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Flash Liquid Chromatography for Isolation of Oligostilbenes from the Methanol Extract of *Dipterocarpus semivestitus* (Dipterocarpaceae)

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Abstract

A flash liquid chromatography purification system, which is a preparative liquid chromatography (PLC) was used in a semi-preparative scale for the isolation and purification of oligostilbenes from the methanol extracts of *Dipterocarpus semivestitus* leaves. A method for optimum chromatographic condition was developed on an ultra-high performance liquid chromatography (UHPLC) system. The method was transferred to the PLC with up-scaling modifications. Two stilbene trimers ampelopsin C, and α-viniferin were isolated from the fractions. The purities of the compounds were determined by the UHPLC.

Keywords – *Dipterocarpus semivestitus*, oligostilbenes, preparative-LC, UHPLC, α-viniferin ampelopsin C

INTRODUCTION

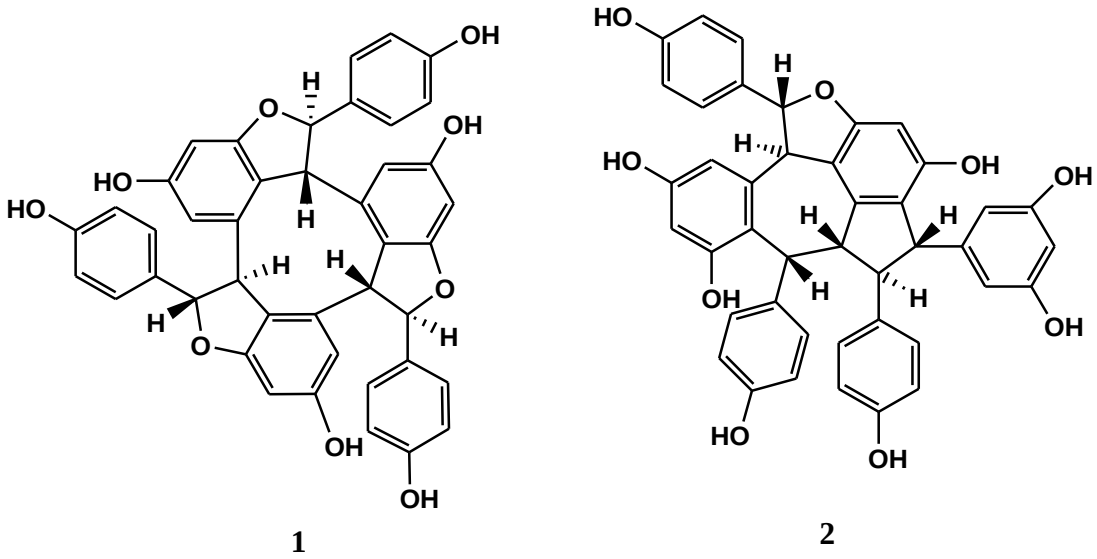
Dipterocarpaceae comprises about 17 genera and approximately 600 species,[1] found predominantly in Southeast Asia and they dominantly distributed in Malaysia with the greatest diversity in Borneo.[2] *Dipterocarpus semivestitus* is a plant from this family, endemic to Peninsular Malaysia and Sumatera, Indonesia. The plants have been listed under the category of critically endangered species by the Red list and International Union of Conservation of Nature (IUCN). In Malaysia, they are only about 53 trees in an area inside the Universiti Teknologi MARA (UiTM) campus in Sri Iskandar, Perak.[3]

Besides providing valuable woods for timber industry, dipterocarpaceous plants produce phytochemicals especially oligostilbenes that has various biological

activities. The compounds from this group have received widespread attention as either a potential therapy or as a preventive agent for numerous diseases.[4] They are however, are not being tested to human.[5] The most widely studied biological effect is anti-inflammatory activity, [6,7,8] followed by cytotoxicity.[9,10,11] Antimicrobial[12,13] and antioxidant activities[14] have also been investigated widely, and their anti-viral activities against HIV[15] and influenza A (H1N1),[16] and their neuroprotective activity[17] were also studied.

The obvious challenge conventional way of isolating chemical constituents from plants was to use open column, gravity chromatography. The nature of the complex structures of the oligostilbenes, many with the same skeleton, but having different stereo centres makes their separation difficult using this method. In many cases they co-elute, and consequently the purification process of stilbene stereoisomers sometimes concludes with mixtures. This process is also time-consuming and requires a high volume of organic solvents. High performance liquid chromatography (HPLC) analysis plays a major role in phytochemical analysis including identification of crude plant extracts.[18] Optimization of HPLC conditions and other important perspectives during method development are conducted to provide simple, precise, rapid and accurate analysis of plants. In order to isolate the compounds, the HPLC analysis is extended to a preparative method by up-scaling the analysis parameters.

In our previous study, we had successfully isolated oligostilbenes from *Neobalanocarpus heimii*[19,20] and *Dryobalanops* spp.[21] using HPLC. We also reported the isolation of four resveratrol oligomers, from the wood of *D. semivestitus*.[22,23] In this report, we describe the separation and purification of two trimeric stilbenes, α-viniferin (**1**) and ampelopsin C (**2**). The structures are as in Figure 1. The compound was isolated from leaves extract of *D. semivestitus*, a dipterocarpaceous plant which is now in the brim of extinction.



1 **2**

Figure 1: Compounds isolated from the leave extract of *D. semivestitus*.

MATERIALS AND METHODS

General experimental procedure

Solvent for extraction was of analytical grade, and chromatographic solvents are of HPLC grade from Fischer Scientific, Waltham, MA, USA. The ultra-pure water was purified at 18 MΩ.cm^{-1} by ELGA PURELAB® Option water purification system from Veolia Water Technologies, Paris, France. The LC-MS grade acetonitrile and water were from JT Baker, Center Valley, PA, USA.

UHPLC analysis of the isolated compounds was conducted on a Dionex™ Ultimate® 3000 Thermo Scientific™ system, Waltham, MA, USA, fitted with a vacuum degasser, a quaternary pump, an automated liquid sampler, and a DAD detector. Preparative HPLC was carried out on Gilson PLC 2000 system, St. Middleton, WI, USA, equipped with a binary pump, a variable wavelengths UV detector, and a fraction collector.

The UHPLC analyses were done on a Phenomenex® Luna 5 µm C18 column (150 X 4.6 mm) equipped with a guard column of similar chemistry. The separations were achieved through a Phenomenex® Gemini-NX 5 µm C18 column (150 X 21.2 mm).

The NMR spectra were measured on Bruker Avance 600 FT-NMR, Billerica, MA, USA, in acetone-d_6 without TMS.

Plant materials and sample extraction

D. semivestitus was collected in a freshwater swamp forest in UiTM Kampus Sri Iskandar, Perak, Malaysia. A voucher specimen was taken and identified by a certified botanist. The leaves was sorted, dried under shade and reduced into powder (2.0 kg). The sample was extracted with petroleum ether (6 L) overnight at room temperature to remove chlorophyll and non-polar constituents. Further extraction (3 x 24 h) with methanol (6 L for each cycle) at room temperature yielded a phenolic-rich extract (35.2 g). The crude methanol extract was fractionated over a vacuum liquid chromatography (VLC), to yield 32 fractions. Fraction no. 10 and 23 were selected to be further isolated.

Samples, fractions, and pure compounds were filtered through 0.45 µm PTFE prior to chromatographic analysis. Chromatographic method was developed on a UHPLC system prior to isolation and purification on a preparative HPLC (PLC). Purity check was done on a UHPLC system.

Development of chromatographic method on UHPLC

Fraction no. 10 and 23 were subjected to an analytical C18 column for profile analysis. The analyses were carried out by adjusting the gradient slopes until a

sufficient resolution was achieved. The analysis was initiated by a full-range gradient of ACN:H_2O (5:95 to 95:5 for 15 minutes) at 1.0 mL/min, detected at 215, 254, and 283 nm. The gradient slope was gradually changed by adjusting the solvent composition at the beginning and/or end of the chromatographic-run until a base-line resolution is achieved.

The accepted chromatographic condition was a gradient of ACN:H_2O (30:70 to 70:30 for 15 minutes) for fraction no. 10 and ACN:H_2O (30:70 to 60:40 for 15 minutes) for fraction no. 23, both at 1.0 ml/min, detected at 215, 254, and 283 nm. The layout of the chromatographic system is shown in Diagram 1.

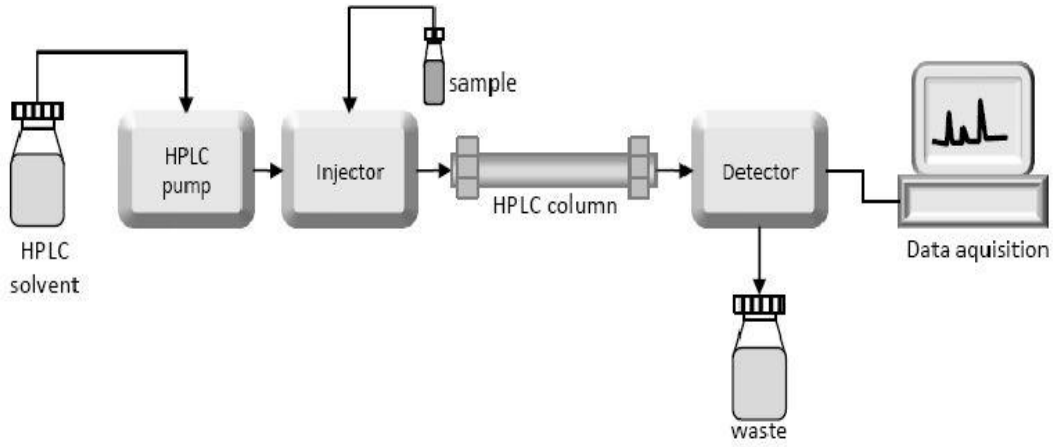


Diagram 1 The layout of HPLC components. For up-scaling from analytical scale to preparative, the difference is the size of column diameter.

Preparative liquid chromatography (PLC) conditions

The scaling up for preparation chromatography was calculated using the following formula:

$$\text{Scaling factor} = \left(\frac{\text{Internal diameter column PLC}}{\text{Internal diameter column UHPLC}}\right)^2$$

$$= \left(\frac{21.2\ \text{mm}}{4.6\ \text{mm}}\right)^2$$

$$= 21.2$$

The value ‘21.2’ is used as a scaling factor for all parameter upscaling from analytical to preparative scale. For a test-run of the PLC on scaled-up parameters, 0.25 ml of

sample was injected into a 5.0 ml loop by syringe to check the sample profile. Some of the conditions were slightly modified to suit the system. Fractions no. 10 and 23 were purified using a gradient of ACN:H_2O (30:70 to 70:30 and 30:70 to 60:40, respectively, for 15 minutes) at 18.0 ml/min, detected at 215 and 254 nm.

For efficient compound separation, 3.0 ml sample was injected into the preparative column, which was overloaded by the sample concentration. The collection was performed by a fraction collector, which is able to collect up to 150 fractions at a time, 20 ml each. Eluent from fraction 10 was collected by peak areas from minute-6 to minute-12, yielding 16 sub-fractions. For fraction 23, the eluent was collected every 0.5 minutes, from minute-2 to minute-8, yielding 12 sub-fractions for each chromatographic run.

Purity check for isolated compound

The isolated compound was subjected to a Phenomenex® Kinetex XB-C18 column (4.6 x 100 mm, 2.6 µm) for a purity check. The chromatographic conditions were a gradient of ACN:H_2O (30:70 to 60:40 for 15 minutes) at 0.7 ml/min. with were detection at 215, 254, and 283 nm.

RESULTS AND DISCUSSION

Fraction no. 10 and 23 from *D. semivestitus* leaves extract were eluted from a VLC and tested on a UHPLC. Their chromatographic profile however, revealed a mixture of many compounds in small amounts. Attempts to further separate the compounds using a conventional VLC would not be very helpful. The samples were then subjected to UHPLC and a method was developed for separation and purification using a PLC.

A fast gradient method of ACN:H_2O (5:95 to 95:5 for 10 minutes) at 1.0 ml/min, which covered a full range of polarity from the very low to high polarity, was chosen as an origin of a method development. This chromatographic condition shows peaks correspond to all compounds in the sample. Chromatogram for fraction no. 10 shows 2 major and 3 minor peaks at a narrow range of retention time (10-11.3 min). Fraction no. 23 shows a major peak at minute-8.5, accompanied by 6 minor peaks and several small groups of overlapping peaks spread from minute-7.0 to minute-11.0 in its chromatogram. The chromatograms are shown in Figure 2.

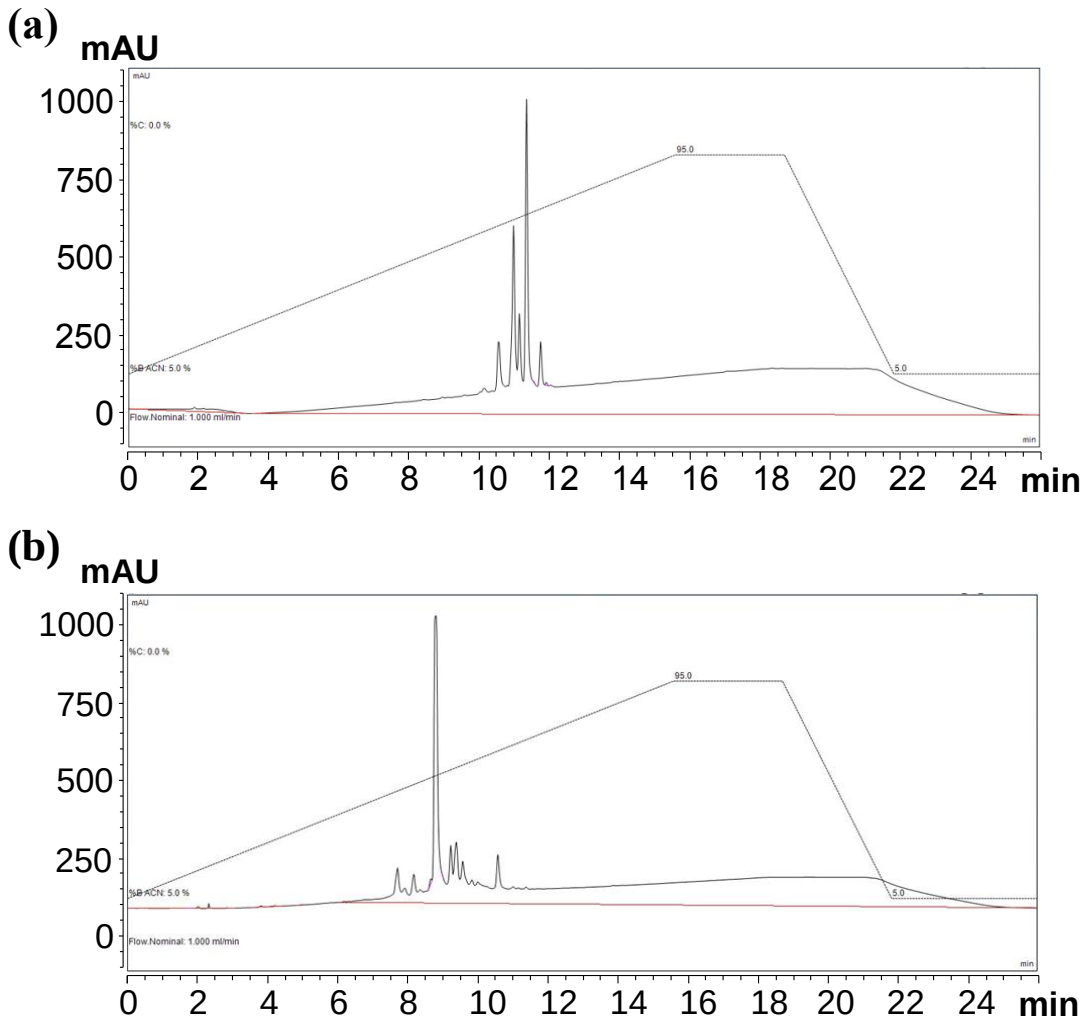


Figure 2: Chromatograms for a full range of polarity gradient (a) fraction no. 10; (b) fraction no. 23.

The gradient slope was gradually changed by adjusting the solvent composition at the end of the chromatographic-run until a base-line resolution is achieved. These adjustments, however, resulted in longer retention time for the compounds to be eluted. The gradient was again adjusted, this time by changing the solvent composition at the beginning of the chromatographic run. This adjustment caused the compounds to retain less in the column and prompt the elution time. The chromatograms for the accepted profiles are shown in Figure 3.

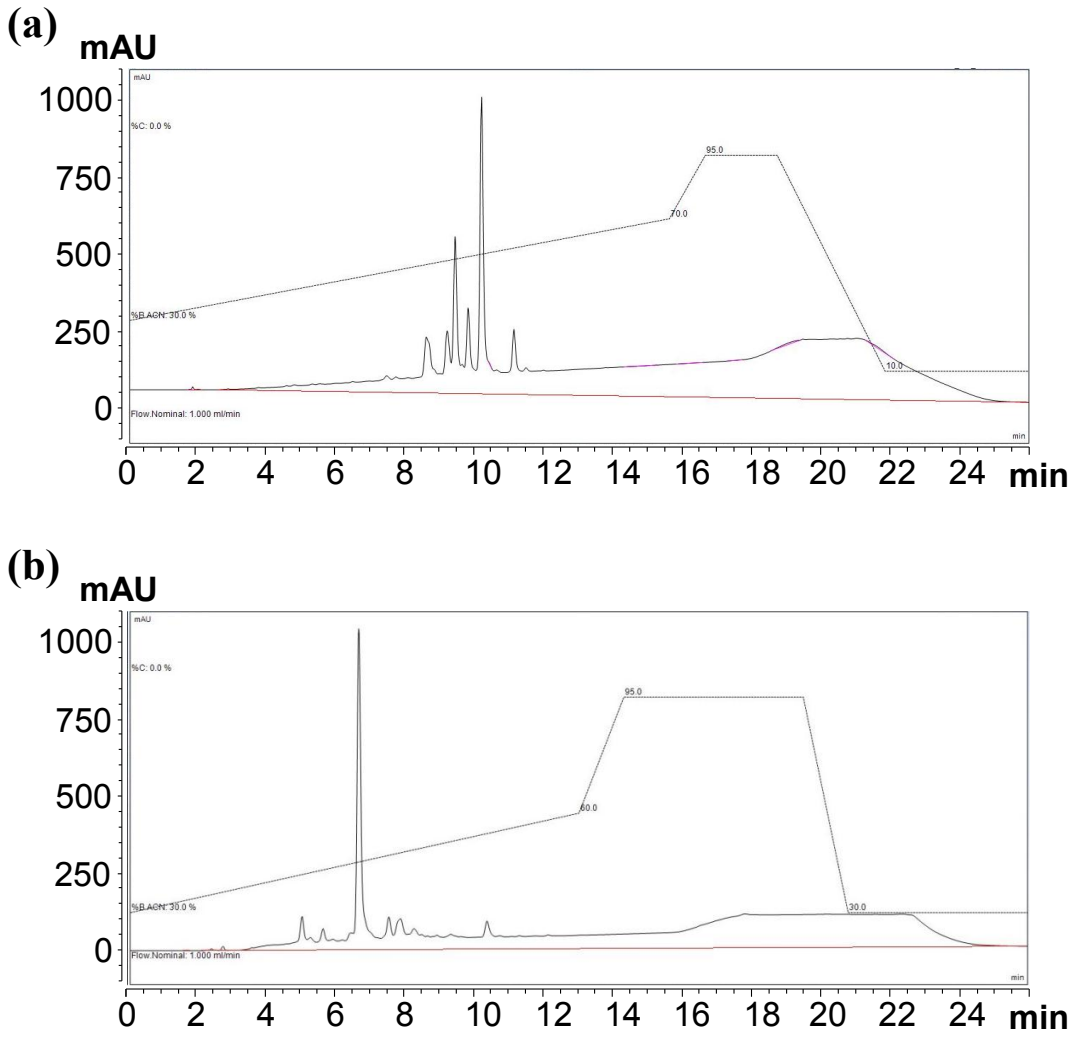


Figure 3: Chromatographic profiles of chemical component in (a) fraction no. 10; (b) fraction no. 23.

The chromatograms in figure 4 show the chromatographic profile of fraction no. 10 and 23 on a PLC system. The chromatographic conditions were kept similar to those of analytical scale with increment of flow-rate and injection volume. The flow rate was set at 18 ml/min for despite the calculation showed that up-scaling factor is 21.2. This is due to the limitation of the pump in the PLC system. As a result, the profiles are not exactly similar to those of analytical scale. Purity check for all collected sub-fractions shows that only sub-fraction 8 of fraction no. 10 (compound **1**) and sub-fraction 4 and 5 of fraction no. 23 are pure enough for NMR analysis. Further investigation revealed that sub-fraction 4 and 5 contain the same compound (compound **2**).

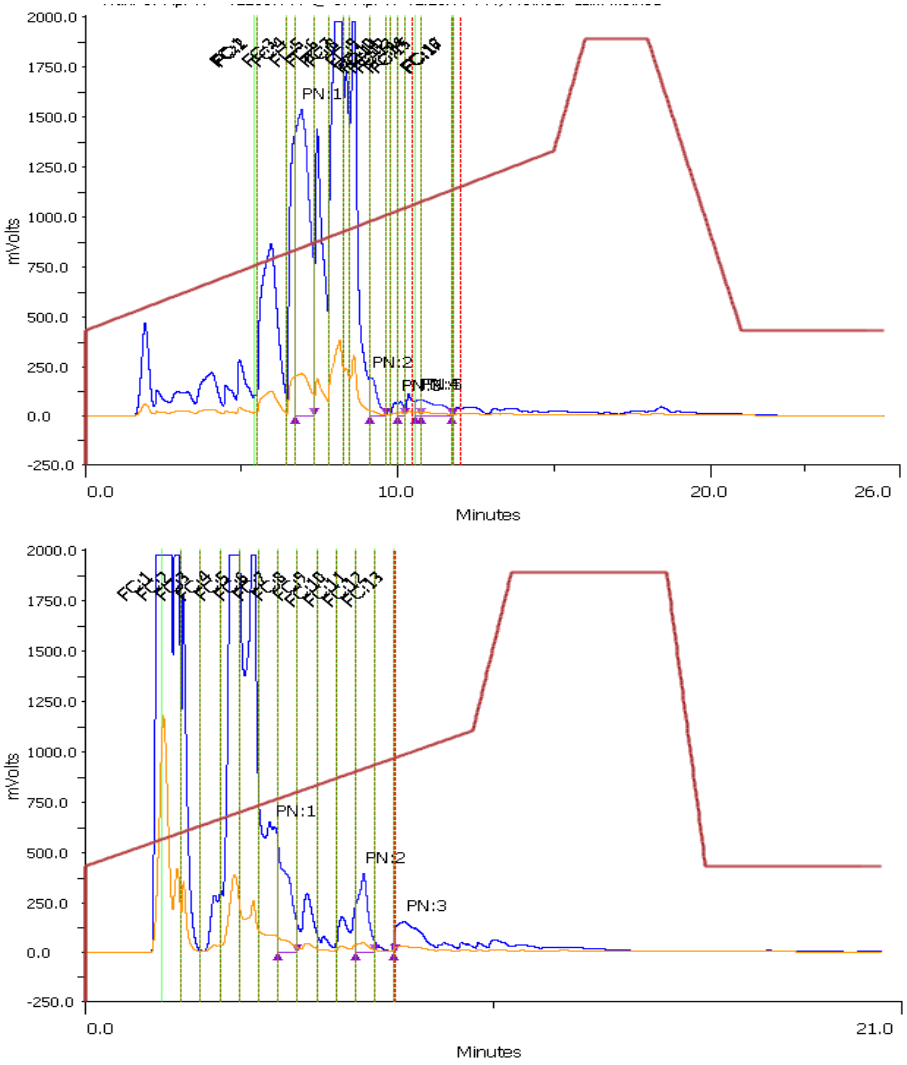


Figure 4: Collection of sub-fractions from (a) fraction no. 10; (b) fraction no. 23

The ^{1}H NMR spectrum of **1** shows six signals in the form of pseudo doublets typical of AA'BB' aromatic systems, a typical characteristic of trimer stilbenes. They are assignable to 4-hydroxyphenyl groups. It also shows signals from 3 sets of meta-coupled aromatic protons based on a tetrasubstituted benzene ring, assignable to resorcinol moiety of a resveratrol. The aliphatic protons are shown mutually coupled in 3 spin systems. The structure **1** was identified by comparing their ^{1}H NMR data to those of published reports. The compound is α-viniferin, which was the first time isolated from *Vitis vinifera*.[24]

The ^{1}H NMR spectrum of **2** shows the presence of six signals in the form of pseudo doublets typical of AA'BB' aromatic systems, assignable to 4-hydroxyphenyl groups. This is also a trimer stilbene. They are It also shows signals from a set of meta-coupled aromatic protons based on a tetrasubstituted benzene ring, one set of signal due to a 3,5-dihydroxyphenyl group and an aromatic proton signal of a

pentasubstituted benzene ring. They are assignable to resorcinol moiety of a resveratrol. The aliphatic protons are shown mutually coupled in 2 spin systems. Comparison with ^{1}H NMR data to those from published reports revealed that compound 2 is ampelopsin C, previously isolated from *Ampelopsis brevipedunculata*.[25] Later, it was isolated from *Vatica pauciflora*,[26] *Upuna borneensis*[27] and *Vitis thunbergii*.[28] Table 1 shows the data from ^{1}H NMR and published reports.

Table 1: ^{1}H NMR data of compounds 1 and 2 (600 MHz, in acetone-d_6).

^{1}H	**1**	**α-viniferin***	**2**	**Ampelopsin C****
H-2,6	6.79 (d, 8.4)	6.77 (d, 8.4)	7.19 (d, 8.4)	7.20 (d, 8.5)
H-3,5	7.05 (d, 8.4)	7.03 (d, 8.4)	6.70 (d, 8.4)	6.70 (d, 8.5)
H-7	6.08 (s)	6.07 (brs)	5.28 (d, 2.5)	5.29 (d, 3.5)
H-8	3.98 (s)	3.97 (brs)	3.67 (brd, 9.0)	3.67 (brd, l2.0)
H-12	6.01 (d, 1.8)	5.99 (d, 1.8)	6.19 (s)	6.17 (s)
H-14	6.29 (d, 1.8)	6.22 (d, 1.8)		
H-2',6'	7.24 (d, 8.4)	7.22 (d, 8.4)	6.82 (d, 8.4)	7.28 (d, 8.5)
H-3',5'	6.81 (d, 8.4)	6.77 (d, 8.4)	6.81 (d, 8.4)	6.82 (d, 8.5)
H-7'	5.97 (d, 9.6)	5.95 (d, 9.7)	5.85 (d, 12.0)	5.85 (d, 12.0)
H-8'	4.72 (d, 10.2)	4.71 (d, 9.7)	4.48 (d, 12.0)	4.48 (d, 12.0)
H-12'	6.74 (d, 2.4)	6.73 (d, 1.8)	6.36 (d, 2.4)	6.37 (d, 2.0)
H-14'	6.25 (d, 2.4)	6.25 (d, 1.8)	6.19 (s)	6.18 (brs)
H-2",6"	7.07 (d, 9.0)	7.08 (d, 8.4)	7.04 (d, 8.4)	7.03 (d, 8.5)
H-3",5"	6.74 (d, 9.0)	6.79 (d, 8.4)	6.77 (d, 8.4)	6.75 (d, 8.5)
H-7"	4.92 (d, 6.6)	4.90 (d, 6.4)	4.33 (dd, 1.2, 8.4)	4.26 (d, 9.5)
H-8"	4.63 (d, 6.6)	4.61 (d, 6.4)	3.71 (dd, 8.4, 12.0)	3.78 (dd, 12.0, 9.5)
H-10",14"	6.61 (d, 1.8)	6.59 (d, 1.8)	6.33 (d, 1.9)	6.22 (d, 2.0)
H-12"	6.24 (d, 1.8)	6.22 (d, 1.8)	6.40 (t, 1.8)	6.20 (t, 2.0)

*400 MHz, in acetone-d_6

**500 MHz, in acetone-d_6

CONCLUSIONS

This study showed that the use of different HPLC techniques is an effective method for the separation of closely eluted compounds. The ability of the systems to develop a suitable chromatographic condition prior to the actual separation process ensures high purity for the isolated compounds. The operational technique of the PLC is simple, as it is dedicated for preparative work, without complicated software to run the system.

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