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Supercritical fluid extraction of aurentiamide acetate from *Patrinia villosa* Juss and subsequent isolation by silica gel and high-speed counter-current chromatography

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Abstract

Supercritical fluid extraction (SFE) of aurentiamide acetate from *Patrinia villosa* Juss was performed. The optimization of parameters was carried out using an analytical-scale supercritical fluid extraction (SFE) system. Then the extraction was scaled up by 100 times using a preparative SFE system under the optimized conditions of 55 °C, 35 MPa and modified CO_2 with 10% methanol. Then, the crude extract I obtained by SFE was chromatographed on silica gel and the solvent system composed of petroleum ether–ethyl acetate (5:1, v/v) was used to produce the crude extract II, which was further isolated and purified by high-speed counter-current chromatography (HSCCC) with a two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water (1:1.2:1.2:1, v/v/v/v). One hundred fifty-five milligrams of aurentiamide acetate was obtained from 400 mg crude extract II (contained 42% target) with a purity of 99.3% determined by HPLC and 92.3% recovery in one-step elution, and identification was performed by UV, MS, ^{1}H NMR and ^{13}C NMR. As far as we know, this is the first report of discovering aurentiamide acetate from the plant of *Patrinia genius*.
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Keywords: *Patrinia villosa* Juss; Aurentiamide acetate; Preparative chromatography; Counter-current chromatography

1. Introduction

Patrinia, a genus of about 20 species, is mainly distributed in central to east of Asia and northeast of North America, 10 of which growing in China. *Patrinia* species have been used as medicinal plants for more than 2000 years from *ShenNongBenCaoJing*, a famous ancient Chinese medicinal literary, and some of them still used in folk medicine as anti-virus and anti-bacteria [1,2], especially two species, *Patrinia scabiosaefolia* Fisch and *P. villosa* Juss (*BaiJiangCao* in Chinese).

With regard to the chemical constituents of this genus, we have found more research about *P. scabiosaefolia* Fisch [3], *P. scabra* [4] *and P. gibbosa* [5] than *P. villosa Juss*. A literature search did not yield any reference to early report on the chemical study from *P. villosa* Juss except for some iridoids [6,7]. So, chemical research is urgently needed for quality control, pharmacological research, and exploring new TCM products to the plant of *P. villosa* Juss.

Aurentiamide acetate (*N*-benzoylphenylalaninoyl-phenlyalaninolacetate) is a peptide derivative (structure shown in Fig. 1) and has been isolated from *Coroton hieronymi* [8], H*yaloseris andrade-limae* [9], *Caloglossa leprieurii* [10], *Dicria fel-tarrae Lour*. [11]. Pure materials are often obtained by some conventional protocols of extraction and separation techniques, such as using organic solvents to extract and column chromatography including silica gel and high-performance liquid chromatography (HPLC) to isolate, in which organic solvents are unfriendly to our environment and the conventional separation methods are tedious, time consuming, needing multiple steps, and

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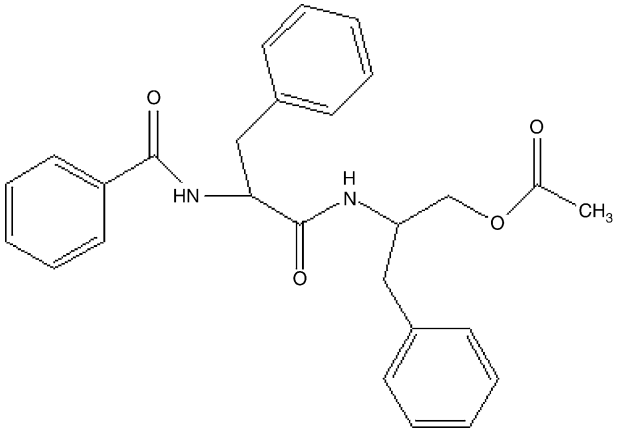

Fig. 1. The chemical structure of aurentiamide acetate.

worse still the sample are adsorbed onto the stationary phase irreversibly.

Two new techniques, supercritical fluid extraction and high-speed counter-current chromatography are widely used to extract and separate natural products from medicinal plants [12–18]. The former uses CO_2 instead of organic solvent and possesses unusual properties such as high compressibility, liquid-like density, high diffusivity, low viscosity and low surface tension. So, supercritical fluid shows a greater ability to diffuse into the ultrafine matrix than the conventional organic solvents, thus improving extraction yield of desired materials from complex matrices. The later, a support free liquid-liquid partition chromatographic technique, eliminates irreversible adsorption of the sample onto solid support [19], has an excellent sample recovery and permits directly introduction of crude samples into the column without more preparation. But there have no reports of using SFE to extract and HSCCC to isolate aurentiamide acetate from natural plants.

The aim of the present paper, therefore, was first to optimize the extraction conditions of aurentiamide acetate by an analytical-scale SFE system using an orthogonal test design. Then, the extraction was scaled up by 100 times by a preparative-scale SFE system. The crude extract I obtained by SFE was cleaned up by silica gel to obtain the crude extract II, which was further isolated and separated by high-speed counter-current chromatography. To our best knowledge, this is the first report of discovering aurentiamide acetate from the plant of *P. genus*.

2. Experimental

2.1. Reagents

Carbon dioxide (CO_2, 99.95%) was obtained from Beijing Analytical Instrument Factory. All solvents and other chemicals including hexane, tetrahydrofuran, isopropanol, ethyl acetate, methanol and acetic acid were analytical grade and purchased from WuLian Chemical Factory, Shanghai, China.

Table 1
L_9 $(3)^3$ orthogonal test design

Test no.	Factors					
	A Pressure (MPa)		B Temperature (°C)		C Modifier (methanol %)[a]	
Matrix 1	A1	15	B1	45	C1	0
2	A1	15	B2	55	C2	5%
3	A1	15	B3	65	C3	10%
4	A2	25	B1	45	C2	5%
5	A2	25	B2	55	C3	10%
6	A2	25	B3	65	C1	0
7	A3	35	B1	45	C3	10%
8	A3	35	B2	55	C1	0
9	A3	35	B3	65	C2	5%

[a] Modifier (methanol %) = volume of added methanol (ml)/sample mass (g).

While acetonitrile used for HPLC was HPLC grade (Merck, Germany). Reverse osmosis Milli-Q water (18 MΩ) (Millipore, USA) was used for all solutions and dilutions.

The *P. villosa* Juss was purchased from a local drug store and identified by Doctor Luping Qin (Department of Pharmacognosy, College of Pharmacy, the Second Military Medical University, Shanghai, China).

2.2. Optimization of SFE extraction

A Suprex HA111-05-20 system (Hua An SFE Company, Nan Tong, Jiang Su Province, China) in the SFE mode was used for optimization the extraction conditions. In this study, extractions were accomplished with 10 ml volume extraction vessel. Nine extractions were carried out at temperature of 45, 55 and 65 °C, pressure of 15, 25 and 35 MPa and two different concentrations of methanol (5 and 10%) were used as modifier. Table 1 shows the SFE experimental conditions for the extraction of aurentiamide acetate from *P. villosa* Juss. After 1 h of static extraction (no liquid flow), the sample was subjected to dynamic extraction for 1 h by flowing liquid CO_2 at a rate of 0.4 ml/min. The extract was trapped into a collection vessel containing about 25 ml tetrahydrofuran–isopropanol (1:1, v/v), and then analyzed by HPLC.

2.3. Scaling-up SFE and preparation of the crude extract

Under the optimized SFE conditions determined above, the extraction was scaled up by 100-fold using a preparative HA221-50-02 SFE system made by the same company. A 200 g amount of sample was placed into an extraction vessel with a 1000 ml capacity, and extracted statically for 1 h and then dynamic extraction for 2.5 h by flowing liquid CO_2 at a rate of 40 kg/h, and the extract in supercritical fluid was depressed directed into separate vessels. The crude extract I from *P. villosa* Juss was light yellow semisolid (4.6 g) and then cleaned up by silica gel column chromatography (glass column: 3.0 cm × 60 cm contained 100 g silica gel). Different volume ratios of petroleum ether–ethyl acetate were used as elution solvents, and the fraction eluted by

petroleum ether–ethyl acetate (5:1, v/v) was collected and evaporated to dryness under reduced pressure at 60 °C, then the crude extract II was obtained, which was used for subsequent HSCCC isolation and separation.

2.4. HSCCC separation procedure

Preparative HSCCC was carried out with a model TBE-300 A high-speed counter-current chromatography (Shenzhen, Tauto Biotech, China). The apparatus equipped with a polytetrafluoroethylene three preparative coils (diameter of tube, 2.6 mm, total volume, 300 ml) and a 20 ml sample loop. The revolution radius or the distance between the holder axis and central axis of the centrifuge (R) was 5 cm, and 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). The HSCCC system was equipped with a model S constant-flow pump, a model UV-II detector operating at 254 nm, and a model N2010 workstation (Zhejiang University, Hangzhou, China).

2.5. Measurement of partition coefficient

Approximately 2 mg of the crude extract II was weighted in a 10 ml test tube to which 4.0 ml of each phase of the equilibrated two-phase solvent system was added. The tube was shaken vigorously for 2 min to equilibrate the sample thoroughly with the two phases. Then the two-phase was separated and evaporated to dryness under reduced pressure. The residue was analyzed by HPLC. The partition coefficient (K) value was expressed as the peak area of target components in the upper phase divided by that in the lower phase.

2.6. Preparation of two-phase solvent system and sample solution

Two-phase solvent systems were used in the present study, *n*-hexane–ethyl acetate–methanol–water (1:1.2:1.2:1, v/v/v/v) was prepared. The solvent mixture was thoroughly equilibrated in a separated funnel at room temperature and the two phases were separated shortly before use.

The sample solution was prepared by dissolving the sample in the 10 ml lower phase of solvent system for isolation and purification.

2.7. HPLC analysis and CCC peak identification

The analytical HPLC system used throughout this study consisted of 515 pump and 2487 detector (Waters), and a model N2000 workstation (Zhejiang University, Hangzhou, China). The crude sample and peak fraction obtained by HSCCC were analyzed by HPLC. The column used was a reversed-phase Lichrospher C_{18} (6.0 mm × 150 mm i.d. 5 μm) (Hanbang Science, Jiang Su Province, China) with a pre-column equipped with the same stationary phase, the mobile phase was CH_3CN–H_2O (80:20, v/v). The flow rate was 1.0 ml/min, and the effluent was monitored at 254 nm.

Identification of the CCC peak fraction was carried out by UV, MS, ^{1}H NMR and ^{13}C NMR.

3. Results and discussion

3.1. Optimization of the SFE conditions

The first step in the SFE of aurentiamide acetate is to optimize the operating conditions to obtain an efficient extraction of target compound and avoid the co-extraction of undesired compounds such as fatty acids and their esters.

Since various parameters potentially affect the extraction process, the optimization of the experimental conditions is a critical step in the development of a SFE method. In fact, the fluid pressure, temperature and modifier are generally considered as the most important factors. The optimization of the method can be carried out step-by-step or by using an experimental design. In the present study, all selected factors were examined using an orthogonal L_9 $(3)^3$ test design.

The extract obtained from each test in SFE was quantitatively analyzed by HPLC for the amount of aurentiamide acetate. The results presented in Table 2 indicated that

Table 2
L_9 $(3)^3$ test results

Test no.	A 1	B 2	C 3	Extraction yield (%)[a]	Concentration (%)[b] aurentiamide acetate	Extraction yield (mg/g)[c] aurentiamide acetate
1	A_1	B_1	C_1	0.13	0.24	0.03
2	A_1	B_2	C_2	1.56	1.38	0.22
3	A_1	B_3	C_3	1.06	1.57	0.17
4	A_2	B_1	C_2	1.29	2.63	0.34
5	A_2	B_2	C_3	1.65	2.96	0.49
6	A_2	B_3	C_1	1.30	2.86	0.37
7	A_3	B_1	C_3	0.69	2.44	0.17
8	A_3	B_2	C_1	2.01	6.71	1.35
9	A_3	B_3	C_2	1.00	2.12	0.21

[a] Extraction yield (%) = the amount of crude extract I/sample mass.
[b] Concentration (%) = the amount of aurentiamide acetate/crude extract I.
[c] Extraction yield (mg/g) = the amount of aurentiamide acetate in crude extract I/sample mass.

Table 3
Analysis of L_9 $(3)^3$ test results

	Aurentiamide acetate yield (mg/g)		
	A	*B*	*C*
K_1	0.39[a]	0.51	0.70
K_2	1.20	2.06	0.76
K_3	1.73	0.75	1.86
k_1	0.13[b]	0.17	0.23
k_2	0.40	0.69	0.25
k_3	0.58	0.25	0.62
R	0.45[c]	0.52	0.39
Optimal level	A_3	B_2	C_3

[a] $K_i^A = \Sigma$ amount of aurentiamide acetate in crude extract I at A_i.
[b] $k_i^A = \frac{K_i^A}{3}$.
[c] $R_i^A = \max\{k_i^A\} - \min\{k_i^A\}$.

maximum extraction yield of the crude extract I was 2.01%, the maximum concentration of aurentiamide acetate in crude extract I was 6.71% and the maximum yield of aurentiamide acetate was 1.35 mg/g.

In our experiment, using two concentration of methanol solution as modifier, extraction efficiencies at different sets of temperature and pressure were examined under L_9 $(3)^3$ test design. The results shown in Table 2 and great difference between each set of SFE conditions were revealed, and the SFE data was analyzed and listed in Table 3.

The influence of temperature to the extraction yield of aurentiamide acetate was the most significant, but high temperature seems unfavorable for our aim. The extraction yield was increased from 45 to 55 °C, and decreased from 55 to 65 °C. High pressure and high concentration of modifier can produce more aurentiamide acetate. Effects of each key factor on the extraction yield of aurentiamide acetate are shown in Fig. 2.

The best SFE condition was $A_3B_2C_3$, according to the extraction yield of aurentiamide acetate. The extraction yield was found to be increased by high concentration of modifier, high pressure and moderate temperature. Under the optimal conditions of 35 MPa, 55 °C and CO_2 fluid modified with 10% methanol, the extraction yield of aurentiamide acetate can reach 1.46 mg/g, which is higher than that of the maximum value in L_9 $(3)^3$ test. The extraction yield would increase much if methanol is added more, while 10% methanol was selected as modifier in our paper mainly considering the co-extraction of undesired compounds and the limitation of the added modifier in SFE (methanol $\leq$20%).

3.2. Preparative-scale SFE

Under the above optimized SFE extraction conditions, 4.6 g crude extract I was obtained from 200 g sample. HPLC analysis in Fig. 3A shows that the crude extract II contained 12.2% of aurentiamide acetate.

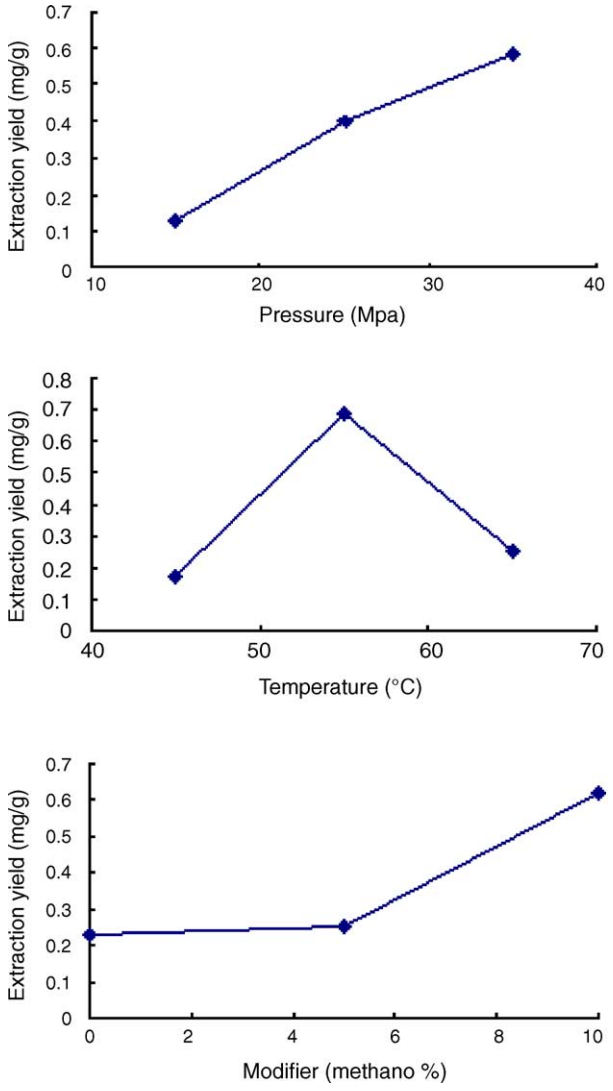

Fig. 2. Effects of temperature, pressure and modifier concentration on extraction yield.

3.3. Comparison of SFE with traditional solvent extraction

About 20 g amount powder of *P. villosa* Juss was soxhlet extracted with 300 ml hexane for 8 h. The yield of crude extract I was found to be 2.54%, which was higher than that of SFE extraction, but the extraction yield of aurentiamide acetate (1.12 mg/g) was much lower than that of SFE extraction (Fig. 4).

3.4. Silica gel step

4.6 g Crude extract I after preparative-scale SFE was first cleaned up by silica gel, and 0.86 g crude extract II, used for further HSCCC isolation, was obtained and analyzed by HPLC (shown in Fig. 3B). The result indicated that the crude extract II contained 42% of the target. The recovery in this step was 64.5%.

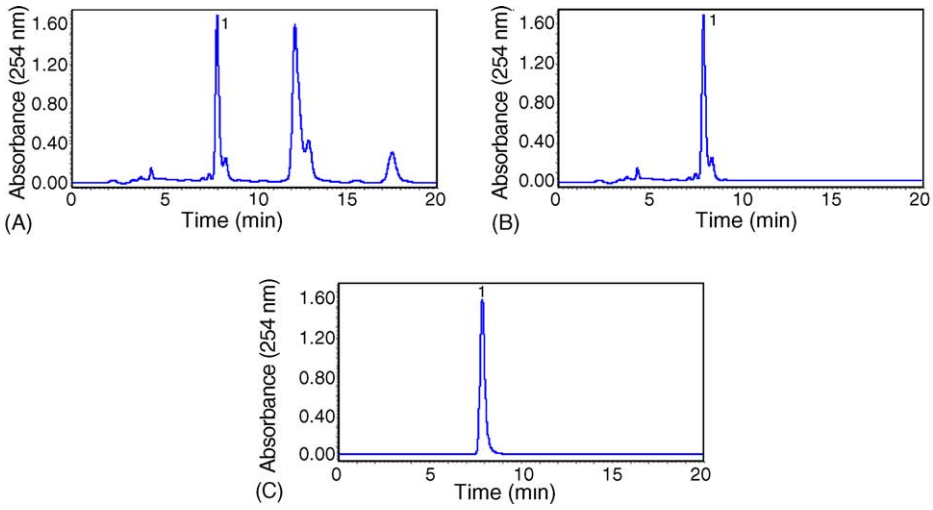

Fig. 3. HPLC chromatogram of the samples. (A) Crude extract I before silica gel step; (B) crude extract II after silica gel step; (C) fraction (155 mg) obtained by HSCCC; column: reversed-phase Lichrospher C $_{18}$ (6.0 mm × 150 mm i.d. 5 μm); mobile phase: CH_3CN–H_2O (80:20, v/v); flow rate: 1.0 ml/min; UV wavelength: 254 nm; peak 1: aurentiamide acetate.

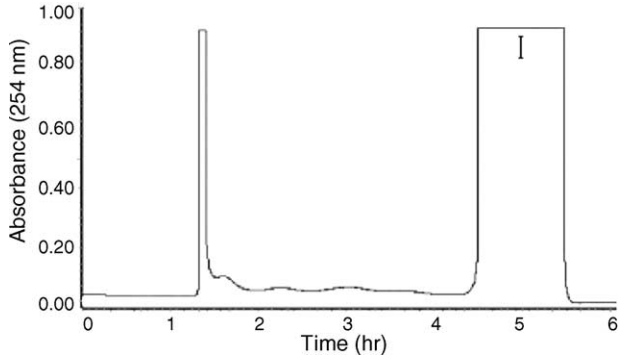

Fig. 4. Chromatogram of the crude extract II by preparative HSCCC. Solvent system: *n*-hexane–ethyl acetate–methanol–water (1:1.2:1.2:1, v/v/v/v); stationary phase: upper phase; mobile phase: lower phase; flow rate: 2.0 ml/min; revolution speed: 800 rpm; sample size: 400 mg; retention of stationary phase: 63%; sample loop: 20 ml; detection wavelength: 254 nm.

3.5. HSCCC separation of aurentiamide acetate

In our research, the two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol– water at different volume ratios (1:0.5:0.5:1, 1:0.8:0.8:1, 1:1:1:1, 1:1.2:1.2:1, 1:1.5:1.5:1, 1:2:2:1, v/v/v/v) were examined, and the *K* values are listed in Table 4. Among them, three kinds of volume ratios (1:0.5:0.5:1, 1:0.8:0.8:1, 1:1:1:1, v/v/v/v) had large *K* values, and two kinds (1:1.5:1.5:1, 1:2:2:1, v/v/v/v) had small *K* values, which were unsuitable for isolation aurentiamide acetate. At last, *n*-hexane–ethyl acetate–methanol–water at a volume ratio of 1:1.2:1.2:1 (v/v/v/v) was selected for our experiment.

Table 4
The *K* values of aurentiamide acetate in different solvent systems

n-Hexane–ethyl acetate–methanol–water	*K*-values
1:0.5:0.5:1	1.95
1:0.8:0.8:1	1.67
1:1:1:1	1.28
1:1.2:1.2:1	0.76
1:1.5:1.5:1	0.099
1:2:2:1	0.027

Using the selected solvent system, one fraction (fraction "I") was obtained in one-step elution and less than 6 h, and the retention of stationary was 63%. One hundred fifty-five grams of aurentiamide acetate was produced from 400 mg crude extract II with a purity of 99.3% determined by HPLC (Fig. 3C) and 92.3% recovery. The over recovery of the compound in our research was 59.5% (multiplication of 64.5% in silica step and 92.3% in HSCCC step), which was lower than that of the HSCCC step. It was obviously that HSCCC has the ability to overcome the irreversible adsorption of sample only in its own isolation procedure, not in silica step.

However, when the crude extract I was directly used for HSCCC separation without silica gel step, which was important for further HSCCC isolation, but the result was not satisfactory and the purity of the obtained material was only 65%, which was much lower than 99.3%, and the retention of the stationary phase decreased a lot because of sits lose. At the same time, different small sample size of the crude extract I and other solvent systems were also tested to separate the pure target without silica gel step, but the outcomes were all bad. So, we can say there is no silica gel step, there is no high pure aurentiamide acetate in the present research.

3.6. Chemical structure identification

The pure product was needed chemical structure identification, which was carried out by UV, MS, ^{1}H NMR and ^{13}C NMR spectra as follows: UVλ_{max}^{MeOH}: 254 nm. TOF-MS: 467.16 $[M + Na]^+$, 911.29 $[2M + Na]^+$, HR-MS: 444.1947. It showed the molecular was 444, which is in agreement with the molecular formula $C_{27}H_{28}N_2O_4$. Comparing with the

reported data, the ^{1}H NMR and ^{13}C NMR data are in agreement with that of aurentiamide acetate in the literature [11].

4. Conclusion

The extraction of aurentiamide acetate by SFE can be improved under optimization extraction conditions by applying low pressure, high concentration of modifier and moderate temperature. It was obvious that under a set of optimized SFE conditions of 55 °C, 35 MPa and modifying supercritical CO_2 fluid with 10% methanol, the extraction yield of aurentiamide acetate can reach 1.46 mg/g, which was much higher than that of solvent extraction. Subsequently, the crude extract I after SFE was cleaned up by silica gel, which was critical for our separation, and then the crude extract I after silica step was further isolated by HSCCC. The content of the compound increased from 12.2% in crude extract II to 42% in crude extract II , and at last reached to 99.3% after HSCCC step. Our study demonstrated that pure aurentiamide acetate can be obtained by SFE and subsequent purified by silica gel and HSCCC from *P. villosa* Juss, and SFE and HSCCC are two powerful techniques of extraction and isolation natural products from raw materials.

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