

# Isolation, Characterization, and Antitumor Activity of a Novel Heteroglycan from Cultured Mycelia of *Cordyceps sinensis*

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## Key words

- *Cordyceps sinensis*
- Clavicipitaceae
- heteroglycan
- characterization
- antitumor activity
- apoptosis
- *Cordyceps unilateralis* subclade

## Abstract

▼  
A novel heteroglycan, *Cordyceps sinensis* polysaccharide 1 (molecular weight  $1.17 \times 10^5$  Da), was isolated and purified from mycelia of the fungus *C. sinensis* obtained by solid-state culture. Structural characterization by chemical analysis, GC-MS, FTIR, and NMR spectroscopy showed that *C. sinensis* polysaccharide 1 was mainly composed of (1 → 6)-linked  $\alpha$ -D-Glc and  $\alpha$ -D-Gal, with minor  $\beta$ -(1 → 4)-D-Xyl and  $\beta$ -(1 → 4)-D-Man residues probably located in the side chains with a trace amount of  $\alpha$ -(1 → 3)-L-Rha residue. In biological assays, *C. sinensis* polysaccharide 1 significantly inhibited proliferation of sarcoma 180 cells and induced apoptosis in a dose-dependent manner. Further studies will elucidate the antitumor

mechanism of *C. sinensis* polysaccharide 1 and promote its utilization for the development of novel, effective anticancer drugs.

## Abbreviations

- ▼
- CSPS-1: *Cordyceps sinensis* polysaccharide 1
  - FACS: fluorescence activated cell sorter
  - FTIR: Fourier transform infrared spectroscopy
  - NSCLC: non-small cell lung cancer
  - S-180: sarcoma 180

**Supporting information** available online at <http://www.thieme-connect.de/products>

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## Introduction

▼  
Natural products have been heavily used in cancer chemotherapy for the past several decades. Mann [1] reported that natural products (including those from plant and microbial sources) and their structural relatives comprise ~50% of the drugs used for cancer chemotherapy. Fungi (particularly mushrooms) have been used by humans as food and as sources of medicinal compounds for thousands of years. A large number of molecules having antitumor properties have been identified from various mushroom species. *Cordyceps sinensis* (Berk.) Sacc. has been classified as belonging to the family Clavicipitaceae, clade B, *Cordyceps unilateralis* subclade [2]. As a famous edible and medicinal mushroom in China, *C. sinensis* possesses many important biological properties, such as antioxidant [3], immunomodulating [4], and antitumor [5], and has been the subject of many research studies. Various bioactive metabolites with antitumor properties have been extracted from *C. sinensis*; these include sterols

[6], cordycepin [7], and polysaccharides [8]. The practical applicability of *C. sinensis* compounds depends not only on their biological properties but also on their biotechnological availability [9]. *C. sinensis* grows slowly and only in high-altitude habitats, and supplies are generally insufficient to meet demand [10]. During the past two decades, culture techniques have been developed to provide abundant mycelia or fruiting bodies as sources of medicinal compounds [11]. Recent attention has focused on polysaccharides as abundant and useful components [12]. Previous studies have succeeded in extracting polysaccharides from mycelia, fruiting bodies, or culture broth of *C. sinensis*, and the polysaccharides vary in their structural characteristics and biological properties. Wasser [9] showed that polysaccharide structures may depend on the composition of the culture medium as well as on the *Cordyceps* species. In the present study, a polysaccharide fraction termed CSPS-1 was extracted, purified, and structurally characterized (molecular weight, mono-

**Table 1** Result of the monosaccharide composition of CSPS-1 using GC-MS.

Sample	Composition
Standards	Rha (21.103 min), Ara (21.853 min), Fuc (22.104 min), Xyl (24.396 min), Man (29.063 min), Glc (29.633 min), Gal (30.230 min), and inositol (34.007 min) <sup>a</sup>
CSPS-1	Rha (20.864 min), Xyl (24.305 min), Man (28.981 min), Glc (29.721 min), Gal (30.429 min), and inositol (33.954 min)

<sup>a</sup> Inositol was used as an internal standard

saccharide composition, polymer linkages) from *C. sinensis* mycelia harvested through a solid-state culture. The antiproliferative effect of CSPS-1 against S-180 cells was investigated *in vitro*.

## Results

Crude polysaccharides from mycelia of solid-state cultured *C. sinensis* were obtained by a series of procedures, including hot water extraction, degreasing, deproteinization, and lyophilization. The polysaccharide sample was separated on a DEAE cellulose-52 column. Two major fractions were detected by spectrophotometry (490 nm) with a phenol-sulfuric acid method (Fig. 1S, Supporting Information). Fraction 1 was further purified on a Sephadex G-100 column, yielding the single polysaccharide CSPS-1 (Fig. 1S, Supporting Information). The molecular weight of CSPS-1 was estimated as  $1.17 \times 10^5$  Da using the calibration curve described in Materials and Methods.

HPLC revealed CSPS-1 as a single homogeneous peak (excluding the solvent peak; Fig. 2S, Supporting Information). The ultraviolet spectrum of CSPS-1 showed no absorbance at 260 and 280 nm, indicating the absence of nucleic acids and proteins (Fig. 2S, Supporting Information). No sulfate groups were revealed by the turbidimetric method. The FTIR spectrum of CSPS-1 showed major peaks at  $3403 \text{ cm}^{-1}$  (O-H stretching),  $2843\text{--}2922 \text{ cm}^{-1}$  (C-H stretching),  $1636\text{--}1733 \text{ cm}^{-1}$  (C-O stretching),  $1384 \text{ cm}^{-1}$  (asymmetrical C-H bending of the  $\text{CH}_2$  group),  $1029\text{--}1204 \text{ cm}^{-1}$  (C-O stretching of C-O-C and C-H-C),  $878 \text{ cm}^{-1}$  (absorption peak of  $\beta$ -glucoside bond), and  $759 \text{ cm}^{-1}$  (symmetric ring stretching of pyranose) (Fig. 2S, Supporting Information). The aldonitrile acetate derivatives of CSPS-1 and monosaccharide standards were analyzed by GC (Fig. 3S, Supporting Information). Based on the retention times of the standards, five kinds of monosaccharide were identified in the CSPS-1 sample: Rha, Xyl, Man, Glc, and Gal (Table 1). Based on peak areas, the monosaccharide molar ratio of CSPS-1 is Glc: Gal: Xyl: Man: Rha = 30.67: 13.37: 5.40: 2.39: 1.0, indicating that CSPS-1 is a heteroglycan, and is composed mainly of Glc and Gal.

Stable absorption at 233 nm was obtained after treatment for seven days, suggesting that complete periodate oxidation had occurred. The oxidation process consumed 1.43 mol periodate per mol glucose residue and yielded 0.53 mol formic acid (Table 2). The formic acid revealed that CSPS-1 contained a 1  $\rightarrow$  6 linkage. The excessive consumption of periodate indicated that CSPS-1 also contained a 1  $\rightarrow$  2 and/or 1  $\rightarrow$  4 linkage. Following transformation of the periodate oxidation/Smith degradation product of CSPS-1 into aldonitrile acetate derivatives, the GC spectrum showed three peaks (excluding the internal standard inositol): glycerol, erythritol, and glucose in a ratio of 10.165:1.949:1 (Fig. 4S, Supporting Information). The large amount of glycerol corresponded to 1  $\rightarrow$  2 and/or 1  $\rightarrow$  6 glycosidic linkages, while the trace amounts of erythritol and glucose corresponded to 1  $\rightarrow$  4 and 1  $\rightarrow$  3 glycosidic linkages, respectively. These findings indicate that (1  $\rightarrow$  2) and/or (1  $\rightarrow$  6) should be ascribed to the main chain linkages, and a low content of (1  $\rightarrow$  3) and (1  $\rightarrow$  4) exists in the main chain or branch.

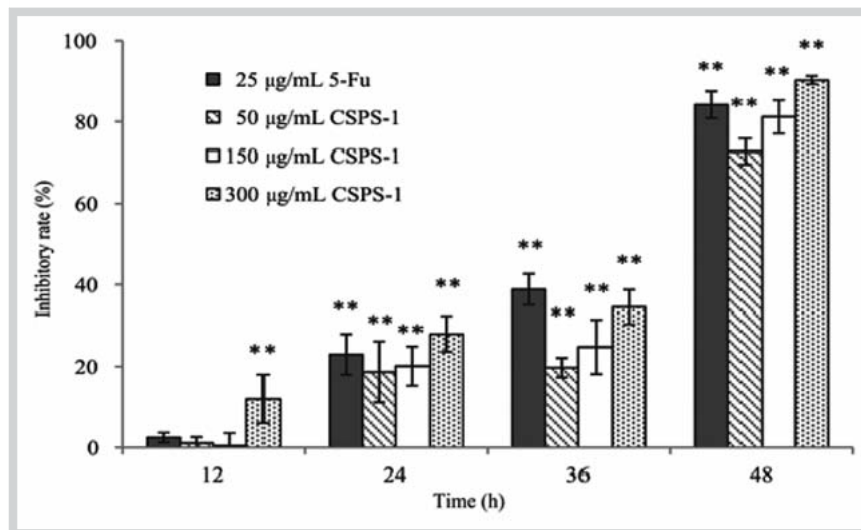
The  $^1\text{H}$  NMR spectrum of CSPS-1 (Fig. 5S A, Supporting Information) mainly contained signals for five anomeric protons at  $\delta$  5.10 ppm, 5.05 ppm, 4.99 ppm, 4.91 ppm, and 4.86 ppm. A chemical shift at  $\delta$  4.66 ppm corresponded to residual HDO (25 °C), with other protons on the glycosidic ring appearing in the region of  $\delta$  3.40–4.40 ppm. In the anomeric carbon region, signals at  $\delta$  5.10 ppm and 5.05 ppm could be attributed to the  $\alpha$  configuration, and the  $\beta$  conformation was confirmed at 4.99 to 4.86 ppm. The  $^{13}\text{C}$  NMR spectrum (shown in Fig. 5S B) also showed multiple resonances of  $\alpha$ - and  $\beta$ -glycosidic bonds. Combined with the aforementioned results, the signals between  $\delta$  107.66 and 106.24 ppm were derived from C-1 of  $\beta$ -(1  $\rightarrow$  4)-D-Xyl. The signals at  $\delta$  102.16 ppm assigned to C-1 of  $\alpha$ -(1  $\rightarrow$  3)-L-Rha,  $\delta$  101.47–100.20 ppm assigned to C-1 of  $\beta$ -(1  $\rightarrow$  4)-D-Man, and  $\delta$  99.65–97.75 ppm assigned to C-1 of  $\alpha$ -(1  $\rightarrow$  6)-D-Glc and  $\alpha$ -(1  $\rightarrow$  6)-D-Gal, respectively [13–15]. Thus, CSPS-1 is a branched-type heteroglycan with both  $\alpha$ - and  $\beta$ -glycosidic linkages. It was supposed to have a backbone of (1  $\rightarrow$  6)-linked  $\alpha$ -D-Glc and  $\alpha$ -D-Gal, and also contain minor  $\beta$ -(1  $\rightarrow$  4)-D-Xyl and  $\beta$ -(1  $\rightarrow$  4)-D-Man residues in the branches. The trace amount of  $\alpha$ -(1  $\rightarrow$  3)-L-Rha residue is probably located randomly in the side chains.

The effect of CSPS-1 on *in vitro* S-180 cell proliferation as a function of time is summarized in Fig. 1. Incubation for 12 h with a high concentration (300  $\mu\text{g}/\text{mL}$ ) of CSPS-1 caused a highly significant ( $p < 0.01$ ) reduction of S-180 proliferation in comparison with the control group. Treatment of cells with CSPS-1 at concentrations of 50, 150, and 300  $\mu\text{g}/\text{mL}$  for durations of 24, 36, and 48 h also caused highly significant decreases in proliferation. After 48 h treatment, the percentages of inhibition in the 50, 150, and 300  $\mu\text{g}/\text{mL}$  groups were 72.74%, 81.22%, and 90.24%, respectively. Proliferation in the positive control group (treated with 25  $\mu\text{g}/\text{mL}$  5-Fu) was significantly lower than that in the nor-

Linkage	Consumption of periodate (mol/mol Glc)	Yield of formic acid (mol/mol Glc)	Products of Smith degradation
1 $\rightarrow$ 2	1	nd <sup>a</sup>	glycerol
1 $\rightarrow$ 3	nd	nd	glucose/mannose
1 $\rightarrow$ 4	1	nd	erythritol
1 $\rightarrow$ 6	2	1	glycerol
CSPS-1 sample	1.43	0.53	glycerol:erythritol:glucose = 10.165:1.949:1

<sup>a</sup> nd: not detected

**Table 2** Periodate oxidation/Smith degradation of CSPS-1.



**Fig. 1** Effect of CSPA-1 on S-180 cell proliferation *in vitro*. 5-Fu: 5-Fu-treated group; CSPA-1: groups treated with CSPA-1 at three dosages (50, 150, or 300 µg/mL). The values shown are mean ± SD (n = 6). \*\*P < 0.01 compared with the normal control group.

mal control group at 24, 36, and 48 h, but not at 12 h. These findings indicate that CSPA-1 displays antitumor activity against S-180 cells *in vitro*.

The apoptotic effect of 24 h treatment with CSPA-1 on S-180 cells was assayed by flow cytometry (► Fig. 2A). The rates of apoptosis for all three CSPA-1 groups and the 5-Fu group were significantly higher than for the normal control group (► Fig. 2B). The apoptosis rates of the 150 and 300 µg/mL CSPA-1 groups were 3.96% and 53.17% higher, respectively, than that of the 5-Fu group. These findings indicate that the antitumor effect of CSPA-1 is exerted via apoptosis.

## Discussion

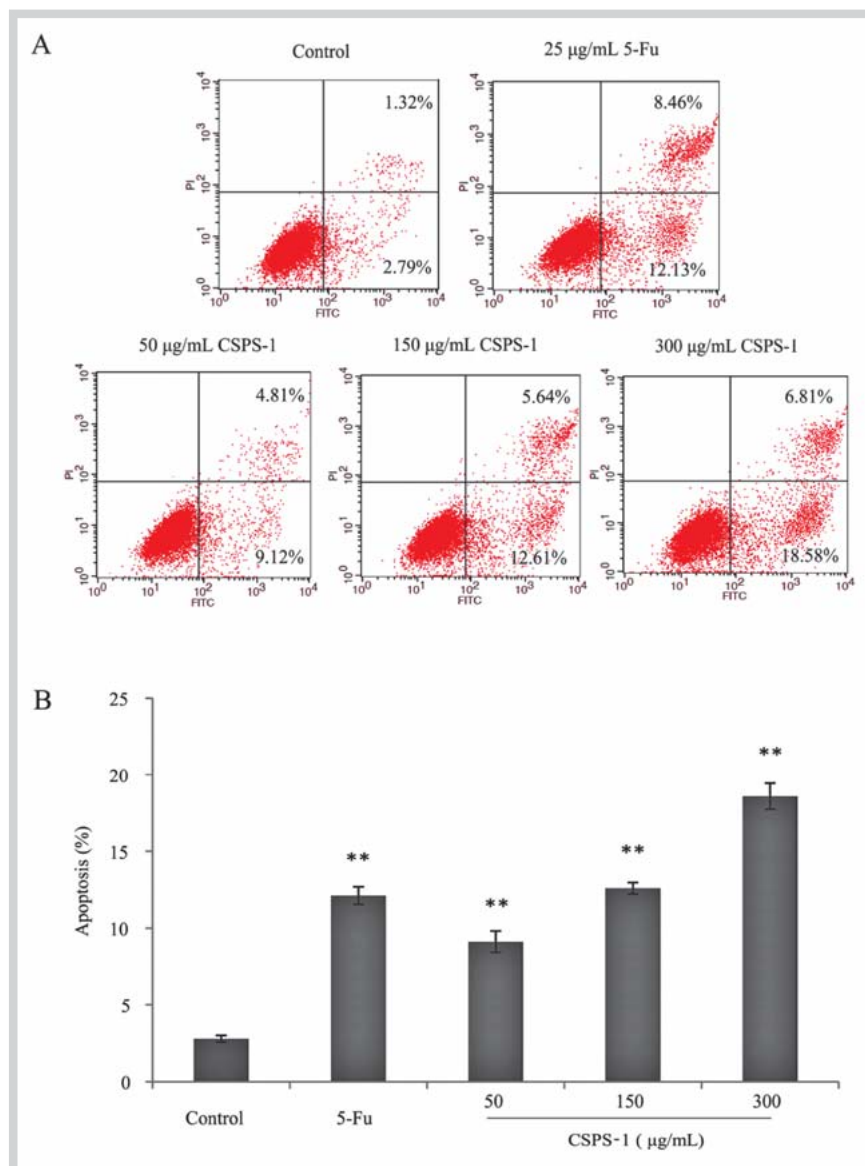
The potentially important role of polysaccharides in tumor/cancer treatment was first recognized over 100 years ago. Antitumor effects have been documented for numerous polysaccharides isolated from 22 mushroom species and 50 types of culture media [16]. The genus *Cordyceps* has received considerable research attention because it is a well-known and popular food source in China.

Several polysaccharides have been isolated from the fruiting bodies, cultured supernatant, or mycelia of *C. sinensis*, and their structures have been characterized in previous studies. SCP-I (MW  $1.84 \times 10^5$  Da), a homoglycan from *C. sinensis* mycelia, was shown to have a backbone of (1 → 4)-D-Glc residues and to carry a single (1 → 6)-linked D-Glc residue [17]. Sheng et al. [4] extracted an exopolysaccharide fraction, EPS ( $1.04 \times 10^5$  Da), from the cultured supernatant of *C. sinensis*. EPS contained Man, Glc, and Gal in a molar ratio of ~23 : 1.0 : 2.6, according to GC analysis. Nie et al. [18] reported that the structure of a bioactive hydrophilic polysaccharide (CBHP; MW  $2.6 \times 10^5$  Da) from cultured *C. sinensis* consisted mainly of Glc (95.19%) plus trace amounts of Man (0.91%) and Gal (0.61%), with a backbone composed of glucopyranose (Glc) joined by 1 → 4 and 1 → 3 linkages. Wang et al. [19] isolated an acidic exopolysaccharide (AEP-1; MW 36.3 kDa) from a mycelial culture of *C. sinensis* and found that it was composed of Glc and pyrano-glucuronic acid (GlcUp) in a molar ratio of 8 : 1 plus a trace amount of Man, and had a linear backbone of (1 → 3)-linked α-D-Glc residues with two branches, α-D-Glc and α-D-GlcUp.

In the present study, we purified CSPA-1, a heteroglycan of MW  $\sim 1.17 \times 10^5$  Da composed of Glc, Gal, Man, Xyl, and Rha in a molar ratio of 30.67 : 13.37 : 5.40 : 2.39 : 1.0, from a hot water extract of *C. sinensis* mycelia. The structural properties of CSPA-1 are strikingly different from those of the other polysaccharides mentioned above. Differences in culture medium, culture conditions, and extraction and purification procedures account for the variability of isolated polysaccharides, including differences in MW, monosaccharide composition, and polymer chain linkages [10, 16, 20]. Mycelial cultures in the previous studies were maintained primarily in liquid culture media and conditions. In contrast, a solid-state culture was used in the present study to obtain large amounts of mycelia.

The biological activities of polysaccharides are affected by their structural properties [20, 21]. Ji et al. [22] studied the adjuvant action of a polysaccharide from *C. sinensis* in the treatment of NSCLC. The proliferation of NSCLC cells treated with this polysaccharide for 48 h was not significantly different from that of the control group. In the present study, the proliferation of S-180 cells was significantly reduced by 24, 36, or 48 h incubation with CSPA-1. After 48 h treatment, the percentages of inhibition in the 50, 150, and 300 µg/mL CSPA-1 groups were 72.74%, 81.22%, and 90.24%, respectively.

Structural features such as (1 → 3)-β-linkages in the glucan backbone have been proposed as important factors in antitumor activity [23, 24]. However, other types of chemical structures were also found in polysaccharides having antitumor effects. A series of water-soluble polysaccharides (GTM1, GTM2, and GTM3) extracted from *Ganoderma tsugae* mycelia displayed high antitumor activity (inhibition ratio > 50%) against S-180 [25]. Structural analyses showed that GTM1 and GTM2 are heteropolysaccharide-protein complexes whose fraction 2 is composed of Gal, Glc, Man, and bound proteins. In this study, CSPA-1 was supposed to have a backbone of (1 → 6)-linked α-D-Glc and α-D-Gal, and contain minor β-(1 → 4)-D-Xyl and β-(1 → 4)-D-Man residues located in the side chains with a trace amount of α-(1 → 3)-L-Rha residue. Previous studies showed that polysaccharides display antitumor activity not only by stimulating proliferation of T lymphocytes, cytokine secretion, and immune function *in vivo* [26, 27], but also have direct effects on tumor cells *in vitro* [28–30]. In the present study, the antiproliferative effect of CSPA-1 on S-180 cells was exerted through an apoptotic pathway *in vitro*. The caspase family



**Fig. 2** CSPS-1 inhibits the proliferation of S-180 cells via the induction of apoptosis. **A** Detection of apoptotic cells by annexin V and PI double staining. **B** Column bar graph of apoptosis. Control: normal control group; 5-Fu: 5-Fu-treated group; CSPS-1: groups treated with CSPS-1 at three dosages (50, 150, or 300 µg/mL). The values shown are mean  $\pm$  SD (n = 6). \*\*P < 0.01 compared with the control group. (Color figure available online only.)

and Bcl protein family play important roles in apoptotic processes [31]. Xu et al. [32] reported that chitooligosaccharides induced apoptosis of human hepatocellular carcinoma cells in a dose-dependent manner *in vitro* by upregulating the pro-apoptotic protein Bax and triggering the initiation of an apoptosis program in the cells. The growth of breast cancer cells was inhibited by an extract from *Phellinus linteus* through the upregulation of p27, resulting in S-phase cell cycle arrest [33]. The antitumor activity of a polysaccharide from *C. jiangxiensis* was exerted through the up-modulation of caspase-3 and down-modulation of Bcl-2 [28]. Further studies will elucidate the antitumor mechanism of CSPS-1 and promote its utilization for the development of novel, effective anticancer agents.

## Materials and Methods

### Materials and reagents

*C. sinensis* was obtained from the Microbial Genetic Stock Center of Huazhong Agricultural University (Wuhan, China). Dried mycelia were harvested through a solid-state culture. DEAE cellu-

lose-52, Sephadex G-100, and T-series dextran molecular weight standards (T-10, T-20, T-40, T-70, T-100) were from Pharmacia Co. D-glucose (Glc), D-galactose (Gal), D-mannose (Man), D-arabinose (Ara), D-xylose (Xyl), D-fucose (Fuc), L-rhamnose (Rha), inositol, and erythritol (purity of standards  $\geq$  99%) were from Sigma-Aldrich. 5-Fu (99%) was purchased from Aladdin Industrial Corporation. Other reagents and chemicals were of analytical grade and from Sinopharm Chemical Reagent Co.

### Extraction and purification of *Cordyceps sinensis* polysaccharide 1

After solid-state fermentation, the mycelia of *C. sinensis* were harvested, dried to a constant weight at 60°C, and pulverized. The dried mycelia powder was obtained. Subsequently, 100 g of mycelia powder was extracted twice with hot water for 3.5 h at 80°C and treated with sufficient  $\alpha$ -amylase to remove starch at 60°C. Ethanol was added to the resulting solution (final concentration 70%), stirred overnight at 4°C, and centrifuged at 5000  $\times$  g for 30 min. The crude polysaccharides obtained were deproteinized with Sevag reagent (chloroform/1-butanol 1:4 v/v) and centrifuged at 3000  $\times$  g for 15 min. The resulting supernatant so-

lution was loaded onto a DEAE cellulose-52 column (2.6 × 40 cm) pre-equilibrated with double-distilled water, and the column was then eluted with double-distilled water and NaCl (2 mol/L) at a flow rate of 30 mL/h. Eluates were collected with a fraction collector and assayed for carbohydrates by the phenol-sulfuric acid method [34]. The first peak sample (3 mg) was purified further by gel filtration chromatography on a Sephadex G-100 column (2.6 × 60 cm) and eluted with deionized water at a flow rate of 30 mL/h. The resulting purified polysaccharide fraction, termed CSPS-1, was lyophilized and subjected to a series of analyses as described below.

### Homogeneity and molecular weight determination

The homogeneity of CSPS-1 was evaluated using an HPLC system (model 2695, Waters Corp.) equipped with an Agilent column (4.6 mm × 250 mm × 5 μm). The presence of proteins and nucleic acids was detected by an ultraviolet spectrophotometer (model 752, Shanghai Optical Instrument Factory Co.). The sulfate content was measured by the turbidimetric method of Dodgson and Price [35]. The molecular weight of CSPS-1 was determined using a calibration curve obtained by plotting the weights of T-series dextran standards against the retention time.

### Fourier transform infrared spectroscopy and nuclear magnetic resonance

The FTIR spectrum of a CSPS-1 sample was collected from KBr pellets using a FT-IR spectrophotometer (model Nexus 470, Thermo Scientific Nicolet) over the wavelength range of 400–4000 cm<sup>-1</sup> [36]. For NMR analysis, a CSPS-1 sample was lyophilized in 99.96% D<sub>2</sub>O, according to the method described by Wang et al. [19]. <sup>1</sup>H and <sup>13</sup>C-NMR were performed with an Agilent 400MR DD2 spectrometer at 25 °C.

### Monosaccharide composition

The monosaccharide composition of CSPS-1 was analyzed by the GC method of Wang et al. [37], with minor modifications. A solution of 10 mg CSPS-1 powder and 6 mL TFA (2 mol/L) was hydrolyzed at 120 °C for 2 h, evaporated, and methanol was added. The dried hydrolysate was mixed with hydroxylamine hydrochloride (10 mg) and pyridine (0.6 mL) and incubated at 90 °C for 30 min, with inositol (5 mg) as the internal standard. The mixture was cooled to room temperature, 1 mL acetic anhydride was added, and the resulting aldononitrile acetate derivatives were analyzed by GC (model 6890 N, Agilent Technologies) with a phenyl methyl siloxane capillary chromatographic column (Hp-5, 30.0 μm × 320 μm × 0.25 μm), and detected by a flame ionization detector. The column temperature was maintained at 120 °C for 3 min and then 210 °C for 4 min. The flow rate was 1.0 mL/min. Seven monosaccharide standards were labeled by the same procedure.

### Periodate oxidation and Smith degradation

CSPS-1 was subjected to periodate oxidation and Smith degradation by the method of Linker et al. [38], with minor modifications. A CSPS-1 sample (20 mg) was mixed with 20 mL sodium periodate (15 mM) at 4 °C in the dark. The reaction was monitored by spectrophotometry at 223 nm. Ethylen glycol (0.4 mL) was added to deoxidize surplus sodium periodate, and the reaction was stopped after 30 min. The solution was reduced with NaBH<sub>4</sub> overnight, pH adjusted to 5.5–7.0 with 0.1 M acetic acid, dialyzed with deionized water for 48 h, and concentrated to dryness by the addition of 2.0 mL methanol. The product obtained was subjected to GC analysis.

### In vitro antitumor assay

The antitumor effect of CSPS-1 was evaluated using S-180 cells (American Type Culture Collection). These cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum (Gibco BRL), penicillin (100 U/mL), and streptomycin (100 U/mL) in a humidified incubator (37 °C, 5% CO<sub>2</sub>). The cells were divided into five groups. The normal control group was treated with the medium only, and 5-fluorouracil (5-Fu) (25 μg/mL) was used as a positive control. The other three groups were treated with sterilized CSPS-1 samples (final concentrations 50, 150, and 300 μg/mL). After incubation for 24 h, S-180 cells (1 × 10<sup>5</sup> cells/well) containing various drugs were placed on a 96-well plate and cultured for 12, 24, 36, or 48 h. Cell proliferation was estimated by the MTT (Sigma) assay. The inhibition rate was evaluated according to the following formula:

$$\text{Inhibition rate (\%)} = (1 - \text{OD}_{\text{exp}}/\text{OD}_{\text{con}}) \times 100\%$$

where OD<sub>exp</sub> and OD<sub>con</sub> are the optical densities of the experimental groups and normal control group, respectively.

### Cell apoptosis assay

The apoptotic effect of CSPS-1 on S-180 cells was assayed by flow cytometry. Following 24 h administration of various drugs in the aforementioned five groups, cells were harvested and examined with an annexin V-FITC/PI apoptosis detection kit (Wuhan Shangcheng Biotechnology Co.). The cells were then pelleted and subjected to FACS analysis (Becton-Dickinson).

### Statistical analysis

The experimental data are expressed as the mean ± SD. Statistical differences between means were evaluated using the program SPSS for Windows, v. 19.0 (SPSS, Inc.). Differences with *p* < 0.05 or *p* < 0.01 were considered significant or highly significant, respectively.

### Supporting information

Purification, HPLC profile, Ultraviolet absorption curve, FTIR, <sup>1</sup>H-NMR, and <sup>13</sup>C-NMR spectra of CSPS-1 as well as the aldononitrile acetate derivative profiles of a mixture of monosaccharide standards and CSPS-1, and their GC spectra following periodate oxidation/Smith degradation are available as Supporting Information.

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### Conflict of Interest

▼ It should be understood that none of the authors have any financial or scientific conflicts of interest with regard to the research described in this manuscript.

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