การวิเคราะห์หาปริมาณสารสำคัญในรากมะยม โดยวิธีเกรเดียน โครมาโตกราฟีของเหลว

Determination of Active Constituents in Dried Root of *Phyllanthus acidus* Skeels by Gradient Liquid Chromatography

สุภาวดี ดาวดี (Supawadee Daodee)[1] *ฉวี เย็นใจ (Chavi Yenjai)*[2]
จินดา หวังบุญสกุล (Jinda Wangboonskul)[3] *ประสาท กิตตะคุปต์ (Prasat Kittakoop)*[4]

บทคัดย่อ

Phyllanthusols A และ B เป็นส่วนประกอบหลักที่พบในรากมะยม และพบว่ามีความเป็นพิษต่อเซลล์ ดังนั้นเพื่อเป็นการพัฒนาสารทั้ง 2 ชนิดนี้ ซึ่งอาจจะเป็นยาต่อไปในอนาคต จึงเห็นความสำคัญที่จะต้องทำการวิเคราะห์หาปริมาณ เพื่อให้ทราบถึงความแน่นอนของปริมาณสารทั้ง 2 ชนิดนี้ในรากมะยมต่อระดับการออกฤทธิ์ทางชีวภาพ โดยการประยุกต์ใช้เทคนิคของโครมาโตกราฟีของเหลวสมรรถนะสูง ซึ่งจะวิเคราะห์ Phyllanthusols A และ B ในส่วนสกัดของเมทธานอล โดยจะทำการวิเคราะห์ร่วมกับการใช้เครื่องตรวจวัดชนิดไดโอด แอเรย์ การแยกสารจะใช้คอลัมน์ Hypersil C18 และตรวจวัดที่ความยาวคลื่น 230 และ 270 นาโนเมตร สภาวะของโครมาโตกราฟีที่ใช้จะเป็นสารผสมของสารละลายฟอสเฟตบัฟเฟอร์ เตตร้าเอธิลแอมโมเนียมโบรไมด์ และ เตตร้าไฮโดรฟูแรน กับเมทธานอล ซึ่งอัตราส่วนจะแปรจาก 80:20 จนถึง 10:90 ในเวลา 60 นาที ซึ่งพบว่าสภาวะนี้จะทำให้แยกสารทั้ง 2 ชนิดออกจากกัน และแยกจากสารประกอบอื่น ๆ ได้อย่างสมบูรณ์ ตัวอย่างรากมะยมแห้งทั้งหมด 14 ตัวอย่างที่เก็บรวบรวมจากพื้นที่ต่าง ๆ ในประเทศไทยได้ถูกสกัดและวิเคราะห์หาปริมาณโดยเทคนิคนี้และพบว่าปริมาณของ Phyllanthusols A จะมีความแตกต่างกันมากถึง 3 เท่า ในขณะที่ Phyllanthusols B มีความแตกต่างกันถึง 4 เท่า

Abstract

Phyllanthusols A and B are the main active constituents,generally found in the dried root of *Phyllanthus acidus* Skeels. These two compounds showed the cytotoxic activity. To develop these compounds which could be a medicine in the future, it is important to determine the amount of these compounds in order to ensure the consistency of the biological activity. The fingerprints from HPLC can be applied for this kind of documentation. Phyllanthusols A and B in the methanolic extract were determined by HPLC in coupling with diode array detector. The separation was carried out using hypersil,C18 column and detect at wavelength 230 and 270 nm. The gradient chromatographic condition using the mixture of a phosphate buffer solution, tetraethylammoniumbromide and tetrahydrofuran : methanol (the ratio varies from 80:20 to 10:90 in 60 min.) was found to allow complete resolution of Phyllanthusols A and B and from other extracted components. Dried root of *Phyllanthus acidus* (14 samples) collected from different part of Thailand were extracted and determined by this technique. It was found the amount of Phyllanthusols A vary up to three folds and Phyllanthusols B vary up to four folds.

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[1] *ผู้ช่วยศาสตราจารย์ ภาควิชาเภสัชเคมี คณะเภสัชศาสตร์ มหาวิทยาลัยขอนแก่น*
[2] *รองศาสตราจารย์ ภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยขอนแก่น*
[3] *รองศาสตราจารย์ ภาควิชาเภสัชเคมี คณะเภสัชศาสตร์ มหาวิทยาลัยขอนแก่น*
[4] *ศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติ สคลองหลวง จ.ปทุมธานี*

Introduction

The extract of this Thai plant, *Phyllanthus acidus* Skeels, has been investigated with respect to biological activity. The root is the part of this plant used for medicinal purposes. It has been shown to have antibacterial activity against fish and shrimp pathogenic bacteria (Direkbusarakom et al,1998). In the past, Thai folk medicine used this plant as an antipyretic (Kittikajorn, 1983 and Pavovat et al, 1987) and in the treatment of chronic constipation (Kittikajorn, 1983 and Pavovat et al, 1987). Phyllanthusols A and B are the main active constituents, generally found in the dried root of *Phyllanthus acidus* and have been characterised as bisabolane glycoside (Vongvanich et al, 2000). These two bisabolane glycosides were water soluble and had the evidence to confirm that it have the cytotoxic activity (Vongvanich et al, 2000). We therefore focused our research on the determination of the level of these two active ingredients. The selective and accurate analytical method is necessary to serve this purpose. We realized that even the constituents from the same herb may vary depending on harvest season, plant origin, drying process and other factors. Consequently, it is necessary to define all the phytochemical constituents of botanical extracts in order to ensure the reliability and consistency of the biological activity.

In this paper we propose a HPLC (Lindsay, 1991) in coupling with diode array detector to determine the amount of Phyllanthusols A and B in the extract of *Phyllanthus acidus* dried root and compare the level of these two active constituents in the dried root which were collected from different part of Thailand.

Experimental

2.1 Chemicals and Materials

Chemicals were obtained from the following sources: Methanol and Tetraethylammoniumbromide from Merck,Germany, Tetrahydrofuran from Carlo Erba, Italy. All solvents and chemicals were of analytical grade.The standard of Phyllanthusols A and B were kindly supplied by the National Centre for Genetic Engineering and Biotechnology,Thailand. Samples of Phyllanthus acidus root (14 samples) were collected form different part of Thailand.

2.2 Apparatus

A Hewlett Packard series LC 1100 with photodiode array detector (Waldbronn, Germany) have been used in the analysis.

2.3 HPLC condition

The separation of *Phyllanthus acidus* dried root was carried out using Hypersil, C18 column (250x4 mm, 5 µm particle size) and detect at wavelength 230 nm for Phyllanthusols B and 270 nm for Phyllanthusols A at flow rate 1ml/min. The gradient chromatographic condition used a buffer of 20 mM disodium hydrogen phosphate, 10 mM tetraethylammoniumbromide and 1% tetrahydrofuran at pH 2 and methanol (the ratio of buffer and methanol vary from 80:20 to 10:90 in 60 min.)

2.4 Sample preparation

Plant material was dried in the oven under the temperature of 50°C to constant weight and an accurately weighed (10 g) of Phyllanthus acidus root was extracted with 50 ml methanol. The plant material was sonicated for 30 minute. The sample was filtered and dilute with a buffer of 20 mM disodium hydrogen phosphate,10 mM tetraethyl ammonium bromide and 1% tetrahydrofuran and methanol.

2.5 Standard preparation

The standard solutions were freshly prepared exactly weighing an amount of about 7 mg Phyllanthusols A and B standard. They were transfered in a 5 ml volumetric flask and solubilized to volume with methanol. By opportune dilution of these methanolic solution in a solvent of mobile phase in HPLC condition. A series of solutions at different concentrations were prepared and used to verity the linearity of calibration curves in HPLC.

2.6 Internal standard preparation

Carbamazepine was used as the internal standard in this method because of its appropriate retention time and also the complete resolution from extract components. Internal standard was freshly prepared exactly weighing an amount of about 20 mg and was transfered in a 25 ml volumetric flask and solubilized to volume with methanol.

2.7 Quantitative analysis

For quantitative analysis a 10 g sample of dried root was extracted with 50 ml methanol as described above. Carbamazepine was used as an internal standard in the analytical procedure by incorporating at the same amount in both the extract and the standard solutions. The range of solution concentrations, obtained by opportune dilution of standard solution, was from 8 to 280 µg/ml for Phyllanthusols A , 9 to 150 µg/ml for Phyllanthusols B. These were prepared in a buffer of 20 mM disodium hydrogen phosphate, 10 mM tetraethylammoniumbromide and 1% tetrahydrofuran at pH 2 and methanol (50:50) by dilution from stock solution. The final concentration of these solutions were filtered by a 0.45 µm. A 20 µl of each solution were injected to HPLC. Chromatogram of each standard solution were obtained and the peak areas were recorded. Calibration curves were prepared by regression of the mean peak area ratios of Phyllanthusols A and B to the internal standard on each concentration. The amount of Phyllanthusols A and B in each extracts of *Phyllanthus acidus* root obtained from calculation of peak ratio on the least square equation of the calibration lines.

2.8 Validation method

The validation data obtained from the analysis by HPLC have been reported in Table 1. The repeatability (relative standard deviation) of the proposed methods to determine Phyllanthusols A and B, on the basis of peak area ratios for ten replicate injections of each sample, was shown in Table 1 at concentrations of 17.5, 70.0 and 140 µg/ml for within day precision. The between day percentage relative standard deviation for ten replicate injection was also shown in this table. The reproducibility of the extraction procedure, determined by carrying out repeated extractions for five replication of a single root sample was also shown in Table 1. Calibration lines were obtained from injecting five standard solutions of both Phyllanthusols and calculated in peak area ratio compared with the peak area of the internal standard. The representative regression equation for both Phyllanthusols were shown in Table 1. The accuracy was determined by calculating the percentage recovery of both Phyllanthusols which were spiked into the solutions. The mean recovery and the percentage relative standard deviation were shown in Table 1. The limit of quantification, determined as the lowest concentration that could be estimated with the signal/noise ratio ($S/N \cong 10$) and limit of detection, determined by injecting successively lower concentrations of each component that could be estimated with the signal/noise ratio ($S/N \cong 3$) were shown in Table 1.

Results and Discussion

The reverse phase C18 stationary phase were tested for the separation of Phyllanthusols A and B. This stationary phase allowed a good separation among Phyllanthusols A and B and the other components which appeared in the extract. The addition of tetraethylammoniumbromide and tetrahydrofuran in the mobile phase can improve the resolution and peak shape of the chromatogram. The gradient chromatographic condition using a buffer of 20 mM disodium hydrogen phosphate, 10 mM tetraethylammoniumbromide and 1% tetrahydrofuran at pH 2 and methanol (the ratio of buffer and methanol vary from 80:20 to 10:90 in 60 min.) was found to allow complete resolution of Phyllanthusols A and B and from other extracted components within 30 minutes. These can also improve the time consumed for the separation. This chromatographic method allows

to obtain a precise qualitative and quantitative analytical profile. Actually the good separation of all compounds allowed the purity control of each peak, detected by diode array detector. We carried out the quantitative analysis using an internal standard, Carbamazepine, and the peak areas normalization. The internal standard was used to eliminate the possible differences in the amount injected, which increase the repeatability of the method. Figure 1 shows the chromatogram of these two standards and internal standard at two wavelength, 230 and 270 nm.

The calibration curve was plotted analyzing solutions at different concentrations of Phyllanthusols A and B,added with a fixed amount of Carbamazepine. The linearity, obtained by plotting the peak area ratio versus concentration of both substance analyzed, gave a good correlation coefficients in excess of 0.993 and intercept below 2 percent of the maximum calibrated range.

The repeatability of this proposed methods, on the basis of peak area ratios for ten replicate injection of each sample ,were below 2 percent relative standard deviation for both of within day precision and between day precision and the precision of extraction method was also below 2 percent relative standard deviation for 5 replication.

The quantitative data obtained from the sample extracts (14 samples), which were collected from different part of Thailand, had been reported in Table 2 and the chromatograms was shown in figure 2. The extrapolation of peak ratio on the calibration lines were used to determine the amount of both Phyllanthusols in 14 samples.

Conclusion

The method that we proposed has been described for the determination of Phyllanthusols A and B in *Phyllanthus acidus* roots. Dried root of *Phyllanthus acidus* (14 samples) collected from different parts of Thailand were extracted and determined. It was found that the amount of Phyllanthusols A vary up to three folds (from 1.97-7.02 mg/g dried root) and Phyllanthusols B vary up to four folds (from 0.26-1.40 mg/g dried root). This can cause the inconsistence of the biological activity.

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Table 1 The validation parameters for the analytical method.

Validation details	Phyllanthusols A	Phyllanthusols B
Mean recovery(%,n=4)	99.08(%RSD= 3.20)	98.03(%RSD=2.14)
Within day precision(n=5)		
%RSD,conc.17.5 µg/ml	0.52	1.63
%RSD,conc.70.0 µg/ml	1.40	1.48
%RSD,conc.140 µg/ml	0.84	1.37
Between day precision		
%RSD (n=10)	0.76	1.70
Precision of extraction method		
%RSD (n=5)	0.76	1.71
Linearity (n=4)		
Mean r^2	0.9985(±0.0011)	0.9935(±0.0052)
Mean slope	0.0293(±0.0021)	0.0159(±0.0002)
% intercept	1.79	1.40
Limit of quantification(µg/ml,n=3)	3.50	7.50
Limit of detection(µg/ml,n=3)	1.75	3.75

Table 2 The amount of Phyllanthusols A and B in the extract of *Phyllanthus acidus* root collected from different part of Thailand.

Sample	Phyllanthusols A(mg/g)	Phyllanthusols B(mg/g)
Mahasarakam	1.97	0.26
Khonkaen, area 1	6.05	1.40
Khonkaen, area 2	4.19	0.53
Khonkaen, area 3	3.66	0.36
Khonkaen, area 4	3.22	0.37
Khonkaen, area 5	5.02	0.90
Khonkaen, area 6	5.56	1.04
Khonkaen, area 7	4.16	0.79
Khonkaen, area 8	1.77	0.28
Khonkaen, area 9	3.74	0.66
Chaiyapum	5.03	0.70
Nontaburee	5.75	0.81
Chiangmai	1.67	0.37
Uthaitanee	7.02	0.81