

## RESEARCH ARTICLE

# Steroidal Saponins from *Paris polyphylla* Suppress Adhesion, Migration and Invasion of Human Lung Cancer A549 Cells Via Down-Regulating MMP-2 and MMP-9

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### Abstract

**Background:** Tumor metastases are the main reasons for oncotherapy failure. *Paris polyphylla* (Chinese name: Chonglou) has traditionally been used for its anti-cancer actions. In this article, we focus on the regulation of human lung cancer A549 cell metastases and invasion by *Paris polyphylla* steroidal saponins (PPSS). **Materials and Methods:** Cell viability was evaluated in A549 cells by MTT assay. Effects of PPSS on invasion and migration were investigated by wound-healing and matrigel invasion chamber assays. Adhesion to type IV collagen and laminin was evaluated by MTT assay. Expression and protease activity of two matrix metalloproteinases (MMPs), MMP-2 and MMP-9, were analyzed by Western blotting and gelatin zymography, respectively. **Results:** PPSS exerted growth inhibitory effects on A549 cells, and effectively inhibited A549 cell adhesion, migration and invasion in a concentration-dependent manner. Western blotting and gelatin zymography analysis revealed that PPSS inhibited the expression and secretion of MMP-2 and MMP-9 in A549 cells. **Conclusions:** PPSS has the potential to suppress the migration, adhesion and invasion of A549 cells. PPSS could be a potential candidate for interventions against lung cancer metastases.

**Keywords:** *Paris polyphylla* - steroidal saponins - lung cancer - metastasis - MMP-2 - MMP-9

*Asian Pac J Cancer Prev*, **15** (24), 10911-10916

### Introduction

Lung cancer is the leading causes of cancer death worldwide (Siegel et al., 2014), mainly due to the difficulty of early diagnosis and the highly metastatic potential, which has being the hotspot of lung cancer research (Li et al., 2014). In fact, the metastasis has already developed before the diagnosis of lung cancer in most cases, for example, approximately 30 to 40% of patients with advanced lung cancer will develop bone metastases (Sone and Yano, 2007; Al Husaini et al., 2009). More than 90% of lethality in cancer patients is caused by metastasis and the occurrence of distant metastases resulting in a significant poor prognosis of lung cancer patients (Cheng et al., 2014).

For metastasis, tumor cells invade through the basement membrane, enter the vasculature, survive without adhesion, disseminate into the circulation, and finally establish a new tumor in another organ. For migration, the cell body must modify its shape and stiffness to interact with the surrounding tissue structures. Hereby, the extracellular matrix (ECM) provides the substrate, as well as a barrier towards the advancing cell body (Friedl and Wolf, 2003; Yilmaz et al., 2007; Al Husaini et al., 2009). Depending on the tumor type and the surrounding

tissue, cell migration involves different cellular strategies to overcome the physical resistance of three-dimensional tissue networks. One important step is invasion into tissues, which requires proteolytic degradation of ECM components. Matrix metalloproteinases (MMPs) are a large family of zinc dependent neutral endopeptidases collectively capable of degradation almost all extracellular matrix components (Guo et al., 2012). MMPs are overexpressed in a variety of malignant tumor types that can promote cancer progression by increasing cancer cell growth, migration, invasion, metastasis and angiogenesis, therefore, inhibition of the function of MMPs, especially MMP-2 and MMP-9, in the ECM is being most attractive for anticancer therapy (Hidalgo and Eckhardt, 2001; Cao et al., 2011).

Many cancer chemotherapy drugs clinically used are derived from natural products which are still the hotspots for discovery of novel leads (Newman and Cragg, 2012). *Paris polyphylla* (Chinese name: Chonglou) is a well-known traditional Chinese medicine with heat-clearing and detoxicating (qing re jie du) functions. It has been traditionally used for the treatment of inflammation and cancer, especially lung cancer (Committee, 2010; Li et al., 2013). Many studies showed that the main active

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ingredients of Paris polyphylla are steroidal saponins (Wang et al., 2007; Yan et al., 2009).

The present study is designed to evaluate the tumor invasive cascade in the metastatic process and investigate the effect of paris polyphylla steroidal saponins (PPSS) on the adhesion, migration and invasion of human lung cancer A549 cells. Moreover, the effect of paris polyphylla steroidal saponins on enzymatic degradation of ECM were also investigated to understand the anti-metastasis mechanisms underlying.

## Materials and Methods

### Chemicals and reagents

Fetal bovine serum (FBS) was obtained from Zhejiang Tianhang Biological Technology Co., Ltd. (Zhejiang, People's Republic of China); RPMI 1640 was obtained from HyClone (Logan, UT, USA). 3- (4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA). Protease inhibitor cocktail and phosphatase inhibitor cocktail were purchased from Roche (CA, USA). Polyclonal antibodies against matrix metalloproteinases-2 (MMP-2), matrix metalloproteinases-9 (MMP-9), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Electrochemiluminescence (ECL) reagent was from Thermo Scientific (Rockford, IL, USA).

### Plant material

Steroidal saponins were prepared from Paris polyphylla Smith var. chinensis (Franch.) Hara. by our lab. The dried root and rhizome of paris polyphylla were extracted with 70% ethanol for three times. Then the solvent was removed under reduced pressure, and the ethanol extract was suspended in H<sub>2</sub>O, subjected to macroporous resin (D101, Sunresin New Materials Co. Ltd., Xi'an, China) column chromatography and eluted with increasing amounts of ethanol (i.e. 0% ethanol, 20% ethanol, 40% ethanol, 60% ethanol, 80% ethanol, and 95% ethanol). The 60%-ethanol fraction was collected and the solvent was removed under reduced pressure, finally yield paris polyphylla steroidal saponins (PPSS) after freezing drying.

### Cell culture

A549, human lung cancer cells, were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were cultured in RPMI1640 medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin in a humidified atmosphere with 5% CO<sub>2</sub> at 37°C. All cells in the exponential phase of growth were used in the experiments.

### Cell viability assay

A549 cells were dispensed in 96-well flat bottom cell culture clusters (Corning, NY, USA) at a density of 6×10<sup>3</sup> cells per well and cultured for 24 h. Thereafter the cells were treated with various concentrations of PPSS for 24 h. After that, the cells were washed with ice-cold

PBS twice and incubated with 5.0 mg/mL MTT solution at 37°C for 2 h. The resulting crystal was dissolved in 150 µL DMSO and the optical density was measured by MTT assay using a micro-plate reader (Bio-Tek ELX800, Bio-Tek, VT, USA). The percentage of cell viability was calculated as follows:

$$\text{Cell viability (\%)} = \frac{(A492_{\text{sample}} - A492_{\text{blank}})}{(A492_{\text{control}} - A492_{\text{blank}})} \times 100$$

### Adhesion assay

The 24-well culture plates (Corning, NY, USA) were coated with type IV collagen (10 µg/ml) (Millipore, MA, USA) and laminin (10 µg/ml) (Becton, Dickinson and Company, NJ, USA), incubated for 24 h at 4°C, the residual liquid in the wells was discarded, and then the wells were blocked with 0.5% BSA in PBS for 30 min. A549 cells were incubated with PPSS (0, 20, 40, 80 mg/L), or RPMI 1640 medium with 1% BSA as control, for 24 h. Cells were then suspended in RPMI 1640 with 0.5% BSA and plated into 24-well culture plates and cultured for 1 h. Medium was then carefully discarded, and the plate was washed with PBS to remove the non-adherent cells, and adherent cells were measured using MTT assay.

### Wound-healing assay

A549 cells (2×10<sup>5</sup> cells/well) were seeded into 6-well culture plates (Corning, NY, USA), incubated and grow to 70% confluency in complete RPMI 1640 medium. Then the cells were starved in serum free medium for another 24 h, the cell monolayers were carefully scratched an artificial "wound" with a pipette tip at 0 h, the wounded monolayers were washed to remove floating cells and observed by using a phase contrast microscope (Olympus, Tokyo, Japan), then incubated in RPMI 1640 medium with various concentrations of PPSS. Cell migration into the wound surface and the average distance of migrating cells were observed in different times.

### MMP gelatin zymography

The activities of MMPs in medium were detected by SDS-PAGE gelatin zymography protease assays. To obtain the conditioned media, A549 cells were incubated with PPSS (0, 20, 40, 80 mg/L) in serum-free media for 24 h. The collected media were centrifuged for 10 min at 4°C at 2000 rpm, 20 µL of the supernatant were then loaded on 10% SDS-PAGE gels that had been copolymerized with 1 mg/ml gelatin. The samples were not activated before running which allowed the latent and active forms of each enzyme to be visualized. Gels were run at 100 V for 2 h at 4°C. The gels were soaked in 2.5% Triton X-100 for 3 h to remove the SDS for deactivate the enzymes, and were incubated in a collagenase buffer (50 mM Tris-HCl pH 7.6, 10 mM CaCl<sub>2</sub>) at 37°C for 40 h to allow proteinase digestion of its substrate. Gels were rinsed again in distilled water, stained with 0.5% Coomassie brilliant blue for 30 min at room temperature and destained with 7% acetic acid until revealing clear bands containing proteolytic activity on a dark blue background. The metalloproteinases was indicated by an unstained proteolytic zone in the substrate according to their molecular weights (92 kDa and 72 kDa, corresponding to MMP-9 and -2 respectively). Bands were

quantified using an image quantitative analysis system (Image-Pro Plus, USA).

#### Migration and invasion assays

Cell migration assays were performed by using transwell chambers (8  $\mu$ m pore size; Millipore, MA, USA). A549 cells were allowed to grow to 75-80% confluency, and incubated in serum-free medium for 24 h. Then the cells were detached and resuspended in serum-free medium. Cell suspension ( $2 \times 10^5$  cells/ml) was added to the upper chamber with or without PPSS (0, 20, 40, 80 mg/L) in 400  $\mu$ L of 1% BSA RPMI 1640 medium. The bottom chamber contained medium with 10% FBS RPMI 1640 medium to serve as a chemoattractant to induce invasion. After 24 h incubation, the non-migrated cells were removed from the upper face of the filters using cotton swabs. The migrated cells were fixed with 100% methanol and then stained with 0.2% crystal violet (Beijing Chemical Works, China). Images were captured from each membrane of three different fields and the migrated cells were counted using a phase contrast microscope. In the invasion assay, invasive potential were determined similarly inserts coated with 100  $\mu$ L (1 mg/ml) matrigel (Becton, Dickinson and Company, NJ, USA).

#### Western blot analysis

A549 cells were treated with or without PPSS for 24 h, then were harvested and lysed in RIPA lysis buffer containing protease inhibitor cocktail and phosphates inhibitor cocktail at 4°C for 60 min. Lysates were centrifuged at 12,000 $\times$ g for 15 min, and the protein content of the supernatant was determined by the Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA). Equal amounts of total protein were separated by 12% SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to Immobilon®-P Transfer Membrane (Millipore Corporation, Billerica, MA, USA). The membranes were soaked in blocking buffer (5% skimmed milk). Proteins were detected with the primary antibodies against MMP-2, MMP-9, and GAPDH followed by horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology) and visualized by using ECL as the HRP substrate.

#### Statistical analysis

All the presented data and results were confirmed in at least three independent experiments and were expressed as mean $\pm$ S.D. Statistical comparisons were made by Student's t-test and One-way ANOVA followed by Tukey's post hoc test.  $p < 0.05$  was considered to represent a statistically significant difference.

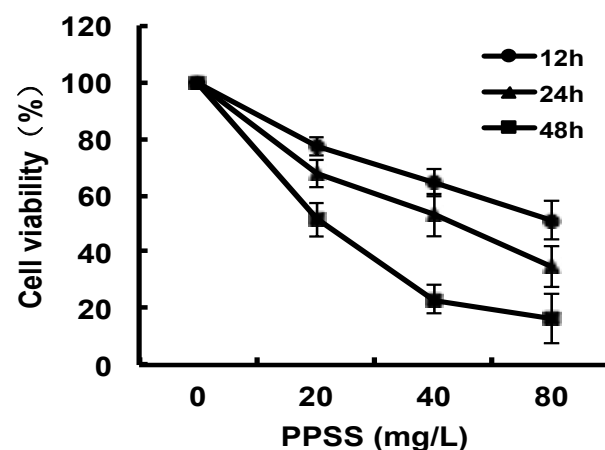
## Results

#### PPSS suppressed A549 human lung cancer cell growth

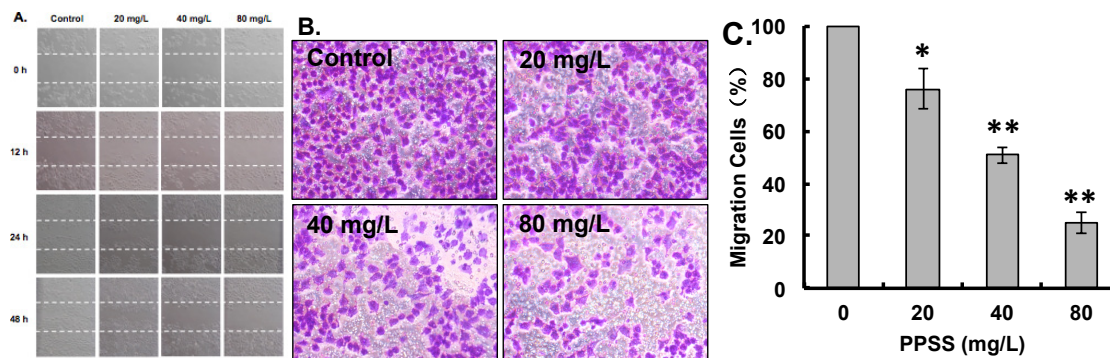
A549 cells were cultured with 0, 20, 40, 80 mg/L PPSS for 12, 24 and 48 h. PPSS decreased the proportion of viable cells in a time- and concentration-dependent manner with IC<sub>50</sub> at 12, 24 and 48 h were 72.55, 49.96, 21.01 mg/L (Figure 1). Compared with the control group, a significant anti-proliferative effect was noted.

#### PPSS inhibited the migration of A549 cells

To investigate the effect of PPSS on cell migration, wound-healing (scratch motility) and transwell invasion assays were used. Monolayers of A549 cells were scratched to form wounds, then cultured in the absence



**Figure 1. Effect of PPSS on Viability of A549 Cells.** A549 cells were treated with various concentrations of PPSS for 12, 24 and 48 h. Cell viability was measured by MTT assay. Data were represented as means $\pm$ S.D. of three independent experiments

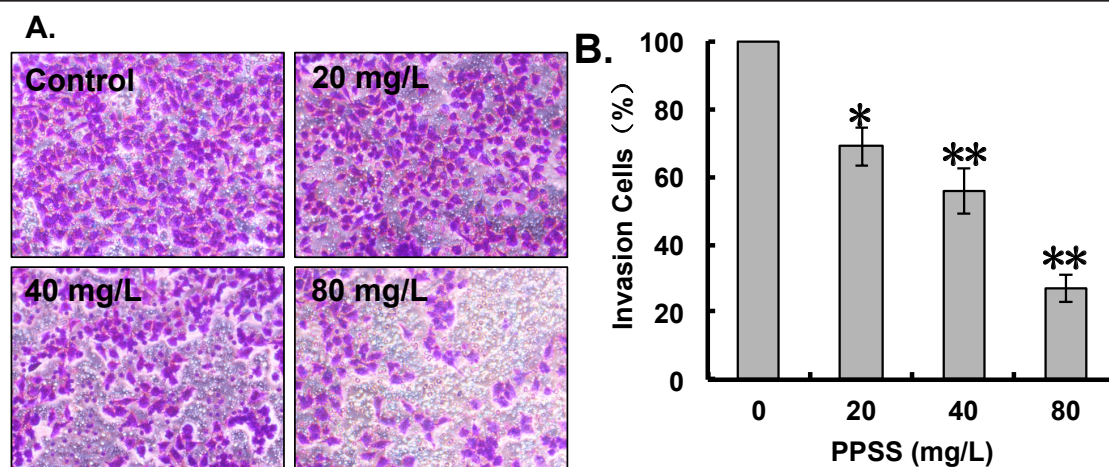


**Figure 2. Effect of PPSS on A549 Cell Migration in Vitro.** A) A549 cells were incubated with 0, 20, 40 and 80 mg/L PPSS for 12, 24 and 48 h, wounded cells were observed by phase contrast microscopy (200 $\times$ magnification). B) The cell migration through the polycarbonate membrane treated with various concentrations of PPSS stained by 0.2% crystal violet. The inhibitory effect of PPSS on the migration of the cells was in a concentration-dependent manner. C) Quantification of the number of cells migrating through the polycarbonate membrane. Means  $\pm$  S.D. of three independent experiments. Statistically significant changes at \* $p < 0.05$ , \*\* $p < 0.01$  vs. control group

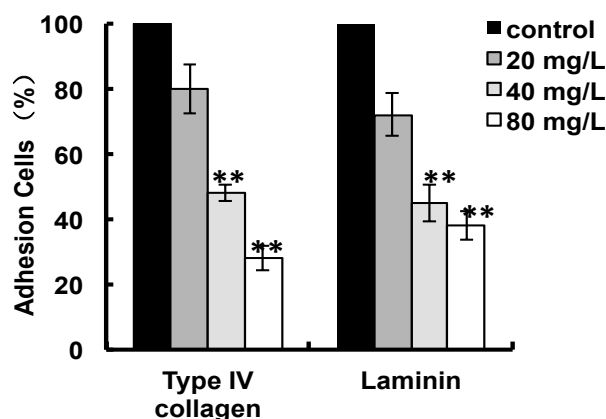


**Table 1. Influence of PPSS on the Average width of wound of A549 Cells**

	Control	20 mg/L	40 mg/L	80 mg/L
12h	152.93±14.65	106.32±8.33	115.74±6.59	143.63±11.23
24h	65.73±14.96	79.85±2.90	139.06±4.01	133.63±8.18
48h	29.61±2.03	71.98±12.20	111.90±6.92	149.93±12.69



**Figure 3. Effect of PPSS on A549 Cell Invasion in Vitro.** A) A549 cell invasion through the matrigel-coated polycarbonate membrane stained by 0.2% crystal violet. The inhibitory effect of PPSS on the invasion of the cells was in a concentration-dependent manner. B) Quantification of the cell invasion assay. Data were represented as means±S.D. of three independent experiments. \* $p<0.05$ , \*\* $p<0.01$  vs. control group



**Figure 4. Effect of PPSS on A549 Cell Adhesion in Vitro.** Pretreatment with different concentrations of PPSS (0, 20, 40 and 80 mg/L) significantly inhibited A549 cell adhesion to the ECM proteins (type IV collagen and laminin) were tested in a concentration-dependent manner, respectively. Mean±S.D. of three separate experiments, \*\* $p<0.01$  compared with the control group

or presence of various concentrations of PPSS (20, 40, 80 mg/L), and observed at 12, 24 and 48 h after cell monolayers had been wounded. The average width of wound was measured after treatment with PPSS (Table 1). A549 cells in PPSS group moved slowly compared with the control group in a concentration-dependent manner (Figure 2A). The transwell invasion assay also showed the similar results. Millicell was used to determine the inhibitory effect of PPSS on A549 cell migration. After 24 h of treatment with 0, 20, 40, 80 mg/L PPSS, the cell number on the lower surface of the membrane decreased in a concentration-dependent manner (Figure 2B-C). All the results showed that PPSS could impair A549 cell migration.

#### PPSS suppressed the invasion of A549 cells

The possible effect of PPSS on cell invasion was examined using matrigel-coated chambers. Cells were treated with 20, 40, 80 mg/L PPSS or vehicle for 24 h in the upper side, and then allowed to migrate through a membrane coated with matrigel. As shown in Figure 3, PPSS inhibited the invasion ability of A549 cells in a concentration-dependent manner. These data were consistent with the results above.

#### PPSS suppressed the adhesion of A549 cells

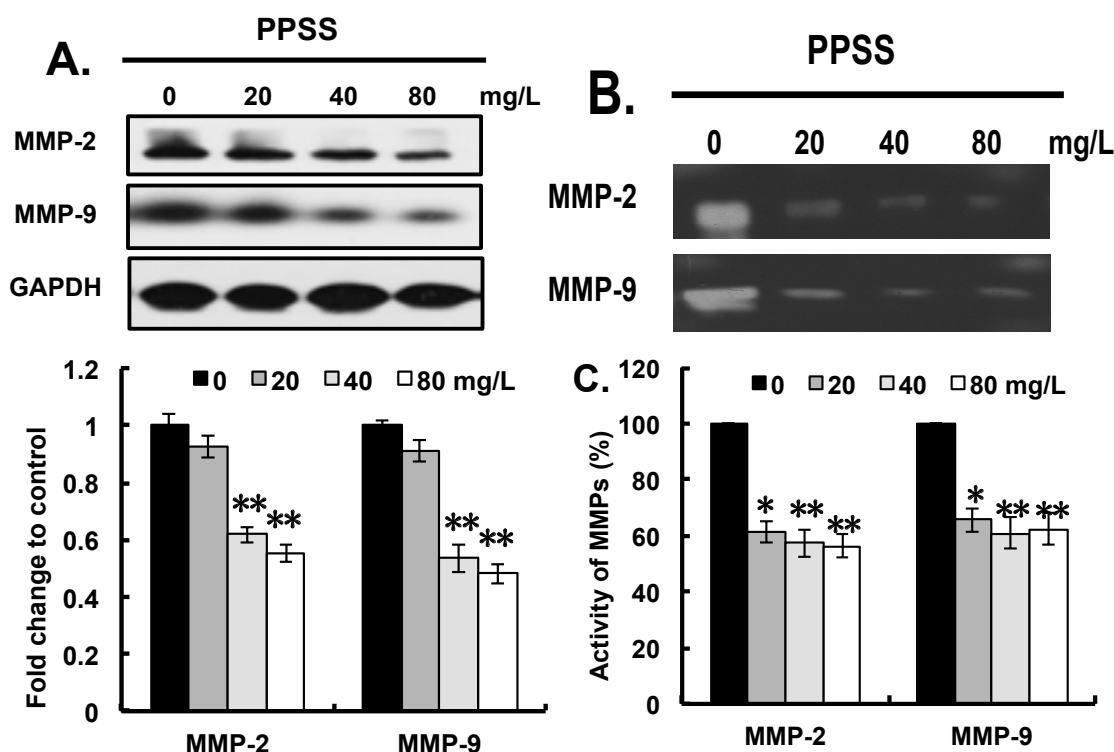
The formation of tumor invasion involves cell adhesion, migration, and degradation of extracellular matrices (ECM). Type IV collagen and laminin were the main components of ECM. In this study, the effect of PPSS on the adhesion of A549 cells to the substrates precoated with type IV collagen and laminin was investigated. Pretreatment with PPSS (20, 40, 80 mg/L) significantly inhibited A549 cell adhesion to type IV collagen and laminin (Figure 4).

#### PPSS down-regulated the protein levels of MMP-2 and MMP-9 in A549 cells

The protein levels of MMP-2 and MMP-9 in A549 cells treated with PPSS (0, 20, 40, 80 mg/L) for 24 h were detected by western blot analysis. As shown in Figure 5A, the concentration-dependent down-regulation of MMP-2 and MMP-9 were detected in PPSS treated cells. Thus protein expression levels of MMP-2 and MMP-9 could be down regulated by PPSS in A549 cells.

#### PPSS suppressed the activity of MMP-2 and MMP-9 in A549 cells

The potential effects of PPSS pretreatment on MMP-2 and MMP-9 production by A549 cells were detected by



**Figure 5. Effects of PPSS on Proteins Expressions and Activities of MMP-2 and MMP-9 in A549 Cells.** A) The cells were lysed after treatment with various concentrations of PPSS for 24 h and the protein levels of MMP-2 and MMP-9 were determined by western blot analysis. It showed that PPSS could inhibit the MMP-2 and MMP-9 expression in a concentration-dependent manner. GAPDH was used as a loading reference. Band density of the specific protein was analyzed with Quantity One image software and the results were expressed as average density to GAPDH. B) Gelatin zymography analysis of serum-free media conditioned by A549 cells treated with 0, 20, 40 and 80 mg/L of PPSS. Results indicated that PPSS could exert an inhibitive effect on the secretion of MMP-2 and MMP-9 in a concentration-dependent manner compared with the control group. C) Quantification of the gelatin zymography assay.  $n=3$ , means $\pm$ S.D. \* $p < 0.05$ , \*\* $p < 0.01$  vs. control group

gelatin zymography. PPSS pretreatment had significant inhibitory effects on the levels of MMP-2 and MMP-9 activity in A549 cells (Figure 5B-C). These results demonstrated that changes in gelatinase secretion or activation by PPSS inhibited the cell invasion.

## Discussion

Lung cancer is one of the most common malignancies and the leading cause of cancer-related death in the world (Liu et al., 2014). After diagnosis of the disease, the average 5 year survival rate is reported to be 5-10%. Metastasis is the final stage in tumor progression from a normal cell to a fully malignant cell, and is the cause of 90% of all deaths from cancer (Yilmaz et al., 2007). An important portion (40%) of the diagnosed lung cancer patients develops distant organ metastasis in thorax, brain, adrenal glands, liver, bone and kidneys, these are the reason for high mortality of lung cancer (Silvestri et al., 2003). Therefore, researchers continue to search for anti-cancer drugs that place extra emphasis on the metastasis.

Traditional Chinese medicine has its boundedness that was due to active ingredients being unclear in cancer treatment. However, with the development of separation and analysis technology, nature extracts provide leads for the potential anti-cancer agents. Actually, more than 50% of anti-cancer drugs are directly or indirectly derived from natural plants (Lee, 1999).

In this study, we have found that paris polyphylla steroidal saponins (PPSS) suppressed A549 human lung cancer cell growth. The mechanism of this effect was investigated and we demonstrated for the first time that PPSS played a remarkable role in inhibiting metastasis via down regulation of MMP-2 and MMP-9 in lung cancer A549 cells.

The effect of PPSS on A549 cell motility was investigated. The wound healing and millicell assays indicated that PPSS reduced the migration of A549 cell in a concentration- and time-dependent manner. The ability of migrate through uncoated porous filters in response to a chemotactic stimulus in A549 cells was examined in a Transwell migration assay. These findings indicate that PPSS inhibited the migration of A549 cells.

The effect of PPSS on A549 cell invasion was investigated by a matrigel-coated chamber invasion assay. Treatment of PPSS displayed obvious inhibition of invasion in a concentration-dependent manner.

An *in vitro* adhesion assay revealed that A549 cells adhere well to extracellular matrices protein collagen type IV and laminin. Pretreatment with PPSS effectively inhibited A549 cell adhesion to collagen type IV and laminin in a concentration-dependent manner.

MMPs are well documented ECM-degrading enzymes and its activity is associated with tumor invasiveness (Yadav et al., 2014). We examined whether the anti-invasive mechanism was related to MMPs' level.

Gelatin zymography assay was used to measure MMPs' activities. The expression and activity of MMPs against matrix macromolecules have been associated with the development of malignant phenotypes and the promotion of cell invasiveness and metastasis. MMP-2 and MMP-9 were two major MMPs mediating the degradation of the ECM (Wang et al., 2014). In our study, PPSS treatment not only reduced the protein expression but also suppressed the enzymatic activity of MMP-2 and MMP-9. These results reveal that PPSS's anti-invasive action is associated with diminishing the ability of lung cancer cells to degrade the components of ECM by modulating MMP-2 and MMP-9 expression and activity.

In conclusion, these results indicated that PPSS was able to inhibit lung cancer cell adhesion, migration and invasion, the mechanism underlying was attributed to attenuation of the activity and expression of MMP-2 and MMP-9. With the undermining of the interrelation, PPSS would be a potential candidate for interventions against lung cancer metastases.

## Acknowledgements

This work was supported by the Fund of the Educational Department of Shaanxi Province, P.R. China (14JK1625).

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