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ORIGINAL ARTICLE

HPLC Determination of Anti-cancer Components Isolated from *Rabdosia rubescens*

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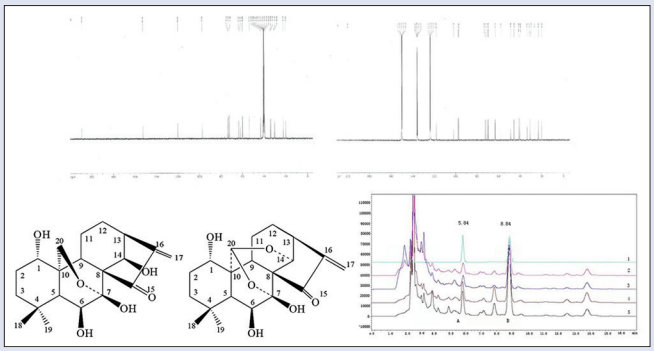
ABSTRACT

Background: *Rabdosia rubescens* is a traditional herbal medicine, commonly known as an anti-cancer plant. A large number of literatures have reported that the diterpenoids in *R. rubescens* have strong anti-tumor effects. The purpose of this study was to isolate the anti-cancer active ingredients from *R rubescens*, identify their structures by nuclear magnetic resonance (NMR), and establish high-performance liquid chromatography (HPLC) method for the determination of content of the oridonin and the ponicidin in *R. rubescens*. **Materials and Methods:** The anti-cancer components of *R. rubescens* were isolated using silica gel column chromatography, and their structures were identified by NMR technology. HPLC method was established for the determination of the content of the oridonin and the ponicidin in *R. rubescens* collected from July to October in Taihang Mountain, China. **Results:** Two anti-cancer components—the oridonin and the ponicidin—were isolated and identified. HPLC methods for the determination of the oridonin and the ponicidin were established. This method could be used to complete the chromatographic analysis of the oridonin and the ponicidin within 10 min, and the chromatographic peaks of each component reached the baseline separation. The contents of the oridonin and the ponicidin in the whole grass of *R. rubescens* collected from July to October were 0.469%, 0.618%, 0.625%, 0.448% and 0.124%, 0.203%, 0.216%, 0.127%, respectively. The results showed that August to September was the best harvest time, and the contents of the oridonin and the ponicidin were the highest during this time. The specific harvest time was determined by the climate and precipitation of the year. **Conclusion:** The HPLC method established in this study was simple, rapid, accurate, reliable, and reproducible, which provided a reference for drug application and resource utilization of *R. rubescens* and could be used for quantitative analysis of anti-cancer active ingredients in *R. rubescens*.

Key words: Anti-tumor components, ^{13}CNMR, HPLC, oridonin, ponicidin, *Rabdosia rubescens*

SUMMARY

- Two anti-cancer components of oridonin and ponicidin were isolated and identified.
- HPLC methods for the determination of oridonin and ponicidin were established. The method could be used for quantitative analysis of anti-cancer active ingredients in *Rabdosia rubescens*.



Abbreviations used: NMR: Nuclear magnetic resonance; DEPT: Distortionless enhancement by polarization transfer; ESI-MS: Electrospray ionization mass spectrometry; HPLC: High-performance liquid chromatography; TLC: Thin-layer chromatography; RSD: Telative standard deviation; TMS: Tetramethylsilane; DMSO: Dimethyl sulfoxide; C_5D_5N: Deuterium substituted of pyridine.

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INTRODUCTION

Rabdosia rubescens (Hemsl.) is the dry aboveground part of Hara, a perennial herb of the genus *Chamellia* in the Labiaceae family. *Rabdosia rubescens* is traditional herbal medicine, commonly known as an anti-cancer plant, which is rich in plant resources in China, mainly distributed in the Yellow River Basin and the vast areas south of China.[1,2] The chemical composition of *R. rubescens* is complex, including flavonoids, organic acids, alkaloids, terpenoids, and so on. Terpenoids contain a series of substances from monoterpenoids and sesquiterpenoids to diterpenoids and triterpenoids. Oridonin and ponicidin are tetracyclic diterpenoid anti-cancer active ingredients isolated from *R. rubescens*.[3,4] In their chemical structure, the structure of cyclopentyl ketone conjugated with extracyclic methylene is the center of its physiological activity; the effect disappears when the ring is split or methylene saturated.[5–8]

Oridonin has a broad spectrum of anti-tumor effects. It has obvious cytotoxic effects on Hela cells, human esophageal cancer 109 cells, and liver cancer BEL-7402 cells *in vitro* and has obvious anti-tumor activities against a variety of transplanted animal tumors such as ECA, S180, P388, L1210, liver cancer, and ARS. It is widely used in the treatment of liver cancer, esophageal cancer, and pancreatic cancer and has achieved certain efficacy in clinical practice.

Ponicidin has an obvious cytotoxic effect on Ehrlich ascites cancer cells *in vitro* and has a definite anti-tumor effect on various transplanted tumors.[9–12] *Rabdosia rubescens*, with its huge resource advantages and good pharmacological activity, has become a commonly used Chinese

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herbal medicine; it is a promising anti-cancer medicinal resource. In this paper, the high-performance liquid chromatography (HPLC) method was established, and the content determination and analysis of the effective anti-cancer components of *R. rubescens* were carried out. The method provided a reference for more objective and effective control and evaluation of the quality of *R. rubescens*.

INSTRUMENT AND MATERIALS

AVANCE DMX-500MHz Nuclear Magnetic Resonance Spectrometer (TMS as internal standard, Bruker company), Q-TOF Micro Mass spectrometer (Bruker company, ESI-MS), Vacuum flash concentration device,[13] LC-20AT High-Performance Liquid Chromatography instrument (Shimadzu Corporation). The chromatographic column was Inertsil ODS-SP C_{18} (4.6 mm × 250 mm, 5 μm), WRS-1B digital melting point tester (Shanghai Precision Scientific Instruments Co., LTD.), Millipore Simplicity ultra-pure water apparatus (Millipore company), AUTO SCIENCE Solvent filter device, RV8 rotary evaporator (IKA company). SHZ-D (III) Circulating vacuum pump, KQ2200DE numerical control ultrasonic instrument. Silica gel for column chromatography (160~200, 200~300 mesh, Qingdao Ocean Chemical Company), Silica gel G and GF254 (Qingdao Ocean Chemical Company) for TLC. Methanol is chromatographic pure, water is ultrapure water, all other reagents were analytically pure. The samples of *Rabdosia rubescens* were collected from July to September 2018 in Taihang Mountain, China, and identified as *Rabdosia rubescens* by Lou Luhuan, professor of medicinal plant taxonomy, Zhejiang A and F University. The sample was dried at 40°C, crushed, and screened through 60 mesh. Oridonin and ponicidin were determined by ^{13}CNMR and ESI-MS, and their structures were confirmed by comparison with those reported in literature. The purities were above 98% by HPLC peak area normalization method.

METHODS AND RESULTS

Extraction and separation

Five kilograms of dry powder of *Rabdosia rubescens* was extracted with 10 times the 95% ethanol for 3 times, and each time for 8 h the extract was combined. The vacuum film concentrator was used for flash concentration at 60°C to 1/5th of the original volume, and an appropriate amount of activated carbon was added for decolorization for two times, with each decolorization for 2 h. The filtrate was filtered under pressure reduction, activated carbon was removed, and then the filtrate was concentrated to no alcohol taste by a vacuum film concentrator; the condensed total extract was 245 g. The total extract was mixed with silica gel, moistened with methanol, and stirred until it was a dry powder. Silica gel (160~200 mesh) column chromatography was used for dry column chromatography separation, and petroleum ether-ethyl acetate system was used for gradient elution, TLC thin layer detection, combined with the same flow fraction. Compound 1 (355 mg) and compound 2 (182 mg) were obtained by petroleum ether-ethyl acetate (8:2) elution system. Compound 1 and compound 2 were recrystallized repeatedly to obtain monomer crystals of compound 1 and compound 2.

Structural characterization

Compound 1 is a white needle-like crystal with melting point of 248°C–250°C. It is insoluble in water, soluble in methanol and acetone, soluble in chloroform and ethyl acetate, and has purplish-red fluorescence at 254 nm UV lamp. Electrospray ionization mass spectrometry (ESI-MS) showed that the molecular ion peak was 364 and the confirmed molecular weight was 364. ^{13}CNMR (125 MHz, DMSO) indicates the presence of 20 carbons, δ: 73.71 (C-1), 29.88 (C-2), 38.87 (C-3), 33.88 (C-4), 59.50 (C-5), 72.97 (C-6), 97.47 (C-7), 62.08 (C-8), 53.53 (C-9), 40.99 (C-10), 19.84 (C-11), 30.55 (C-12), 43.24 (C-13), 72.19 (C-14), 209.08 (C-15), 152.52 (C-16), 119.88 (C-17), 33.28 (C-18), 22.26 (C-19), 63.25 (C-20). Distortionless Enhancement by Polarization Transfer (DEPT) spectrum shows the grade number of carbon in the molecule. The carbon atoms with δ values of 43.24, 59.90, 53.53, 72.97, 72.19, 73.71 are hypomethyl (CH), and the carbon atoms with δ values of 19.84, 29.88, 30.55, 38.87, 63.25, 119.88 are methylene (CH_2). The carbon atoms with δ values of 22.26, 33.28 are methyl (CH_3), and the rest are five quaternary carbons (C) and one ketone carbonyl carbon. C_{15} is ketone carbonyl carbon; C_{16} and C_{17} are alkene carbon; C_7 are carbon carrying two oxygens; and C_1, C_6, C_{14} and C_{20} are oxygen-carrying carbons. That tells us that this molecule has six oxygens and 20 carbons. According to the molecular weight, there are 28 hydrogens, so the molecular formula is $C_{20}H_{28}O_6$. ^{13}C-NMR data were basically consistent with those reported in the literature.[14,15] Thin-layer chromatography (TLC) expansion of the sample under more than two different expansion conditions showed a spot in both, so compound 1 was identified as oridonin.

Compound 2 was a white needle-like crystal with mp of 238°C –240°C. It is insoluble in water, soluble in methanol and acetone, soluble in chloroform and ethyl acetate, and has purplish-red fluorescence at 254 nm UV lamp. ESI-MS showed that the molecular ion peak was 362 and the confirmed molecular weight was 362. ^{13}CNMR (125 MHz, C_5D_5N) indicates the presence of 20 carbons, δ: 72.01 (C-1), 30.26 (C-2), 39.89(C-3), 33.50(C-4), 63.21 (C-5), 72.95 (C-6), 105.75 (C-7), 57.65 (C-8), 45.63 (C-9), 48.77 (C-10), 19.89 (C-11), 26.72 (C-12), 40.99 (C-13), 69.79 (C-14), 200.35 (C-15), 150.23 (C-16), 117.90 (C-17), 30.92 (C-18), 23.06 (C-19), 97.22 (C-20). DEPT spectrum shows the grade number of carbon in the molecule. The carbon atoms with δ values of 40.99, 45.63, 63.21, 69.79, 72.01, 72.95, 97.22 are hypomethyl (CH), and the carbon atoms with δ values of 19.89, 26.72, 30.26, 39.89, 117.90 are methylene (CH_2); the carbon atoms with δ values of 23.06 and 30.92 are methyl (CH_3); and the rest are five quaternary carbons (C) and one ketone carbonyl carbon. C_{15} is ketone carbonyl carbon, C_{16} and C_{17} are alkene carbon, C_7 and C_{20} are carbon carrying two oxygens, and C_1, C_6 and C_{14} are oxygen-carrying carbons. That tells us that this molecule has six oxygens and 20 carbons. According to the molecular weight, there are 26 hydrogens, so the molecular formula is $C_{20}H_{26}O_6$. ^{13}C-NMR data were basically consistent with those reported in the literature.[16,17] TLC expansion of the sample under more than two different expansion conditions showed a spot in both, so compound 2 was identified as ponicidin. ^{13}CNMR data of compounds 1 and 2 are shown in Table 1. ^{13}CNMR spectrum diagrams are shown in Figures 1 and 2, and the structure of compounds 1 and 2 are shown in Figure 3.

Content determination by HPLC

Chromatographic condition

LC-20AT High-Performance Liquid Chromatography instrument (Shimadzu Corporation, Japan), chromatographic column: Inertsil ODS-SP C18 chromatographic column (4.6 mm × 250 mm, 5 μm), mobile phase: methanol-water (55:45), flow rate: 0.2 mL·min^{-1}, test wavelength: 239 nm, column temperature 23°C, injection volume: 10 μL. The retention time of ponicidin was 5.84 min, and the retention time of oridonin was 8.84 min. HPLC chromatogram of reference substance and samples are shown in Figure 4.

Preparation of mixed reference solution

Nine point seven eight milligrams of oridonin and 9.50 mg of the ponicidin, the reference substance, were precisely weighed by placing them in a 50 mL volumetric flask, dissolving them in methanol, and diluting them to the scale and shaking them well. The mixed reference

solution with a concentration of the oridonin was 195.6 μg·mL^{-1} and the ponicidin was 190.0 μg·mL^{-1}.

Preparation of test sample solution

Two grams of *Rabdosia rubescens* powder collected from different months and passed through a 60-mesh sieve were precisely weighed and placed in a Soxhlet extractant, and 100 mL of methanol was added to extract until it was nearly colorless (8 h). After filtration, the filtrate was concentrated to dry by vacuum film concentrator.[13] The appropriate amount of petroleum ether soak was added to decolorize. After drying petroleum ether, the residue was dissolved with methanol, and the volume was fixed to 25 mL volumetric flask, and the filter with 0.45 μm microporous membrane. The sample solution of each test was obtained.

Table 1: 13C-NMR data of compounds 1 and 2

The number of the carbon	Compound 1		Compound 2	
	^{13}C (δ)	DEPT	^{13}C (δ)	DEPT
1	73.71 (d)	CH	72.01 (d)	CH
2	29.88 (t)	CH_2	30.26 (t)	CH_2
3	38.87 (t)	CH_2	39.89 (t)	CH_2
4	33.88 (s)		33.50 (s)	
5	59.90 (d)	CH	63.21 (d)	CH
6	72.97 (d)	CH	72.95 (d)	CH
7	97.47 (s)		101.75 (s)	
8	62.08 (s)		57.65 (s)	
9	53.53 (d)	CH	45.63 (d)	CH
10	40.99 (s)		48.77 (s)	
11	19.84 (t)	CH_2	19.89 (t)	CH_2
12	30.55 (t)	CH_2	26.72 (t)	CH_2
13	43.24 (d)	CH	40.99 (d)	CH
14	72.19 (d)	CH	69.79 (d)	CH
15	209.08 (s)		200.35 (s)	
16	152.52 (s)		150.23 (s)	
17	119.88 (t)	CH_2	117.90 (t)	CH_2
18	33.28 (q)	CH_3	30.92 (q)	CH_3
19	22.26 (q)	CH_3	23.06 (q)	CH_3
20	63.25 (t)	CH_2	97.22 (d)	CH

Linear relation test

The mixture of reference solutions 1, 3, 5, 7, 9 μL was taken and the integral values of the peak area of each component was determined according to the above chromatographic conditions. The regression equation of the oridonin was Y=14259.5X-9639.12, r = 0.9999. The regression equation of the ponicidin was as follows: Y=11010.7X-12363.8, r = 0.9999. The results showed that the linear relationship of the oridonin and the ponicidin were good.

Methodology investigation on the analysis method

Precision test: A precise amount of 10 μL of mixed reference solution with a certain concentration was taken and the sample was injected repeatedly for six times according to the above chromatographic conditions. The peak area values were determined respectively. The data in HPLC content determination were determined for three times, and then the average value was taken. The relative standard deviation of the average value was expressed as relative standard deviation (RSD).

The RSD of the chromatographic peak area of the oridonin was 0.25% and the RSD of the ponicidin was 0.36%. The results showed that the precision of the instrument was good.

Repeatability test: Precision weighing 1.0 g dried R. rubescens powder, 6 parts respectively. The test sample solution was prepared according to the above method, and the samples were analyzed according to the above chromatographic conditions. The RSD of the

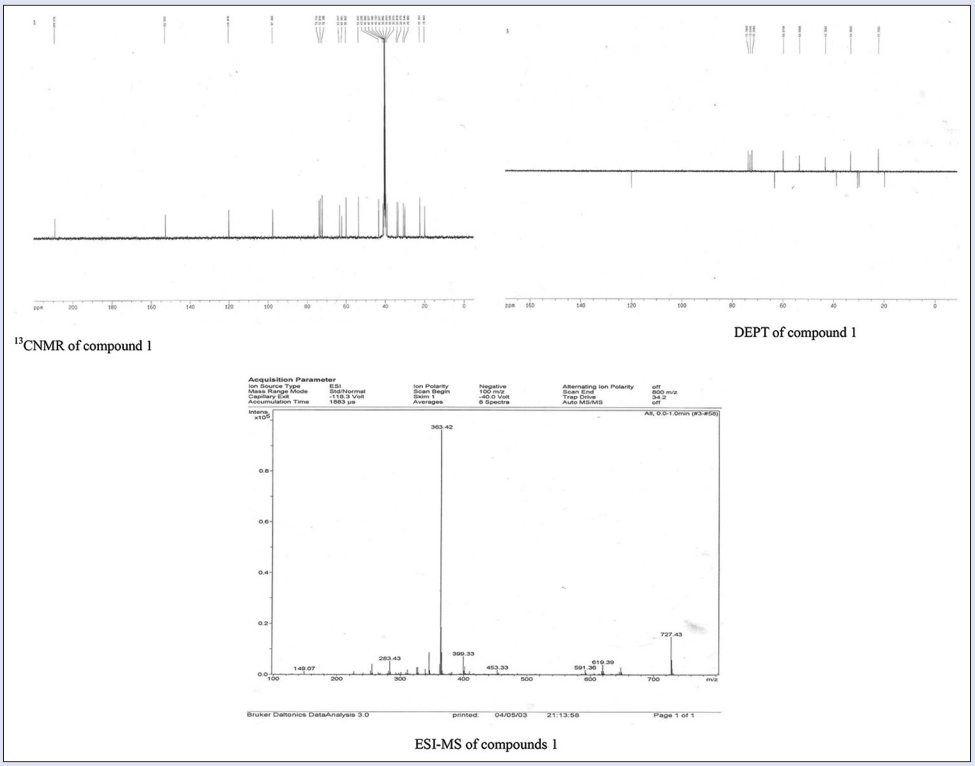

Figure 1: ^{13}CNMR spectrum and MS spectrum of compound 1

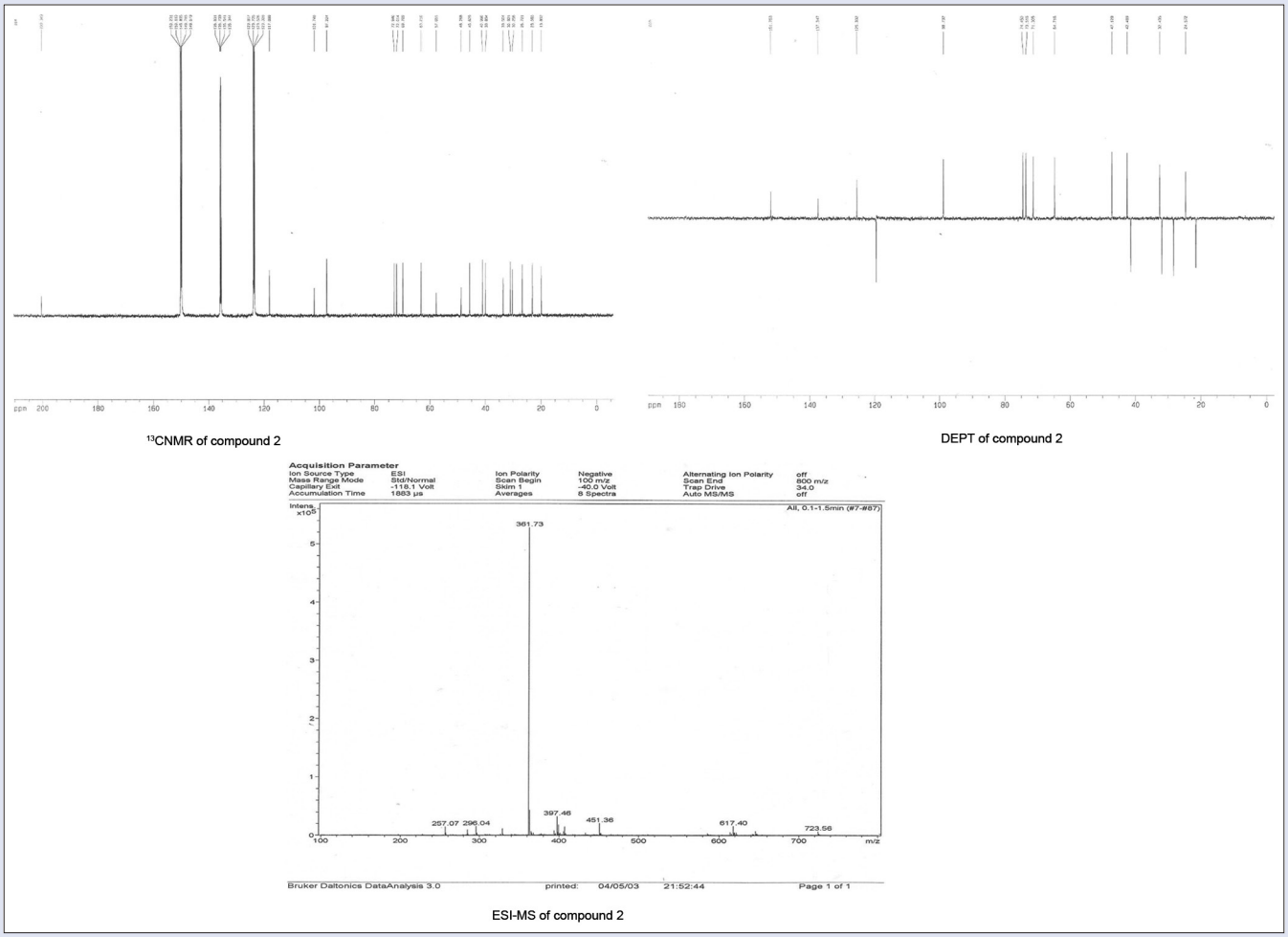

Figure 2: ^{13}CNMR spectrum and MS spectrum of compound 2

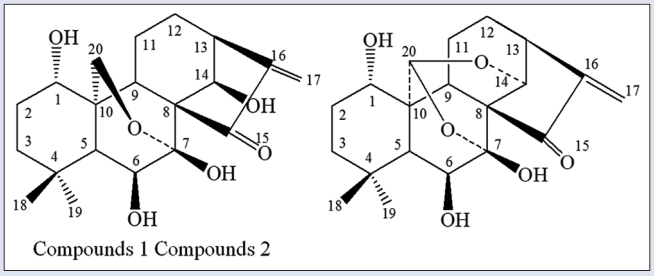

Figure 3: Structure of compound 1 (oridonin) and compound 2 (ponicidin)

content average of the oridonin and the ponicidin were 0.55% and 0.34%, respectively.

Stability test: Ten microliters of test sample solution was precisely measured and placed at room temperature. According to the above chromatographic conditions, the sample was injected and determined once every 2 h, and the determination was repeated five times for 10 h. The peak area values of the oridonin and the ponicidin were recorded, and the content determination results were calculated. The results showed that the RSD of the content average of the oridonin and the ponicidin in the tested sample solution were 0.85% and 0.30%, respectively. The results showed that the tested sample solution was stable within 10 hours.

Content determination of sample

About 2.0 g of *Rabdosia rubescens* powder collected from different months (July to October) was carefully weighed and prepared according to the above preparation method of test sample solution. Four test sample solutions were obtained. The contents of the oridonin and the ponicidin were calculated according to the above chromatographic conditions. The calculation results are shown in Table 2.

DISCUSSION

Determination of preparation method of test sample solution by HPLC method

In order to determine the preparation methods of test sample solution, different polar solvents, including methanol, ethanol, and acetone and different extraction methods, including ultrasonic extraction, soxhlet extraction, and maceration extraction were investigated, and the extraction time was optimized. The results found that continuous reflux extraction with methanol as a solvent for 8 h had the best effect. The extraction rate of the oridonin and the ponicidin were relatively the highest.

Selection of mobile phase in HPLC method

When selecting the mobile phase, methanol-water and methanol-phosphoric acid-aqueous solution systems were used.[18–20] It

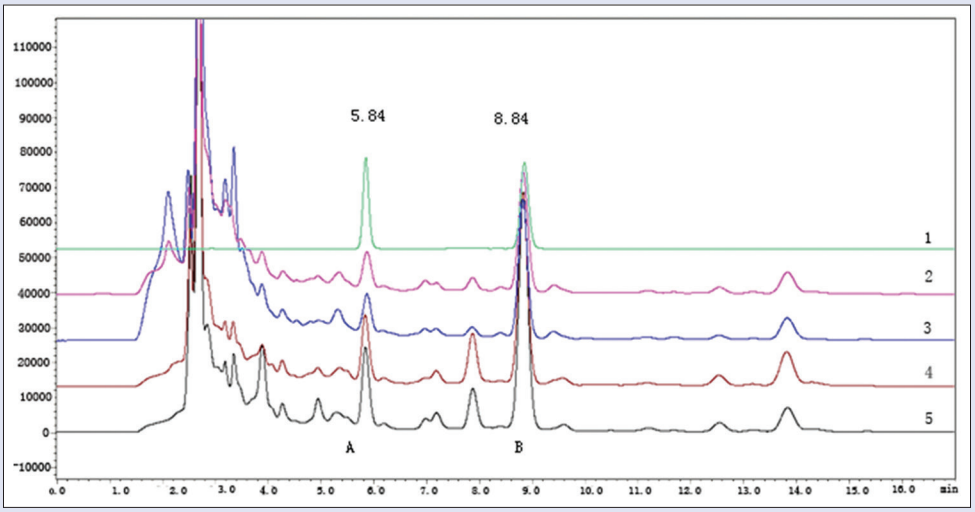

Figure 4: HPLC of reference substance and samples. (A: oridonin, the retention time is 5.84 min. B: ponicidin, the retention time is 8.84 min. 1: reference substance; 2: sample on July; 3: sample in October; 4: sample on August; 5: sample in September)

was found that the addition of acid had little effect on the separation degree and peak pattern. Therefore, methanol-water gradient elution was adopted. it can make the separation degree of the oridonin and the ponicidin reach more than 1.5, so methanol-water system was used in this experiment.

HPLC analysis of determination results

It has been reported[21] that the content of oridonin and the ponicidin in *R. rubescens* leaves is much higher than that in the whole *rubescens*, so the leaves of *R. rubescens* are used as medicinal parts in this study. It has been reported[22] that the best ripening and harvesting season of *R. rubescens* is from July to October every year, and the content of effective components is relatively highest. Therefore, in this study, leaves of *R. rubescens* at different periods from July to October were collected. The contents of the anti-cancer active ingredients oridonin and ponicidin were determined.

In this study, the chemical constituents of *R. rubescens* were extracted, isolated, and structure identified. and the oridonin and the ponicidin of the anti-tumor active components were used as reference substances to establish HPLC method. The content of *R. rubescens* collected from different periods was determined by HPLC method. The results showed that the best harvest time for *R. rubescens* was from August to September every year, and the content of the oridonin and the ponicidin was relatively highest. The specific harvest time was determined by the climate and precipitation of the year. The HPLC method established in this study was simple, rapid, accurate, reliable and reproducible, which provided a reference for drug application and resource utilization of *R. rubescens* and could be used for quantitative analysis of anti-cancer active ingredients in said herb.

Liu *et al.*[23,24] proved through experiments that the inhibition rate of cell growth of NB4 cells and leukemia HL-60 cell line cultured *in vitro* was treated with different concentrations of oridonin, and the results showed that oridonin could inhibit cell growth and induce cell apoptosis. Wang *et al.*[25] showed that *R. rubescens* extract could induce massive necrosis and apoptosis of ascites hepatoma H22 cells by intragastric administration in mice. Liu *et al.*[26] studied the mechanism of oridonin promoting phagocytosis of apoptotic bodies by macrophages differentiated from human lymphoma cells U937. Xiao *et al.*[27] found that oridonin had a significant inhibitory effect on the growth of human nasopharyngeal carcinoma cell line CNE cells. Liu *et al.*[28] observed the apoptosis of human colon cancer HCT8 cells induced by oridonin *in vitro*, and the apoptosis rate increased with the increase of concentration. Yang *et al.*[29] explored and found that oridonin has significant anti-DNA mutation effect. Guan *et al.*[30] showed that oridonin injection had a satisfactory effect on reducing the tumor size of liver cancer, relieving symptoms and improving the quality of life of patients. Oridonin has an obvious inhibitory killing effect on many cancer cell lines. Ponicidin had obvious cytotoxicity to ascites carcinoma cells cultured *in vitro*. Ponicidin has an inhibitory effect on a variety of transplanted tumors. It has an obvious anti-tumor effect on ascites cancer, S180 liver cancer, and L1 ascites cancer in mice, which significantly prolongs the survival time and makes some animals survive for a long time. It also has an obvious anti-tumor effect on reticulosarcoma and solid liver cancer.[31] Oridonin and ponicidin have good anti-cancer activity and low toxicity to normal cells, so they have good clinical application prospect. At present, there is no monomer preparation of oridonin and ponicidin in China except for the total extract preparation of *R. rubescens* in the early market. It is of great significance for the development and utilization of the abundant anti-tumor plant resources of *Rabdosia rubescens*.

Table 2: Contents of two anti-tumor active components in different harvesting time of *Rabdosia rubescens* (%, *n*=3)

Harvesting month	Planting area	Content of oridonin	Content of ponicidin
On July 18	Jiyuan city, Henan province	0.469%	0.124%
On August 20	Jiyuan city, Henan province	0.618%	0.203%
On September 20	Jiyuan city, Henan province	0.625%	0.216%
On October 18	Jiyuan city, Henan province	0.448%	0.127%

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Conflicts of interest

There are no conflicts of interest.

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