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EFFECTS OF *VIETNAMESE SOPHORA* ROOT ON GROWTH, ADHESION, INVASION AND MOTILITY OF MELANOMA CELLS

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Abstract

Background: *Vietnamese Sophora* Root mainly contains active constituents such as alkaloids, and it has anti-tumour, antibacterial, and anti-inflammatory effects. The objective of the paper was to study the effects of *Vietnamese Sophora* Root on growth, adhesion, invasion and motility of mouse melanoma $B_{16}BL_6$ cells, and to preliminarily explore its mechanism of action.

Materials and Methods: MTT assay was used to detect the effect of *Vietnamese Sophora* Root aqueous extract on $B_{16}BL_6$ cell proliferation. Cell adhesion assay, reconstituted basement membrane invasion assay and chemotactic motility assay were used to observe the effects of *Vietnamese Sophora* Root aqueous extract on adhesion, invasion and motility of $B_{16}BL_6$ cells.

Results: Different concentrations of *Vietnamese Sophora* Root aqueous extracts had different degrees of inhibitory effects on $B_{16}BL_6$ proliferation. With the decrease of concentration, the proliferation inhibitory effect decreased and even turned to promoting effect. The extract significantly inhibited the adhesion of $B_{16}BL_6$ cells to the basement membrane component LN, and had a significant effect on both the invasive and migratory capacities of $B_{16}BL_6$ cells through the basement membrane.

Conclusion: We concluded that the aqueous extract of *Vietnamese Sophora* Root can inhibit the proliferation of melanoma cells, as well as their adhesion and movement.

Keywords: *Vietnamese Sophora* Root; $B_{16}BL_6$; adhesion; invasion

Introduction

Vietnamese Sophora Root is the dried root and rhizoma of *Sophora tonkinensis Gapnep*. in family Leguminosae of class Dicotyledoneae, which is mainly grown in Guangxi. It has heat-clearing and detoxifying, swelling-diminishing and sore-throat relieving efficacies (Chinese, 2005). It can cure throat abscess, sore-throat, throat obstruction, swollen and aching gums, heat cough with panting and fullness, jaundice, diarrhoea, haemorrhoids, heat swelling, favus of the scalp, scabies, and snake, insect and dog bites (Jiangsu, 1977). Studies have found that *Vietnamese Sophora* Root mainly contains active constituents such as alkaloids, flavonoids, saponins and polysaccharides. The alkaloids isolated from *Vietnamese Sophora* Root (Dong et al., 2001; Institute, 1996) include matrine, oxymatrine, and anagyrine, etc. The flavonoids isolated from *Vietnamese Sophora* Root include flavone, flavanone, genistein, etc., and the polysaccharide components include SSa-1, SSa-2, SSa-3, SSb-1, SSb-2, etc. (Dou et al., 1998; Dong et al., 2001). Researches have demonstrated a variety of pharmacological effects of *Vietnamese Sophora* Root, such as anti-tumour effect (Xiao et al., 2000), antibacterial effect (Ding et al., 2002), anti-inflammatory effect (Du et al., 2008) and immunoregulatory effect (Shuai et al., 2009).

Materials and methods

Medicinal materials

Chinese medicinal herb *Vietnamese Sophora* Root, was purchased from Baidu Medicine Company, Shenyang，China. The herb was identified by Professor Kang Yangguo, and was placed in the pharmacy centre. ID number is 2011-3-311.

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Cell lines

Melanoma $B_{16}BL_6$ cells were provided by the Affiliated Hospital of China Medical University.

Reagents and instruments

The following reagents and instruments were used: RPMI 1640 medium, purchased from GIBCO, USA; fetal bovine serum, purchased from Sijiqing Bioengineering Materials Co., Ltd., Hangzhou; MTT, purchased from Sigma, USA; DMSO, purchased from Damao Chemical Instrument Supply Station, Tianjin; DMEM, purchased from Gibco; pyrrolidone-free polycarbonate membrane PVPF (diameter 13 mm, pore size 8.0 μm), purchased from Whatman; Transwell chamber, purchased from Costar; Eukitt mounting medium, purchased from Fluka; Olympus inverted microscope, purchased from OLYMPUS, Japan; continuous wavelength microplate reader, purchased from Bio-RAD; clean bench, purchased from Suzhou Purification Equipment Co., Ltd.; CO_2 incubator, purchased from SANYO, Japan.

Preparation of *Vietnamese Sophora* Root aqueous extract

In reference to the method in Xiao et al. (2000), *Vietnamese Sophora* Root was pulverised, added with distilled water and kept overnight, followed by centrifugation at 3500 rpm for 20 min. The supernatant was concentrated to 1/10 of its original volume and centrifuged at 20000 rpm for 30 min. The supernatant was then freeze-dried to obtain *Vietnamese Sophora* Root extract.

Cell cultivation

Cells were cultured in RPMI 1640 medium added with 10% fetal bovine serum under conditions of 37 ℃ and 5% CO_2. The culture medium was replaced every 2 ~ 3 d.

$B_{16}BL_6$ cell proliferation inhibition assay

With reference to Carmichael's MTT method in Carmichael et al. (1987), $B_{16}BL_6$ cells in the logarithmic growth phase were collected and digested with 0.25% trypsin. The cell concentration was adjusted to 5×10^4/ml, and the cells were seeded in 96 well plates at 100 μL per well. 6 duplicate wells were set up. After culturing for 24 h, 100 μL of drug-containing culture media were added. Final concentrations of *Vietnamese Sophora* Root extracts were 400, 500, 600, 700 and 800 μg/ml respectively. The negative control group was added with equal volume of culture medium. After incubation at 37 ℃ for 48 h, each well was added with 20 μl of MTT (2 mg/ml), and the incubation was continued for another 4 h. Then, the supernatant was discarded, and each well was added with 100 μl of DMSO to dissolve crystals. Afterwards, OD values were measured at wavelength of 570 nm using microplate reader. The inhibition rate (IR) of the drug on cell growth was calculated according to the following formula:

IR (%) = (1 - average OD value of experimental group / average OD value of control group) × 100%.

$B_{16}BL_6$ cell adhesion assay (Trapp et al., 2010)

With reference to the literature, 96-well plates were coated with Matrigel at 2 μg/well, placed on a super clean bench and air-dried for later use. The plates were blocked with 1% BSA at 40 μL per well, and incubated at 37℃ for 1 h. They were then washed 3 times with PBS for later use. B16BL6 melanoma cells in the logarithmic growth phase were collected and the cell concentration was adjusted to 1×10^6/mL. The cells were added to the above 96-well plates at 90 μL per well. Afterwards,10 μL of DMEM media containing different concentrations of *Vietnamese Sophora* Root extracts were added to each well, and the plates were incubated at 37℃ for 1 h. The plates were then washed twice in PBS, added with 20 μL of MTT solution (2 mg/ml) and cultured for an additional 4 h in the incubator. The supernatant was discarded and 100 μL of DMSO solution was added to each well. After mixing well by shaking, OD value was measured at 570 nm using microplate reader, and inhibition rate (IR) of the drug on cell adhesion was calculated according to the following formula:

IR (%) = (1 - average OD value of experimental group / average OD value of control group) × 100%

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$B_{16}BL_6$ cell reconstituted basement membrane invasion assay (Gu et al., 2007)

PVPF filter membrane was attached to the Transwell chamber. The outer surface of the membrane was coated with 5 μg of FN, and inner surface with 10 μg of Matrigel. The membrane was dried overnight at room temperature to form a matrix barrier layer. $B_{16}BL_6$ cells, which were pre-treated with drug for 24 h, were digested with 0.25% trypsin, and re-suspended in serum-free DMEM medium containing 0.1% BSA at a density of 2×10^6/ml. 100 μL of the above cell suspension was added to the Transwell chamber. The chamber was placed in a 24-well plate, and the plate was added with 600 μl of serum-free DMEM medium containing 0.1% BSA, and incubated at 37℃ for 4 h. The membrane was fixed in methanol for 1 min, stained with haematoxylin and eosin. Cells that did not permeate the membrane on one side of Matrigel were wiped off, and the filter membrane was sealed on the slide with Eukitt. 5 different fields of the membrane, namely up, down, left, right and centre, were selected under a 400x optical microscope. The number of invasive cells in each field was counted, and the average value was calculated. The inhibition rate (IR) of the drug on cell invasion was calculated according to the following formula:

IR (%) = (1 - average number of invasive cells of experimental group / average number of invasive cells of control group) × 100%

$B_{16}BL_6$ chemotactic motility assay (Zheng et al., 2003)

The steps were the same as the invasion assay except for not coating Matrigel on the inner surface of PVPF membrane. The inhibition rate (IR) of the drug on cell motility was calculated according to the following formula:

IR (%) = (1 - average number of motile cells of experimental group / average number of motile cells of control group) × 100%

Statistical analysis

The experimental results were analysed using SPSS 10.0 statistical software. The count data were compared with the χ^2 test, and measurement data were compared using the t test. $P<0.05$ indicated statistically significant difference.

Results

Effect of *Vietnamese Sophora* Root aqueous extract on proliferative capacity of $B_{16}BL_6$ cells

With the decreasing concentration of *Vietnamese Sophora* Root aqueous extract, inhibition rate of B16BL6 cell proliferation gradually decreased. The proliferation was even promoted at lower concentrations. When the concentration of *Vietnamese Sophora* Root extract was 800 μg/ml, the inhibition rate was 27.09%, which showed a significant difference. When the concentration was decreased to 500 μg/ml, the proliferation promoting effect was present. The results are shown in Table 1.

Table 1: Determination of the effect of *Vietnamese Sophora* Root on $B_{16}BL_6$ cell proliferation by MTT assay (n=6, $\overline{X}\pm S$)

Concentration μg/ml	OD value	Inhibition rate (%)
Control group	1.078±0.153	
800	0.786±0.058**	27.09
700	0.899±0.082	16.60
600	1.036±0.126	3.90
500	1.093±0.104	-1.39
400	1.125±0.147	-4.36

Comparison with control group, ** $P<0.01$, * $P<0.05$

Effect of *Vietnamese Sophora* Root on adhesion capacity of $B_{16}BL_6$ cells to the basement membrane component

Experimental results showed that when the tumour cells had been adherent to the matrix, that is, when the tumour cells have completed binding adhesion to the matrix, *Vietnamese Sophora* Root was added, and it was found that the *Vietnamese Sophora* Root inhibited the adhesion

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between tumour cells and matrix Matrigel. When the concentration was 800 μg/ml, the inhibition rate reached 20.93%, and when the concentration was 400 μg/ml, the inhibition rate also reached 3.82%. The results are shown in Table 2.

Effect of *Vietnamese Sophora* Root on reconstituted basement membrane invasion of $B_{16}BL_6$ cells

The results showed that compared with the control group each *Vietnamese Sophora* Root concentration group can significantly inhibit the number of melanoma cells and basement membrane-invasive cells, and the difference was significant. Moreover, the inhibitory effect increased with increasing drug concentration. The results are shown in Table 3.

Table 2: Effect of *Vietnamese Sophora* Root on adhesion capacity of $B_{16}BL_6$ cells to LN (n=6, $\overline{X}$±S)

Concentration μg/ml	OD value	Inhibition rate %
Control group	0.497±0.013	
800	0.393±0.016**	20.93
700	0.407±0.021**	18.11
600	0.428±0.015**	13.88
500	0.462±0.012*	7.04
400	0.478±0.028*	3.82

Comparison with control group, ** P<0.01, * P<0.05

Table 3: Effect of *Vietnamese Sophora* Root on reconstituted basement membrane invasive capacity of $B_{16}BL_6$ cells (n=6, $\overline{X}$±S)

Concentration μg/ml	Number of invasive cells	Inhibition rate %
Control group	40.42±3.53	
800	11.52±2.53**	71.50
700	13.85±3.85**	65.73
600	22.62±2.69**	44.04
500	29.95±4.22*	25.90
400	36.69±3.84*	9.23

Comparison with control group, ** P<0.01, * P<0.05

Effect of *Vietnamese Sophora* Root on chemotactic motility of $B_{16}BL_6$ cells

Vietnamese Sophora Root aqueous extract can significantly inhibit the motility of B16BL6 cells through the basement membrane at high concentrations. The results are shown in Table 4.

Table 4: Effect of *Vietnamese Sophora* Root on chemotactic motility of $B_{16}BL_6$ cells (n=6, $\overline{X}$±S)

Concentration μg/ml	Number of motile cells	Inhibition rate %
Control group	45.26±4.85	
800	24.48±4.15**	45.91
700	29.94±3.93**	33.85
600	33.82±5.28**	25.28
500	37.83±4.69*	16.42
400	42.31±5.27	6.52

Comparison with control group, ** P<0.01, * P<0.05

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Discussion

Metastasis of malignant cells is a complex multi-step process, blocking each link of the tumour cell adhesion, invasion and migration process and could inhibit the tumour metastasis (Webb et al., 2000). Adhesion is the initiating step of tumour cell invasion. At first, tumour cells bind to the basement membrane, matrix components laminin, fibronectin and collagen through membrane surface receptors. Then, the tumour cells secrete or use a variety of proteases in the cell membrane to degrade the basement membrane and matrix. At last, tumour cells directionally pass through the damaged basement membrane and matrix parts to achieve metastasis (Kohn et al., 1995).

The results of this experiment showed that *Vietnamese Sophora* Root aqueous extract can significantly inhibit the adhesion of melanoma $B_{16}BL_6$ cells to the basement membrane component LN, and has a significant inhibitory effect on invasion and motility of $B_{16}BL_6$ cells. In view of the above effects, it is speculated that the *Vietnamese Sophora* Root aqueous extract may be able to inhibit the metastasis of melanoma $B_{16}BL_6$ cells; the details remain to be investigated in further experiments.

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