Malaysian Journal of Chemistry, 2022, Vol. 24(4), 127-134
(Special Issue: *International Conference on Natural Products 2022*)

Efficient Technique for the Isolation of Oligostilbene from Polar Extract of *Dipterocarpus semivestitus*

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A plant from the Dipterocarpaceae family, *Dipterocarpus semivestitus* was studied for its oligostilbene components, as they exhibit a wide range of biological activities. The objective of this study is to determine a fast and effective method for the isolation of the oligostilbenes. Two methanol extracts of plant parts, the stem and leaves were subjected to ultra-high-performance liquid chromatography (UHPLC) for profile analysis and preparative high-performance liquid chromatography (prep HPLC) to isolate the compounds. Vaticaphenol A, a stilbene tetramer was isolated, and its structure was elucidated and characterized by nuclear magnetic resonance (NMR) spectral data analysis. The compound was eluted at 12.7 minutes (t_R), by applying a gradient mobile phase of ACN: H_2O (15:85 to 95:5 for 14 minutes) at 1 ml/min on an analytical scale. Apart from the flow rate, the chromatographic condition was remained similar for preparative scale. The use of different HPLC techniques is an effective method for separating 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.

Key words: Dipterocarpaceae; oligostilbenes; HPLC; vaticaphenol A

Received: September 2022; Accepted: November 2022

Oligostilbenes are special polyphenolic compounds polymerized from a stilbene. One of the widely known stilbene derivatives is resveratrol [1]. Oligostilbenes have many complex structures yet to be discovered. These compounds are rich in the dipterocarps species such as *Dipterocarpus semivestitus.* Dipterocarpaceae is a family of large African and South Asia timber trees, which comprises seventeen genera [2]. Of all the genera, *Dipterocarpus*, *Dryobalanops*, *Hopea*, *Shorea*, and *Vatica* are the most common studied for their oligostilbene content.

Dipterocarpaceae is one of the main timber-producing families in the tropical rain forests of Southeast Asia and contributes a high proportion of the emergent and main canopy strata of the forest. The highest species diversity of dipterocarps is known in evergreen rainforests in Peninsular Malaysia, Sumatra, and Borneo [3]. This genus is known to be endangered as only 53 plants exist in its habitat.

The Red List and International Union of Conservation of Nature (IUCN) have listed the *Dipterocarpus semivestitus* under the category of critically endangered species. Due to this reason, rapid and numerous studies must be done by the researchers to identify and elucidate the structure of unknown compounds in the plant. With these efforts, we can obtain essential information on their nature and discover their biosynthesis. Oligostilbenes are reported to have anti-tumor [4], anti-microbial [5], anti-inflammatory and other significant biological activities [6,7].

To date, there are approximately 100 out of more than 400 stilbene derivatives were extracted and identified from the *Dipterocarpaceae*. Oligostilbenes are polar compounds, therefore, it is difficult to use the conventional method to isolate the compounds. Fortunately, in this high-tech era, various innovations have been developed in the qualitative analysis of unknown compounds making the isolation process easier. In this study, we used preparative HPLC in isolating oligostilbenes from *D. semivestitus* plant.

Preparative HPLC is advantageous as it gives a large amount of high purity yields [8-11]. Recent studies have also shown successful isolation of oligostilbenes from *D. semivestitus* by semi-preparative HPLC. In the previous study, oligostilbenes were successfully isolated from *Neobalanocarpus heimii* [12,13] and *Dryobalanops* spp. [14] using HPLC. The isolation of four resveratrol oligomers from the wood of *D. semivestitus* was reported [15,16]. This report describes the separation and purification of a tetrameric stilbene, vaticaphenol A (**1**). The compound was isolated from leaves extract of *D. semivestitus*.

†Paper presented at the International Conference on Natural Products 2022 (ICNP 2022)

EXPERIMENTAL

Plant Materials and Sample Extraction

D. semivestitus stem and leaves were collected in a freshwater swamp forest in UiTM Sri Iskandar, Perak, Malaysia. A voucher specimen (IK 032DS) was taken and identified by Prof Dr Mohd Nazip Suratman, a certified botanist from the Faculty of Plantation and Agrotechnology, UiTM Selangor, Malaysia. The dried stem (2 kg) was extracted with *n*-hexane (6 L) overnight at room temperature to remove the non-polar constituents. Further extraction (3 x 24 h) with acetone (6 L for each cycle) at room temperature yielded a phenolic-rich extract. The solvent was evaporated to yield the crude extract (42 g). Meanwhile, the leaves (2 kg) were sorted, dried under the shade, and reduced into powder. 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 g). The crude extracts were fractionated between water and ethyl acetate by liquid-liquid extraction to further remove the non-polar components.

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 wavelength UV detector, and a fraction collector. The chromatographic analyses were carried out on a Phenomenex® Luna 2.7 µm C18 column (100 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 (250 X 10.0 mm). The NMR spectra were measured on Bruker Avance 600 FT-NMR, Billerica, MA, USA, in acetone-d_6 without TMS.

Sample Preparation

The leaves (85.3 mg) and stem (64.6 mg) extracts were weighed and dissolved in methanol to reach the concentration of 10 mg/ml. All samples, fractions, and pure compounds were filtered through 0.20 µm PTFE prior to chromatographic analyses. The chromatographic method was developed on a UHPLC system prior to the isolation and purification on a semi-preparative HPLC. Purity check was done on a UHPLC system.

Development of the Chromatographic Method on UHPLC

Extracts from stem and leaves were subjected to an analytical C18 column for profile analysis. The analyses were carried out by adjusting the gradient slopes for the best resolution. The analysis was initiated by a full-range gradient of ACN: H_2O (5:95 to 95:5 in 14 minutes) at 0.7 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 baseline resolution was achieved. The accepted chromatographic condition for both extracts was a gradient of ACN: H_2O (15:85 to 95:5 in 14 minutes).

Semi-preparative Liquid Chromatography (p-HPLC) Conditions

The scaling up for semi-preparative chromatography was calculated using the formula by Heuera et al. (1996) [17]. The flow rate and sample load for the semi-preparative scale were calculated as follows.

$$= analytical\ scale \times \frac{DP^2}{DA^2} \times \frac{SP}{SA} \times \frac{LP}{LA}$$

Where;
DP = diameter of semi-preparative column
DA = diameter of analytical column
SP = particle size of semi-preparative column
SA = Particle size of analytical column
LP = particle size of semi-preparative column
LA = Particle size of analytical column

The flow rate was adjusted to 10 ml/min as a scaling factor for all parameters upscaling from analytical to semi-preparative scale. This is due to the limitation of the HPLC pump on the semi-preparative system. For a test-run of the p-HPLC on scaled-up parameters, 0.25 ml of the sample was injected into the column to check the sample profile. Some of the conditions were slightly modified to suit the system. For efficient compound separation, a 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 can collect up to 150 fractions at a time, 20 ml each. Eluents were collected by peak areas from minute-6 to minute-12.

Purity checks for the 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 14 minutes) at 0.7 ml/min. with were detection at 215, 254, and 283 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 ^{1}H-NMR spectrum and comparison with the reported spectral data.

RESULTS AND DISCUSSION

Development of the Chromatographic Method

The method development was started by setting up a fast gradient of ACN: H_2O (5:95 to 95:5 for 14 minutes) at 1.0 ml/min. This gradient covered a full range of polarity from the very high to low. This chromatographic condition would show peaks corresponding to all compounds in the sample. The gradient slope was gradually changed by adjusting the solvent composition at the end of the chromatographic run 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 of the chromatographic run. This adjustment caused the compounds to retain less in the column and prompt the elution time.

Chromatograms for the extract from the stem show four apparent peaks at a narrow range of retention time (1.5 mins), accompanied by unresolved minor peaks and several small groups of overlapping peaks spread from minute-7.0 to minute-13.0 in chromatogram. Figure 1 shows the improvements in the retention time and peak resolutions with the change of the solvent composition.

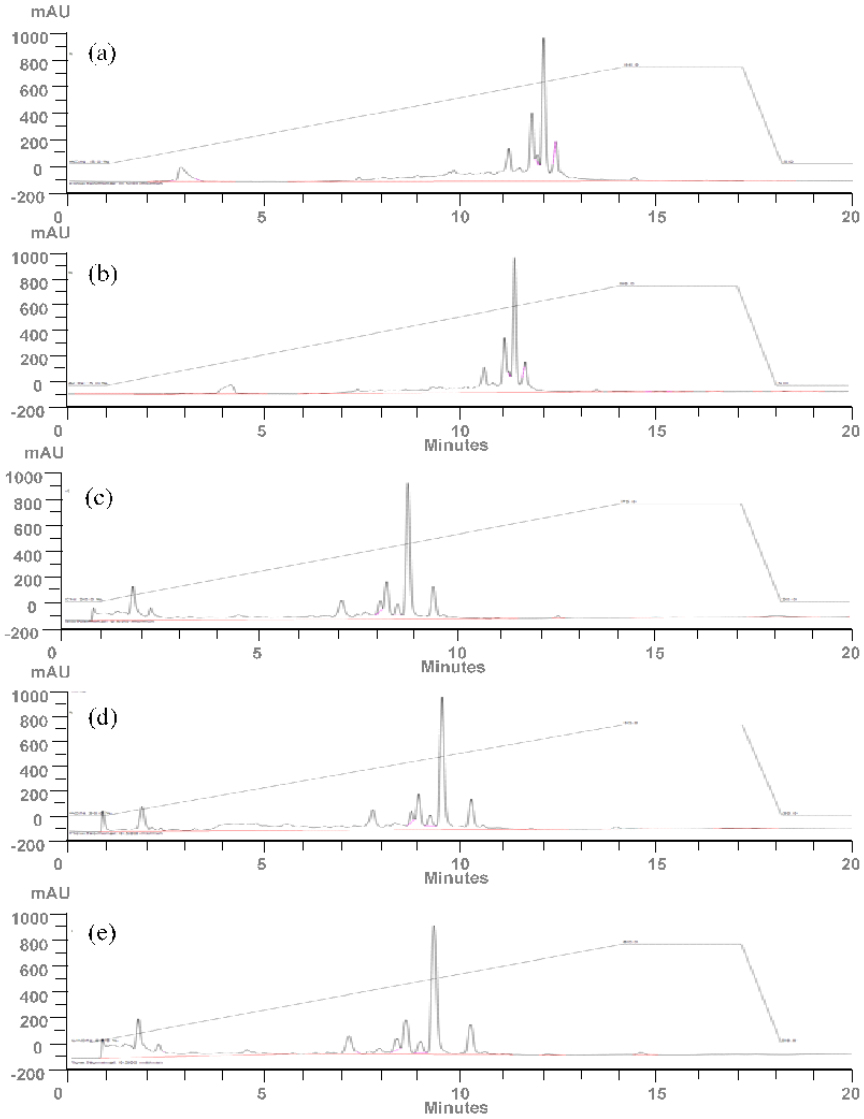


Figure 1. Chromatograms of stem extract on UHPLC; (a) ACN:H_2O (5:95 to 95:5), (b) ACN:H_2O (5:95 to 85:15), (c) ACN:H_2O (20:80 to 95:5), (d) ACN:H_2O (15:85 to 95:5), (e) ACN:H_2O (15:85 to 85:15) in 14 minutes. The series of profiles show adjustment of solvent composition and improvement of peak resolution as well as decreasing elution time.

The fast gradient solvent composition (Figure 1a) shows all peaks were eluted in a narrow chromatographic window. The solvent strength was increased by reducing the polarity at the end of the chromatographic run (Figure 1b), resulting in a slight improvement in retention time, but the resolution remained the same. Another attempt was to increase the solvent strength by reducing the polarity at the beginning (Figure 1c). This attempt showed improvement in both retention time and peak resolution. Figure 1(d-e) show some minor adjustments to increase the quality of the chromatographic profiles.

The chromatograms in Figure 2 show the profile of the leaves extract. Using the fast gradient solvent composition (Figure 2a) for the stem extract, the peaks are well resolved despite the rapid change of polarity. There are only two apparent peaks observed accompanied by approximately ten small peaks, which are buried at the baseline. Since these two peaks are well resolved, it is expected that faster elution would not jeopardize their resolution. Figure 2(b-d) shows how the chromatographic resolution improved by changing the gradient slope. The steepness was changed by adjusting the solvent composition at the beginning and/or end of the chromatographic run. The acceptable mobile phase composition was decided to be at ACN: H_2O (15:85 to 95:5 for 14 minutes). This adjustment caused the compounds to retain less in the column and prompt the elution time. The resolution, however, is not compromised (Figure 2d).

Previous studies [18-20] 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 [21], column temperature [22], and types of stationary phases [23].

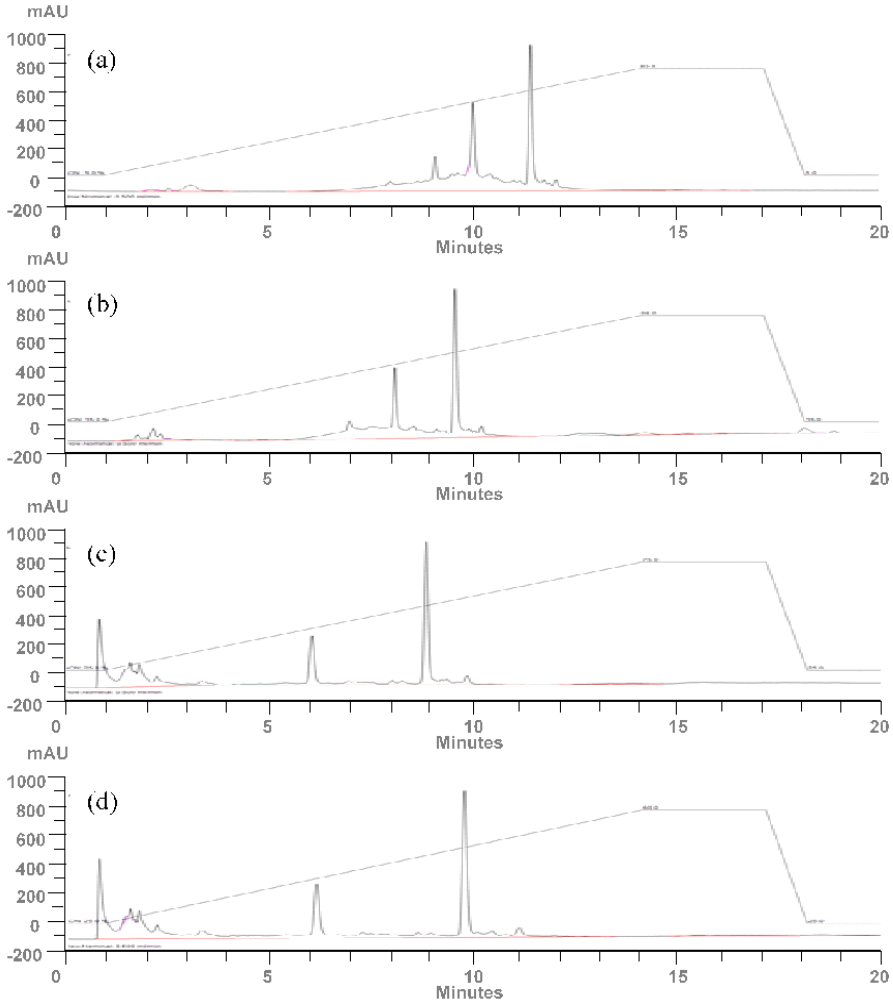


Figure 2. Chromatogram of leaves extract on UHPLC; (a) ACN:H_2O (5:95 to 95:5), (b) ACN:H_2O (5:95 to 85:15), (c) ACN:H_2O (20:80 to 95:5), (d) ACN:H_2O (15:85 to 95:5) in 14 minutes. The series of profiles show adjustment of solvent composition and improvement of peak resolution.

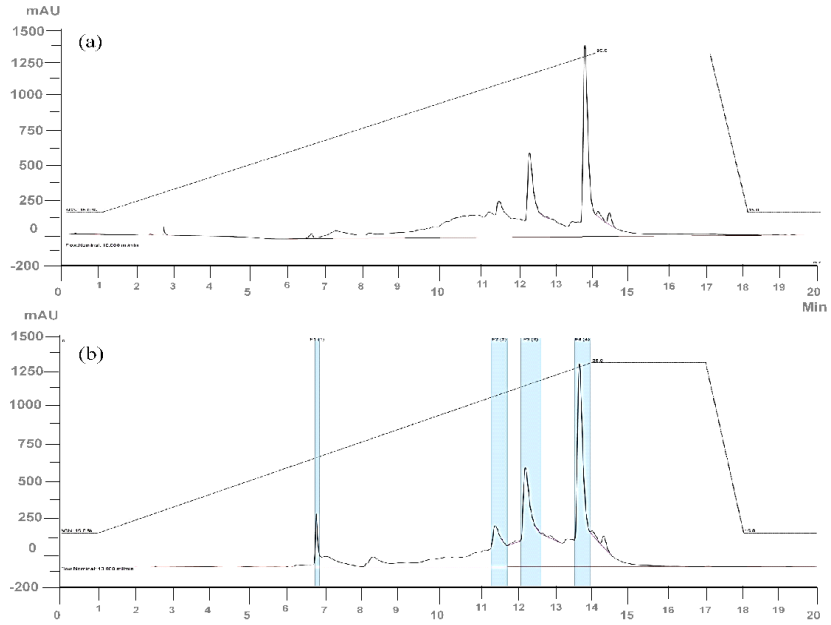


Figure 3. Chromatogram of leaves extract when up scaled to; (a) semi-preparative and (b) collection of its constituents, both at ACN: H_2O (20:80 to 95:5) in 14 minutes.

Semi-preparative Liquid Chromatography

The chromatograms in Figure 3 show the profile of the leaves extracts on a semi-preparative HPLC system. The chromatographic conditions were kept similar to those of the analytical scale with an increment of flowrate and injection volume. The flow rate was set at 10 ml/min despite the calculation showed that the up-scaling factor is 15.8 ml/min. This is due to the limitation of the pump in the semi-preparative system. As a result, profiles are not exactly similar to those of the analytical scale, despite the effort to elute faster while keeping the resolution (Figure 3a). The elution was collected based on the peaks (Figure 3b). The peak-based collection is more favourable than a time-based method for well resolved components. In this method, elution is collected when the detector detects an increasing in chromatogram slope and stop collecting when the peak reaches the baseline. This collection method however neglects small peaks under the collection threshold and unresolved peaks. Multiple injections were executed to collect a sufficient amount of samples for the NMR analyses.

In other studies, when the chromatographic system is of lower performance, medium pressure liquid chromatography (MPLC) [24] or column with larger particle sizes [25], the time-based collection technique was preferable, due to poorly resolved component, lest losing the sample constituents.

Purity Checks for Isolated Compound

Purity checks for all collected eluents show that the only compound eluted at minute-12 in the preparative scale is pure enough for NMR analysis. Figure 4 shows a chromatogram of the isolated compound, the peak appears at earlier retention time due to different column dimensions used for the purity check. A chromatogram of a single peak is an indication of a pure compound. The ^{1}H-NMR spectrum later confirmed the purity of the sample.

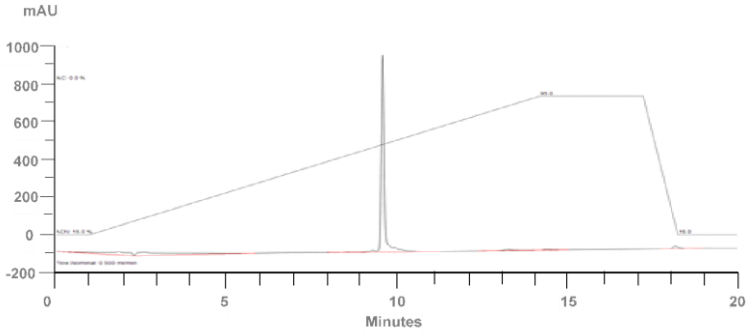


Figure 4. Chromatogram of a single peak, anticipated of a pure compound.

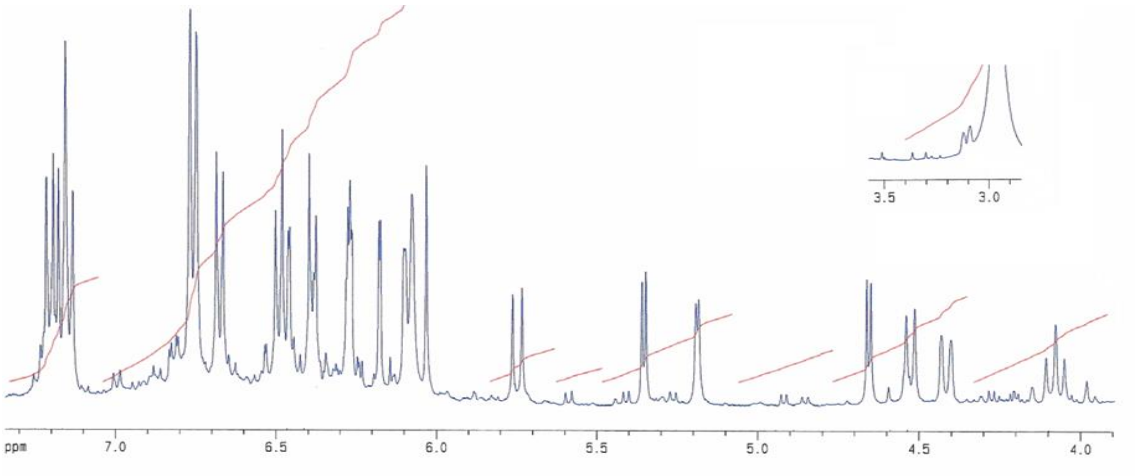
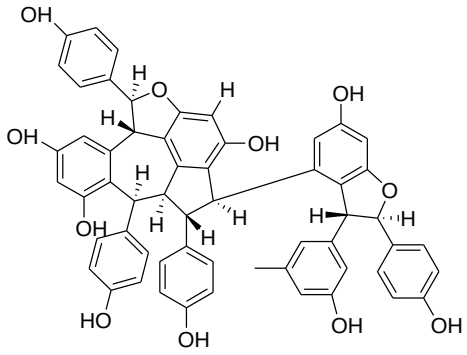


Figure 5. 1H-NMR of isolated compound. The experiment was run at 600 MHz in *d*-acetone.

Spectroscopic Analysis and Structural Identification

The ^{1}H-NMR (Figure 5) spectrum shows four sets of *ortho*-coupled aromatic protons in AA'BB' spin systems at δ_H 7.22, 6.76 (2H, d, J=8.5 Hz), 7.16, 6.76 (2H, d, J=8.5 Hz), δ_H 7.14, 6.67 (2H, d, J=8.5 Hz) and 6.49, 6.38 (2H, d, J=8.5 Hz) indicating the presence of four monosubstituted *p*-hydroxyphenyl groups. Two sets of *meta*-coupled aromatic protons in AX spin systems resonated at δ_H 6.10, 6.27 (1H, d, J=1.7 Hz, each) and δ_H 6.46, 6.17, (1H, d, J=1.8 Hz, each) were assignable to two disubstituted resorcinol moieties. Two signals of *meta*-coupled aromatic protons in the AA'B spin system were observed at δ_H 6.08 (2H, *brs*), 6.27 (1H, d, J=2.4 Hz), suggesting the presence of an independent 2,5-dihydroxyphenyl group. Finally, an aromatic proton of a penta-substituted benzene ring resonated at δ_H 6.03 (1H, s).

The analysis of the ^{1}H NMR spectrum also revealed eight aliphatic methine groups. Two pairs of signals at δ_H 5.75, 4.42 (1H, d, J=11.6 Hz, each) and 5.35, 4.66 (1H, d, J=5.1 Hz, each) were attributed to two diaryl-dihydrobenzofuran moieties. The other two pairs of aliphatic ^{1}H NMR signals, at δ_H 5.19, 3.10 (1H, d, J=3.5 Hz, each) and 4.08, 4.52 (1H, d, J=10.7 Hz, each) showed correlations as one spin system.



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The structure (Figure 6) for the isolated compound was identified by comparing their ^{1}H NMR data to those of published reports. The compound is vaticaphenol A (**1**), which was first isolated from *Vatica diospyroides* [26], followed by *Vatica oblongofolia* [27]. It was also isolated from a non-dipterocarpaceous plant, *Vitis vinifera* [28].

The development of chromatographic conditions prior to isolation reduces the need for repetitive isolation processes as the parameters for optimal separation were determined beforehand [29,30]. 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.

CONCLUSION

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 before the actual separation process ensures high purity for the isolated compounds. The operational technique of the p-HPLC is simple, as it is dedicated for preparative work, without complicated software to run the system. The isolated compound was identified as vaticaphenol A by ^{1}H-NMR spectral analysis and comparison with the reported data.

ACKNOWLEDGEMENTS

The authors acknowledge the Institute for Research Management and Innovation (IRMI), Universiti Teknologi MARA, Malaysia for technical support and the Ministry of Higher Education Malaysia for a research funding under the Fundamental Research Grant Scheme (FRGS/1/2019/STG03/UITM/02/6).

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