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Highlights

A purified cell-bound exopolysaccharides (CPS) were extracted from *Bacillus velezensis* SN-1. in Da-jiang.

The primary structure of CPS was identified by GPC, FT-IR, GC-MS, NMR.

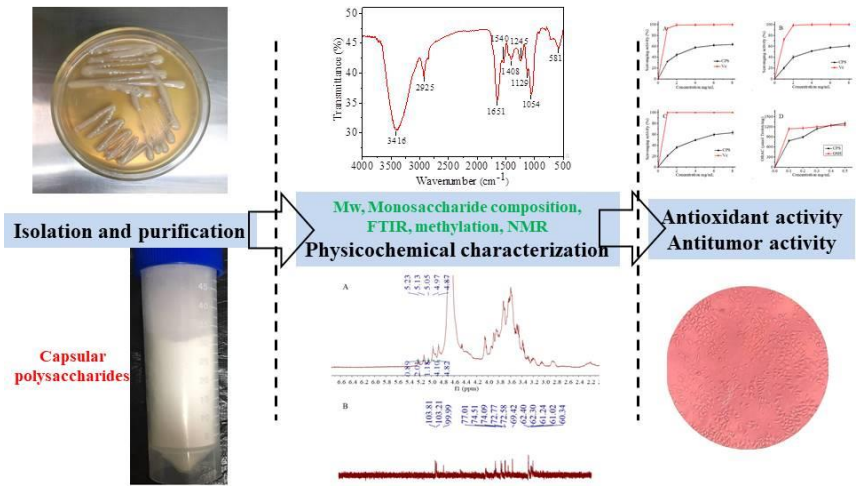
The CPS exhibited potent antioxidant activity in vitro.

The CPS had significant antitumor activities on HepG-2 tumor cells in vitro.

Abstract

A capsular polysaccharides (CPS) producer *Bacillus velezensis* SN-1 (*B. velezensis* SN-1) was isolated in Da-jiang, China. We used ultrasonic extraction to obtain CPS from a culture of *B. velezensis* SN-1 at a yield of 755 mg/L. Using gel permeation chromatography (GPC), CPS was separated into a single peak with a molecular weight of 1.46×10^5 Da. Its structures were characterized by gas chromatography (GC), methylation, fourier transform infrared spectroscopy (FTIR), and nuclear magnetic resonance (NMR). The CPS was identified as a polysaccharide with a highly branched main chain of mannose with (1→3) connections. Moreover, our results revealed that CPS has the capacity to scavenge DPPH radical, hydroxyl radical, ABTS radical and·oxygen radical in a manner that relied on concentration. Anti-neoplastic analysis showed that CPS displayed significant anti-tumor activity towards HepG-2 tumor cells. Above findings indicate that CPS generated by *B. velezensis* SN-1 may be adapted for use as a natural antioxidant in foodstuffs and as an anti-tumor drug.

Graphical abstract



Keywords: *Bacillus velezensis* SN-1; Capsular polysaccharides (CPS)

; Antioxidant activity in vitro; Antitumor activity in vitro

1. Introduction

Exopolysaccharides (EPS) are among the principal metabolites made by several bacterial species, and come in two forms, based on their location: capsular polysaccharides (CPS), where the polymer binds tightly to the cell envelope, and released exopolysaccharides, which are secreted into the ambient medium[1]. Exopolysaccharides protect microorganisms from harmful environments, such as osmotic pressure, dryness, and toxic compounds [2]. In addition, they play a vital role in the colonization of different ecological systems. For example, CPS promotes the colonization of the probiotics to the intestinal mucosa, hence, circumventing the host's immune system activity [3]. *Bacillus*-secreted EPS possess individual rheological and physical features that promote the rheology, essence, and "taste" of fermented dairy products [4]. Moreover, EPS also possess a variety of physiological functions, including anticancer, antioxidant, immunity promoting, cholesterol lowering, anti-hypertension and anti-microbial [5, 6]. In 2015, in China alone 4.8 million cancer cases and 2.8 million cancer-related deaths occurred [7]. Currently, there are no new treatments for sarcomas beyond chemotherapy, which is often characterized by serious side effects. Therefore, it is urgent to extract and identify antineoplastic agents from natural sources. EPS from secure natural sources, for instance, *Bacillus*, might be a good alternative to synthetic antineoplastic drugs.

A mass of EPS-producing *Bacillus* have been studied, focusing on dairy products of technological interest, for instance, *Bacillus paralicheniformis*, *Bacillus thuringiensis*

and *Bacillus tequilensis* [8-10]. In our previous study, we isolated *Bacillus velezensis* SN-1 (*B. velezensis* SN-1) from Da-jiang, a traditional fermented food popular in Northeastern part of China[11-13]. *B. velezensis* produces bioflocculants which aggregate colloidal and cellular substances, and are mainly composed of polysaccharides[14]. Thus, *B. velezensis* SN-1 can be used as a new potentially probiotic strain and a textural agent.

In our previous researches, we have isolated strains that produced EPS *B. velezensis* SN-1 from Chinese Da-jiang, and collected CPS it produced. In the recent work, CPS from *B. velezensis* SN-1 were made and characterized by gas chromatography (GC), fourier transform infrared spectroscopy (FT-IR) and nuclear magnetic resonance (NMR). In addition, their antioxidant and antitumor activities against HepG-2 were evaluated.

2. Materials and methods

2.1. Microorganism and chemicals

B. velezensis SN-1 was isolated from naturally fermented Da-Jiang (Chaoyang, China), and kept in a semisynthetic medium (1000 mL) including 2% KH_2PO_4, 16% K_2HPO_4, 2% $MgSO_4 \cdot 7H_2O$, 1% $CaSO_4 \cdot 2H_2O$, 2% $FeCl_3$, 5% yeast extract and 2% sucrose, respectively (pH 7.0). Sephadex G-100 and DEAE -52 cellulose were from yuanye Bio-Technology Co.,Ltd (Shanghai, China). Mannose, arabinose, fucose, fructose, glucose, xylose and galactose were from MREDA (China). All other reagents used were analytically pure. α-Diphenyl-β-picrylhydrazyl (DPPH) and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was bought from Sigma-Aldrich Co.,Ltd (Shanghai, China). Dimethyl sulphoxide (DMSO) and phosphate buffered saline (PBS) were from Solarbio Science Technology Co., Ltd (Beijing, China).

2.2. Extraction methods of CPS

After getting vaccinated for 48 h, the cultures were centrifuged (10000 ×*g*, 4℃, 15 min) to collecting cell precipitates. The sediment was washed two times with 0.85% NaCl and centrifuged, the sediment was suspended in 1 M NaCl. The sediment was treated with Scientz-IID ultrasonic instrument (Ningbo, China) at 20 W, 4℃ for 5 minutes; The obtained CPS concentrates was then removed proteins by 4% (w/v) trichloroacetic acid (TCA). Three times the volume of absolute ethanol was added to the concentrate for 24 h to precipitate the polysaccharide followed by centrifugation.

Dialyzed precipitates (Mw cut-off: 8000-14,000 Da) were freeze-dried and collected as crude CPS.

2.3. Analysis of bacterial growth and EPS production

B. velezensis SN-1 was grown in 100 mL LB (Lysogeny broth) medium and cultivated under hypoxic conditions at 37°C for 48 h. Samples (1.0 mL) were taken at different time intervals (0-48 h). The survival of the microbial cells was assessed by two successive dilutions. Each dilution was transferred to LB medium, cultivated at 37°C for 48 h under hypoxic conditions, and the pH was measured.

2.4. Purification of CPS

The samples were subjected to DEAE-Sepharose Fast Flow (2.6 cm × 20 cm) and eluted with NaCl stepwise gradient (0, 0.05, 0.10, and 0.20 M). The EPS were further purified using a Sephadex G-100 column (1.6 cm × 50 cm). Purified water served as eluent at a flow rate of 0.2 ml per minute. Active fractions were then collected, dialyzed and freeze-dried.

2.5. Estimation molecular weight (Mw) of CPS

The Mw and number average molecular weight (Mn) of EPS-SN-1 were concluded by gel permeation chromatography (GPC) with the Breeze system (Waters 1525, USA) accompany with the Waters Ultrahydrogel Linear column kept at 35°C and coupled to the refractive index (RI) detector. CPS were made elution with 0.1 M $NaNO_3$ at a

flow rate of 1 ml/min. The pullulan norm scope from 6100 to 642,000 MP (peak molecular weight) (Sigma-Aldrich, USA) for estimating the Mw of CPS. According to the definition of the International Union of Pure and Applied Chemistry (IUPAC), the polydispersion index (PDI) is calculated as follows: Dm=Mw / Mn.

2.6. Monosaccharide composition of CPS

5 mg of purified CPS was added to 4 ml (2 mol/l) of trifluoroactic acid (TFA) and hydrolyzed for 2 h at 110 ℃. Then, the hydrolyzed products were turned into derivatized into acetaldehydes in acetonitrile followed by gas chromatography (GC) to determine monosaccharide composition. GC was carried out on Agilent 6890N GC with a flame ionization detector (FID) and a HP-5 capillary column (30 m×0.25 mm×250 μm). The running conditions were as follows: N_2 carrier gas speed was 1.0 ml/min; The injection temperature and detector temperature were 250℃ and 280℃, respectively; The initial column temperature was 120℃ for 3 min, then the temperature was increased to 210℃ at 15 ℃/min and kept at 210℃ for 4 min. Eight common sugars (d-mannose, d-xylose, l-fucose, d-glucose, d-galactose, l-arabinose, d-fructose, l-rhamnose) were prepared as standards for comparison.

2.7. Methylation analysis

CPS was methylated three times by the improved Ciucanu way [15]. The product was further hydrolyzed with 2 M TFA at 100°C for 2 hours, then decreased with $NaBH_4$, and then acetylated. Partially methylated alditol acetates (PMAAs) was made analyzed by gas chromatography-mass spectrometry (GC-MS). The GC-MS system

(Agilent Technologies, Palo Alto, California) is accompany with a DB-5 column (30.00 m × 0.25 mm × 0.25 μm). The temperature was programmed to be 160-210°C at 2 °C/min. Then it goes up to 210-240°C at 5 °C/min.

2.8. FT-IR and NMR spectroscopy analysis

Feature groups and glycoside bonds in molecules were assessed with fourier transform infrared spectroscopy (FT-IR) (Nicolet iS10, Thermo, USA). In addition, the CPS was ground with KBr powder (CPS: KBr around 1:100) and flattened into transparent sheets for infrared spectra of 800 cm^{-1} and 4000 cm^{-1}.

The analysis of ^{1}H and ^{13}C nuclear magnetic resonance (NMR) was determined with the Ascend 500 HD (Bruker, USA). The dried species were redissolved in deuterium oxide (D_2O, 99.9%) at a concentration of approximately 10 mg/mL. The chemical shifts was expressed as “parts per million” (ppm).

2.9. Antioxidant activity of CPS

2.9.1. DPPH radical ($DPPH^{\bullet}$) scavenging effect

The ability of CPS to clear $DPPH^{\bullet}$ radicals was assessed by previously described methods [16] with minor modifications. Briefly, 1.0 ml of a CPS solution with various concentration (1.0-8.0 mg/mL) was mixed with 0.2 mL $DPPH^{\bullet}$ solution (0.4 mM) and 2.0 mL deionized water. The absorbance of the solution was recorded at 517 nm. A lower absorbance rate is associated with higher free radical clearing power. The below

equation was used to calculate the ability of CPS to clear DPPH$^{\bullet}$:

$$Scavenging\ activity(\%) = \frac{A_0 - A}{A_0} \times 100$$

Where to take 95% ethanol mixed with DPPH, measured A (OD value at 517 nm), the OD value A_0 (A_0 is a blank solution that replaces the sample with distilled water).

2.9.2. Hydroxyl radical ($^{\bullet}$OH) scavenging activity

The ability of CPS to clear hydroxyl radicals was determined based on a previously described method with minor modifications [17]. In short, 160 μL of a CPS solution at different concentrations (1.0-8.0 mg/mL) was incubated at 37℃ for 30 min with 40 μL of 9.0 mM $FeSO_4$, 40 μL of 0.03% H_2O_2 and 20 μL of a 9.0 mM salicylic acid-ethanol solution. A CPS solution (160 μL) was blended with 100 μL of distilled water as the control group. The variation in absorbance produced by salicylic acid was evaluated at 510 nm. Ascorbic acid (Vc) was applied as a reference material. The role of hydroxyl radical scavenging was determined as indicated below.

$$Scavenging\ activity(\%) = \frac{A_s - A_0}{A_c - A_0} \times 100$$

where A_S is the OD vale of the exopolysaccharide; A_0 is the OD vale of the blank solution with distilled water replacing the sample; A_c is the OD vale of a control solution in the shortage of H_2O_2.

2.9.3. ABTS radical ($ABTS^{\bullet+}$) scavenging activity

As mentioned previously [18], the antioxidant activity of pure EPS can be determined

using $ABTS^{\bullet+}$ clearance test. A 7 mM ABTS solution was blended with a 2.45 mM $K_2S_2O_8$ solution and incubated in the dark for 16 hours at indoor temperature to form $ABTS^{\bullet+}$ radicals. CPS samples at various concentrations were mixed with the $ABTS^{\bullet+}$ working solution. The above mixtures were incubated at 37°C for 8 minutes in the dark and their absorbance evaluated at 734 nm. Vc was applied as a positive control and deionized water as blank. The equation used to determine free radical clearing activity was as follows:

$$Scavenging\ activity(\%) = \frac{Abs_{blank} - Abs_{sample}}{Abs_{blank}} \times 100$$

where Abs_{blank} is the suction rate of the specimen with the ABTS working solution; Abs_{sample} is the absorbance of the specimen background solution with the ABTS working solution.

2.9.4. Oxygen radical ($O_2^{-\bullet}$) absorbance capacity assay

$O_2^{-\bullet}$ scavenging capacity was determined by the oxygen radical absorbance capacity (ORAC) method. Briefly, 200 μl fluorescein sodium solution was dispensed into each well of a 96-well plate, followed by 20 μl 0.5 mg/ml EPS sample. The plate was shaken for 5 min and incubated at 37°C for 10 min. After adding 20 μl 2,2'-azobis (amidinopropane) dihydrochloride, the fluorescence was measured at 1 min intervals (fn) at excitation 485 nm and emission 535 nm. To determine the time for the abscissa, ordinate is EPS fluorescence decay samples. The measurement results with the content of the sample is equivalent Trolox (50 μM) representation. Glutathione (GSH) was used as the positive control. Area under the curve (AUC) fluorescence recession

was calculated as the sum of each trapezoidal area using the below equation:

$$AUC = 0.5 \times (f_0 + f_1) \times \Delta t + 0.5 \times (f_1 + f_2) \times \Delta t + \ldots + 0.5 \times (f_x + f_{x+1}) \times \Delta t + 0.5 \times (f_{n-1} + f_n) \times \Delta t$$

where f_n is relative fluorescence intensity on behalf of the n-th measurement point and Δt is the time between consecutive time points. ORAC value of Trolox equivalents (μM Trolox equivalents/mg) was calculated as follows:

$$ORAC_{vale} = \frac{AUC_{Sample} - AUC_{AAPH}}{AUC_{Trolox} - AUC_{AAPH}} \times \frac{molarity\ of\ Trolox}{molarity\ of\ sample}$$

Where AUC_{sample} under fluorescent antioxidants recession area under the curve; AUC_{AAPH} fluorescent radical action when no antioxidant is present recession area under the curve; AUC_{Trolox} fluorescence under standard antioxidants recession area under the curve; molarity of Trolox concentration (μmol/g); molarity of sample concentration (mg/mL).

2.10. Antitumor activity of CPS

To study the anti-tumor activity of CPS, human liver cancer HepG-2 cells were incubated in culture medium in the presence of 10% fetal calf serum, penicillin (100 U/mL) and futostrep (100 mg/L) in a humidified atmosphere containing 5% CO_2 at 37℃. In vitro anti-tumor activity of CPS against HepG-2 cells was evaluated by MTT assay [19]. Briefly, 100 μL of tumor cells (2×10^5 cells/mL) were seeded onto a 96-well plate. The tumor cells were treated with diverse concentrations of CPS (50, 200, 500, 1000 and 2000 μg/mL) or fluorouracil (FU, 50 μg/mL) for 24, 48, or 72 hours, respectively, under a humidified atmosphere including 5% CO_2 at 37℃. At the end of each incubation period, 10 μL (5 mg/mL) of MTT were added and the

incubation was continued for another 4 hours. The media were then aspirated and replaced with 100 μL DMSO to dissolve the crystals. The absorbance was measured at 570 nm using a UV-VIS spectrophotometer. Cell growth inhibition was expressed as follows:

$$Cell\ inhibition\ ratio\ (\%) = \left[1 - \frac{A_{treated} - A_{blank}}{A_{control} - A_{blank}}\right] \times 100$$

Where $A_{control}$ and A_{blank} were the absorbance of the system without adding CPS and without cells, respectively.

For colony formation assay, cells were seeded into 6-well plates at 1×10^5 cells per well and incubated in the existence of diverse concentrations of CPS (50, 200, 500，1000, and 2000 μg/mL). After 24 hours of culture, the medium was aspirated and the same volume of fresh medium including various concentrations of CPS was added. The culture was continued for 48 hours and then observed under an inverted microscope TS 100 (Japan).

2.11. Statistical analysis

Data are expressed as average value ± SEM. Significant diversities in average values were determined by ANOVA, and $p < 0.05$ was supposed to statistically significant.

3. Results and discussion

3.1. Preparation of CPS

Fig. 1 indicates the growth of *B. velezensis* SN-1 at diverse fermentation times, the pH values of the medium, and the yield of CPS. The results showed that the output of CPS increased rapidly during the first 28 hours to a maximum concentration of 270.34 mg/L, while subsequently the output decreased with continued fermentation. Our results also showed that during the first 32 hours of culture, *B. velezensis* SN-1 grew rapidly while the pH of the medium rapidly decreased, but that thereafter the growth rate of *B. velezensis* SN-1 slightly decreased while the pH of the culture medium dropped further. After 48 hours of culture, the final pH of the medium was about 3.1. Our results are consistent with previous research showing that the yield of CPS decreased upon prolonged incubation [20]. The results indicated the bacteria entered the late stage of stabilization. With the consumption of nutrients in the culture medium and the accumulation of harmful metabolites, the bacteria began to decompose the capsules in order to adapt to the harmful environment, as a result, the CPS production began to decline[1].

3.2. Purification and physicochemical characteristics of CPS

The crude CPS was purified by DEAE Cellulose DE-52 further purification chromatographic column (Fig. 2A). Two peaks were eluted. The first peak was strong, showing the presence of a major fraction of EPS. The fractions corresponding to the first peak were combined, dialyzed, freeze-dried and further purified on a Sephadex

G-100 column. Thereafter, a pure product was obtained (Fig. 2B), showing that ultrasonic extraction of CPS yields a homogeneous polysaccharide.

GPC analysis confirmed the homology and molecular weight (Mw) of polysaccharides derivatives. Mw is the total weight of the sample divided by the weight of the individual molecules, while Mn is the total weight of the sample divided by the number of molecules[21]. As shown in Fig. 3. the Mw and Mn of CPS were calculated to be 1.46×10^5 Da and 6.68×10^4 Da respectively. In addition, the PDI of the CPS was 2.18, indicating dispersed rather than uniformly sized particles. According to previous studies, the Mw of CPS made by *Paenibacillus polymyxa* EJS-3 and *Bacillus licheniformis* PASS26 are 8.5×10^5 Da and 5.6×10^5 Da, separately. These are larger than the molecular weight of CPS obtained in this study.

As shown in Fig. 4, GC analysis of monosaccharide composition showed that CPS was made up of different monosaccharides containing mannose and glucose in an approximate molar ratio of 0.546 : 0.453, respectively.

3.3. Methylation analysis of CPS

Methylation analysis (Table 1) of CPS showed the presence of T-Man, 1,3-linked-Glc, 1,3-linked-Man and 1,3,6-linked-Man units. The result indicated the presence of four components, namely 2,3,4,6-Me_4-Man, 2,4,6-Me_3-Glc, 2,4,6-Me_3-Man, and 2,4-Me_2-Man in molar ratio of 14.5 : 44.5 : 36.7: 4.3. The content of glucose in the

partly methylated mixture of CPS takes up approximately 44.5% of the total carbohydrate, which is accordance to the findings of the monosaccharide composition. In addition, except for the final residues, the number of above residues took up 85.5% of the total methylated sugars, indicating that CPS was a greatly branched polysaccharide.

3.4. FT-IR spectroscopy

The FT-IR spectra of purified CPS of *B. velezensis* SN-1 showed the same functional base in all media (Fig. 5), a wide peak at approximately 3416 cm^{-1} (range 3600-3200 cm^{-1}) corresponding to the O-H elastic vibration of polysaccharides [22] as well as a weak C-H stretching band at 2925 cm^{-1}. The peak at 1651 cm^{-1} corresponds to the C=O elastic vibration of the N-acetyl group or a protonated carboxylic acid[23]. In addition, a peak was found at 1540 cm^{-1}, which corresponded to the N-H deformation and vibration of the amine group [24]. In addition, the peak at 1408 cm^{-1} is equivalent to the C-O bond of C=O stretch and a carboxyl group [25]. The peaks at 1245 cm^{-1} may be due to the elastic vibration of the acetyl group [26]. The energy band in the 900-1150 cm^{-1} area was attributed to the vibration of a C-O-C bond [27].

3.5. ^{1}H NMR and ^{13}C NMR analysis of CPS

We further analyzed CPS by NMR spectra. The ^{1}H NMR spectra indicated the heterocyclic signal of CPS generated by SN-1 in *B. velezensis*. This spectrum reveals the complexity and heterogeneity of CPS. The proton signal of α-anomeric pyranose

is greater than 4.8 ppm, and the proton signal of β-anomeric pyranose is less than 4.8 ppm [28] . In ^{1}H NMR (Fig. 6A), six heterocyclic proton signals appeared in the lower field (δ 4.93-5.3) , showing the presence of an α-configuration bond. In addition, the coupled constants of anomeric proton signals in δ 4.94-4.98 (*J=4.10* Hz) and δ 4.85-4.89 (*J=4.82* Hz) was in the scope of 4-8 Hz [29], showing the presence of a β-anomeric center.

The ^{13}C NMR spectrum indicated three anomeric carbon signals at δ103.81, 103.21, and 99.99 ppm, and other signals were located at δ 60-74 ppm (Fig. 6B). Since the alpha carbon signal of α-pyranose is generally δ 97-101 ppm, and β-pyranose residueis generally δ 101-105 ppm[30]. Therefore, the anomeric carbon signal at δ 103.81 and 103.21 represented the β-D-mannose. The peaks at δ 74.51 and 74.09 ppm revealing the possibility of substitution at C-2 or C-3 in the hexose residues[31], According to the methylation analysis, this might be the signal of C-3 substitution of glucose and mannose respectively. The chemical shift appearing at 60-70 ppm was assigned to the C-6 signal in the units. As reavealed by the results, CPS is mainly made up of α configuration glycosidic bond and a small amount of β-glycosidic linkage. The backbone chain might be composed of →3-Man-1→ and →3-Glc-1.

3.6. Antioxidant activities of CPS

3.6.1. DPPH free radical (DPPH$^{\bullet}$) scavenging

The DPPH$^{\bullet}$ scavenging activity of CPS was assessed at different concentrations (0-8.0

mg/mL). The scavenging activity of CPS increased with increasing concentration (Fig. 7A) reaching a maximum clearance capacity of 63.0% at a CPS concentration of 8 mg/mL. The potent antioxidant capacity of CPS can be attributed to its ability to donate electrons or hydrogen to stop the $DPPH^{\bullet}$ chain reaction[32]. In comparison, EPS produced by *Lactobacillus plantarum* JLAU103 reached a maximum $DPPH^{\bullet}$ scavenging capacity at a concentration of 10.0 mg/mL, indicating that CPS produced by *B. velezensis* SN-1 had a better $DPPH^{\bullet}$ scavenging capacity than EPS produced by *Lactobacillus*.[33]. Compared with *Lactobacillus* EPS, CPS has higher proton contribution capacity [16].

3.6.2. Hydroxyl radical ($^{\bullet}OH$) scavenging

Hydroxyl radicals produced by Fenton reactions readily pass through cytomembranes and cause severe damage to the majority of biomacromolecules (including carbohydrates, proteins, lipids and deoxyribonucleic acids) in living cells, resulting in tissue injury or apoptosis [34]. Fig. 7B shows that CPS possessed good $^{\bullet}OH$ scavenging activity, as their maximum scavenging percentage 60.6% at a concentration of 8.0 mg/mL. Hydrogen atoms present in CPS can combine with hydroxyl groups to form water, and carbon free radicals can be further oxidized to peroxide free radicals, which eventually decompose into harmless products[35]. Similar interpretation was reported by Zhang et al [36] in *Chlorella pyrenoidosa* FACHB-9 with a maximum of 63.1% at 8.0 mg/ml.

3.6.3. ABTS radical ($ABTS^{\bullet+}$) scavenging

ABTS radical scavenging is commonly applied to test the antioxidant activity of natural products. As indicated in Fig. 7C, increasing the concentration of CPS results in a gradual raise in its $ABTS^{\bullet+}$ scavenging activity, which proves its antioxidant features. In the presence of Vc at 1 mg/mL, the clearance rate was 99.7%. However, when the concentration was greater than 6.0 mg/mL, the $ABTS^{\bullet+}$ clearance percentage of CPS was more than 50.0%, while when the concentration was 8.0 mg/mL, the clearance rate of CPS reached 63.3%. These findings showed that CPS has the capacity of scavenging ABTS free radicals and therefore may serve as a potential antioxidant.

3.6.4. Assay of Oxygen Radical Absorbance Capacity (ORAC)

The ORAC assay has traditionally been used to evaluate the donor ability of hydrogen atoms. ORAC values acquired for CPS and GSH are indicated in Fig. 7D. At the concentration of 0.4 mg/mL, CPS showed the highest ORAC value (1243.4 μmol TE/g), even above GSH (1238.4 μmol TE/g, 0.4 mg/mL). The ability to scavenge $O_2^{-\bullet}$ might be due to the surface-capping exopolysaccharides on CPS, which enhanced their capacity to deliver hydrogen to $O_2^{-\bullet}$ because of the weak dissociation energy of the O-H bond[8]. Compared with the antioxidant activity of other foods, the ORAC values of rice bran and red grape juice were 18.0 μmol TE/mL and 25.0 μmol TE/mL[37, 38], separately, indicating that the antioxidant activity of CPS in this study was far ahead.

3.7. In vitro antitumor activity of CPS

Fig.8 summarizes the inhibitory effect of CPS on HepG-2 cell growth at various concentrations (50-2000 μg/mL) and incubation periods (24, 48, 72 h). The inhibitory effect of CPS on HepG-2 cells increased significantly with increasing concentrations and incubation times ($p < 0.05$). At the highest concentration (2000 μg/mL) and maximum incubation time (72 h), CPS suppressed the growth of HepG-2 cells by 81.33% ± 1.12%, i.e. its activity was significantly superior to that of the positive control FU. This indicated that CPS had anti-tumor capacity to HepG-2 cells. In addition, we also investigated the effects of the CPS on colony formation of human liver cancer cells HepG-2 through an inverted microscope (Fig. 8). At low densities, healthy HepG-2 cells were observed to have a single morphology, tight cell connections, with large individual cells and a relatively full morphology. After 48 hours of CPS treatment, the number of HepG-2 cells gradually decreased, the intercellular space became larger, some cells expanded and shrunk, and the amount of cell debris increased. This indicates that the colony formation of HepG-2 cells was inhibited which was consistent with the results of the MTT experiments. Furthermore, the morphological changes indicated that the CPS inhibits the proliferation of HepG-2 cells. Moreover, the inhibitory effects of CPS on HepG-2 cells were dose and time dependent.

4. Conclusions

In the present work the CPS from *B. velezensis* SN-1 was prepared and characterized. The CPS was consisted of glucose and mannose with an estimated Mw of 1.46×10^5 Da. Using FT-IR, NMR and methylation analysis, CPS was identified as a highly branched polysaccharide with a backbone of (1→3)-linked Man. Moreover, our results also revealed that CPS has the potential capacities to scavenge $DPPH^{\bullet}$, $^{\bullet}OH$, $ABTS^{\bullet+}$ and $\cdot O_2^{-\bullet}\cdot$ in a concentration dependent way. In vitro anti-tumor analysis showed that CPS had strong anti-tumor activity against HepG-2 tumor cells. Our findings indicated that CPS generated by *B venzensis* SN-1 may be adapted for use as a natural antioxidant in functional diets and as an anti-tumor drug.

Conflict of interest

The authors declare no conflict of interest

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Competing interests

The authors declare that they have no competing interests.

Figure Captions

Fig. 1. Production kinetic curve of EPS-producing *Bacillus velezensis* SN-1 CPS at 37°C up to 48 h. The results are presented as three independent analysis (mean ± SD, n=3).

Fig. 2. DEAE-cellulose-52 anion exchange chromatogram (A) and Sephadex G-100 (B) gel filtration chromatogram of CPS.

Fig. 3. Hydrolysis analysis by gel permeation chromatography (GPC) of CPS.

Fig. 4. GC chromatograms of monosaccharides composition of CPS

Fig. 5. FTIR spectrum of CPS.

Fig. 6. NMR spectra of the polysaccharide EPS-SN-1. ^{1}H NMR spectrum of CPS (A); ^{13}C NMR spectrum of CPS (B);

Fig. 7. In vitro antioxidant activity of EPS-SN-1 from *B. velezensis* SN-1 using ascorbic acid (Vc) and glutathione (GSH) as reference, respectively. DPPH radical scavenging ability (A); Hydroxyl radical scavenging ability (B); ABTS radical scavenging activity (C); Oxygen radical absorbance capacity (ORAC) (D).

Fig. 8. Antitumor effects of CPS on HepG-2 cells. All values were expressed as means ± standard deviation (SD) of three replications.

Table Captions

Table 1 GC-MS data analysis of partial *O*-methylated alditol acetates of sample.

Peak No.	Methylated sugars	Linkages types	Relative molar ratio
1	2,3,4,6-Me_4-Man	Manp-(1→	14.5
2	2,4,6-Me_3-Glc	→3)Glcp(1→	44.5
3	2,4,6-Me_3-Man	→3)Manp(1→	36.7
4	2,4-Me_2-Man	→3,6)Manp(1→	4.3

Table 1 GC-MS data analysis of partial *O*-methylated alditol acetates of sample.

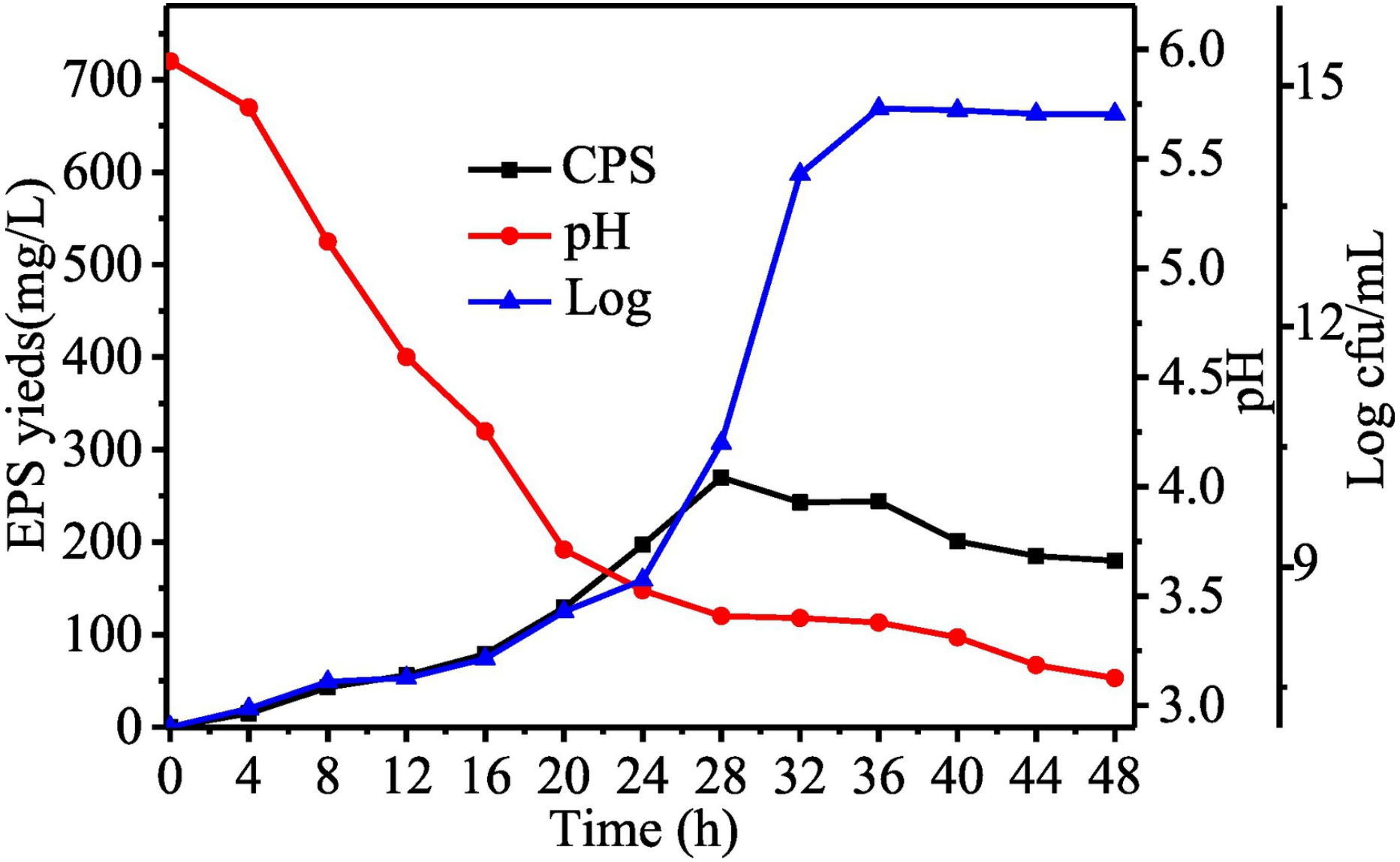

EPS yieds(mg/L)
pH
Log cfu/mL
Time (h)
CPS
pH
Log
0
100
200
300
400
500
600
700
3.0
3.5
4.0
4.5
5.0
5.5
6.0
9
12
15
0
4
8
12
16
20
24
28
32
36
40
44
48

Figure 1

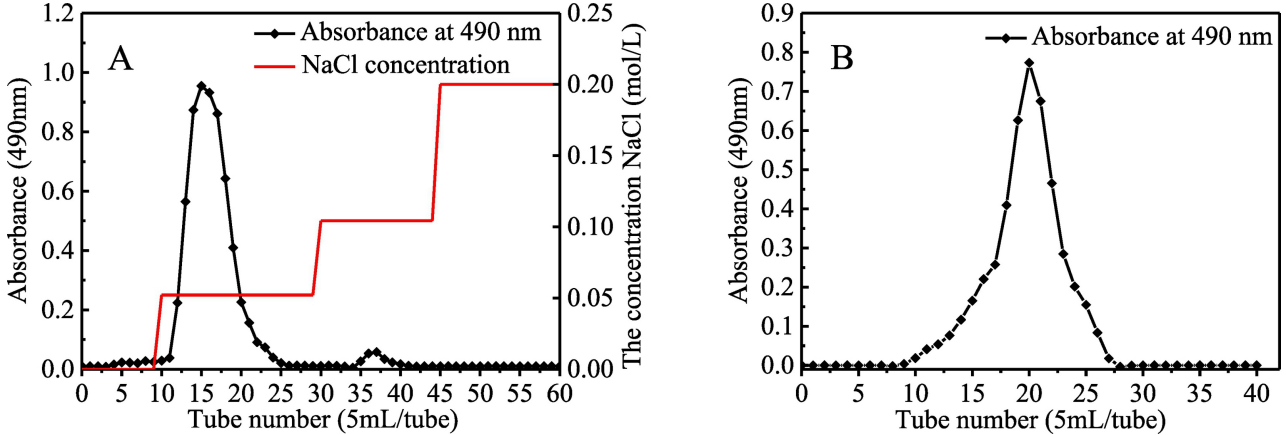
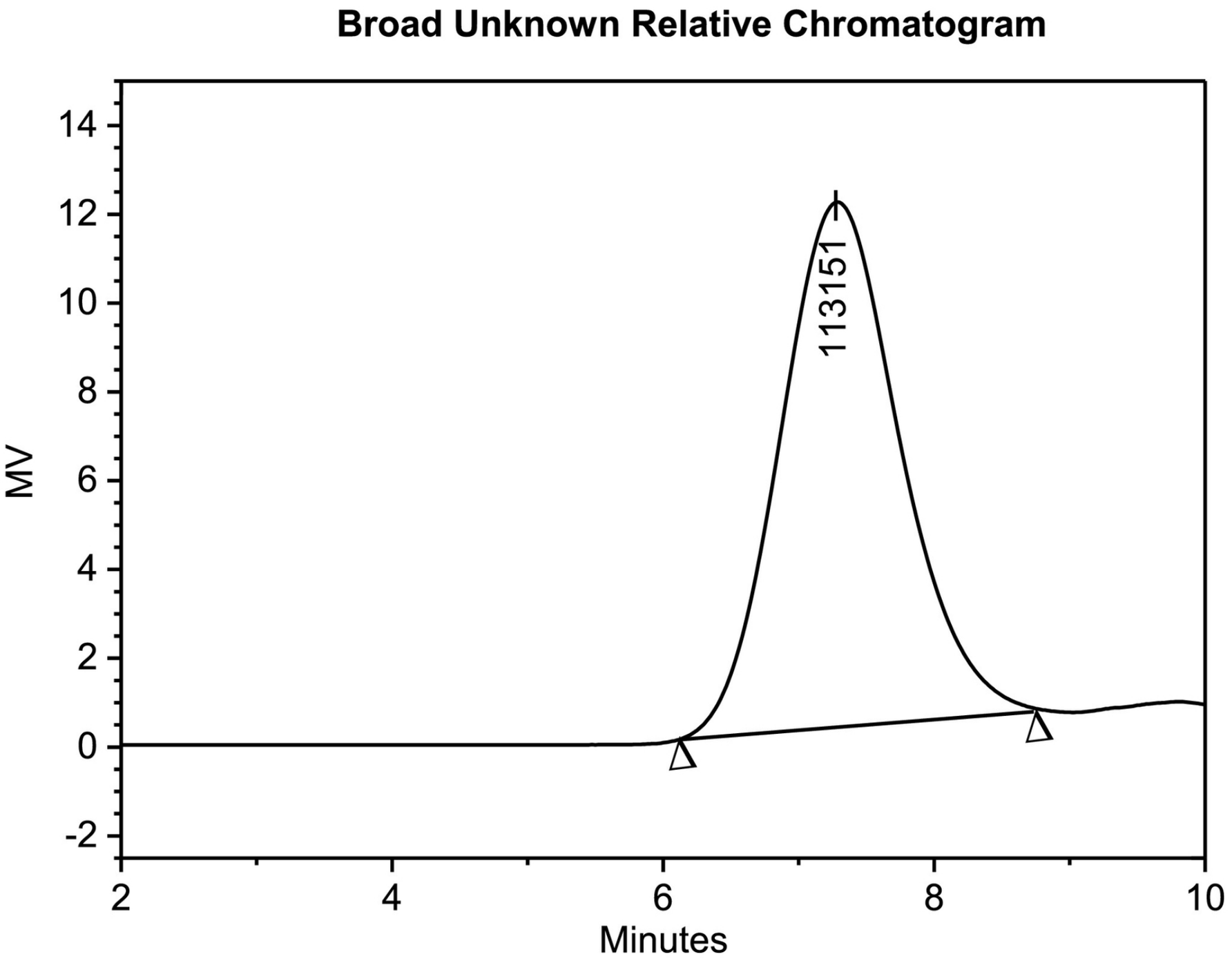

A
Absorbance at 490 nm
NaCl concentration
Absorbance (490nm)
The concentration NaCl (mol/L)
Tube number (5mL/tube)
B
Absorbance at 490 nm
Absorbance (490nm)
Tube number (5mL/tube)

Figure 2



	Distribution Name	Mn (Daltons)	Mw (Daltons)	Polydispersity
1		66827	145883	2.18299

Figure 3

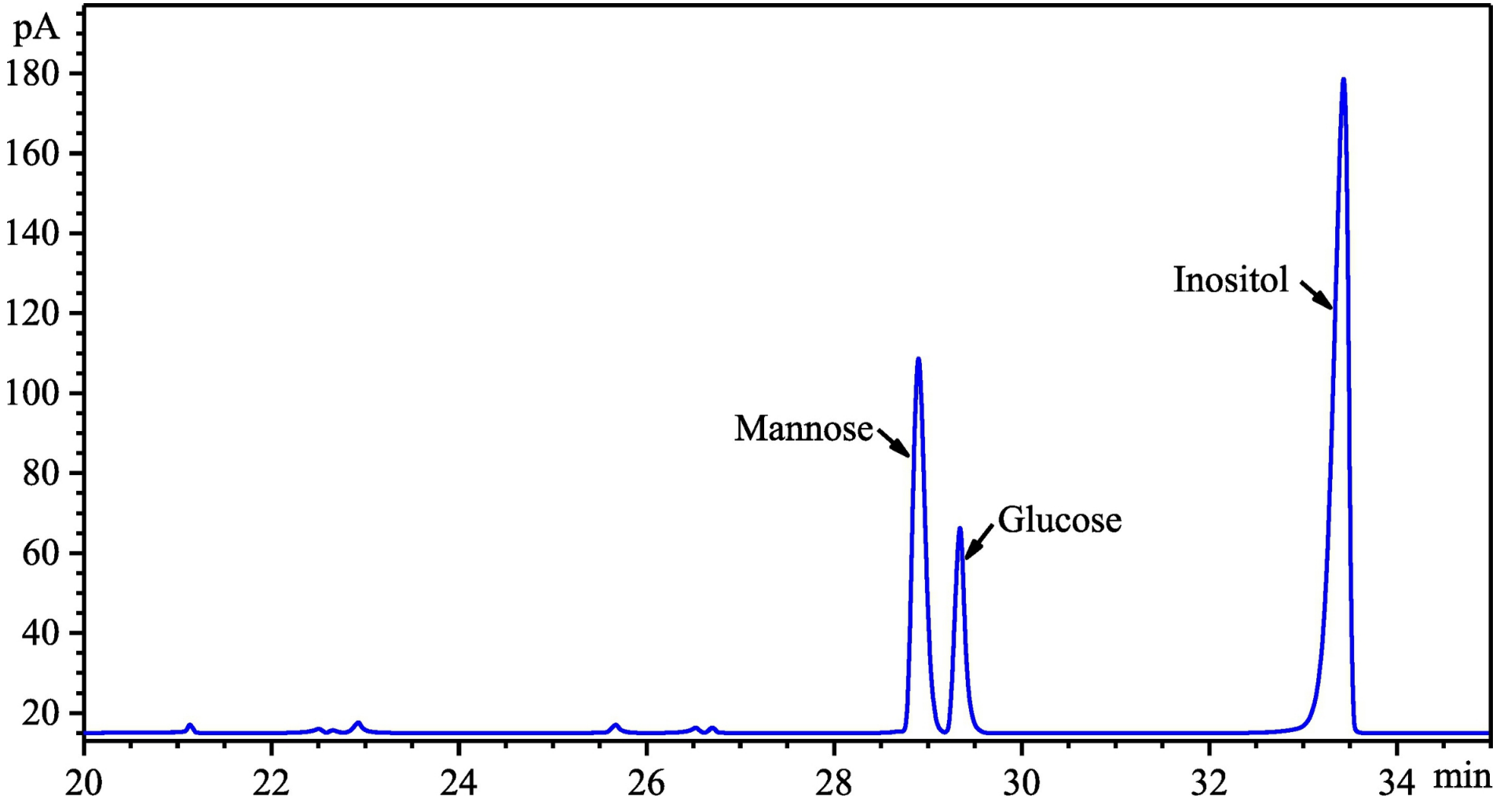

pA
180
160
140
120
100
80
60
40
20
Mannose
Glucose
Inositol
20
22
24
26
28
30
32
34
min

Figure 4

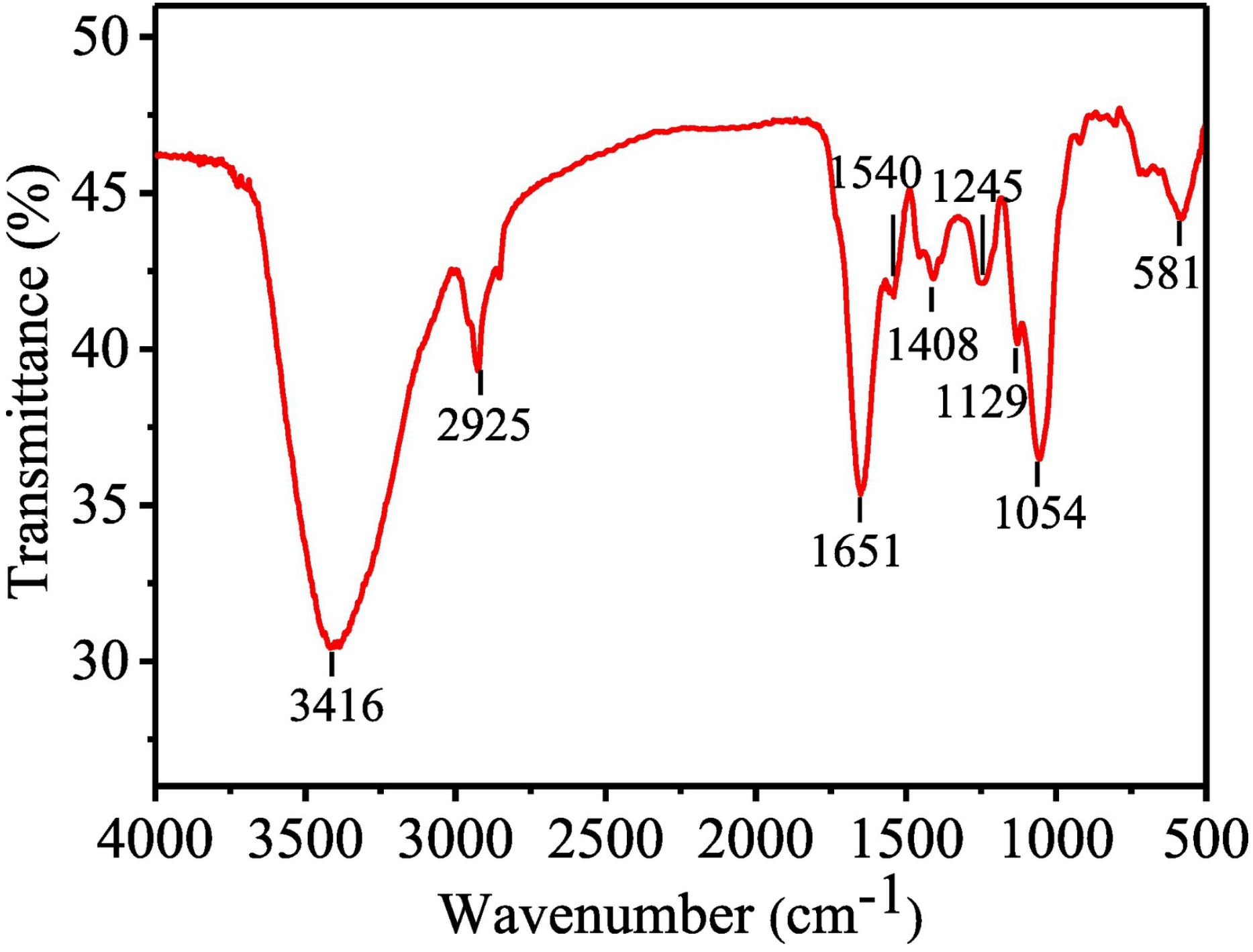

Transmittance (%)
50
45
40
35
30
3416
2925
1651
1540
1408
1245
1129
1054
581
4000
3500
3000
2500
2000
1500
1000
500
Wavenumber (cm-1)

Figure 5

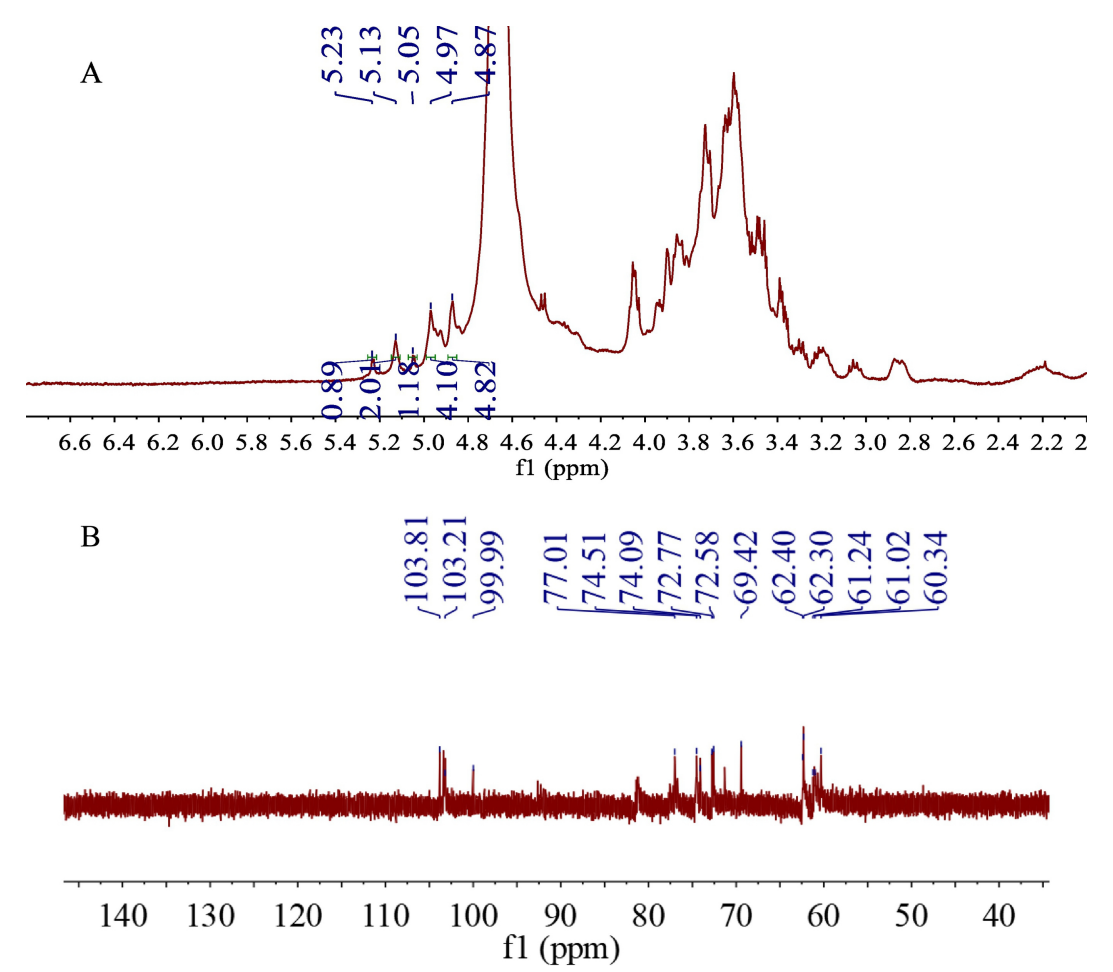

A
5.23
5.13
5.05
4.97
4.87
0.89
2.01
1.18
4.10
4.82
6.6 6.4 6.2 6.0 5.8 5.6 5.4 5.2 5.0 4.8 4.6 4.4 4.2 4.0 3.8 3.6 3.4 3.2 3.0 2.8 2.6 2.4 2.2 2
f1 (ppm)
B
103.81
103.21
99.99
77.01
74.51
74.09
72.77
72.58
69.42
62.40
62.30
61.24
61.02
60.34
140 130 120 110 100 90 80 70 60 50 40
f1 (ppm)

Figure 6

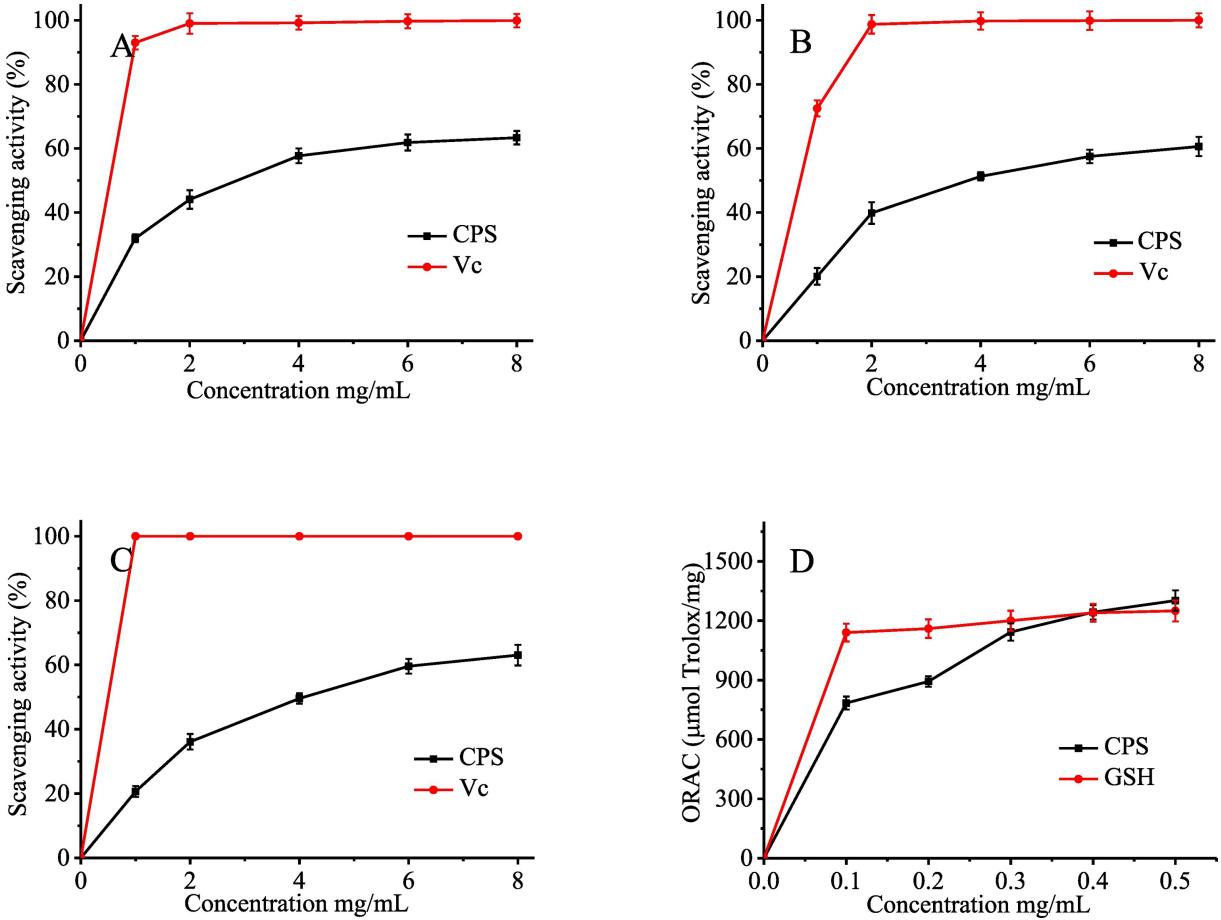

A
Scavenging activity (%)
Concentration mg/mL
CPS
Vc
B
Scavenging activity (%)
Concentration mg/mL
CPS
Vc
C
Scavenging activity (%)
Concentration mg/mL
CPS
Vc
D
ORAC (μmol Trolox/mg)
Concentration mg/mL
CPS
GSH

Figure 7

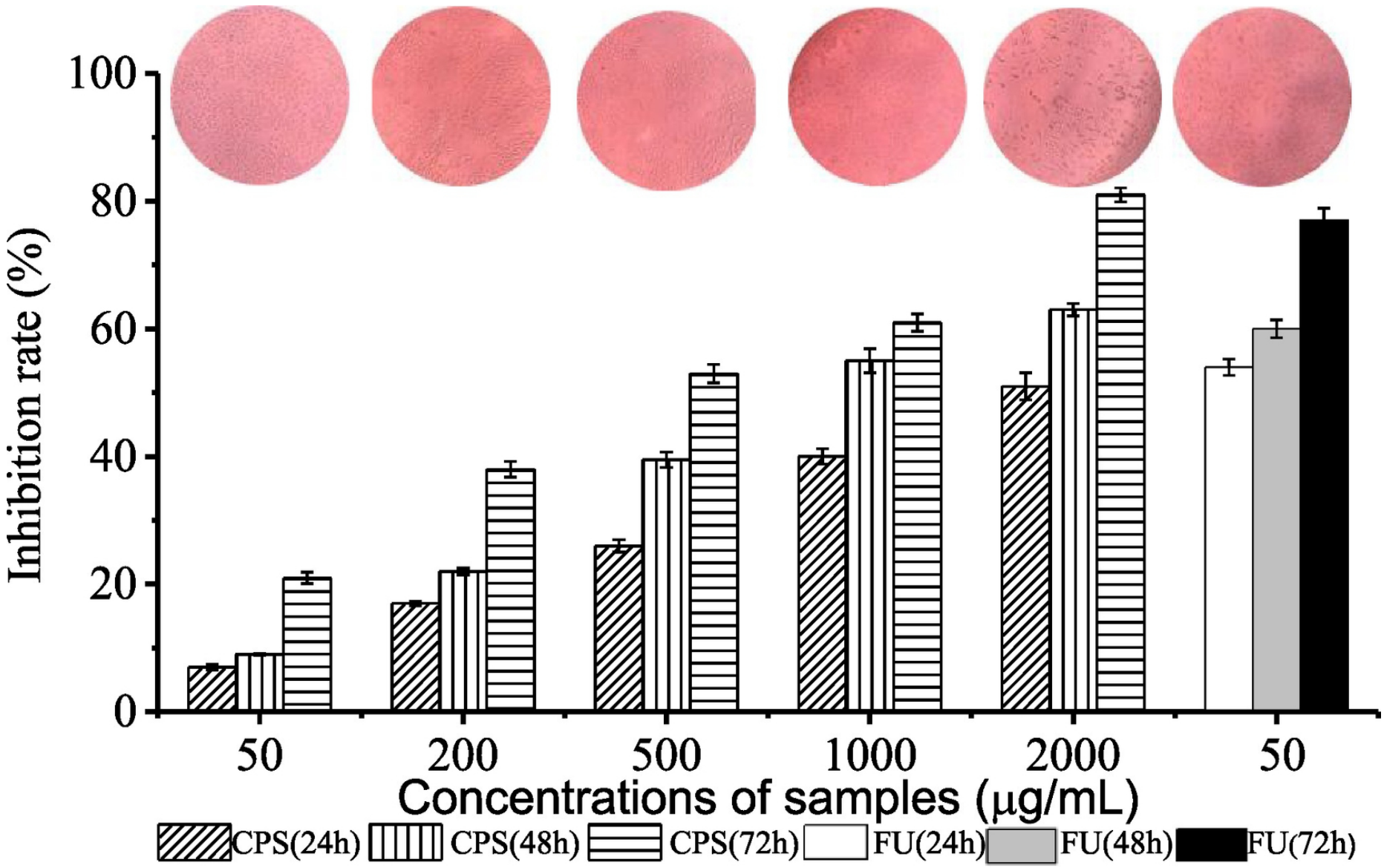

100
80
60
40
20
0
Inhibition rate (%)
50
200
500
1000
2000
50
Concentrations of samples (μg/mL)
CPS(24h)
CPS(48h)
CPS(72h)
FU(24h)
FU(48h)
FU(72h)

Figure 8