Immunomodulatory Proanthocyanidins from *Ecdysanthera utilis*

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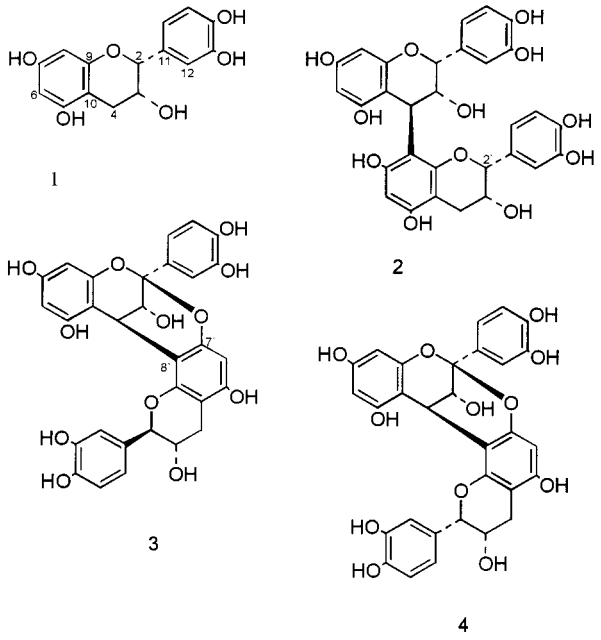
Two new A-type proanthocyanidins have been isolated from *Ecdysanthera utilis* and identified as epicatechin-(4β→8,2β→O→7)-epicatechin-(4β→8)-epicatechin (**5**) and epicatechin-(4β→8)-epicatechin-(4β→8,2β→O→7)-epicatechin-(4β→8)-epicatechin (**6**), respectively. The structure-related components epicatechin (**1**), procyanidin B2 (**2**), proanthocyanidin A1 (**3**), proanthocyanidin A2 (**4**), and aesculitannin C (**7**) were also isolated. All of these compounds were identified and evaluated for immunopharmacological activity. Human peripheral blood mononuclear cells (PBMC) were used as target cells, and cell proliferation was determined by ^{3}H-thymidine uptake. The results indicated that compound **3** suppressed PBMC proliferation activated with phytohemagglutinin (PHA). The inhibitory mechanisms may involve the blocking of interleukin-2 (IL-2) and interferon-γ (IFN-γ) production, since compound **3** attenuated IL-2 and IFN-γ production of PBMC in a dose-dependent manner. Therefore, it is suggested that immunomodulatory agents are present in *E. utilis*.

Ecdysanthera utilis Hayata & Kawakami (Apocynaceae) is a large climbing shrub scattered in forests at low altitudes in Taiwan.[1] Its roots and stems have been used as analgesic, antiphlogistic, and spasmolytic agents in Taiwan folk medicine.[2] A literature survey shows that the crude extract of *E. utilis* possessed antiinflammatory effects.[3] Previous chemical study of this plant has resulted in the isolation of steroids and triterpenoids.[4]

The inflammatory response provides early protection in restricting tissue damage at the site of infection or tissue injury.[5] Both leukocytes, including lymphocytes and monocytes, and cytokines have important roles in clearance of the antigen and healing of the tissue.[6] Interferon-γ (IFN-γ) is an important inflammatory factor for the attraction of macrophages.[7] Interleukin-2 (IL-2) stimulates proliferation of T-lymphocytes and also acts on natural killer cells and macrophages to induce other cytokines involved in inflammatory responses.[8] Paradoxically, the inflammatory response to invasive organisms, if sufficiently intense or inappropriately prolonged, could aggravate the injury or even cause death. The use of antiinflammatory medications must therefore be done carefully.[9] Blockade of lymphocyte activation and proliferation is one antiinflammatory mechanism[10] that has potential therapeutic benefit. In the present study, human peripheral blood mononuclear cells (PBMC) were used as target cells, and *E. utilis* was selected for evaluation of immunopharmacological activity. A systemic study of the bioactive part of *E. utilis* stems has resulted in the isolation of five A-type proanthocyanidins and one procyanidin and epicatechin. This paper deals with the structure elucidation of the proanthocyanidins by spectroscopic and chemical means. Furthermore, the results of the immunopharmacological evaluations of individual components are also presented.

Results and Discussion

E. utilis stems were extracted with acetone, and the resulting extract was granulated by Celite. The granule of extract was then eluted successively with *n*-hexane, $CHCl_3$, EtOAc, and acetone. The bioactive EtOAc extract fraction was subjected to sequential column chromatography on Sephadex LH-20 and Cosmosil 140 C_{18}-OPN to give seven components. Compounds **1**−**4** were readily identified as epicatechin, [11] procyanidin B2,[12] proanthocyanidin A1,[13] and proanthocyanidin A2,[13] respectively, by comparison of their spectral data with those of authentic samples.



Compounds **5**−**7** yield an orange coloration on reaction with anisaldehyde-sulfuric acid reagent, which is thought to be characteristic of proanthocyanidins.[14] Compounds **5** and **7** exhibited ion peaks at *m*/*z* 865 $[M + H]^+$ and 863 $[M + H]^+$, respectively, in the fast atom bombardment mass spectra (FABMS), corresponding to a trimeric constitution for both compounds. The ^{13}C NMR spectrum of **5** shows two signals at δ 80.0 and 78.6 attributable to flavan C-2 carbons. In addition, the appearance of a ketal carbon signal at δ 100.0 indicated that **5** possesses an A-type unit in the molecule. On degradation with acid in the presence of α-toluenethiol, **5** gave epicatechin (**1**) and 4′-benzylthioproanthocyanidin A-2 (**8**). Therefore, **5** was determined to be a coupled product of procyanidin A-2 and epicatechin.

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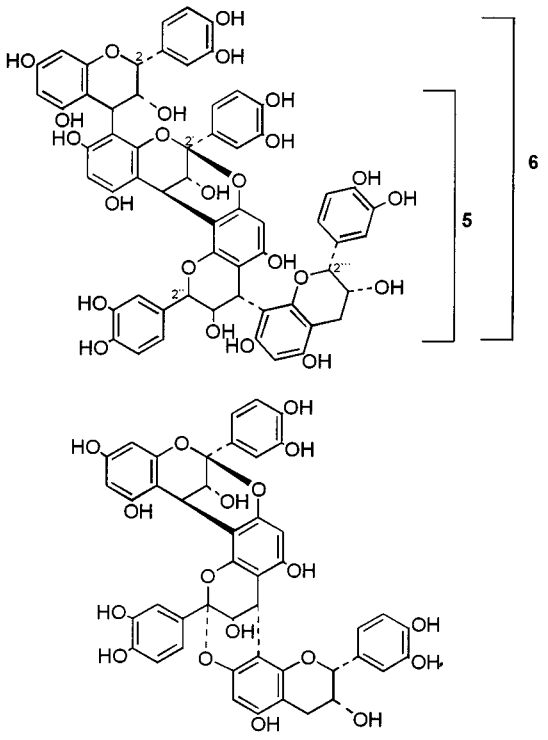

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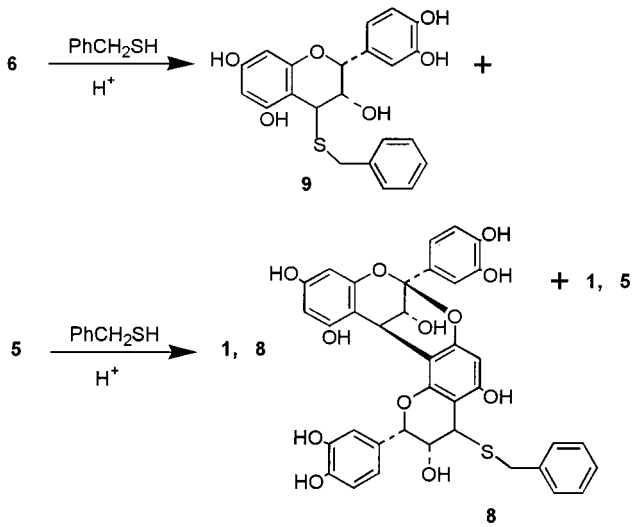
In the ^{1}H NMR spectrum, signals of H-3 and H-2″ were apparent at δ 3.31 and 4.40, respectively. These remarkable upfield shifts are attributable to the magnetic anisotropic effects of the B″-ring and the A′-ring, which can be located just in front of the protons only when **5** possessed a (4′β-8) linkage.[15] Therefore, the structure of **5** was determined as epicatechin-(4β→8,2β→O→7)-epicatechin-(4β→8)-epicatechin.[16] The ^{13}C NMR data of **7** showed a C-2 methine signal at δ 81.4 and two ketal carbon signals at δ 100.0 and 100.7, indicating that trimeric **7** possesses an additional a C−O interflavanoid linkage relative to **5**. By comparison of the published data, the structure of **7** was determined as aesculitannin C.[17]



In the FABMS, **6** gave a $[M + H]^+$ ion peak at *m*/*z* 1053 corresponding to a tetrameric constitution. Comparison of the ^{1}H and ^{13}C NMR spectra of **6** with those of **5** revealed that **6** contains an additional epicatechin molecule relative to **5**. On thiolytic degradation, **6** gave 4-benzylthioepicatechin (**9**), epicatechin-(4β→8,2β→O→7)-epicatechin-(4β→8)-epicatechin (**5**), 4′-benzylthioproanthocyanidin A-2 (**8**), and epicatechin (**1**). ^{13}C NMR signals at δ 75.6, 70.4, and 36.8, attributable to C-2, C-3, and C-4, respectively, were similar to those of procyanidin B2 (**2**). These results implied that **6** possesses an epicatechin as the upper unit with the 4β−8 interflavonoid linkage to **5**. Therefore, the structure of **6** was determined as epicatechin-(4β→8)-epicatechin-(4β→8,2β→O→7)-epicatechin-(4β→8)-epicatechin. The isomeric compounds of **5** and **6** had been isolated from *Cinnamomum zeylanicum*.[18]

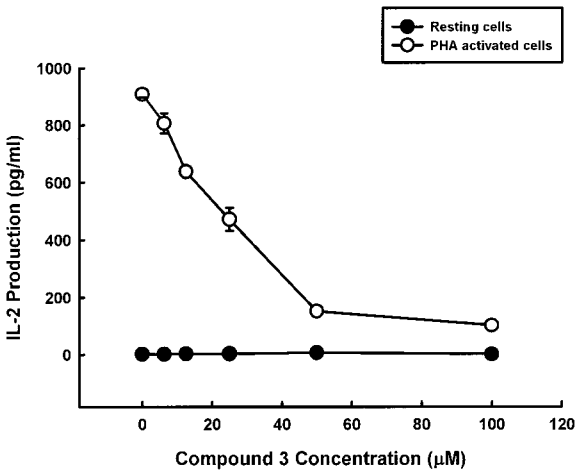


Figure 1. IL-2 production in PBMC cultures treated with compound **3**. PBMC (2×10^5/well) were treated by 0, 6, 12.5, 25, 50, and 100 μM of compound **3** with or without PHA (5 μg/mL) for 3 days. The cell supernatants were then collected and IL-2 concentration was determined by EIA. Each point is the mean of three independent experiments.

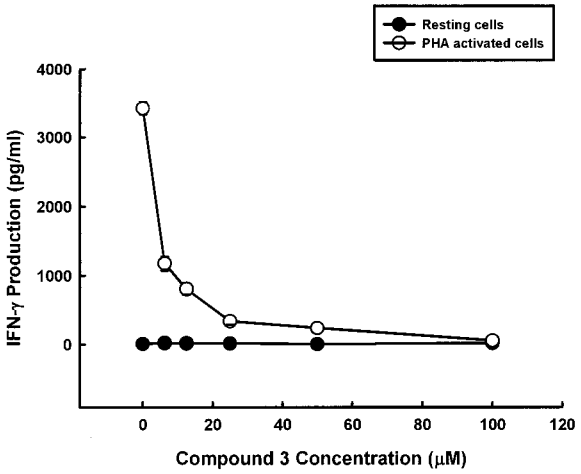


Figure 2. Impairment of IFN-γ production in PBMC cultures treated with compound **3**. PBMC (2×10^5/well) were treated by 0, 6, 12.5, 25, 50, and 100 μM of compound **3** with or without PHA (5 μg/mL) for 3 days. The cell supernatants were then collected and IFN-γ concentration was determined by EIA. Each point is the mean of three independent experiments.

All of the isolated compounds were evaluated for their effects on PBMC proliferation. With the exception of **3**, no inhibitory activity on PBMC proliferation was detected. The results indicated that the ^{3}H-thymidine uptake in the resting or PHA-stimulated states was not affected by DMSO (0.1%) treatment. Although similar to cyclosporin A in having little effects on ^{3}H-thymidine uptake in resting PBMC, both cyclosporin A and **3** significantly suppressed the enhanced uptake observable in activated cells. The IC_{50} of **3** on activated PBMC proliferation was 30.6 μM.

To determine whether the impairment of activated PBMC proliferation was related to cytokines production, the cell supernatants were collected and the production of IL-2 and IFN-γ were assayed by EIA. As shown in Figure 1 and Figure 2, the stimulated production of IL-2 and IFN-γ in activated PBMC was significantly inhibited by **3** in a concentration-dependent manner. The IC_{50} of **3** on IL-2 and IFN-γ production in PBMC activated with PHA were 25 and 5.1 μM, respectively. It is unlikely that **3** suppressed PBMC proliferation through cytotoxic effects, because the results of trypan blue staining indicated no significant difference (data not shown) in cell viabilities of PBMC treated with or without **3** for 4 days. The inhibitory mechanisms may involve the impairments of IL-2 and IFN-γ production. These results indicated that **3** is an immunomodulatory agent.

Experimental Section

General Experimental Procedures. Melting points were determined with a Yanaco micro melting point apparatus and are uncorrected. IR spectra were obtained as KBr pellets on a Nicolet Avatar 320 IR spectrometer. UV spectra were obtained on a Hitachi U-3200 spectrophotometer in MeOH. ^{1}H, ^{13}C, and 2D NMR spectra were measured with a Varian Inova-500 spectrometer. EIMS, HREIMS, and APCIMS were recorded on a Finnigan MAT 95S and a Finnigan LCQ spectrometer, respectively.

Plant Materials. The stems of *E. utilis* were collected at Shihting, Taipei, Taiwan, in October 1999. A voucher specimen (No. 199508) has been deposited in the herbarium of the Department of Botany of the National Taiwan University.

Extraction and Isolation. The milled stems (10.0 kg) were extracted with acetone (70 L × 3). The concentrated acetone extract (378 g) was granulated with Celite (2.8 kg). The granule of extract was then eluted successively with *n*-hexane (8 L), $CHCl_3$ (8 L), EtOAc (8 L), and acetone (8 L). The bioactive EtOAc extract fraction (38 g) was subjected to column chromatography on Sephadex LH-20 (10 × 100 cm) using a solvent system (MeOH−H_2O) of increasing MeOH percentage yielding eight fractions. Fraction 3 (10.3 g) was further chromatograph on Cosmosil 140C_{18}-OPN (4 × 60 cm) and eluted with 10% MeOH−H_2O followed by increasing MeOH portion to give compounds **1** (330 mg), **2** (194 mg), and **6** (644 mg). Fractions 4 (7.8 g), 6 (3.4 g), and 8 (2.5 g) were purified in the same manner as described above. Compound **5** (5.08 g) was obtained from fraction 4, compounds **3** (1.06 g) and **4** (28 mg) were obtained from fraction 6, and compound **7** (16 mg) was obtained from fraction 8, respectively.

Epicatechin-(4*β*→8, 2*β*→O→7)-epicatechin-(4*β*→8)-epicatechin (5): amorphous powder; $[\alpha]_D^{26}$ 63.5° (*c* 1.15, acetone); ^{1}H NMR (CD_3OD) δ 2.91 (2H, m, H-4″), 3.31 (1H, s, H-3), 3.87 (1H, s, H-3″), 4.14 (1H, s, H-4), 4.17 (1H, d, J = 3.0 Hz, H-3′), 4.40 (1H, s, H-2″), 4.57 (1H, s, H-4′), 5.72 (1H, s, H-2′), 5.82 (1H, s, H-6″), 5.98 (1H, d, J = 2.0 Hz, H-8), 6.04 (1H, d, J = 2.0 Hz, H-6), 6.12 (1H, s, H-6′), 6.73−7.33 (9H, C, C′, C″ ring protons); ^{13}C NMR (CD_3OD) δ 28.7 (C-4), 29.6 (C-4″), 38.1 (C-4′), 67.0 (C-3), 67.3 (C-3″), 72.3 (C-3′), 78.6 (C-2′), 80.0 (C-2″), 96.0/96.5/98.3 (C-6/-8/6′/-6″), 100.0 (C-2), 100.1 (C-10′), 104.9 (C-10), 106.2 (C-10″), 106.6 (C-8″), 108.7 (C-8′); FAB MS *m*/*z* 865 $[M + H]^+$.

Thiolytic Degradation[15] of 5. A solution of **5** (500 mg) in EtOH (10 mL) containing α-toluenethiol (2 mL) and acetic acid (3 mL) was refluxed 20 h under N_2 atmosphere. The reaction mixture was concentrated to give an oily residue, which was chromatographed over Sephadex LH-20. Elution with 30% MeOH−H_2O furnished epicatechin (**1**) and 4′-benzylthioproanthocyanidin A-2 (**8**)[15] as an off-white amorphous powder: ^{1}H NMR (CD_3OD) δ 3.91 (1H, s, H-3′), 3.97 (2H, s, −CH_2−S−), 4.06 (2H, m, H-3, 4′), 4.37 (1H, d, J = 3.5 Hz, H-4), 5.28 (1H, s, H-2′), 6.01/6.06/6.11 (each 1H, s, H-6/-8/-6′); ^{13}C NMR (CD_3OD) δ 29.3 (C-4), 38.1 (−CH_2−S−), 44.4 (C-4′), 68.1 (C-3), 71.3 (C-3′), 77.5 (C-2′), 96.6 (C-8), 97.0 (C-6′), 98.3 (C-6), 100.1 (C-2), 102.5 (C-10′), 104.0 (C-10), 107.1 (C-8′); ESI MS *m*/*z* 697 $[M - H]^-$.

Epicatechin-(4*β*→8)-epicatechin-(4*β*→8,2*β*→O→7)-epicatechin-(4*β*→8)-epicatechin (6): amorphous powder; $[\alpha]_D^{26}$ 48.6° (*c* 1.83, acetone); ^{1}H NMR (acetone-d_6) δ 2.66 (2H, m, H-4‴), 3.26 (1H, d, J = 3.5 Hz, H-3′), 3.61 (1H, s, H-3‴), 4.02 (2H, s, H-3, -3″), 4.08 (1H, s, H-2‴), 4.16 (1H, d, J = 3.5 Hz, H-4′), 4.30 (1H, s, H-4‴), 4.32 (1H, s, H-4), 4.67 (1H, s, H-2), 5.60 (1H, s, H-2″), 5.83/5.89/5.91/6.02 (5H, H-6/-8/-6′/-6″/-6‴), 6.31−7.32 (12H, C, C′, C″, C‴ ring protons); ^{13}C NMR (acetone-d_6) δ 27.8 (C-4′), 29.0 (C-4‴), 36.8 (C-4), 37.8 (C-4″), 65.9 (C-3′), 66.1 (C-3‴), 70.4 (C-3), 71.0 (C-3″), 75.6 (C-2), 77.6 (C-2″), 79.2 (C-2‴), 95.4/96.0/97.7 (C-6/-8/6′/6″/6‴), 98.1 (C-2), 99.3 (C-2′); FAB MS *m*/*z* 1153 $[M + H]^+$.

Thiolytic Degradation of 6. Treatment of compound **6** (300 mg) with α-toluenethiol (2 mL) and acetic acid (3 mL) in ethanol (10 mL) in the same manner as described for **5**, followed by separation on a Sephadex LH-20 column with 30% MeOH−H_2O as eluant, gave 4-benzylthioepicatechin (**9**), epicatechin-(4*β*→8,2*β*→O→7)-epicatechin-(4*β*→8)-epicatechin (**5**), 4′-benzylthioproanthocyanidin A-2 (**8**), and epicatechin (**1**). Compound **9**: ^{1}H NMR (CD_3OD) δ 3.84 (1H, d, J = 2.0 Hz, H-4), 3.95 (2H, s, −CH_2−S−), 4.04 (1H, d, J = 2.0 Hz, H-3), 5.22 (1H, s, H-2′), 5.89/5.96 (each 1H, d, J = 2.0 Hz, H-6/-8); ^{13}C NMR (CD_3OD) δ 36.8 (C-4), 42.8 (−CH_2−S−), 70.5 (C-3), 74.4 (C-2), 94.6/95.7 (C-6/-8), 99.0 (C-10); ESI MS *m*/*z* 411 $[M - H]^-$.

Aesculitannin C (7): amorphous powder; $[\alpha]_D^{26}$ 64.3° (*c* 0.42, acetone); ^{1}H NMR (CD_3OD) δ 2.83 (1H, d, J = 15.5 Hz, H-4′), 2.97 (1H, dd, J = 15.5, 5.0 Hz, H-4′), 3.99 (1H, d, J = 3.5 Hz, H-3), 4.11 (1H, d, J = 3.5 Hz, H-3′), 4.27 (1H, br s, H-3″), 4.54 (1H, d, J = 3.5 Hz, H-4′), 4.69 (1H, d, J = 3.5 Hz, H-4), 4.94 (1H, s, H-2″), 6.03 (1H, d, J = 2.0 Hz, H-6), 6.07 (1H, d, J = 2.0 Hz, H-8), 6.11 (1H, s, H-6″), 6.18 (1H, s, H-6′), 6.79−7.43 (9H, C, C′, C″ ring protons); ^{13}C NMR (CD_3OD) δ 29.7/29.8/30.0 (C-4/-4′/-4″), 67.1 (C-3″), 68.1 (C-3′), 68.6 (C-3), 81.4 (C-2″), 96.3/96.4/97.8/98.4 (C-6/-8/-6′/-6″), 100.0 (C-2), 100.7 (C-2′), 102.4 (C-10″), 103.7 (C-10), 105.9 (C-10′), 107.7 (C-8″), 108.9 (C-8′); FAB MS *m*/*z* 863 $[M + H]^+$.

Preparation of PBMC. Heparinized human peripheral blood (20 mL) was obtained from healthy donors. PBMC was isolated by the Ficoll-Hypaque gradient density method as described previously.[19] The 20 mL of peripheral blood was centrifuged at 2000 rpm, 4 °C for 10 min, to remove the plasma. Blood cells were diluted with PBS buffer, then centrifuged in a Ficoll-Hypaque discontinuous gradient at 1500 rpm for 30 min. The PBMC layers were collected and washed with cold distilled water and 10× Hanks' buffer saline solution (HBSS) to remove red blood cells. The cells were resuspended to a concentration of 2 × 10^6 cells/mL in RPMI-1640 medium supplemented with 2% fetal calf serum (FCS), 100 U/mL penicillin, and 100 μg/mL streptomycin.

Lymphoproliferation Test. The lymphoproliferation test was modified from that previously described.[20] The density of PBMC was adjusted to 2 × 10^6 cells/mL before use. A 100 μL sample of cell suspension was divided into a 96-well flat-bottomed plate (Nunc 167008, Nunclon, Raskilde, Denmark) with or without 5 μg/mL PHA (Sigma, St. Louis, MO). Various concentrations of components isolated from *E. utilis* were added to the cells. The plates were incubated in 5% CO_2-air humidified atmosphere at 37 °C for 3 days. Subsequently, ^{3}H-thymidine (1 μCi/well; NEN) was added into each well. After 16 h incubation, the cells were harvested on glass fiber filters by an automatic harvester (Dynatech, Multimash 2000, Billingshurst, U.K.). Radioactivity in the filters was measured by a scintillation counting. The inhibitory activity of each component on PBMC proliferation was calculated by the following formula:

$$\text{inhibitory activity (\%)} = \frac{\text{control group (CPM)} - \text{experiment group (CPM)}}{\text{control group (CPM)}} \times 100$$

Determination of IL-2 and IFN-γ Production. PBMC (2 × 10^5 cells/well) were cultured with PHA alone or in

combination with varying concentrations of compound **3** for 3 days. The cell supernatants were then collected and assayed for IL-2 and IFN-γ concentrations by the enzyme immunoassays (EIA; R&D systems, Minneapolis, MN). The EIA assays used here are not reported to exhibit detectable cross-reactivity with the other cytokines we tested.[21]

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