



# Preparative separation of oligostilbenes from *Vitis thunbergii* var. *taiwaniana* by centrifugal partition chromatography followed by Sephadex LH-20 column chromatography

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## ABSTRACT

*Vitis thunbergii* var. *taiwaniana* is a vitaceous native folk medicinal plant in Taiwan. The root of *V. thunbergii* var. *taiwaniana* is used to treat hepatitis and arthritis. Centrifugal partition chromatography (CPC) and Sephadex LH-20 gel filtration column chromatography were successively used to isolate and purify three oligostilbenes, (–)-vitisin B, (+)-vitisin A, and ampelopsin A, from the root of *V. thunbergii* var. *taiwaniana*. The two-phase solvent system of CPC composed of chloroform–methanol–water (6:7:4, v/v/v) was used for the preparative separation of the methanolic extract of *V. thunbergii* var. *taiwaniana*. Using CPC and Sephadex LH-20 column chromatography, about 1 g of the crude extract was separated, yielding 28.9 mg of (–)-vitisin B, 18.7 mg of (+)-vitisin A, and 18.2 mg of ampelopsin A at respective purities of 93%, 91%, and 90%.

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## 1. Introduction

Oligostilbenes are bioactive natural products contained in the families Vitaceae, Cyperaceae, and Leguminosae, as well as other medicinal plants [1,2]. However, the genus *Vitis* contains a great diversity of oligostilbenes which are biogenetically synthesized by oxidative coupling of resveratrol or (+)- $\epsilon$ -viniferin [3]. Vitisin A, heyneanol A, vitisin B, and other tetrameric stilbenes exhibit many potent biological activities such as protective effects against  $\beta$ -amyloid-induced neurotoxicity [4], 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibition [5], influenza A virus-induced airway inflammatory response inhibition [6], DNA topoisomerase II inhibition [7], and antifungal activities [8].

The root of *Vitis thunbergii* Sieb. & Zucc. var. *taiwaniana* Lu (Vitaceae), an indigenous wild grape of Taiwan, is used as a hepatoprotective, anti-inflammatory, and antiarthritis folk medicine for

treatment of hepatitis, jaundice, diarrhea, and arthritis [9]. Its fruits are also used for making grape juice and wines by some aboriginal people of Taiwan.

In a previous report, several resveratrol derivatives were isolated from the root of *V. thunbergii* [10]. However, the separation is time-consuming and requires repeated column chromatography. Many interesting compounds are irreversibly adsorbed onto the solid stationary phase causing low yields. Counter-current chromatography with the benefits of no irreversible adsorption, high recovery of samples, and low solvent consumption was applied for natural product isolation [11,12]. Preparative high-performance liquid chromatography (HPLC) or column chromatography was used to purify the centrifugal partition chromatographic (CPC) fractions in the literature [13]. However, the procedure is time-consuming and needs large amounts of mixed solvents which are difficult to recycle. Sephadex LH-20 is derived from Sephadex G-25, a bead-form crosslinked dextran matrix, with hydroxypropylation. The gel filtration property of Sephadex LH-20 has been used to isolate natural products [14].

The successful preparative separation of three oligostilbenes, (+)-vitisin A, (–)-vitisin B, and ampelopsin A (Fig. 1), from the methanolic extract of the roots of *V. thunbergii* var. *taiwaniana* by CPC and Sephadex LH-20 column chromatography is described in this paper.

**Abbreviations:** CPC, centrifugal partition chromatography; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; TLC, thin-layer chromatography; ESI-MS, electrospray ionization mass spectrometer; NMR, nuclear magnetic resonance.

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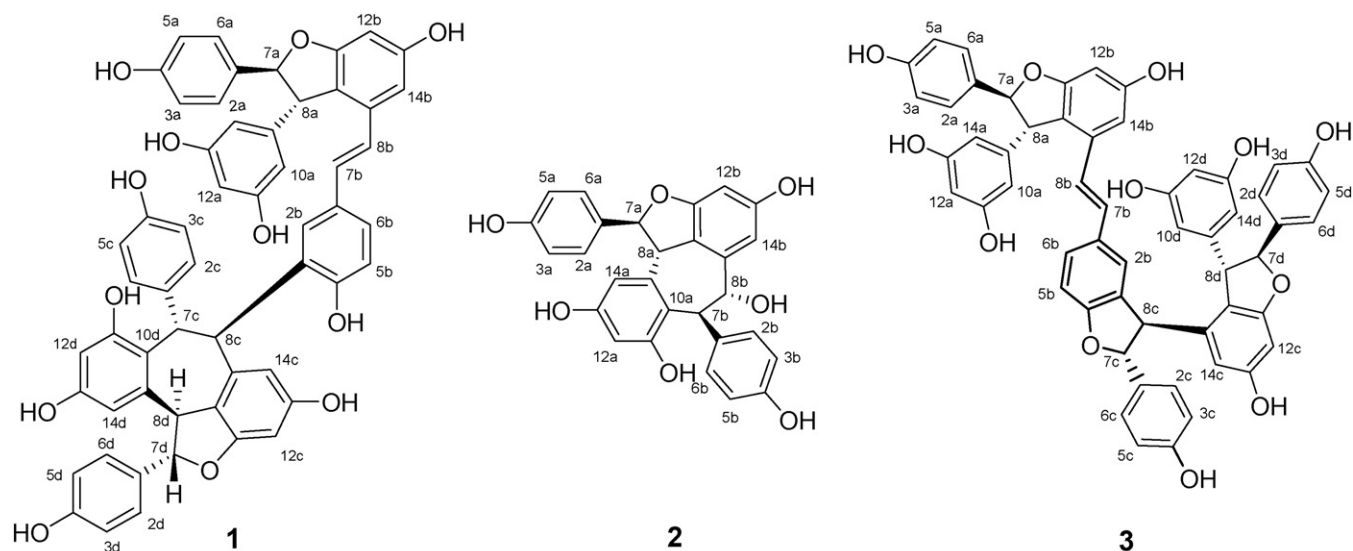


Fig. 1. Chemical structures of (+)-vitisin A (1), ampelopsin A (2), and (-)-vitisin B (3).

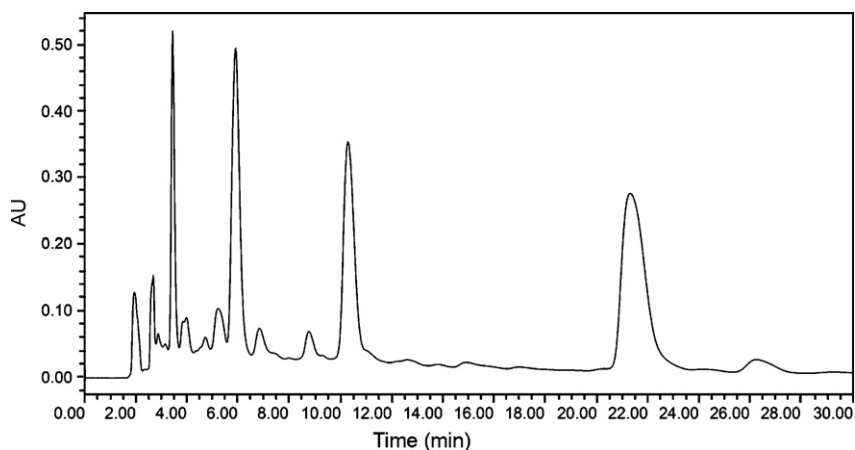


Fig. 2. HPLC analyses of the crude methanolic extract from the roots of *Vitis thunbergii* var. *taiwaniana*. HPLC conditions: reversed-phase LiChrospher 100 RP-18e column (4 mm × 250 mm, 5 μm, Merck); column temperature: 40 °C; mobile phase: 0.05% TFA–acetonitrile (68:32); flow rate: 1.0 ml/min; monitored at 280 nm.

## 2. Experimental

### 2.1. Apparatus

The experiments were performed using a CPC model LLB-M (Sanki Engineering, Kyoto, Japan) with 12 disks, 2136 partition channels (178 partition channels/disk) and a total volume of 230 ml. The maximum speed of rotor is up to 2000 rpm. A 4-way mode switching valve allows operating in either the descending and ascending mode. The CPC equipment used was a preparative liquid chromatograph LC-8A (Shimadzu, Kyoto, Japan), a CPC model LLB-M, a UV-vis detector SPD-10A (Shimadzu), a Super Fraction Collector SF-2120 (Advantec, Tokyo, Japan), and a Chromatography Data Station SISC-Lab (32) vers. 2.1 (Scientific Information Service Corporation, Taipei, Taiwan). The sample was manually injected using a Rheodyne valve model 7725i (Rheodyne, Cotati, CA, USA) through a 2.4 ml loop.

Waters HPLC equipment (Milford, MA, USA) used was a 1525 binary HPLC pump, a 2487 dual λ absorbance detector, an in-line degasser AF, a 717 plus autosampler, and a Millennium<sup>32</sup> vers. 3.20.

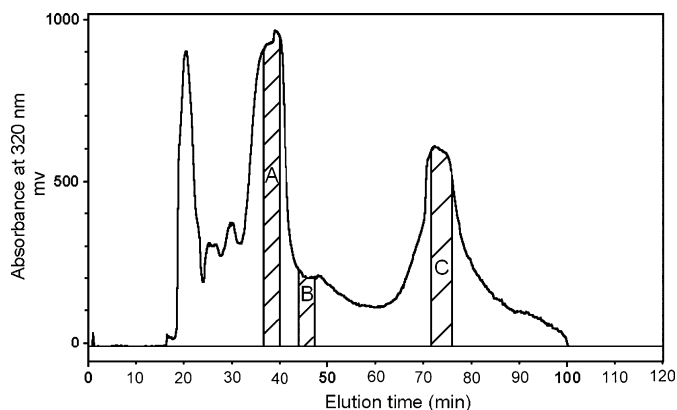


Fig. 3. Chromatogram of the crude extract from the roots of *Vitis thunbergii* var. *taiwaniana* by CPC. Solvent system: chloroform–methanol–water 7:6:4; stationary phase: upper aqueous phase; mobile phase: lower organic phase; flow rate: 10.0 ml/min; revolution speed: 1200 rpm, monitored at 320 nm.

## 2.2. Reagents

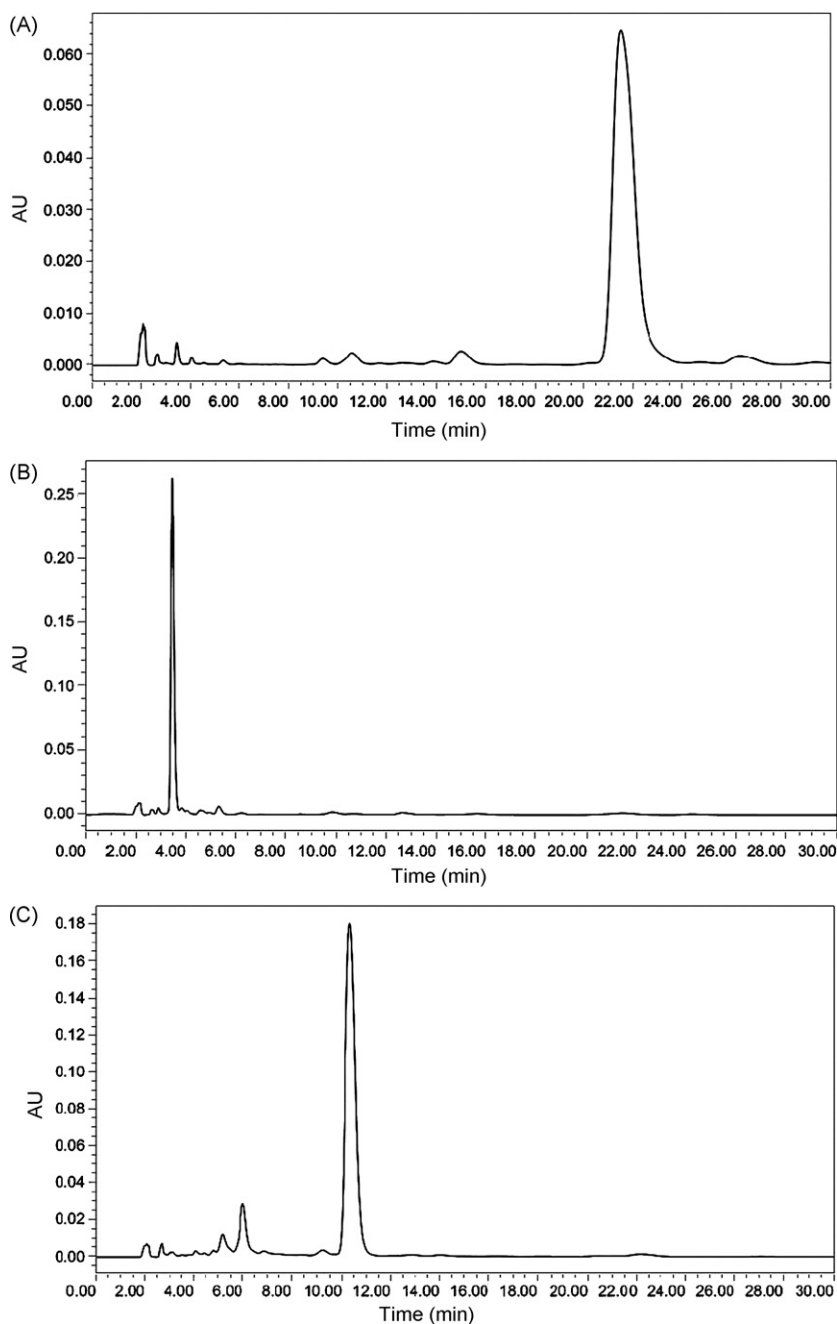
Chloroform, methanol, and other organic solvents used for sample preparation and CPC were of analytical grade and were purchased from Mallinckrodt Chemicals (Phillipsburg, NJ, USA). Acetonitrile and trifluoroacetic acid (TFA) used for the HPLC analysis were of chromatographic grade and were purchased from J.T. Baker (Phillipsburg, NJ, USA). Sephadex LH-20 was purchased from Amersham Pharmacia Biotech AB (Uppsala, Sweden). Roots of *V. thunbergii* var. *taiwaniana* were purchased in a herbal store in Hualian, Taiwan in December 2006. A voucher specimen was deposited in the Graduate Institute of Biomedical and Biopharmaceutical Sciences, National Chiayi University, Chiayi, Taiwan.

## 2.3. Preparation of samples

The roots of *V. thunbergii* var. *taiwaniana* were cut into small pieces and dried in a blowing oven at 45 °C for 3 days before use. The dried roots (2.2 kg) were extracted with methanol (20 l) under reflux for 1 h. The filtrate was concentrated in a rotary evaporator at 45 °C and freeze-dried to obtain the methanolic extract (224 g).

## 2.4. Preparation of the two-phase solvent and sample solution

The solvent system utilized in the present study was prepared by mixing chloroform, methanol, and water (6:7:4, v/v/v), and thoroughly equilibrating the mixture in a separatory funnel at room



**Fig. 4.** HPLC chromatogram of fractions obtained by CPC. HPLC conditions: reversed-phase LiChrospher 100 RP-18e column (4 mm × 250 mm, 5 μm, Merck); column temperature: 40 °C; mobile phase: 0.05% TFA–acetonitrile (68:32); flow rate: 1.0 ml/min, monitored at 280 nm. (A) Fraction A chromatogram: **3** (89.86% purity), (B) fraction B chromatogram: **2** (79.52% purity), (C) fraction C chromatogram: **1** (80.82% purity).

temperature; the two-phase solvent was separated shortly before use. The sample solution for CPC was prepared by dissolving the crude extract in the upper phase (aqueous layer) at a concentration of 1.0 g/2 ml. The sample solution for Sephadex LH-20 gel filtration chromatography was prepared by dissolving the dried peak fraction containing (–)-vitisin B, (+)-vitisin A, or ampelopsin A obtained from CPC with methanol.

### 2.5. Separation procedure

CPC separation was performed as follows: the cartridge was first entirely filled with the upper phase without rotation. Then the lower phase was pumped into the cartridge in descending mode at a flow rate of 10.0 ml/min, while the apparatus was run at a revolution speed of 1200 rpm. After equilibrium was established, as indicated by the emergence of the mobile phase in the effluent, the sample dissolved in the stationary phase was injected through the injection valve. Continuous UV detection of the effluent from the outlet of the cartridge was monitored at 320 nm.

Fractions obtained from CPC were chromatographed on a Sephadex LH-20 column (1.5 cm i.d. × 36 cm) eluted with methanol at flow rate of 4.0 ml/min and the eluate were collected with test tubes (20 ml/tube). For elution chromatogram monitor, 300 μl of eluted solution from each test tube were diluted with 2.7 ml of methanol and the UV absorptions were measured at 254, 280, and 320 nm. HPLC was used to monitor the desired compounds. The purity of fractions in each characterized compound was estimated by an area normalization method from the integration of these chromatograms (% of total area).

### 2.6. HPLC analyses of oligostilbenes

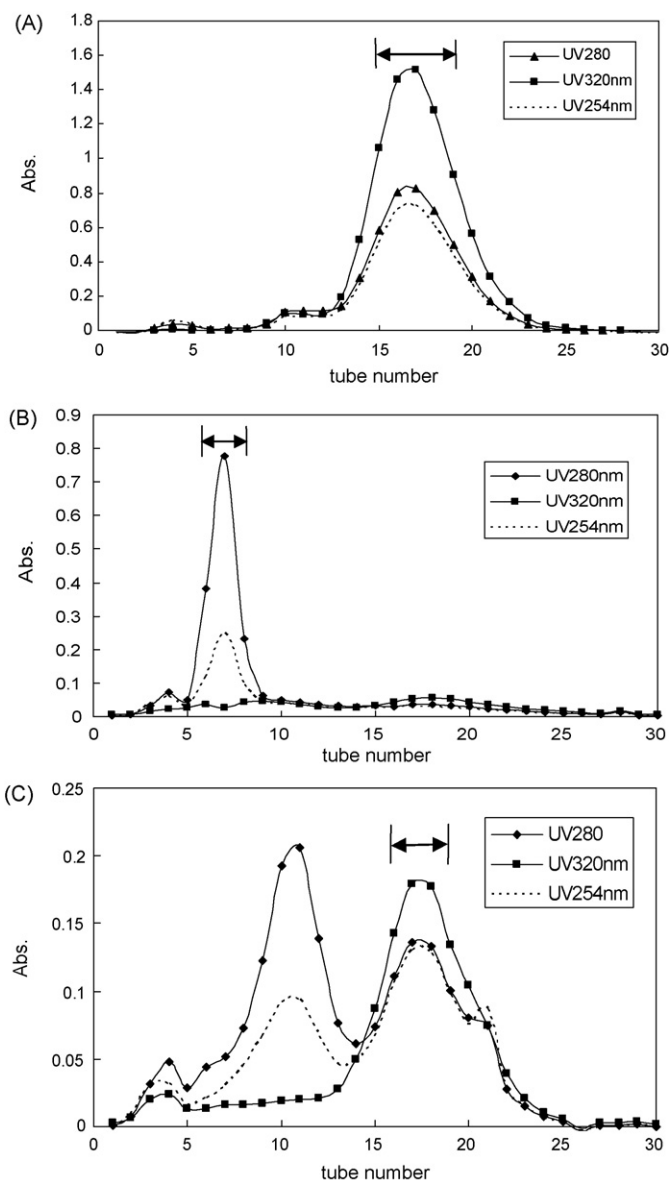
HPLC conditions of the crude extract from *V. thunbergii* var. *taiwaniana* and obtained from CPC peak fractions were as follows: The analysis was performed using a LiChrospher 100 RP-18e column (4 mm × 250 mm, 5 μm, Merck, Darmstadt, Germany). The mobile phase was composed of 0.05% TFA in water–acetonitrile (68:32, v/v). The flow rate was 1.0 ml/min, and 10 μl portions were injected into the column. The column temperature was set to 40 °C. The oligostilbenes were detected by absorbance at 254 nm. The retention times of ampelopsin A, (+)-vitisin A, and (–)-vitisin B were 3.43, 6.98, and 21.5 min, respectively.

### 2.7. Thin-layer chromatography

The CPC condition were chosen by thin-layer chromatography (TLC) on an aluminium sheet coated by silica gel 60F<sub>254</sub> (Merck, Darmstadt, Germany) using the lower layer of the variable ratio of chloroform–methanol–water as the mobile phase. After TLC developed, the spots were visual by UV irradiation at 254 and 365 nm.

## 3. Results and discussion

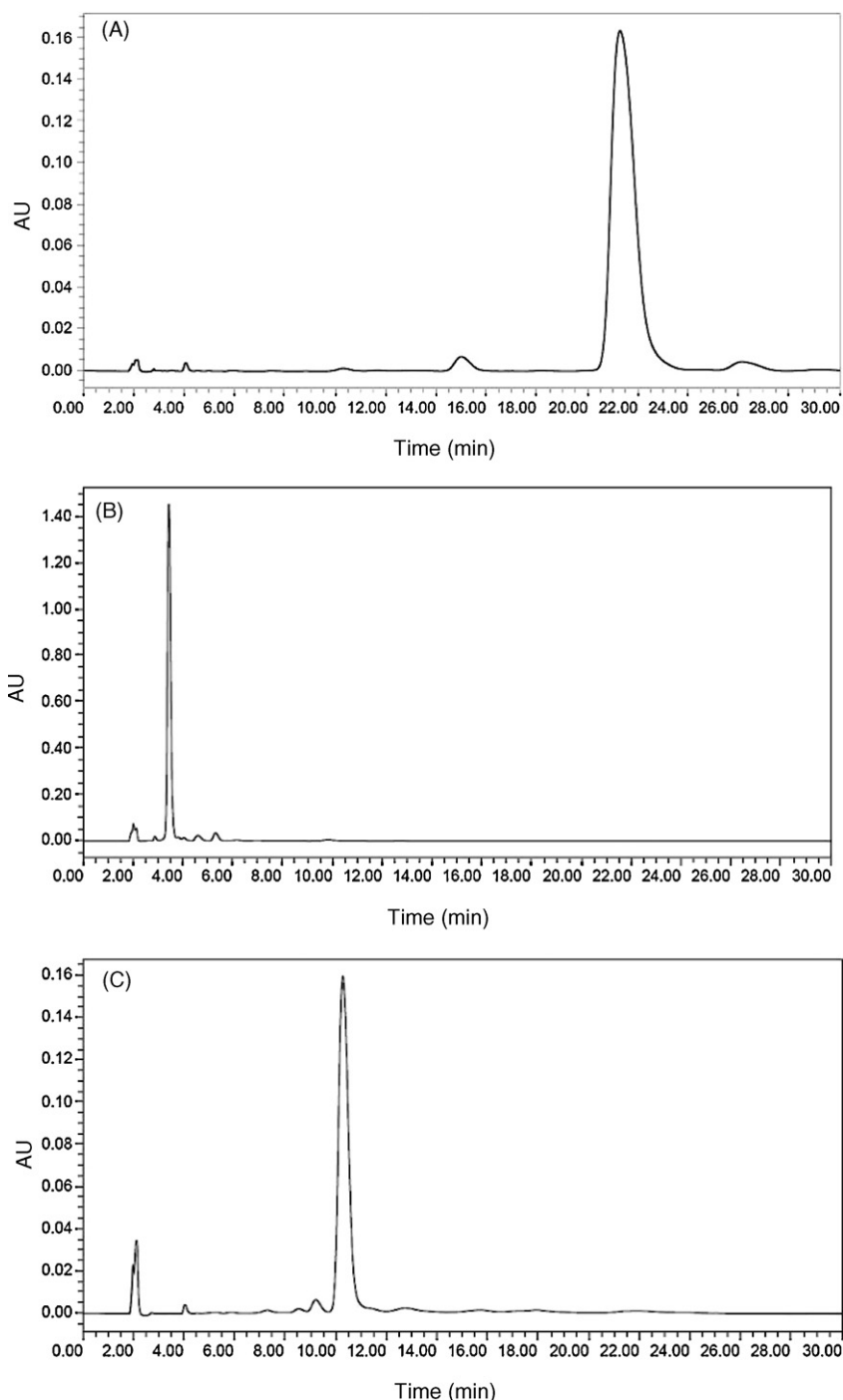
The HPLC analyses of the methanolic crude extract of *V. thunbergii* var. *taiwaniana* roots, shown in Fig. 2, indicated that several compounds were contained in the extract. In order to isolate the oligostilbenes, the lower layer of the two-phase solvent system of chloroform–methanol–water was examined by silica gel TLC to analyze the crude extract. The volume ratio of 6:7:4 showed three major spots in the developed TLC at R<sub>f</sub> values of 0.2, 0.3 and 0.48, and this ratio was chosen for CPC separation of the crude extract. CPC separation of the crude extract led to three fractions (Fig. 3). Due to the maximum absorption wavelength of compounds at 280 nm will cause chromatogram over scale and cannot distinguish each peak; the wavelength was set at 320 nm for the detection of target compounds. Each shaded part of the CPC chromatogram



**Fig. 5.** Typical chromatogram of Sephadex LH-20 column after CPC fractionation. (A) Chromatogram of CPC fraction A; (B) chromatogram of CPC fraction B; (C) chromatogram of CPC fraction C. Column chromatography condition: Sephadex LH-20 column (1.5 cm i.d. × 36 cm) eluted with methanol at flow rate of 4.0 ml/min and the eluate was collected with test tubes (20 ml/tube). For elution chromatogram monitor, 300 μl of eluate from each test tube was diluted with 2.7 ml of methanol and the UV absorptions were measured at 254, 280, and 320 nm, respectively. The symbol ↔ indicated the combine fractions for each compounds.

was concentrated to obtain 88.4 mg of fraction A, 28.2 mg of fraction B, and 39.8 mg of fraction C with respective purities of 88.5%, 76.6%, and 79.5% (Fig. 4). Fractions A and C contained tetrameric resveratrols which were identified by negative-mode electrospray ionization mass spectrometer (ESI-MS) with quasi-molecular ion of *m/z* 905 Da. Fraction B contained the dimeric resveratrol, which was identified by negative-mode ESI-MS with quasi-molecular ion of *m/z* 469 Da. However, the dark brown color of each fraction suggested that the purity was not very good.

Fractions A–C were then purified on a Sephadex LH-20 (1.5 cm i.d. × 36.5 cm) column eluted with methanol. The typical Sephadex LH-20 elution chromatograms, as shown in Fig. 5, display some impurity can be removed from fractions A–C. However, the reversed-phase HPLC chromatograms were not change distinctly (Fig. 6). The reason perhaps due to the impurity cannot elute with



**Fig. 6.** HPLC chromatogram of CPC fractions after Sephadex LH-20 column chromatography. HPLC conditions were the same as those in Fig. 4. (A) Fraction A chromatogram: **3** (93% purity), (B) fraction B chromatogram: **2** (90% purity), (C) fraction C chromatogram: **1** (91% purity).

isocratic mobile phase or the impurity cannot detect at 280 nm. This drawback may occur when using area normalization method for compound purity calculation, some impurity will be omitted. After Sephadex LH-20 column purification, we obtained 28.9 mg of (–)-vitisin B (**1**), 18.2 mg of ampelopsin A (**2**), and 18.7 mg of (+)-vitisin A (**3**) at respective purities of 93%, 90%, and 91% (Fig. 6).

The structural identifications of (–)-vitisin B, ampelopsin A, and (+)-vitisin A were carried out by examining ESI-MS,  $^1\text{H}$  nuclear magnetic resonance (NMR), and  $^{13}\text{C}$  NMR spectra as follows.

(–)-Vitisin B  $[\alpha]_{\text{D}}^{25} = -171.04^\circ$  (*c* 0.6, MeOH); ESI-MS  $m/z$ : 905  $[\text{M}-\text{H}]^-$ ;  $^1\text{H}$  NMR (500 MHz, acetone- $\text{d}_6$ )  $\delta$  (ppm): 7.26 (2H, d,  $J = 8.6$  Hz, H-2a, H-6a), 7.20 (2H, d,  $J = 8.5$  Hz, H-2d, H-6d), 7.14 (1H,

d,  $J = 8.3$  Hz, H-6b), 6.90 (2H, d,  $J = 8.6$  Hz, H-3a, H-5a), 6.83 (2H, d,  $J = 8.6$  Hz, H-3d, H-5d), 6.76 (1H, d,  $J = 16.8$  Hz, H-8b), 6.70–6.62 (5H, m, H-5b, H-2b, H-14b, H-2c, H-6c), 6.60 (1H, d,  $J = 16.8$  Hz, H-7b), 6.58 (2H, d,  $J = 8.6$  Hz, H-3c, H-5c), 6.31 (2H, brs, H-12c), 6.24–6.20 (5H, m, H-12b, H-10d, H-14d, H-12d, H-14c), 6.19 (1H, d,  $J = 2.1$  Hz, H-12a), 6.12 (2H, d,  $J = 2.2$  Hz, H-10a, H-14a), 5.53 (1H, d,  $J = 4.7$  Hz, H-7c), 5.41 (2H, m, H-7d, H-7a), 4.53 (1H, d,  $J = 4.5$  Hz, H-8a), 4.46 (1H, d,  $J = 5.4$  Hz, H-8d), 4.32 (1H, d,  $J = 4.7$  Hz, H-8c).  $^{13}\text{C}$  NMR (125 MHz, acetone- $\text{d}_6$ )  $\delta$  (ppm): 162.4 (C-11b), 162.3 (C-11c), 160.2 (C-13c), 160.0 (C-4b), 159.8 (C-11a, C-13a), 159.4 (C-11d, C-13d), 158.1 (C-13b), 157.9 (C-4d), 157.7 (C-4a), 147.3 (C-4c), 142.1 (C-9a), 136.3 (C-9d), 134.3 (C-9c), 134.2 (C-8b), 133.8 (C-1a), 132.5 (C-1d), 132.3

(C-1b, C-1c), 131.7 (C-3b), 130.3 (C-8b), 127.9 (C-2d, C-6d), 127.8 (C-2a, C-6a), 127.5 (C-2c, C-6c), 126.4 (C-6b), 125.6 (C-2b), 123.9 (C-7b), 119.8 (C-10b), 119.7 (C-10c), 116.3 (C-3a, C-5a), 116.1 (C-3d, C-5d), 115.8 (C-3c, C-5c), 110.5 (C-5b), 107.0 (C-14c, C-10d), 106.9 (C-14d), 106.8 (C-10a, C-14a), 104.4 (C-14b), 102.4 (C-12a), 102.1 (C-12d), 96.8 (C-12b), 96.4 (C-12c), 94.0 (C-7d), 93.8 (C-7a), 91.1 (C-7c), 57.0 (C-8d), 56.8 (C-8a), 52.1 (C-8c).

Ampelopsin A  $[a]_D^{25} = -216.7^\circ$  (c 0.09, MeOH); ESI-MS  $m/z$ : 469  $[M-H]^-$ ;  $^1H$  NMR (500 MHz, acetone- $d_6$ )  $\delta$  (ppm): 7.09 (2H, d,  $J=8.6$  Hz, H-2a, 6a), 6.88 (2H, d,  $J=8.2$  Hz, H-2b, 6b), 6.76 (2H, d,  $J=8.4$  Hz, H-3a, 5a), 6.62 (2H, d,  $J=8.6$  Hz, H-3b, 5b), 6.60 (1H, d,  $J=2.2$  Hz, H-14b), 6.41 (1H, d,  $J=2.2$  Hz, H-12a), 6.21 (1H, brs, H-14a), 6.14 (1H, d,  $J=2.2$  Hz, H-12b), 5.74 (1H, d,  $J=11.4$  Hz, H-7a), 5.43 (1H, d,  $J=4.8$  Hz, H-7b), 5.39 (1H, d,  $J=4.8$  Hz, H-8b), 4.14 (1H, d,  $J=11.4$  Hz, H-8a).  $^{13}C$  NMR (125 MHz, acetone- $d_6$ )  $\delta$  (ppm): 160.2 (C-13a), 158.9 (C-11b), 158.8 (C-13b), 158.4 (C-4b), 157.2 (C-4a), 156.0 (C-11a), 143.1 (C-9a), 140.4 (C-9b), 132.7 (C-1a), 130.9 (C1b), 129.9 (C-2a, C-6a), 128.73 (C-2b, C-6b), 118.9 (C-10a), 118.3 (C-10b), 115.9 (C-3a, C-5a), 115.4 (C-3b, C-5b), 110.4 (C-14b), 105.4 (C-14a), 101.4 (C-12a), 97.0 (C-12b), 88.4 (C-7a), 71.1 (C-8b), 49.6 (C-8a), 43.9 (C-7b).

(+)-Vitisin A  $[a]_D^{25} = +363.5^\circ$  (c 0.8, MeOH); ESI-MS  $m/z$ : 905  $[M-H]^-$ ;  $^1H$  NMR (500 MHz, acetone- $d_6$ )  $\delta$  (ppm): 7.19 (2H, d,  $J=8.5$  Hz, H-2a, 6a), 7.14 (2H, d,  $J=8.5$  Hz, H-2c, H-6c), 7.02 (2H, d,  $J=8.3$  Hz, H-2d, H-6d), 6.87 (1H, dd,  $J=8.4, 2.2$  Hz, H-6b), 6.81 (2H, d,  $J=8.5$  Hz, H-3a, 5a), 6.77 (2H, d,  $J=8.5$  Hz, H-3c, H-5c), 6.68 (1H, d,  $J=8.4$  Hz, H-5b), 6.65 (2H, d,  $J=8.3$  Hz, H-3d, H-5d), 6.50 (1H, d,  $J=2.2$  Hz, H-14b), 6.38 (2H, brs, H-7b, H-8b), 6.25 (1H, d,  $J=2.2$  Hz, H-12b), 6.23 (1H, d,  $J=2.2$  Hz, H-14c), 6.21 (1H, d,  $J=2.2$  Hz, H-12a), 6.16 (2H, d,  $J=2.2$  Hz, 10a, 14a), 6.08 (1H, d,  $J=1.8$  Hz, H-2b), 6.07 (1H, d,  $J=2.2$  Hz, H-12d), 6.04 (1H, d,  $J=1.8$  Hz, H-14d), 6.01 (1H, d,  $J=2.2$  Hz, H-12c), 5.87 (1H, d,  $J=11.6$  Hz, H-7c), 5.47 (1H, d,  $J=3.3$  Hz, H-8d), 5.38 (1H, d,  $J=3.3$  Hz, H-7d), 5.35 (1H, d,  $J=5.3$  Hz, H-7a), 4.40 (1H, d,  $J=5.3$  Hz, H-8a), 4.23 (1H, d,  $J=11.6$  Hz, H-8c).  $^{13}C$  NMR (125 MHz, acetone- $d_6$ )  $\delta$  (ppm): 162.5 (C-11b), 160.3 (C-13d), 159.7 (C-11a, C-13a), 159.4 (C-13b), 158.7 (C-11d), 158.4 (C-4c), 158.1 (C-4a), 157.8 (C-11c), 156.9 (C-13c), 155.9 (C-4d), 155.1 (C-4b), 147.2 (C-9a), 142.3 (C-9c), 141.2 (C-9d), 136.5 (C-9b), 135.3 (C-1d), 133.9 (C-1a), 132.7 (C-3b), 132.5 (C-2b), 131.1 (C-1c), 131.0 (C-8b), 130.1 (C-2c, C-6c), 128.9 (C-1b), 128.8 (C-2d, C-6d), 127.9 (C-2a, C-6a), 123.6 (C-6b), 122.6 (C-7b), 120.3 (C-10c), 120.0 (C-10d), 119.1 (C-10b), 116.0 (C-3a, C-5a), 115.9 (C-3c, C-5c), 115.4 (C-3d, C-5d, C-5b), 110.0 (C-14d), 106.8 (C-10a, C-14a), 104.9 (C-14c), 104.3 (C-14b), 102.0 (C-12a), 100.7 (C12c), 96.5 (C-12b), 96.0 (C-12d), 93.8 (C-7a), 88.4 (C-7c), 57.1 (C-8a), 49.4 (C-8c), 41.3 (C-8d), 40.7 (C-7d).

The results were similar to those in the literature [15–17].

Reversed-phase preparative HPLC with trifluoroacetic acid (TFA) or acidic buffers was used to purify the counter-current chromatography fraction. It improved the peak tailing of the

polyphenolic compounds in the chromatographic separation. As reported previously, (–)-vitisin B was converted to (+)-vitisin A, a seven-membered ring isomer, by treatment with hydrochloric acid at room temperature [3]. The same degradation occurred when using preparative liquid chromatography with 0.05% TFA–CH<sub>3</sub>CN (65:35) as the mobile phase to purify (–)-vitisin B. After concentration in a rotary evaporator, (–)-vitisin B was partially transformed to (+)-vitisin A, and the purity decreased (data not shown).

In the present study, a Sephadex LH-20 column eluted with methanol was used to purify the CPC fractions. Solvent consumption was lower. After evaporating each fraction, it was easy to obtain compounds with good purity and decreased degradation. Methanol can be reused for environmental benefits.

This method is useful for high-speed purification of oligostilbenes from *V. thunbergii* var. *taiwaniana* with high purity and can be used for the large-scale preparation of natural products by industry.

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