

A PVP-extract fungal protein of *Omphalia lapideacens* and its antitumor activity on human gastric tumors and normal cells

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Abstract. *Omphalia lapidescens* is an important medicinal fungus as well as traditional Chinese medicine used for disease treatment. It is mainly used as a vermifuge for anthelmintic therapy, but it has not been hitherto reported to possess antitumor activity. In this study, a purified bioactive protein in *O. lapidescens* (pPeOp) was obtained using polyvinylpyrrolidone (PVP) followed by gel filtration chromatography. To evaluate the *in vitro* antitumor activity of pPeOp in human gastric tumor cells (MC-4 and SGC-7901) and normal cells (MC-1), MTT assay and FCM assay were used and the morphological changes, cell viability, cell death rate and cell apoptosis rate of MC-4, SGC-7901 and MC-1 cells were estimated. The results showed that pPeOp could significantly reduce the cell viability of MC-4 and SGC-7901 cells in a concentration-dependent manner, with IC₅₀ values of 236.05 and 156.28 µg/ml, respectively. The morphological observation also indicated a similar result. In FCM assays, a significant increase of cell death rate and cell apoptosis rate of the tumor cells were observed, indicating probable necrosis-inducing effects and/or apoptosis-inducing effects of pPeOp. Importantly, there was no significant effect of pPeOp on MC-1 cells in each assay, showing that pPeOp has no adverse effects on the normal cells. In conclusion, pPeOp is a newly discovered bioactive protein in *O. lapidescens* and this is the first report on antitumor activity of such a fungal protein. This may provide a meaningful basis for developing a new protein drug for treatment against cancer, especially gastric cancer.

Introduction

Cancer is a malignant disease induced by disorganized cell cycle and uncontrollable tumor cell growth, which has become

a major public health problem for the whole world. The tumor cells form a mass of tissues which proliferate rapidly, spread throughout the body and may eventually cause death of the host (1). The main strategies for treating cancer are surgery and radiation, even if these are rarely successful unless the tumor is diagnosed at an early stage. For advanced tumors, chemotherapy is the primary choice of treatment. However, this is not an ideal choice since the chemical drugs used are associated with severe adverse events and drug resistance (2). This prompted us to seek for new therapeutic options with better efficacy and less side effect for tumor treatment. Recently, medicinal fungi have attracted much attention and generated great interest for its potential applications in the cancer therapy field (3-6). This kind of natural products are promising candidates which display little toxicity and low side effect, suggesting their certain advantage over the chemotherapeutic agents (7).

Omphalia lapidescens Schroeter (Lei-wan in Chinese), a member of traditional Chinese medicines (TCM), is one of the most important medicinal fungi in China and has been widely used for long time in clinical practices of anthelmintic therapy. It is well known as a vermifuge, possessing medicinal activity against many kinds of parasites such as roundworms, cestodes and ancylostomes (8,9). This fungus was originally reported in Shennong Materia Medica, the earliest Pharmacopoeia of China in Eastern Han Dynasty (24-220 AD), and was recently described in the Chinese Pharmacopoeia (2010 edition) (10). According to the Pharmacopoeia description, *O. lapidescens* is attributed to the stomach and large intestine meridians, indicating its potential effects in these two organs (11). Modern pharmacological studies have demonstrated that a *O. lapidescens* protein (neutral metalloprotease) can exert anthelmintic activity whilst its polysaccharide (a fungal (1→3)-β-D-glucan) possesses limited antitumor activity against murine solid and ascites tumors (12,13). However, no report of any antitumor protein of *O. lapidescens* has been published, except our preliminary one in which a crude antitumor protein extract was found (14).

Polyvinylpyrrolidone (PVP) is a multifunctional macromolecular product developed by Reppe (15,16) in his study of acetylene chemistry. This water-soluble high polymer possesses many interesting properties such as non-toxicity, high solubility, chemical stability, and physiological compatibility (17). It was initially utilized as a plasma extender and

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a carrier or vehicle for various substances in the blood stream (18). Recently, it has been found to possess protective effect on enzymes and proteins against polyphenol-induced denaturation, via its high affinity to polyphenols (19). This activity has resulted in a positive application of PVP in the extraction of active proteins or enzymes from the plants (20,21).

In the present study, a PVP-extracted protein of *O. lapidescens* was purified and characterized. Its *in vitro* antitumor activity was also evaluated by assessing cytotoxicity against two human gastric carcinoma cell lines (MC-4 and SGC-7901), using MTT cell viability assay and flow cytometry (FCM) assay.

Materials and methods

Materials and reagents. The fruiting body of *O. lapidescens* was purchased from Fang Hui Chun Tang Traditional Chinese Medicine Drugstore (Hangzhou, China) and authenticated by the authors for protein extraction. Fetal bovine serum (FBS) was provided by Hangzhou Sijiqing Biological Engineering Material Company (Hangzhou). Trypsin, Dulbecco's modified Eagle's medium (DMEM) and RPMI-1640 medium were purchased from Gibco Invitrogen Corp. (Paisley, UK). Hoechst 33258 Kit was obtained from Jiangsu Beyotime Institute of Biological Technique (Nantong, China). Annexin V-FITC Apoptosis Detection Kit I was purchased from BD Pharmingen (San Diego, USA). BCA Protein Assay Reagent Kit was provided by Nanjing KeyGen Biotechnology Company (Nanjing, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, CA, USA). PVP (K30) was obtained from Shanghai Bio Life Science and Technology Co. (Shanghai, China). Other reagents and solvents were of analytical grade and commercially available.

Protein extraction and purification. A mass of *O. lapidescens* fruiting body (200 mg) was washed with 4°C distilled water three times, followed by freeze-drying. It was then ground in liquid nitrogen to fine powders and placed into 0.875 ml iced PVP-extraction buffer (15% 1.0 M Tris-HCl, pH 8.0; 2% PVP; 25% glycerol) for 4 h. After centrifugation (12000 × g, 20 min) at 4°C, the supernatant was collected and kept for the next step.

Gel filtration chromatography was employed to purify proteins from the above extract. The supernatant was loaded onto a Sephadex G-50 column (Pharmacia Biotechnology, Uppsala, Sweden) which was pre-equilibrated with 50 mM Tris-HCl buffer (pH 8.5). The flow rate was 0.2 ml/min, and the absorbance was monitored at 280 nm. There were three $A_{280\text{ nm}}$ peak fractions, named G-1, G-2 and G-3, of which G-2 was ultrafiltrated by Millipore ultrafiltration tube (cut-off of 3 kDa) at 4000 rpm for 30 min and prepared as a purified PVP-extracted *O. lapideacens* protein (pPeOp) for further analysis.

Protein assay and tricine-SDS-PAGE. Overall protein contents of the crude *O. lapideacens* extract and pPeOp were determined by the standard BCA protein assay (22). Possible components in the extract before and after purification were analyzed by tricine-SDS-PAGE (23) using Hoefer miniVE

vertical electrophoresis system (Amersham Pharmacia Biotech, Uppsala, Sweden). Briefly, 10 μ l aliquots of the sample (1 mg/ml) were loaded on tricine-SDS gels (4% stacking gel and 16% resolving gel). The stacking gel was run at 10 mA and the resolving at 20 mA. After electrophoresis, protein band was detected by Coomassie brilliant blue staining method (24). Conspicuous protein component present in pPeOp was selected as a target for further study.

Tumor cell lines and culture condition. Human normal gastric epithelial (MC-1) cells and carcinoma (MC-4 and SGC-7901) cells were provided by Zhejiang Provincial Center for Disease Control and Prevention (Zhejiang CDC) (Hangzhou, China). The MC-1 and MC-4 cells were cultured in DMEM containing 10% fetal calf serum (FCS), 20 mM L-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin, while the SGC-7901 cells were incubated in RPMI-1640 medium supplemented with 100 ml/l FCS, 20 mM L-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin. All cell lines were maintained in a humidified incubator at 37°C and 5% CO₂ atmosphere.

Antitumor activity of pPeOp *in vitro*. Cultured cells (MC-1, MC-4 and SGC-7901) taken in the stage of logarithmic growth were detached with 0.25% trypsin and cell suspensions were made in a medium described above. Their cell viabilities were shown to be in excess of 98% by trypan blue exclusion test. Aliquots of the suspension of each cell strain containing 5×10^4 cells were seeded in each well of two culture plates (96-well plate for MTT assay and 12-well plate for FCM assay) and incubated at 37°C under 5% CO₂ for 24 h. Then DMEM-diluted (for MC-1 and MC-4) and RPMI-1640-diluted (for SGC-7901) pPeOp suspensions (120, 90, 60, 45 and 30 μ g/ml), which has been sterilized by filtration using 0.22 μ m Millipore filter, were separately used to treat the cells in each well for each cell strain. The PVP (120 μ g/ml) treated cells was used as the negative control and untreated cells as normal control. After the treatment, cells in each plate were incubated for 18 h at 37°C with 5% CO₂, awaiting the following tests.

Morphological analysis. The cytotoxicity of pPeOp against human gastric carcinoma cells was evaluated from a morphological perspective. A light microscope was adopted to observe the pPeOp-induced cell damage. The incubated cells were stained with Hoechst 33258 using apoptotic nuclear chromatin staining kit, followed by PBS washes for three times. The cells were examined and photographed by Leica DMIRE2 inverted fluorescence microscope system (Leica Microsystems Corp., Bensheim, Germany).

MTT assay. The MTT assay was employed to further evaluate the pPeOp cytotoxicity on the tumor cells. After the last incubation, 10 μ l of MTT solution (5 mg/ml) was added to each well of the 96-well plate and the plate was incubated at 37°C for 4 h. The supernatant was carefully removed and to each well 150 μ l DMSO was added. The plate was gently shaken for 10 min and the optical density (OD) at 490 nm of each well was determined by a microplate reader (Wellsan MK3; Thermo labsystems, Helsinki, Finland). The cytotoxic activity of pPeOp is expressed as IC₅₀ (i.e., the concentration inhibiting cell proliferation by 50% compared with the untreated

control). The cell viability of the treated groups was expressed as a percentage of normal control groups, which was assumed to be 100%. Three experimental replicates were carried out for each sample.

FCM assay. The pPeOp cytotoxicity was also evaluated by FCM assay. After the last incubation, cells in the 12-well plate were collected and rinsed twice with phosphate-buffered saline (PBS), and then were suspended in the binding buffer to reach a concentration of 1×10^6 cells per ml. Each suspension (100 μ l) was stained with 5 μ l FITC-labeled Annexin V and 5 μ l propidium iodide (PI) using Annexin V-FITC Apoptosis Detection Kit I as instructed by the manufacturer. A FCM analysis was carried out to detect fluorescent cells using Beckman Coulter Epics Altra flow cytometer (Beckman Coulter Corp., CA, USA) for assessing cytotoxicity of pPeOp. Three experimental replicates were performed for each sample.

Statistical analysis. All measurements were expressed as the mean \pm standard deviation and subjected to one-way analysis of variance (ANOVA), followed by Fisher's least significant difference (LSD) comparison. P-value of <0.05 was considered statistically significant and $P < 0.01$ very significant. All analyses were performed using an updated version of DPS software (25).

Results

Isolation and purification of *O. lapidescens* proteins. The PVP extracted protein components of *O. lapidescens* were distinguished and presented on the tricine-SDS-PAGE profile, the major bands of which were seen below 20 kDa (Fig. 1, lane 1). After purification by molecular-sieve chromatography, the extracted proteins were separated into three fractions (G-1, G-2 and G-3) through Tris-HCl elution on Sephadex G-50 column (Fig. 2). Of the three, the highest peak of protein fractions was found in G-2, indicating that it might be the first major fraction with a highest protein concentration. A tricine-SDS-PAGE analysis of G-2 was carried out and the result showed that only one protein band (about 16 kDa) was retained after the purification (Fig. 1, lane 2). This protein was defined as pPeOp.

Morphological changes induced by pPeOp. A light microscopy was applied to analyze the morphological changes of the normal cells (MC-1) and tumor cells (MC-4 and SGC-7901) as well as their cell nuclei. As shown in Fig. 3A and C, untreated MC-1 and MC-4 in the normal control displayed normal cell shape and natural cell apoptosis, indicating the normal condition of these cells. When treated with the PVP buffer, MC-4 still remained normal as compared to its normal control (Fig. 3D), showing that PVP had no effect on the tumor cells. However, after exposure to a concentration gradient of pPeOp (30, 45, 60, 90 and 120 μ g/ml), MC-4 was significantly damaged by cell shrinkage, membrane blebbing, boundary split or aggregation, and underwent an increasing cell death with increasing pPeOp concentrations (Fig. 3E-I). In contrast, with the highest level of pPeOp (120 μ g/ml), no significant cell death was seen in MC-1 compared to its normal control (Fig. 3B), indicating that pPeOp at that level had no cytotoxicity

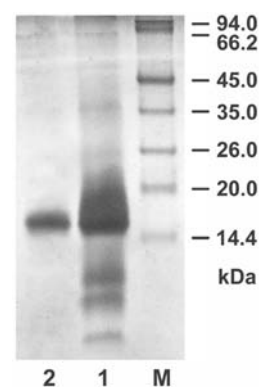


Figure 1. The tricine-SDS-PAGE of protein extracts of *O. lapidescens* before and after purification. M, molecular mass protein markers; lane 1, unpurified protein extract; lane 2, pPeOp.

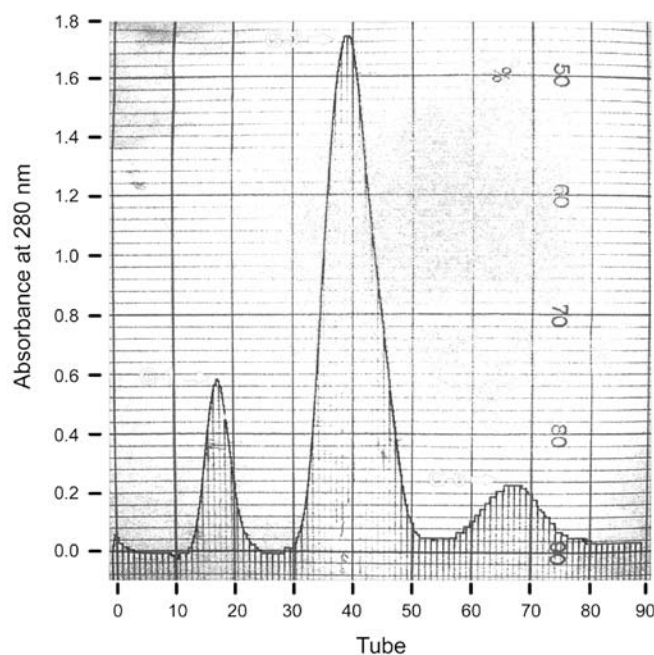


Figure 2. The curve of *O. lapidescens* proteins separated by Sephadex G-50 chromatography. Column specification, 10 x 600 mm; equilibrate liquid, Tris-HCl buffer (pH 8.5, 50 mM); sample, protein extracts of *O. lapidescens*; detection wavelength, UV 280 nm; flow rate, 0.2 ml/min; collection rate, 1 ml/tube.

on the normal gastric cells. The morphological changes of SGC-7901 displayed a similar pattern to MC-4 (data not shown).

Under a fluorescence microscopy, morphological changes of cells and cell nuclei were further observed after Hoechst 33258 staining. In the normal control group, MC-1 and MC-4 were normally round in shape and homogeneously stained, with uniform chromatin across the nuclei (Fig. 4A and C). A similar morphological profile was found in the PVP-treated MC-4 group (Fig. 4D). However, after the same treatments on MC-4 with pPeOp concentration gradient as indicated above, typical morphological changes such as cell shrinkage or collapse, boundary split or aggregation, nuclei condensation, and nucleus fragmentation were observed to intensify along

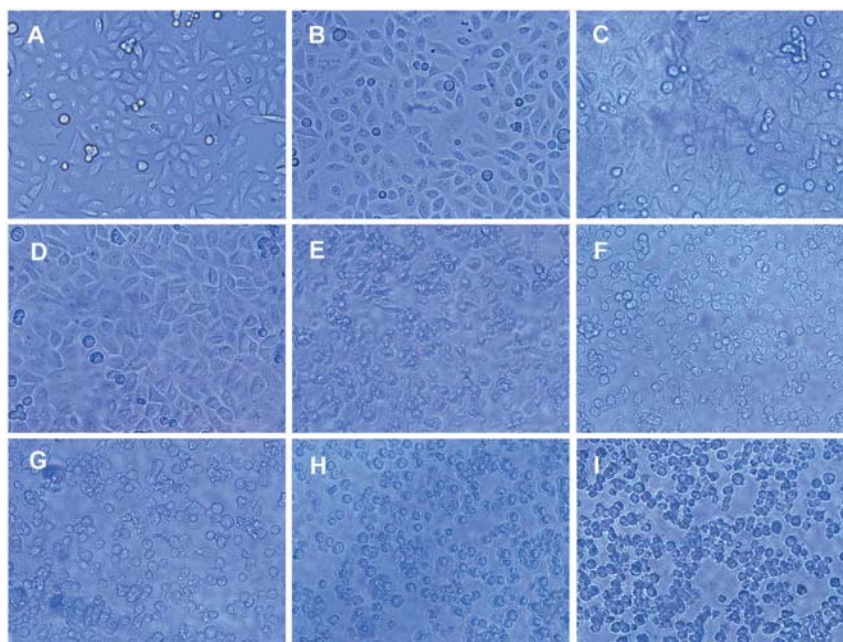


Figure 3. Morphological views of the cultured tumor cells (MC-4) and normal cells (MC-1) with concentration-gradient treatment of pPeOp (x200). (A) Normal control of MC-1 cells; (B) MC-1 cells treated with 120 $\mu\text{g/ml}$ pPeOp; (C) normal control of MC-4 cells; (D) PVP treated MC-4 cells; (E-I) MC-4 cells treated with pPeOp of gradient concentrations (30, 45, 60, 90 and 120 $\mu\text{g/ml}$).

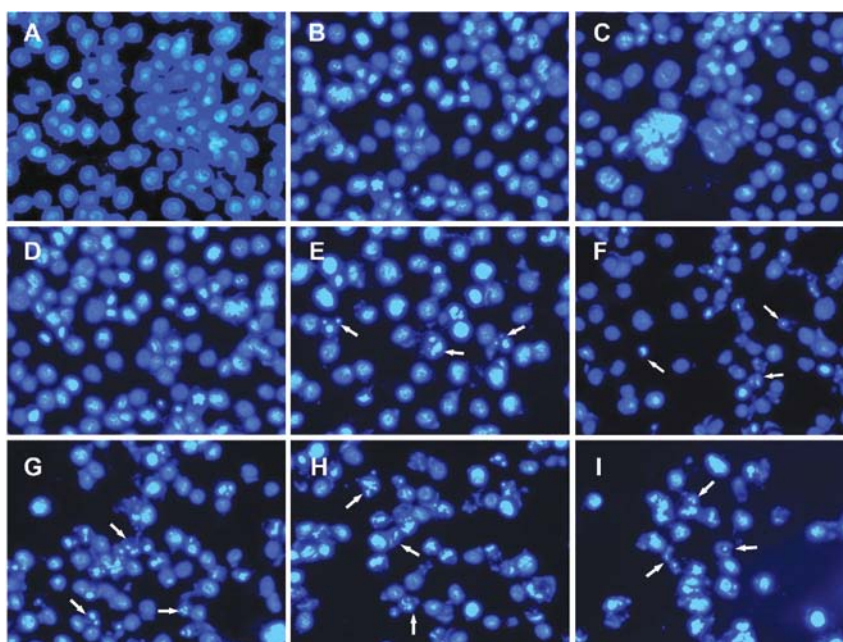


Figure 4. Morphological analysis of Hoechst 33258 stained tumor cells (MC-4) and normal cells (MC-1) with concentration-gradient treatment of pPeOp (x200). (A) Normal control of MC-1 cells; (B) MC-1 cells treated with 120 $\mu\text{g/ml}$ pPeOp; (C) normal control of MC-4 cells; (D) PVP treated MC-4 cells; (E-I) MC-4 cells treated with pPeOp of gradient concentrations (30, 45, 60, 90 and 120 $\mu\text{g/ml}$).

with the increasing pPeOp concentrations (Fig. 4E-I). But contrarily, pPeOp at the highest concentration failed to cause any significant morphological change of MC-1 when compared to its normal control (Fig. 4B). These results, together with the similar findings from the light microscopy, suggested that pPeOp itself could induce apoptosis-like cell death only of the MC-4 rather than MC-1, thereby indicating that pPeOp might have the capability of inhibiting gastric tumor cells with no effect on normal gastric cells.

Cytotoxicity of pPeOp tested by MTT assay. Given the cytotoxicity of pPeOp derived from the morphological analysis, dose-dependent MTT assay was performed to measure the cell proliferation inhibition activity of pPeOp which was expressed by cell viability and the IC_{50} . As illustrated in Fig. 5, when compared with the normal controls, the decrease of cell viability in MC-4 and SGC-7901 became more significant with increasing concentration of pPeOp ($P < 0.05$ and $P < 0.01$) and no more than 70% cell viability were seen in their 120 $\mu\text{g/}$

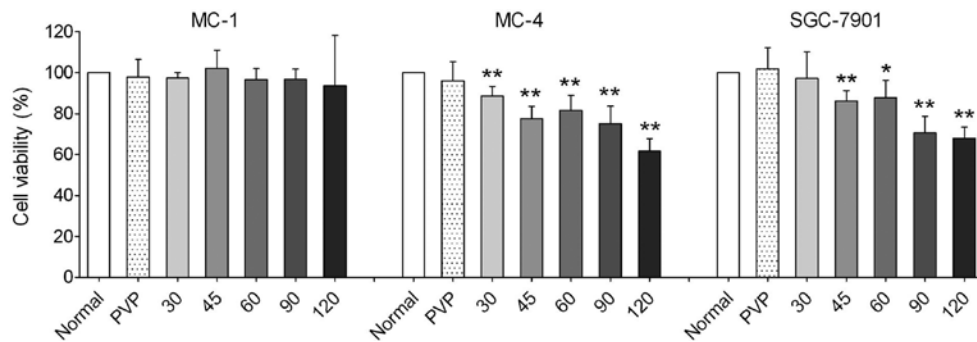


Figure 5. Cytotoxicity of pPeOp on the viability of normal cells (MC-1) and tumor cells (MC-4 and SGC-7901) determined by MTT assays. Results are presented as a percentage of control group viability. Values are the mean \pm SD. * $P < 0.05$ and ** $P < 0.01$ vs. normal control group.

Table I. Assessment of pPeOp-inducing cell death or apoptosis of normal cells (MC-1) and tumor cells (MC-4 and SGC-7901) by FCM assay.

Groups	MC-1		MC-4		SGC-7901	
	Death (%)	Apoptosis (%)	Death (%)	Apoptosis (%)	Death (%)	Apoptosis (%)
Normal	1.4 \pm 0.92	1.2 \pm 0.76	1.8 \pm 1.15 ^c	4.3 \pm 2.83 ^b	4.3 \pm 2.26 ^b	3.8 \pm 1.34 ^b
PVP	1.6 \pm 1.41	1.1 \pm 0.44	2.1 \pm 0.58 ^c	3.1 \pm 1.76 ^b	4.2 \pm 0.53 ^b	2.6 \pm 0.88 ^b
30 μ g/ml pPeOp	1.5 \pm 0.96	4.4 \pm 1.07	1.9 \pm 0.48 ^c	9.5 \pm 6.35 ^{a,b}	24.3 \pm 7.93 ^a	8.6 \pm 3.80 ^b
45 μ g/ml pPeOp	1.3 \pm 0.15	5.2 \pm 1.59	3.5 \pm 2.45 ^{b,c}	17.2 \pm 1.25 ^a	17.0 \pm 9.93 ^{a,b}	11.7 \pm 7.84 ^b
60 μ g/ml pPeOp	1.8 \pm 0.92	5.8 \pm 2.06	8.1 \pm 1.97 ^{a,b,c}	14.6 \pm 8.50 ^a	18.2 \pm 8.35 ^{a,b}	18.0 \pm 13.00 ^{a,b}
90 μ g/ml pPeOp	1.7 \pm 1.10	5.5 \pm 4.72	12.0 \pm 7.18 ^{a,b}	18.4 \pm 10.03 ^a	26.6 \pm 17.84 ^a	13.9 \pm 9.43 ^b
120 μ g/ml pPeOp	2.6 \pm 2.49	5.3 \pm 1.27	17.3 \pm 11.44 ^a	9.2 \pm 2.19 ^{a,b}	17.3 \pm 3.55 ^{a,b}	35.2 \pm 20.02 ^a
F-test	-	-	3.99	3.31	2.86	2.96
LSD test	$P > 0.05$	$P > 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$

Values are the mean \pm SD. Table entries with different lowercase letters in each column differ significantly (LSD, $P < 0.05$).

ml pPeOp treated cultures (61.67% in MC-4 and 68.10% in SGC-7901), while none of the pPeOp concentrations had significant effect on the cell viability of MC-1 (all $P > 0.05$). Moreover, PVP at a high concentration (120 μ g/ml) failed to cause any significant reduction of cell viability in each cell line under the same experimental condition (all $P > 0.05$). In MC-4, pPeOp exerted significant inhibitory effect on the cell viability over its whole concentration range from 30 to 120 μ g/ml (all $P < 0.01$) with IC_{50} value of 236.05 μ g/ml. In SGC-7901, the cell viability was significantly inhibited by pPeOp over a range from 45 to 120 μ g/ml ($P < 0.01$ for 45, 90 and 120 μ g/ml; $P < 0.05$ for 60 μ g/ml), with IC_{50} value of 156.28 μ g/ml.

Cytotoxicity of pPeOp tested by FCM assay. FCM assay was employed using Annexin V-FITC and PI double staining to further evaluate the cytotoxicity of pPeOp on normal gastric cells (MC-1) and gastric tumor cells (MC-4 and SGC-7901). Complete cell death of each group was represented by the upper left quadrant of the cell plot (FITC Annexin V/PI⁺) and apoptosis by both the upper right quadrant (late apoptosis/necrosis, FITC Annexin V/PI⁺) and lower right quadrant (early apoptosis, FITC Annexin V/PI⁻), while viable cells

of each group were present in the lower left quadrant (FITC Annexin V/PI⁻) (Fig. 6). Of these, the FITC Annexin V⁺ cells (right quadrants) were considered as apoptotic cells whilst the FITC Annexin V/PI⁺ cells (upper left quadrant) as dead one. The mean values of the apoptotic cell rate and dead cell rate of each cell line were calculated from three independent experimental replicates of FCM assay. The LSD multiple comparison test was employed to statistically analyze the significant difference between the normal control and each treated groups for each cell line. As shown in Table I, pPeOp at any concentration as well as PVP at 120 μ g/ml had no marked effect on MC-1 since no significant difference of either cell death or cell apoptosis can be seen between the normal and any other treated cells (both $P > 0.05$). However, treatment on MC-4 resulted in significant differences of both cell death and cell apoptosis between the normal groups and treated groups (both $P < 0.05$), of which pPeOp treatment from 45 to 120 μ g/ml induced significant increase of cell death in a concentration-dependent manner and that from 30 to 120 μ g/ml also increased cell apoptosis significantly. A similar result was seen in SGC-7901 that pPeOp was effective on increasing cell death significantly over its whole concentration range ($P < 0.05$) and could also exert a

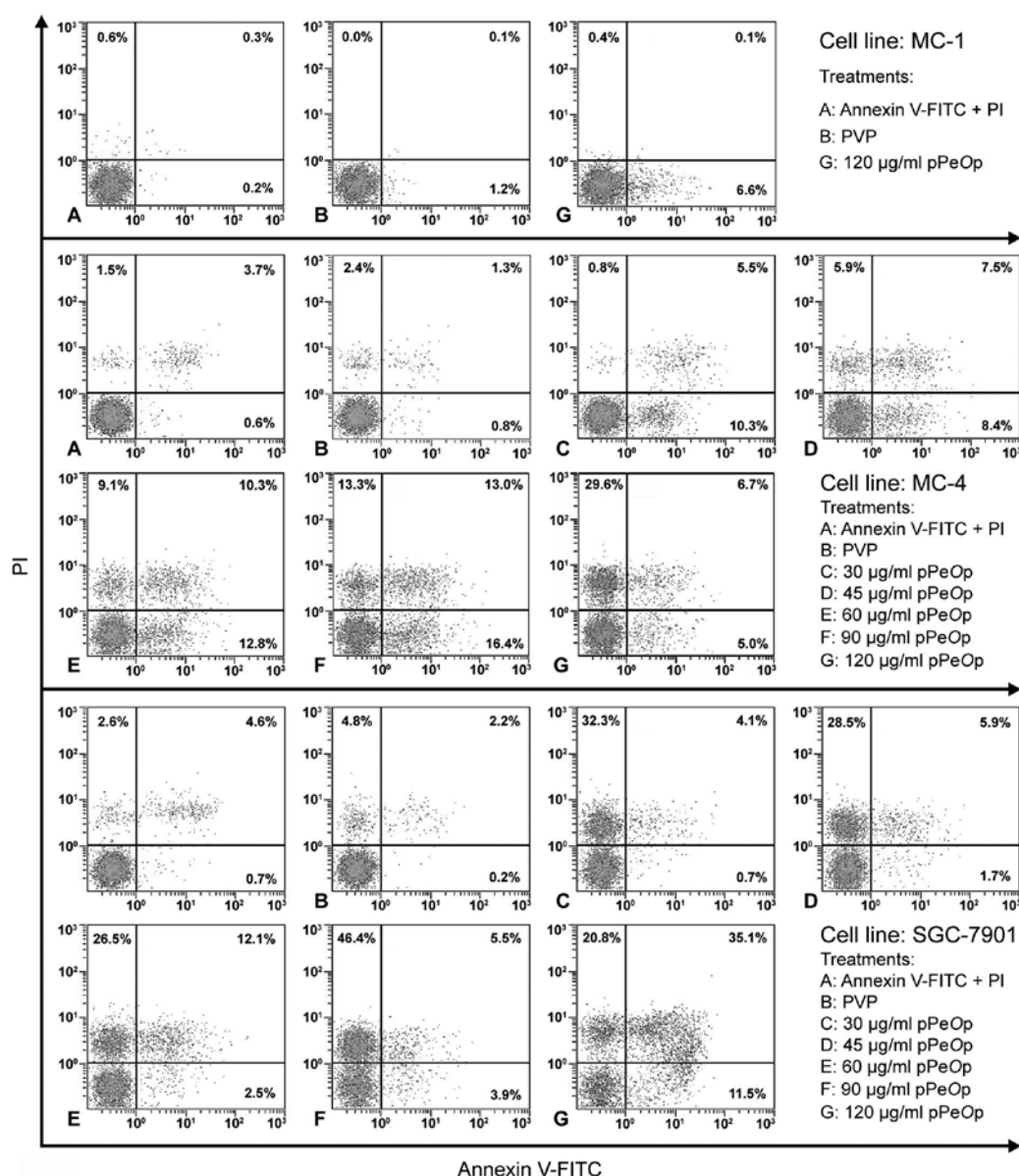


Figure 6. Cytotoxicity of pPeOp on normal cell line (MC-1) and tumor cell lines (MC-4 and SGC-7901) determined by FCM assay. (A) Normal controls; (B) PVP treated negative controls; (C-G) cells treated with pPeOp of gradient concentrations (30, 45, 60, 90 and 120 µg/ml). Data shown in each quadrant represent the percentage of cells treated in each group and are representative of three independent experiments.

significant apoptosis-inducing effect at its concentration of 60 and 120 µg/ml ($P < 0.05$). Further, no obvious effect of PVP on the cell death or cell apoptosis could be found in MC-4 or SGC-7901. These findings indicated that pPeOp can substantially induce cell death and cell apoptosis in the gastric tumor cell lines without any negative effect on the normal gastric cells.

Discussion

O. lapidescens regarded as both the TCM and medicinal fungus is widely used to treat diseases in clinic for thousands of years (26). Traditionally, its fruiting body, in which many bioactive ingredients such as polysaccharides and proteins are involved, is used for treatment. Those ingredients have attracted growing interest for their therapeutic effects (12-14).

However, no studies have hitherto reported on the antitumor activity of any protein of *O. lapidescens*. To date, only one protein classified as neutral metalloprotease has been found possessing anthelmintic activity (13). However, recently, our preliminary study has discovered that a crude protein extract of *O. lapidescens* fruiting body possessed inhibitory effect against tumor cell growth (14). Accordingly, in this study, we purified one protein (pPeOp) from the protein extract and performed MTT assay and FCM assay to evaluate its anti-tumor activity on human gastric tumor cells as well as normal gastric cells.

After a preliminary screening, PVP was chosen as an extract agent for the protein extraction, due to its favorable property of non-toxicity, high solubility and chemical stability (17). Moreover, it was reported to possess a high polyphenol-affinity which could indirectly protect protein activity from

polyphenol-induced denaturation (19). Lee and Yun (27) have demonstrated that there are diverse fungal polyphenols in medicinal fungi such as *Phellinus linteus* and *Inonotus xeranticus*, and the polyphenols composed the yellow pigment produced in the fungal fermentation broth. In this study, we found the crude extract of *O. lapidescens* displayed yellow pigmentation, indicating that the yellow pigment composed of polyphenols may exist in this fungus. It can be deduced that, during the extraction, the polyphenol-induced denaturation may occur and the activity of fungal proteins may thereby be inhibited. Thus, applying PVP to the extraction in this study was expected to result in a counteraction of the polyphenol-induced denaturation. To our knowledge, this is the first study reporting the application of PVP for the fungal-protein extraction.

pPeOp with molecular weight of 16 kDa was purified from the PVP-extracted protein mixture of *O. lapidescens* by gel filtration chromatography. The chromatographic profile showed no interference among the three purified fractions G-1, G-2 and G-3 (Fig. 2), indicating that the purification was well accomplished and each peak of the fraction represented a pure protein component of *O. lapidescens*. In fact, pPeOp (G-2) as the highest-concentration component in the PVP-extracted protein mixture was reported for the first time in this study. Although its amino acid sequence has not been determined, it will not be the known protein (neutral metalloprotease) of *O. lapidescens* because: i) the known metalloprotease was extracted with 95% ethanol rather than PVP, indicating that pPeOp might be denatured by polyphenols or not be extracted successfully; ii) the metalloprotease was obtained from the cultured mycelia of *O. lapidescens* strain but not from the fruiting body in which pPeOp existed; and iii) the molecular weight of metalloprotease (40 kDa) was much higher than that of pPeOp (16 kDa). Therefore, this is the first discovery of such a protein of *O. lapidescens*, even though it has not been identified yet. However, whether it is a novel fungal protein or what amino acid sequence it has, requires further study.

Both MTT assay and FCM assay, accompanied with morphological observation, were employed in this study. The morphological changes, cell viability, cell death rate and cell apoptosis rate of the tumor cells (MC-4 and SGC-7901) and normal cells (MC-1) were assessed to determine whether pPeOp could exert significant and specific antitumor activity. The results of both the morphological observation and MTT assay showed that pPeOp possessed a significant inhibitory effect on gastric tumor cells in a concentration-dependent manner with an insignificant effect on normal gastric cells. With its highest concentration (120 $\mu\text{g/ml}$), most of the tumor cells suffered necrosis and/or apoptosis with cell shrinkage, boundary split, and nucleus fragmentation, under the light microscopy and fluorescence microscopy (Figs. 3 and 4), and the decrease of each tumor cell viability of more than 30% (38.33% in MC-4 and 31.90% in SGC-7901) was found by the MTT assay (Fig. 5). The FCM assay also revealed a similar result that pPeOp could induce tumor cell death or apoptosis significantly (Table I). The statistically significant difference between the treated and untreated cells were evaluated using LSD test, in which the different lower case letters meant significant difference between each variable ($P < 0.05$). However, no concentration-dependent manner of pPeOp's

apoptosis-inducing effect on tumor cells was observed. It may be due to the fact that this assay cannot completely distinguish the late apoptosis and cell death in the upper right quadrant of the cell plot, neither could we distinguish the apoptotic death and non-apoptotic death in the upper left quadrant. Therefore, the cell apoptosis rate and cell death rate might not be exactly measured, resulting in the irregularity of the data. Anyway, there still existed a trend toward an increase of cell apoptosis and cell death with increased concentrations of pPeOp (Table I). During all these tests, MC-1 was employed for assessing cytotoxicity of pPeOp on normal cells, by which the adverse effects of pPeOp could be somewhat evaluated. The results indicated that pPeOp might do no significant harm to normal gastric cells from the aspects of morphology, cell viability and cell apoptosis/death rate. Nevertheless, as shown in Table I, there was an insignificant increase of MC-1 cell apoptosis after pPeOp treatments, as compared with untreated cells ($P > 0.05$), indicating that pPeOp might trigger slight apoptosis of normal gastric cells. Whether such an adverse effect of pPeOp is indeed present needs further evaluation and confirmation. Furthermore, according to the TCM theory, *O. lapidescens* as a TCM is attributed to the stomach meridian, indicating that this fungus can exert medicinal effect on gastric diseases (10,11). That is why we have preferentially chosen gastric tumors for this study. Fortunately, the present results have supported our hypothesis that such an *O. lapidescens* protein might be effective on gastric tumors and also have supported the above TCM theory about meridian tropism to some extent.

At present, many proteins from different sources have been found to possess antitumor activity (28-32). Of these, fungal proteins are the major one, in which fungal immunomodulatory protein (FIP), ribosome-inactivating protein (RIP), lectins, and glycoproteins, are involved, due to their favorable effect against a variety of tumors (33). In this study, pPeOp, as a newly discovered antitumor protein of *O. lapidescens*, could successfully inhibit gastric tumor cell proliferation *in vitro*, providing a promising candidate of fungal protein for the treatment against cancer, especially gastric cancer. However, what kind of protein pPeOp is and whether it is effective *in vivo* or even in the clinic, require further exploration. The next step will be to analyze the gene sequence and amino acid structure of pPeOp, and meanwhile it will proceed to *in vivo* experiments.

In conclusion, based upon our preliminary study, a crude antitumor protein extract from *O. lapidescens* was found (14). In this study, we isolated and purified pPeOp with 16-kDa molecular weight from that extract and *in vitro* evaluated its antitumor activity by morphological analysis, MTT assay and FCM assay. Collectively our results obtained from the present study, we can conclude that pPeOp possesses a significant cytotoxic effect against human gastric tumors (MC-4 and SGC 7901) in a concentration-dependent manner, with no side effect on normal gastric cells (MC-1). This is a newly discovered antitumor protein of *O. lapidescens*, owing to the first application of PVP in the fungal protein extraction as well as the first report on such a bioactive protein. Additionally, the anticancer potential of pPeOp indicates for the first time that *O. lapidescens* may be a promising resource for new

drug development against cancer, especially gastric cancer. Further studies are required to identify pPeOp and evaluate its antitumor effect *in vivo*.

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