http://dx.doi.org/10.4314/ajtcam.v10i3.17

EFFECT OF PARIS SAPONIN ON ANTITUMOR AND IMMUNE FUNCTION IN U14 TUMOR-BEARING MICE

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Abstract

We evaluated the effect of Paris saponin on inhibition of cervical cancer in mice and on immune regulation in tumor-bearing mice. MTT assay was used to examine the effect of Paris saponin on U14 cell proliferatiosn *in vitro*; the ascites tumor model of U14 cervical cancer was established to observe the effect of Paris saponin on inhibition of the tumor and on survival time of mice; and serum IL-4 and IFN-γ levels in tumor-bearing mice were detected. The Paris saponin showed significant inhibitory effect on growth of cervical cancer U14 cells both *in vitro* and *in vivo*, prolonged the survival time of mice, increased the serum IFN-γ level of tumor-bearing mice, and reduced the serum IL-4 level. The Paris saponin can inhibit U14 cell growth and prolong survival time of mice; it is speculated that the Paris saponin may express its anti-tumor activity by improving the body's immune system.

Key words: Paris saponin, antitumor, cytokine

Introduction

Rhizoma Paridis is the dried rhizome of *Paris polyphylla* Smith var. *yunnanensis* (Franch.) Hand.-Mazz. or *Paris polyphylla* Smith var. *chinensis* (Franch.) Hara, which belongs to the Liliaceae plant family (Chinese Pharmacopoeia Commission,. 2010). It is bitter, slightly cold, mildly toxic, and can clear heat and remove toxicity, relieve swelling and pain, cool the liver and arrest convulsion. Pharmacological studies have found that its active ingredients have the haemostatic effect (Fu, 2007), anti-tumor effect (Liu, 2008), antibacterial effect (Li, 2009), anti-inflammatory effect (Zhang, 2008), brain and kidney protection effect (Huang, 2008), antioxidant effect (Gao, 2007), and uterine contraction effect (Cong, 2005). At present, the chemical constituents extracted from Rhizoma Paridis are mainly steroidal saponins and free amino acids, of which Paris saponins account for approximately 80% of the total number of compounds, most of them are diosgenin, which is an active anti-tumor ingredient, including Paris saponin I (diosgenin 3-O-α-L-rha-(1-2)-[α-L-arab-(1-4)]-β-D-glu),Paris saponin II (diosgenin3-O-α-rha-(1-4)-α-L-rha-(1-4)-[α-L-rha-(1-2)]-β-D-glu), Paris saponin III, diosgenin and C22-hydroxy-protodioscin, C22-methoxy-protodioscin, C22-hydroxy-protopolyphyllin I, C22-methoxy-protopolyphyllin I, C22-methoxy- protopolyphyllin II, etc. (Wu., 2004). Isolated PSII has potent inhibitory effects on the growth of tumor cells without deleterious effects on different normal cell types or benign neoplastic derived cells. (Xiao et al., 2012).

Materials and methods

Medicinal materials

Rhizoma Paridis was purchased from Dalian Baidu Pharmaceutical Company.

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Apparatuses and reagents

Refrigerated centrifuge (Eppendorf, Germany), clean bench (Suzhou Purification Equipment Co., Ltd.), CO_2 incubator (SANYO, Japan), continuous wavelength microplate reader (Bio-RAD), electronic balance (Shanghai Precision Instrument Co., Ltd.), IFN-γ and IL-4 ELISA test kit (purchased from Nanjing Jiancheng Reagent Company), MTT (purchased from Sigma-Aldrich Corp), cyclophosphamide (purchased from Jiangsu Hengrui Medicine Co., Ltd.)

Cell lines and experimental animals

Cervical cancer cell lines (U14) was purchased from the Chinese Academy of Medical Sciences. Female Kunming mice weighing 18 ± 2 g were purchased from the Laboratory Animal Center of Dalian Medical University.

Preparation of Paris saponin extract

Referencing to the method of Zhu Li-li (Zhu., 2006), *Rhizoma Paridis* was soaked in a 10-fold amount of 70 % ethanol overnight, and extracted three times with heat reflux extraction at 88 ^{o}C for 4 hours, the filtrates were combined and ethanol was recovered, then they were dried *in vacuo* and Rhizoma Paridis total saponins were obtained.

***In vitro* experiment**

The intraperitoneally passaged ascites were extracted from mice under sterile conditions using MTT assay, dispersed into single cell suspension using culture solution, seeded in a 96-well plate with 100 µl each well, and cultured for 24 hours. Culture solution was discarded, and drug-containing culture medium was added (three replicate wells for each drug concentration). Meanwhile, blank control group which was only added with the complete culture solution was set up. After culturing in a 37 ^{o}C incubator with 5 % CO_2 and saturated humidity for 48 h and 72 h, each well was added with 20 µL of MTT solution, the culturing was continued for an additional 4 h, then the supernatant was discarded. 150 µL of DMSO was added to each well, and the plate was shaken in a micromixer for 10 min, after the crystalline particles were fully dissolved, absorbance value (A value) of each hole was measured at 490 nm, and the growth inhibition rate (IR) was calculated according to the following formula:Cell growth inhibition rate (%) = (1 - average A value of drug group / average A value of control group) × 100.

***In vivo* test (Li et al, 2009)**

The intraperitoneally passaged ascites were extracted from mice under sterile conditions, the concentration of collected cells was adjusted to 1×10^{6}/mL using sterile saline solution, and 0.2 ml was inoculated intraperitoneally into each mouse. 24 h after inoculation, the mice were weighed, grouped and administered; mice were randomly divided into 5 groups, with 10 mice in each group, they were negative control group, positive control group as well as Paris saponin high- and low-dose treatment groups respectively. Positive control group was administered by intraperitoneal injection of 20 mg/kg of cyclophosphamide according to body weight, negative control group was intragastrically administered normal saline, and Paris saponin high- and low-dose groups were intragastrically administered 100 mg/kg and 50 mg/kg of Paris saponin respectively according to body weight for 8 consecutive days. On the 9th day, mice were blood-sampled from eyeballs and sacrificed, ascites of each group were extracted, and the number of ascites tumor cells was counted.

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IFN-γ and IL-4 levels in serum of tumor-bearing mice

Blood was sampled from eyeballs of mice in experimental groups and control groups, and centrifuged at 3000 r/min for 20 min, serum was collected, and IFN-γ and IL-4 levels in serum of each group were measured according to kit instructions.

Effect of Paris saponin on survival time of tumor-bearing mice

Modeling and grouping method was as described above, after continuous administration for 15 days and drug withdrawal, survival time of mice in each group was recorded, and life prolongation rate was calculated according to the following formula.

Life prolongation rate (%) = [(mean survival time of mice in drug treatment group - mean survival time of mice in negative control group) / mean survival time of mice in negative control group] × 100.

Statistical analysis

The results obtained were analyzed using Origin 7.5 statistical software.

Results

The results for in-vitro inhibitory effect of Paris saponin on U14 cells are as follows. The results showed that different doses of Paris saponin (5μg/ml-80μg/ml) had inhibitory effect on U14 cells *in vitro*, in a concentration-dependent manner, inhibition rate increased with increasing of concentrations, at the same time, it also had certain time-dependency, which was significantly different ($p<0.05$) compared with the control group (Table 1).

Table 1: Inhibitory effect of Paris saponin on U14 cells

	Treatment time (h)	
	48	72
Concentration (μg/ml) Control group	Inhibition rate (%)	Inhibition rate (%)
5	15.12	7.48
10	24.75	31.41
20	57.65	67.15
40	67.47	74.55
80	71.22	76.35

The results for in-vivo anti-tumor effect of Paric saponin in U14 ascites tumor bearing mice are shown in Table 2. The results showed that, after intragastric administration of different doses of Paris saponin in tumor-bearing mice, numbers of ascites tumor cells in mice were all significantly reduced, which were significantly different compared with the negative control group, indicating that Paris saponin had an anti-tumor effect *in vivo*.

Table 2: Effect of Paris saponin on inhibition of in-vivo ascites tumor growth ($\bar{X}\pm S$)

Group	Drug concentration (mg/k	Number of animals Start	End	Number of tumor cells×10^8cell/ml	Inhibition rate (%)

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	g)				
Control group	Normal saline	10	10	8.10±0.42	
Cyclophosphamide	20	10	10	2.72±0.34**	66.4
Paridis total saponins	50	10	10	4.78±0.24**	41.0
	100	10	10	3.91±0.56**	51.7

Note: Comparison with the negative control group, ** P<0.01

The results for effect of Paris saponin on serum IFN-γ and IL-4 levels in tumor-bearing mice are as follows (Table 3).

Table 3: Effect of Paris saponin on serum IFN-γ and IL-4 levels in tumor-bearing mice ($\overline{X}$ ±S)

Group	Drug concentration mg/kg	IFN-γ pg/ml	IL-4 pg/ml
Control	Normal saline	81.57±7.16	13.84±2.74
Cyclophosphamide	20	76.11±8.45	14.94±2.96
Paris saponin	50	61.71±6.78**	19.87±2.77**
	100	47.36±7.15**	21.57±2.89**

Note: Comparison with the negative control group, ** *P*<0.01

The results show that, compared with the negative control group, serum IFN-γ level in mice of Paris saponin group significantly increased, while IL-4 level significantly decreased.

Effect on survival time of mice

Table 4: Effect of Paris saponin on survival time of tumor-bearing mice ($\overline{X}$ ±S)

Group	Drug concentration (mg/kg)	Number of animals	Survival time (Days)	Life prolongation rate (%)
Control	Normal saline	10	14.4±1.8	-
Cyclophosphamide	20	10	26.7±2.5**	85.4
Paris saponin	50	10	19.6±1.9*	36.1
Paris saponin	100	10	23.4±2.0**	62.5

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

Compared with the negative control group, survival times of mice in cyclophosphamide group and Paris saponin group were significantly prolonged; mean survival times in Paris saponin groups were 19.6±1.9 and 23.4±2.0 d, and life prolongation rates were 36.1 % and 62.5 % respectively.

Discussion

The experimental results show that the Paris saponin had significant inhibitory effect on U14 cells *in vitro*, while *in vivo*, it could also significantly inhibit number of tumor cells in tumor-bearing mice; in addition, serum IFN-γ level in mice was significantly increased, while IL-4 level was significantly reduced, thereby activating the immune cells to generate immune responses. It is probable that the tumor inhibition effect of Paris saponin is achieved via activation of immune factors in the body (Li., 2008).

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Many literatures have reported that the antitumor mechanism of Rhizoma Paridis may be through the inhibition of protein and DNA synthesis in tumor cells, inhibition of tumor cell mitosis, thereby inhibiting cell proliferation (Li., 2010), regulation of the body's immune (Bian et al, 2002) as well as induction of apoptosis and anti-angiogenesis (Lee et al, 2006; Shimizu et al, 2005; Hu et al, 2008). Rhizoma Paridis has complex chemical composition, and varied pharmacological effects, especially the tumor inhibition effect; clinical researches on active ingredients of Rhizoma Paridis should be carried out for the development and use of Rhizoma Paridis.

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