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Research article

Isolation and high-performance liquid chromatography analysis of chettaphanin I in extracts from *Cladogynos orientalis* roots

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Abstract

Importance of the work: *Cladogynos orientalis* Zipp. ex Span. is a Thai traditional plant that has been used for antiflatulence and anti-stomach pain effects.

Objectives: To isolate and quantitative analysis of chettaphanin I in *C. orientalis* root extracts and fractions.

Materials & Methods: Chettaphanin I was isolated from the root extract of *C. orientalis* using chromatographic techniques and structurally identified using spectroscopic and spectrometric techniques. A high-performance liquid chromatography (HPLC) method was developed and validated for the quantitative analysis of chettaphanin I in the root extracts and fractions from this plant.

Results: It was found that the root ethanol extract contained a higher amount of chettaphanin I than the root decoction extract (2.97 ± 0.14% weight per weight (w/w) and 1.82 ± 0.12% w/w, respectively, in the dried extract). Using a solvent-solvent extraction process from the root decoction extract, the obtained aqueous fraction contained a significant amount of chettaphanin I (6.19 ± 0.36% w/w of dried extract) while the dichoromethane fraction contained a very low amount.

Main finding: Chettaphanin I was a compound mainly found in the roots of *C. orientalis* which could be used as a marker for the quality control of raw materials and root extracts of this plant. HPLC method was developed and validated for quantitative analysis of chettaphanin I.

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Introduction

Cladogynos orientalis Zipp. ex Span. or *Croton crassifolius* Geisel., which is called 'chettaphangki' in Thai, is a plant in the Euphorbiaceae family, of which the roots have been listed in Thailand National List of Essential Medicines 2018, as a component in a formulation to treat flatulence and colic (Ministry of Public Health, 2018). The whole plant of *C. orientalis* exhibited anti-dengue virus properties (Klawikkan et al., 2011) while the leaf extract provided effective inhibition of human hepatocarcinoma (HepG2) (Machana et al., 2011). The leaf, stem and root extracts of this plant showed a low 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging effect while the root and stem extracts also promoted low-to-intermediate antibacterial activity against *Staphylococcus intermedius* (Sithisarn et al., 2015). Ethanol extracts from the roots, stems, and leaves of *C. orientalis* exhibited anti-hepatitis C virus activities determined by an immunofluorescence assay, from which the leaf extract was found to inhibit NS5B expression (Thongsri et al., 2019).

Terpenoid compounds, including ent-halimane diterpenes such as chettaphanin I and chettaphanin II, sesquiterpenes such as 8-hydroxy-alpha-guaiene, spathulenol, cyperenoic acid, and triterpenes such as taraxerol and acetoxyaleuritolate have been previously reported from *C. orientalis* (Sato et al., 1970, 1971; Kanlayavattanakul et al., 2005; Liu et al., 2016; Yuan et al., 2017a) Some phenolic acids and flavonoids (including chlorogenic acid, isovitexin, apigenin glycosides, epicatechin, quercetin and rutin) and a coumarin (scopoletin) have been reported in *C. orientalis* (Kanchanapoom, 2007; Machana et al., 2011; Sithisarn et al., 2015). Unusual aromatic diglycosides, including 4′-O-galloyl-violutoside and 4′′-O-galloyl-benzyl-*O*-α-L-rhamnopyranosyl-(1→6)-*β*-D-glucopyranoside) have been found in the aerial parts (Kanchanapoom, 2007). Clerodanoids, including crassins A–H, crassifolins A–G, crassifolius A–C, cracrosons E–H, were isolated from the roots of this plant (Wang et al., 2012; Tian et al., 2017; Yuan et al., 2017b; Qiu et al., 2018). In addition, halimane diterpenoid (crassifoliusin A), meroditerpenoids (norcrassin A and cracroson D) and sesquiterpenoids (crocrassins A and B) have been isolated from the root of this plant (Zhang et al., 2014; Yuan et al., 2017b; Qiu et al., 2018; Zhang et al., 2018). Pyran-2-one derivatives, including crotonpyrones A–C, were reported from the root of this plant (Li et al., 2014; Huang et al., 2016). Some diterpenes isolated from the roots of this plant, especially penduliflaworosin, showed antiangionic activity demonstrated by the inhibition of vessel formation on Tg (fli1a: EGFP)y1-type zebrafish embryos (Wang et al., 2016). Crassin H showed cytotoxic effects against the HL-60 and A549 cell lines (Yuan et al., 2017b), while crassifolius A promoted cytotoxic effects against the Hep3B and HepG2 cell lines (Tian et al., 2017) and Cracrosons D and E showed cytotoxic effects against the T24 and A549 cell lines (Qiu et al., 2018). Chettaphanin I, which was found in the roots and stems of this plant, was reported to promote low antioxidant activity with an inhibitory effect on *Staphylococcus intermedius* (Sithisarn et al., 2015). This compound was also reported in *Adenochlaena siammensis* Ridl (Euphorbiaceae) (Sato et al., 1970, 1971). Despite the pronounced activities of *C. orientalis* extracts, standardization of herbal drugs is an essential task for reproducible efficacy. Comprehensive HPLC fingerprint analysis, including determination of total phenolic and total flavonoid contents, have been developed in our previous work (Sithisarn et al., 2015). While it is practically impossible to take account of all constituents and the therapeutic components have not been obtained, the most abundant ones in a herbal material could be used for qualitative and quantitative assessment (Li et al., 2008). Therefore, a simple and rapid analysis method for the quantitation of chettaphanin I is needed, as a major constituent in *C. orientalis*. The present study was conducted to isolate and identify chettaphanin I from the root extract of *C. orientalis*. An HPLC method was developed and validated for quantitative analysis of this compound in the root extract of *C. orientalis*. The present study could provide basic information for quality control and standardization of raw materials and root extracts of *C. orientalis*.

Materials and Methods

Plant materials and extraction

The roots of *C. orientalis* were purchased in Muang district, Nakhon Phanom province, Thailand, in October 2013. Plant samples were identified according to their botanical and taxonomical characteristics using the identification key presented in Flora of Thailand (Santisuk and Larsen, 2011). The plant samples were cleaned and dried in hot-air oven (50°C) for 6 hr and powdered using an electronic mill (20-mesh sieve). Then, the extract was prepared using the following process:

Decoction

Dried root powder of *C. orientalis* was boiled (80°C) with distilled water (plant-to-water ratio 1:10 weight per volume, w/v) for 3 hr and then filtered. The extraction process was repeated twice. The filtrates were combined and dried using

a freeze-drying machine (SciQuip Ltd., UK) to obtain the dried root decoction extract (CORD).

Soxhlet extraction using 95% ethanol

The dried root powders of *C. orientalis* were extracted with 95% ethanol (plant/water ratio 1:10 w/v) using a Soxhlet apparatus at 70°C until the extraction was completed (28 hr). The extract solution was then dried using a water bath to obtain the dried root ethanol extract (CORE).

Solvent-solvent extraction of dried root decoction extract

A sample (10 g) of CORD was fractionated using a solvent-solvent extraction technique with distilled water and dichloromethane (extract-to-each solvent ratio 1:10 w/v) for 30 min. The fractionation process was repeated twice. The aqueous and dichloromethane fractions were separately combined. Each fraction was dried using a water bath to yield a dried aqueous fraction from the root decoction extract (CORDA) and a dichloromethane fraction from the root decoction extract (CORDD).

Chemicals and reagents

HPLC grade methanol and dichloromethane were obtained from Labscan (Thailand). Deionized water was purified using an Ultra Clear™ system (Siemens Water Technologies Corp.; Germany) Commercial ethanol was distilled before use. All reagents were of analytical grade, if not stated otherwise.

Isolations of chettaphanin I

The root decoction extract of *C. orientalis* (20 g) was re-extracted using methanol. The insoluble part was discarded and the remaining solution was dried under vacuum using a rotary evaporator to yield 6.15 g of the extract. Then, the extract was continuously separated using conventional column chromatography (Merck silica gel 60, 70–230 mesh) with hexane, ethyl acetate, and methanol at increasing polarity (approximately 30–50 mL for each fraction). The fractions were monitored using thin layer chromatography (TLC) detected with an anisaldehyde sulfuric acid spray reagent. The fractions eluted with ethyl acetate and the mixture of ethyl acetate and methanol were combined. The combined fraction (658 mg) was subjected to column chromatography (Merck silica gel 60, 70–230 mesh) and eluted with hexane, ethyl acetate, and methanol at increasing polarity (approximately 30–50 ml for each fraction). The fraction eluted with hexane-ethyl acetate (40:60 to 30:70, v/v) yielded 229 mg of the compound. Structural elucidation of the isolated compound was done using spectroscopic and spectrometric techniques, consisting of nuclear magnetic resonance spectroscopy (NMR), and mass spectrometry (MS). The purity was assessed by TLC and HPLC.

High performance liquid chromatography apparatus and conditions

HPLC was performed on an Agilent 1260 Series (Agilent Technologies; USA) equipped with a 1260 Quat pump VL quaternary pump, 1260 ALS autosampler, 1260 TCC column thermostat and 1260 DAD VL diode array detector (DAD). A Hypersil BDS-C18 column (4.6mm internal diameter × 15 cm, 3.5 µm) was used for the quantitative analysis. A gradient mobile phase system was performed with water (solvent A) and methanol (solvent B) at a flow rate of 1 mL/min. The gradient program was adjusted from 50% to 100% solvent B during 12 min and kept at 100% solvent B for 8 min. The column was equilibrated using 100% solvent A for 10 min via a prior injection. The column temperature was 25°C with an injection volume of 5 µL. Ultraviolet detection was performed at 248 nm.

Stock and working solutions of standard compound

Stock standard solution of chettaphanin I, with purity of more than 95% as determined by HPLC, was prepared by accurately weighing and dissolving it in methanol to obtain a concentration of 1,000 µg/mL.Working standard solutions were obtained by the appropriate dilutions of the stock solution using methanol to obtain the desired concentration.

Method validation

Validation of the method was done according to the International Conference on Harmonization guidelines (ICH 1996/2005). The method was validated for linearity, precision, accuracy, limit of detection (LOD) and limit of quantitation (LOQ).

Linearity

The linearity of the method was evaluated by analyzing a series of varying concentrations of chettaphanin I. Samples (each 5 µL) of the seven standard solutions containing 1.56–100 µg/mL of chettaphanin I were analyzed using HPLC. The standard curve of chettaphanin I was obtained by plotting the concentrations of the standard versus the peak areas. Then, the slope and intercept values were determined. The correlation coefficient was calculated using the least-square linear regression method. Peak purity was investigated using the DAD.

Precision

The intra-day precision was performed by analyzing the chettaphanin I at a concentration of 20 µg/mL on the same day (n = 7). Inter-day precision was carried out in the same manner as the intra-day precision but on three different days (n = 3). The relative standard deviation (RSD) was calculated.

Accuracy

The accuracy of the HPLC method was evaluated using the recovery of chettaphanin I in the *C. orientalis* root extracts. Chettaphanin I at three different concentrations (50 µg/mL, 100 µg/mL and 150 µg/mL) that were spiked into the *C. orientalis* root extracts and analyzed under the optimized HPLC conditions. Then, the percentages of recovery were calculated.

Limit of detection and limit of quantitation

Determination of the signal-to-noise ratio was calculated under the proposed chromatographic condition. LOD was consider as 3:1 and LOQ as 10:1.

Statistical analysis

The data on the chettaphanin I contents in *C. orientalis* roots were analyzed using one-way analysis of variance. Then the least significant difference was used to compare means. The tests were considered significant when $p < 0.05$. All analyses were performed using the SPSS for Windows software package (version 16.0; SPSS Inc.; USA).

Results and Discussion

Dried root powder of *C. orientalis* was extracted using different methods and solvents, namely water decoction and Soxhlet extraction with 95% ethanol. The dried root decoction extract was fractionated using dichloromethane and water that yielded a dried aqueous fraction from the root decoction extract and a dichloromethane fraction from the root decoction extract. The yields of the obtained extracts and fractions are shown in Table 4. In order to get an overview of characteristic compound profiles, comparative HPLC analyses were carried out of the crude extracts of the root of *C. orientalis* from different extractions. Then, isolation of the major compound was undertaken, yielding chettaphanin I (Fig. 1). Its structure was elucidated using NMR and MS analyses and the spectroscopic data were compared with published data (Sato et al., 1970, 1971; Marcos et al., 2003; Kanlayavattanakul, 2004; Kanlayavattanakul et al., 2005) as follows:

Chettaphanin I ($C_{21}H_{26}O_6$, M.W. 374.43 g/mol).

HRMS (ESI-QTOF), m/z (% rel. intensity), $[M+Na]^+$ = 397.1617 (*calc.* 397.1627).

^{1}H NMR (400 MHz, $CDCl_3$), δ (ppm) = 8.01 (s, 1H, Furan-*H*-H16), 7.43 (s, 1H, Furan-*H*-H15), 6.66 (s, 1H, Furan-*H*-H14), 5.85 (s, 1H, C*H*=C), 3.72 (s, 3H, OCH_3), 3.29 (d, J_{AB} = 18.9 Hz, 1H, C*H*H), 3.15 (d, J_{AB} = 18.9 Hz, 1H, CH*H*), 2.72 (d, J_{AB} = 17.2 Hz, 1H, C*H*H), 2.56 (d, J_{AB} = 17.2 Hz, 1H, CH*H*), 2.41 (td, J = 14.0, 3.5 Hz, 1H, C*H*HCH$_2$), 2.26-2.16 (br. m, 1H, CH$_2$C*H*HCH,\), 2.02 (br. t, 14.5 Hz, 1H, CH*H*CHH), 1.89-1.65 (br. m, 1H, CH$_2$C*H*HCH, and 1H, O*H*), 1.53 (dd, J = 13.6, 3.2 Hz, 1H, C*H*CH$_2$), 1.39 (s, 3H, CH_3), 1.21 (s, 3H, CH_3), 0.91 (d, J = 6.8 Hz, 3H, CHCH_3).

^{13}C NMR (100 MHz, $CDCl_3$), δ (ppm) = 197.8 (*C*=O), 190.9 (*C*=O), 174.6 (O=*C*O), 167.5 (*C* = CH), 146.3, 144.1, 128.0, 125.7, 108.4, 72.8, 53.0, 52.4, 47.9, 43.3, 41.3, 35.3, 31.9, 25.9, 25.1, 19.5, 16.8.

An HPLC method was developed for analysis of the contents of chettaphanin I in the *C. orientalis* extract. Then, optimization of the mobile phase compositions was undertaken. A reversed phase C18 column, which is generally used in pharmaceutical analysis, was used in this study. From the various mobile phases trialed, the system containing a gradient system using water and methanol provided the most symmetrical peaks and the most efficient separation and speed. A wavelength of 248 nm, yielding the maximum absorbance capacity, was used for detection.

The validation for the quantitative analysis of chettaphanin I was conducted as described above. The correlation coefficient (R^2) value was 0.9998 for the linearity curve (linear equation of Y = 6.7464X + 5.9295 with a linear range of 1.56–100 µg/mL) of chettaphanin I (Table 1), confirming the linearity of the method. Peak purity was investigated using the DAD and there was no indication of co-elution or impurities.

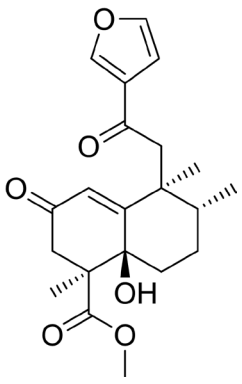

Fig. 1 Chemical structure of chettaphanin I

Table 1 Method validation parameters for quantification of chettaphanin I

Parameter	Result
Regression equation	Y = 6.7464X+5.9295
Correlation of determination	0.9998
Linear range, µg/mL	1.56–100
LOD, µg/mL	0.20
LOQ, µg/mL	0.60

LOD = limit of detection; LOQ = limit of quantitation; X = concentration of standard in micrograms per milliliter; Y = peak area at 248 nm.

For precision, the intra-day precisions (% RSD) of chettaphanin I at days 1, 2 and 3 were 0.53, 1.36 and 0.66%, respectively (average 0.85 ± 0.45%). The inter-day precision (%RSD) of chettaphanin I was 1.23 (Table 2). Therefore, this method could be regarded as precise. Accuracy was tested based on the percentages of recovery of chettaphanin I at the spiked concentrations of 50, 100 and 150 µg/mL having values of 100.14 ± 0.61%, 99.71 ± 2.15% and 100.25 ± 0.18%, respectively, with an average percentage of recovery of 100.03 ± 0.01% (Table 3). The results were in compliance with the acceptable recovery limit of AOAC guidelines for dietary supplements and botanicals (95–102%) (Association of Official Analytical Chemists, 2019). Therefore, this HPLC method was accurate for the analysis of this compound in *C. orientalis* root extracts. The LOD and LOQ values of chettaphanin I were 0.20 µg/mL and 0.60 µg/mL (Table 1), respectively, indicating the high sensitivity of the method.

Table 2 Intra-day and inter-day precisions of chettaphanin I (% relative standard deviation)

Compound	Intra-day			Inter-day
	Day 1	Day 2	Day 3	
Chettaphanin I	0.53	1.36	0.66	1.23

Table 3 Recovery study of chettaphanin I

Level	Theoretical[a] (µg/mL)	Found[b] (µg/mL)	Recovery[b] (%)
1	22.55	22.58±0.15	100.14±0.61
2	31.25	31.16±0.39	99.71±2.15
3	38.03	38.13±0.03	100.25±0.18
	Average		100.03±0.01

[a] = theoretical value which is the amount calculated based on original amount plus spiked amount;

[b] = expressed as mean ± SD (n = 3)

The HPLC method was successfully used for the quantitative analysis of chettaphanin I in extracts from *C. orientalis*. The HPLC chromatograms of chettaphanin I and the extract from roots of *C. orientalis* are shown in Fig. 2. The DAD absorbance spectrum of the compound from the peak at retention time 5.6 min showed λ_{max} at 248 nm, corresponding to the chemical characteristics of chettaphanin I (Fig. 2F), which in turn corresponded to a report on the presence of chettaphanin I in *C. orientalis* root extracts (Sithisarn et al., 2015). The content of chettaphanin I in *C. orientalis* extracts is shown in Table 4. The chettaphanin I content in the root ethanol extract (CORE) was higher than the content in the root decoction extract (CORD) (2.97% w/w and 1.82% w/w, respectively, of dry extract). However, the aqueous fraction obtained from the solvent-solvent extraction process of the decoction extract (CORDA) contained quite a high amount of chettaphanin I (6.19% w/w of dry extract) while the dichloromethane fraction (CORDD) contained a very low amount (lower than 0.06 µg/mL). Chettaphanin I was tested for cytotoxic activity toward a human, small-cell, lung-cancer cell line (NCI-H187) and for antituberculosis activity using the Mycobacterium tuberculosis $H_{37}Ra$ but it showed no significant cytotoxicity (IC50 < 5 µg/mL) nor antituberculosis activity (MIC < 12.5 µg/mL) (Kanlayavattanakul et al., 2005). In addition, this compound had exhibited an inhibitory effect against *Staphylococcus intermedius*, with a low antioxidant effect determined using a DPPH scavenging assay and also that both the decoction and ethanol extracts from various parts of *C. Orientalis* (leaves, roots and stems) showed somewhat similar antioxidant effects, with rutin as the active compound (Sithisarn et al., 2015). However, the antibacterial effects against *S. intermedius* were found in the root and stem extracts, which were reported to contain chettaphanin I, while this compound was not found in the leaf extracts, which promoted no antibacterial effect against *S. intermedius* (Sithisarn et al., 2015). The zones of inhibition of the root extracts and fractions against *S. intermedius* were in the range 8–14 mm, while the zones of inhibition of chettaphanin I were in the range 7–9 mm (Sithisarn et al., 2015). From all the information, it could be suggested that chettaphanin I is a major compound and could play an important role in the antibacterial effects of the root extracts of C. *orientalis*. This compound could be used as a marker for the quality control of raw materials and extracts from the roots of *C. orientalis*.

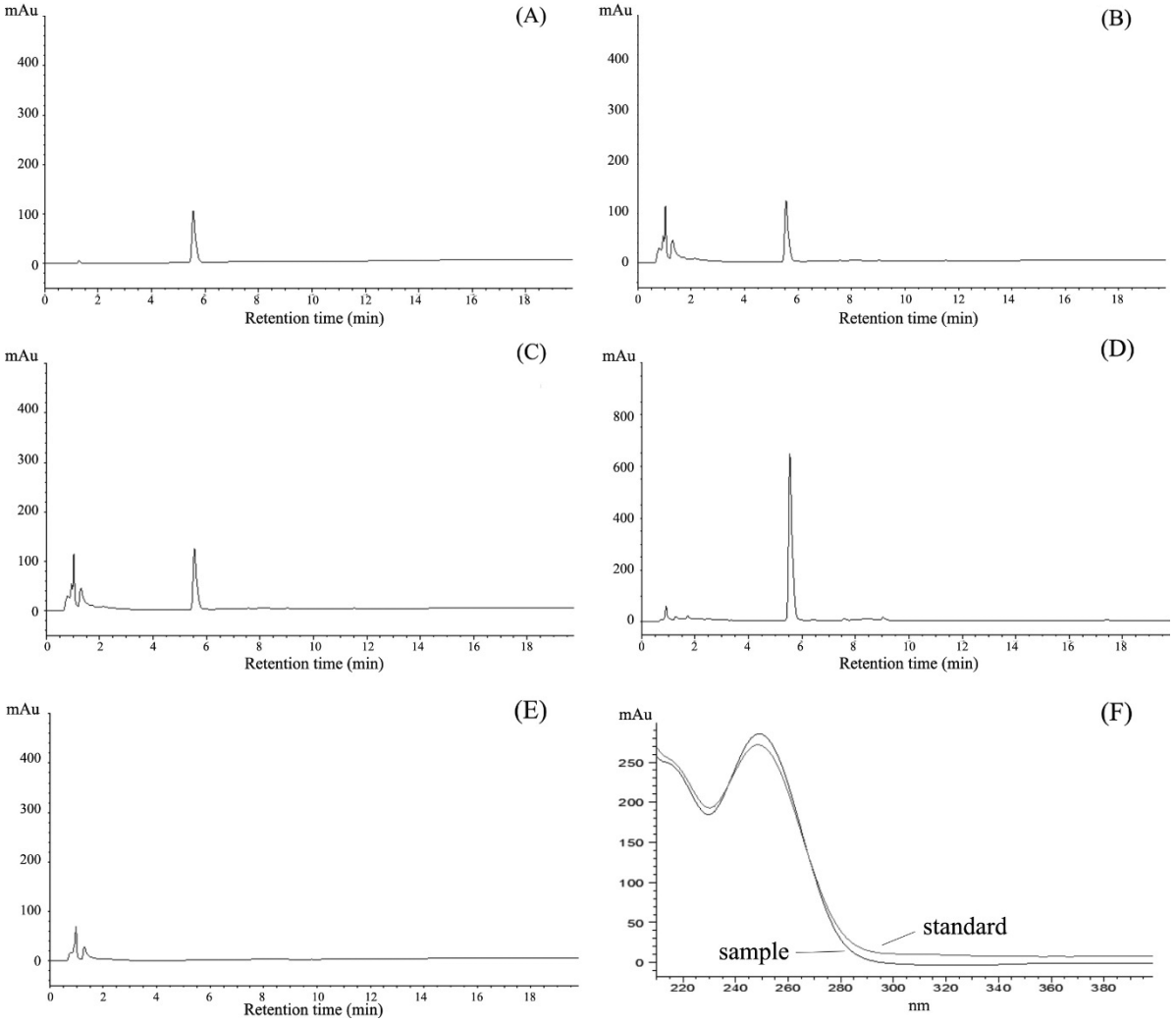

Fig. 2 High performance liquid chromatography chromatograms of: (A) chettaphanin I standard; (B) root decoction extract of *Cladogynos orientalis*; (C) root ethanol extract of *C. orientalis*; (D) aqueous fraction from root decoction extract of *C. orientalis*; (E), dichloromethan fraction from root decoction extract of *C. orientalis*; (F) ultraviolet spectral comparison of peak chettaphanin I standard and sample, where peak identification at retention time of 5.6 min = chettaphanin I

Table 4 Quantitative analysis of chettaphanin I contents in *Cladogynos orientalis* extracts

Sample	% Yield extract (g/100 g dried plant)	Amount of chettaphanin I	
		g/100 g dried extract	g/100 g dried plant
CORD	5.52	1.82±0.12[a]	0.101±0.006[a]
CORE	4.50	2.97±0.14[b]	0.133±0.006[b]
CORDA	0.88	6.19±0.36[c]	0.054±0.003[c]
CORDD	4.64	< 0.06	< 0.003

CORD = root decoction extract; CORE = root ethanol extract; CORDA = aqueous fraction from the root decoction extract; CORDD = dichloromethane fraction from root decoction extract.

Mean ± SD in the same column superscripted with different lowercase letters are significantly ($p < 0.05$) different.

In conclusion, chettaphanin I was isolated and identified from the root decoction extract of C. *orientalis*. The HPLC method was developed and validated for the quantitative analysis of chettaphanin I in the root extracts and fractions from C. *orientalis*. The ethanol root extract contained higher amounts of chettaphanin I than the decoction extract. However, by using the solvent-solvent extraction process from the root decoction extract, the obtained aqueous fraction was found to contain a significant amount of chettaphanin I (approximately 6% w/w of dried extract). This is the first report of the quantitative analysis of this compound in plant extracts using the HPLC method. Chettaphanin I could be used as a marker for quality control and the standardization of raw materials and root extracts in the future.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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