Original Article

การวิเคราะห์หาปริมาณคาร์บาโซลแอลคาลอยด์ 5 ชนิดจากรากส่องฟ้าดง โดยวิธีโครมาโทกราฟีแบบของเหลวสมรรถนะสูง

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บทคัดย่อ

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บทนำ: *Clausena harmandiana*เป็นพืชสมุนไพรชนิดหนึ่งที่มีฤทธิ์ทางชีวภาพที่สำคัญ ดังนั้นในการศึกษาครั้งนี้จึงมีวัตถุประสงค์เพื่อพัฒนาวิธีการวิเคราะห์หาปริมาณคาร์บาโซล แอลคาลอยด์ทั้ง 5 ชนิดจากส่วนของราก คือ clausine-K clausine-E lansine 7-methoxymukonal และ 7-hydroxyheptaphylline **วัสดุและวิธีการทดลอง:** วิธีโครมาโทกราฟีแบบของเหลวสมรรถนะสูงที่มีเครื่องตรวจวัดชนิดไดโอด แอเรย์ได้ถูกนำมาประยุกต์ใช้ในการวิเคราะห์หาปริมาณและแยกสารโดยใช้คอลัมน์ชนิด Hypersil ODS พร้อมทั้งตรวจวัดที่ความยาวคลื่น 254 นาโนเมตร **ผลการทดลอง:** พบว่าอาซีโตไนไตรล์และน้ำในอัตราส่วน 40 ต่อ 60 เป็นสภาวะเคลื่อนที่ที่เหมาะสมและสามารถใช้ในการแยกสารสำคัญทั้ง 5 ชนิดออกจากกันได้อย่างชัดเจนทั้งในส่วนสกัดหยาบของเอทธิล อะซีเตทและเอทานอล ทั้งนี้วิธีการวิเคราะห์ได้ถูกตรวจสอบว่าสามารถนำไปใช้ในการวิเคราะห์ได้อย่างถูกต้องและน่าเชื่อถือ โดยการทดสอบครอบคลุมทั้ง ความแม่น ความเที่ยง ความเป็นเส้นตรง ขีดจำกัดการตรวจพบและขีดจำกัดในการหาปริมาณ **สรุปผล:** วิธีโครมาโทกราฟีแบบของเหลวสมรรถนะสูงที่ใช้ในการวิเคราะห์ครั้งนี้สามารถนำไปใช้ในการหาปริมาณสารสำคัญจากส่วนรากของพืชชนิดนี้ได้เป็นอย่างดีและสามารถใช้ในการควบคุมคุณภาพได้โดยเป็นวิธีที่ใช้สภาวะของการวิเคราะห์ที่ง่ายและใช้เวลาในการวิเคราะห์สั้น

คำสำคัญ: *Clausena harmandiana*, โครมาโทกราฟีแบบของเหลวสมรรถนะสูง, คาร์บาโซล แอลคาลอยด์

Abstract

Determination of five carbazole alkaloids from the root of *Clausena harmandiana* by High Performance Liquid Chromatography.

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Introduction:*Clausena harmandiana* Pierre is a herbal plant which has important biological activities. Thus, the aim of this study was to develop an analytical method for determination of five active carbazole alka-

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loid constituents in the root bark of this plant which were clausine-K, clausine-E, lansine, 7-methoxymukonal and 7-hydroxyheptaphylline. **Materials and Method:** High Performance Liquid Chromatography (HPLC) with diode array detector was developed and used in this study. Separation was carried out using a Hypersil ODS column and detection at 254 nm wavelength. **Results:** Chromatographic conditions using a mixture of acetonitrile and water with constant ratio of 40:60 was found to give complete resolution of these five active constituents and from other components in the extract of ethyl acetate and ethanol. This analytical method was validated for accuracy, precision, linearity, limit of detection and limit of quantitation and could give reliable results which could be used for the determination of these five active constituents in the root bark of this herbal plant. **Conclusion:** The HPLC method developed in this study provided a good determination of the active constituents in the root bark of this plant and should be used for the purpose of the quality control. The advantage of this analytical method was the simple HPLC conditions and short analysis time.

Keywords: *Clausena harmandiana*, HPLC, Carbazole alkaloids

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Introduction

Clausena harmandiana (Pierre) Pierre ex Guillaumin is a herbal plant in family Rutaceae (Smittinan, 2001) also known in Thai as “Song-fa” or “Song-fa-dong”. This plant is a perennial shrub erect with 39-67 cm height, 5-8 mm in diameter and hairless stem. It grows naturally in communal grazing land, mixed deciduous forest and sandy to sandy-loam soil in Yasothon, Buriram, Khon Kaen, Udonthani and Surat Thani province. The young leaves and leaves are used as fodder for cattle. Young leaves are also used by humans for food and as medicinal plant. The roots of *C. harmandiana* have been used as a folk remedy medicine to treat headache and fever and used in combination with some herbal plants to treat broncholitis and abdominal discomfort.Some researchers have reported the isolation (Wangboonskul *et al.,* 1984) and biological activities of this plants which could show anti-TB activity against *Mycobacterium tuberculosis*, antimalarial activity against *Plasmodium falciparum*, antifungal activity against *Candida albican* and cytotoxicity against Vero cells (Chaichantipyath *et al.,* 1988; Yenjai *et al.,* 2000; Sunthitikawinsakul *et al.,* 2003). A carbazole alkaloid is the main active constituents in this plant which has been previously isolated and tested (Yenjai *et al.,* 2000). It is quite important to know the amount of these carbazoles to further study about the relationship between the biological activity and the amount of each active constituent. This study aims to find and develop a method which can be used to determine the amount of active constituents in the root of *C. harmandiana*. The interesting active constituents are clausine-K, clausine-E, lansine, 7-methoxymukonal and 7-hydroxyheptaphylline (Figure 1). Clausine-K and clausine-E are the main active compounds and were previously isolated from the stem of *Clausena excavata* (Wu *et al.,* 1996) and can inhibit rabbit platelet aggregation and had vasocontraction effect. Moreover, it was found that clausine-K and 7-methoxymukonal had antimycobacterial activity against *Mycobacterium tuberculosis* (Sunthitikawinsakul *et al.,* 2003; Thongthoom *et al.,* 2010). Thus, the amount of these compounds is the key to further study about this plant in the future.

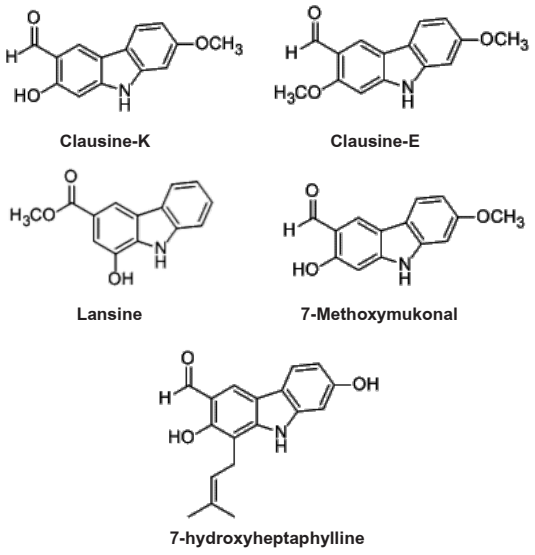

Figure 1 The chemical structures of clausine-K, clausine-E, lansine, 7-methoxymukonal and 7-hydroxyheptaphylline

Materials and Methods

The pure compounds used as the standard of five carbazole alkaloids were obtained from the isolation of *C. harmandiana* root which had been characterized and identified by Dr. Chavi Yenjai using TLC, NMR and MS from the laboratory of the Chemistry Department, Faculty of Sciences, Khon Kaen University. The roots of *C. harmandiana* were collected from Khon Kaen Province. A botanically identified voucher specimen (KK9807179) was deposited at the herbarium of The Forest Herbarium. All solvents were of AR and HPLC grade and were purchased from Merck® (Darmstadt, Germany). A Hewlett Packard® LC-1100 gradient liquid chromatography instrument, equipped with auto sample system and a photodiode array detector was used in the determination of these compounds under the following operating conditions; acetonitrile-water (40:60, %v/v) as mobile phase, flow rate 1.0 mL/min. The analysis was performed on a Hypersil® ODS column (250x4.0 mm, 5µm) and these compounds were detected at 254 nm.

Preparation of stock standard solution

Clausine-K, clausine-E, lansine, 7-methoxymukonal and 7-hydroxyheptaphylline were accurately weighted for 0.00069, 0.00010, 0.00055, 0.00028 and 0.00048 g respectively. Then, all compounds were individually dissolved with 500 µL methanol and sonicated until completely dissolved.

Preparation of standard solution

Each stock standard solution of five compounds were pipetted, diluted and then were prepared at the same time at each concentration of standard solution (1-5) for which the final concentrations are shown in Table 1. Fifty percent of acetonitrile in water was used as the solvent for each concentration.

Validation of HPLC method

The optimized HPLC method was validated for accuracy, precision, linearity, limit of detection and limit of quantitation. Accuracy of this HPLC method could be defined by the percent deviation of concentration found from true value. Within-day and between-day precision were done to assure the precision of the method. Linearity was tested by using linear regression analysis and the coefficient of determination was then calculated. For detection limit and limit of quantitation, standard solutions were diluted until signal/noise ratio were approximately 3 and 10, which was detection limit and limit of quantitation respectively.

Table 1 The concentrations of standard clausine-K, clausine-E, lansine, 7-methoxymukonal and 7-hydroxyheptaphylline solutions.

Standard solution	Concentration of clausine-K solution (µg/mL)	Concentration of clausine-E solution (µg/mL)	Concentration of lansine solution (µg/mL)	Concentration of 7-methoxy mukonal solution (µg/mL)	Concentration of 7-hydroxyhepta-phylline solution (µg/mL)
1	3.45	0.50	2.75	1.40	2.40
2	6.90	1.00	5.50	2.80	4.80
3	13.8	2.00	11.0	5.60	9.60
4	20.7	3.00	16.5	8.40	14.4
5	27.6	4.00	22.0	11.2	19.2

Table 2 The validation results of clausine-K, clausine-E, lansine, 7-methoxymukonal and 7-hydroxyheptaphylline.

Validation criterias	**clausine-K**	**clausine-E**	**lansine**	**7-methoxy mu-konal**	**7-hydroxy hepta-phylline**
Accuracy					
%deviation from true value	0.0003-0.01% (at concentration 3.45-27.6 µg/mL)	0.0001-0.002% (at concentration 0.50-4.0 µg/mL)	0.0021-0.0059% (at concentration 2.75-22.0 µg/mL)	0.0023-0.0054% (at concentration 1.40-11.2 µg/mL)	0.0007-0.0043% (at concentration 2.40-19.2 µg/mL)
Precision Within-day precision (n=5)					
% RSD	‹2%	‹2%	‹3%	‹2%	‹3%
Between-day precision (n=5)					
% RSD	‹2%	‹2%	‹2%	‹3%	‹3%
Linearity (n=5)					
intercept	-78.16 (±1.58)	23.23 (±3.07)	35.59 (±1.57)	-7.96 (±3.02)	-83.50 (±4.63)
Slope (±SD)	46.68 (±0.07)	446.65 (±3.37)	69.86 (±1.22)	89.04 (±0.91)	74.51 (±2.83)
r^2(±SD)	0.9913 (±0.0001)	0.9873 (±0.0013)	0.9976 (±0.0009)	0.9875 (±0.0016)	0.9979 (±0.0017)
Limit of detection (n=3)					
concentration (signal/noise ratio)	1.73 µg/mL (S/N =2.8)	0.25 µg/mL (S/N =4.3)	0.69 µg/mL (S/N =2.5)	0.70 µg/mL (S/N =3.3)	1.20 µg/mL (S/N =2.8)
Limit of quantitation (n=3)					
concentration (signal/noise ratio)	3.45 µg/mL (S/N =12.7)	0.50 µg/mL (S/N =15.34)	1.37 µg/mL (S/N =8.9)	1.40 µg/mL (S/N =10.2)	2.40 µg/mL (S/N =9.3)

Assay amount of five carbazole compounds in the extract of C. harmandiana

For this study, the root extracts of *C. harmandiana* were prepared by using ethyl acetate and ethanol as the solvent. Roots of *C. harmandiana* were dried to constant weight and an accurately weighed sample of powder (1.3 kg) was extracted with ethyl acetate (2X2 L) or ethanol (2X2 L). Then, the solutions were evaporated until dry at 50 degree Celsius using rotary evaporator. Both crude extract solutions were prepared by accurately weighing approximately 2 mg of dried crude extract and dissolving in 500 µL methanol and then adding with 600 µL of a mixture of acetonitrile and water (50:50). Three replications of the prepared crude extract solutions were analyzed by injecting 20 µL into the HPLC system under the described chromatographic conditions. The amounts of the five carbazole alkaloids were calculated by comparing the peak area to the calibration curve of each standard solution.

Results and discussion

The chromatographic conditions in this study could give a good resolution for the analysis of five carbazole compounds which are shown in figure 2 for the mixture of standard solutions. The retention times of these five compounds were 6.51, 11.10, 12.20, 13.97 and 28.90 min for clausine-K, clausine-E, lansine, 7-methoxymukonal and 7-hydroxyheptaphylline respectively. In addition, these conditions were found to allow complete resolution of five carbazole compounds from other extracted components which is shown in Figure 3.

This HPLC method was accurate, reliable and could be used for the determination of clausine-K, clausine-E, lansine, 7-methoxymukonal and 7-hydroxyheptaphylline as the validation results show that the percent deviations from true value were lower than 0.01%, and the within-day and between-day precision were lower than 3 % RSD. The assay was linear over the range from 3.45 to 27.60 µg/mL (clausine-K), 0.50 to 4.00 µg/mL (clausine-E), 2.75 to 22.0 µg/mL (lansine), 1.40 to 11.2 µg/mL (7-methoxymukonal) and 2.40 to 19.20 µg/mL (7-hydroxyheptaphylline) with r^2›0.99 for all five carbazole compounds. Limits of detection were 1.73, 0.25, 0.69, 0.70 and 1.20 µg/mL and limits of quantitation were 3.45, 0.50, 1.37, 1.40 and 2.40 µg/mL for clausine-K, clausine-E, lansine, 7-methoxymukonal and 7-hydroxyheptaphylline respectively. The validation results of these compounds are shown in Table 2.

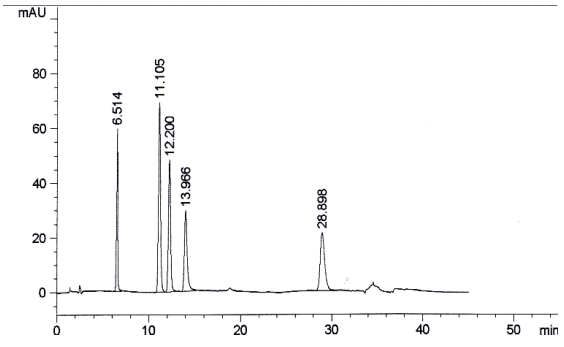

Figure 2 HPLC Chromatogram of the standard mixture solution of clausine-K, clausine-E, lansine, 7-methoxymukonal and 7-hydroxyheptaphylline (in order of elution) using acetonitrile and water (40:60) as mobile phase at a flow rate of 1 mL/min.

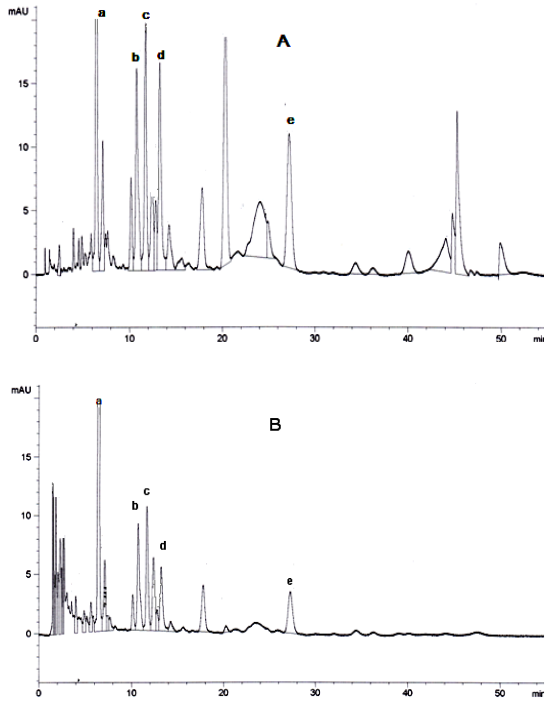

Figure 3 HPLC Chromatogram of ethyl acetate and ethanol crude extract extracted from the root of C. harmandiada using acetonitrile and water (40:60) as mobile phase at a flow rate of 1 mL/min (A= ethyl acetate crude extract, B= ethanol crude extract; a = clausine-K, b = clausine-E, c = lansine, d = 7-methoxymukonal and e = 7-hydroxyheptaphylline).

Table 3 The amount of clausine-K, clausine-E, lansine, 7-methoxymukonal and 7-hydroxyheptaphyllin in the crude extract of ethyl acetate and ethanol.

compounds	amount in ethylacetate crude extract (%w/w±SD)	amount in ethanol crude extract (%w/w±SD)
Clausine-K	5.36(±0.02)	4.59(±0.02)
Clausine-E	0.17(±0.01)	0.07(±0.001)
Lansine	1.41(±0.01)	0.69(±0.01)
7-methoxymukonal	1.05(±0.005)	0.24(±0.01)
7-hydroxyheptaphylline	1.71(±0.01)	0.65(±0.01)

In this study, we only focused on the compounds found in the root part because it has been used as the folk remedy medicine to treat some symptoms. For this reason which we would like to know the amount of the active compounds in the root part of this plant and establish a method to analyze these compounds.

The HPLC method which was developed and validated was appropriate to determine the amount of these five active constituents in the root part of *C. harmandiana*. The use of this plant for the folk remedy medicine is prepared in water or ethanol and the active constituents may be presented differently in the different polarities. Therefore, two crude extracts from the root of this plant were prepared by using ethyl acetate or ethanol as the solvent. The content of these five carbazole compounds in these two extracts were analyzed in three replications using the method described above and calculated. The amount of these compounds are shown in Table 3.

It was found that the content of each compound varied in these two extract solvents which might be due to different polarity of the five carbazole compounds and their solubility. Clausine-K was presented as the major constituent in these two extracts. The total amount of five compounds found in the extract of ethyl acetate was higher than in the extract of ethanol. Thisresult showed the efficiency of using ethyl acetate as the solvent for the extraction of these compounds.

Conclusion

The rational of the uncertainty in effectiveness of herbal plant products shown in some studies may be caused by the high variation of the level of its active constituents (Daodee *et al.,* 2006). This leads to the requirement to find methods for determination of the active constituents in each herbal plant. Thus, in this study, the quantitation of the active constituents in the root bark of *C. harmandiana* allows for further study about the relationship between their biological activities

and the amount. The reliable, accurate and sensitive method to determine the amount of five carbazole compounds in the root bark of this plant is needed in the drug development processes. The HPLC method developed in this study provided a good determination of the active constituents in the root bark of this plant and should be used for the purpose of the quality control. The advantage of this analytical method was the simple HPLC conditions and short analysis time.

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