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Combination of High-Performance Liquid Chromatography (HPLC) Methods for Isolation of Ampelopsin C, a Trimeric Oligostilbene

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ABSTRACT

Ampelopsin C is a trimeric oligostilbene found in a limited number of plant families including Dipterocarpaceae. It shows anti-inflammatory effects in experimental models of psoriasis, induces apoptosis in HepG2 human hepatoma cell line, inhibits human glioma through inducing apoptosis and autophagy and other biological properties. The present work oversees a method for isolation of ampelopsin C from the methanol extract of *Dipterocarpus semivestitus* leaves. Different chromatographic techniques were employed, including an ultra-high performance liquid chromatography (UHPLC) analysis for method development, a preparative high performance liquid chromatography (prep-HPLC) for isolation and a recycling high performance liquid chromatography (r-HPLC) for purification of the compound. The isolated compound was identified and characterized using nuclear magnetic resonance (NMR). The spectroscopic data confirmed the structure of ampelopsin C.

Keywords: Dipterocarpaceae, *Dipterocarpus Semivestitus*, Oligostilbenes, Ampelopsin C, Chromatography.

Introduction

Ampelopsin C, a trimer oligostilbene, has been reported to inhibit cell growth and/or induce apoptosis in various types of tumors.[1] It was first isolated from *Ampelopsis brevipedunculata*, a Vitaceae family.[2] The oligostilbenes are a group of polyphenolic compounds that are polymerized from stilbene units. Oligostilbenes exist in different oligomerisation; including resveratrol dimers, trimers, tetramers, hexamers, heptamers and octamers.[3] The most common stilbene monomer is resveratrol, found abundantly in vines and grapes. Various articles reported a wide range of bioactivities by oligostilbenes, include antibacterial,[4] antioxidant,[5] cytotoxic,[6] anti-hyperlipidemic[7] and anti-HIV effect.[8]

Apart from Vitaceae family, oligostilbenes distributed in limited plant families, including Dipterocarpaceae, Cyperaceae, Gnetaceae and Leguminosae. These compounds are well known to play an important role to protect the plants from bacterial and fungal invasion.[3] In the previous study, oligostilbenes were isolated from *Neobalanocarpus heimii* [9,10] and *Dryobalanops* spp.,[11] both are Dipterocarpaceae.

The Dipterocarpaceae family includes 16 genera with approximately 600 species. It is a large tropical plant family, widely distributed in the Southeast Asia regions especially in Malaysia and Indonesia.[12-14] *Dipterocarpus semivestitus* is the endemic species of this genus distributed in Peninsula Malaysia and Kalimantan, Indonesia. Due to its endemicity, the population of the plant is very limited, and it is prone to extinction. Many years ago, the forest reserve was de-gazetted and being converted for land use developments. The current population status of *D. semivestitus* is reported to be declined due to its habitat undergone extensive exploitation and loss.[15]

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The present Red List shown that the species is almost extinct. It is intended as the first critical step toward prioritizing national conservation measures. Rescue operations are encouraged which, apart from rescuing threatened populations, would also increase *ex situ* collections of Dipterocarpaceae.[16]

The plant is locally known as *Keruing padi* and the word '*padi*' refers to its small leaves. It confines in the lowland forest, low-lying and sometimes swampy grounds. This species is rarely explored and reported to be having an extremely small population. In Peninsular Malaysia, *D. semivestitus* is restricted to the freshwater swamp forest.[17] *D. semivestitus* has a significant plank buttress. The bark formation includes grey bole and scales. The twig is slender, glabrous, sometimes glabrescent and drying black with glabrous buds. The leaves characteristics are small and thin, simple, alternate arranged and glabrous on both surfaces. The leaves margin is wavy, the shape is elliptic oblong, contains secondary veins with unique reddish brown colour on the under surface. The ridges between the nerve inconspicuous. The petiole is slender, the fruits are small, with undulate ridges on the calyx tube obovate and sparsely golden hairy. The fruits also have three undeveloped and two developed wings.[18]

The purpose of this study is to isolate ampelopsin C from the *D. semivestitus*. Previously, four resveratrol oligomers were isolated from the wood of *D. semivestitus*.[19,20] In this research, a combination of high performance liquid chromatography (HPLC) techniques was employed for the isolation procedure, and the confirmation of its identity using nuclear magnetic resonance (NMR).

Materials and Methods

Plant materials and sample extraction

D. semivestitus was collected in a freshwater swamp forest in Perak, Malaysia in June 2013. A voucher specimen was taken and identified by Prof Dr Mohd Nazip Suratman, a certified botanist from the Faculty of Plantation and Agrotechnology, UiTM Selangor, Malaysia. The leaves were sorted, dried under the shade, and reduced into powder (2 kg). The sample was extracted with petroleum ether (6 L) overnight at room temperature to remove chlorophyll and the 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

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g). The crude extracts were fractionated between water and ethyl acetate to further remove the non-polar components.

HPLC apparatus and chromatographic conditions

HPLC analysis of the fractionated extracts and 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. The chromatographic profiles and the integrated data were recorded using Chromeleon Chromatography software. The separations were achieved through a Phenomenex® Luna 5 µm C18 column (150 X 4.6 mm) equipped with a guard column of similar chemistry.

Isolation of compound was carried out on a PLC2050 purification system by Gilson International, Germany. It is a stand-alone chromatographic system with a binary pump, manual injection port, a UV-Vis detector, and a fraction collector. The chromatographic run and fractions collection were controlled by Gilson Glider Prep software. The separations were through a Phenomenex® Gemini-NX 5 µm C18 column (150 X 21.2 mm) equipped with a guard column of similar chemistry.

Recycling HPLC was performed on JAI recycling HPLC, LC-9103, Yokohama, Japan, equipped with a liquid feed pump type L-7150, a UV-3702 UV detector, a connecting unit involving a recycle valve, and a selector valve. The column was a JAIGEL-ODS 10 µm C18 column (20 x 250 mm) with a guard column.

The NMR spectra were measured on Bruker Avance 600 FT-NMR, Billerica, MA, USA, in acetone-*d*6 without TMS.

Sample preparation

For HPLC analysis on an analytical scale, plant extract (1.0 mg) was weight accurately and dissolved in methanol to obtain the concentration of 1 mg/ml according to the weight. The solution was filtered through a 0.22 µm PTFE filter membrane into a HPLC vial. 20 µL of the solution was injected into the UHPLC system. The chromatographic conditions were adjusted accordingly to achieve satisfactory peak resolution at suitable run-time.

Compound isolation was performed on a preparative high performance liquid chromatography (prep-HPLC) system. 100 mg of plant extract was dissolved in 10 ml solvent (MeOH:ACN; 1:1) to achieve the concentration of 10 mg/ml according to the weight. The sample was filtered through a 0.45 µm polytetrafluoroethylene (PTFE) membrane filter. The solution was injected manually into the prep-HPLC system, twice, 5 ml each injection. The chromatographic condition was as of the UHPLC analysis.

Development of the chromatographic method on UHPLC

A method development was initiated by a full-range gradient of ACN:H2O (5:90 to 95:5), enables the elution of all compounds in sample ranging from polar to non-polar compounds. The run-time was initially set for 25 minutes. From the chromatogram of the full-range analysis, the chromatographic conditions were determined by gradually altering the solvent composition, gradient slope and chromatographic run time. By keeping the initial solvent composition of ACN:H2O at 5:90, the water component was gradually reduced at the end of chromatographic run, until the retention time of the last peak achieve maximum chromatographic run, and/or an isocratic gradient is achieved. The run-time was adjusted to accommodate the retention time of all compounds in the samples. Each chromatogram was analysed and the best solvent composition was decided. The analyses were continued by keeping the end solvent composition of selected condition, while gradually increasing acetonitrile percentage in the initial composition. Using this strategy, the solvent compositions were adjusted until an isocratic gradient was achieved. An optimum chromatographic condition was selected to be adopted in the isolation process using preparative HPLC.

Isolation of Compound on a prep-HPLC system

The plant extract was isolated using a gradient of ACN:H_2O (15:85 to 60:40, for 15 minutes) at 18.0 ml/min, detected at 215 and 280 nm. The eluent was collected by peak areas from minute-2 to minute-12, yielding 7 sub-fractions.

Purity Check for Isolated Compound Using UHPLC

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 (15:85 to 60:40 for 15 minutes) at 0.7 ml/min. with the detection at 215, 254, and 283 nm.

Purification of compound using recycling HPLC

The purification of the isolated compound was performed on a recycling HPLC system equipped with JAIGEL-ODS-AP preparative column (20 x 250 mm, 10 µm). The flow rate was set as 4 ml/min with gradient elution of ACN:H_2O (5:95) and ACN:H_2O (45:55). The detection was at 215 nm.

Spectroscopic analysis and structural identification

The isolated compound was dried under vacuum, dissolved in deuterated acetone, and transferred into NMR tube for NMR analysis. The structure of the isolated compound was identified by the interpretation of the 1H-NMR spectrum and later comparing the data with reported spectral data.

Results and Discussion

Development of the chromatographic method

The method development was started with setting up a fast gradient of ACN: H_2O (5:95 to 95:5 for 20 minutes) at 1.0 ml/min. This gradient covered a full range of polarity from the very high to the lowest possible. This chromatographic condition shows peaks corresponding to all compounds in the sample (Figure 1). Based on this result, the analyses were continued by gradually changed the gradient slope. This is carried out by adjusting the solvent composition at the end of the chromatographic run, while maintaining the initial composition, until a baseline resolution is achieved. These adjustments, however, resulted in a longer retention time for the compounds to be eluted. The gradient was again adjusted, this time by changing the solvent composition at the beginning and maintaining the composition at the end of the chromatographic run. This adjustment caused the compounds to retain less in the column and prompt the elution time. The selection was made based on the peak resolution and the chromatographic run-time (Figure 2). Previous studies[21-23] showed significant changes in chromatographic profile even when only the solvent proportions were changed. Other factors may contribute to the improvement of chromatographic separation, such as solvent flow rate,[24] column temperature,[25] and types of stationary phases.[26]

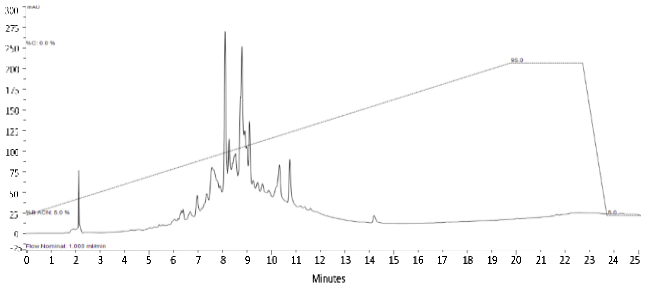

Figure 1: Chromatogram of UHPLC analysis at a full range gradient

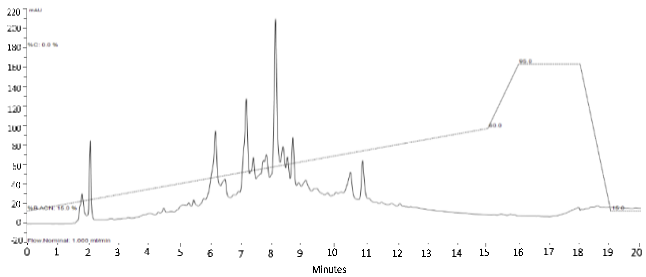

Figure 2: Chromatogram of selected chromatographic condition to be used in preparative HPLC for isolation procedure

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Isolation of compound by preparative high performance liquid chromatography (prep-HPLC)

For isolation of compound, the optimum chromatographic condition that has been developed in method development was used; which are solvent gradient of ACN:H_2O (15:85 to 60:40) in 15 minutes and the flushing took 5 minutes, made a total chromatographic run in 20 minutes.

The prep-HPLC column can be loaded with more sample than UHPLC column and thus, the concentration of 100mg/10ml of sample was used in this analysis. This is because the internal diameter of a prep-HPLC column is larger compared to that of UHPLC. As the column is larger, more volume of solvent and extra amount of sample can be employed and at higher velocity of mobile phase. In this analysis, flow rate was set at 12 ml/min.

There was a slight difference in the retention time when the compounds eluted from the column used in prep-HPLC analysis compared to UHPLC analysis. The selectivity of the prep-HPLC and UHPLC are different, thus the difference in retention time. The selectivity factor, α, is the ability of the chromatographic system to chemically distinguish between sample components. It is usually measured as a ratio of the retention factors, k, of the two peaks in a chromatogram and can be visualized as the distance between the apices of the two peaks.

Based on the peak and time observed in the chromatogram, the collection was based on the peak observed. In figure 3, the eluents were collected by the fraction collector at minute-2.5 until minute-13. Seven fractions were collected for further analysis.

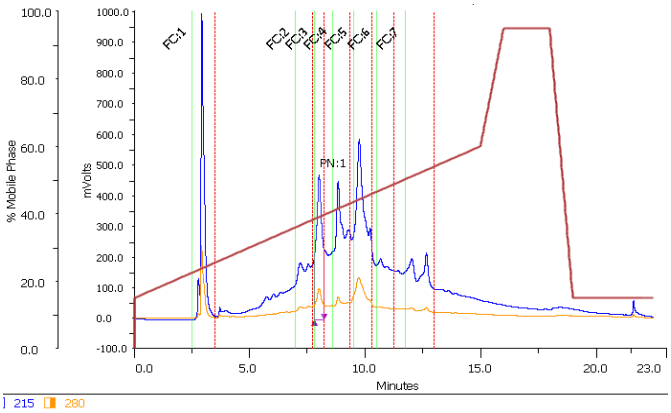

Figure 3: Chromatogram of preparative liquid chromatography (prep-HPLC) shows collection of seven fractions

Purity checks for the eluted fractions

Purity checks for all collected eluents from the prep-HPLC show that fractions 4,5 and 6 worth further isolation. Figure 4 shows chromatograms of the collected fractions. The peak appears at earlier retention time is due to different column dimensions of the UHPLC. The samples were subjected to a recycling HPLC (r-HPLC) for further purification. A chromatogram of a single peak is an indication of a pure compound.[27]

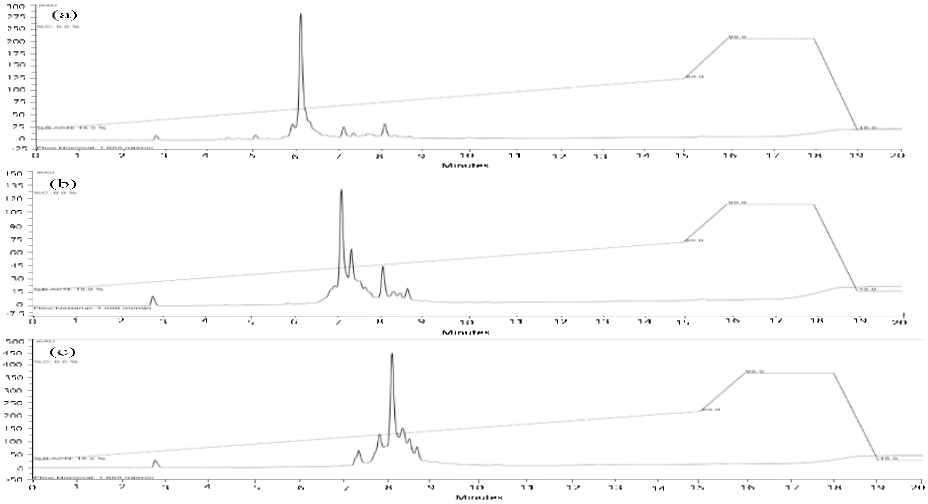

Figure 4: Chromatograms of fractions collected from prep-HPLC; (a) fraction 4, (b) fraction 5, and (c) fraction 6

Compound purification on a recycling HPLC

The chromatogram in Figure 5 shows the chromatographic cycles in the separation process of a fraction collected from prep-HPLC eluent. On sample injection, the eluent was drained to remove any compounds other than the compounds of interest. When the compound peaks appeared at 38 minutes, the valve was then switched for the eluent to be channelled back to the column. When all the anticipated metabolites were completely eluted, the eluent was drained again to remove the remaining impurities. At every cycle, the impurities were drained until the sample contained only the targeted compound. The chromatographic cycles were repeated until a base line resolution was achieved. Each cycle took 38 minutes to complete. After achieving good resolution, the eluent was collected manually on the peak observation.

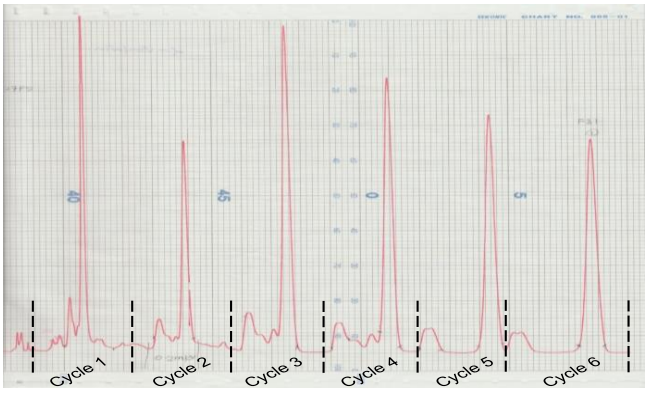

Figure 5: Chromatogram from recycling HPLC shows cycles of chromatographic run

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Spectroscopic analysis and structural identification

The analysis of ^{1}H NMR spectrum (Figure 6) showed the presence of six signals in the form of pseudo doublets typical of AA'BB' aromatic systems at δ 7.24, 6.80 (2H, d, J=8.5 Hz, each), δ 7.16, 6.66 (2H, d, J=8.5 Hz, each) and δ 6.99, 6.71 (2H, d, J=8.5 Hz, each). They were assignable to three 4-hydroxyphenyl groups. Signals from a set of meta-coupled aromatic protons based on a tetrasubstituted benzene ring was observed at δ 6.33 and 6.15 (1H, d, J=1.9 Hz, each). Further analysis of the ^{1}H NMR spectrum revealed one set of signals due to a 3,5-dihydroxyphenyl group at δ 6.18 (2H, d, J=2.0 Hz) and 6.17 (1H, t, J=2.0 Hz). An aromatic proton signal of a penta-substituted benzene ring was observed at δ 6.13 (1H, *s*).

Analysis of ^{1}H NMR also revealed a spin system of mutually coupled aliphatic protons, which were identified from their coupling constant value at δ 5.271H, d, J=4.2 Hz), 3.63 (1H, *br d*, J=11.6 Hz) 3.74, (1H, *pt*, J=9.6 Hz) and 4.22 (1H, d, J=9.6 Hz). The splitting pattern suggesting coupling between methine protons H-7c and H-8b indicated that resveratrol unit B and C were connected through an H-8b/H-7c linkage. The remaining two signals for aliphatic protons at δ 5.82 and 4.44 (1H, d, J=11.9 Hz, each) were mutually coupled to each other. The structure for the isolated compound was identified by comparing their ^{1}H NMR data to those of published reports.[2]

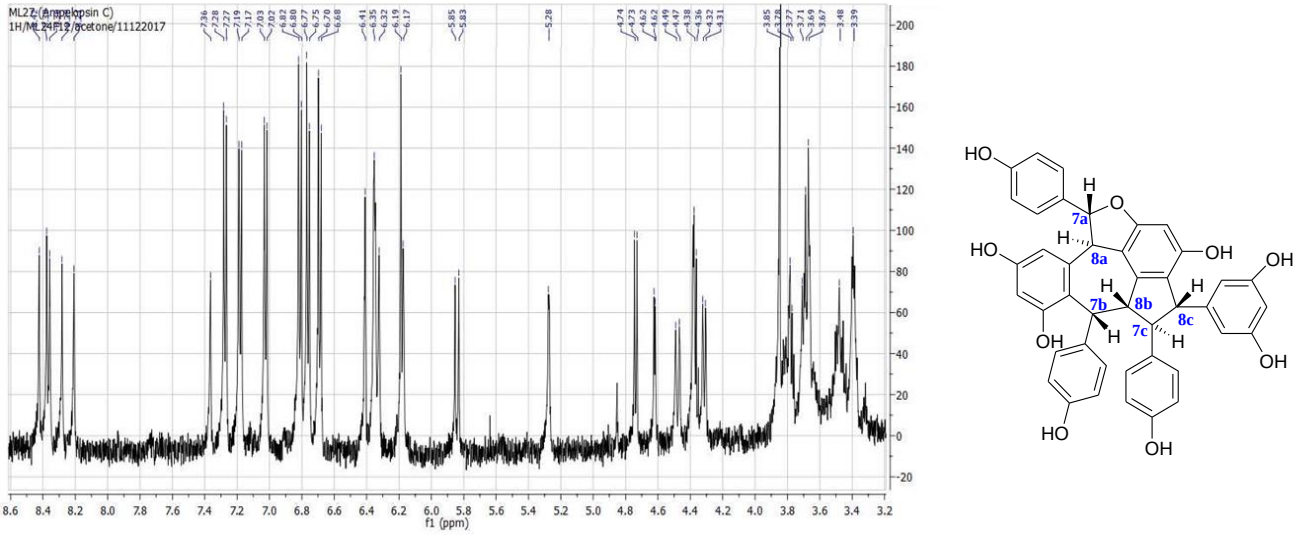

Figure 6: ^{1}H-NMR spectrum and the structure of ampelopsin C

Conclusion

The use of multiple HPLC systems and technologies to isolate a compound is proven to be effective in this research as it is convenient, sensitive, and efficient compared to conventional chromatographic method such as TLC and open column. The development of chromatographic conditions prior to isolation reduces the need for repetitive isolation processes as the parameters for optimal separation were determined beforehand. It is also very handy to quickly isolate plant chemical constituents with high purity. This method could be applied not only in chemical isolation and purification but in quantitative analyses for accurate measurement. The ability of HPLC systems to develop a suitable chromatographic condition before the actual separation process ensures efficient isolation. The robustness of the prep-HPLC system affords faster isolation process and the recycling HPLC saves time and solvent consumption. The sensitivity of the UHPLC detects impurities at their very low concentrations, yields high purity isolated compounds. The isolated compound was identified as ampelopsin C by ^{1}H-NMR spectral analysis and comparison with the reported data.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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