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Preparative isolation and purification of amides from the fruits of *Piper longum* L. by upright counter-current chromatography and reversed-phase liquid chromatography

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Abstract

A versatile counter-current chromatography (CCC) with upright type-J multilayer coil planet centrifuge, named upright CCC, was applied to the isolation and purification of amides from *Piper longum* L., which is widely used as an anodyne and a treatment for stomach disease in China. After the saponification by KOH of the ethanol extracts solution of 15 kg of crude drug "Piper Longi Fructus", the fruits of *P. longum* L., the solution was extracted with light petroleum and 500 g of red crude oil was obtained. Using 2.5 g of red crude oil as sample, the preparative upright CCC with a two-phase system composed of light petroleum (bp 60–90 °C)–ethyl acetate–tetrachloromethane–methanol–water (1:1:8:6:1, v/v) was successfully performed, which yielded nine fractions. Then these fractions were further purified by use of reversed-phase liquid chromatography (RPLC) with a glass column of 500 × 10 mm i.d. packed with reversed-phase silica gel. As a result, nine target amides with over 95% purity, i.e., 50 mg of (2*E*,4*E*)-*N*-isobutyl-eicosa-2,4-dienamide, 150 mg of (2*E*,4*E*,14*Z*)-*N*-isobutyl-eicosa-2,4,14-trienamide, 110 mg of (2*E*,4*E*,12*Z*)-*N*-isobutyl-ocatadeca-2,4,12-trienamide, 50 mg of guineensine, 60 mg of pipernonaline, 75 mg of pellitorine, 63 mg of piperine, 45 mg of piperanine, and 40 mg of piperlonguminine were isolated, respectively. Structures of all compounds were identified by electrospray ionization MS, electron impact ionization MS, one- and two-dimensional NMR spectra.

Keywords: Counter-current chromatography; *Piper longum*; Plant materials; Amides

1. Introduction

The genus *Piper*, belonging to the Piperaceae, has been received considerable attention in recent years because of its reputation of chemical and biological properties. Various *Piper* species, widely distributed in the tropical and subtropical regions of the world, have been used as a spice and also as a folk medicine [1,2]. These interesting properties prompted us to study the chemical components of piper species. *Piper Longum* L., a *Piper* species plant, is widely used as an anodyne and a treatment for stomach disease in China [3,4]. The extract of crude drug "Piperis Longi Fructus", the fruits of *P. longum* L., is frequently used as anti-inflammatory insecticidal agents [2,5,6]. Most of the earlier works on *Piper* species seem to suggest that the major insecticidal active components are amides [7–12].

The preparative separation and purification of amides from plant materials by conventional methods is tedious and usually requires multiple chromatography steps, such as gravity-field column chromatography (CC) and thin-layer chromatography (TLC). Furthermore, basic amides can suffer from peak tailing and poor efficiency on silica-based columns. Thus, it is very difficult to obtain high pure amides using traditional chromatography. Fortunately, owing to no complications caused by solid supports, such as adsorptive sample loss and deactivation, tailing of solute peaks, and contamination, counter-current chromatography (CCC) has been an area of intense research since the first introduction of CCC in 1970 [13], and various apparatus and broad applications have been advanced [14–16]. Recently, we have developed a versatile CCC method with upright type-J multilayer coil planet centrifuge, named upright CCC [17]. Thus, the primary fractionation of the red crude

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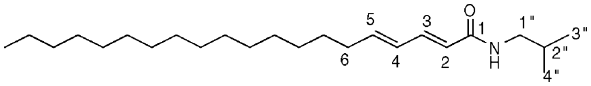
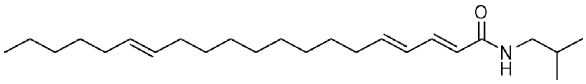
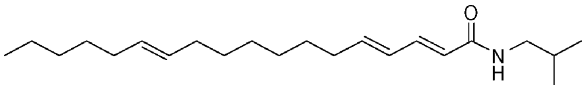
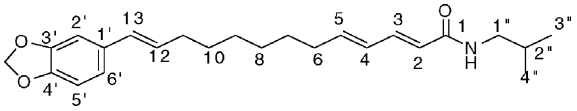
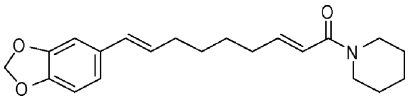
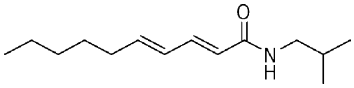
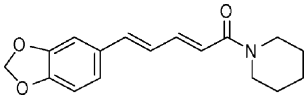
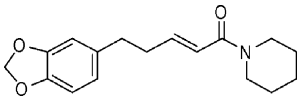
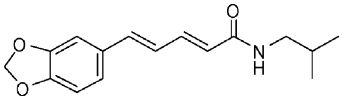
Table 1
Chemical structure of amides purified from *Piper longum* L.

Compound	Name	Chemical structure
1	(2*E*,4*E*)-*N*-Isobutyleicosa-2,4-dienamide	
2	(2*E*,4*E*,14*Z*)-*N*-Isobutyleicosa-2,4,14-trienamide	
3	(2*E*,4*E*,12*Z*)-*N*-Isobutylocatadeca-2,4,12-trienamide	
4	Guineensine	
5	Pipernonaline	
6	Pellitorine	
7	Piperine	
8	Piperanine	
9	Piperlonguminine	

oil from the fruits of *P. Longum* L. was performed with the upright CCC apparatus in order to preparation of nine target amides **1**–**9** (Table 1), which have various biological activities [4,7–12,18–23]. Consequently, these fractions obtained from upright CCC containing the target amides were respectively further purified by a simple reversed-phase liquid chromatography (RPLC). The purpose of this study, therefore, is to develop a simple and efficient method for the isolation and purification of the amides from the fruits of *P. longum* L. by CCC and RPLC.

2. Experimental

2.1. Apparatus

The CCC isolation and purification of amides from the fruits of *P. longum* L. was first performed by upright CCC. Its design principle and dimensions were described in the literature [17]. The upright CCC apparatus holds four identical multilayer coil columns in the symmetrical positions around the rotary frame at distance of 9 cm from the central axis of the centrifuge to maintain perfect balance of centrifuge system without the use of a counterweight. Each separation column was made by winding a single piece of 4 mm i.d. and 1 mm wall thickness polytetrafluroethylene (PTFE) tubing directly onto the holder hub of 5 cm diameter forming three layers of right-handed and left-handed coils alternating in each layer between a pair of flanges spaced 35 cm apart. The β-value (ratio of helical radius of the coil and revolution radius) of the multilayer coil varies from 0.28 at the internal terminal to 0.48 at the external terminal ($\beta = r/R$, where r is the distance from the coil to the holder shaft, and R, the revolution radius or the distance between the holder axis and central axis of the centrifuge). These multilayer coils are connected in series on the rotary frame using a flow tube (PTFE, 1.6 mm i.d. and 0.7 mm wall thickness) to give a total capacity of 1600 ml while the unique gear arrangement on the rotary frame establishes a twist-free mechanism of the flow tubes so that continuous elution can be performed without the use of rotary seal.

The apparatus can be operated up to maximum speed of 800 rpm with a speed Sunwind control unit (Shenduo Electric Corp., Shanghai, China) and up to 60 °C with a temperature control unit. In addition, this CCC system is equipped with a Type-J-W metering pump (Zhijiang

Petroleum Equipment, Hangzhou, China), a HD-9704 UV spectrometer operating at 254 and 280 nm, Shimadzu C-R1B Chomatopac recorder, BSZ-100 fraction collector, a sample injection valve with a 30 ml sample loop and NT2000 data analysis system (Institute of Automation Engineering, Zhejiang University, Hangzhou, China).

The fractions of upright CCC were further purified by a RPLC system, which employed a BT01-100 isocratic pump (Long-Pump Co., Baoding, China), a glass column of 500 × 10 mm i.d. packed with the reversed-phase silica gel (LiChroprep RP-18, 40–63 μm, Damstadt, Merck), a HD-9704 UV spectrometer operating at 254 and 280 nm, BSZ-100 fraction collector and NT2000 data analysis system. The bottom of the glass column is fitted with a flat fritted glass bed (1.5 mm layer) above the stopcock to prevent the silica gel from escaping from column through the stopcock. The top of the column is topped with 14/23 standard taper glass joint to connect with pump through a Teflon tube (1 mm i.d. and 0.5 mm wall thickness).

The high-performance liquid chromatography used was an Agilent 1100 system including a G1312A BinPump, G1314A variable-wavelength detector (VWD), a model 7725 injection valve with 20 μl loop, a PT100 column oven and an Agilent ShemStation for LC. The column used was a reversed-phase C_{18} column (YMC-PACK ODS-A column, 150 × 4.6 mm i.d., 5 μm, 120 Å).

2.2. Reagents

All organic solvents used for CCC and RPLC were of analytical grade and purchased from Huadong Chemicals, Hangzhou, China. Reverse osmosis Milli-Q water (18 MΩ) (Millipore, Bedford, MA, USA) was used for all solutions and dilutions. Methanol used for HPLC analysis was of chromatographic grade and purchased from Merck, Darmstadt, Germany.

The crude drug "Piperis Longi Fructus" was purchased from Huadong Medicinal Corp., Hangzhou, China. A voucher specimen with reference number 021109 is kept in the Institute of Organic and Pharmaceutical Chemistry, Zhejiang University, Hangzhou, China.

2.3. Preparation of crude sample

Dried and powdered crude drug "Piper Longi Fructus" (15 kg) was extracted three times with 95% ethanol. The ethanol extracts were combined and evaporated to dryness by a rotary evaporator (Shenshun Biotech. Corp., Shanghai) under reduced pressure of −0.1 MPa and 40 °C, which yielded 1.2 kg of red oil syrup. The syrup was allowed to stand to give crystals, which were recrystallised to yield 158 g 95% pure piperine, a major amide of *P. longum* L. The remaining extract was suspended in 15% KOH (2000 ml) and incubated at 60 °C for 60 min. The saponified solution was extracted with 6 l light petroleum (bp 60–90 °C) three times. The petroleum extract solutions were combined and washed with distilled water until the pH of solution approaches to neutral. The neutral light petroleum solution was concentrated under reduced pressure and 40 °C. As a result, 500 g red crude oil was obtained.

2.4. Preparation of two-phase solvent system and sample solutions

The two-phase solvent system used was composed of light petroleum–ethyl acetate–tetrachloro–methanemethanol–water at various volume ratios (8:6:1:1:1, 15:15:11:0:6, 15:10:0:2.5:2.5, 15:10:2.5:0:2.5, v/v). The solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and the two phases were separated shortly before use.

The sample solutions were prepared by dissolving 2.5 g red crude oil in 7.5 ml the upper phase and 7.5 ml lower phase.

2.5. CCC separation procedure

The preparative CCC was similarly performed with a common high-speed CCC instrument as follows: the four upright multilayer coiled columns connected in series were first entirely filled with upper phase as the stationary phase, and then 15 ml sample solution containing 2.5 g of red crude oil was injected through the sample port and the lower organic phase as mobile phase was pumped into the column at a flow rate of 4.0 ml min^{-1} while the column was rotated at desired rotary speed (100, 300, or 500 rpm). The effluent from the outlet of the column was monitored with a UV detector at 280 nm and automatically collected in 40 ml test tube per 5 min using a BSZ-100 fraction collector. Peak fractions were collected according to the elution profile, TLC, and HPLC detection.

Using the two-phase solvent system composed of light petroleum–ethyl acetate–tetrachloromethane–methanol–water at various volume ratios (8:6:1:1:1, v/v) at 300 rpm, the optimum upright CCC was performed, which yielded nine fractions I–IX containing compounds **1**–**9**, respectively.

2.6. RPLC separation

The fractions I–IX obtained from upright CCC were further purified by RPLC. The glass column packed with reversed-phase silica gel (500 × 10 mm, 40–63 μm, Merck) is first balanced with mobile phase at a flow rate of 2 ml min^{-1} for 1 h. The mobile phase is methanol and water. Its volume ratios changed with fraction samples in order to save solvents and time. For isolations of CCC fractions I–III, the mobile phase is methanol–water (90:10, v/v). For CCC fractions IV and V, the volume ratio of methanol and water is 80:20. For CCC fractions VI–IX, the ratio is 70:30. For each isolation, 100–250 mg of fraction sample was redissolved in mobile phase to form sample solution with the concentration of 300 mg ml^{-1}. After the column was

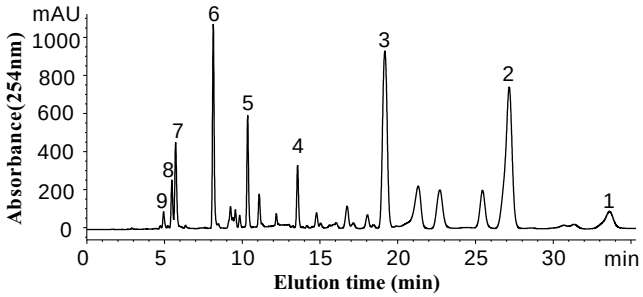


Fig. 1. Chromatogram of red crude oil from *P. longum* L. by HPLC analysis: (**1**) (2*E*,4*E*)-*N*-isobutyleicosa-2,4-dienamide, (**2**) (2*E*,4*E*,14*Z*)-*N*-isobutyleicosa-2,4,14-trienamide, (**3**) (2*E*,4*E*,12*Z*)-*N*-isobutyl-ocatadeca-2,4,12-trienamide, (**4**) guineensine, (**5**) pipernonaline, (**6**) pellitorine, (**7**) piperine, (**8**) piperanine, and (**9**) piperlonguminine. Conditions: column, YMC-Pack ODS-A (150 × 4.6 mm i.d., 5 μm, 120 Å); column temperature, 25 °C; mobile phase, solvent A (methanol) and solvent B (water) in a linear gradient: A from 70 to 90% and B from 30 to 10% for 10 min; flow rate, 1 ml min^{-1}; detection, 254 nm; sample concentration, 2 μg μl^{-1}; injection volume, 20 μl.

balanced by mobile phase, the top glass joint of the column is opened and the solvents above the reversed-phase silica gel were removed while the sample solution was added onto the top of column. Consequently, the mobile phase was pumped at a flow rate of 2 ml min^{-1}. The effluent from the outlet of the column was monitored with a UV detector at 254 nm and automatically collected in test tube with 12 ml capacity per 5 min using a fraction collector. Peak fractions were collected according to the elution profile and HPLC detection. After the target amide was collected, the column is eluted with the mobile phase of methanol–water (95:5, v/v) for 1.5 h to remove the other impurities.

2.7. Analysis and identification of CCC peak fractions and RPLC fractions

The crude sample and each peak fraction obtained from upright CCC and RPLC were analyzed by TLC and HPLC. TLC is run on glass supported silica gel F-254 plates (0.25 mm layer, Qingdao Marine Chemical Inc., Qingdao, China) and developed by solvent composed of light petroleum–ethyl acetate–acetone with volume ratio of 6:1:1. The HPLC analyses were performed with an YMC-Pack ODS-A column (150 × 4.6 mm i.d., 5 μm, 120 Å). The mobile phase was methanol and water at appropriate volume ratio as shown in each HPLC chromatogram. The flow-rate was 1.0 ml min^{-1}, and the effluent was monitored at 254 nm.

Identification of the CCC peak fraction was carried out by electron impact ionization (EI) MS, electrospray ionization (ESI) MS, one- and two-dimensional NMR. EI-MS was carried out on HP5898B mass spectrometer. ESI-positive MS was performed using Bruker Esquire 3000 plus spec-

Table 2
HPLC analyses of amides purified from *Piper longum* L. by UCCC and RPLC

Compound	Purity (% area)		
	Red crude oil	First step by UCCC	Second step by RPLC
1	3.7	25.2	99.5
2	21.8	76.0	99.6
3	18.5	88.3	96.7
4	2.7	49.5	99.2
5	4.3	57.8	96.2
6	7.6	95.0	100.0
7	3.9	80.7	99.5
8	1.8	56.9	95.2
9	0.7	44.0	97.5

Table 3
Partition coefficients (*K*) of amides purified from *P. longum* L. (25 °C)

Compound	*K*[a] value (C_{upper}/C_{lower})			
	Solvent A[b]	Solvent B[c]	Solvent C[d]	Solvent D[e]
1	0.031	–[f]	–[f]	–[f]
2	0.078	0.009	0.021	0.015
3	0.124	0.029	0.034	0.034
4	0.232	0.130	0.080	0.096
5	0.218	0.198	0.089	0.091
6	0.656	0.918	0.302	0.513
7	0.557	1.280	0.273	0.403
8	0.447	1.145	0.223	0.342
9	1.257	4.625	0.634	1.471

[a] Partition coefficient of solute measured with "shake-flask" method, the quantitative analysis carried out by HPLC.
[b] Solvent A: tetrachloromethane–methanol–light petroleum–ethyl acetate–water (8:6:1:1:1, v/v).
[c] Solvent B: tetrachloromethane–methanol–water–light petroleum (15:15:11:6, v/v).
[d] Solvent C: tetrachloromethane–methanol–water–ethyl acetate (15:10:2.5:2.5, v/v).
[e] Solvent D: tetrachloromethane–methanol–water–light petroleum (15:10:2.5:2.5, v/v).
[f] The compound is largely dissolved in lower phase so that it was not found in upper phase by HPLC.

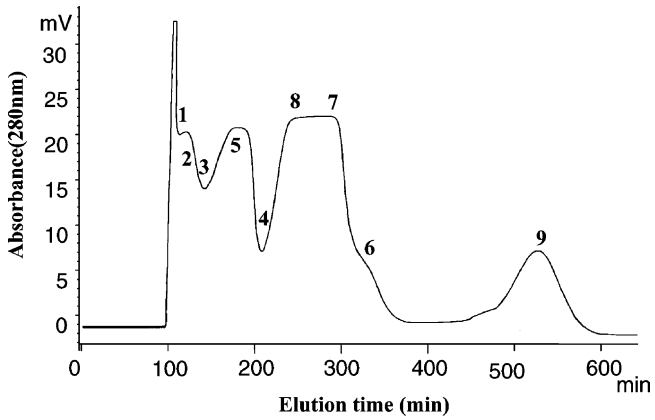


Fig. 2. Chromatograms of 2.5 g red crud oil from *P. longum* L. by upright CCC separation. Conditions: column, multilayer coil of 4.0 mm i.d. PTFE tube with a total capacity of 1600 ml; rotary speed, 300 rpm; column temperature, 35 °C; solvent system, light petroleum (bp 60–90 °C)–ethyl acetate–tetrachloromethane–methanol–water (1:1:8:6:1, v/v); mobile phase, lower phase; flow rate, 4 ml min^{-1}; detection, 280 nm; sample size, 2.5 g; retention of the stationary phase, 75.2%. Peak numbers as in Fig. 1.

trometer with an ESI interface. One- and two-dimensional NMR. experiments were carried out using a Bruker Advance DMX 500 NMR spectrometer with chloroform ($CDCl_3$) as solvent and tetramethylsilane (TMS) as internal standard.

3. Results and discussion

3.1. HPLC analyses and preparative CCC separation

The red crude oil from crude drug "Piper Longi Fructus" was first analyzed by HPLC. The result indicated that it contained several compounds including target amides **1**–**9** and unknown compounds as shown in Fig. 1. The contents of the target amides **1**–**9** in the red crude oil were shown in Table 2.

In order to achieve an efficient separation of target compounds, the two-phase solvent system of tetrachloromethane–methanol–light petroleum (bp 60–90 °C)–ethyl acetate–water at various volume ratios (8:6:1:1:1, 15:15:11:0:6, 15:10:0:2.5:2.5, 15:10:2.5:0:2.5, v/v) was examined using the upright CCC apparatus. The results indicated that the system composed of tetrachloromethane–methanol–light petroleum (bp 60–90 °C)–ethyl acetate–water at volume ratio of 8:6:1:1:1 was most suitable for separation at lower and

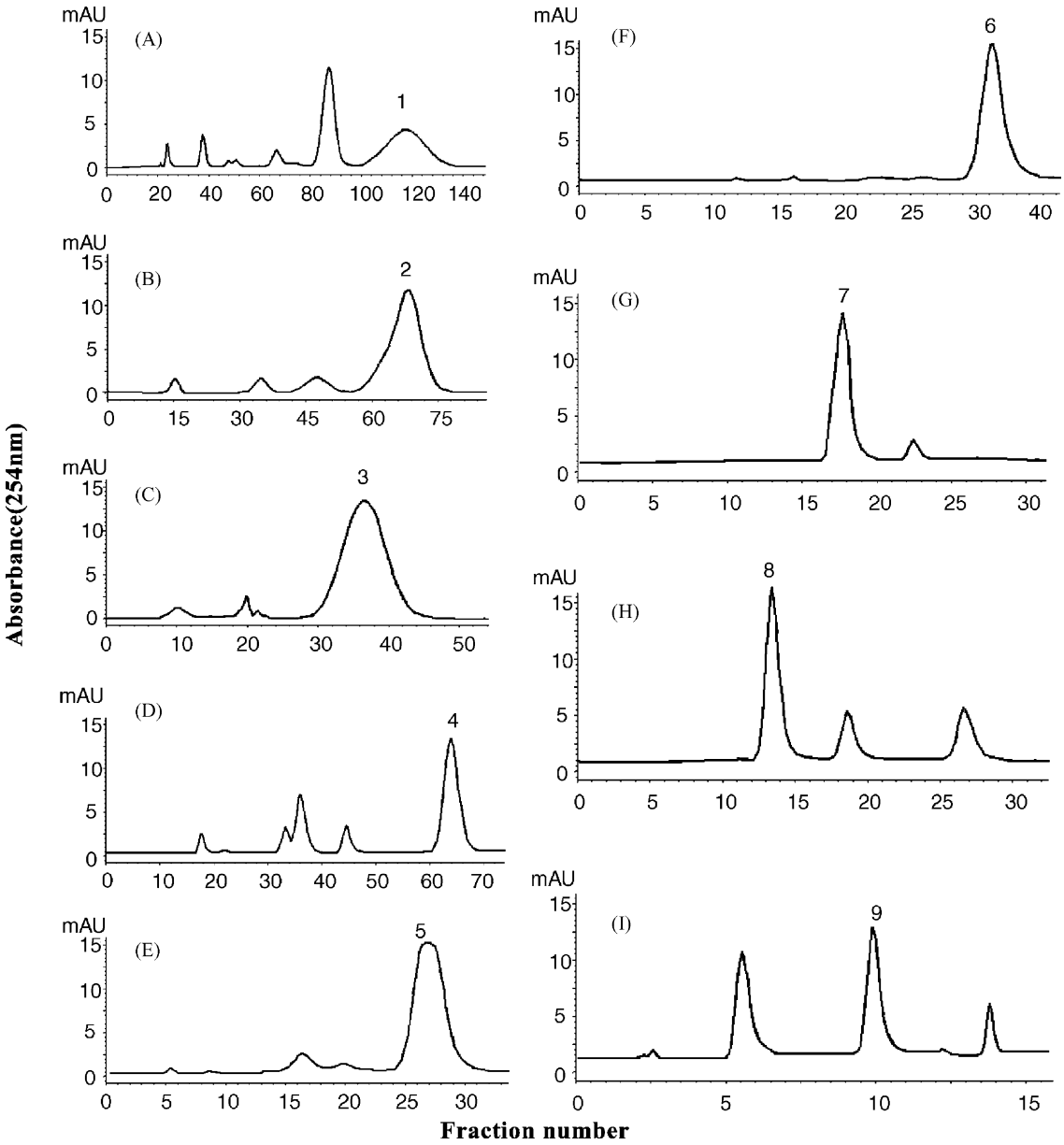


Fig. 3. Chromatograms of fractions I–IX by RPLC separation: (A) fraction I; (B) fraction II; (C) fraction III; (D) fraction IV; (E) fraction V; (F) fraction VI; (G) fraction VII; (H) fraction VIII; (I) fraction IX. Conditions: glass column packed with reversed-phase silica gel (500 × 10 mm, 40–63 μm); column temperature, 25 °C; mobile phase, (A), (B) and (C) 90% methanol and 10% water; (D) and (E) 80% methanol and 20% water; (F), (G), (H) and (I) 70% methanol and 30% water; flow rate, 1 ml min^{-1}; detection, 254 nm. Peak numbers as in Fig. 1.

higher rotary speed. In addition, the high partition efficient and high retention of stationary phase (55.1% retention at 100 rpm and 75.2% retention at 300 rpm) were obtained by using this two-phase system. While using the solvent system tetrachloromethane–methanol–light petroleum (bp 60–90 °C)–ethyl acetate–water (15:10:2.5:2.5, v/v), the good retention of stationary phase also can be obtained at rotary speed of 100 rpm, but not obtained at higher rotary speed of 300 rpm (53.1% retention at 100 rpm and 25.4% retention at 300 rpm). While using other two solvent systems, the high retention and partition could not be obtained. This may be partly due to the fact that the emulsification of sample and solvent system at high rotary speed is larger than one at low rotary speed. This also showed that

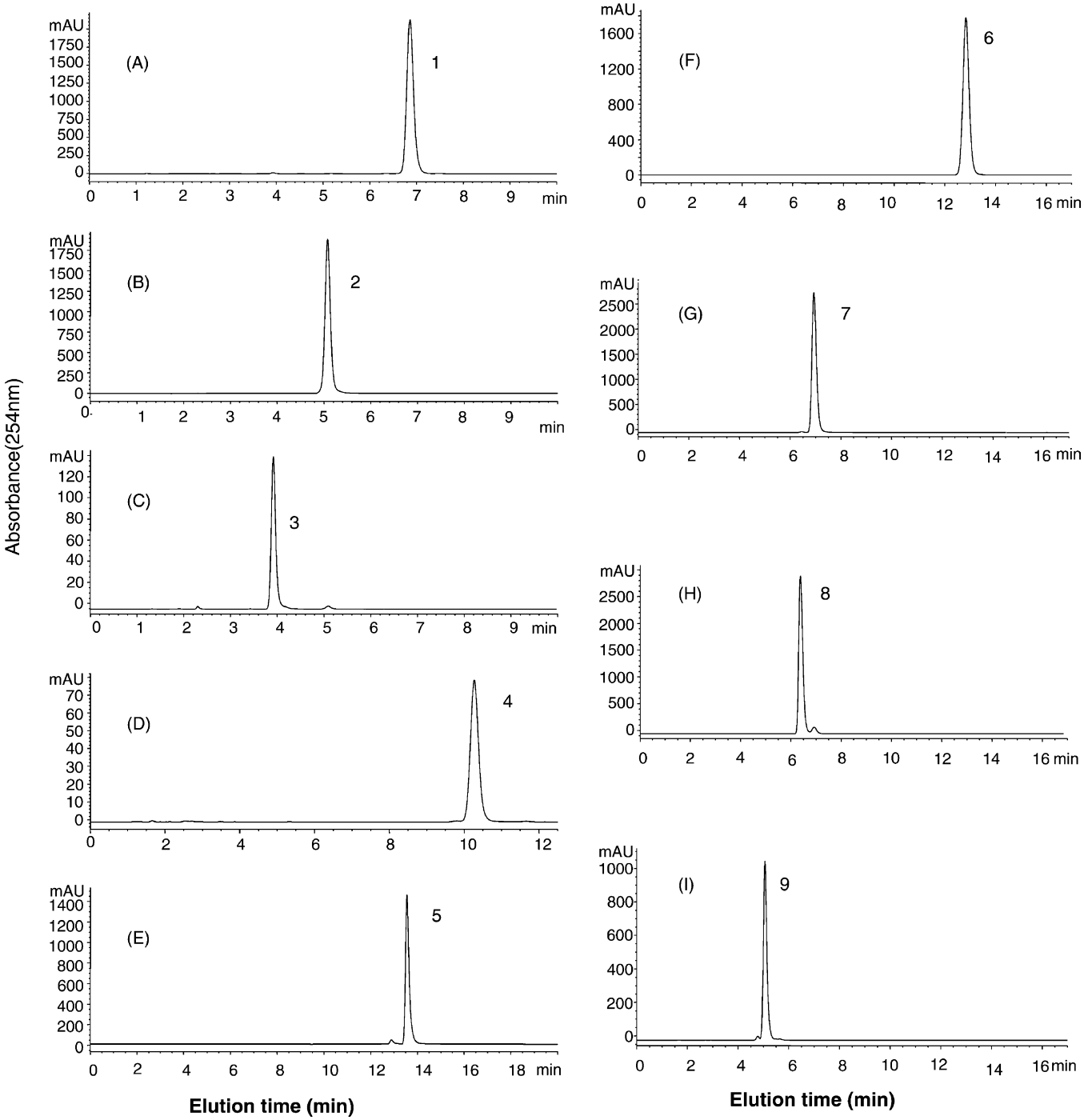


Fig. 4. HPLC chromatograms of amides purified from *P. longum* L. by two-step separation of CCC and RPLC. Conditions: column, YMC-PACK ODS-A (150 × 4.6 mm i.d., 5 μm, 120 Å); column temperature, 25 °C; mobile phase, (A), (B) and (C) 98% methanol and 2% water; (D) 85% methanol and 15% water; (E) 80% methanol and 20% water; (F), (G), (H) and (I) 70% methanol and 30% water; flow rate, 1 ml min^{-1}; detection, 254 nm.

the system tetrachloromethane–methanol–light petroleum (bp 60–90 °C)–ethyl acetate–water (15:10:2.5:2.5, v/v), is more suitable for low-speed separation. Of course, high rotary speed and high retention always result in good partition efficiency and high resolution. Thus, the system tetrachloromethane–methanol–light petroleum (bp 60–90 °C)–ethyl acetate–water (8:6:1:1:1, v/v) is favorable.

The *K* (partition coefficient) values of various amides in several solvent systems were measured according to the literature [16] as shown in Table 3. Because of low polarity of the lipophilic amides, the ideal partition coefficient *K* (0.5–2.0) for each amide could not be obtained in these solvent systems. Comparatively, the amides in the solvent system tetrachloromethane–methanol–light petroleum

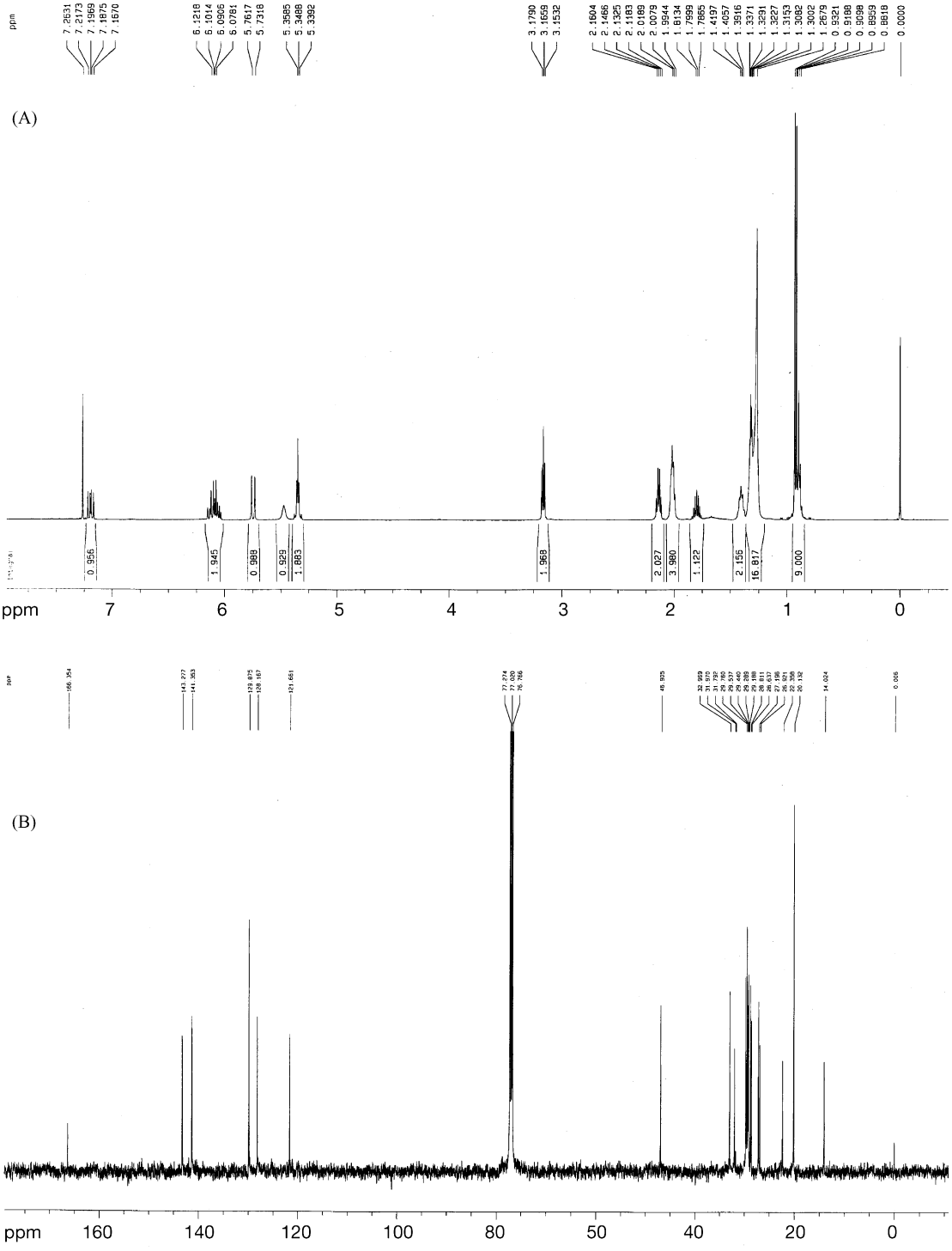


Fig. 5. (A) ^{1}H and (B) ^{13}C NMR spectra of compound **2** ($CDCl_3$, 500 MHz).

(bp 60–90 °C)–ethyl acetate–water (8:6:1:1:1, v/v), have appropriate *K*. The results agree with above separation experiments using upright CCC.

Using the system composed of tetrachloromethane–methanol–light petroleum (bp 60–90 °C)–ethyl acetate–water (8:6:1:1:1, v/v), preparative CCC separation was performed with the upright CCC apparatus at 300 rpm. Fig. 2 shows the chromatogram of CCC separation of 2.5 g of the red crude oil. Peak fractions were collected according to the elution profile and HPLC detection. As a result, nine fractions, i.e., 250 mg fraction I, 210 mg fraction II, 160 mg fraction III, 140 mg fraction IV, 130 mg fraction V, 110 mg fraction VI, 100 mg fraction VII, 130 mg fraction VIII, 125 mg fraction IX, were obtained. These nine fractions contained the target compounds **1**–**9**, respectively. Table 2 shows the purities of the CCC fractions by HPLC detection, which indicate high partition coefficient and high resolution were obtained under the operational condition. In this one step CCC separation, the purity of the compound **6**, pellitorine, was 95%, and the purities of the compounds **2**, **3** and **7** were over 75% while the other compounds **1**, **4**, **5**, **8** and **9** were also richened. These results indicate the upright CCC method is powerful for preparation of these target amides.

3.2. RPLC separation

Then, the fractions I–IX obtained from above the CCC separation were further purified by RPLC. The fractions were isolated respectively in successively one-step RPLC separation, yielded the target amides **1**–**9** with over 95% purity based on HPLC (Table 2). Fig. 3 shows the RPLC chromatograms of the CCC fractions I–IX. From CCC fraction I, 50 mg compound **1** with 99.45% purity was obtained by collecting the RPLC fraction 101–136 (Fig. 3A). From CCC fraction II, 150 mg compound **2** with 99.64% purity was obtained by collecting the RPLC fractions 63–71 (Fig. 3B). From CCC fraction III, 110 mg compound **3** with 96.74% purity was obtained by collecting the RPLC fractions 33–39 (Fig. 3C). From CCC fraction IV, 50 mg compound **4** with 99.23% purity was obtained by collecting the RPLC fractions 61–66 (Fig. 3D). From CCC fraction V, 60 mg compound **5** with 96.23% purity was obtained by collecting the RPLC fractions 25–27 (Fig. 3E). From CCC fraction VI, 75 mg compound **6** with 100% purity was obtained by collecting the RPLC fractions 30–36 (Fig. 3F). From CCC fraction VII, 63 mg compound **7** with 99.51% purity was obtained by collecting the RPLC fractions 17–19 (Fig. 3G). From CCC fraction VIII, 45 mg compound **8** with 95.2% purity was obtained by collecting the RPLC fractions 13–14 (Fig. 3H). From CCC fraction IX, 40 mg compound **9** with 97.51% purity was obtained by collecting the RPLC fractions 9–11 (Fig. 3I). The HPLC chromatograms of purified amides are shown in Fig. 4. The purity of these compounds were further confirmed by ^{1}H and ^{13}C NMR experiments. Fig. 5 shows the ^{1}H and ^{13}C NMR spectra of (2*E*,4*E*,14*Z*)-*N*-isobutyleicosa-2,4,14-trienamide (**2**), which clearly indicate the compound purified by two-step separation of CCC and RPLC is very pure. These NMR spectra also showed our HPLC data of these purified compounds are very reliable.

Table 4
One-dimensional NMR data of (2*E*,4*E*)-*N*-isobutyleicosa-2,4-dienamide (**1**)

Atom	^{13}C NMR δ (ppm)	DEPT[a]	^{1}H NMR δ (ppm)
1	166.633	q[b]	
2	121.877	CH	5.74 (1H, d, J = 15.00 Hz)
3	141.597	CH	7.19 (1H, m)
4	128.391	CH	6.09 (1H, m)
5	143.531	CH	6.07 (1H, m)
6	33.042	CH_2	2.14 (2H, m)
7–17	28.854–29.918	CH_2	1.40–1.25 (22H, m)
18	32.01	CH_2	1.40–1.25 (2H, m)
19	22.92	CH_2	1.40–1.25 (2H, m)
20	14.347	CH_3	0.87 (3H, t, J = 7.05 Hz)
1″	47.146	CH_2	3.64 (2H, t, J = 6.40 Hz)
2″	28.856	CH	1.78 (1H, m)
3″	20.343	CH_3	0.92 (3H, d, J = 6.50 Hz)
4″	20.343	CH_3	0.92 (3H, d, J = 6.50 Hz)
–NH–			5.49 (1H, br, s)

[a] DEPT 90 and DEPT 135 experiments.
[b] Quaternary carbon.

3.3. Structural identification of isolated compounds

The structures of the compounds corresponding to the CCC peak fractions and RPLC peak fractions was characterized by spectroscopic analyses, including the ESI-MS, EI-MS, one- and two-dimensional NMR spectra, and by comparison with published data.

(2*E*,4*E*)-*N*-Isobutyleicosa-2,4-dienamide (**1**) is a white amorphous solid. The ESI-MS spectrum showed the characteristic ions at *m*/*z* 364 and 386 due to $[M + H]^+$ and $[M + Na]^+$ corresponding to the molecular formula $C_{24}H_{45}NO$, with two degrees of unsaturation. One-dimensional NMR data (Table 4) are identical to the reported data [4,18].

(2*E*,4*E*,14*Z*)-*N*-Isobutyleicosa-2,4,14-trienamide (**2**) is a white amorphous solid. The ESI-MS spectrum showed the $[M + H]^+$ and $[M + Na]^+$ ions at *m*/*z* 362 and 384 corresponding to the molecular formula $C_{24}H_{43}NO$, with three degrees of unsaturation. Our ^{1}H and ^{13}C NMR data (Table 5) are identical to the reported data [19]. Here, we give the two-dimensional NMR-based unambiguous assignments for all the protons and carbons as shown in Table 5.

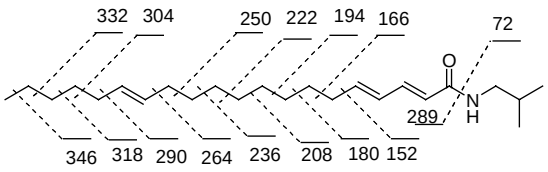


Fig. 6. Mass fragmentation patterns of compound **2**. M^+, *m*/*z* 361.

Table 5
One- and two-dimensional NMR data of (2*E*,4*E*,14*Z*)-*N*-isobutyleicosa-2,4,14-trienamide (**2**)

Atom	^{13}C NMR δ (ppm)	DEPT[a]	HMQC δ (ppm)	HMBC correlation with	H–H COSY correlation with
1	166.354	q[b]		H-3, H-2, H-1′′	
2	121.661	CH	5.75 (1H, d, J = 15.00 Hz)		H-3
3	141.353	CH	7.19 (1H, m)	H-4, H-5	H-2, H-4
4	128.167	CH	6.10 (1H, m)	H-5, H-6, H-3	H-5, H-3
5	143.277	CH	6.08 (1H, m)	H-3, H-6	H-6, H-4
6	32.969	CH_2	2.14 (2H, m)	H-7	H-7, H-5
7	28.811	CH_2	1.41 (2H, m)	H-6	H-6
8–12	29.760–29.188	CH_2	1.27 (10H, m)		
13	26.921	CH_2	2.09 (2H, m)	H-14, H-15	H-14
14	129.875	CH	5.34 (1H, m)	H-13	H-13, H-15
15	129.875	CH	5.34 (1H, m)	H-16	H-14, H-16
16	27.196	CH_2	2.00 (2H, m)	H-15, H-14	H-15, H-17
17	29.760–29.188	CH_2	1.27 (4H, m)		
18	31.97	CH_2	1.31 (2H, m)	H-20	H-19
19	22.358	CH_2	1.32 (2H, m)	H-18, H-20	H-20, H-18
20	14.024	CH_3	0.89 (3H, t, J = 7.05 Hz)		H-19
1′′	46.905	CH_2	3.17 (2H, t, J = 6.55 Hz)	H-3′′, H-4′′	NH, H-2′′
2′′	28.638	CH	1.79 (1H, m)	H-1′′, H-3′′, H-4′′	H-1′′, H-3′′, H-4′′
3′′	20.132	CH_3	0.92 (3H, d, J = 6.65 Hz)	H-1′′, H-4′′	H-2′′
4′′	20.132	CH_3	0.92 (3H, d, J = 6.65 Hz)	H-1′′, H-3′′	H-2′′
–NH–			5.48 (1H, br, s)		1′′

Abbreviations: DEPT, Distortionless enhancement by polarization transfer; HMQC, Heteronuclear multiple-quantum correlation; HMBC, Heteronuclear multiple-bond connectivities; COSY, Correlated Spectroscopy; DEPT 90, Spectra is acquired with a 90° proton pulse; DEPT 135, Spectra is acquired with a 135° proton pulse.
[a] DEPT 90 and DEPT 135 experiments.
[b] Quaternary carbon.

Two-dimensional NMR including ^{1}H–^{1}H COSY, HMQC and HMBC confirmed the assignment of two allylic methylene carbons adjacent to an isolated double bond to be at δ 26.921 and 27.196. The upfield shift of these signals indicated the geometry of this double bond to be *Z* [19]. The location of the isolated *Z*-double bond was presumed on the basis of the EI-MS fragmentation pattern. As shown in Fig. 6, the fragments with a difference of 14 mass units (CH_2) were from *m*/*z* 152 to *m*/*z* 264, followed by a fragment ion at *m*/*z* 290 instead of *m*/*z* 278, indicating a Δ^{14} double

Table 6
One- and two-dimensional NMR data of (2*E*,4*E*,12*Z*)-*N*-isobutylocatadeca-2,4,12-trienamide (**3**)

Atom	^{13}C NMR δ (ppm)	DEPT[a]	HMQC δ (ppm)	HMBC correlation with	H–H COSY correlation with
1	166.399	q[b]		H-3,H-2,H-1′′	
2	121.681	CH	5.75 (1H, d, J = 15.00 Hz)	H-4	H-3
3	141.339	CH	7.19 (1H, m)	H-4, H-5	H-2, H-4
4	128.219	CH	6.13 (1H, m)	H-2	H-5, H-3
5	143.251	CH	6.06 (1H, m)	H-3, H-6	H-6, H-4
6	32.955	CH_2	2.13 (2H, m)	H-4	H-7, H-5
7	28.802	CH_2	1.41 (2H, m)	H-6	H-6
8–10	28.71429.718	CH_2	1.28 (6H, m)		
11	27.161	CH_2	2.00 (2H, m)	H-12, H-13	H-12
12	129.918	CH	5.34 (1H, m)	H-11	H-11
13	129.81	CH	5.34 (1H, m)	H-14	H-12, H-14
14	26.916	CH_2	2.00 (2H, m)	H-13, H-12	H-13
15	28.714-29.718	CH_2	1.28 (2H, m)		
16	31.959	CH_2	1.30 (2H, m)	H-17	H-17
17	22.664	CH_2	1.31 (2H, m)	H-18	H-18, H-16
18	14.123	CH_3	0.88 (3H, t, J = 6.95 Hz)		H-17
1′′	46.915	CH_2	3.16 (2H, t, J = 6.35 Hz)	H-3′′, H-4′′	NH, H-2′′
2′′	28.627	CH	1.79 (1H, m)	H-3′′, H-4′′	H-1′′, H-3′′, H-4′′
3′′	20.124	CH_3	0.92 (3H, d, J = 6.95 Hz)	H-1′′	H-2′′
4′′	20.124	CH_3	0.92 (3H, d, J = 6.95 Hz)	H-1′′	H-2′′
–NH–			5.52 (1H, br, s)		1′′

[a] DEPT 90 and DEPT 135 experiments.
[b] Quaternary carbon.

Table 7
One-dimensional NMR data of guineensine (**4**) and pipernonaline (**5**)

Atom	Guineensine (**4**)			Pipernonaline (**5**)		
	^{13}C NMR δ (ppm)	DEPT[a]	^{1}H NMR δ (ppm)	^{13}C NMR δ (ppm)	DEPT[a]	^{1}H NMR δ (ppm)
1	166.379	q[b]		165.445	q[b]	
2	121.754	CH	5.76 (1H,d, J = 14.98 Hz)	120.382	CH	6.27 (1H, d, J = 15.00 Hz)
3	141.312	CH	7.22 (1H, m)	145.521	CH	6.84 (1H, m)
4	129.369	CH	6.13 (1H, m)	32.27	CH_2	2.25 (2H, m)
5	129.342	CH	6.09 (1H, m)	27.821	CH_2	1.52 (2H, m)
6	32.85	CH_2	2.19 (2H, m)	28.851	CH_2	1.52 (2H, m)
7	29.005	CH_2	1.45 (2H, m)	32.545	CH_2	2.20 (2H, m)
8	28.735	CH_2	1.35 (2H, m)	128.782	CH	6.06 (1H, m)
9	28.949	CH_2	1.35 (2H, m)	129.466	CH	6.30 (1H, d, J = 15.20 Hz)
10	29.343	CH_2	1.45 (2H, m)			
11	32.901	CH_2	2.19 (2H, m)			
12	143.108	CH	6.06 (1H, m)			
13	128.272	CH	6.30 (1H, d, J = 15.75 Hz)			
1′	132.494	q		132.229	q	
2′	105.407	CH	6.92 (1H, s)	105.274	CH	6.90 (1H, d, J = 1.6 Hz)
3′	146.928	q		146.469	q	
4′	147.928	q		147.808	q	
5′	108.226	CH	6.76 (1H, m)	108.091	CH	6.75 (1H, d, J = 8.0 Hz)
6′	120.203	CH	6.76 (1H, m)	120.099	CH	6.77 (1H, dd, J = 1.5, 8.0 Hz)
OCH_2O	100.908	CH_2	5.96 (2H, s)	100.788	CH_2	5.95 (2H, s)
1″	46.938	CH_2	3.19 (2H, t, J = 6.43 Hz)	42.85	CH_2	3.56 (2H, m)
2″	28.645	CH	1.82 (1H, m)	26.512	CH_2	1.67 (2H, m)
3″	20.13	CH_3	0.95 (3H, d, J = 6.69 Hz)	24.536	CH_2	1.58 (2H, m)
4″	20.13	CH_3	0.95 (3H, d, J = 6.69 Hz)	25.589	CH_2	1.58 (2H, m)
5″				46.458	CH_2	3.56 (2H, br, s)
–NH–			5.50 (1H, br, s)			

[a] DEPT 90 and DEPT 135 experiments.
[b] Quaternary carbon.

bond. In the light of one- and two-dimensional NMR data, the assignments of all protons and carbons were elucidated.

(2*E*,4*E*,12*Z*)-*N*-Isobutylocatadeca-2,4,12-trienamide (**3**) is a white amorphous solid which gave similar spectral characteristics to those of compound (**2**), except for its molecular ion peak. The ESI-MS spectrum showed the $[M + H]^+$ ion at *m*/*z* 334 corresponding to the molecular formula $C_{22}H_{39}NO$, with three degrees of unsaturation. The geometry and location of an isolated double bond were determined to C–12Z on the basis of the EI-MS fragmentation pattern, one- and two-dimensional NMR measurements. All data of one- and two-dimensional and assignments are

Table 8
One- and two-dimensional NMR data of pellitorine (**6**)

Atom	^{13}C NMR δ (ppm)	DEPT[a]	HMQC δ (ppm)	HMBC correlation with	H–H COSY correlation with
1	166.651	q[b]		H-3, H-2, H-1″	
2	121.938	CH	5.75 (1H, d, J = 15.00 Hz)	H-4	H-3
3	141.543	CH	7.18 (1H, m)	H-5	H-4, H-2
4	128.416	CH	6.12 (1H, m)	H-2, H-6	H-3, H-5
5	143.461	CH	6.06 (1H, m)	H-3, H-6, H-7	H-4, H-6
6	33.133	CH_2	2.14 (2H, m)	H-5, H-4, H-7	H-5, H-7
7	28.704	CH_2	1.42 (2H, m)	H-6, H-8	H-8, H-6
8	31.586	CH_2	1.29 (2H, m)	H-6, H-7, H-9, H-10	H-9, H-7
9	22.691	CH_2	1.29 (2H, m)	H-8, H-10	H-10, H-8
10	14.221	CH_3	0.87 (3H, t, J = 7.05 Hz)		H-9
1″	47.153	CH_2	3.16 (2H, t, J = 6.40 Hz)	H-2″, H-3″, H-4″	H-2″, NH
2″	28.851	CH	1.78 (1H, m)	H-1″, H-3″, H-4″	H-1″, H-3″, H-4″
3″	20.341	CH_3	0.90 (3H, d, J = 8.65 Hz)	H-1″, H-2″, H-4″	H-2″
4″	20.341	CH_3	0.90 (3H, d, J = 8.65 Hz)	H-1”, H-2”, H-3”	H-2″
–NH–			5.55 (1H, br s)		

[a] DEPT 90 and DEPT 135 experiments.
[b] Quaternary carbon.

Table 9
One-dimensional NMR data of piperine (**7**), piperlonguminine (**8**) and piperlongumine (**9**)

Atom	Piperine (**7**)			Piperanine (**8**)			Piperlonguminine (**9**)		
	^{13}C NMR δ (ppm)	DEPT[a]	^{1}H NMR δ (ppm)	^{13}C NMR δ (ppm)	DEPT	^{1}H NMR δ (ppm)	^{13}C NMR δ (ppm)	DEPT	^{1}H NMR δ (ppm)
1	165.7	q[b]		165.495	q		166.173	q	
2	120.221	CH	6.43 (1H, d, $J = 14.60$ Hz)	121.233	CH	6.22 (1H, d, $J = 15.28$ Hz)	122.593	CH	5.91 (1H, d, $J = 14.74$ Hz)
3	142.81	CH	7.31 (1H, m)	144.045	CH	6.83 (1H, m)	141.022	CH	7.36 (1H, m)
4	125.58	CH	6.74 (1H, m)	34.498	CH_2	2.47 (2H, m)	124.684	CH	6.67 (1H, m)
5	138.529	CH	6.75 (1H, m)	34.553	CH_2	2.71 (2H, t, $J = 7.37$ Hz)	138.824	CH	6.77 (1H, m)
1′	131.25	q		135.015	q		130.905	q	
2′	105.916	CH	6.98 (1H, d, $J = 1.30$ Hz)	108.163	CH	6.69 (1H, d, $J = 1.23$ Hz)	105.732	CH	6.97 (1H, d, $J = 1.35$ Hz)
3′	148.36	q		145.754	q		148.225	q	
4′	148.424	q		147.593	q		148.225	q	
5′	108.723	CH	6.78 (1H, d, $J = 8.05$ Hz)	108.862	CH	6.74 (1H, d, $J = 7.89$ Hz)	108.506	CH	6.76 (1H, d, $J = 8.03$ Hz)
6′	122.746	CH	6.89 (1H,dd, $J = 1.35$,8.05 Hz)	121.419	CH	6.64 (1H, dd, $J = 1.37$, 7.91 Hz)	123.221	CH	6.88 (1H, dd, $J = 1.34$, 8.03 Hz)
OCH_2O	101.499	CH_2	5.97 (2H, s)	100.789	CH_2	5.93 (2H, s)	101.309	CH_2	5.97 (2H, s)
1″	43.24	CH_2	3.58 (2H, br, m)	43.212	CH_2	3.50 (2H, br, s)	47.022	CH_2	3.90 (2H, t, $J = 6.48$ Hz)
2″	25.69	CH_2	1.60 (2H, m)	25.887	CH_2	1.57 (2H,m)	28.66	CH	1.82 (1H, m)
3″	24.885	CH_2	1.66 (2H, m)	24.642	CH_2	1.65 (2H, m)	20.16	CH_3	0.93 (3H, d, $J = 6.70$ Hz)
4″	26.303	CH_2	1.60 (2H, m)	25.92	CH_2	1.57 (2H, m)	20.16	CH_3	0.93 (3H, d, $J = 6.70$ Hz)
5″	46.9	CH_2	3.58 (2H, br, m)	46.692	CH_2	3.50 (2H, br, s)			

[a] DEPT 90 and DEPT 135 experiments.
[b] Quaternary carbon.

illustrated in Table 6. Our ^{1}H and ^{13}C NMR data (Table 6) are identical to the reported data [19].

Guineensine (**4**) showed the prominent fragment ion at *m*/*z* 384 and 406 due to $[M + H]^+$ and $[M + Na]^+$ in the positive ESI-MS spectrum. This corresponds to the molecular formula $C_{24}H_{33}NO_3$. ^{1}H and ^{13}C NMR data were showed in Table 7, which are in agreement with the published data [4,8,9,20].

Pipernonaline (**5**) had the prominent fragment ion at *m*/*z* 342, 364 and 380 due to $[M + H]^+$, $[M + Na]^+$ and $[M + K]^+$ in the positive ESI-MS spectrum. This corresponds to the molecular formula $C_{21}H_{27}NO_3$. ^{1}H and ^{13}C NMR data were showed in Table 7, which are in agreement with the published data [4,11,21].

Pellitorine (**6**) is a white amorphous solid. The ESI-MS spectrum showed the characteristic ions at *m*/*z* 224 and 246 due to $[M + H]^+$and $[M + Na]^+$ corresponding to the molecular formula $C_{14}H_{25}NO$, with two degrees of unsaturation. ^{1}H and ^{13}C NMR data (Table 8) are identical to the reported data [7,10,12,20]. Table 7 also showed its two-dimensional NMR data.

Piperine (**7**) is a pale yellow crystals which had the characteristic ions at *m*/*z* 286 and 308 due to $[M + H]^+$and $[M + Na]^+$ corresponding to the molecular formula $C_{17}H_{19}NO_3$. ^{1}H and ^{13}C NMR data (Table 9) are identical to the reported data [4,22].

Piperanine (**8**) had $[M + H]^+$, $[M + Na]^+$ ions at *m*/*z* 288 and 310 corresponding to the molecular formula $C_{17}H_{21}NO_3$. ^{1}H and ^{13}C NMR data (Table 9) are identical to the reported data [7,12].

Piperlonguminine (**9**) showed the characteristic ions at 274 and 296 due to $[M + H]^+$, $[M + Na]^+$ in the positive ESI-MS spectrum, which corresponds to the molecular formula $C_{16}H_{19}NO_3$. ^{1}H and ^{13}C NMR data (Table 9) are identical to the reported data [4,23].

4. Conclusion

In conclusion, large-scale preparative isolation and purification of amides from crude drug “Piper Longi Fructus”, the fruits of *P. longum* L., were successfully made by use of upright CCC and RPLC. The present study indicated that upright CCC and RPLC are very useful for the preparative separation of amides from *P. longum* L.

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