}H_{12}O_7$ was deduced for compound **9**. In the ^{1}H NMR spectrum, a 3,5,6,7,4'-substituted flavanonol skeleton was suggested, by the appearance in the aromatic region of two doublet signals at δ_H 7.35 (2H, d, J = 8.4 Hz) and 6.83 (2H, d, J = 8.4 Hz), assigned to H-3′, H-5′ and H-2′, H-6′ respectively, indicative of a 4′-substitution on ring B, a one-proton singlet at δ_H 5.86 (1H, s) typical of H-8 on a 5,6,7-trihydroxy-substituted ring A, and two characteristic 1H doublets for H-2 and H-3 at δ_H 4.97 (1H, d, J = 11.6 Hz) and 4.57 (1H, d, J = 11.6 Hz), respectively. In the ^{13}C NMR spectrum, C-2, C-3 and C-4 resonances appeared at δ_C 85.1, 73.8 and 198.7, respectively, as expected in 2,3-*trans* flavanonols with aryl and hydroxyl substituents at C-2 and C-3 that are equatorially oriented.[21] Thus, compound **9** was identified as 3,5,6,7,4′-pentahydroxyflavanonol.[22] Compounds **10–12** were isolated and identified as acacetin (**10**), (+)-catechin (**11**), and (−)-catechin (**12**), respectively, by detailed comparison of their ^{1}H, ^{13}C NMR and MS data, with those published in the literature.[23]

Due to the development of life, the diversity of foods and busy life, peoples have no much more time for improving their body health. Some of them are not able to do or they do not know how to do. As the result, many peoples are underdeveloped and suffered myocardial infarction and ischemic stroke induced by arterial thrombosis. The interaction between platelets and blood vessels is important in the development of thrombosis and cardiovascular diseases. Uncontrolled platelet aggregation is critical in arterial thrombosis, leading to ischemia; and may cause life-threatening disorders, such as heart attacks and stroke. Hence, in the treatment and prevention of these cardiovascular diseases, the inhibition of thrombus formation is of fundamental importance. Although it is well established that aspirin still provides an effective secondary prevention of ischemic cardiovascular disorders, this drug can produce hemorrhagic events and upper gastrointestinal bleeding as major drawbacks. Unfractionated heparin (UFH), low molecular weight heparin (LMWH) and fondaparinux (an AT-dependent factor Xa [FXa] inhibitor) bind to antithrombin (AT), enhance its protease inhibition activity, and exert anticoagulant effects. However, its use is accompanied by some side effects, frequently requiring monitoring of partially activated thromboplastin time, and resulting in other hemorrhagic complications. Therefore, the search for alternative anticoagulants from natural sources with reduced side effects is still needed, and urgent.

In our study, we followed activity-guided isolation using an *in vitro* thrombin time assay to search for anti-blood coagulants from the EtOAc fraction (the only active fraction) of the stem of *P. tricuspidata.* Successfully, we isolated 12 compounds from this extract and evaluated their antithrombotic effect. The anticoagulant action of these isolated compounds **1–12** was determined using an *in vitro* thrombin time (TT) assay, and the results are presented in Figure 2(a). The cytotoxicity of the isolates was also determined using MTT assay on JT/Neo cells, and no cytotoxic activity was found after treatment with tested compounds for 4 h incuba-

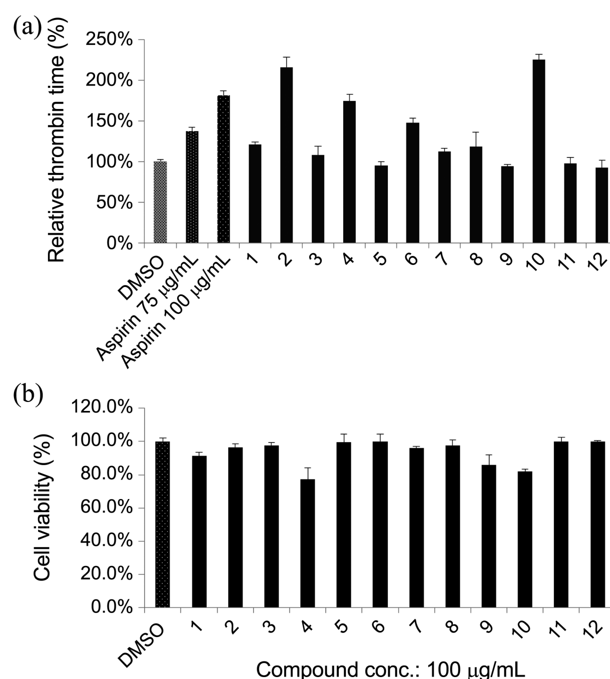

Figure 2. (a) Inhibitory effects of compounds (**1**–**12**) isolated from *P. tricuspidata* against blood coagulation in thrombin time (TT) assay. (b) Inhibitory effects of isolated compounds (**1**–**12**) on JT/Neo cells. The JT/Neo cells were seeded in 96 well cell culture plates with 1×10^5 cells/each well. And the cells were incubated with or without compounds (100 μg/mL) for 4 h. Cell viability was detected using MTT reagent, and the values shown are means ± SD of three independent experiments.

Table 1. Inhibition of LPS-induced NO production of isolated compounds **1**–**12** in macrophage RAW264.7 cells

Compounds	IC_{50} value (μM)[a]	Cytotoxicity (% Inhibition)[b]
1	> 50	NT
2	11.9 ± 0.3	NT
3	47.3 ± 1.1	NT
4	2.9 ± 0.2	NT
5	> 50	NT
6	29.0 ± 0.6	NT
7	> 50	NT
8	> 50	NT
9	> 50	NT
10	45.6 ± 1.5	NT
11	> 50	NT
12	> 50	NT
Celastrol[c]	1.0 ± 0.1	–

[a]The inhibitory effects are represented as the molar concentration (mM) giving 50% inhibition (IC_{50}) relative to the vehicle control. These data represent the average values of three repeated experiments. [b]Data showed no cytotoxic effect of compounds **1**–**12** on cell viability. RAW264.7 cells were incubated with or without compounds (5–50 μM) and LPS (1 μg/mL). The data shown are means ± SD of three independent experiments. [c]The compound was used as positive control.

tion, except for compounds **4** and **10** given a little 22 and 18% inhibition at 100 μg/mL (Fig. 2(b)). Among the isolates, compounds **2**, **4** and **10** possessed the most potency with 216, 174 and 225% inhibition, respectively; while aspirin, used as positive control, displayed only 181% inhibition, at the same concentration of 100 μg/mL. Compound **6**, with 148% inhibition, displayed stronger activity than aspirin with 138% inhibition, at concentration of 75 μg/mL. Furthermore, compound **10** was found to be a major compound in total EtOAc fraction, thus, we are now preparing further investigation on its anti-coagulant and anti-platelet effects in *in vivo*.

To assess the effect of the isolates (**1**–**12**) on LPS-induced NO production in RAW264.7 cells, cells were treated with LPS (1 μg/mL) for 24 h after treatment, with/without tested compounds for 1 h. Neither LPS nor samples were added to the control group. Cell culture media were harvested post-treatment; and NO levels were quantified, using the Griess reaction. As shown in Table 1, compounds **2**, **4** and **6** displayed inhibitory potency, with IC_{50} values of 11.9, 2.9 and 29.0 μM, respectively. Compounds **3** and **10** showed moderate effects, with IC_{50} values of 47.3 and 45.6 μM, respectively; while the others were weak, or not active. The cytotoxic effects of isolated compounds were also evaluated in the presence of LPS using MTT assay, and these compounds showed no cytotoxicity, even at concentrations of 50 μM (Table 1). Accordingly, we used 1, 3, 10 and 30 μM of compounds **2**, **4** and **6**, to further investigate the inhibitory effects on the LPS-induced productions of the inflammatory mediators NO in RAW264.7 cells, regardless of concentration. Neither LPS nor samples were added to the control group. Thus, the inhibitory effects of these compounds on

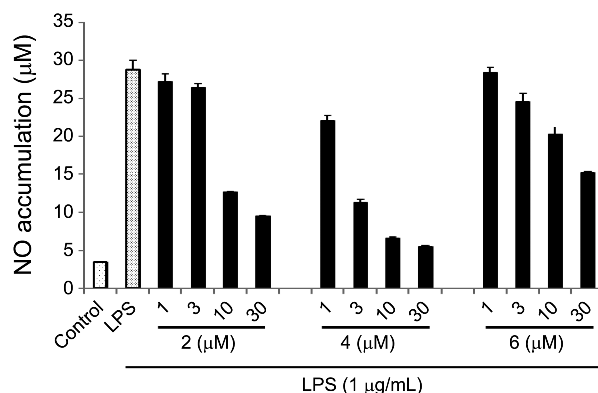

Figure 3. Inhibitory effect of compounds **2**, **4** and **6** on LPS-induced NO production in RAW264.7 macrophages. RAW264.7 cells were pretreated with different concentrations (1, 3, 10 and 30 μM) of compounds for 1 h, then with LPS (1 μg/mL), and incubated for 24 h. Control values were obtained in the absence of LPS and compounds.

NO production were not attributable to any cytotoxic effect. As shown in Figure 3, after LPS (1 μg/mL) stimulation, NO production increased by approximately 12-fold after 24 h. Compounds **2**, **4** and **6** reduced the NO production 24 h after LPS stimulation, in a dose-dependent manner.

When investigating the structural activity relationship (SAR), we found that compounds that possessed a methoxy moiety (compounds **2**, **4**, **6** and **10**) afforded stronger inhibitory activities on both blood coagulation and NO production. In the caffeic acid derivatives **1**–**6**, the methoxy group attached to the carbonyl carbon of the caffeoyl moiety, to produce methyl esterification. The formation of this methyl ester may be responsible for the inducement of inhibitory activity of these derivatives. In addition, compound **10**, with a methoxy moiety attached at C-4', also possessed stronger inhibitory activities, than simple hydroxyl flavonoids (compound **9**, **11**–**12**). This observation indicated that substitution of this methyl unit may play an important role in inhibiting the aggregation of thrombus in blood vessel, as well as the production of nitrite oxide induced by lipopolysaccharide in macrophages. Overall, our active compounds provided significant meaning (potential antithrombotic and anti-inflammatory effects) and could be considered as new lead compounds for development of agents against arterial thrombosis, ischemia, and possibly myocardial disease.

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