Bioactive Constitutents from the Stems of *Annona montana*

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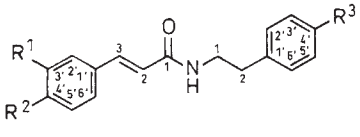
Abstract

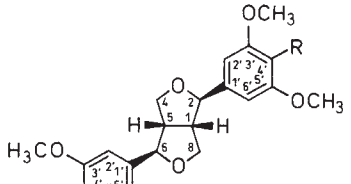
Three structurally related acid amides, *N-trans*-feruloyltyramine (**1**), *N-p*-coumaroyltyramine (**2**), and *N-trans*-caffeoyltyramine (**3**), one lignan, (−)-syringaresinol (**4**), one aromatic aldehyde, syringaldehyde (**5**), and two steroids, β-sitosterol and β-sitosterol-β-D-glucoside were isolated from the stem parts of *Annona montana* (Annonaceae). *N-trans*-Caffeoyltyramine (**3**) is a new natural compound. These compounds and their acetate derivatives were subjected to the antiplatelet aggregation and cytotoxicity bioassay where some of them showed significant activities.

Key words

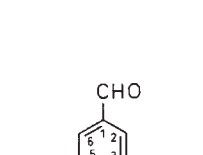
N-trans-Caffeoyltyramine, Annonaceae, *Annona montana*, cytotoxicity, antiplatelet aggregation.



	R^1	R^2	R^3
1	OCH_3	OH	OH
1a	OCH_3	OAc	OAc
2	H	OH	OH
2a	H	OAc	OAc
3	OH	OH	OH
3a	OAc	OAc	OAc



4 R = OH
4a R = OAc



5 R = OH
5a R = OAc

Introduction

Annona montana Macf. (Annonaceae) is widely distributed from the West Indias to southern Brazil and is cultured for its fruit in Taiwan (1). In the previous paper (2), the leaves of this plant were found to contain a novel cytotoxic phenanthrene alkaloid, annoretine [3-hydroxy-4-methoxy-*N*-methyltetrahydropyrido[4,3-*a*]phenanthrene], together with two cytotoxic alkaloids, argentinine and liriodenine, as well as a new oxoaporphine alkaloid, annolatine and one steroid, sitosterol-β-D-glucoside, which are inactive. As part of our continuing search for novel plant bioactive agents, the MeOH extract of stem parts of *A. montana* was found to show significant inhibitory effects on the platelet aggregation induced by several aggregating agents and cytotoxicity against the P-388 murine lymphocytic leukemia system in cell culture. Bioassay-directed fractionation traced the active fractions to active components. We report herein on the isolation, identification and antiplatelet aggregation activity as well as cytotoxicity of compounds **1**–**5**.

Materials and Methods

General procedure

All melting points were made on a Mel-Temp II apparatus and are uncorrected. Optical rotations were taken on a Jasco DIP-370 Digital Polarimeter. Ultraviolet absorption (UV) and infrared (IR) spectra were measured on a Hitachi U-2000 spectrophotometer and a Hitachi 260-30 spectrophotometer, respectively. ^{1}H-NMR and ^{13}C-NMR spectra were recorded with a Varian Gemini NMR spectrometer at 200 MHz and 50 MHz using TMS as internal standard. Mass (MS) spectra were run on a JEOL JMS-HX 110 mass spectrometer. Active carbon (Wako) and silica gel 60 (Merck, 230–400 mesh) were used for CC, and pre-coated silica gel plates (Merck, Kieselgel 60 F-254, 0.50 mm) were used for preparative TLC.

Plant material

The stems of *A. montana* were collected from Chie Shan, Kaohsiung Hsien, Taiwan, in December 1990. A voucher specimen (YC 9007) is deposited in the Graduate Institute of Natural Products, Kaohsiung Medical College, Kaohsiung, Taiwan, Republic of China.

Extraction and separation

The air-dried and powdered stems (8.85 kg) were extracted 5 times with MeOH (10 l) at room temperature. The combined MeOH extracts (50 l) were evaporated under reduced pressure and partitioned to give $CHCl_3$, *n*-BuOH, and aqueous so-

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lutions, respectively. The *n*-BuOH layer was concentrated to 1/5 volume in vacuo to afford a precipitate (67 g) which was chromatographed on an active carbon column (40 × 2 cm) eluted with a gradient of MeOH in $CHCl_3$. In the MeOH-$CHCl_3$ (7 : 3) eluent, three fractions were present according to TLC. These were rechromatographed on a silica gel column (35 × 2 cm) eluted with $CHCl_3$-MeOH [18 : 1 (2 l)] to give 43.2 mg of **2** and 68.1 mg of **3**, respectively. The $CHCl_3$ layer was extracted with 3 % HCl to remove alkaloids. The neutral $CHCl_3$ solution was dried (K_2CO_3) and evaporated to leave a brownish viscous residue (143 g) which was chromatographed on a silica gel column (70 × 6 cm) eluted with $CHCl_3$-EtOAc-MeOH mixtures of gradually increasing polarity. In the $CHCl_3$-EtOAc-MeOH (4 : 1 : 1) eluent, four fractions were present according to TLC. These were rechromatographed on a silica gel column (27 × 2 cm) eluted with acetone (2.5 l) and $CHCl_3$-MeOH [2 : 1 (5.4 l)] to give 40.2 mg of **6** and 115.7 mg of **1**, 86.2 mg of **4**, 26.7 mg of **5** and 55.7 mg of **7**, respectively.

Acetylation of **1**, **2**, **3**, **4**, *and* **5**

Each compound (10 mg) was acetylated with acetic anhydride (1 ml) and pyridine (1 ml) at room temperature for 24 h, respectively. The end products were partitioned with $CHCl_3$ and H_2O, then the $CHCl_3$ layer was concentrated and directly chromatographed on a silica gel column (16 × 2 cm) eluted with $CHCl_3$ (2.5 l) to afford the acetyl derivatives **1a** (10.3 mg), **2a** (9.7 mg), **3a** (9.5 mg), **4a** (8.9 mg), and **5a** (9.1 mg), respectively.

N-trans-Caffeoyltyramine (**3**): m.p. 214–215 °C; $C_{17}H_{17}NO_4$; EIMS *m/z* (rel. int): 299 ([M]$^+$, 100), 180 (53), 163 (76), 120 (20), 107 (9); HREIMS: found 299.1162 (calcd. 299.1158); UV λ_{max}^{MeOH} nm (log ε): 221 (4.15), 240sh (4.00), 294 (4.04), 321 (4.08); UV $\lambda_{max}^{MeOH+NaOH}$ nm (log ε): 208 (4.09), 225 (4.12), 242 (4.01), 305 (3.85), 349 (4.08), 360 (4.10); IR ν_{max}^{KBr} cm^{-1}: 3400 (br, OH), 1650 (CONH), 1590, 1515, 1440; ^{1}H-NMR (200 MHz, CD_3OD) δ: *caffeoyl moiety*: 6.33 (1H, d, J = 15.7 Hz, H-2), 6..76 (1H, d, J = 8.3 Hz, H-5′), 6.89 (1H, dd, J = 8.3, 2.0 Hz, H-6′), 6.99 (1H, d, J = 2.0 Hz, H-2′) and 7.38 (1H, d, J = 15.7 Hz, H-3); *tyramine moiety*: 2.74 (2H, t, J = 7.3 Hz, H-2), 3.45 (2H, t, J = 7.3 Hz, H-1), 6.72 (2H, d, J = 8.6 Hz, H-3′ and H-5′) and 7.05 (2H, d, J = 8.6 Hz, H-2′ and H-6′). ^{13}C-NMR (50 MHz, CD_3OD) δ: *caffeoyl moiety*: 115.12 (1C, C-2′), 116.50 (1C, C-5′), 118.49 (1C, C-2), 122.12 (1C, C-6′), 128.38 (1C, C-1′), 142.19 (1C, C-3), 146.74 (1C, C-3′), 148.76 (1C, C-4′) and 169.31 (1C, C-1); *tyramine moiety*: 35.85 (1C, C-2), 42.58 (1C, C-1), 116.30 (2C, C-3′ and C-5′), 130.76 (2C, C-2′ and C-6′), 131.37 (1C, C-1′) and 156.95 (1C, C-4′).

N-trans-Caffeoyltyramine triacetate (**3a**): m.p. 163–164 °C; $C_{23}H_{23}NO_7$; EIMS *m/z* (rel. int.): 425 ([M]$^+$, 3), 383 (2), 341 (15), 221 (7), 205 (41), 163 (81), 120 (100), 107 (8); UV λ_{max}^{MeOH} nm (log ε): 211 (4.52), 217 (4.53), 273 (4.55); IR ν_{max}^{KBr} cm^{-1}: 3400 (NH), 1770 (C=O), 1740 (C=O), 1660 (CONH), 1620, 1500, 1440; ^{1}H-NMR (200 MHz, CD_3OD) δ: *caffeoyl moiety*: 2.27 (3H, s, $OCOCH_3$), 2.28 (3H, s, $OCOCH_3$), 6.53 (1H, d, J = 15.7 Hz, H-2), 7.24 (1H, d, J = 8.3 Hz, H-5′), 7.42 (1H, d, J = 2.0 Hz, H-2′), 7.45 (1H, dd, J = 8.3, 2.0 Hz, H-6′) and 7.48 (1H, d, J = 15.7 Hz, H-3); *tyramine moiety*: 2.25 (3H, s, $OCOCH_3$), 2.86 (2H, t, J = 7.3 Hz, H-2), 3.53 (2H, t, J = 7.3 Hz, H-1), 7.02 (2H, d, J = 8.6 Hz, H-3′ and H-5′) and 7.26 (2H, d, J = 8.6 Hz, H-2′ and H-6′).

(−)-Syringaresinol (**4**): m.p. 158–160 °C; $C_{22}H_{26}O_8$; $[\alpha]_D^{24}$: −6.1° ($CHCl_3$, *c* 0.1); EIMS *m/z* (rel. int.): 418 ([M]$^+$, 24), 182 (34), 181 (100), 167 (34); HREIMS: found 418.1622 (calcd 418.1627); ^{13}C-NMR (50 MHz, $CDCl_3$) δ: 54.32 (2C, C-1 and C-5), 56.37 (4C, OCH_3 × 4), 71.81 (2C, C-4 and C-8), 86.07 (2C, C-2 and C-6), 102.78 (4C, C-2′ and C-6′), 132.08 (2C, C-1′), 134.36 (2C, C-4′) and 147.22 (4C, C-3′ and C-5′).

(−)-Syringaresinol diacetate (**4a**): m.p. 174–175 °C; $C_{26}H_{30}O_{10}$; EIMS *m/z* (rel. int.): 502 ([M]$^+$, 3), 460 (52), 418 (100), 181 (6), 167 (5); UV λ_{max}^{MeOH} nm (log ε): 206 (4.97), 220 (4.43), 271 (3.60); IR ν_{max}^{KBr} cm^{-1}: 1760 (C=O), 1600, 1500, 1450, 1420; ^{1}H-NMR (200 MHz, $CDCl_3$) δ: 2.34 (6H, s, $OCOCH_3$ × 2), 3.1 (2H, m, H-1 and H-5), 3.84 (12H, s, OCH_3 × 4), 3.94 (2H, dd, J = 9.2, 3.7 Hz, Ha-4 and Ha-8), 4.32 (2H, dd, J = 9.2, 6.8 Hz, Hb-4 and Hb-8), 4.78 (2H, d, J = 4.3 Hz, H-2 and H-6) and 6.60 (4H, s, H-2′ and H-6′).

Assay method for antiplatelet aggregation

Thrombin (bovine) was purchased from Park Davis Co. (Detroit, U.S.A.) and dissolved in 50 % glycerol to give a stock solution of 100 NIH units/ml. Collagen (type I, bovine Achilles tendon), obtained from Sigma Chem. Co. (St. Louis, U.S.A.) was homogenized in 25 mM acetic acid and stored at −70 °C. Platelet-activating factor (PAF, mixture of C-16 and C-18 PAF), arachidonic acid, EDTA and bovine serum albumin were purchased from Sigma Chem. Co. (St. Louis, U.S.A.).

Platelet-rich plasma (PRP) was obtained from blood collected from the marginal ear vein of a rabbit of either sex, anticoagulated with disodium EDTA (1 : 14, v/v, 100 mM), and centrifuged for 10 min at 90 × *g* and room temperature. Rabbit platelet suspension was obtained from EDTA-anticoagulated PRP according to the washing procedure described (3). Platelet numbers were counted by a Coulter Counter (Model ZM) and adjusted to 4.5×10^8 platelets/ml and suspended in Tyrode's solution of the following composition (mM): NaCl (136.9), KCl (2.7), $NaHCO_3$ (11.9), $MgCl_2$ (2.1), NaH_2PO_4 (0.4), $CaCl_2$ (1.0 mM), and glucose (11.1) with bovine serum albumin (0.35 %). Aggregation was measured by the turbidimetric method (4) using a Lumi-aggregometer (Chrono-Log Co. U.S.A.); the absorbance of platelet suspension was assigned as 0 % aggregation and the absorbance of platelet-free Tyrode's solution as 100 % aggregation. All glassware was siliconized. The platelet preparation was warmed at 37 °C for 3 min and stirred at 1200 rpm before the addition of inducers. In order to eliminate the effect of the solvent on the aggregation, the final concentration of DMSO was fixed at 0.5 %. Results are given in Tables **1** and **2**.

Assay method for cytotoxic activity

P-388, KB, A-549, HT-29, and HL-60 were purchased from the American Type Culture Collection. The P-388, KB, A-549, and HT-29 cell lines were cultured in Rosewell Park Memorial Institute (RPMI) 1640 medium containing 10 % heat-inactivated fetal bovine serum (FBS). The HL-60 cell line was cultured in Iscove's modified Dulbecco's medium (IMDM) with 20 % heat-inactivated FBS. All the cell lines were maintained in an

Table 1 Effects of the compounds **1**–**3** and their acetate derivatives **1a**–**3a** and compound **4** on the aggregation of rabbit platelets induced by thrombin, arachidonic acid, collagen, and PAF[a].

Compound	% Aggregation			
	Thrombin	Arachidonic acid	Collagen	PAF
Control	87.6 ± 0.5	89.5 ± 2.1	89.9 ± 0.9	88.9 ± 1.0
1	84.3 ± 2.0	0.0 ± 0.0[d]	34.2 ± 3.8[d]	78.0 ± 2.4[d]
1a	82.0 ± 1.4[d]	8.7 ± 7.1[d]	47.5 ± 8.5[d]	73.9 ± 1.0[d]
2	84.4 ± 1.9	0.0 ± 0.0[d]	36.3 ± 2.6[d]	85.5 ± 1.6
2a	80.7 ± 1.0[d]	32.9 ± 15.3[d]	76.8 ± 4.1[d]	79.7 ± 3.1[b]
3	85.0 ± 1.5	0.0 ± 0.0[d]	33.6 ± 4.5[d]	86.4 ± 1.9
3a	81.7 ± 1.7[c]	8.3 ± 6.8[d]	36.6 ± 3.1[d]	39.9 ± 5.7[d]
4	86.4 ± 1.7	7.2 ± 6.2[d]	67.2 ± 6.4[d]	72.9 ± 3.7[d]

[a] Platelets were preincubated with each compound (300 μM) or 0.5 % DMSO (control) at 37 °C for 3 min, then the inducer thrombin (0.1 U/ml), arachidonic acid (100 μM), collagen (10 μg/ml), or PAF (4 nM) was added. Values are presented as means ± S.E. (n = 3–5).

[b] $P < 0.05$.

[c] $P < 0.01$.

[d] $P < 0.001$ as compared with the respective control.

Table 2 Effects of the compounds **1–3** and **1a** on the aggregation of rabbit platelets induced by arachidonic acid[a].

Compound		% Aggregation Arachidonic acid
Control		92.5 ± 1.0
1	150 μM	0.0 ± 0.0[d]
	60 μM	15.7 ± 7.6[d]
	30 μM	85.1 ± 1.1
1a	150 μM	24.2 ± 19.8[d]
	60 μM	73.5 ± 6.1
	30 μM	89.1 ± 3.5
2	150 μM	0.0 ± 0.0[d]
	60 μM	0.0 ± 0.0[d]
	30 μM	85.7 ± 0.9
3	150 μM	0.0 ± 0.0[d]
	60 μM	46.0 ± 17.0[b]
	30 μM	86.6 ± 2.2
Aspirin	50 μM	0.0 ± 0.0[d]
	20 μM	43.1 ± 16.9[c]
	10 μM	90.5 ± 1.3

[a] Platelets were preincubated with each compound (150, 60, 30 μM), aspirin (50, 20, 10 μM) or 0.5% DMSO (control) at 37 °C for 3 min, then the inducer arachidonic acid (100 μM) was added. Values are presented as means ± S.E. (n = 3–5).
[b] $P < 0.05$.
[c] $P < 0.01$.
[d] $P < 0.001$ as compared with the respective control.

Table 3 Cytotoxicity[a] of compounds **1–5** and their derivatives **1a–5a**.

Compound	ED_{50} (μg/ml) P-388	KB	A-549	HT-29	HL-60
1	7.97	–	–	–	2.90
1a	2.68	–	–	–	–
2	2.26	–	–	–	–
2a	7.71	–	–	–	–
3	2.92	–	–	–	–
3a	1.51	–	–	–	–
4	0.67	–	–	–	–
4a	3.78	–	–	–	–
5	3.28	–	–	–	–
5a	–	–	–	–	–

[a] For significant activity of pure compounds, an ED_{50} value of ≤ 4.0 μg/ml is required (4).
–: ED_{50} value ≥ 10 μg/ml.

incubator at 37 °C in humidified air containing 5% CO_2. For routine cytotoxicity assays, all five cell lines were adapted to one single medium, RPMI 1640 supplemented with 10% FBS and 1 mM glutamate.

The cytotoxic activities of the tested compounds against P-388, KB, A-549, HT-29, and HL-60 were assayed using standard protocols (5, 6) and with modification of the MTT {3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide} colorimetric method described by Alley et al. (7). For P-388 cells, 200 μl of culture were established at 1500 cells/well in 96-well tissue culture plates (Falcon). Tested compounds were dispensed subsequently to the established culture plate at eight concentrations each with three repetitions. After 3 days of incubation, P-388 cells were enumerated with MTT.

To measure the cytotoxic activities of the pure compounds or the crude fractions against KB, A-549, HT-29, and HL-60, each cell line was initiated at 2000, 750, 750, and 1000 cells/well, respectively, in 96-well microtiter plates. Three to eight concentrations encompassing 8- to 128-fold range were performed on each cell line. KB, A-549, HT-29, and HL-60 cells were enumerated using MTT after the exposure to tested samples for 3, 7, 7, and 7 days, respectively. Fifty μl of 1 mg/ml MTT were added to each well, and plates were incubated at 37 °C for a further 4 h. Supernatant was aspirated with a Dynatec Automatic Waser. Formazan crystals were redissolved in DMSO (Merck) for 10 min with shaking, and the plate was read immediately on an enzyme-linked immunosorbant assay reader (Microplate Reader, BioRad) at a wavelength of 540 nm. The ED_{50} was defined as 50% reduction of absorbance in the no drug control assay. Results are given in Table 3.

Results and Discussion

The MeOH extract of stem parts of *A. montana* was fractionated by solvent partitioning followed by *in vitro* antiplatelet aggregation and P-388 cytotoxicity tests. Further separation and purification by chromatography furnished the principal active components **1**, **2**, **3**, **4**, and **5**. Compounds **1**, **2**, **4**, and **5** were readily identified by UV, IR, MS, ^{1}H-NMR, and ^{13}C-NMR spectral data analysis and comparison with the reported data of the known compounds (8–17). Moreover, acetylation of *N-trans*-feruloyltyramine (**1**), *N-p*-coumaroyltyramine (**2**), (–)-syringaresinol (**4**), and syringaldehyde (**5**) with Ac_2O and pyridine gave *N-trans*-feruloyltyramine diacetate (**1a**), *N-p*-coumaroyltyramine diacetate (**2a**), (–)-syringaresinol diacetate (**4a**) and syringaldehyde acetate (**5a**), respectively. Among them, **1a**, **2a** and **5a** were identified by comparison (UV, MS and ^{1}H-NMR) with literature data (8, 10, 11) and the ^{1}H- and ^{13}C-NMR spectral data of **4a** were also consistent with the proposed structure. This is the second report of compounds **1** and **2**, and first report of syringaldehyde (**5**) occurring in the Annonaceae family (8).

N-trans-Caffeoyltyramine (**3**) was obtained from MeOH as a colorless amorphous powder, m.p. 214–215 °C (lit. 219.5–222.5 °C) (9). Its molecular formula was established as $C_{17}H_{17}NO_4$ by HREIMS (found 299.1162, calcd. 299.1158) and ^{13}C-NMR. The presence of hydroxy groups and an amide function in the *N-trans*-caffeoyltyramine molecule was indicated by two IR bands at 3400 and 1650 cm^{-1} and a signal appearing at δ 169.31 (s) in the ^{13}C-NMR spectrum. Its UV spectrum exhibited several absorption maxima in the same regions as *N-trans*-feruloyltyramine (**1**); these underwent bathochromic shifts on adding base, suggesting that *N-trans*-caffeoyltyramine (**3**) should be a phenolic derivative of an amide. The ^{1}H-NMR spectrum of **3** revealed the presence of seven aromatic protons at δ 6.99 (1H, d, J = 2.0 Hz), 6.89 (1H, dd, J = 8.3, 2.0 Hz), and 6.76 (1H, d, J = 8.3 Hz) corresponding to H-2′, 6′, and 5′ on the caffeic acid moiety, respectively, while the signals at δ 7.05 (2H, d, J = 8.6 Hz) and 6.72 (2H, d, J = 8.6 Hz) represent H-2′, 6′ and H-3′, 5′ on the tyramine moiety, respectively, which was also in full agreement with the ^{13}C-NMR data. The assignment of the ^{13}C-NMR chemical shift data of **3** was achieved by the application of ^{1}H-^{13}C heteronuclear shift correlation 2D experiments. Proof of the phenolic character of *N-trans*-caffeoyltyramine was obtained by preparation of the *O*-acetyl derivative (**3a**). Comparison of the ^{1}H-NMR spectra of *N-trans*-caffeoyltyramine and its *O*-acetyl derivative **3a** indicated the downfield acetylation shift of the all proton resonances, strongly suggesting that the phenolic groups are located at C-3′ and C-4′ of the caffeic acid moiety, and C-4′ of the tyramine moiety, re-

spectively. Therefore, it is proposed that the structure of **3** should be *N-trans*-caffeoyltyramine. Following the structural elucidation of *N-trans*-caffeoyltyramine (**3**) by spectral means, we recognized that this compound has been prepared synthetically by Okuyama et al. (9). Comparison of the spectral data (UV, IR, MS and ^{1}H-NMR) of natural *N-trans*-caffeoyltyramine (**3**) with those of the synthetic compound (9) proved they are identical.

As shown in Table **1**, three acid amides and their acetyl derivatives **1a–3a** and the lignan, (−)-syringaresinol (**4**) showed significant inhibitory effects on the aggregation of rabbit platelets induced by thrombin, arachidonic acid, collagen, and PAF. Especially, complete inhibition was observed in arachidonic acid-induced platelet aggregation by compounds **1–3** (300 μM). They gave marked inhibitory effects against arachidonic acid even at 60 μM (Table **2**). Moreover, compounds **1–5** and their acetyl derivatives **1a–5a** showed potent and selective cytotoxicity against the P-388 cell line and compound **1** also showed significant cytotoxicity against the HL-60 cell line (Table **3**).

Acknowledgements

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