Analysis of *Pandanus* Extracts by Reversed Phase High Performance Liquid Chromatography

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ABSTRACT

The Pandanus species (Pandanaceae family) was investigated, in order to set up a library of their chromatographic profiles. From a literature review, the most common Pandanus plant that was greatly examined was P. amaryllifolius (the fragrant screw pine), followed by P. sanderi. Here, the chromatographic data from an extract of a relatively unfamiliar Pandanus is also presented. The leaves of P. monotheca were extracted by using methanol. Later, the extract was filtered, prior to a Reversed-Phase High Performance Liquid Chromatography (RP-HPLC) experiments. The automated system was set to run by gradient elution (acetonitrile:water). The flow rate was maintained at 1 ml/min, otherwise stated in a number of attempts. The ultra-violet absorbance of the Pandanus' constituents was measured via Multiple Wavelength Detector. From the chromatogram, the major peaks for Pandanus' compounds were eluted (λ = 220, 260 and 360 nm) and the retention times were recorded. The lower absorbance readings of other minor peaks could be due to various minor secondary metabolites, such as the furolignan. The different elution time depends on the polarity of the composition. In short, Pandanus' phytochemicals could be obtained via alcoholic extraction. The utilisation of modern chromatographic applications enables the scientists to discover and subsequently, purify single component of the complex natural mixtures. This finding can be beneficial to the research areas in botanical study, pharmacognostical assessment and biotechnology industry. It is aimed that more Pandanus species could be learnt for the future.

Keywords: *chromatography, flavonoid, liquid, Pandanus, reversed-phase*

INTRODUCTION

Pandan species (genus: *Pandanus*, family: Pandanaceae) is among the herbs, used for commercial planting. The most popular species is the fragrant screw pine or *P. amaryllifolius*, which was also referred as *P. odorus* [1, 2]. It is commonly cultivated in South East Asia such as Malaysia, Indonesia, India and Thailand [3]. This plant is widely used due to its multipurpose usage in traditional Malay dishes, flavouring and colouring agents, due to its sweet and pleasurable taste [4]. Since plants contain numerous secondary metabolites which are important in health maintenance, research is conducted to relate these secondary metabolites found in *Pandanus* species that possess the medicinal properties. The information about the activity of secondary metabolites in *P. amaryllifolius* are still inadequate. Therefore, an investigation was initiated to provide information of the phytochemicals via High Performance Liquid Chromatography (HPLC) analysis. The separation of *Pandanus'* alcoholic extract through HPLC will produce purified compounds that could show single, isolated, chromatographic peaks and may represent a type of polar secondary metabolites such as the flavonoids. Previous reports mentioned that *Pandanus* fractions with hypoglycaemic effect contains phenolics (e.g. 4 hydroxybenzoic acid, flavonoids) and lignans that were obtained from the polar components of two *Pandanus* species (*P. odoratissimus* and *P. odorus*) [5, 6].

From the literature [4], the separation of flavonoid composition was performed by using Reversed-Phase HPLC (RP-HPLC), which include a Model 1100 pump supplied with a multi solvent delivery system, an Agilent C18 (5 µm, 4.6 x 250 mm) column and an L-7400 ultraviolet (UV) detector. The mobile phase that was used in the HPLC analysis are 2% acetic acid (CH_3COOH) and 0.5% acetic acid-acetonitrile (CH_3CN), (50:50 v/v). *Pandanus* samples were taken from different localities, which include Pontian, Klang and Bachok. Meanwhile, in this study, other areas were selected, which consisted of Sri Kembangan (Selangor), Sungai Besar (Selangor) and Cameron Highland (Pahang) (Table 1). It is aimed that the *Pandanus* samples could be extracted by using methanol/ethanol, and their chromatographic profile could be obtained via RP-HPLC, in a neutral condition.

METHODOLOGY

Extract Preparation

Nine samples were obtained which were then labelled as A1 to A9 (Table 1). The plant materials were dried and then cut into small pieces. Later, they were macerated in 20 ml of methanol for three days. The extracts were filtered by using a filter paper before evaporating the solvent by rotary evaporator. Sample A1 and A2 were originated from the same plant, thus, were duplicate samples. The extracts were subjected to RP-HPLC analysis. The chromatogram peaks were monitored whether the following techniques were repeatable, or if there was any inconsistency in the sample preparation.

Table 1: Samples from *Pandanus* Species

Label	*Pandanus* species	Plant Part	Source
A1	*P. amaryllifolius*	Root	Sri Kembangan, Selangor
A2	*P. amaryllifolius*	Root	Sri Kembangan, Selangor
A3	*P. amaryllifolius*	Leaves	Cameron Highland, Pahang
A4	*P. amaryllifolius*	Leaves	Sungai Besar, Selangor
A5	Tea samples which include *P. amaryllifolius* and *Cymbopogon* species, known as lemongrass	Leaves	Perai, Pulau Pinang
A6	*P. amaryllifolius*	Leaves	Bandar Utama, Kuala Lumpur
A7	*P. monotheca*	Leaves	University of Malaya, Kuala Lumpur
A8	*P. amaryllifolius*	Subterranean root	Sri Kembangan, Selangor
A9	*P. sanderi*	Stilt root	Sri Petaling, Kuala Lumpur

Separation by Reversed-Phase HPLC (RP-HPLC)

One mL of extract was transferred into a vial through 0.45 µm membrane filter. This step was done to each sample. The vial was then be placed in the autosampler. The solvent that used was acetonitrile (CH_3CN) and water. The system was set up to run in a gradient elution as follows: 0 min, 10:90; 3 min, 10:90; 33 min, 90:10; 35 min, 90:10; 36 min, 10:90; and 45 min, 10:90, otherwise stated in respective trials. The system used for separation is a reverse-phase system (temperature of the column = 30°C). The rate of flow was set as 1 mL/min and the UV absorbance was set to measure at 220, 260 and 360 nm [4]. The peaks in the chromatograms for all samples were observed, recorded and reviewed. A total of seven trials were made and the results were discussed.

RESULT AND DISCUSSION

All samples were extracted and analysed with RP-HPLC, in order to obtain their chromatographic profiles. Unfortunately, the experiments were not proceeded for certain samples, due to the compound degradation.

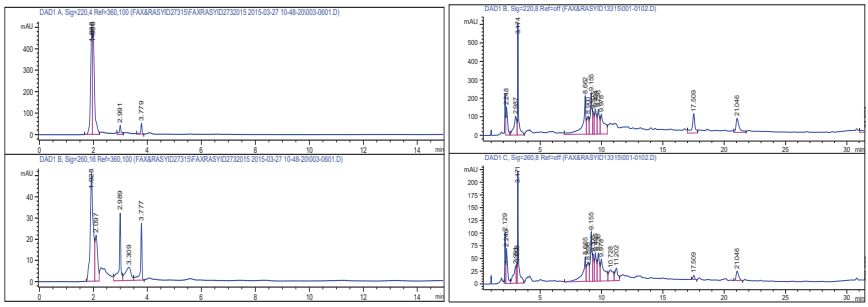
First Attempt of RP-HPLC Analysis

In the beginning, A1 to A6 were investigated and the UV absorbance was recorded at $\lambda = 220$ and 260 nm. It was found that the compounds with the highest absorbance values were eluted within five minutes, whereby the solvent ratio is 10:90 (CH_3CN:H_2O). In this initial trial, the same solvent gradient were applied for A1 to A6. This step was performed in order to observe the effect of similar solvent gradient in each chromatogram. Since water is more polar than CH_3CN, this combination of mobile phases was a comparatively polar mixture. The compounds which eluted earliest were more polar, compared to the furthest eluted. It was found that each A1 to A6 chromatogram showed numerous peaks that were not well isolated. Nevertheless, this could be improved by modifying either the solvent gradient or the injection volume, or both parameters. Therefore, this preliminary result will become a reference for the following attempts.

Second Attempt of RP-HPLC Analysis

In this second attempt, some of the parameters were modified in order to obtain more separated peaks. The modifications for the solvent gradient were applied individually on selected samples, which include A1, A2, A3 and A6. The modification was based on the outcomes of the chromatograms. It was unlucky that sample A4 and A5 had to be excluded for further RP-HPLC optimisation, due to sample decomposition. For sample A1, the injection volume was increased from 10 µl to 20 µl in order to increase the amount of absorption. The concentration gradient was modified by starting the separation at 60% CH_3CN. The intention was to obtain better isolated peaks for the compounds which eluted at minute 24 to 35. As a result, there were some increment on the absorbance for the compounds that were eluted in minute 24 to 35.

For sample A2, the absorbance in the first trial was too high due to high sample concentration. To improve the chromatogram, the injection volume was reduced from 10 µl to 5 µl while the solvent gradient was maintained as in the initial trial. Even though there was a decrease in the absorbance, the peaks were still not separated. Isolating the peaks could be time consuming because the retention time for most of peaks were too close from one another. Hence, sample A2 is not an ideal choice for next sample injection. For sample A3, the first attempt gave a chromatogram that showed most of the peaks were located within the first five minutes after the sample injection. Therefore, initiatives were made to focus on the peaks by isolating them isocratically. The solvent was maintained at 10% CH_3CN for ten minutes. The injection volume was increased from 10 µl to 20 µl to increase the absorbance unit. As a result, more isolated peaks were obtained and are presented in Figure 1(a).



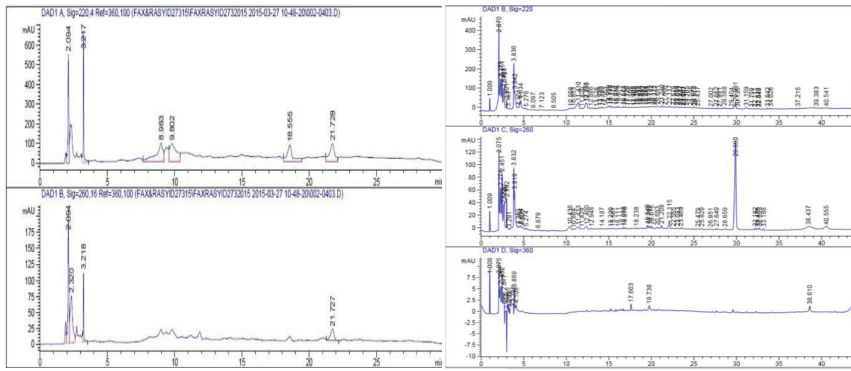
(a) (b)

Figure 1: The chromatogram of (a) A3 and (b) A6 (λ = 220 and 260 nm)

For sample A6, an attempt to obtain better separation for compounds which were eluted in the first 25 minutes was performed by altering the solvent gradient and increasing the injection volume from 10 µl to 20 µl. In the second attempt, the solvent gradient was set and the result is represented in Figure 1(b). More isolated peaks were observed especially for two compounds which were eluted at 17.509 and 21.046 minutes. However, compounds within 8 to 12 minutes were still clustered together. The clustering may be due to the slightly small polarity differences within each compound, thus making these compounds difficult to be isolated.

Third Attempt of RP-HPLC Analysis

By comparison, A6 showed the finest chromatographic profile amongst all samples (A1-A6). The chromatograms from the first and second attempts showed that A6 was likely to contain pure, isolated peaks/compounds. However, compounds which were eluted within eight to 12 minutes are still clustered. Therefore, a new solvent gradient was made in order to separate these compounds. For the third attempt, the injection volume was still maintained at 20 µl while the new solvent gradient was set. The chromatogram is represented in Figure 2 (a).



(a) (b)

Figure 2: The chromatogram of (a) A6 (λ = 220 and 260 nm) and (b) A9 (λ = 220, 260 and 360 nm)

In the third attempt, the compounds which were eluted within eight to 12 minutes showed minor changes. Firstly, the absorbance was decreased, compared to the previous chromatogram in Figure 1, even though the injection volume was the same. The depletion may be due to compound transformation, owing to the improper storage of the sample. Secondly, there was no improvement on the separation by comparison with chromatogram in Figure 2. This may as well, be related to the compound degradation. Different collection sites for Pandanus samples would result in unrelated amount of detected phenolics [4]. Here, it could be suggested that A3 and A6 would contain significant natural constituents, compared to others.

Fourth Attempt of RP-HPLC Analysis

The methanolic extracts from miscellaneous parts of three *Pandanus* species were involved. They include the leaves of *P. monotheca* (A7), the subterranean root of *P. amaryllifolius* (A8) and the stilt root of *P. sanderi* (A9). Their chromatograms were quite similar in pattern for the first five minutes (10% CH_3CN, 90% water). This shows that these three samples might possess similar chemical compositions. However, there were slightly different pattern for the following 40 minutes. The chromatogram of A9 [Figure 2(b)] showed a sharp, single, isolated peak at the retention time of 29.89 minutes that do not occur in the chromatograms of A7 and A8. This peak might show the presence of a compound that only exist in the *P.*

sanderi. However, this method was time consuming and required 45 minutes to complete. The peak was quite low, indicating a low absorbance unit and the compound was eluted too early. Therefore, some HPLC parameters were changed in the next attempt, in order to retain the peak.

Fifth Attempt of RP-HPLC Analysis

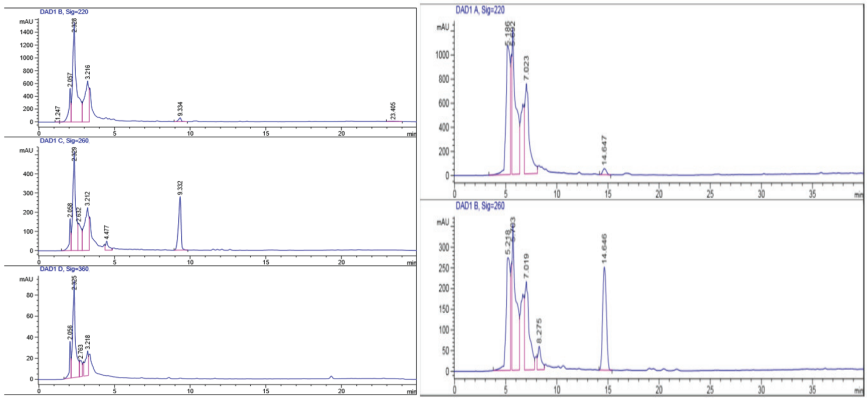
The samples were subjected to standard chromatographic parameters, mainly on the gradient system and the sample volume. The sample volume was increased to 20 μL because the samples were too dilute in the previous analysis. Some modifications were made to the previous solvent's gradient system. During the first injection, there are compounds which are eluted very fast. Referring to the gradient system, those compounds might be very polar. Therefore, considering the presence of those highly polar compounds, the gradient system was altered in terms of solvent percentage into a lesser polar solvent combination. This reduced the possibility of the compound from being eluted early. The solvent's gradient system was then, started from 60% to 90% CH_3CN. The previous analysis took 45 minutes to complete one cycle of analysis and it is very time consuming. In this attempt, the time had been reduced to 24 minutes per cycle. The chromatogram from the fifth attempt for A7 to A9 met the objectives. The compounds did not elute too early, thus, this procedure was not time consuming and the absorbance unit (sample concentration) has increased. All three samples (A7-A9) show similar chromatographic pattern to each other. They show comparable chromatographic profile in the first five minutes. This finding might indicate the presence of the same compositions. These compounds might be the major moiety of *Pandanus* species. However, *P. sanderi* (A9) showed a bit difference in a later stage. It indicated the presence of a pure compound via the occurrence of a single, symmetrical peak at the retention time of 9.332 minutes (λ = 260 nm, Figure 3). There was no similar peak seen in the other extracts.

Sixth Attempt of RP-HPLC Analysis

Only one extract was involved in the sixth attempt, which was A9. This extract was selected since it showed a very promising candidate of having

a relatively pure chemical component. This mainly due to the presence of a high resolution, sharp peak in the chromatogram. In this attempt, there were some changes made to the parameters. The first change was the flow rate. The flow rate was reduced to 0.5 ml/min. This reduction in the flow rate was done mainly to resolve the compounds that were present in the first five minutes of the previous injection. The second change was the wavelength. It was recorded at dual wavelengths of λ = 220 and 260 nm. There were also some changes made to the solvent's gradient system. The other parameters involving the RP-HPLC analysis were remained as the common factors.

The changes made on the gradient system include the prolongation, in term of the time. As the flow rate was reduced by half, the retention time for each compound was expected to be higher. Therefore, the time taken for one cycle of HPLC analysis to complete was increased. The chromatogram showed some unresolved compounds in the first five minutes. Then, one distinct and sharp peak was seen at the retention time of 14.646 min [Figure 3(b)]. This compound was eluted in between 60-80% of CH_3CN. This shows that the compound is relatively a non-polar compound. This analysis used a longer time to complete for each cycle, which was 36 minutes, as compared in the fifth trial, which only took 25 minutes. Therefore, it is considered as laborious. Some adjustment to the parameters can be performed to improve the RP-HPLC analysis for further experimental use. Any drifts or unstable chromatogram baselines should be avoided.



(a) (b)

Figure 3: The chromatogram of A9 (a) in the fifth (λ = 220, 260 and 360 nm) and (b) sixth attempt of RP-HPLC analysis (λ = 220 and 260 nm)

Seventh Attempt of RP-HPLC Analysis

The sample involved in this trial was A9, the methanolic extract of the stilt root of *P. sanderi*. For this injection, some alterations were made to the parameters mainly to obtain the best chromatographic conditions. The first alteration was the increment of the flow rate. The reduction in the flow rate would be able to broaden the peak in the first five minutes. However, it caused the isolated peak to be eluted at retention time of 14.646 min. This led to a non-efficient analysis and too much of time consuming. Therefore, the increment of the flow rate to 1 ml/min might be better to reduce the compound's retention time. Other adaptations can be done to improve this study, which include the time to complete one cycle. The reason to decrease the time was to reduce the solvent consumption during the preparative HPLC. Later, it was found that the time to complete one full cycle was reduced to 26 minutes, rather than 36 minutes in the sixth attempt. The isolated peak was found to be in 60-80% of CH_3CN. Therefore, the solvent percentage would only reach a maximum of 80% of CH_3CN, where the compound was able to elute. The chromatogram (Figure 4) showed the complete cycle of HPLC analysis of 26 minutes. A shift in the retention time was observed, whereby one sharp, isolated peak gave a retention time of 7.344 min. Therefore, it is considered suitable to be used in a preparative RP-HPLC. Based on this chromatogram, it is suggested as the best chromatogram for *P. sanderi* extract.

Eighth Attempt of RP-HPLC Analysis

The leaves of *P. monotheca* (A7) received additional attention, due to its rarity within the list of *Pandanus'* publications. Later, it was found that this plant is in fact, belongs to *Benstonea ornata. P. monotheca* is therefore, treated as a synonym [8]. From the chromatogram, the major peaks for the compounds were eluted (λ = 260 and 360 nm) and the retention time was recorded within 8 minutes after the HPLC injection. The lower absorbance readings of other minor peaks could be due to various minor secondary metabolites, such as the furolignans [9]. The elution time greatly depends on the polarity of this Pandanus' composition. Based on this chromatogram (Figure 4), it is believed that the profiling of this extract could be further improved, in order to obtain the optimised chromatogram for *P. monotheca.*

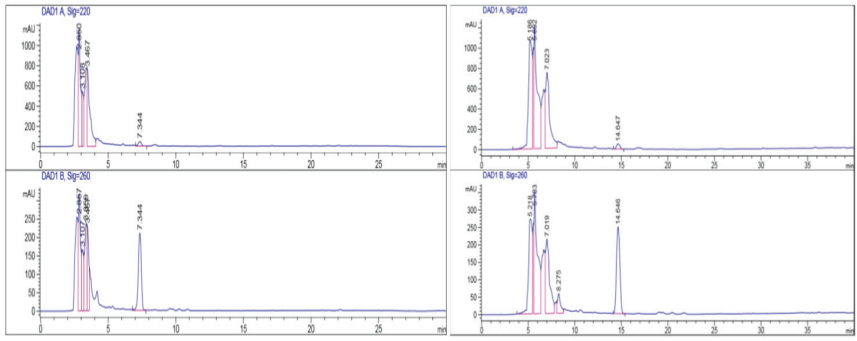


Figure 4: The chromatogram of (a) A9 (λ = 220 and 260 nm) and (b) A7 (λ = 220 and 360 nm)

CONCLUSION

It is recommended that further studies concerning *Pandanus* extracts would include the utilisation of two-dimensional liquid chromatography. Obtaining isolated phytochemical content from *Pandanus* could prove the existence of health promoting materials which will benefit consumers. This study is hoped to contribute as a reference for future purposes. In the meantime, the occurrence of phytochemicals (e.g. naringenin, Figure 5) from *Pandanus* species should also be kept in view, due to their biological properties. The preparative RP-HPLC procedure is in progress, in order to isolate, purify and consequently, to characterise the natural compositions. This RP-HPLC can be utilised as the final tool for compound identification, on a condition that, the standards or reference compounds were included in the analysis. The RP-HPLC reproducibility would be much effected by the storage of those extracts.

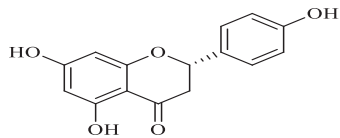


Figure 5: Naringenin, an example of a flavonoid from *Pandanus* species

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