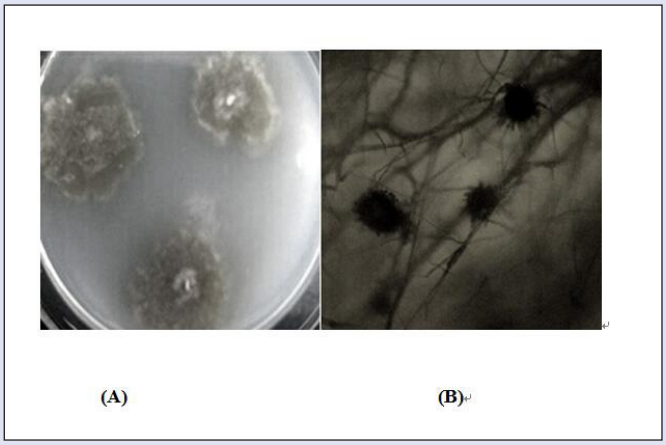

Figure 1: Colony morphology (A) and morphology of mycelial and asexual spore formation (B) of endophytic *Chaetomium* sp. JY25: (A) representative images were taken after 6 days' cultivation; (B) representative images were taken at the magnification of 400-fold.

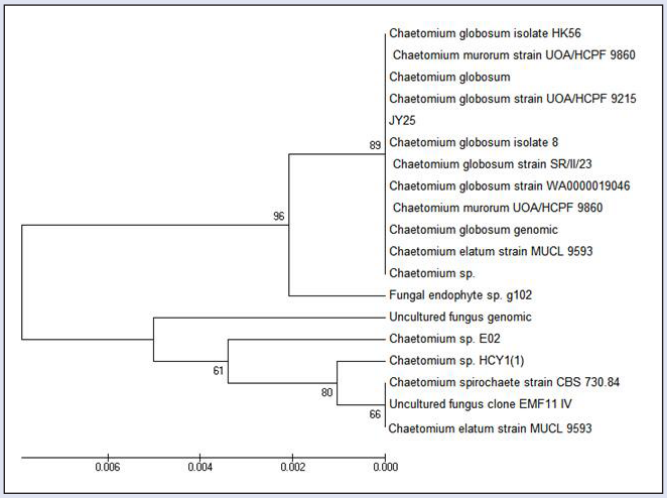

Figure 2: The phylogenetic dendrogram for *Chaetomium* sp. JY25 and related strains based on the ITS rDNA sequence. Numbers in parentheses are accession numbers of published sequences. Bootstrap values were based on 1000 replicates.

UV-2550 spectrophotometer (Shimadzu, Kyoto, Japan). The scavenging activity was calculated using the following equation:

OH radical scavenging rate (%) = $(A_{sample} - A_{blank})/(A_{control} - A_{blank}) \times 100\%$,

where A_{sample}, $A_{control}$, and A_{blank} were defined as absorbances of the sample, control (without EPS), and blank (without H_2O_2 and EPS), respectively.

Regarding the DPPH radical scavenging activity assay, 2 mL of 0.1 g/L DPPH in 50 % ethanol was added to 2 mL of the EPS solution. The absorbance was measured at 517 nm after 20 min of incubation at 25°C. In addition, instead of DPPH, 50% ethanol was used for the blank, whereas distilled water was used for the control instead of sample. The scavenging activity of DPPH radicals by the sample was calculated according to the following equation:

DPPH radical scavenging activity (%) = $1 - (A_{sample} - A_{blank}])/(A_{control}) \times 100\%$,

where A_{sample}, $A_{control}$, and A_{blank} were defined as absorbances of the sample, blank (without DPPH), and control (without EPS), respectively.

In both assays, the EPS samples were predissolved in water and tested at various concentrations in parallel with vitamin C (Vc) as an antioxidant reference (positive control).

Antiproliferation activity

A 549A cell line is widely used as a pulmonary epithelial cell model for drug metabolism *in vitro*.[18] For the antiproliferation effect study, human lung carcinoma A549 cells were obtained from American Type Culture Collection and cultured in the RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) in a humidified 5% CO_2 atmosphere at 37°C.[19] The proliferation of A549 cells was determined using the colorimetric MTT assay described by Mosmann (1983).[20] Briefly, cells were seeded at a density of 3 × 104 cells/well in a 100 µL volume of the medium in 96-well plates and allowed to attach. After 24 h, the additional medium containing the EPS was added to the wells to achieve the final concentration from 0.075 to 5.0 mg/mL. Ten µL MTT (0.4 %) was added after 48 h. After incubated at 37°C for another 4 h, the supernatant was aspirated and then 150 µL DMSO was added to each well. Absorbance was measured at 490 nm by a 96 well microplate reader (Tecan, GENios ELIASA Co., Austria). All results *in vitro* were expressed as the inhibition rate of tumor cell proliferation as follows:

Inhibition rate (%) = $(1 - A_{sample}/ A_{control}) \times 100\%$, (1)

where A_{sample} and $A_{control}$ were defined as absorbances of the sample and control (without extract), respectively. And IC_{50} was determined by nonlinear regression analysis using the Graph Pad Prisme statistics software package (Ver. 2.0; San Diego, CA).

Thermogravimetric analysis of EPS

Thermogravimetric analysis (TGA) of the polysaccharide was conducted in a TA Q5000IR TGA apparatus using the 15 mg EPS fraction of the test material. The TGA curve plots the TGA signal (converted to the percent weight change on the *Y*-axis) against the reference material temperature (on the *X*-axis).

Statistical analysis

The results were expressed as the mean value ± standard deviation (SD) from triplicates. The results were analyzed for statistical significance by the one-way analysis of variance (ANOVA) test using the Statistical Package of the Social Science (SPSS) version 11.0 (SPSS Inc., Chicago, IL). Group means were considered to be significantly different at $P < 0.05$, as determined by the technique of protective least-significant difference (LSD).

RESULTS AND DISCUSSION

Identification of the endophytic fungus JY25

After growth on nutrient agar at 37°C for 3 d, the colonies of endophytic fungus JY25 were opaque and circular and about 4-6 cm in diameter with an irregular margin and cream-colored. However, after 5 d, the colonies changed to nacarat, even to yellowish-brown color [Figure 1A]. Furthermore, the mycelial and conidial morphologies were observed on a light microscopy. The mycelia are transparent, thick, and septate, and the ascospore could be observed from the spherical perithecium [Figure 1B]. The ITS rDNA gene sequence of endophytic fungus JY25 was identified by the polymerase chain reaction, sequenced and compared to all sequences in GenBank. The ITS fragments of 599 bp were amplified and sequenced, and the GenBank accession number was JN180937.1. The nucleotide sequence was blasted in Gen bank (Megablast) and revealed the closest match to that of *Chaetomium* sp. with a homology of 89% [Figure 2]. Therefore, based on the combined

analysis of the fungal morphological characters, the strain JY25 could be identified as *Chaetomium* sp. The strain was the first endophyte to have been reported from *G. pentaphyllum* and was kept in the Henan Province Microbiological Culture Collection Center (HPMCC no. 255534)

Purification and characterization of the EPS

The EPS (1.45 g/L) were obtained from the fermentation broth by the method of ethanol precipitation. In gel filtration chromatography of the culture filtrate on Sepharose CL-6B, only 1 fraction of EPS was coeluted as shown in Figure 3. Figure 3 also showed the presence of protein peaks. However, electrophoresis analysis would need to be conducted in order to ascertain whether the proteins are bound to the polysaccharide (forming protein–polysaccharide complexes) or just have similar molecular weight as compared to EPS (they are eluted at the same time) according to the different charges between polysaccharides and proteins. The detailed monosaccharide compositions from the carbohydrate fraction in the purified EPS could be worked out from trifluoroacetic acid hydrolysis and GC-MS analysis as illustrated in Table 1. Results indicated that the purified EPS was mainly composed of glucose, mannose, arabinose, and galactose with the molecular ratio of 78.29:8.99:8.64:4.08. The result showed that glucose was the major monosaccharide.

FT-IR has been a potent and very useful tool for observing structural and functional groups in polysaccharides. The FT-IR spectra of the EPS were shown in Figure 4. The strong and wide stretching peak at 3263.6 cm^{-1} was O-H stretching vibrations. C-H stretching vibration and bending vibration were at 2931.9 and 1390.3 cm^{-1}, respectively. The peak at 1582.7 cm^{-1} which was attributed to the stretching vibration of the carbonyl bond (C = O). Two stretching peaks at 1145.0 and 1020.3 cm^{-1} indicated the presence of C-O bonds. Furthermore, the peaks at 879.4 and 612.9 cm^{-1} were assigned to the skeletal mode of pyranose ring.[21] The results showed the characteristic absorbance of polysaccharides.[22]

Table 1: Carbohydrate composition in the purified EPS produced from submerged culture of endophytic *Chaetomium* sp. JY25 in a stirred-tank reactor

	Monosaccharide composition %
Glucose	78.29 ± 3.6
Arabinose	8.64 ± 0.8
Mannose	8.99 ± 0.9
Galactose	4.08 ± 0.6

Table 2: Relevant molecular parameters of EPS produced by the submerged culture of endophytic *Chaetomium* sp. JY25 in MALLS analysis.

Parameters	EPS (error %)
M_n (g mol^{-1})	1.067×10^4(5)
M_w (g mol^{-1})	1.961×10^4(4)
M_z (g mol^{-1})	9.603×10^4(9)
M_w/M_n	1.838 (6)
R_n (nm)	45.4 (9)
R_w (nm)	44.8 (8)
R_z (nm)	48.2 (7)

a M_n, M_w, and M_z refer number-, weight-, z-average molecular weight, respectively. M_w/M_n means the polydispersity ratio. R_n, R_w, and R_z refer number-, weight-, z-average square mean radius of gyration, respectively.

The molecular properties of EPS were determined using SEC/MALLS are summarized in Table 2. The average molar mass weight (Mw) of EPS was determined to be 1.961×104 g/mol. Polydispersity values (M_w/M_n), as a measure of the width of molecular mass distribution, are important due to the relevance and significant influence of molecular weight distribution on the functional properties of polysaccharides. The medium value (1.838) of the polydispersity ratio for the EPS fraction means that the EPS molecule exists dispersed in the aqueous solution forming the aggregates.[23] The RMS radii ranged from 44.8 to 48.2 nm with no clear trends.

The decomposition behavior of the polysaccharide is important to ascertain the thermochemical stability.[24] Experimental results for the TGA analysis of purified EPS have been included in Figure 5. According to the TGA curve of EPS, the degradation temperature of EPS was determined to be 143.7°C. This finding suggested that the stability of the EPS fractions is compromised at temperatures above the observed

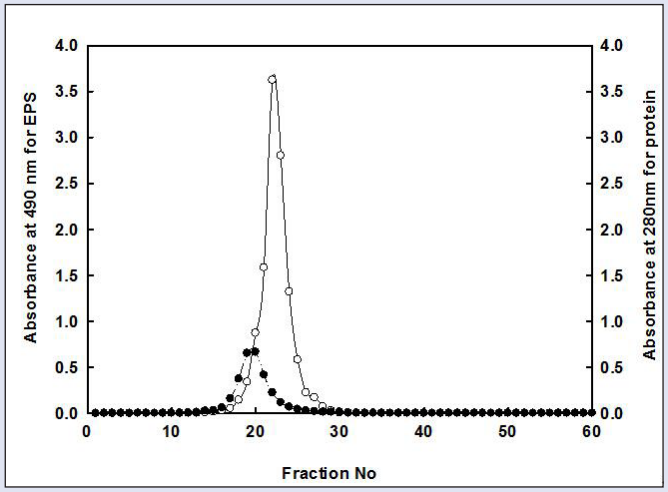

Figure 3: Elution profiles of the EPS in Sepharose CL-6B chromatography. Eluate was analyzed by measuring the absorbance at 490 nm for carbohydrate (o) and the absorbance at 280 nm for protein (•).

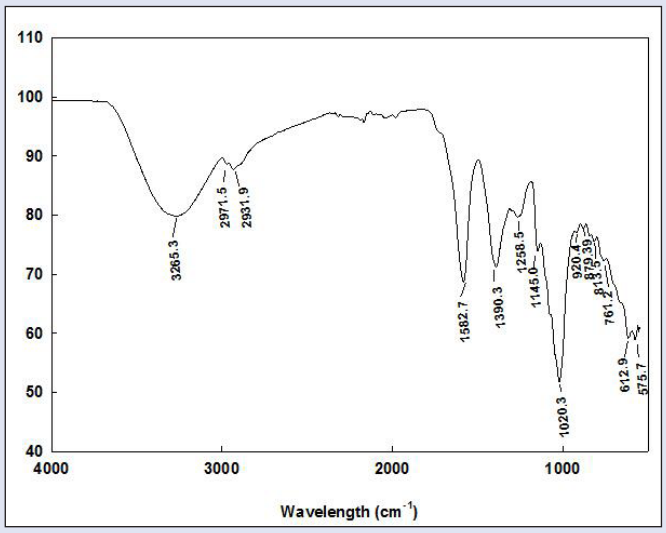

Figure 4: The FT-IR spectra of the purified EPS produced by the submerged culture of endophytic *Chaetomium* sp. JY25.

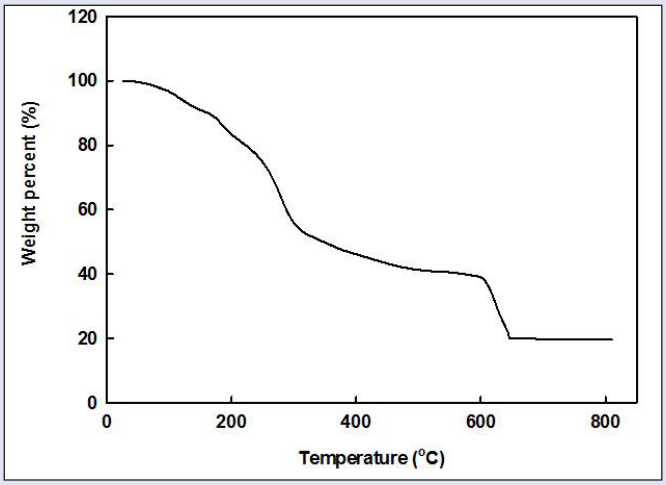

Figure 5: TGA thermogram of the purified EPS from endophytic *Chaetomium* sp. JY25.

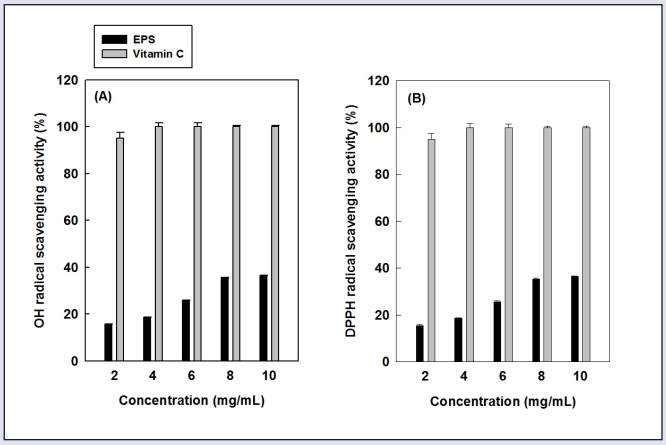

Figure 6: sp. JY25. The results represent mean ± S.D. (n = 3). OH (A) and DPPH (B) radical scavenging activity of EPS from endophytic *Chaetomium* sp. JY25.

degradation temperature. Furthermore, a significant mass loss was recorded in each fraction at temperatures around 305°C and gradually decreased to leave a final residue of ca. 19.2% of the original EPS mass. In any case, TGA confirmed that the EPS generally possessed a high thermal stability.

Antioxidant properties analysis

Antioxidants are the substances that can scavenge free radicals and provide protection against diseases and prolong the shelf life of food.[25] Among various antioxidants, polysaccharides in general have strong antioxidant activities and can be explored as novel potential antioxidants.[17] *In vitro* antioxidant capacities of EPS from submerged culture of *Chaetomium* sp. were evaluated using hydroxyl and DPPH radical scavenging assays.

Hydroxyl radical is the most reactive radical, which can react with most bio-macromolecules functioning in living cells and induce severe damage to adjacent biomolecules.[26] The results of hydroxyl radical scavenging activities summarized in Figure 6A showed that

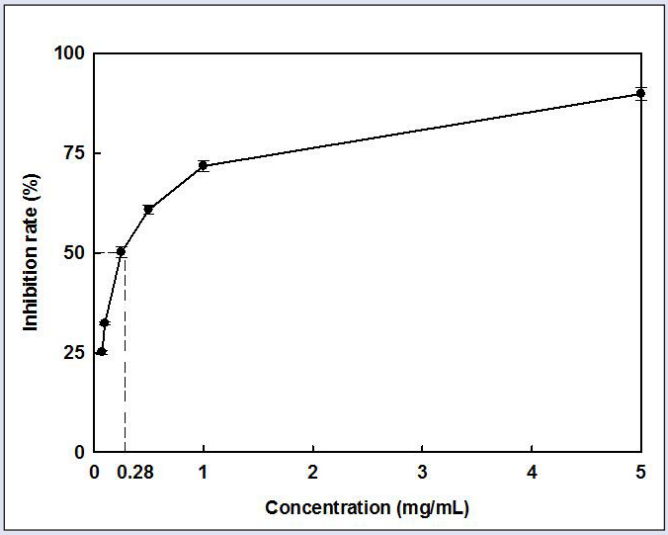

Figure 7: Effect of concentration of purified EPS produced from the submerged culture of endophytic *Chaetomium* sp. JY25 on tumor A549 cells.

hydroxyl radical scavenging activities were improved at increasing concentrations of EPS. At a concentration of 10 mg/mL, EPS exhibited the antioxidant activity of a 100%, which is the same as the scavenging activity of vitamin C. This result indicated that the EPS had a strong hydroxyl radical scavenging activity, which was obviously higher than that of polysaccharides from endophytic *Paenibacillus polymyxa* EJS-3.[27] DPPH radical methods can also evaluate the free radical scavenging ability of natural compounds. In this experiment, scavenging rates of EPS depicted in Figure 6B proved that radical scavenging activity was also concentration dependent and reached a maximum of 36.6% at 10 mg/mL, which was in good agreement with hydroxyl radical scavenging properties though it was lower than that of vitamin C [Figure 6A]. The data implied that the EPS had certain scavenging activity for DPPH radical scavenging effect, which was higher than that of the EPS from the submerged culture of *Chaetomium* sp.[28] Leung *et al.* (2009) suggested that antioxidant activity of polysaccharides might be attributed to their hydroxyl groups and other functional groups, such as C=O and -O-, which can donate electrons to reduce the radicals to a more stable form or react with the free radicals to terminate the radical chain reaction.[29]

Antiproliferative properties analysis

In this work, to evaluate the inhibition on the cancer cell proliferation, various concentrations of EPS from endophytic fungus JY25 (0.0075 mg/mL-5.0 mg/mL) was added to a culture medium of A549 cells. It shows that all extracts have the inhibitary effect on the A549 cell proliferation [Figure 7]. The EPS possessed good inhibitary effects and the effects were significantly dose-dependent. At 5.0 mg/mL and 48 h after medicating, the inhibition rate of A549 cell was 89.8%. The 50% inhibitory concentration (IC_{50}) was calculated to be 0.282 mg/mL. The similar results were observed by Chen *et al.*,[6] who found that the EPS from endophyte *Bacillus amyloliquefaciens* sp. isolated from *Ophiopogon japonicas* displayed concentration-dependent inhibitory effects against the MC-4 and SGC-7901 cells, with an IC_{50} of 19.7 and 26.8 mg/mL, respectively. Liu *et al.*[27] also found EPS from endophytic *P. polymyxa* EJS-3 had the similar antiproliferative activity with the 55.4%

inhibition rates against human gastric carcinoma BGC-823 cells at the concentration of 0.4 mg/mL.

CONCLUSIONS

It was the first time that endophytic fungus *Chaetomium* sp. was isolated for EPS production from medicinal plant *G. pentaphylla*. The EPS with a yield of 1.45 g/L was obtained from culture filtrates of this fungus. Preliminary molecular characterization of the EPS was analyzed. Antioxidant and MTT assays in this study suggest that EPS from the endophytic fungus *Chaetomium* sp. could be explored as a valuable candidate for the discovery of a new drug or healthy food. Further works on other bioactivities of the EPS are in progress using more sensitive experimental modes in our laboratory.

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Conflicts of interest

There are no conflicts of interest

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