Simultaneous determination of nine analytes in *Clausena harmandiana* Pierre. by new developed high-performance liquid chromatography method and the influence of locations in Thailand on level of nordentatin and dentatin

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ABSTRACT

Background: *Clausena harmandiana* Pierre. (CH) contains various bioactive analytes with pharmacological benefits. Most researches were focused on carbazole analytes determined by isocratic high-performance liquid chromatography (HPLC), only few were focused on coumarin analytes and harvested location. **Objective:** To develop and validate gradient HPLC method to analyze the variance of nine target analytes contained in roots of CH grown naturally in four different provinces of Thailand. **Materials and Methods:** The analytical method was undertaken by gradient HPLC with 3% tetrahydrofuran in acetonitrile, and 0.05% phosphoric acid in water as mobile phases, on Hypersil ODS column (4.0 × 250 mm, 5 µm), at flow rate 1.0 mL/min and detected at wavelength 280 nm. The method was validated for system linearity, limit of detection, limit of quantitation, precision and accuracy. **Results:** The new-developed method was able to detect the nine target analytes in CH root. The validation showed the reliability of the method. All system suitability parameters were within the satisfied limits. The linear responses of method were observed at $r^2 \geq 0.999$ for all analytes. The obtained amount of nine analytes showed the biodiversity of contents in different provinces. Of the nine target analytes, the level of nordentatin and dentatin in coumarin groups were considerably high in plants collected from one specific province of Thailand. **Conclusion:** This study has shown that the new-developed method is reliable, precise, accurate and sensitive to determine and quantify the nine target analytes in CH. Nordentatin and dentatin obviously show the higher level in one specific province of Thailand.

Key words: *Clausena harmandiana* Pierre, dentatin, high-performance liquid chromatography, nordentatin, Thailand

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INTRODUCTION

Clausena is a plant in the *Rutaceae* family with various pharmacological benefits. The pharmacological activities of *Clausena* have been studied in either crude extract or purified extract form. Chakraborty *et al.*[1] and Sohrab *et al.*[2] discovered that the extract of *Clausena heptaphylla* inhibited the activities of some bacteria, similar to the pharmacological activities of *Clausena harmandiana* (CH) reported by Chatchawanchonteera *et al.*[3] antineurodegeneration[4] and anticancer,[5] were the pharmacological activities observed in CH as well.

The antinociceptive activity of *Clausena excavate* and CH crude extract was observed by Rahman *et al.*[6] and Wangboonskul *et al.*,[7] respectively with no detection of toxicological activities in the animal model.[8] Adebajo *et al.*[9] and Rajesh *et al.*[10] reported the activities of *Clausena lansium* and *Clausena dentate* crude extract on hepatotoxicity inhibition. The antioxidant and antiinflammatory activities of plants in this species have been continuously reported

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in both crude and purified extract such as *C. lansium*,[9,11,12] *Clausena guillauminii*[13,14] and CH.[15] The antidiabetic activity was also found in *C. lansium*[9] and CH.[16]

Many analytes of plants in *Clausena* species were determined. The studies of Wu and Furukawa,[17] Chakraborty *et al*.,[18] Ito *et al*.,[19] Yenjai *et al*.,[20] Booyarat *et al*.,[4] Nakamura *et al*.,[13,14] Prasertcharoensuk *et al*.[15] and Prasad *et al*.[12] reported various analytes such as heptaphylline, 7-methoxyheptaphylline, osthol, dentatin, nordentatin, xanthoxyletin, clausenol and clausenal in *Clausena* plants. The recent researches have been focusing on two analytes of dentatin and nordentatin, the compounds in coumarin group that show the distinguished activities to protect the neurodegenerative condition.[4] In addition, these two analytes showed the activity of bacterial inhibition[17] such as antimycobacterial tuberculosis and antifungal.[21] Yenjai *et al*.[20] also found that three analytes of dentatin, clausarin and heptaphylline displayed the antimalarial activities of *Plasmodium falciparum*.

The variation of bioactive analytes found in plants has been considered as a barrier of herbal research. Therefore, the study of locations that influences the amount of analytes in CH is required to determine the suitable rangeland to harvest this plant.

Plants in *Clausena* species contain two core groups of carbazole alkaloid and coumarin. The determination of carbazole alkaloid has been previously studied by new isocratic high-performance liquid chromatography (HPLC) method and found that this method was efficient to determine and quantify the carbazole analytes.[22] However, the essential analytes in coumarin group such as nordentatin and dentatin and some other essential analytes of carbazole alkaloid have never been focused. Therefore, the new gradient HPLC has been developed for these target analytes in this study.

The aim of the present study was to develop and validate the gradient HPLC method to determine, quantify and demonstrate the variance of nine target analytes which were clausine-k, lansine, 7-methoxymukonal, xanthoxyletin, 7-hydroxyheptaphylline, nordentatin, 7-methoxyheptaphylline, heptaphylline and dentatin, contained in roots of CH Pierre. grown naturally in four different provinces of Thailand.

MATERIALS AND METHODS

Materials

Nine pure compounds; clausine-k, lansine, 7-methoxymukonal, xanthoxyletin, 7-hydroxyheptaphylline, nordentatin, 7-methoxyheptaphylline, heptaphylline and dentatin were kindly provided by Assoc. Prof. Chavi Yenjai, PhD., Faculty of Sciences, Khon Kaen University, Thailand. The purity of all standards was over 98% as indicated by the peak purity of chromatographic profile by HPLC. HPLC-grade acetonitrile was purchased from Merck Co., (Merck, Darmstadt, Germany). Deionized water was purified by Milli-Q system (Millipore, Bedford, MA, USA). Other chemicals were of reagent grade.

Methods

Chromatographic condition

High-performance liquid chromatography analysis was carried out on an HP 1100 series system (Germany), equipped with a quaternary gradient pump, vacuum degasser, autosampler, column oven, a diode array detector and controlled with the Agilent Chemstation software (Agilent Technologies, USA). Chromatographic separation was performed at the room temperature using a Hypersil ODS analytical column (4.0 × 250 mm, 5 μm) with a guard column supplied by Agilent Technologies. The mobile phase consisted of the mixture of 3% tetrahydrofuran in acetonitrile (A) and 0.05% phosphoric acid in water (B). The initial composition was 40% A and 60% B. The gradient program was as follows; 0-10 min, 40% A; 10-15 min, 50% A; 15-23 min, 60% A; 23-35 min, 100% A; 35-37 min, 100% A; 37-40 min, 40% A; 40-45 min, 40% A. The stop time and post time were set at 45 and 3 min, respectively. The mobile phase was delivered to the column at a flow rate of 1.0 mL/min and the eluate was detected at 280 nm. Ten microliters of sample were injected into the HPLC system. All data acquired were proceeded by Agilent Chemstation Rev. A. 10.02 software (Agilent Technologies, USA).

High-performance liquid chromatography-ultraviolet optimization

The HPLC method was optimized to develop the simultaneous assay. The 0.1 mg of each of nine pure analytes extracted from the root of CH was accurately weighted into a 10 mL volumetric flask, dissolved with methanol and adjusted to the volume. Due to the variation in the contents of analytes in herbal samples, the chromatographic profile obtained from the filtrate solution prepared from 0.5 g of the ground sample macerated in 10 mL ethanol, was compared with the chromatographic profile obtained from the standard solution prior to the validation. The ratios of the level among the analytes were calculated for the establishment of calibration curves. The spectra of each pure analyte obtained from the diode array detector were recorded.

Method validation

The system linearity was performed using gradient HPLC-diode array method. Six concentrations,

covered 80-120% of the expected levels were prepared in methanol and injected into HPLC. The calibration curves were plotted and coefficient of determination was calculated. The limit of detection (LOD) and limit of quantification were determined in triplicate at a signal-to-noise ratio (S/N) of 3:1 and 10:1, respectively. The within day precision (repeatability) was performed by repeated analysis for 10 injections on a single day. The between day precision was carried out on 3 different days. Variations were expressed by the relative standard deviations (RSD). For accuracy, two concentrations at high and low level, of the known value of nine analytes, were prepared and injected in 10 replicates. The found concentration of each bioactive analyte was analyzed within the same day.

Plant sample preparation

Samples of natural growth CH were collected from four different provinces of Thailand; Khon Kaen, Mahasarakum, Roei and Nan province. The roots were separated and dried at 40°C in hot air oven. The samples were subsequently ground into powder. Dried root powders of 0.5 g were accurately weight and 10 mL of 95% ethanol were added following by ultrasonication. The extraction was done for 10 min without any heat in every 24 h for 5 days. The extract was filtered through a 0.45 μm polytetrafluoroethylene syringe filter (Waters, Milford, MA, USA) before injecting into an HPLC.

RESULTS AND DISCUSSION

Optimization of chromatographic method

The optimization of gradient HPLC-ultraviolet (UV) system to separate nine pure analytes extracted from the root of CH that were clausine-k, lansine, 7-methoxymukonal, xanthoxyletin, 7-hydroxyheptaphylline, nordentatin, 7-methoxyheptaphylline, heptaphylline and dentatin was done successfully as shown in Figure 1. The resolutions between each adjacent peak were adequate for simultaneous quantification of each analyte. The spectra of each peak was determined using diode array detector proceeded by Agilent Chemstation Rev. A. 10.02 software as shown in Figure 2.

Method validation

System linearity

The correlation coefficients at $r^2 \geq 0.999$ indicated the acceptable correlations between concentrations of investigated analytes and their peak areas within the test ranges are shown in Table 1. It was observed that the correlation coefficients were ≥0.999 in all calibration plots of nine analytes indicating the linearity of the system.

The limits of detection and limit of quantitation

The LOD (signal: Noise ratio = 3:1) was ranged from 17 to 75 ng/mL whereas the limit of quantitation (signal: Noise ratio = 10:1) was ranged from 66 to 223 ng/mL for all nine analytes as illustrated in Table 1.

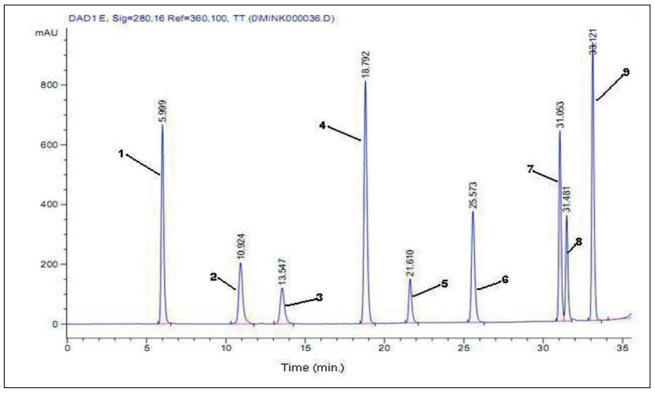

Figure 1: Chromatographic profile obtained from the mixture of nine pure analytes of clausine-k (1), lansine (2), 7-methoxymukonal (3), xanthoxyletin (4), 7-hydroxyheptaphylline (5), nordentatin (6), 7-methoxyheptaphylline (7), heptaphylline (8) and dentatin (9)

Table 1: The system linearity of calibration plot of nine analytes shown as correlation coefficient and least square equation using gradient HPLC method and the LOD and LOQ

Analytes	Test range (μg/mL)	Least square equation[a]	Correlation coefficient (r^2)	LOD[b] (ng/mL)	LOQ[c] (ng/mL)
Clausine-k	3.1-620.0	y=25003x+85.918	0.9993	17	66
Lansine	0.9-170.0	y=44385x+45.47	0.9990	22	77
7-methoxy mukonal	0.6-125.0	y=38801x+5.6153	0.9993	52	137
Xanthoxyletin	4.8-950.0	y=24663x−187.54	0.9998	41	129
7-hydroxy heptaphylline	0.7-135.0	y=28982x+19.321	0.9991	35	153
Nordentatin	1.7-335.0	y=31769x+75.271	0.9988	51	120
7-methoxy heptaphylline	3.0-590.0	y=22023x+104.5	0.9985	40	223
Heptaphylline	0.9-170.0	y=42731x+37.989	0.9992	58	192
Dentatin	4.1-810.0	y=23323x+115.45	0.9992	75	185

[a]y=Peak area; x=Concentration (μg/mL); [b]S/N=3:1, [c]S/N=10:1. HPLC: High-performance liquid chromatography; LOD: Limit of detection; LOQ: Limit of quantitation; S/N: Signal-to-noise ratio

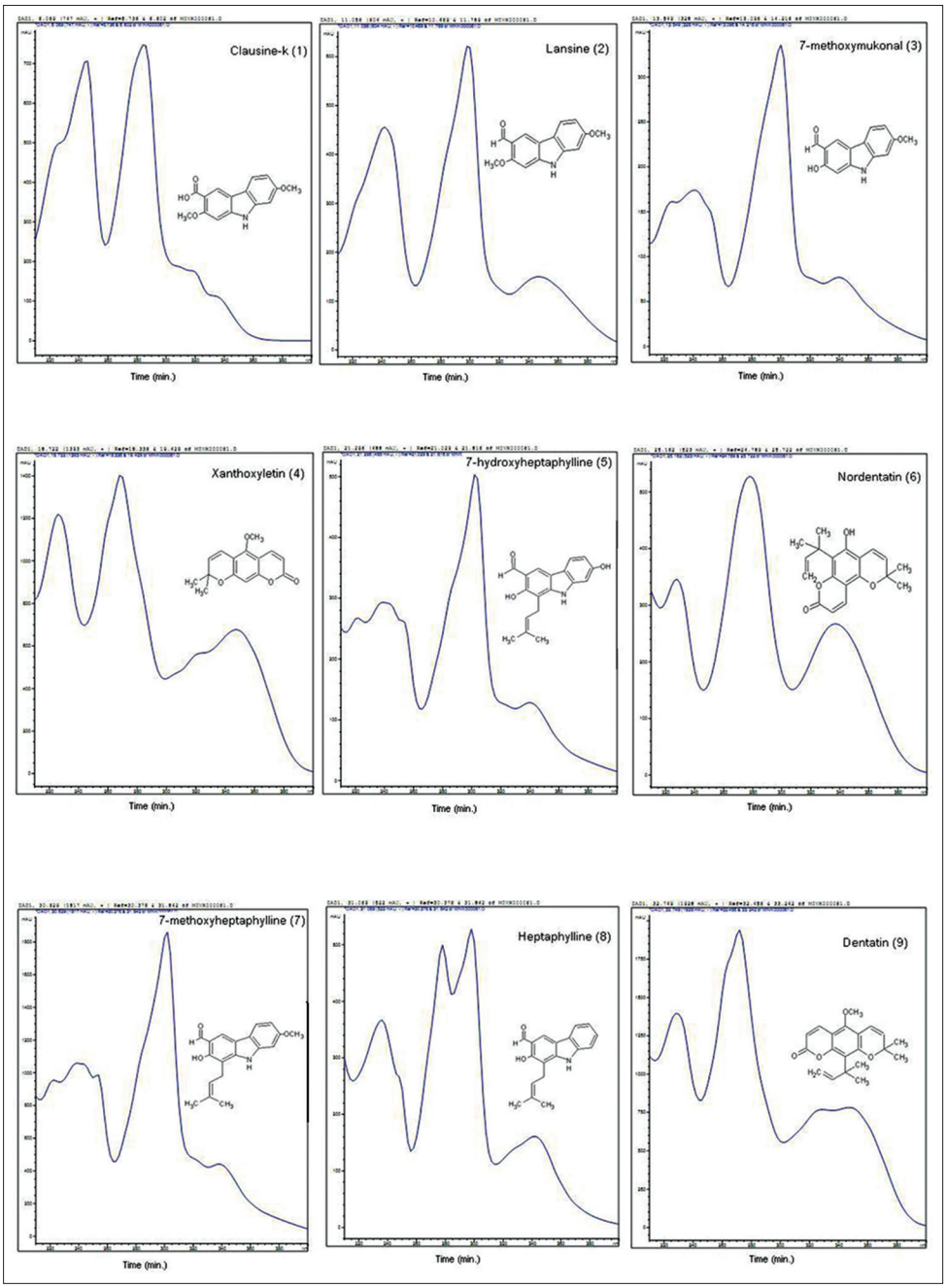

Figure 2: Spectra of nine pure analytes of clausine-k (1), lansine (2), 7-methoxymukonal (3), xanthoxyletin (4), 7-hydroxyheptaphylline (5), nordentatin (6), 7-methoxyheptaphylline (7), heptaphylline (8) and dentatin (9) by the diode array detector proceeded by Agilent Chemstation Rev. A. 10.02 software

Precision of peak response within a day and between days

The method showed good repeatability of the peak response of all nine analytes. The precision of retention time, precision within day (n = 10) and between days (3 days) (n = 14) of the peak area of all analytes were <0.87, 0.62 and 3.51% RSD, respectively as shown in Table 2.

Accuracy

Good accuracy of the method was shown by the overall recovery of 98.35-100.89%, with the % RSD ranging from 0.15 to 0.60 as illustrated in Table 3.

These results indicated that this gradient HPLC-UV method is precise, accurate and sensitive for separation and quantification of nine analytes in CH Pierre. root.

Chromatographic profile and compounds quantification

The chromatographic profile obtained from the ethanolic extract of the root of CH is shown in Figure 3. The spectra of all nine analytes were apparently observed. The spectra of lansine and 7-methoxymukonal were clearly detected although the peak purity was low and the resolution was relatively inadequate. However, the spectra

Table 2: Precision within day and between days of nine analytes in CH by gradient HPLC method

Analytes	Average retention time in 1-day (min)±SD (%RSD) (*n*=3)	Test concentrations (µg/mL)	Precision within day±SD (%RSD) (*n*=10)	Precision between 3 days±SD (%RSD) (*n*=14)
Clausine-k	5.72±0.05 (0.81)	310	7353±32 (0.44)	7479±223 (2.98)
		155	3742±11 (0.29)	3845±137 (3.51)
Lansine	10.18±0.08 (0.78)	85	3704±16 (0.43)	3758±100 (2.66)
		42.5	1868±5 (0.27)	1889±37 (1.96)
7-methoxymukonal	12.55±0.11 (0.87)	62.5	2351±9 (0.38)	2387±64 (2.68)
		31.25	1169±5 (0.43)	1184±28 (2.36)
Xanthoxyletin	18.03±0.03 (0.15)	475	10788±54 (0.50)	10924±258 (2.36)
		237.5	5465±8 (0.15)	5523±102 (1.85)
7-hydroxy heptaphylline	20.79±0.11 (0.52)	67.5	1916±9 (0.47)	1944±52 (2.67)
		33.75	970±6 (0.62)	981±20 (2.04)
Nordentatin	24.65±0.19 (0.77)	167.5	5269±31 (0.59)	5344±139 (2.60)
		83.75	2670±4 (0.15)	2699±52 (1.93)
7-methoxy heptaphylline	30.25±0.15 (0.50)	295	6494±33 (0.51)	6580±161 (2.45)
		147.5	3286±10 (0.30)	3321±66 (1.99)
Heptaphylline	30.70±0.14 (0.45)	85	3573±19 (0.53)	3622±89 (2.46)
		42.5	1806±10 (0.55)	1827±38 (2.08)
Dentatin	32.39±0.11 (0.35)	405	9323±40 (0.43)	9428±205 (2.17)
		202.5	4749±11 (0.23)	4795±82 (1.71)

CH: *Clausena harmandiana*; HPLC: High-performance liquid chromatography; RSD: Relative standard deviation; SD: Standard deviation

Table 3: Accuracy shown as percentage of recovery of nine analytes in CH at high and low concentrations (*n*=10) by gradient HPLC method

Analytes	Known concentration (µg/mL)	Found concentration (µg/mL)	% recovery±SD (%RSD)
Clausine-k	310.00	308.25±1.37	99.44±0.44 (0.44)
	155.00	156.38±0.45	100.89±0.29 (0.29)
Lansine	85.00	83.77±0.35	98.56±0.42 (0.42)
	42.50	42.17±0.12	99.22±0.27 (0.28)
7-methoxymukonal	62.50	61.87±0.24	98.99±0.38 (0.39)
	31.25	30.77±0.14	98.46±0.46 (0.47)
Xanthoxyletin	475.00	467.85±2.34	98.50±0.49 (0.50)
	237.50	236.28±0.35	99.47±0.15 (0.15)
7-hydroxy heptaphylline	67.50	66.45±0.31	98.45±0.45 (0.46)
	33.75	33.53±0.19	99.34±0.57 (0.57)
Nordentatin	167.50	164.98±0.99	98.50±0.59 (0.60)
	83.75	83.23±0.14	99.38±0.17 (0.17)
7-methoxy heptaphylline	295.00	290.62±1.47	98.52±0.50 (0.51)
	147.50	146.57±0.44	99.37±0.30 (0.30)
Heptaphylline	85.00	83.72±0.44	98.50±0.52 (0.53)
	42.50	42.18±0.23	99.25±0.55 (0.55)
Dentatin	405.00	398.30±1.72	98.35±0.42 (0.43)
	202.50	202.01±0.48	99.76±0.24 (0.24)

CH: *Clausena harmandiana*; HPLC: High-performance liquid chromatography; RSD: Relative standard deviation; SD: Standard deviation

of other analytes, especially, nordentatin and dentatin were noticeably determined.

The level in gram(s) of clausine-k, lansine, 7-methoxymukonal, xanthoxyletin, 7-hydroxyheptaphylline, nordentatin, 7-methoxyheptaphylline, heptaphylline and dentatin in 100 g of dried ground samples collected from four different provinces of Thailand were ranged from 0.8-1.02, 0.05-0.09, 0.02-0.07, 0.16-0.22, 0.03-0.15, 0.06-0.67, 0.16-0.27, 0.05-0.15 and 0.08-0.48, respectively as shown in Figure 4. The levels of lansine, 7-methoxymukonal were also calculated in approximate and displayed in Figure 4 as well. The amount of target analytes, nordentatin and dentatin, for the dementia patient could be found in quite a high level in plants collected specifically from "Nan" province when compare to other provinces. Additionally, clausine-k showed very high and similar level among plants collected from four different provinces of Thailand. These results indicated the suitable rangeland in Thailand to harvest the CH.

It was observed that the chromatographic profiles of samples collected from four different provinces of Thailand: Nan,

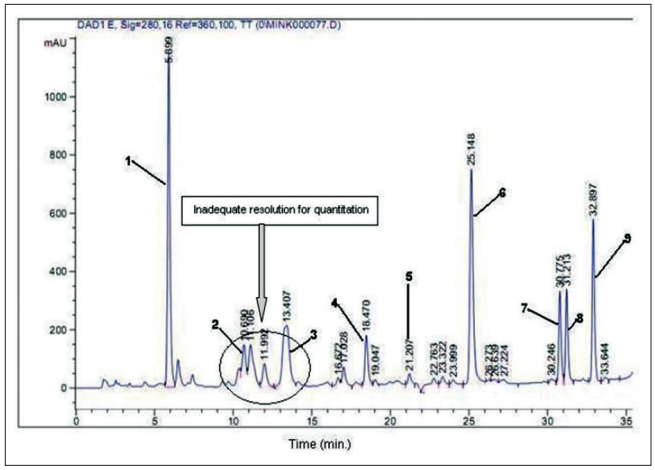

Figure 3: Chromatographic profile obtained from the ethanolic extract of Clausena harmandiana root of clausine-k (1), lansine (2), 7-methoxymukonal (3), xanthoxyletin (4), 7-hydroxyheptaphylline (5), nordentatin (6), 7-methoxyheptaphylline (7), heptaphylline (8) and dentatin (9)

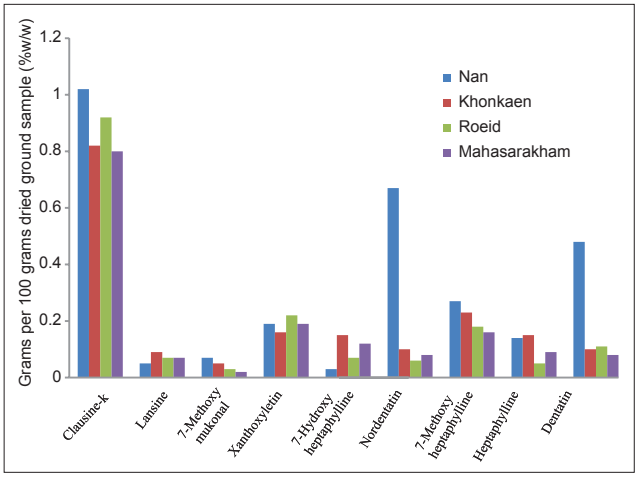

Figure 4: Level of nine analytes; clausine-k (1), lansine (2), 7-methoxymukonal (3), xanthoxyletin (4), 7-hydroxyheptaphylline (5), nordentatin (6), 7-methoxyheptaphylline (7), heptaphylline (8) and dentatin (9) in root of *Clausena harmandiana* 100 g of dried ground samples (Bars represent province)

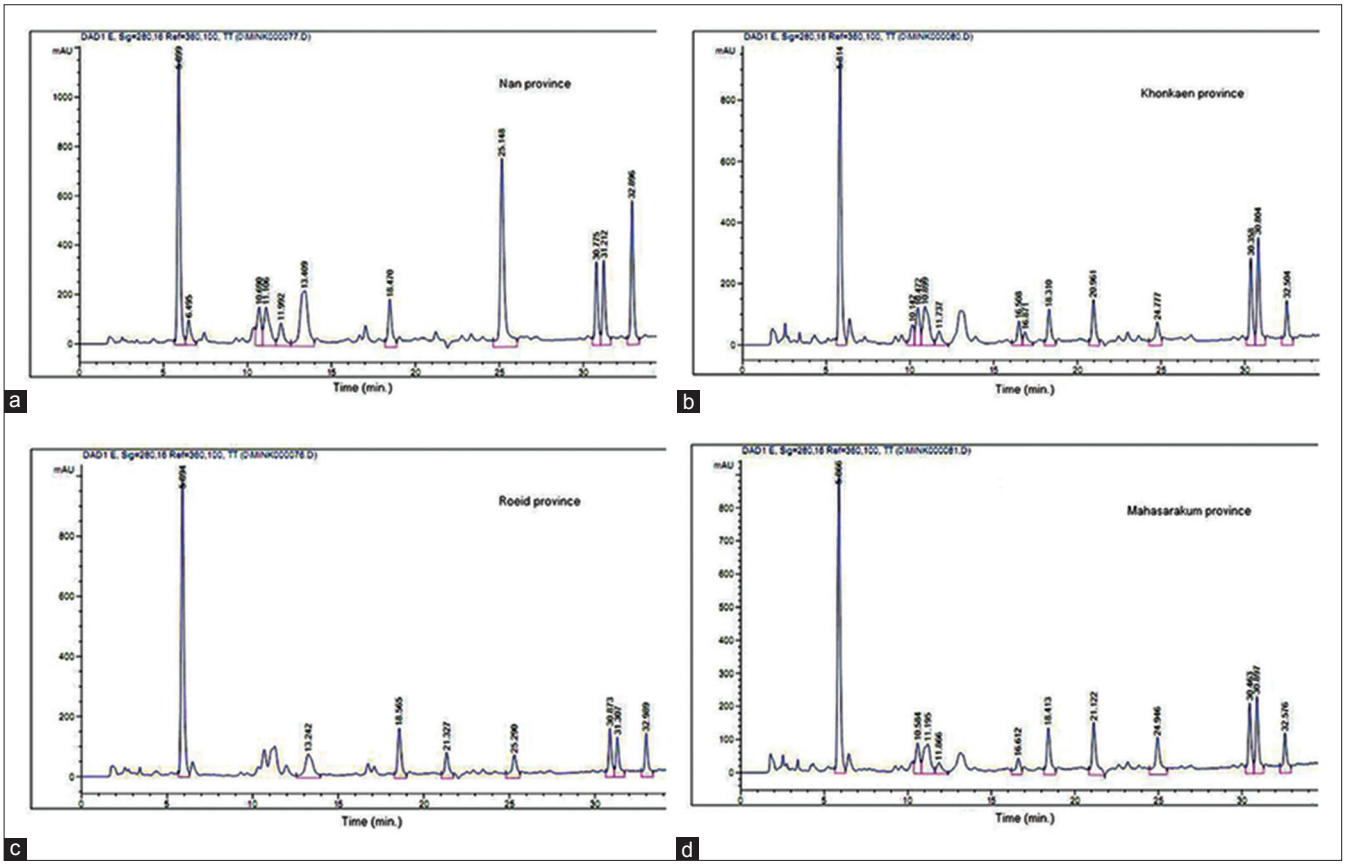

Figure 5: Chromatographic profile of *Clausena harmandiana* collected from Nan (a), Khon Kaen (b), Roeid (c) and Mahasarakum (d) provinces of Thailand

Khon Kaen, Roeid and Mahasarakum, gave similar patterns as shown in Figure 5a-d, respectively. As a consequence of pattern similarity, it can be concluded that HPLC chromatographic profile can be used to identify CH plant although the level of the analytes varied from place to place.

CONCLUSIONS

The new developed HPLC method was reliable, precise, accurate and sensitive to determine the nine target analytes in CH root harvest from different locations of Thailand

quantitatively and qualitatively. The amount of two target analytes, nordentatin and dentatin, was observed in high level in CH roots collected from one specific province of Thailand. The feasible rangeland to harvest CH for the high level of nordentatin and dentatin was in "Nan" province of Thailand according to this study. The developed method and the obtained results could be helpful for the quality control of the CH plants, especially plant grown in different locations.

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