Australian Journal of Basic and Applied Sciences

2020 May; 14(5): pages 12-18
DOI: 10.22587/ajbas.2020.14.5.2

Original paper AENSI Publications

# Chromatographic Conditions for Baseline Resolution of *Dipterocarpus semivestitus* Extracts

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**Received date: 22 February 2020, Accepted date: 25 April 2020, Online date: 25 May 2020**



**Abstract**

**A HPLC-based chromatographic method was developed for a separation of *Dipterocarpus semivestitus extracts.* Two samples obtained from the stem and leaves of the plant were used and being analysed on a reversed-phase column chromatography. The optimized parameter in development of UHPLC method was the gradient composition of the mobile phase. Only the gradient of mobile phase was altered, while the flow rate, column temperature and injection volume were keep constant. The results showed that the optimum chromatographic condition was achieved when a gradient elution was adopted, with the solvent composition of ACN:$H_2O$ from 30:70 to 60:40 in 14 minutes for the stem extract, whereas for the leaves extract, the solvent composition was at ACN:$H_2O$ from 25:75 to 75:25 during the same chromatographic-run time. The injection volume of 5 µL is best for a 1 mg/ml sample concentration. The temperature was maintained at 35°C and the flow rate was at 0.5 ml/min. Apart from good quality of separation and resolution, the elution time was also taken into consideration. The result shows that UHPLC is able to provide baseline resolution within a short chromatographic run.**

**Keywords:** *Dipterocarpus semivestitus,* chromatography, UHPLC, reversed-phase, gradient

## INTRODUCTION

*Dipterocarpus* is the third largest genus of Dipterocarpaceae family. *Dipterocapus semivestitus* are found in Kalimantan and peninsular Malaysia (Wiart, 2016). Dipterocarpaceae family was reported to produce high diversity of oligostilbene structures, ranging from dimeric to octameric stilbenes. Oligostilbenes are the most phytochemical studies on this family due to their abundance in the plants which can be isolated from their leaves, bark, wood, heartwood and seeds. Oligostilbenes have attracted attention due to the bioactivities of crude extracts, fractions, as well as the isolated pure compounds including anti-inflammatory, cytotoxicity, antimicrobial and antioxidant activities (Ito, 2011; Zuraida, *et al*. 2011; Siti Azima *et al*., 2017).

As the isolation and characterization techniques for plant compounds developed, investigations of oligostilbenes became more abundant and more new compounds from various families were characterized. In our previous report, we have isolated five new oligostilbenes (Bayach, *et al*. 2015) and four known compounds from *Neobalanocarpus heimii* (Manshoor, *et al*., 2016). We also isolated five known from *D. semivestitus* (Ramli *et al*., 2017).

The principle of chromatographic separation based on the relative interaction of a solute with these two phases is describe by partition (K) or distribution (D) coefficient which is the ratio of concentration of solute in stationary phase to concentration of solute in mobile phase. The difference in their partitioning behavior between mobile liquid phase and stationary phase makes the different components in the column pass at different rates (Lindholm, 2014). It is important to consider several factors for a purpose of phytochemical profiling such as the mechanism of separations, bed shape, chemical and physical properties, chemical nature and chemical compositions.

Chromatographic technique is now being used beyond its original purpose, which is to separate and purify a mixture of compounds. It is also used to identify and quantify the individual components of mixtures in the phytochemical and analytical

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chemistry (Boligon and Athayde, 2014). HPLC analysis plays a major role in phytochemical analysis including identification of crude plant extracts (Bucar, Wube and Schmid, 2013).

Method development for HPLC requires decisions regarding choice of column, mobile phase, detectors, and method quantitation (Prathap, *et al*. 2013). Optimization of HPLC conditions and other important perspectives during method development are conducted to provide simple, precise, rapid and accurate analysis of plants (Jalal *et al*., 2018; Muis *et al*., 2017; Fatin *et al*., 2016).

The column is selected depending on the nature of the solute and the information about the analytes. The ability of reversed phase to handle compounds of diverse polarity and molecular mass causes over 65% of all HPLC separations has been estimated carried out in reversed phase mode. It can separate the molecules that possess some degree of hydrophobic character with excellent recovery and resolution (Amersham, 1999). The obvious challenge in chromatographic profiling of plant extracts is repetitive method development works whenever working with new samples. More often than not, the chromatographic method differs for every sample. Thus, chromatographic method is developed for one particular sample only at one time. This leads to the increase consumption of money and time. We were suggesting a dereplication strategy to identify compounds as early as in a crude mixture in order to work faster (Manshoor and Weber, 2015a; 2015b).

In developing a method, it requires decisions regarding the choice of column, mobile phase, detectors and method of qualification (Prathap, *et al*. 2013). It involves selection of the most suitable mobile phase, the right detector, the best column, an appropriate column length, a suitable stationary phase and a proper internal diameter for the column in developing a new HPLC method (Kirkland, 1993). Knowledge of psychochemical properties of the compounds is also taken into consideration in development of an HPLC method.

More often than not, acetonitrile is used as an organic modifier in a reverse phase HPLC. The analyte of interest is detected by using suitable detector after the chromatographic separation (Chandrul and Srivastava, 2010). The aim of analysis of each HPLC method may vary for each developmental area. In phytochemical studies, HPLC are frequently applied for quantitative and qualitative analysis of organic compounds in natural plant extracts (Hajnos and Sherma, 2010). A number of HPLC methods have been developed for the quantification of samples such as sildenafil in human plasma (Al-Hroub, *et al*. 2015), acetazolamide, furosemide and phenytoin in suspensions (Sila-on, *et al*. 2015) and alkaloids from *Nicotiana spp.*(Moghbel, Ryu, and Steadman, 2015).

Phytochemical studies of Dipterocarpaceae by using HPLC are conducted to determine the composition of the plant such as resveratrol dimer O-glucosides with enantiomeric aglycones (Ito, *et al*. 2013), bergenin phenylpropanoates (Ito, *et al*. 2012), oligostilbenoids and 3-ethyl-4-phenyl-3,4-dihydroisocoumarins (Morikawa, *et al*. 2012).

The purpose of conducting this research is to develop a chromatographic method by using UHPLC and optimized a chromatographic condition in UHPLC separation technique to produce a reasonably good separation and better resolution in order to separate and purify the plant components, Dipterocarpaceae crude extracts.

## MATERIAL AND METHODS

Sample of *D. semivestitus* stem and leaves extracts are available at Atta-ur-Rahman Institute for Natural Product Discovery (AuRINs), Universiti Teknologi MARA, Malaysia. Organic solvents used for extraction were of analytical grade. Acetonitrile used for HPLC analyses were of chromatographic grade from RCI Labscan. Ultrapure water (18 MΩ $cm^{-1}$) was obtained from PURELAB® Option water purification system (ELGA).

1 g of each sample was extracted in 10 ml of acetone. Extraction was carried out by maceration under sonication. For each sample, the extraction process was repeated 3 times. The extracts were filtered and reduced under vacuum. The samples were transferred into vials and dried over nitrogen gas.

The HPLC system is a Dionex Ultimate 3000 from Thermo Scientific. The system is equipped with an ultra-pressure pump, a degasser, an auto sampler and a diode array detector (DAD). The chromatographic profiles and the integrated data were recorded using Chromeleon Chromatography software. The separations were achieved through a Phenomenex® Luna 2.5 µm C18 column (100 X 4.6 mm) equipped with a guard column of similar chemistry.

The UHPLC analyses for Dipterocarpaceae crude extracts were established at the wavelengths of 215 nm, 254 nm and 283 nm. The column temperature was maintained at 35°C and the flow rate was set at 0.5 ml/min. The injection volume was 1 µL. the mobile phase were acetonitrile: water (ACN:$H_2O$) at different compositions at gradient elution mode. Prior to analyses, the system was purged to free from air bubbles, the column was conditioned for 30 minutes. After each chromatographic run, the column was flushed with 95% acetonitrile for 5 minutes, followed by a 5-minute post-run at solvent composition of the next injection. The injections were programmed on an autosampler.

Extracts from stem and leaves were subjected to an analytical C18 column for initial profile analysis. The analysis for stem extract 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 (figure 1a). The solvent gradient for leaves extract was initiated at ACN:$H_2O$ (20:80), while the rest of the parameters were kept similar to those of the stem (figure 1b).

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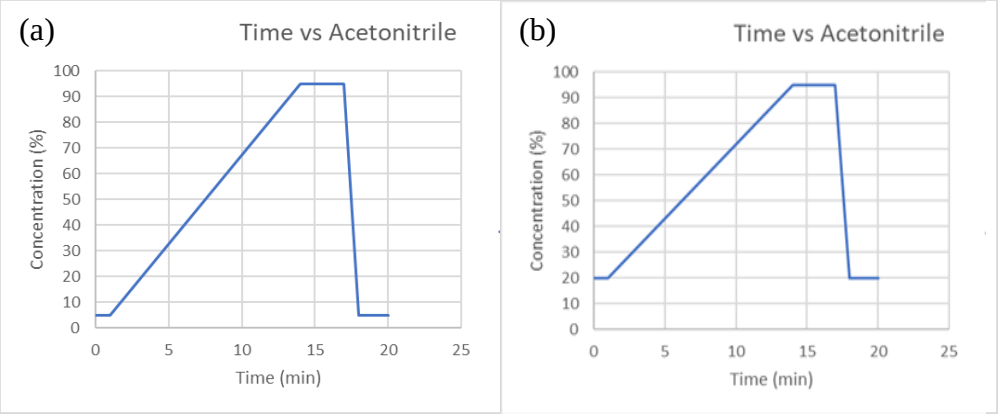


**Figure 1** The initial gradient solvent composition for stem (a) and leaves (b) extracts.

The analyses were carried out by gradually changed the solvent composition at the beginning and/or end of the chromatographic-run, adjusting the gradient slopes. The acceptable resolution is determined when a base-line resolution is achieved. The progression is summarizes in Table 1.

**Table 1:** Solvent compositions for development of chromatographic condition in separation of components in the extracts.

| Chromatographic conditions | Stem extract | Leaves extract |
|---|---|---|
| | $t_0$ - $t_{14}$ **(ACN:$H_2O$)** | $t_0$ - $t_{14}$ **(ACN:$H_2O$)** |
| 1 | (5:95 to 95:5) | (20:80 to 95:5) |
| 2 | (15:85 to 95:5) | (20:80 to 85:15) |
| 3 | (20:80 to 95:5) | (25:75 to 75:25) |
| 4 | (20:80 to 85:15) | (25:75 to 65:35) |
| 5 | (25:75 to 75:25) | (25:75 to 50:50) |
| 6 | (30:70 to 75:25) | - |
| 7 | (30:70 to 65:35) | - |
| 8 | (30:70 to 60:40) | - |

## RESULTS AND DISCUSSIONS

Both samples underwent a full-range gradient method to obtain the overall performance of their chromatographic profiles. The full-range gradient method is also a standard gradient elution scouting run to determine the best elution mode to be choose in this study. Dolan (2000) had suggested that the scouting run can be a guide tool to determine the best elution mode for a specific sample, column and eluent system.

The composition of acetonitrile controls the solvent strength, thus it is used to determine the composition of the strong solvent for faster elution of the compounds from the column. The analyses were started by setting the solvent composition at ACN:$H_2O$ (5:95 to 95:5). The initial composition later adjusted by increasing the percentage of acetonitrile at the beginning of a chromatographic run. The chromatograms in figure 2 show the decreasing of retention times when the solvent strength was increased. The separation, however was not improved as the slopes were decreased. Further adjustments were performed in order to achieve a desirable resolution.

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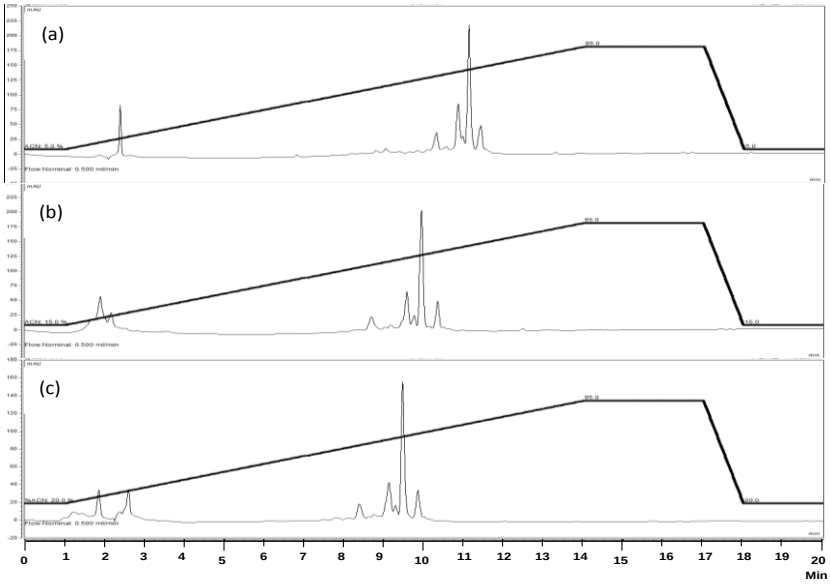


**Figure 2** Chromatogram of the stem extract of *D. semivestitus*. (a) ACN:$H_2O$ (5:95 to 95:5); (b) ACN:H2O (15:85 to 95:5); (c) ACN:H2O (20:80 to 95:5).

The second set of chromatographic adjustment was by keeping the initial composition at 20% acetonitrile and changing the end-composition. Figure **3** shows the chromatographic profiles of the extract when the composition was change from ACN:H2O (20:80 to 95: 5) to ACN:H2O (20:80 to 85:15). From this observation, the solvent slope was decreased by setting the solvent condition as of ACN:H2O (25:75 to 75:25). The chromatogram shows improvement in peaks separation, but does not achieve a baseline resolution. The retention time is acceptably good, however there is still more room to improve, considering there is no compound elutes in the first 10 minutes.

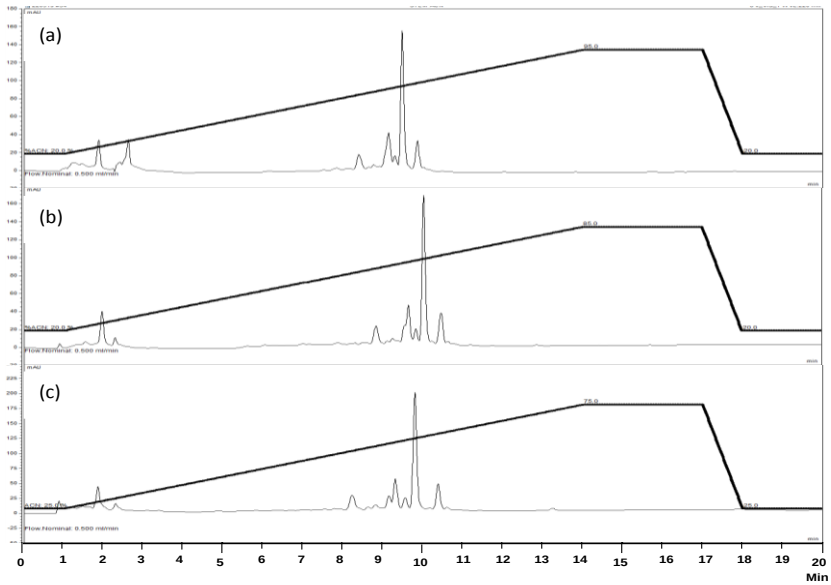


**Figure 3** Chromatogram of the stem extract of *D. semivestitus*. (a) ACN:H2O (20:80 to 95:5); (b) ACN:H2O (20:80 to 85:15); (c) ACN:H2O (25:75 to 75:25).

In order to decrease the column retention, the solvent strength was increase by 10% at the beginning of the chromatographic run (ACN:H2O ; 30:70). At the end of the chromatographic run, the solvent compositions were varied at ACN:H2O (75:25, 70:30 and 65:35). This way, the solvent slope was gradually decreased, resulting in reduction of solvent strength. This strategy is to quickly elute the extract content at early retention time, but later slowly decrease the elution speed to provide enough time for the constituents to resolve in the column. Figure **4** shows a set of three chromatograms demonstrating the profiles resulting from each solvent composition. The increment of the initial gradient from ACN:H2O (25:75) to ACN:H2O (30:70) reduces the elution time from 9.2 to 7.0 minutes. The peaks spread in wider retention time range, from around 2 minutes to approximately 3.2 minutes. The peak resolution for the condition of ACN:H2O (30:70 to 60:40) is acceptably good and most peaks achieved baseline resolution (figure 4c).

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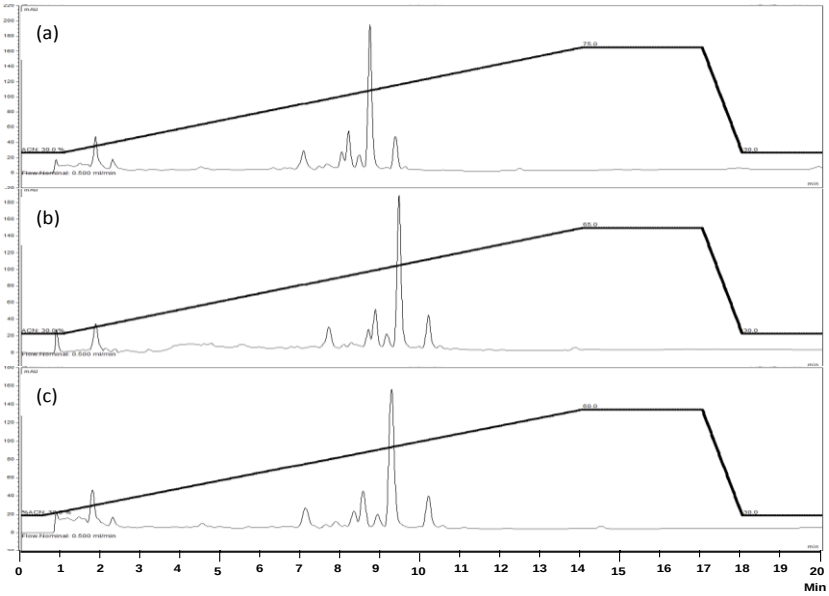


**Figure 4** Chromatogram of the stem extract of *D. semivestitus*. (a) ACN:H2O (30:70 to 75:25); (b) ACN:H2O (30:70 to 65:35); (c) ACN:H2O (30:70 to 60:40).

The analyses for *D. semivestitus* leaves extract were started at ACN:$H_2O$ (20:80 to 95:5 in 14 minutes). Two significant peaks were observed; together with some undeterminable small peaks spread from minute-5 to minute-10 of the chromatographic run. The peaks are very low in intensity that most of them are buried at the baseline. For these analyses, only the two visible peaks are considered for their separation and resolution.

The analyses were continued by decreasing the slope from ACN:$H_2O$ (20:80 to 95:5) to ACN:$H_2O$ (20:80 to 85:25). Figure 5 shows the improvement in elution time and resolution. For this sample, only two peaks corresponding to oligostilbenes are considered, and they are very well resolved. The method development is now focused on improving the elution time.

In the second set of analysis, the initial solvent composition was set at ACN:$H_2O$ (25:75) and the final compositions were ACN:$H_2O$ (95:5 and 85:15). The elution times however do not improve, although it gives better resolution. Figure 6 (a and b) shows there is only a slight different in resolution, despite the 10% difference in solvent composition.

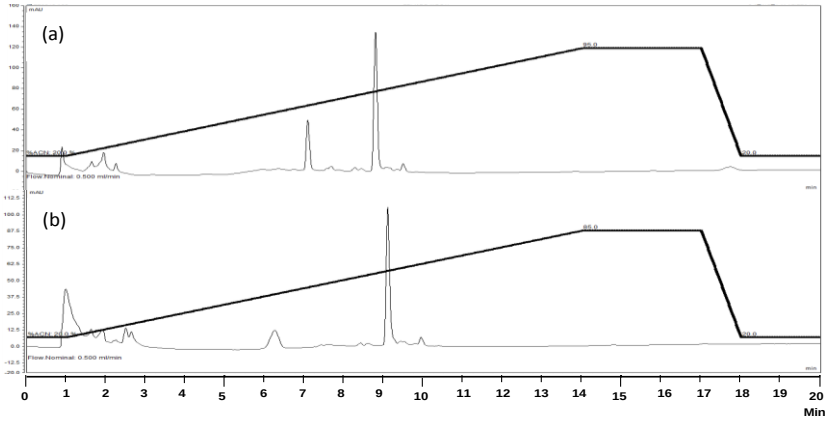


**Figure 5** Chromatogram of the leaves extract of *D. semivestitus*. (a) ACN:H2O (20:80 to 95:5); (b) ACN:H2O (20:80 to 85:15).

The slope was then abruptly change by setting the final composition at ACN:$H_2O$ (50:50). The profile shows a sharp change in both resolution and elution time (figure 6c)

A gradient method is preferable over an isocratic method as by applying gradient solvent composition, separation is faster and the target is to achieve a chromatographic run-time within 14 minutes. Some analytes may be poorly retained when the range of analyte polarities is broad. Thus the resolution might lost with the peaks eluting at or near the void volume. The range of possible alteration of mobile phase composition is broader in gradient elution, as both initial and end composition could be adjusted indefinitely. The initial composition of mobile phase is altered to resolve early eluting analytes while the final composition of mobile phase is altered to ensure elution of all compounds of interest from the column within a reasonable time.

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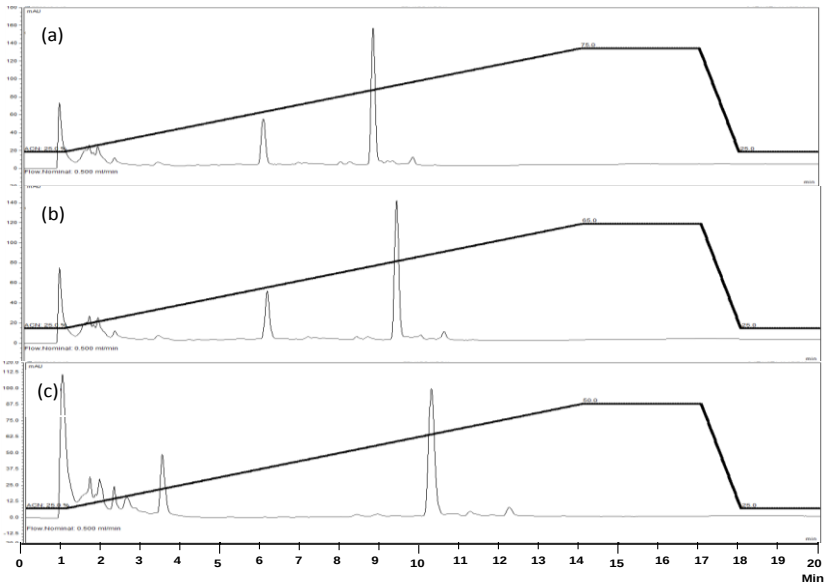


**Figure 6** Chromatogram of the leaves extract of *D. semivestitus*. (a) ACN:H2O (25:75 to 75:25); (b) ACN:H2O (25:75 to 65:35); (c) ACN:H2O (25:75 to 50:50).

In a partition chromatography as on a C18 column, the solvent polarity plays a crucial role in separation of compounds. The solvent trapped in between the 18-chained hydrocarbon and behave as a stationary phase. The analytes interact with the trapped solvent and are released as the solvent polarity changes. This justifies the better performance of gradient elution than as of the isocratic.

The choice of column was based on the fact that Dipterocarpaceae family is rich in oligostilbenes. The compounds include hydroxyl groups that make the highly polar. The Kinetex 2.5µm XB-C18, 100mm column with particle size of 2.5 µm provides much higher resolution than conventional column of 5µm. The 100mm length column reduces the elution time as compared to conventional column of 150 or 250mm. The choice of this column is only possible for a ultra-performance liquid chromatography (UHPLC) system, which is equipped with high pressure pump.

## CONCLUSION

The results show that a short chromatographic-run is possible in analyzing plant samples, providing the right column choice, a good solvent composition and a reliable chromatographic system. A gradient elution of water-acetonitrile, from 30:70 to 60:40 and 25:75 to 75:25 for the stem and leaves extracts respectively, show acceptably good separations with high resolution. The run time was restricted only up to 14 minutes to save time during analyses of many compounds, besides to demonstrate the performance of a UHPLC system. Other parameters, the injection volume (1µL) the column temperature (35°C) and the flow rate (1.0 ml/min) were kept constant.

## ACKNOWLEDGEMENT

The members of this project would like to thank Atta-ur-Rahman Institute for Natural Product Discovery, for supporting this project by providing laboratory space and administrative assistance. The authors acknowledge the Institute for Research Management and Innovation (IRMI), Universiti Teknologi MARA, Malaysia for student financial support under the Graduate Initiative Program (600-IRMI 5/3/GIP (046/2018)).

## CONFLICT OF INTEREST

Authors declare no conflict of interest in the present work.

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