Isolation and purification of schisandrol A from the stems of *Schisandra chinensis* and cytotoxicity against human hepatocarcinoma cell lines

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ABSTRACT

Background: Schisandrol A, a lignan with anticancer effects, is one of the representative components that identifies *Schisandra chinensis*. **Objective:** A method for purifying schisandrol A from the stems of *S. chinensis* was established using an octadecylsilyl (ODS) column combined with preparative high-performance liquid chromatography (HPLC). **Materials and Methods:** Crude extracts obtained from the stems of *S. chinensis* using 70% ethanol were separated on an AB-8 macroporous resin column and then eluted with a graded ethanol series. After 70% methanol was used in an ODS column separation, preparative HPLC was used for subsequent purification. The structure was identified by electrospray ionization-mass spectrometry, ^{1}H-nuclear magnetic resonance, and ^{13}C-nuclear magnetic resonance. HepG2 and Bel-7402 hepatocellular carcinoma cell lines were used for toxicological evaluation. **Results:** 21.4 mg of schisandrol A with a purity of 95.2% were collected. The cytotoxicity of the ODS-purified sample and schisandrol A were significantly stronger than that of a resin-purified sample. **Conclusion:** Schisandrol A was successfully extracted from the stems of *S. chinensis* and separated with an ODS column combined with preparative HPLC. The samples obtained during the purification process showed different levels of cytotoxicity on the HepG2 and Bel-7402 hepatocellular carcinoma cell lines.

Key words: Cytotoxicity, octadecylsilyl, preparative high-performance liquid chromatography, *Schisandra chinensis*, schisandrol A

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INTRODUCTION

Schisandra chinensis (Turcz.) Baill (Wuweizi in Chinese), a member of the Magnoliaceae family found in northeast Asia,[1] is listed officially as a herbal medicine in the Chinese Pharmacopeia.[2] The fruits and stems of *S. chinensis* are added to functional foods and used to treat various diseases. The main functional constituents of *S. chinensis* are lignans, including schisandrol A (schisandrin), schisandrol B (gomisin A), deoxyschisandrin (schisandrin A), and γ-schisandrin (schisandrin B).[3] Among these lignans, the Chinese Pharmacopeia listed schisandrol A as one of the representative components that identifies *S. chinensis*, and specified that its content for medical purposes should not be <0.4%.[2] As a typical lignan, schisandrol A shows many biological effects. It is reported to have anticancer properties,[4] aid contraction of the corpus cavernosal smooth muscle,[5] inhibit the activity of cytochrome P4503A4,[6] and have synergistic actions with some other drugs.[7] The traditional method of purifying schisandrol A is preparative thin layer chromatography,[8] which is expensive, time-consuming, and unsuitable for large-scale operations.

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Octadecylsilyl (ODS)-type silica gel has been widely used for the separation of natural products.[9] There are many advantages to this separation method: Sample treatment is simple, there is good reproducibility, and large-scale preparation is possible. Kanchanapoom successfully used an ODS column during the separation of lignan glucosides from *Acanthus ilicifolius*.[10]

Hepatocellular carcinoma is a global health problem with a high rate of occurrence and mortality. Ingesting chemopreventive agents in the diet is one-way to combat this disease.[11] Human hepatoma HepG2 and Bel-7402 are

two cell lines that are widely used as experimental models to screen chemopreventive agents. The two cell lines closely resemble human hepatocytes, are well characterized, and are easy to culture. Recently, the cytotoxicity of lignans and the anti-inflammatory and anti-proliferative qualities of many herbal extracts on the two cell lines have been reported.[12-19]

As *S. chinensis* is a well-known traditional herbal medicine that is believed to have hepatoprotective effects,[20] it is of interest to determine whether its extracts can inhibit hepatocellular carcinoma in the lab. The aim of this study was to investigate a method of purifying schisandrol A using an ODS column, combined with preparative high-performance liquid chromatography (HPLC). We report here the structure of schisandrol A and the cytotoxic effects that it possessed at three stages, as measured by its effects on the cancer cell lines HepG2 and Bel-7402.

MATERIALS AND METHODS

Experimental apparatus

The preparative HPLC equipment was an LC-6AD system (Shimadzu, Japan) and model N2000 chromatography workstation (Zhejiang University, China). The analytical HPLC equipment were a 1525 pump and a 2487 detector (Waters, USA) controlled by Breeze software. Electrospray ionization-mass spectrometry was performed using Agilent 1100 Series LC-MS Trap SL (Agilent, USA). The nuclear magnetic resonance spectrometer was the Agilent 400/54 Premium Shielded NMR Magnet System (Agilent, USA). The microplate reader was the Mode 680 type with 96 wells (Bio-Rad, Japan).

Reagents and materials

Stems of *S. chinensis* were obtained from the College of Horticulture, Shenyang Agricultural University (Shenyang, China). Chromatography-grade methanol was used for preparative and analytical HPLC (Merck, Germany). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and dimethyl sulfoxide were purchased from Sigma (St. Louis, USA). Minimum essential medium was purchased from GIBCO Co. (USA). AB-8 macroporous adsorption resin was purchased from the Cangzhou Bon Adsorber Technology Co., Ltd. (Cangzhou, China). ODS with a particle size of 50 μm (ODS-A, AA12S50) was purchased from YMC (Kyoto, Japan). The other chemicals and solvents were analytical reagent grade and were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Preparation of resin-purified sample

Schisandra chinensis stems were dried at a constant temperature of 60°C and pulverized to the 30-mesh stage using a disintegrator. A 1-kg sample of the powder obtained was extracted with 3000 mL of ethanol (70%) at 60°C for 3 h. This procedure was repeated twice, and the extracts were combined together. After it was poured through a ceramic filter, the filtrate was concentrated and ethanol was removed in a vacuum to produce an aqueous fluid. The fluid was then subjected to separation using a glass column (5 × 100 cm) that was packed with 500 mL of macroporous resin AB-8 and eluted with 0%, 30%, and 70% ethanol. The eluent of 70% ethanol was evaporated to dryness in a vacuum. The resulting 15.6 g of dried powder was a resin-purified sample (RS) that was stored in a refrigerator (4°C) for the next separation procedure.

Selection of methanol ratio in octadecylsilyl column separation

A 1-g sample of RS powder was dissolved in 5 mL 30% methanol and subjected to separation by using a ODS column (2.5 × 100 cm, packed with 100 g ODS packing). It was eluted with 30%, 50%, 70%, and 90% methanol to obtain four fractions: 30% methanol extract (228 mg), 50% methanol extract (334 mg), 70% methanol extract (274 mg), and 90% methanol extract (62 mg) after removal of the solvent under reduced pressure. These fractions were analyzed by an analytical HPLC system, and the fraction with the highest concentration of schisandrol A was used as the ODS-purified sample (OS).

Subsequent purification and analysis using high-performance liquid chromatography

After the OS underwent separation in the ODS column, a 100 mg sample of it was further purified using preparative HPLC on a Shim-pack PREP-ODS(H) (250 × 20 mm, 5 μm) column at 25°C. The mobile phase was methanol: Water (55:45, v/v) in isocratic mode, and the sample was injected through a 900 μL loop. The flow rate was constantly maintained at 5.0 mL/min, and the effluent was monitored at 254 nm. HPLC analyses of the RS, OS, and preparative HPLC peak fraction were performed using a Hypersil ODS-2 C18 column (150 × 4.6 mm, 5 μm) at 25°C. The mobile phase was methanol: Water (55:45, v/v) in isocratic mode, with a run time of 20 min. The flow rate was maintained at 1.0 mL/min, and ultraviolet (UV) detection was again set at 254 nm. The injection volume was 10 μL.

Cell lines and cytotoxicity analysis by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay

Two human hepatocellular carcinoma cell lines (HepG2 and Bel-7402) were purchased from the School of Pharmacy of Shenyang Pharmaceutical University (Shengyang, China). The two cell lines were cultured in a humidified incubator at 37°C with 5% CO_2 atmosphere. Minimum essential

medium supplemented with 10% fetal bovine serum, 100 μg/mL streptomycin, and 100 units/mL penicillin was used as the culture medium. The reduction of MTT was detected by mitochondrial dehydrogenase to blue formazan product, which reflected the normal functioning of mitochondria and indicated normal cell viability.[21]

After the cells were harvested from the culture flasks, they were counted on a hemocytometer, and cell viability was determined by the trypan blue dye exclusion method. HepG2 and Bel-7402 were incubated in a 96-well plate with 100 μL of growth medium and 1×10^4 cells and 5×10^3 cells, respectively, per well. After a 24 h adhesion period, the cells were treated with essential oils in medium for another 24 h. Next, 20 μL of 5 mg/mL MTT in phosphate-buffered saline was added to each well and the plate was incubated at 37°C for 4 h. After the medium was removed, 100 μL of dimethyl sulfoxide was added to each well. The solutions underwent incubation at 37°C for 10 min, after which their absorbance at 570 nm was detected by the microplate reader.[22] The cytotoxicity of the solutions was compared with that of the control (treated with 0.1% dimethyl sulfoxide).

Statistical analysis

The data were presented as mean ± standard deviation of triplicates and evaluated by one-way analysis of variance followed by Duncan's multiple-range tests. All statistical analyses were performed using SPSS for Windows, Version 13.0 (SPSS Inc, IBM, Chicago, IL, USA).

RESULTS AND DISCUSSION

Selection of methanol ratio in octadecylsilyl column separation

In this study, macroporous resin could not separate schisandrol A completely, and RS could not achieve the injection requirement of preparative HPLC, but an ODS column may be an appropriate alternative to both these approaches. The results of ODS-purified fractions of different amounts of schisandrol A when screened among four methanol ratios are shown in Table 1. The 70% methanol extract was enriched in 37.14% schisandrol A. However, only 1.12% and 0.31% schisandrol A were detected in the 30% and 90% methanol extracts, respectively. Therefore, we chose the 70% methanol extract as the OS for subsequent purification.

Purification and analysis by high-performance liquid chromatography

A peak fraction (fraction I, 136-167 min) was separated from the OS, and the preparative HPLC chromatogram is shown in Figure 1. The RS, OS, and fraction I were analyzed using analytical HPLC, and their chromatograms are shown in Figure 2. Schisandrol A showed obvious ultraviolet absorption from 200 to 270 nm,[23] and methanol has a cut-off wavelength of 254 nm, so that wavelength was chosen for separation and analysis by HPLC. Isocratic elution was used to obtain the optimum resolution and shorten the analysis time, because the polarity of major lignans was similar. Preparative HPLC showed a good separation effect, and 21.4 mg of fraction I with a purity of 95.2% was collected.

Structural identification

Compound 1 (fraction I) was structurally identified using electrospray ionization-mass spectrometry, ^{1}H nuclear magnetic resonance, and ^{13}C nuclear magnetic resonance. $[M + H]^+$ (m/z 433) and dehydration peak $[M + H - H_2O]^+$ (m/z 415) were quasi-molecular ions in the first-order spectra, which showed dehydration fragment ions formed from the easily lost hydrogen in C-7. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 6.78 (1H, s, 4-H), 6.59 (1H, s, 11-H),

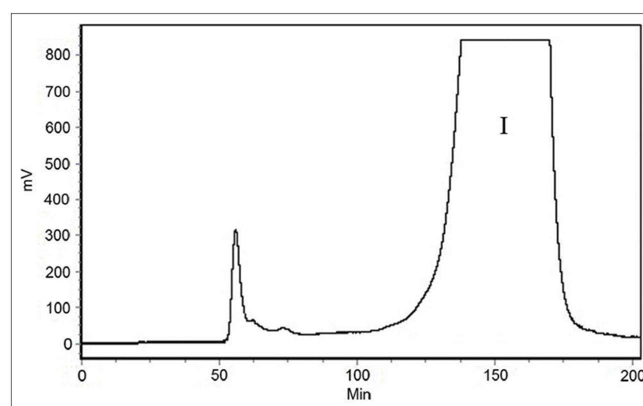

Figure 1: Preparative high-performance liquid chromatography conditions: Column, Shim-pack PREP- octadecylsilyl(H) (250 × 20 mm, 5 μm); mobile phase, methanol:Water (55:45, v/v); flow rate, 5.0 mL/min; detection wavelength, 254 nm. An octadecylsilyl-purified sample was used

Table 1: The concentration of schisandrol A in the RS and four fractions with different methanol ratios produced by an ODS column at 25°C

Sample	RS	Fractions with different methanol ratios (%)			
		30	50	70	90
Schisandrol A ratio (%)	21.72±1.22	1.12±0.04	9.62±0.75	37.14±2.24	0.31±0.02

RS: Resin-purified sample; ODS: Octadecylsilyl

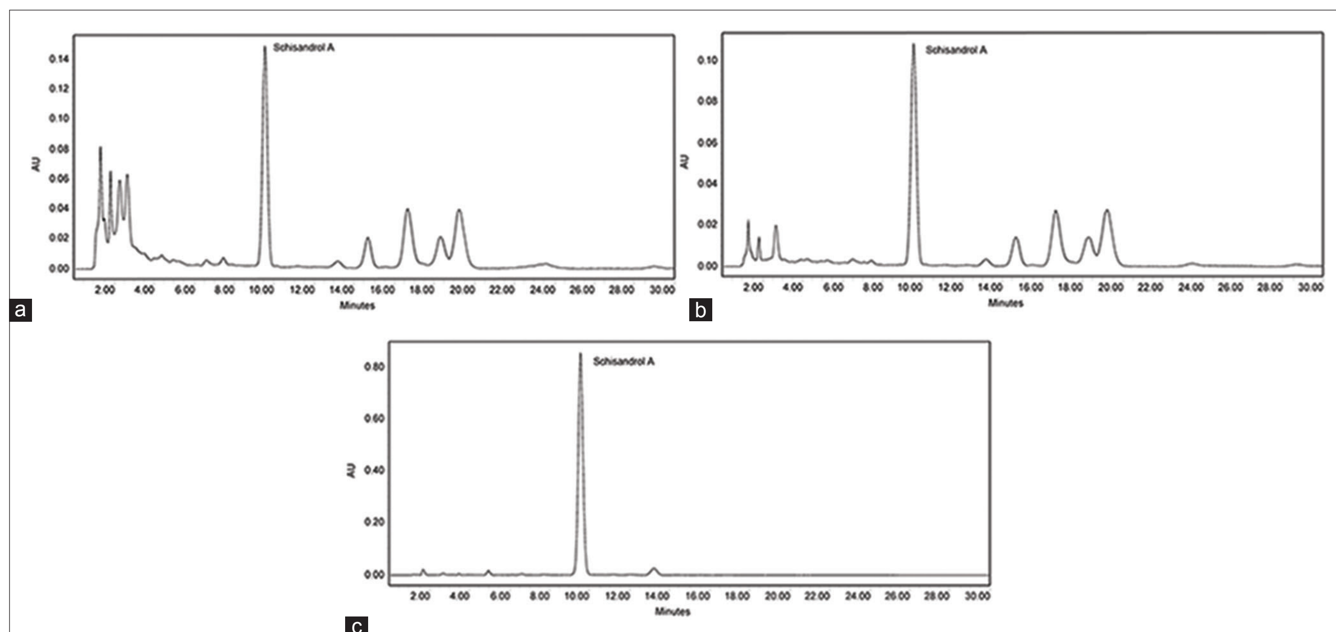

Figure 2: Analysis of high-performance liquid chromatography (HPLC) conditions: Column, Hypersil octadecylsilyl-2 C18 column (150 × 4.6 mm, 5 μm); mobile phase, methanol:Water (55:45, v/v); flow rate, 1.0 mL/min; detection wavelength, 254 nm. Samples were resin-purified sample (a), octadecylsilyl-purified sample (b), and preparative HPLC peak fraction I (c)

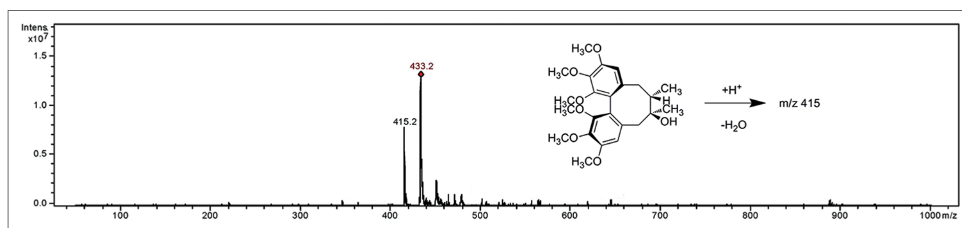

Figure 3: Chemical structure and electrospray ionization-mass spectrometry spectra of schisandrol A

4.06, 3.79 (each 3H, s, 2 × OCH3), 3.67, 3.57 (each 3H × 2, s, 4 × OCH_3), 2.63 (2H, br.d, *J* = 13.52, 6-H), 2.36 (2H, m, 9-H), 1.96 (1H, m, 8-H), 1.61 (1H, br.s, 7-OH), 1.32 (3H, s, 17-CH_3), 0.80 (3H, d, *J* = 7.04, 18-CH_3). ^{13}C NMR (101 MHz, $CDCl_3$): 152.2 (C-1), 140.6 (C-2), 151.3 (C-3), 109.7 (C-4), 134.8 (C-5), 37.5 (C-6), 41.2 (C-7), 34.5 (C-8), 40.1 (C-9), 133.8 (C-10), 111.3 (C-11), 150.8 (C-12), 140.9 (C-13), 151.7 (C-14), 122.1 (C-15), 121.7 (C-16), 11.2 (C-17), 18.6 (C-18), 60.9, 60.7, 60.4, 60.1, 56.2, 55.7 (OCH_3 × 6). By comparing these data with results of previous work,[24,25] we identified compound 1 as schisandrol A. The chemical structure and electrospray ionization-mass spectrometry spectra of schisandrol A is shown in Figure 3.

Cytotoxicity

The antitumor activity of RS, OS, and schisandrol A were tested on the human hepatocellular carcinoma cell lines, HepG2 and Bel-7402. The effects on the two cell lines after 24 h of exposure at dosages from 1 to 100 μg/mL are shown in Table 2. A comparison of the inhibitory concentration of 50 with results of previous work shows that the samples have obvious cytotoxic effects at all three steps on both cancer cell lines.[26] The cytotoxicity of OS and schisandrol A on two cancer cell lines were very similar and were significantly stronger than that of RS from the stems of *S. chinensis*. This result may be attributed to the lower amount of lignans in RS. Besides schisandrol A, some other hepatoprotective lignans appear to exist in OS.[27] The purification method using an ODS column shows great practical value; it is much easier and cheaper than preparative HPLC. Further study of the anticancer mechanism of schisandrol A is merited.

Table 2: Cytotoxicity of the RS, OS, and schisandrol A from the stems of *S. chinensis*, as tested on the HepG2 and Bel-7402 cell lines

Cell lines	IC_{50} (μg/mL)		
	RS	OS	Schisandrol A
HepG2	40.21±1.79	31.89±2.26	27.64±2.64
Bel-7402	37.26±2.31	29.48±1.97	28.33±3.17

S. chinensis: Schisandra chinensis; RS: Resin-purified sample; OS: Octadecylsilyl-purified sample; IC_{50}: Inhibitory concentration of 50

CONCLUSION

Schisandrol A was successfully extracted from the stems of *S. chinensis* and separated with an ODS column combined with preparative HPLC. The samples obtained during the purification process showed different levels of cytotoxicity on the HepG2 and Bel-7402 hepatocellular carcinoma cell lines. These results are very relevant to the development of functional foods containing *S. chinensis*.

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