Determination of a Pyranocoumarin and Three Carbazole Compounds in *Clausena excavata* by RP-HPLC

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Abstract

Clausenidin, *O*-methylmukonal, 3-formyl-2,7-dimethoxycarbazole, and clauszoline-J, isolated from the rhizomes and roots of *Clausena excavata*, exhibit anti-HIV-1 activity in a syncytial assay with EC_{50} values of 5.3, 12.0, 29.1, and 34.2 µM, respectively. Due to the highly active anti-HIV-1 property, quantitative analysis of four compounds are investigated. The direct analysis of these four compounds in the crude extracts of the combined rhizomes and roots of *Clausena excavata* from ten various sources in Thailand by high-performance liquid chromatography is accomplished. Chromatographic separation is achieved on a C_{18} column, and the mobile phase is a mixture of methanol and distilled water in a mode of isocratic or gradient elution detected at 254 nm at a flow rate of 0.6 mL/min for clausenidin, at 274 nm at a flow rate of 0.6 mL/min for *O*-methylmukonal, at 298 nm at a flow rate of 0.4 mL/min for 3-formyl-2,7-dimethoxycarbazole, and at 242 nm at a flow rate of 0.4 mL/min for clauszoline-J. This is the first quantitative analysis of these four anti-HIV-1 compounds from the crude extract without prior isolation and purification steps.

Introduction

Clausena excavata Burm f. (Rutaceae) (*C. excavata*) is a shrub growing mainly in southern and southeastern Asia. Thai folklore preparation of the rhizome and root extracts from this plant, found in Thai whiskey containing approximately 35% ethanol, has been used for AIDS treatment in Thailand. We have previously reported the isolation of nine antimycobacterial constituents (1), four antifungal compounds (1), and five anti-HIV-1 constituents (2,3) from the rhizomes and roots of *C. excavata*. Other biological activities reported in the literatures so far include antinociceptive activity (4), antiplatelets (5,6), and antitumor (7), and antibacterial activities (8). The barks of stems and roots of *C. excavata* also contain some carbazole alkaloids (9) and coumarins (10). Five anti-HIV-1 compounds isolated from rhizomes and roots are a pyranocoumarin, clausenidin, three carbazoles, clauszoline-J, 3-formyl-2,7-dimethoxycarbazole, O-methylmukonal, and one limonoid, clausenolide-1-ethyl ether (Figure 1) (2,3). Clausenidin (EC_{50} = 5.3 µM) showed anti-HIV-1 activity better than the other three compounds, *O*-methylmukonal (EC_{50} = 12.0 µM), 3-formyl-2,7-dimethoxycarbazole (EC_{50} = 29.1 µM), and clauszoline-J (EC_{50} = 34.2 µM), and exhibited potential therapeutic index (PTI) values of 7.0, 56.7, 8.0, and 1.6, respectively. *O*-Methylmukonal has the highest PTI (PTI = 56.7), which means that this compound is a very good indicator of HIV-1 therapy. 3-Formyl-2,7-dimethoxycarbazole (PTI = 8.0) and clausenidin (PTI = 7.0) also showed good indicators of anti-HIV-1 activity. These four compounds did not show any cytotoxic effect against the KB and BC-1 cancer cell lines (3).

It is known that secondary metabolites in the same species of plant are not usually produced in equal amounts in different geographical locations. This encouraged us to develop an analytical method under optimal conditions to measure the amount of the active anti-HIV-1 compounds, clausenidin, *O*-methylmukonal, 3-formyl-2,7-dimethoxycarbazole, and clauszoline-J, in the crude extracts of *C. excavata*. The presence of clausenidin, *O*-methylmukonal, 3-formyl-2,7-dimethoxycarbazole, and clauszoline-J may be used as markers to indicate anti-HIV-1 activity in the traditional extract prepared for AIDS therapy. Furthermore, the quantitative determination of clausenidin, *O*-methylmukonal, 3-formyl-2,7-dimethoxycarbazole, clauszoline-J by high-performance liquid chromatography (HPLC) have not previously been reported, and these four compounds are present in small quantities in the plant rhizomes and roots. So rapid

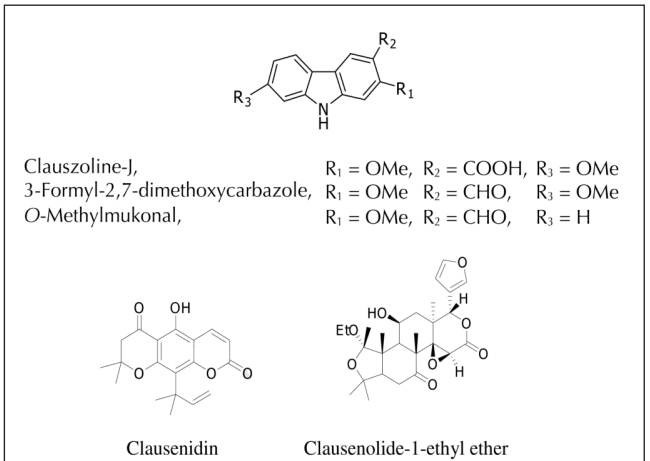

Figure 1. Chemical structures of clausenidin, clauszoline J, 3-formyl-2,7-dimethoxycarbacole, *O*-methylmukonal, and clausenolide-1-ethyl ether.

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Table I. The HPLC Chromatographic Condition Of Each Four Pure Compounds

Standards	Linear range (ppm)	System	Regression equation	R^2	t_R (min)	UV Detected (nm)
Clausenidin	2.5–200 (5 points)	30% H_2O–MeOH, 30 min, 0.6 mL/min	$y = 18.146x - 24.796$	0.9994	19.23 ± 0.27	254
O-methyl-mukonal	0.3125–5.000 (5 points)	50% H_2O–MeOH, 0–35 min 50% H_2O–MeOH–MeOH, 35–40 min MeOH, 40–100 min, 0.6 mL/min (For A, B, E, F, G, H, J) and 0.4 mL/min (For C, D, I)	$y = 236.580x - 5.801.4$	0.9950	24.91 ± 3.10	274
3-formyl-2,7-dimethoxy-carbazole	0.15625–10.000 (5 points)	60% (0.1%HCOOH)–CH_3CN, 0–30 min 60% (0.1%HCOOH)–CH_3CN–CH_3CN, 30–40 min CH3CN, 40–60 min, 0.4 mL/min (For A, B, C, D, E, F, I, J), 0.32 mL/min (For G, H)	$y = 556.377x + 5.810.1$	0.9999	25.74 ± 0.32	298
Clauszoline-J	2.5–100 (5 points)	60% (0.1%HCOOH)–CH_3CN, 0–30 min 60% (0.1%HCOOH)–CH_3CN–CH_3CN, 30–40 min CH_3CN, 40–60 min, 0.4 mL/min (For A, B, C, D, F, G, H, I, J), 0.2 mL/min (For E)	$y = 444.659x + 89.844$	0.9999	18.73 ± 0.71	242

method of quantitative analysis with reproducible result is required. This report presents a HPLC procedure for quantitative analysis of clausenidin, *O*-methylmukonal, 3-formyl-2,7-dimethoxycarbazole, and clauszoline-J in crude *C. excavata* extracts. The plants were from ten representative provinces of five regions of Thailand, i.e., Lampang and Chiangmai (Northern), Chachoengsao and Kanchanaburi, (Central), Chumporn (Southern), Sakonnakhon and Sisaket (Northeastern), Rayong, Trat and Chanthaburi (Eastern). The HPLC quantitative analysis of clausenidin, *O*-methylmukonal, 3-formyl-2,7-dimethoxycarbazole, and clauszoline-J reported herein would be very useful for patients using folklore medicine for AIDS therapy.

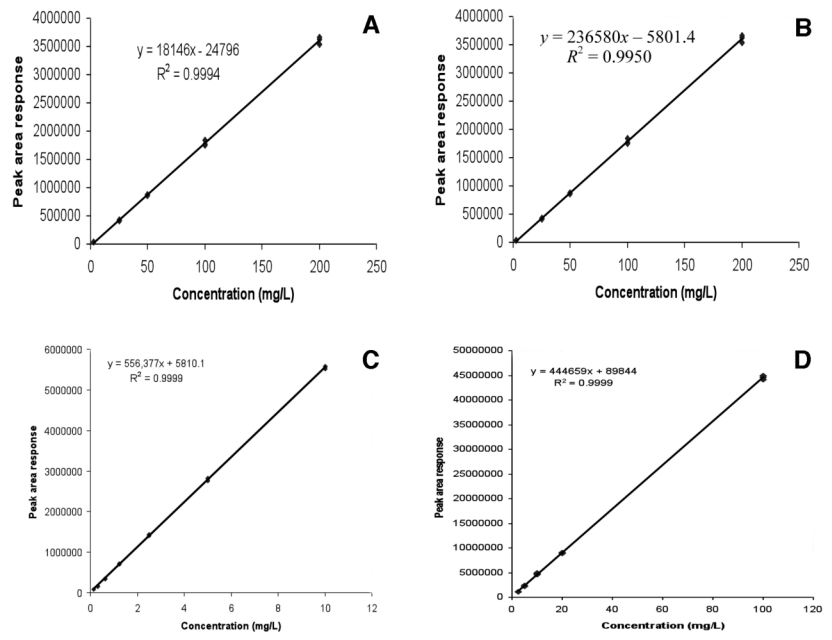

Figure 2. Linear relationship between peak area response and concentration of (A) clausenidin, (B) *O*-methylmukonal, (C) 3-formyl-2,7-dimethoxycarbazole, and (D) clauszoline-J.

Experimental

Materials and chemicals

The rhizomes and roots of *C. excavata* were collected in October 2004 from Trat and Rayong provinces, December 2004 from Chanthaburi province, February 2005 from Sisaket, Chiangmai, and Lampang provinces, March 2005 from Kanchanaburi province, April 2005 from Sakonnakhon province, June 2005 from Chachoengsao province, and July 2005 from Chumporn province. They have been deposited as a voucher specimen, Ngampong Kongkathip-1 (BK63423), at the Bangkok Herbarium (Department of Agriculture,

Table II. Percentage of Four Anti-HIV-1 Constituents in *Clausena excavata* Extracts from Ten Different Sources in Thailand by HPLC Methods

Source	Clausenidin (g % dry wt.)*	*O*-Methylmukonal (g % dry wt.)*	3-Formyl-2,7-dimethoxycarbazole (g % dry wt.)*	Clauszoline-J (g % dry wt.)*
Lampang (Northern)	0.08720 ± 0.00150	ND†	0.00255 ± 0.00011	0.01089 ± 0.00029
Trat (Eastern)	0.03381 ± 0.00090	0.0005 ± 0.00006	0.00119 ± 0.00014	0.00890 ± 0.00021
Chachoengsao (Central)	0.03355 ± 0.00078	0.00280 ± 0.00055	0.01322 ± 0.00051	0.06237 ± 0.00084
Chumporn (Southern)	0.02885 ± 0.00069	0.00177 ± 0.000214	0.00133 ± 0.00003	0.01166 ± 0.00028
Rayong (Easernt)	0.01658 ± 0.00026	ND†	0.00015 ± 0.00001	0.00444 ± 0.00015
Sakonnakhon (Northeastern)	0.01156 ± 0.00008	0.00612 ± 0.00040	0.00897 ± 0.00015	0.04592 ± 0.00092
Chiangmai (Northern)	0.01022 ± 0.00004	0.00057 ± 0.00005	0.00138 ± 0.00003	0.02166 ± 0.00021
Kanchanaburi (Central)	0.00953 ± 0.00006	ND†	0.00069 ± 0.00001	0.00665 ± 0.00007
Sisaket (Northeastern)	0.00693 ± 0.00004	0.00571 ± 0.00029	0.01888 ± 0.00028	0.09301 ± 0.00059
Chanthaburi (Eastern)	0.00611 ± 0.00004	0.00032 ± 0.000019	0.00047 ± 0.00001	0.00371 ± 0.00005

* Average of three injections. † ND = Not Detected.

Bangkok, Thailand). Pure clausenidin, *O*-methylmukonal, 3-formyl-2,7-dimethoxycarbazole, and clauszoline-J were from our laboratory, Natural Products and Organic Synthesis Research Unit (NPOS, Bangkok, Thailand). All solvents/chemicals used were of AR/HPLC-grade and obtained from Merck (Bangkok, Thailand).

Instrumentation and chromatographic conditions

The HPLC system consists of a Constametric 4100 Series Quaternary Solvent Delivery system, model PC 1000 fitted with a C_{18} column (300 × 3.90 mm i.d., 10 µm) (Phenomenex, Torrance, CA) with UV variable-wavelength detector used at wavelengths of 254, 274, 298, 242 nm. (Department of Chemistry, Faculty of Science, Kasetsart University, Thailand). The HPLC chromatographic condition of each pure compound is summarized in Table I.

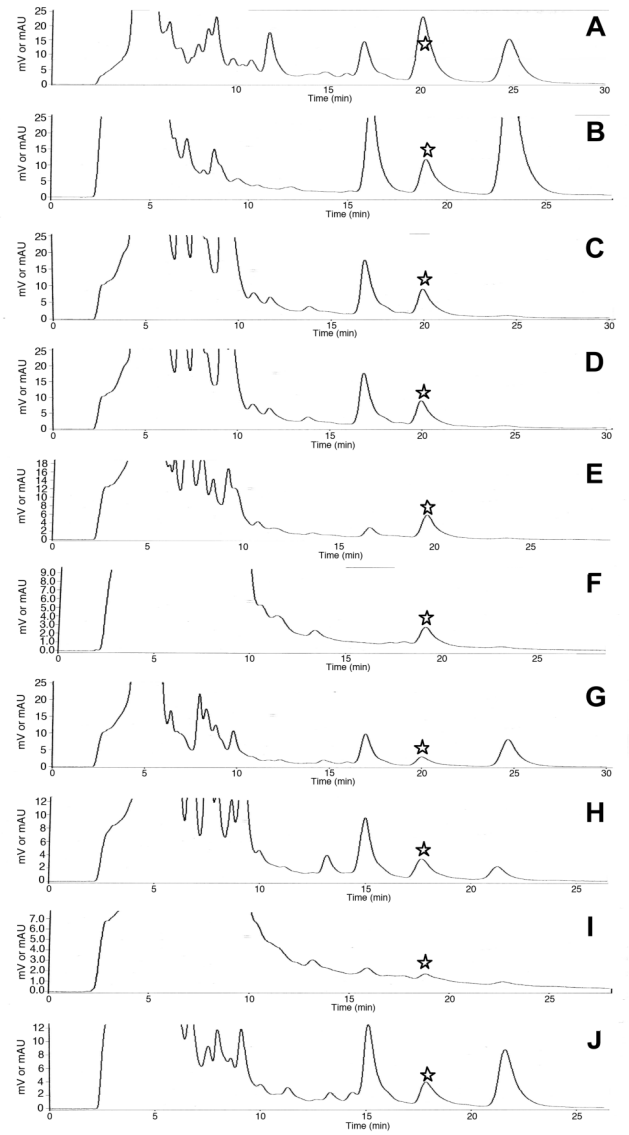

Figure 3. The HPLC Chromatograms of *C. excavata* crude extracts from ten provinces: (A) Lampang, (B) Trat, (C) Chachoengsao, (D) Chumporn, (E) Rayong, (F) Sakonnakhon, (G) Chiangmai, (H) Kanchanaburi, (I) Sisaket, and (J) Chanthaburi. Star denotes clausenidin peak.

Sample and calibration curve preparation

The rhizomes and roots of *C. excavata* (100 g) were chopped into small pieces, dried and ground finely, then macerated in 35% EtOH in water for two weeks. Monitoring four active compounds in the extract every 4 h for 12 h and every three days in three weeks by HPLC, it was found that the concentration of the active compounds increased and two-week maceration time is optimum for obtaining the most increased amount of the active compounds. After two-week maceration, the solution was filtered off and evaporated in vacuo to give a crude extract. The yield of the extracts from Lampang, Trat, Chachoengsao, Chumporn, Rayong, Sakonnakhon, Chiangmai, Kanchanaburi, Sisaket, and Chanthaburi were 6.27, 5.35, 6.66, 6.28, 6.89, 8.38, 6.02, 4.94, 12.24, and 5.04 (g % of dried plant), respectively.

The crude extracts (0.050 g) from the locations mentioned previously were each transferred to a 10-mL volumetric flask,

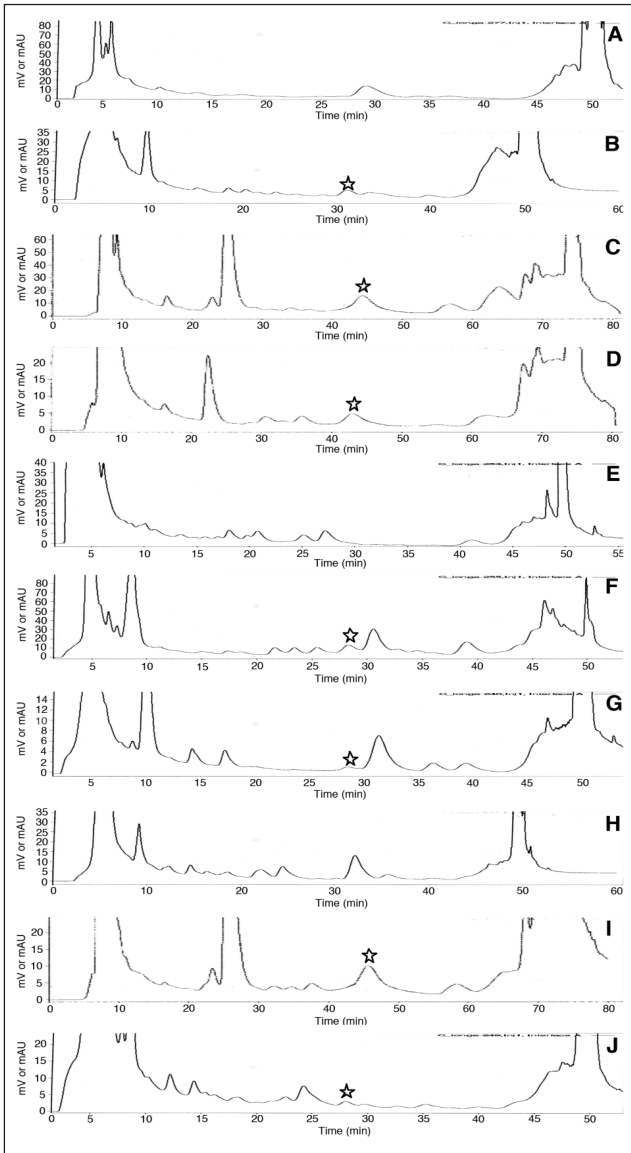

Figure 4. The HPLC Chromatograms of *C. excavata* crude extracts from ten provinces: (A) Lampang, (B) Trat, (C) Chachoengsao, (D) Chumporn, (E) Rayong, (F) Sakonnakhon, (G) Chiangmai, (H) Kanchanaburi, (I) Sisaket, and (J) Chanthaburi. Star denotes *O*-methylmukonal peak.

and the volume was adjusted to 10 mL with methanol.

The linearity of the method was evaluated by analyzing a series of standard solutions of four pure compounds. Ten microliters of each of the five working standard solutions of four pure compounds was injected into the HPLC. Elution was carried out as described in Table I, and a standard calibration curve was obtained by plotting each concentration of four pure compounds versus its respective peak area (Figure 2). The calibration range was chosen to calculate the four pure compounds concentration in *C. excavata* samples.

Determination of four pure compounds in samples

The sample volume was 10 µL. Four pure compounds concentration was calculated on the basis of the linear calibration function. The content of four pure compounds was expressed as grams per 100 g of dried plant material.

Results

Our analysis of the four pure compounds (clausenidin, O-methylmukonal, 3-formyl-2,7-dimethoxycarbazole, and clauszoline-J) by HPLC showed a single peak at the retention time of 19.23 ± 0.27 min, 24.91 ± 3.10 min, 25.74 ± 0.32 min, and 18.73 ± 0.71 min, respectively. Each calibration curve was prepared to determine each pure compound content in the crude samples. This was derived from three injections of five concentrations of four pure compounds versus the peak areas response. Linearity of clausenidin, *O*-methylmukonal, 3-formyl-2,7-dimethoxycarbazole, and clauszoline-J of the dose-response curve were observed in this concentration range with the linear regression equation: $y = 18{,}146x - 24{,}796$, $y = 236{,}580x - 5{,}801.4$, $y = 556{,}377x + 5{,}810.1$, and $y = 444{,}659x + 89{,}844$, respectively, and a very high coefficient of determination (R^2) value at 99.94%,

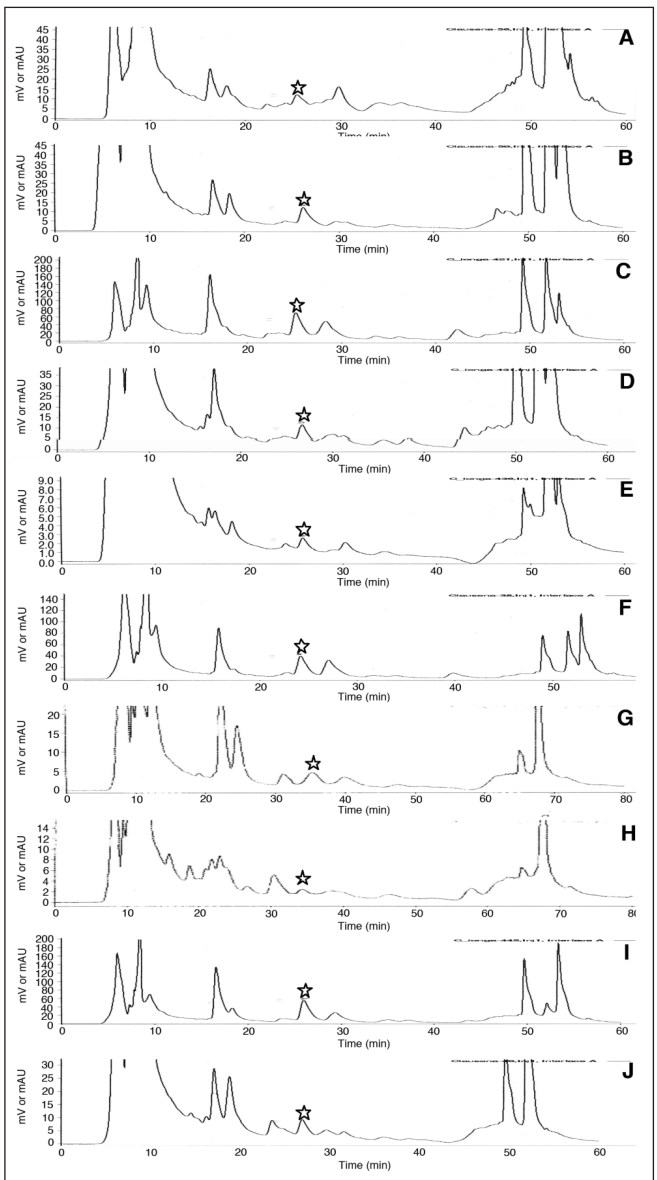

Figure 5. The HPLC Chromatograms of *C. excavata* crude extracts from ten provinces: (A) Lampang, (B) Trat, (C) Chachoengsao, (D) Chumporn, (E) Rayong, (F) Sakonnakhon, (G) Chiangmai, (H) Kanchanaburi, (I) Sisaket, and (J) Chanthaburi. Star denotes 3-formyl-2,7-dimethoxycarbazole peak.

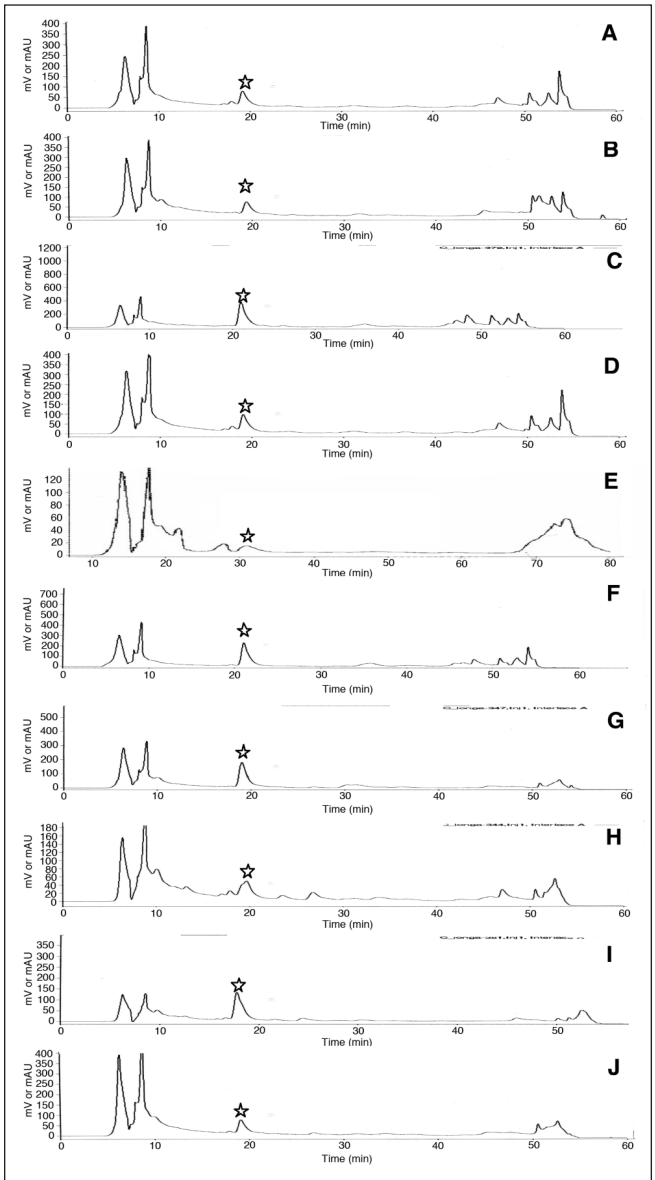

Figure 6. The HPLC Chromatograms of *C. excavata* crude extracts from ten provinces: (A) Lampang, (B) Trat, (C) Chachoengsao, (D) Chumporn, (E) Rayong, (F) Sakonnakhon, (G) Chiangmai, (H) Kanchanaburi, (I) Sisaket, and (J) Chanthaburi. Star denotes clauszoline-J peak.

99.50%, 99.99%, and 99.99%, respectively (Figure 2). The identity of each peak was confirmed by determination of retention time and by spiking with standard.

The contents of four pure compounds in ten different varieties of *C. excavata* by HPLC are summarized in Table II. Figure 3–6 show the HPLC Chromatograms of the peak for four compounds (clausenidin, *O*-methylmukonal, 3-formyl-2,7-dimethoxycarbazole, and clauszoline-J, respectively) in the *C. excavata* extracts from ten various sources in Thailand.

The quantitative analysis of four anti-HIV-1 constituents in the ten various sources in Thailand exhibited the contents of clausenidin in the extracts, estimated from the calibration curve, expressed as $8.72 \times 10^{-2} \pm 1.5 \times 10^{-3}$ to $6.11 \times 10^{-3} \pm 4.0 \times 10^{-5}$ g % of dry wt. whereas the contents of *O*-methylmukonal were $6.12 \times 10^{-3} \pm 4.0 \times 10^{-4}$ to $3.2 \times 10^{-4} \pm 1.9 \times 10^{-5}$ g % of dry wt. The contents of 3-formyl-2,7-dimethoxycarbazole in the extracts were $1.89 \times 10^{-2} \pm 2.8 \times 10^{-4}$ to $1.50 \times 10^{-4} \pm 1.0 \times 10^{-5}$ g % of dry wt. The content of clauszoline-J were $9.30 \times 10^{-2} \pm 5.9 \times 10^{-4}$ to $3.71 \times 10^{-3} \pm 5.0 \times 10^{-5}$ g % of dry wt.

Discussion

The quantitative analysis of clausenidin, *O*-methylmukonal, 3-formyl-2,7-dimethoxycarbazole, and clauszoline-J by HPLC has not been previously reported. Because we have isolated four anti-HIV-1 compounds from the rhizomes and roots of *C. excavata,* we have first tried to analyze these compounds in the crude extracts by HPLC without purification using gradient elution composed of water, methanol, and acetonitrile in various proportions. It was found that this mobile phase was not suitable for separation of all the compounds. There is a report (11) to analyze furanocoumarins by HPLC using 60% MeOH–H_2O as optimal mobile phase. So the mobile phase composed of water and methanol in various proportions were tried for analyzing clausenidin, a pyranocoumarin. The result showed that when using 30%H_2O–MeOH as the mobile phase, good separation of clausenidin was achieved. In the same manner, this mobile phase system was tried to analyze the other three active compounds. It was found that 50%H_2O–MeOH was suitable for *O*-methylmukonal analysis but not for the others. The other eluent systems (various proportions of water and acetonitile) were also tried for 3-formyl-2,7-dimethoxycarbazole, and clauszoline-J, but the results did not show good separation. Then the mobile phase composed of various proportions (0.1% HCOOH)–CH_3CN was used, and it was found that gradient eluent composed of 60% (0.1% HCOOH)–CH_3CN is optimal for 3-formyl-2,7-dimethoxycarbazole and clauszoline-J (Table I).

Table II showed the amount of the four anti-HIV-1 compounds in the *C. excavata* extract from ten different sources in Thailand by HPLC. For the content of clausenidin, the crude extract from Lampang province showed the highest amount, while the crude extract from Chantaburi province has the lowest amount. For the content of *O*-methylmukonal, the crude extract from Sakonnakhon province exhibited the most amount, while the crude extract from Chantaburi province has the lowest amount. The crude extract from Sisaket province possessed the most amount of 3-formyl-2,7-dimethoxycarbazole and clauszoline-J, while the crude extract from Rayong province has the least amount of 3-formyl-2,7-dimethoxycarbazole. The crude extract from Chantaburi province has the least amount of clauszoline-J.

Conclusion

It is concluded that the amount of four anti-HIV-1 constituents, clausenidin, *O*-methylmukonal, 3-formyl-2,7-dimethoxycarbazole, and clauszoline-J, in the *C. excavata* extracts could be analyzed by HPLC with optimal condition. The extract from different location showed different amounts of these four compounds. Thus the HPLC quantitative analysis of clausenidin, *O*-methylmukonal, 3-formyl-2,7-dimethoxycarbazole, and clauszoline-J could be indicators for the presence of anti-HIV-1 activity in the crude extracts of *C. excavata* from any location of Thailand and should be very useful for the preparation of folklore medication for AIDS therapy. This report can be used for routine quality control in the *C. excavata* extracts.

Acknowledgments

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