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Separation and Purification of Z Ligustilide and Senkyunolide A from *Ligusticum chuanxiong* Hort. with Supercritical Fluid Extraction and High-Speed Counter-Current Chromatography

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Abstract: The essential oil was obtained by supercritical fluid extraction from dried roots of *Ligusticum chuanxiong*. Different solvent systems for high-speed

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counter-current chromatography (HSCCC) were compared. A system composed of *n* hexane–ethyl acetate–methanol–water–acetonitrile in the ratio of 8:2:5:5:3 (v/v) was found to be optimum for HSCCC of the essential oil. Z ligustilide and senkyunolide A were separated by HSCCC with purity of 98% determined by GC. The chemical structures of these two components were identified by nuclear magnetic resonance (NMR) and mass spectrometry (MS).

Keywords: Counter-current chromatography, Z ligustilide, Senkyunolide A, *Ligusticum chuanxiong*

INTRODUCTION

The root of *Ligusticum chuanxiong* Hort. is a traditional Chinese medicine named "*chuanxiong*" for the treatment of headaches, ischemic stroke, anemia, and cerebral vascular disease (1–3). In recent years, the essential oil derived from *chuanxiong* has been used as a new medicine for treating strokes. The essential oil contains phthalides in which, Z ligustilide and senkyunolide A were found to be the main active compounds (4, 5). These two compounds can be used as markers for the quality control of *chuanxiong* or any medicine containing *chuanxiong*.

Phthalides are unstable and difficult to purify by conventional liquid–liquid partition and silica gel column chromatography (6). A possible alternative is high-speed counter-current chromatography (HSCCC) which was developed by Ito (7). Being a partition chromatography without solid support, HSCCC eliminates any irreversible adsorption in chromatographic process with solid support. Over the last few years, HSCCC has attracted considerable interest in the separation and purification of natural products. Many compounds with high purity have been separated and purified from various herbs using HSCCC (7–14).

The successful separation by HSCCC necessitates a careful search for a suitable two-phase system which provides an ideal range of the partition coefficient values for the applied sample (8). One may refer to the literature for searching a suitable solvent system. In this study, acetonitrile was added into the solvent system composed of *n* hexane–ethyl acetate–methanol–water, which resulted in the changes of the polarity of the stationary phase and the mobile phase. Therefore the hydrophobic compounds (Z ligustilide and senkyunolide A) could be separated successfully by HSCCC.

EXPERIMENTAL

Apparatus

A TBE-300A Semi-Preparative HSCCC (Shanghai Tauto Biotech Co. LTD., Shanghai, China) was equipped with three polytetrafluoroethylene (PTFE) multilayer coils. The separation column was 150 m in length and 1.5 mm i.d. with a total capacity of 280 mL. The β value varied from 0.5 at the internal terminal to 0.8 at the external terminal ($\beta = r/R$ where r is the distance from the coil to the holder shaft, and R is the revolution radius or the distance between the holder axis and central axis of the centrifuge).

The solvent was pumped into the column with a Model S-1007 constant-flow pump (Beijing Shengyitong Technology Development Co. Ltd., China). A Model 8823B UV detector (Beijing BINTA Instrument Technology Co. Ltd., China) was used to monitor the effluent at 280 nm continuously. A manual sample injection valve with a 20 mL loop was used to introduce the sample into the column.

A Supercritical carbon dioxide apparatus (Nantong Huaan supercritical Extraction Co. Ltd., China) was used for extracting essential oil from *chuanxiong*.

A gas chromatography (GC; Shimadzu Model 14 B) equipped with FID (flame ionization detector) was used for the analysis of the essential oil from *chuanxiong* and the purified peak fractions from the preparative HSCCC separation.

Chemicals and Reagents

All organic solvents used for HSCCC were of analytical grade and purchased from Tianjin Shield Company, Tianjin, China. Water used was distilled water.

The chemical structures of Z ligustilide and senkyunolide A are shown in Fig. 1. Z ligustilide has the same chemical structure as senkyunolide A except for the double bond between C-7 and C-9.

Preparation of Two-Phase Solvent System

The two-phase solvent system was composed of *n* hexane–ethyl acetate–methanol–water–acetonitrile. After thoroughly equilibrating the mixtures in a separation funnel at room temperature, two phases were formed before use where the organic phase was used as the stationary phase and the aqueous phase as the mobile phase.

Preparation of Sample and Sample Solutions

12 g of essential oil was extracted with supercritical carbon dioxide under the pressure of 25 MPa and the temperature of 40°C from 1.0 kg of dried and powdered *chuanxiong*. A 120 mg amount of this oil was dissolved with 4 mL of mixture containing both upper and lower phase for preparative separation.

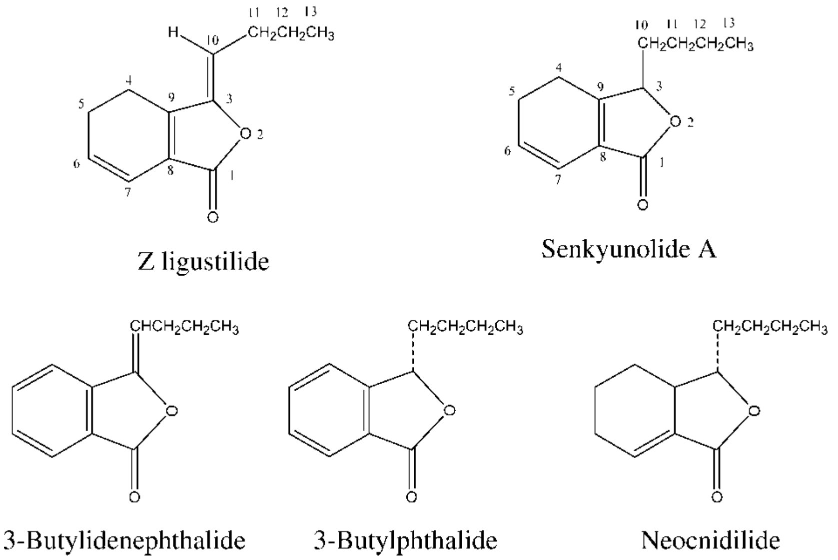

Figure 1. Major phthalides from *Ligusticum chuanxiong* Hort.

Separation Procedure

The coiled column was first filled entirely with the upper organic phase. Then the apparatus was rotated at an optimum speed of 800 rpm, while the lower aqueous phase was pumped into the head end of the column at a suitable flow-rate of 2.0 mL/min. After the mobile phase front emerged and hydrodynamic equilibrium was established in the column, 4 mL of the sample solution containing 120 mg of the essential oil was injected through the injection valve. The effluent from the tail end of the column was continuously monitored with UV detection at 280 nm and the peak fractions were collected according to the elution profile.

TLC Analysis

TLC analysis was carried out on Silica gel 60 F_{254} (Yantai Chemical Industry Research Institute, China). The solvent consisted of *n* hexane–ethyl acetate (10:1), and the sample was dissolved in methanol. Direct detection was done with UV light at 254 nm and 365 nm.

GC Analysis

Each purified peak fraction from the preparative HSCCC separation was analyzed by GC. The column used was an SE-54 capillary column (50 m, 0.2 mm i.d., 0.5 μm film thickness, Dalian Elite Analytical Instruments Co. Ltd., Dalian, China.). The pressure of the carrier gas (N_2) was 210 kPa with a split ratio of 30:1. Samples of 1 μL were injected manually.

NMR and MS Analysis

Identification of HSCCC peak fractions was carried out by EI mass spectrometry, UV, and NMR analysis. ^{1}H NMR and ^{13}C NMR were recorded at 400 MHz at constant temperature in deuterated methanol (CD_3OD).

RESULTS AND DISCUSSION

Optimization of GC Conditions

In order to select an appropriate condition for the GC analysis of senkyunolide A and Z ligustilide, a SE-54 capillary column was used. The oven temperature was 240°C, the temperature of the on-column injector and detector was 280°C. Under these conditions, major peaks could be obtained and each peak got baseline separation. As shown in Fig. 2, the main components of *chuanxiong* are senkyunolide A and Z ligustilide, which was in agreement with the result in the literature (4).

Optimization of Solvent System of HSCCC

Successful separation by HSCCC largely depends on selection of a suitable two-phase solvent system, which provides an optimum range of partition coefficient for a target compound. If the *K* value is much smaller than 1, the solutes will be eluted close to each other near the solvent front, which may result in the loss of peak resolution; if it is much greater than 1, the solutes will be eluted in excessively broad peaks, and may lead to extended elution time (8). Sets of two-phase solvent systems are summarized in the Tables 1 and 2 in the literature (7, 8). Since senkyunolide A and Z ligustilide are hydrophobic, a series of hydrophobic solvent systems were tested according to the previous studies (12–14). Three hydrophobic solvent systems (*n* Hexane–ethyl acetate–methanol–water (8:2:5:5, v/v); *n* Hexane–ethyl acetate–methanol–water (9:1:5:5, v/v); *n* Hexane–methanol–water (10:5:5, v/v)) shown in the literature (7, 8) were also tested. GC was used to measure the sample concentration in each phase, from which the *K* values of the target compounds were calculated.

In the three hydrophobic solvent systems (*n* Hexane–ethyl acetate–methanol–water (8:2:5:5, v/v); (9:1:5:5, v/v); *n* Hexane–methanol–water (10:5:5, v/v)) as shown in the literature (7, 8), the *K* values of senkyunolide

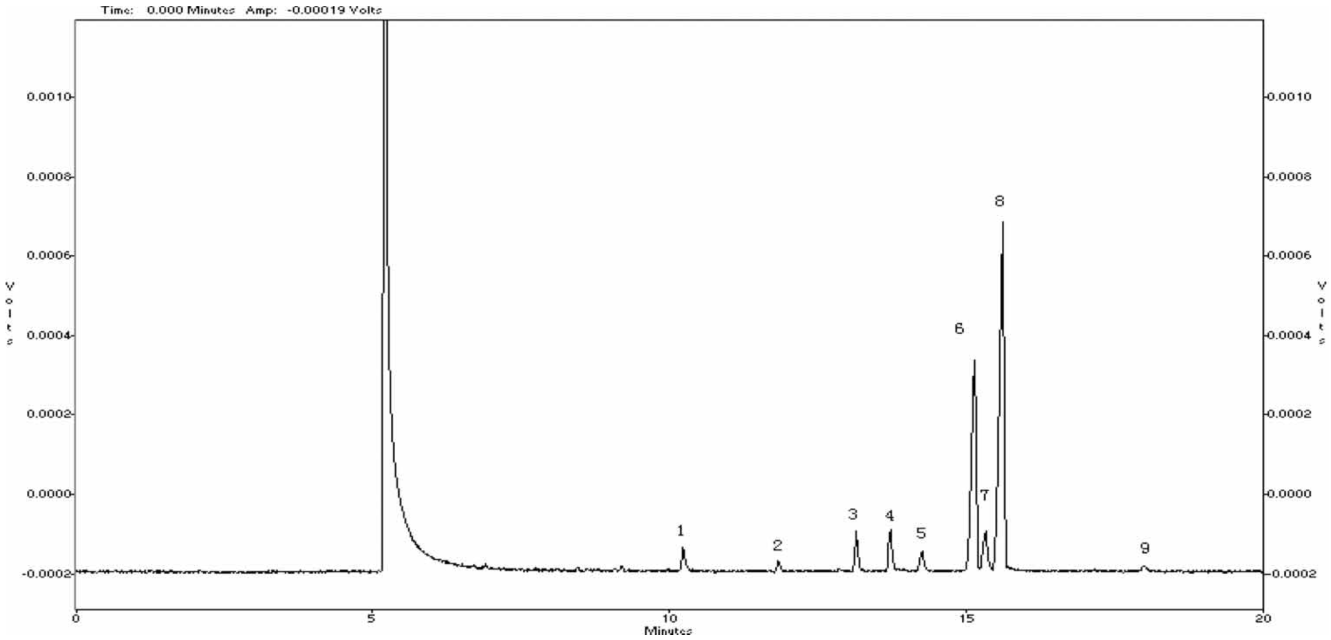

Figure 2. GC analysis of essential oil of *chuanxiong* extracted by supercritical carbon dioxide (Sample in ethanol) 3. 3-butylphthalide; 4. 3-butylidenephthalide; 6. senkyunolide A; 7. neocnidilide; 8. Z ligustilide. Experimental conditions: column: SE-54 (50 m × 0.2 mm i.d., 0.5 μm film thickness); Column temperature: 240°C; split ratio of 30:1; detection: FID; injection volume: 1 μL.

Table 1. The K (partition coefficient) values of senkyunolide A and Z ligustilide in different solvent systems

Solvent system	K value[a] Senkyunolide A	Z ligustilide	Alpha[b] $= K_2/K_1$
n Hexane–ethyl acetate–methanol–water (1:1:1:1, v/v)	6.57	13.12	2.00
n Hexane–ethyl acetate–methanol–water (1:3:2:4, v/v)	33.33	94.09	2.82
n Hexane–ethyl acetate–methanol–water (3:7:5:5, v/v)	6.03	12.86	2.13
n Hexane–ethyl acetate–methanol–water (8:2:5:5, v/v)	5.30	12.15	2.29
n Hexane–ethyl acetate–methanol–water (9:1:5:5, v/v)	5.18	18.37	3.55
n Hexane–ethyl acetate –methanol–water (20:1:10:10, v/v)	9.02	11.95	1.32
n Hexane–methanol–water (10:5:5, v/v)	5.25	14.77	2.81
n Hexane–methanol–water (150:135:8, v/v)	0.21	0.35	1.67
n Hexane–methanol–water (6:5:2, v/v)	0.33	0.78	2.36
n Hexane–methanol (2:1, v/v)	0.24	0.35	1.46
n Hexane–ethyl acetate–methanol–water–acetonitrile (8:2:5:5:1, v/v)	2.87	8.01	2.79
n Hexane–ethyl acetate–methanol–water–acetonitrile (8:2:5:5:2, v/v)	1.73	4.87	2.81
n Hexane–ethyl acetate–methanol–water–acetonitrile (8:2:5:5:3, v/v)	1.05	3.07	2.92

[a] K value was expressed as the peak area of the compound in the upper phase divided by the peak area of the compound in the lower phase.

[b] K_1 was the partition coefficient of senkyunolide A, K_2 was the partition coefficient of Z ligustilide.

A and Z ligustilide were high (Table 1), and the elution time will be rather long. While the *K* values were too low (Table 1) in the more hydrophobic solvent systems (*n* Hexane–methanol–water (150:35:8, v/v), *n* Hexane–methanol–water (6:5:2, v/v), *n* Hexane–methanol (2:1, v/v)), and the peak resolution will be rather poor.

The result (Table 1) indicated the *K* value of the solvent system composed of *n* Hexane–ethyl acetate–methanol–water (8:2:5:5, v/v) is a bit better. So we modified this system by the addition of acetonitrile in a different volume ratio. As shown in Table 1, the partition coefficients of Z ligustilide and senkyunolide A decrease when the relative volume of the acetonitrile increases from 0 to 3. It is obvious that acetonitrile in the solvent system can increase Z ligustilide and senkyunolide A in the mobile phase, thereby shortening the retention time of the two target compounds in the upper phase. When the relative volume of the acetonitrile increases to 4, the retention of the stationary phase was poor. As shown in Table 1, *n* Hexane–ethyl acetate–methanol–water (8:2:5:5) solvent systems gave the similar selectivity α values. The selectivity α values increase from 2.29 to 2.92 when the relative volume of the acetonitrile increases from 0 to 3.

From the above results, it was observed that *n* Hexane–ethyl acetate–methanol–water–acetonitrile (8:2:5:5:3, v/v) was the best choice. The two phases in this simple system reached equilibrium very quickly. The *K* values for senkyunolide A and Z ligustilide in this system were 1.05 and 3.07, respectively, which produced a good separation by the semi-preparative HSCCC.

Figure 3 shows the results of the preparative separation of 120 mg essential oil from *chuanxiong* by HSCCC, which yielded 18 mg of Z ligustilide and 10 mg of senkyunolide A. The purity of senkyunolide A and Z ligustilide were 98.2% and 98.6%, respectively (Fig. 4).

Our study indicated that if the target compound is mostly distributed in the upper organic phase, and the hydrophobic solvent systems listed in Tables 1 of the literature (7, 8) were not satisfied, one may try adding a modifier (such as acetonitrile) into the solvent system so as to change the partition coefficient of the target compounds, and perhaps a good result could be obtained.

Identification of the Components of *Chuanxiong*

^{1}H NMR and ^{13}C NMR were recorded in deuterated methanol (CD_3OD). Senkyunolide A: $[\alpha]_D^{20}$ -57.1° ($c = 0.5$, $CHCl_3$). ^{1}H NMR (400 MHz, CD_3OD) δ (ppm): 0.93 (3H, t, J = 7.1 Hz, H-13), 1.38 (4H, m, H-12, 11), 1.55 (1H, m, H-10), 1.93 (1H, m, H-10), 2.51 (4H, m, H-4, 5), 5.07 (1H, dd, J = 7.2, 3.2 Hz, H-3), 5.97 (1H, m, H-6), 6.11 (1H, d, J = 9.7 Hz, H-7). ^{13}C NMR (75 MHz, CD_3OD) δ (ppm): 14.2 (q, C-13), 21.6 (t, C-4), 23.2 (t, C-5), 23.5 (t, C-12), 27.9 (t, C-10), 32.8 (t, C-11), 84.5 (d, C-3), 117.1 (d, C-7), 125.1 (s, C-9), 130.1 (d, C-6), 164.9 (s, C-8), 173.6 (s, C-1).

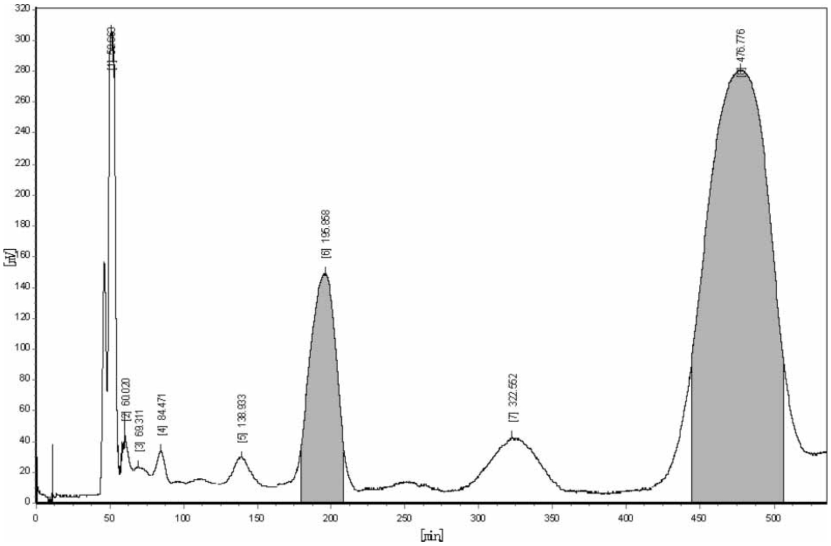

Figure 3. HSCCC separation of essential oil from *chuanxiong* Peaks: 6 = senkyunolide A, 8 = Z ligustilide. Two-phase solvent system: *n* hexane–ethyl acetate–methanol–water–acetonitrile (8:2:5:5:3, v/v); stationary phase: upper organic phase; mobile phase: lower aqueous phase; flow-rate: 2.0 mL/min; revolution speed: 800 rpm; sample size: 120 mg; retention of stationary phase: 64%; UV wavelength: 280 nm.

Z ligustilide: ^{1}H NMR (400 MHz, CD_3OD) δ (ppm): 0.95 (3H, t, J = 7.6 Hz, H-13), 1.50 (2H, m, H-12), 2.38 (2H, q, J = 7.6 Hz, H-11), 2.48 (2H, m, H-5), 2.60 (2H, t, J = 13.5 Hz, H-4), 5.23 (1H, t, J = 8 Hz, H-10), 6.01 (1H, m, H-6), 6.28 (1H, dt, J = 9.5, 1.5 Hz, H-7). ^{13}C NMR δ (ppm): 13.8 (q, C-13), 18.5 (t, C-4), 22.4 (t, C-5), 28.1 (t, C-12), 28.1 (t, C-11), 112.9 (d, C-10), 117.1 (d, C-7), 123.9 (s, C-9), 129.9 (d, C-6), 147.1 (s, C-8), 148.5 (s, C-3), 167.6 (s, C-1).

EI MS was adopted to analyze senkyunolide A and Z ligustilide. The mass data of Z ligustilide, formed as m/z (rel. int.), were 190(M+, 66), 161(100), 148(75), 133(18), 105(51), 77(28), 55(41) and the mass data of senkyunolide A were 192(M+, 23), 135(5), 107(100), 79(22). Compared the data of mass and NMR with that given in reference (4, 15, 16), Senkyunolide A and Z ligustilide could be identified.

CONCLUSION

Two phthalide compounds, senkyunolide A and Z ligustilide, were obtained from the essential oil of the *Ligusticum chuanxiong* Hort. with one step high-speed counter-current chromatography for the first time. The results of

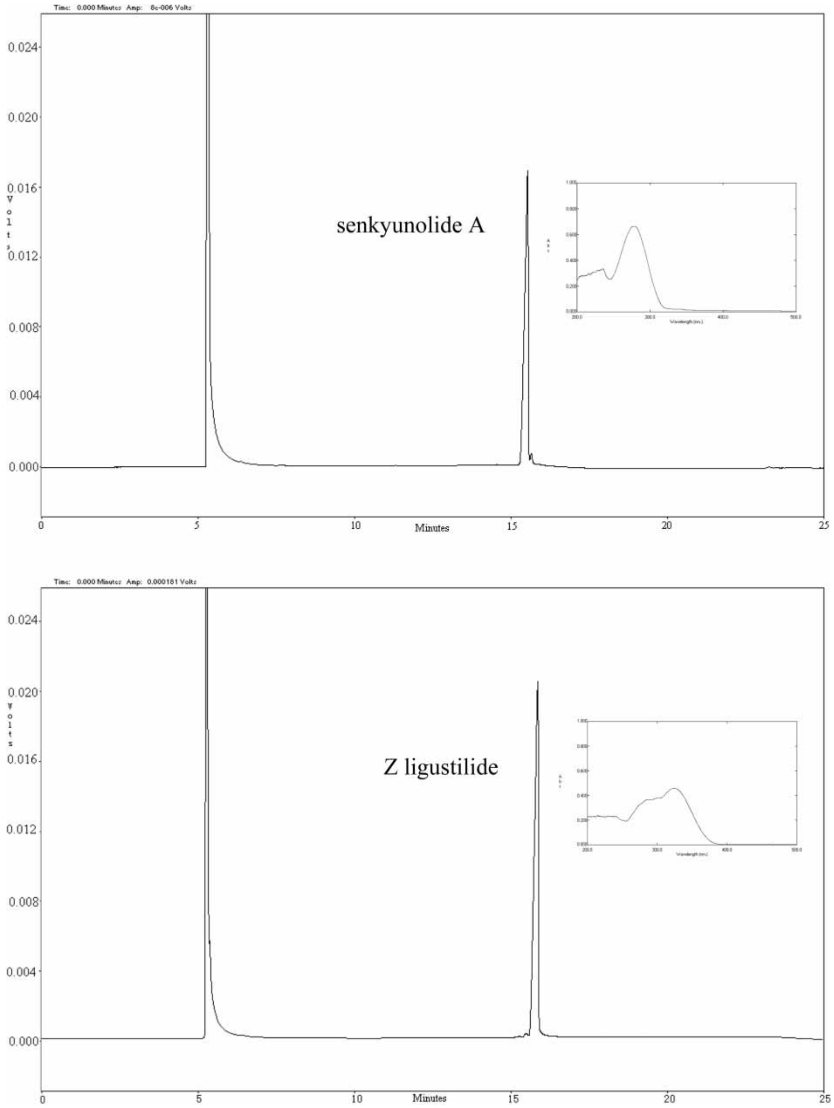

Figure 4. GC analysis and UV spectra of peak fractions from HSCCC separation. (Sample in ethanol) Experimental conditions: column: SE-54 (50 m × 0.2 mm i.d., 0.5 μm film thickness); Column temperature: 240°C; split ratio of 30:1; detection: FID; injection volume: 1 μL.

our studies indicated that acetonitrile could change the polarity of the stationary phase and the mobile phase of the solvent system composed of *n* hexane–ethyl acetate–methanol–water, increase non-polarity compound in the aqueous phase, and shorten the retention time of the non-polarity compounds in the organic phase. The present study indicates that HSCCC is a very powerful technique for the preparative separation and purification of bioactive components from plant materials.

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