Journal of Medicinal Plants Research Vol. 5(17), pp. 4218-4223, 9 September, 2011
Available online at http://www.academicjournals.org/JMPR
ISSN 1996-0875 ©2011 Academic Journals

Full Length Research Paper

Effects of sulfated polysaccharide extracted from *Prunella vulgaris* on endothelial cells

Wu Xiong-Zhi[1]*, Zhang Shuang-Xia[1], Zhu Cong[1] and Chen Dan[2]

[1]Tianjin Medical University Cancer Institute and Hospital, Huan - Hu - Xi - Road, He - Xi - District, Tianjin 300060, China.
[2]Department of Pharmacology, School of Basic Medical Sciences, Tianjin Medical University, Tianjin 300070, China.

Accepted 26 July, 2011

Sulfated polysaccharide extracted from *Prunella vulgaris*, a commonly used traditional Chinese herb, possesses anti-tumor effect. But its underlying mechanism is unclear. The binding reaction of bFGF – *P. vulgaris* sulfated polysaccharide (PVSP) and bFGF - heparan were characterized by surface plasmon resonance assay. The competitive binding between bFGF - PVSP and bFGF - heparan sulfate were determined by solid phase combined assay. PVSP was capable of binding to bFGF in a dose - dependent manner. Moreover, PVSP could competitively inhibit the binding of heparan sulfate to the cell binding domain of bFGF in a dose - dependent manner. PVSP significantly inhibited the proliferation of ABAE cells in a dose - dependent and time - dependent manner. The percentage of apoptosis in adult bovine aortic endothelial cells showed significant difference between PVSP - treated cells and control cells. PVSP down regulated adult bovine aortic endothelial cell migration in a dose - dependent manner. Therefore, PVSP could influence the proliferation, apoptosis and migration of endothelial cells, which can be ascribed to the competitive ability of PVSP to inhibiting the binding of bFGF - heparan sulfate.

Key words: Angiogenesis, endothelial cell, *Prunella vulgaris*, sulfated polysaccharide.

INTRODUCTION

Angiogenesis play an important role in cancer metastasis from the primary tumor to the secondary lesions (Folkman, 1990, 1995). Various angiogenic growth factors are involved in this process. Fibroblast growth factors (FGF) are one of the earliest described angiogenic factor in tumor angiogenesis (Abraham et al., 1986). FGF is secreted by tumor cells and endothelial cells and it accumulates in the extracellular matrix (ECM). The release of FGF may influence solid tumor growth and neovascularization. According to the article documented, FGF influences the proliferation, apoptosis and migration of endothelial cells (Drinane et al., 2006; Tao et al., 2010). Accordingly, neutralizing anti - FGF antibodies could affect tumor growth (Baird et al., 1986; Gross et al., 1993; Hori et al., 1991).

Basic fibroblast growth factors (bFGF), a member of FGFs family, belong to heparan - binding growth factor which typically store in ECM and bind to heparan sulfate proteoglycans (Kiefer et al., 1990). Heparan sulfate proteoglycans regulate bFGF signal pathway by direct binding to bFGF and its receptor (Pellegrini, 2001). Heparan sulfates are necessary to form bFGF - receptor oligomerization (Plotnikov et al., 1999; Schlessinger et al., 2000).

bFGF preferentially bind to heparan sulfates before binding to its specific receptor (Harmer, 2006). Targeting the binding of bFGF - heparan significantly reduces the growth of solid tumor.

Prunella vulgaris, a commonly used Chinese herb, has a wide range of reported medicinal activities (Brindley et al., 2009; Fang et al., 2005; Ryu et al., 2000; Skottová et al., 2004; Xu et al., 1999). *P. vulgaris* sulfated polysaccharide (PVSP) was revealed to be the major content with anti - tumor activity of *P. vulgaris* (Choi et al., 2010; Feng et al., 2010). However, no detailed report can

*Corresponding author. E-mail: ilwxz@163.com. Tel: +862228297282.

Abbreviations: PVSP, *Prunella vulgaris* sulfated polysaccharide; **ABAE,** adult bovine aortic endothelial, **FGF,** fibroblast growth factors; **ECM,** extracellular matrix.

be found about its underlying mechanism. Recently some research has shown that various sulfated polysaccharides have the anti - angiogenesis ability via binding to FGF (Folkman and Shing, 1992). Here, the effects of PVSP on endothelial cells and its underlying mechanism were investigated.

MATERIALS AND METHODS

Cell line and reagents

PVSP was purchased from Ze Lang Nanjing Medical Technology Co., Ltd (Nanjing, China). Heparan degrading enzyme assay kit was obtained from Takara (Japan). The heparin was obtained from solarbio biology (China). The 7-AAD and annexin V for apoptosis test was purchased from Biolegend (US). The adult bovine aortic endothelial (ABAE) cells were kindly provided by Dr. zhang (Nankai university). ABAE cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin (Hyclone, USA) at 37℃ in a humidified 5% CO_2 incubator.

Surface plasmon resonance assay

The kinetics and specificity of the binding reactions of bFGF - PVSP and bFGF - heparin were performed on surface plasmon resonance apparatus (BIAcore X100). Briefly, the first step is the immobilization of bFGF on CM5 sensor chips using the amine coupling kit according to the procedure described by the manufacturer. PBS was used as a mobile phase at a flow rate of 10 µl/min. The carboxymethyl dextran matrix of the sensorchip surface was first activated with an injection of 70 µl of the EDC: NHS reagent mixture. Then, 70 µl of bFGF at 100 µg/ml in 10 mM NaOAc (pH 5.7) was injected and allowed to covalently couple to the sensor surface. Finally, the unreacted sites were blocked by injection of 70 µl of 1M ethanolamine (pH 8.5). A response unit of ~6580 Ru was achieved after immobilization. The second step was to determine the binding affinity for bFGF of PVSP. This was done by injecting 15 µl PVSP at different concentrations (0.25, 0.5, 1 µg/ml) in PBS buffer onto the immobilized bFGF chip. For evaluated the ability of PVSP inhibition of heparin bind to bFGF, 10 µg/ml heparin was added to the chip after PVSP was added and bound to the chip. To correct for nonspecific binding and bulk refractive index changes, a blank channel (FC2) without heparin or PVSP was employed as a control for each experiment. All binding interactions were recorded in real time and the blank channel readings were subtracted from the results before analysis. Changes in mass due to the binding response were recorded as resonance units. All binding experiments were done at 25℃ with a constant flow rate of 10 µl/min HBS - EP. The sensor chip surface was regenerated by an injection of 10 µl of 15 mM HCl plus 0.5 M NaCl and then switched to PBS in between experiments. For determination of association and disassociation rate constants, the real - time binding capacity was recorded. For direct assessment of dissociation constants, the association phase was allowed to proceed to equilibrium. Binding kinetics and stoichiometry were determined by surface plasmon resonance using the BIACORE software.

Solid phase heparan-binding assay

Heparan degrading enzyme assay (code MK412, TAKARA) was applied to research the competitive binding between bFGF - PVSP and bFGF - heparan sulfate with modified protocol. Briefly, 50 µl of different concentrations of PVSP (10, 50 µg/ml) and 50 µl of biotinylated heparan sulfate were mixed and transferred into the well of the cell binding domain of bFGF immobilized 96 - well microtiterplate. Then the microtiterplate was incubated at 37℃ for 15 min. After discarding the reaction solution and washing, 100 µl of avidin POD conjugate was added into each well and incubated at 37℃ for 30 min. Then, 100 µl of POD substrate was added into each well and performed color development at room temperature for 5 to 15 min. Then 100 µl of stop solution was added into each well. After zero adjustment of microplate reader by using distilled water as zero control, the absorbance of each well was measured at 450 nm. The inhibition ratio was determined by using biotinylated heparan sulfate without mixed with PVSP as a control.

Proliferation assays

ABAE cells were seeded in 24 - well plates at 0.4×10^4 cells/ well and incubated with the presence of 10, 100, 200 µg/ ml PVSP and control (absence of sample) groups, respectively. At 24, 48, 72, 96 and 120 h after treatment, the cells were collected respectively. The cell number was counted with a hematocytometer and the viability of the cell was checked by trypan blue exclusion assay.

Analysis of apoptosis

ABAE cells were seeded in 6 - well plates at 2×10^5 cells/ well for 24h. After the supernatant was discarded, blank medium and 10, 100, 200 µg/ ml PVSP were added separately to the different wells of the plates. After 48 hours treatment, the Cells were collected, followed by stained with 7-AAD/ Annexin V-FITC (Biolegend, US). Then, Cell apoptosis was analyzed by flow cytometry (BD FACSCalibur, USA).

Wound-healing assay

ABAE cells were seeded in 24 - well plates at 2×10^5 cells/ well and allowed to grow for 1 day before being exposed to PVSP (10, 50µg/ml, respectively). For the control group, an equal volume of DMEM was added into the medium. A linear wound about 1mm in width was made by scratching the monolayer cell culture with a pipette tip after cell confluency. Then DMEM with different concentration of PVSP and 10% FBS were added for cell growth. Wound healing of the cells was observed under phase-contrast microscope after 0 and 48 h.

Statistical analysis

All experiments were repeated three times, with three replicate samples in each experiment. Single-factor ANOVA was used for data analysis. Statistical calculations were performed by SPSS 10.0. All values are expressed as means ± SD.

RESULTS

Inhibited bFGF-heparin binding

In order to assess the ability of PVSP binding to bFGF *in vitro*, we exploited the surface plasmon resonance assay. As shown in Figure 1A, PVSP was capable of binding to bFGF that had been immobilized on CM5 chip in a dose - dependent manner. Solid phase combined assay was used to ensure a competitive binding between

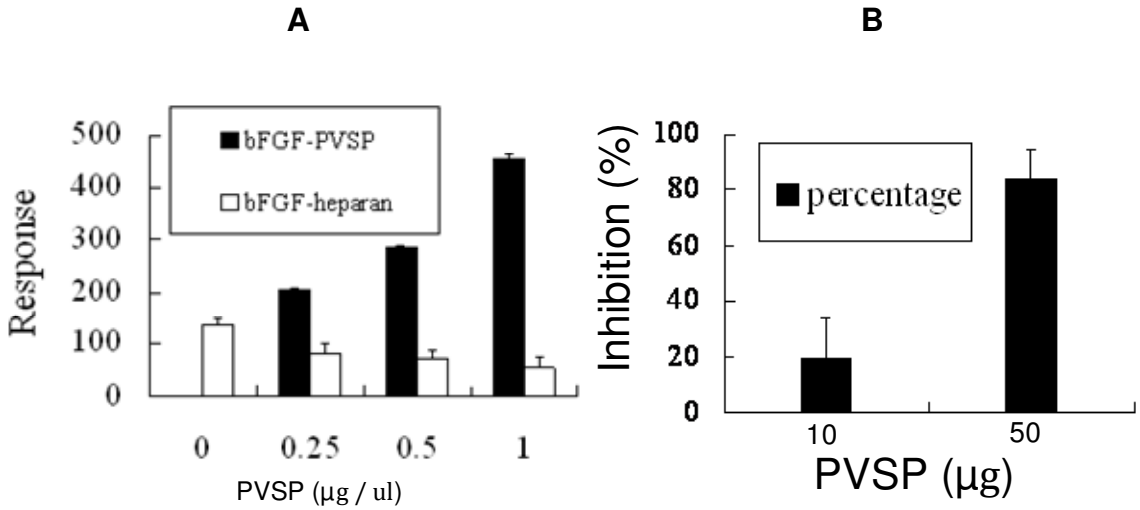

Figure 1. PVSP inhibited the bFGF-heparan binding. A, Surface plasmon resonance analysis. B, solid phase combined assay. PVSP bind to bFGF and the binding ability was concentration-dependent. Moreover, PVSP concentration-dependently inhibited the binding of biotinylated heparan sulfate to CBD-bFGF immobilized on microtiterplate. PVSP: *Prunella vulgaris* sulfated polysaccharides.

bFGF - PVSP and bFGF - heparan sulfate.

Our results indicated that PVSP significantly inhibited the binding of heparan sulfate to the cell binding domain of bFGF that had been immobilized on microtiterplate in a dose - dependent manner (Figure 1B).

Inhibited the proliferation of endothelial cells

The growth curves showed that PVSP significantly inhibited the proliferation of ABAE cells in dose - and time - dependent manner. On 120 h after the addition of PVSP, the cell numbers were 7155 cells/well for control group, 5572 cells/well for 10 µg/ml PVSP, 2133 cells/well for 100 µg/ml PVSP and 500 cells/well for 200 µg/ml PVSP, respectively.

There was significant difference between the treatment and control groups (Figure 2).

Promoted the apoptosis of endothelial cells

The percentage of apoptosis in ABAE cells showed significant difference between PVSP - treated cells and control cells (P<0.01) (Figure 3). The percentage of apoptosis of cells was 11.7 ± 0.5; 17.2 ± 0.78 and 19 ± 0.8% in the group treated with 10, 100 and 200 µg/ml PVSP respectively, which was much higher than that of the control group (3.9 ± 0.7%) (P<0.01).

Inhibited the migration of endothelial cells

We investigated the effect of PVSP on ABAE cells migration using a monolayer wound-healing assay. Considering the major influence of PVSP on ABAE cells apoptosis, we utilized lower concentrations of PVSP (10 and 50 µg/ml) in this study. We found that PVSP exerted inhibitory effect on the migration of ABAE cells even at concentration of 10 µg/ml. Moreover, no obvious migration was observed after 48 h with the treatment of 50 µg/ml PVSP. Our result suggested that PVSP could dramatically down regulate ABAE cell migration in a dose - dependent manner compared to untreated controls (Figure 4).

DISCUSSION

Sulfated oligosaccharides or sulfated polysaccharides play important roles in biological process (Kovensky, 2009; Wu, 2006). They are capable of binding to protein by the interactions mediated by their highly specific sequences (heparin - antithrombin, heparan sulfate - growth factors / herpes simplex virus) or by electrostatic interaction between sulfate groups and cationic sites of proteins. Sulfated polysaccharides are involved inmultiplies pharmacological effects, such as the control of proteolysis, the modulation of the angiogenesis and the oligomerisation of cell growth factors.

Large amount of research work have shown that the polysaccharides was considered to be the major content of *P. vulgaris*, which possesses immunoregulatory, anti - inflammatory and anti - virus activity (Fang et al., 2005; Ryu et al., 2006; Xu et al., 1999; Brindley et al., 2009). Some researches have shown that PVSP have the anti - tumor activity (Choi et al., 2010; Fang et al., 2005; Feng et al., 2010). PVSP is a close structural homologue of

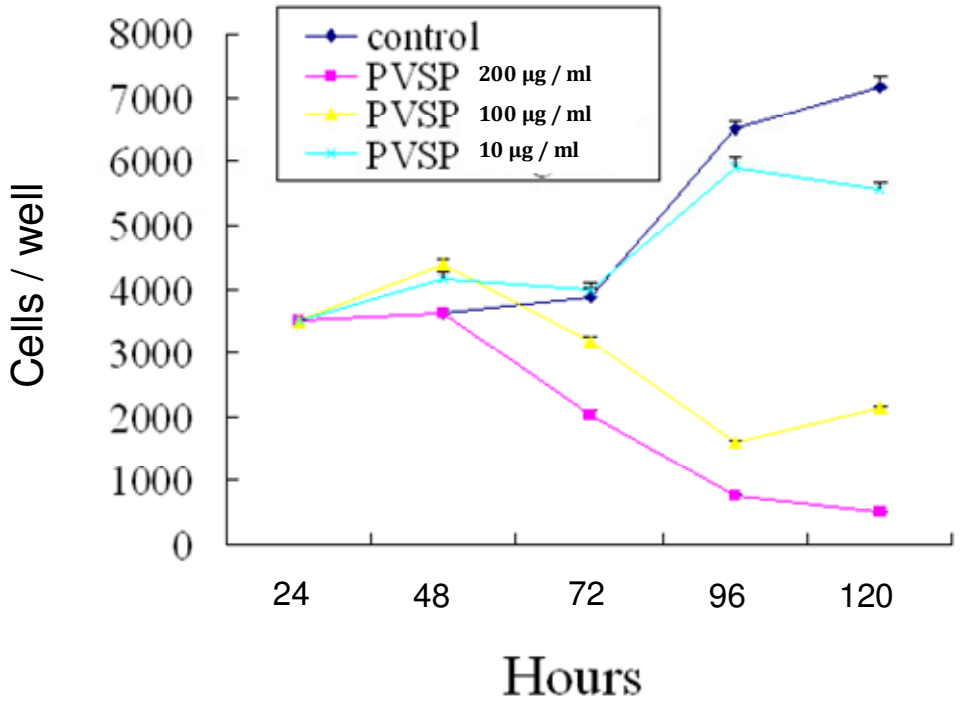

Figure 2. PVSP suppressed the proliferation of endothelial cells. Endothelial cell proliferation was assessed by trypan blue exclusion assay. Data were expressed as means ± SD (n=3). The number of cells was reduced after administrated with PVSP. PVSP: *Prunella vulgaris* sulfated polysaccharides.

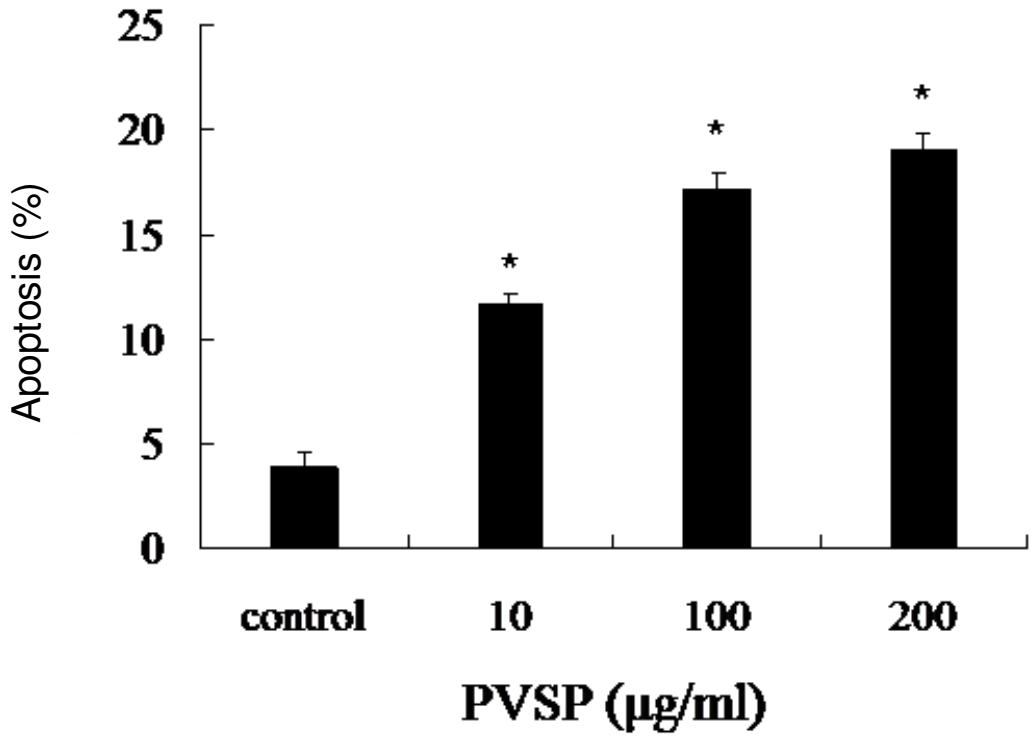

Figure 3. PVSP induced apoptosis of endothelial cells. The apoptosis was assessed by flow cytometry. The level of apoptosis cells increased after administrated with different concentration of PVSP for 48h. Endothelial cell apoptosis is shown as % of all collected cells. Data were expressed as means ± SD (n=3). PVSP: *Prunella vulgaris* sulfated polysaccharides. *: P<0.01 versus control.

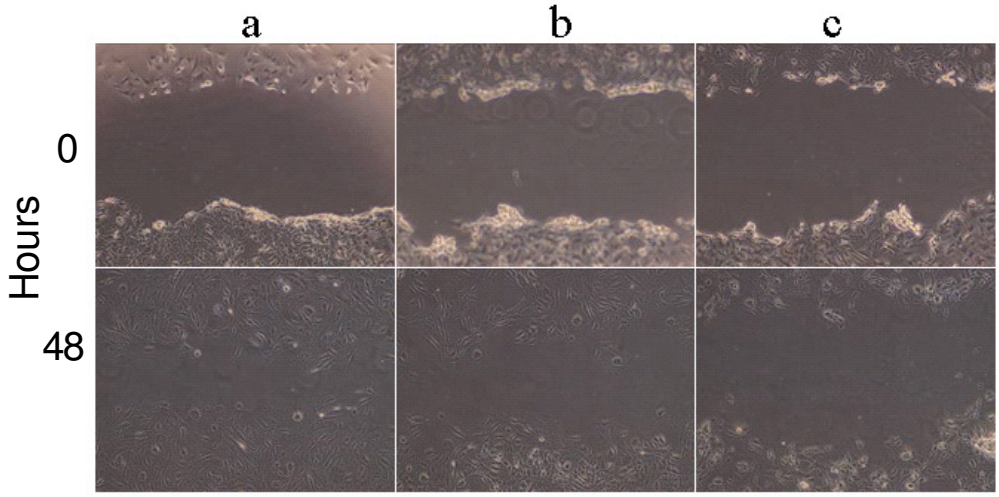

Figure 4. Wound-healing assay of PVSP on endothelial cells. Phase-contrast microscope images were shown (100×). Indicated times (0 h, 48 h) depict the duration after scratching of the monolayer. a: Control group, b:10 μg/ml PVSP, c: 50 μg/ml PVSP. PVSP: *Prunella vulgaris* sulfated polysaccharides.

endogenous sulfated polysaccharides such as heparin and heparan sulfated. The hypothesis is that the binding of PVSP to bFGF may inhibit the binding of bFGF to heparan. Surface plasmon resonance assay and solid phase heparin - binding assay showed that PVSP is capable of binding to bFGF in a concentration- dependent manner. Moreover, PVSP can dose - dependently inhibit the binding of heparin to bFGF.

FGF influence the proliferation, apoptosis and migration of endothelial cells (Drinane et al., 2006; Tao et al., 2010). Human neutralizing FGF antibody is able to inhibit the proliferation, migration and tube formation of human umbilical vein endothelial cells (Drinane et al., 2006). Plasminogen activator inhibitor - 1 is capable of inducing high levels of apoptosis and inhibiting the migration and proliferation of arterial endothelial cells via inhibiting FGF-2 functions (Tao et al., 2010). In this work ABAE cells were used to assess the influence of PVSP on endothelial cells. Dramatically, PVSP inhibited the proliferation and migration of ABAE cells. Moreover, PVSP induced the apoptosis of ABAE cells.

In conclusion, PVSP influence the proliferation, apoptosis and migration of endothelial cells, which can be ascribed to the abilities of binding to bFGF and inhibiting the binding of bFGF - heparan. It indicated that PVSP could potential to be used as adjuvant therapy medicines to the treatment of malignant tumors.

ACKNOWLEDGEMENTS

This study was supported by Grants 30801495 from the National Natural Science Foundation of China. We thank Prof. Qiao Bin from Analysis and Detecting center, School of Chemical Engineering and Technology, Tianjin University for the assistant of Surface plasmon resonance assay.

REFERENCES

Abraham JA, Mergia A, Whang JL, Tumolo A, Friedman J, Hjerrild KA, Gospodarowicz D, Fiddes JC (1986). Nucleotide sequence of a bovine clone encoding the angiogenic protein, basic fibroblast growth factor. Science, 233: 545-548.

Baird A, Mormède P, Böhlen P (1986). Immunoreactive fibroblast growth factor (FGF) in a transplantable chondrosarcoma: inhibition of tumor growth by antibodies to FGF. J. Cell Biochem., 30: 79-85.

Brindley MA, Widrlechner MP, McCoy JA, Murphy P, Hauck C, Rizshsky L, Nikolau B, Maury W (2009). Inhibition of lentivirus replication by aqueous extracts of *Prunella vulgaris*. Virol. J., 20: 6-8.

Choi JH, Han EH, Hwang YP, Choi JM, Choi CY, Chung YC, Seo JK, Jeong HG (2010). Suppression of PMA-induced tumor cell invasion and metastasis by aqueous extract isolated from *Prunella vulgaris* via the inhibition of NF-kappaB-dependent MMP-9 expression. Food Chem. Toxicol., 48: 564-571.

Drinane M, Walsh J, Mollmark J, Simons M, Mulligan-Kehoe MJ (2006). The anti-angiogenic activity of rPAI-1(23) inhibits fibroblast growth factor-2 functions. J. Biol. Chem., 281: 33336-33344.

Fang X, Chang RC, Yuen WH, Zee SY (2005). Immune modulatory effects of *Prunella vulgaris* L. Int. J. Mol. Med., 15: 491-496.

Feng L, Jia XB, Shi F, Chen Y (2010). Identification of two polysaccharides from *Prunella vulgaris* L. and evaluation on their anti-lung adenocarcinoma activity. Molecules, 15: 5093-5103.

Folkman J (1990). What is the evidence that tumors are angiogenesis dependent ? J. Natl. Cancer Inst., 82: 4-6.

Folkman J, Shing Y (1992). Control of angiogenesis by heparin and other sulfated polysaccharides. Adv. Exp. Med. Biol., 313: 355-364.

Folkman J (1995). Angiogenesis in cancer, vascular, rheumatoid and other disease. Nat. Med., 1: 27-31.

Gross JL, Herblin WF, Dusak BA, Czerniak P, Diamond MD, Sun T, Eidsvoog K, Dexter DL, Yayon A (1993). Effects of modulation of basic fibroblast growth factor on tumor growth *in vivo*. J. Natl. Cancer Inst., 85: 121-131.

Harmer NJ (2006). Insights into the role of heparan sulphate in

fibroblast growth factor signalling. Biochem. Soc. Trans., 34: 442-445.
Hori A, Sasada R, Matsutani E, Naito K, Sakura Y, Fujita T, Kozai Y (1991). Suppression of solid tumor growth by immunoneutralizing monoclonal antibody against human basic fibroblast growth factor. Cancer Res., 51: 6180-6184.
Kiefer MC, Stephans JC, Crawford K, Okino K, Barr PJ (1990). Ligand-affinity cloning and structure of a cell surface heparan sulfate proteoglycan that binds basic fibroblast growth factor. Proc. Natl. Acad. Sci. USA, 87: 6985-6989.
Kovensky J (2009). Sulfated oligosaccharides: new targets for drug development? Curr. Med. Chem., 16: 2338-2344.
Pellegrini L (2001). Role of heparan sulfate in fibroblast growth factor signalling: a structural view. Curr. Opin. Struct. Biol., 11: 629-634.
Plotnikov AN, Schlessinger J, Hubbard SR, Mohammadi M (1999). Structural basis for FGF receptor dimerization and activation. Cell, 98: 641-650.
Ryu SY, Oak MH, Yoon SK, Cho DI, Yoo GS, Kim TS, Kim KM (2000). Anti-allergic and anti-inflammatory triterpenes from the herb of *Prunella vulgaris*. Planta. Med., 66: 358-360.
Schlessinger J, Plotnikov AN, Ibrahimi OA, Eliseenkova AV, Yeh BK, Yayon A, Linhardt RJ, Mohammadi M (2000). Crystal structure of a ternary FGF-FGFR-heparin complex reveals a dual role for heparin in FGFR binding and dimerization. Mol. Cell, 6: 743-750.
Skottová N, Kazdová L, Oliyarnyk O, Vecera R, Sobolová L, Ulrichová J (2004). Phenolics-rich extracts from *Silybum marianum* and *Prunella vulgaris* reduce a high-sucrose diet induced oxidative stress in hereditary hypertriglyceridemic rats. Pharmacol. Res., 50: 123-130.
Tao J, Xiang JJ, Li D, Deng N, Wang H, Gong YP (2010). Selection and characterization of a human neutralizing antibody to human fibroblast growth factor-2. Biochem. Biophys. Res. Commun., 394: 767-773.
Wu XZ (2006). Sulfated oligosaccharides and tumor: promoter or inhibitor? Panminerva med., 48: 27-31.
Xu HX, Lee SH, Lee SF, White RL, Blay J (1999). Isolation and characterization of an anti-HSV polysaccharide from *Prunella vulgaris*. Antiviral Res., 44: 43-54.