Exp Oncol 2009
31, 1, 27–32

Experimental ONCOLOGY

IN VITRO AND *IN VIVO* ANTICANCER ACTIVITY OF STEROID SAPONINS OF *PARIS POLYPHYLLA* VAR. *YUNNANENSIS*

*L.L. Yan[1, §], Y.J. Zhang[2, §], W.Y. Gao[1, *], S.L. Man[1], Y. Wang[1]*

[1]*School of Pharmaceutical Science and Technology, Tianjin University, Tianjin 300072, China*

[2]*School of Traditional Chinese Medicine, Tianjin University of Traditional Chinese Medicine, Tianjin 300193, China*

Aim: **To confirm the anticancer activity of steroid saponins isolated from the rhizome of *Paris polyphylla* var. *yunnanensis* and evaluate the structure-activity relationships of these steroid saponins *in vitro* and *in vivo*. *Materials and Methods:* Eight known steroid saponins were isolated from the rhizome of *Paris polyphylla*. The LA795 lung adenocarcinoma cell line from mice was chosen to evaluate cytotoxicity by means of MTT assay, and to study apoptosis by means of AnnexinV-FITC/PI flow cytometry. Diosgenin-3-α-L-arabinofuranosyl(1→4)-[α-L-rhamnopyranosyl(1→2)]-β-D-glycopyranoside (compound 1), the main steroid saponin of *Paris polyphylla*, and diosgenin (Dio), the aglycone of major steroid saponins, were evaluated for antitumor activity on LA795 lung adenocarcinoma in T739 inbred mice. *Results:* The steroid saponins showed remarkable cytotoxicity and caused typical apoptosis in a dose-dependent manner. They were evaluated *in vivo* by their effect on tumor developed in T739 inbred mice. The oral administration to T739 mice bearing LA795 lung adenocarcinoma of compound 1 and diosgenin significantly inhibited tumor growth, by 29.44% and 33.94%, respectively. HE staining showed that lungs and livers of treated mice underwent various levels of histopathological alterations. It was demonstrated by TUNEL assay that apoptosis rate in tumor cells was increased in comparison to cells in control mice. The 3-O-glycoside moiety and spirostanol structure played an important role in the anticancer activity of steroid saponins, and the number and the variety of glycosides of compounds strongly influenced on their anticancer activity. *Conclusion: Rhizoma Paridis* saponins showed anticancer activity against lung adenocarcinoma cell line, both *in vitro* and *in vivo*, and their effect was dependent on compounds' structure in a certain degree.**

Key Words: ***Paris polyphylla* var. *yunnanensis*, steroid saponins, diosgenin, anticancer activity.**

Paris polyphylla var. *yunnanensis* (Fr.) Hand-Mazz. is a perennial medicinal plant from the Euthyra Franch group in the *Trilliaceae* family, mostly distributed in the southwest of China. The rhizome of *Paris polyphylla* is used in traditional Chinese medicine as a haemostatic and antimicrobial agent [1, 2]. Recently, studies of the aqueous, ethanolic and methanolic extracts of *Paris polyphylla* showed their anticancer activity on several types of cancer cell lines. More extensive phytochemical and pharmacological studies further identified steroid saponins as the main antitumor active components [1, 3–6]. *Paris* saponins are a group of plant glycosides consisting of a steroid aglycone, to which one or more sugar chains are attached. They exhibit cell membrane–permeabilizing properties and, thus, have been investigated for their therapeutic potential [7]. Steroid saponins from *Paris polyphylla* are classified in two main groups: diosgenin (Dio) glycosides and pennogenin glycosides. Furthermore, D-glycopyranoside, L-rhamnopyranoside and L-arabinofuranoside are the main glycosides linked in the structure of steroid saponins [8]. However, the relationship between the anticancer activity and structure of the steroid saponins of *Paris polyphylla* had not yet been reported.

Received: November 11, 2008.

*Correspondance: E-mail: metayll@hotmail.com

§Authors contributed equally.

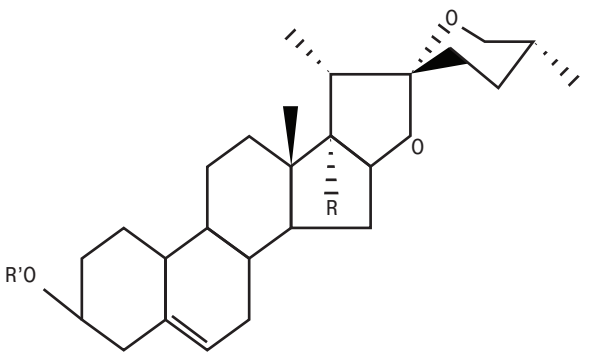
Abbreviations used: CPH – cyclophosphamide; Dio – diosgenin; HE – histological examination; MTT – Methylthiazolyldiphenyl-tetrazolium bromide; PHPLC – preparative high performance liquid chromatography; PTLC – preparative thin layer chromatography; TUNEL – terminal deoxytransferase nick end-labeling.

Preliminary screening of *Paris polyphylla* steroid saponins anti-proliferative activity revealed inhibitory effect of these compounds on cell growth of LA795 lung adenocarcinoma cell line. Eight known steroid saponins were isolated from the rhizome of *Paris polyphylla* [9, 10]. In our experiments, the anticancer activity of these steroid saponins of was investigated. Their cytotoxicity was evaluated by MTT assay, and apoptosis was studied by AnnexinV-FITC/PI staining. We also studied the antitumour effects of Diosgenin-3-α-L-arabinofuranosyl(1→4)-[α-L-rhamnopyranosyl(1→2)]-β-D-glycopyranoside (compound 1), the main steroid saponin from *Paris Polyphylla*, and diosgenin (Dio), the aglycone of compound 1, on inbred strains of laboratory mice (T739) that carried LA795 metastatic lung cancer. The relationships between anticancer activity and structure of *Paris polyphylla* saponins were discussed in this paper.

MATERIALS AND METHODS

Plant material and extracts preparation. The herb *Paris polyphylla* var. *yunnanensis* had been collected from Yunnan Province in the southwestern part of China, and identified by Dr. Gao. Voucher specimen (No.057902) was deposited in School of Pharmaceutical Science and Technology of Tianjin University. The rhizome of *Paris polyphylla* was dried at room temperature, and powdered, and extracted by organic solvents. After using column chromatography on Sephadex LH-20, PTLC and PHPLC, eight steroid saponins, titled compound 1 to 8, were obtained (Fig. 1) [9, 10]. The purity of compound 4 is > 75%,

and the purity of other seven compounds is > 90%. Diosgenin (the purity > 99.9%) was purchased from Sigma-Aldrich Chemie GmbH.



	R	R'
Compound 1	H	-α-L-arabinofuranosyl(1→4)-[α-L-rhamnopyranosyl(1→2)]-β-D-glycopyranoside
Compound 2	OH	-α-L-arabinofuranosyl(1→4)-[α-L-rhamnopyranosyl(1→2)]-β-D-glycopyranoside
Compound 3	H	-α-L-rhamnopyranosyl(1→4)-[α-L-rhamnopyranosyl(1→2)]-β-D-glycopyranoside
Compound 4	OH	-α-L-rhamnopyranosyl(1→4)-[α-L-rhamnopyranosyl(1→2)]-β-D-glycopyranoside
Compound 5	H	-α-L-arabinofuranosyl(1→4)-β-D-glycopyranoside
Compound 6	OH	-α-L-arabinofuranosyl(1→4)-β-D-glycopyranoside
Compound 7	H	-α-L-rhamnopyranosyl(1→4)-[α-L-rhamnopyranosyl(1→4)]-[α-L-rhamnopyranosyl(1→2)]-β-D-glycopyranoside
Compound 8	OH	
Diosgenin	H	H

Fig. 1. Structure of diosgenin and compound 1~8 [9, 10]

***Cell line and culture*.** The mice lung adenocarcinoma cell line (LA795) was purchased from Peking Union Medicial College (Beijing, China). The LA795 cells were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (100 μg/mL) at 37 °C in 5% CO_2. Cells were passaged every 3–4 days.

In vitro assay for cytotoxicity (MTT assay). LA795 cells (3×10^4 cells/mL) were plated on 96-well plates (200 μL of medium per well). After 24 h, cells were synchronized by adding serum-free media. After another 24 h, the cells were treated with various concentrations of DMSO solutions of studied compounds (concentration of DMSO < 0.1%). Each solution was added to eight wells. In another 24 h, 100 μL of 0.5 mg/mL MTT medium was added to each well for another 4 h. The supernatant fluid was then removed, 100 μL per well of DMSO was added, and samples were shaken for 15 min. Absorbance at 570 nm was measured with an enzyme-linked immunosorbent assay plate reader. This experiment was carried out in triplicate. Growth inhibition (GI) was calculated according to the formula: GI = 1 – (A_{570} of treated cells / A_{570} of control cells). IC_{50} values were calculated using a linear regression of dose-dependent curves plotted from at least six points.

Annexin V-FITC/PI staining for apoptosis evaluation. Apoptosis was quantified using flow cytometry to measure the levels of detectable phosphatidylserine on the outer membrane of apoptotic cells. LA795 cells were seeded on 6-well plates (3×10^4 cells/mL), and incubated with the compounds in 0.02% DMSO solutions. The concentration of each compound was equal to one third from respective IC_{50} value. After 24 h, cells were harvested by centrifugation, washed twice with PBS at 4 °C, and re-suspended in diluted binding buffer from the Annexin V-FITC kit at a concentration of 1×10^6 cells/mL. 100 μL of this suspension was added to a 5 mL Flow Cytometry (FC) tube. 5 μL of AnnexinV-FITC and 10 μL of 20 mg/mL propidium iodide (PI) were further added to the tube, followed by 15 min of incubation in the darkness. 400 mL of PBS were then added. Quantitative analysis of apoptotic cells level was performed using a Flow Cytometer (Coulter Epics Altra, Beckman Coulter Inc., USA). The procedure was carried out three times.

In vivo tumor growth inhibition. T739 inbred mice, both male and female, weighting 18.0 ± 2.0 g, were obtained from the Laboratory Animal Breeding and Research Center, Cancer Hospital, Academy of Medical Science (Beijing, China). The animals were kept under pathogen-free conditions, and fed with chow and sterile water *ad lib*. This animal study was approved by the Institutional Animal Care and Use Committee of China, and Institutional guidelines for animal welfare. LA795 cells (1×10^7/mL) were injected subcutaneously into the right armpits of the mice. Tumors grew to a volume of 0.5 to 1.0 cm^3. After sacrificing the mice, the tumor was excised, cut into small pieces (volume 0.5–1.0 mm^3) and dipped in 0.9% Sodium Chloride at 4 °C. After filtration and deliquation the pieces dispersed into cells. Those tumor cells were then injected into recipient mice (2×10^6/mL, 0.2 mL for each mouse). Thus, T739 mice (n = 40) bearing LA795 lung adenocarcinoma were obtained. Five days after injection of LA795 cells, forty T739 mice bearing LA795 lung adenocarcinoma were randomly divided into four groups (n = 10 per group): 1) control group (0.1 ml/10 g distilled water, p. o.), 2) cyclophosphamide (CPH) treated group (20 mg/kg, i. p.), 3) compound 1-treated group (100 mg/kg, p. o.), and 4) diosgenin-treated group (200 mg/kg., p. o.). Drugs were administered daily for 14 days, once a day.

At the first and the last days of the experiment, the body weight of each mouse was measured. The tumor volume was estimated every other day, on the basis of two-dimensional tumor measurements performed with a micrometer, and resorting to the formula suggested by Kato *et al.* (1994): Tumor vol. (mm^3) = $0.5A \times B^2$, where A is the longest diameter and B the shortest diameter of the tumor. The mice were sacrificed at the day 14, and autopsies were performed. Major organs such as lung, liver, kidneys, spleen, and tumor mass were then dissected and their weights were measured. The spleen index was calculated using the formula: spleen index = weight of spleen (mg) / body weight (g). The tumor inhibitory rate was calculated using the formula: tumor inhibitory rate (%) = 100% × (mean tumor weight in control mice minus mean tumor weight in treated mice) / mean tumor weight in control mice.

Histological examination. Dissected organs (lung, liver, kidneys) were fixed in 10% paraformaldehyde (pH 7.5) and then processed for paraffin embedding according to the standard histological procedures. 4 μm-thick sections were prepared and

stained with HE stain. Histopathological examination was completed using Olympus microscopy.

TUNEL assay. Apoptotic cells in the tumor sections were visualized by the terminal deoxyncleotidyl transferase mediated d-UTP nick and labeling (TUNEL) technique according to the manufacturer's instruction (Chemicon International, Inc, USA). Tissue sections were treated with 10 μg/mL proteinase K for 10 min at room temperature. The slides were immersed in a 2% H_2O_2 solution to block endogeneous peroxidase activity. TdT was used to catalyze the addition of biotin-conjugated d-UTP to the 3'-OH ends of DNA fragments. The incorporated biotin was detected by streptavidin conjugated to horseradish peroxidase. The staining was then performed using 3, 3'-diaminobenzidine tetrahydrochloride (DAB) as the chromagen and H_2O_2 as the substrate. Tissue sections were counterstained with hematoxylin and then mounted. Cells with deep-brown stained nuclei were counted as positive.

Statistical analysis. SPSS for Windows (SPSS Inc.) was used to analyze the data and plot curves. Data was expressed as the mean ± standard deviation (SD). The statistical significance of differences observed between each treatment group and the control was determined by the Student's *t*-test (two-tailed). Differences were considered statistically significant if $p < 0.05$.

RESULTS

Inhibition of LA795 cells growth in vitro. Nine compounds (compounds 1–8 and Diosgenin) were screened using MTT assay. These compounds showed remarkable cytotoxicity against LA795 cell in a dose-dependent manner (Fig. 2). Their median inhibitory concentration (IC_{50}) are listed in Table 1.

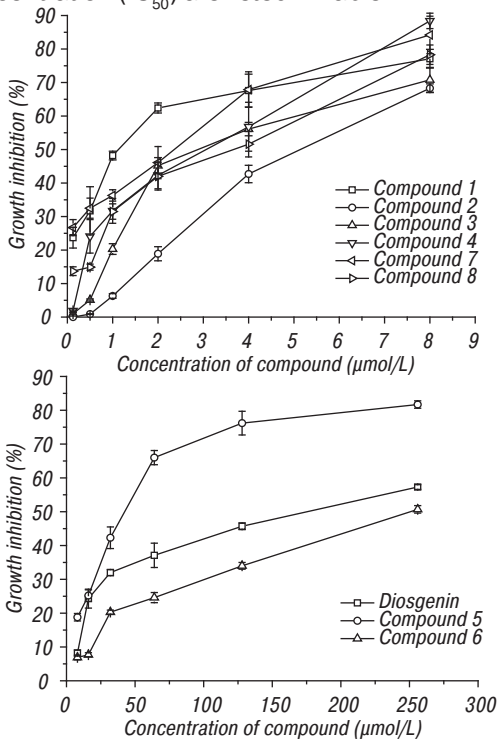


Fig. 2. Inhibitory effect of compound 1~8 and diosgenin on LA795 cell growth. LA795 cells were treated with compounds 1~8 or diosgenin at different concentrations for 24 h, and the growth inhibition was determined using the MTT assay

Table 1. IC_{50} of the compounds ($\bar{x}$ ± S, n = 3)

Compound	IC_{50} (μmol/L)
Compound 1	1.85 ± 0.11
Compound 2	5.14 ± 0.29
Compound 3	3.06 ± 0.33
Compound 4	2.26 ± 0.47
Compound 5	39.00 ± 3.63
Compound 6	319.53 ± 24.77
Compound 7	1.35 ± 0.19
Compound 8	2.71 ± 0.26
Diosgenin	149.75 ± 10.43

Annexin V-FITC assay of LA795 cells apoptosis. Many chemical compounds can inhibit the growth of tumor cells, but not all of them can trigger apoptosis [11]. To determine whether apoptosis was induced by the compounds mentioned above, we performed flow cytometric analysis with Annexin V-FITC conjugated to propidium iodide (PI). Annexin V-FITC-positive, PI-negative (Annexin V-FITC (+) PI (-)) cells were considered to be in an early apoptotic stage, while Annexin V-FITC-positive, PI-positive (Annexin V-FITC (+) PI (+)) cells were considered to be late apoptotic or necrotic. Apoptosis of LA795 cells was induced by compounds added at the concentration equal to one third from their IC_{50} values, although at different degree. The rates of apoptosis induced by *Paris polyphylla* compounds were significantly higher than apoptotic rate in the control group ($p < 0.01$) (Table 2).

Table 2. Flow cytometry analysis of LA795 cells after incubation with drugs for 24 h ($\bar{x}$ ± S, n = 3)

Compound	Dosage (μmol·L^{-1})	Annexin (+) PI (-) (%)	Annexin (+) PI (+) (%)
Control	–	1.1 ± 0.3	4.6 ± 0.7
Compound 1	0.62	12.9 ± 0.5*	36.3 ± 2.6*
Compound 2	1.71	5.4 ± 0.6*	20.0 ± 1.0*
Compound 3	1.02	6.8 ± 0.2*	32.3 ± 3.0*
Compound 4	0.75	8.6 ± 1.0*	34.9 ± 3.1*
Compound 5	13.00	3.7 ± 0.1*	13.5 ± 1.7*
Compound 6	106.51	1.3 ± 0.1	18.2 ± 0.4*
Compound 7	0.45	13.7 ± 0.3*	41.1 ± 2.4*
Compound 8	0.90	10.3 ± 0.9*	39.6 ± 1.9*
Diosgenin	49.92	3.4 ± 0.3*	11.8 ± 1.7*

Notes: Annexin (+) PI (-) (%): the percentage of cells in an early apoptotic stage; Annexin (+) PI (+) (%): the percentage of cells in a late apoptotic or necrotic stage. *$p < 0.01$ scores of drug-administered group *vs* control group.

Anti-tumor effects of the compounds in vivo. Although any reduction of the tumor volume was not observed between the drug treatment groups, tumor masses increased much slowly than in the control group during the period of drug administration (Fig. 3). Eventual tumor weight in all treated groups was obviously lower than in the control group ($p < 0.01$) (Table 3). That indicated CPH, compound 1 and Dio didn't make the solid tumors disappear, but slow down their growth.

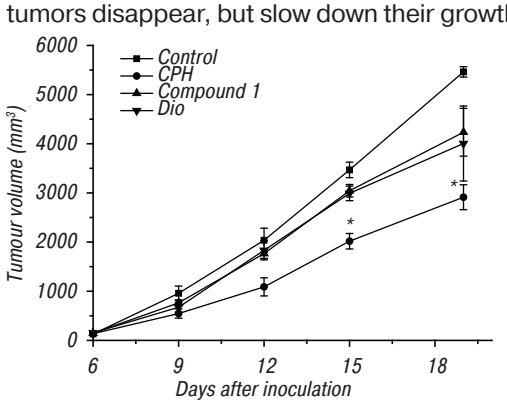


Fig. 3. Effect of drug treatment on tumor volume. CPH: Cyclophosphamide; Dio: Diosgenin. *$p < 0.01$ scores of drug-administered group *vs* control group.

Table 3. Inhibitory effects of drug treatment on LA795 transplanted tumors in T739 mice

Group	Dosage (mg/kg/d)	Tumour volume (mm^3)	Tumour weight (g)	Inhibition of growth of tumour (%)
Control	–	5462.5 ± 105.5	6.3 ± 0.6	
CPH	20	2912.7 ± 252.9*	2.8 ± 0.4*	56.09
Compound 1	100	4234.0 ± 486.4	4.4 ± 0.5*	29.44
Dio	200	4005.1 ± 762.4	4.2 ± 0.5*	33.94

Notes: CPH: Cyclophosphamide; Dio: Diosgenin. *$p < 0.01$ scores of drug-administered group *vs* control group.

Our original purpose of including the cyclophosphamide (CPH) group was to compare the anticancer activity of compound 1 with the CPH group, because CPH is generally used for treatment of various types of cancers. It is a clinically approved anticancer agent that works by slowing or stopping cell growth. Its use is becoming more common in anticancer experimental research, and the dosage (20 mg/kg) of the CPH treated group was referred to previous reports [12, 13].

The solid tumor in the control group grew well and the average tumor weight reached 6.3 g. Meanwhile, CPH exhibited significant anti-tumor activity. In the CPH group (20 mg/kg, i. p.), the average tumor weight was only 2.8 g and the inhibitory ratio was 56.09%. Compound 1 and Diosgenin also exerted a remarkable inhibitory effect on tumor growth. In the compound 1-treated group (100 mg/kg, p. o.) and the Diosgenin-treated group (200 mg/kg, p. o.), the tumor weights were significantly reduced ($p < 0.01$). The inhibitory ratio of tumor growth reached 29.44% and 33.94%, respectively (see Table 3).

The body weight of mice in the treatment groups appeared to be slightly decreased in comparison with the control group, but these results were not statistically-valid ($p > 0.05$) (Table 4). Spleen index in all three treatment groups (CPH, compound 1 and Diosgenin) was reduced compared with the control group, especially the spleen index of mice treated with compound 1.

Table 4. Effects of drug treatment on body weight and spleen index of T739 mice bearing LA795 lung adenocarcinoma

Group	Dosage (mg/kg/d)	Body weight (g)		Spleen index
		Before the treatment	After the treatment	
Control	–	23.7 ± 1.1	23.9 ± 1.0	6.8 ± 1.1
CPH	20	24.4 ± 1.3	23.6 ± 1.3	5.3 ± 0.4
Compound 1	100	24.0 ± 0.8	23.5 ± 1.1	4.5 ± 0.7*
Dio	200	25.0 ± 1.1	24.7 ± 1.1	6.0 ± 0.8

Notes: CPH: Cyclophosphamide; Dio: Diosgenin. *$p < 0.05$ scores of drug-administered group *vs* control group.

Effects of compounds on lung, liver, kidneys, and tumor mass in mice. In order to investigate the toxicity of the treated compounds, lung, liver and kidney sections were prepared and stained by HE. Lesions of various degree were found in the lungs and livers of treatment groups. The kidney microstructure was normal. For the compound 1 group, lungs were seriously damaged with a large area of hemorrhagic infarction (Fig. 4, *a*). For other groups (CPH and Diosgenin), there were congestion and inflammatory infiltration of lungs. Also, the liver lesions in mice treated with compound 1 were the most severe among all three treatment groups. As shown on Fig. 4, *b* and 4, *c*, the normal structure of the hepatic lobules was destroyed, and hydropic degeneration, inflammatory infiltration and fatty degeneration were observed. For other treatment groups, slight hydropic degeneration and inflammatory infiltration was found.

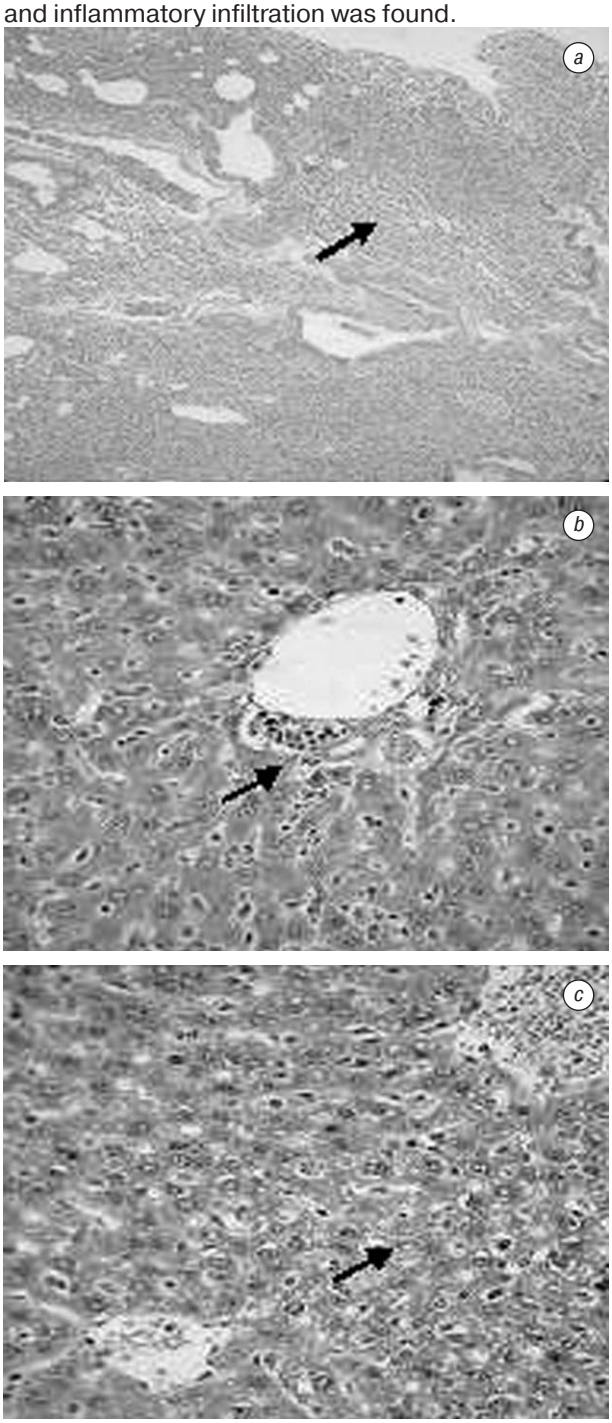


Fig. 4. Pathological changes in lungs and livers of T739 mice bearing LA795 lung adenocarcinoma upon compound 1 treatment. *a*, lung tissue, showing hemorrhagic infarction, 100 ×. *b*, liver tissue, showing inflammatory infiltration, 400 ×. *c*, liver tissue, showing fatty degeneration, 400 ×

TUNEL assay. TUNEL assay of tumor sections was performed to examine the ability of compound 1 to induce cell apoptosis. The apoptosis index is presented on Fig. 5. Compared with the control group, the number of apoptosis cells increased significantly for all three treatment groups. The apoptosis index of the compound 1 group was 6-fold higher than that of the control group, and was almost on the same level as shown for the CPH group.

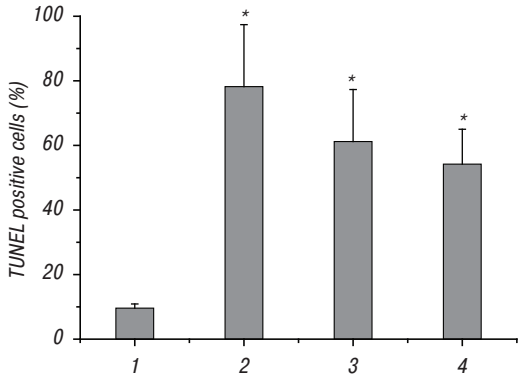


Fig. 5. The number of apoptotic cells in tumor sections were detected by TUNEL assay. Apoptotic cells in tumors increased significantly in drug-treated groups. CPH: Cyclophosphamide; Dio: Diosgenin. *$p < 0.01$ scores of drug-administered group *vs* control group.

DISCUSSION

Currently chemotherapy is regarded as one of the most efficient cancer treatment aproach. Although chemotherapy significantly improves symptoms and the quality of life of patients with lung cancer, only modest increase in survival rate can be achieved. Faced with palliative care, many cancer patients use alternative medicines, including herbal therapies. Among these therapies, traditional Chinese medicine is probably the best established and codified, dating back several thousand years. Traditionally, Chinese herbs are used in combinations of single herbs, or prescriptions — called formulas. Specific herbal extracts, and combinations, have been designed to treat specific diseases including cancer [14–16].

Paris polyphylla var. *yunnanensis* has been traditionally used for centuries. Some medical formula, such as Chonglou-Tang, which includes this Chinese herb as a main ingredient, have been used to treat cancer. Recent studies pointed to steroid saponins as the main anti-tumor components of this herb. Previous studies pointed that *Paris polyphylla* var. *yunnanensis* contained significant amounts of steroid saponins, including polyphyllin D, diosgeninscin, gracillin, trillin, methylmotogracillin, and so on [5, 6, 17–21].

In the experiments *in vitro*, as shown in MTT results, diosgenin saponins had stronger cytotoxicity than pennogenin saponins. These compounds had remarkable cytotoxicity against LA795 cell, and eight steroid saponins (except compound 6) had higher inhibition of cell growth compared with diosgenin.

Compound 7 with four glycosides had shown the highest rate of cell growth inhibition among other studied steroid saponins, while compounds 5 and 6, containing two glycosides, exhibited the lowest inhibition rate both among diosgenin and pennogenin saponins. This data indicates that the number of glycosides correlate with the cytotoxicity of steroid saponins. However, IC_{50} of compound 4 was lower than that of compound 3 and compound 8. This phenomenon could be explained by the fact that the purity of compound 4 was approximately 75%, and the purity of other seven compounds was more than 90%.

Compared with compound 3 and compound 2, compound 1 and 4 showed higher cytotoxicity rate, respectively. Although the number of glycosides in compounds was the same, the varieties of glycosides were different. Besides glycopyranoside and rhamnopyranoside, their main difference was that compounds 1 and compound 2 contain arabinofuranosides, and that compound 3 and 4 contain rhamnopyranosides. When there was used aglycone of diosgenin, the activity of rhamnopyranoside was stronger than of arabinofuranoside-containing compound. But when there was aglycone of pennogenin, the effect was opposite. This indicated that the variety of glycosides of steroid saponins also could affect cytotoxicity.

Altogether, the 3-O-glycoside moiety and spirostanol structure were found to be essential for the proapoptotic activity with regard to anticancer structural relationship of steroid saponins. Their cytotoxic activities were strongly influenced by the number and the variety of steroid saponins.

Compound 1 was the main steroid saponin component in *Paris polyphylla*, and the contents of other seven compounds were very few. So compound 1 and diosgenin (Dio), the aglycone of compound 1 were selected for *in vivo* experiment. And we hope that the anti-tumor activities *in vivo* of other compounds could be estimated through the results of compound 1. In *in vivo* experiment, the molar dose of compound 1 was set to one-quarter of diosgenin, because of the high toxicity of compound 1 for mice. The results showed that compound 1 was as effective as diosgenin with the 4-fold molar dose in inhibition of tumor growth. This further indicated that glycosides of steroid saponins promote the anticancer activity of diosgenin. From the results of tissue sections HE staining, it is seen that compound 1 caused stronger lung and liver damage than diosgenin. Probably the 3-O-glycoside structure may contribute to the toxicity of steroid saponins.

Treatment of tumors is directed not only on inhibition of cell proliferation, but also on induction of apoptosis of tumor cells. More and more attention is paid to the ability of drugs to induce apoptosis in the process of evaluation of anti-tumor agents' effectiveness. That is why we tested the ability of eight steroid saponins extracted from *Paris polyphylla* to induce apoptosis by means of AnnexinV-FITC/PI flow cytometry approach. The rates of apoptosis in the treatment groups were significantly higher than that in the control group, which was shown by AnnexinV-FITC/PI flow cytometry. Additionally, in TUNEL assay of tumor sections, the number of apoptotic cells in tumors treated with compound 1 was increased too. This data suggest that one of anti-tumor mechanisms of steroid saponins is an induction of tumor cell apoptosis. The main biologic activity ascribed to saponins is their membrane permeabilizing property. The main actions are considered changes in membrane permeability and pore formation [22–24]. In apoptotic death, an increase of mitochondrial membrane permeability is considered to be one of the key events. A sudden increase in permeability of the inner mitochondrial

membrane, the so-called mitochondrial permeability transition, is a common feature of apoptosis and is mediated by the mitochondrial permeability transition pore (mtPTP) [25–27]. So the detailed mechanism of the eight steroid saponins extracted requires future study.

In conclusion, steroid saponins of *Paris polyphylla* var. *yunnanensis* could be regarded as promising drugs for cancer therapy, but the mechanisms of their anti-cancer activity and their toxicity should be further addressed. In our studies, we demonstrated that the structure of spirostanol 3-O-glycoside and the number of glycosides were two main factors responsible for compounds' cytotoxicity. Also, we plan to study further the saponins structure-function relationships, and perform structure modifications to design new compounds with strong anti-tumor activity and low toxicity.

ACKNOWLEDGEMENTS

This work was supported by a grant 07JCZD-JC05400 from Tianjin Municipal Science and Technology Commission (Tianjin, China). We also wish to thank Traditional Chinese Medicine Department at Tianjin University of Traditional Chinese Medicine for the use of their equipment.

REFERENCES

1. **Song L, Hong X, Ding Z.** Paris plants. In: Modern Grand Dictionary of Chinese Materia Medica. Beijing: People's Sanitation Press, 2001: 1619–22.

2. **Shanshan W, Wenyuan G, Hongquan D.** Advances in studies on chemical constituents and pharmacological activities of *Rhizoma Paridis*. Chin Trad Herb Drugs 2004; **35**: 344–7 (In Chinese).

3. **Ravikumar PR, Hammesfahr P, Charles J.** Cytotoxic saponins from the Chinese herbal drug Yunnan Bai Yao. J Pharm Sci 1979; **68**: 900–3.

4. **Shi XF, Du DJ, Xie DC.** Effect of steroidal saponins in Rhizoma Paridis on H_{22} animal transplant tumor. J Chin Med Mater 1992; **15**: 33–6.

5. **Wang Y, Zhang Y, Zhu Z, *et al.*** Exploration of the correlation between the structure, hemolytic activity, and cytotoxicity of steroid saponins. Bioorg Med Chem 2007; **15**: 2528–32.

6. **Zhang LT, Zuo YT, Gao WY, *et al.*** Anti-tumor effects of extracts and chemical constituents from *Paris polyphylla* var *yunnanensis*. Chin Trad Herb Drugs 2007; **38**: 422–4 (In Chinese).

7. **Bachran C, Sutherland M, Heisler I, *et al.*** The saponin-mediated enhanced uptake of targeted saporin-based drugs is strongly dependent on the saponin structure. Exp Biol Med 2006; **231**: 412–20.

8. **Tang HF, Zhao YP.** Research survey of plants of Paris L. Chin Trad Herb Drugs 1998; **12**: 839–42 (In Chinese).

9. **Wang Y, Gao WY, Yuan LC, *et al.*** Chemical constituents from rhizome of Paris polyphylla var yunnanensis. Chin Trad Herb Drugs 2007; **38**: 17–20 (In Chinese).

10. **Wang Y, Zhang YJ, Gao WY, *et al.*** Anti-tumor constituents from Paris polyphylla var yunnanensis. Chin J Chin Mater Med 2007; **32**: 1425–8 (In Chinese).

11. **Cozzi P.** The discovery of a new potential anticancer drug: a case history. Farmaco 2003; **58**: 213–20

12. **Zhenhua W, Qiusheng Z, Ke L, *et al.*** Ginsenoside Rh2 Enhances Antitumour Activity and Decreases Genotoxic Effect of Cyclophosphamide. Basic & Clinical Pharmacology & Toxicology 2006; **98**: 411–5.

13. **Ji-fang W, Rui-bin S, Ning W, *et al.*** Inhibitory effect of agmatine on proliferation of tumor cells by modulation of polyamine metabolism. Acta Pharmacologica Sinica 2005; **26**: 616–22.

14. **Douer D, Tallman MS.** Arsenic trioxide: new clinical experience with an old medication in hematologic malignancies. J Clin Oncol 2005; **23**: 2396–410.

15. **Hu J, Jin W, Yang PM.** Reversal of resistance to adriamycin in human breast cancer cell line MCF-7/ADM by beta-elemene. Chin J Oncol 2004; **26**: 268–70.

16. **Yin XL, Zhou JB, Jie CF, *et al.*** Anticancer activity and mechanism of *Scutellaria barbata* extract on human lung cancer cell line A549. Life Sci 2004; **75**: 2233–44.

17. **Cai J, Liu M, Wang Z, Ju Y.** Apoptosis induced by dioscin in Hela cells. Biol Pharm Bull 2002; **25**: 193–6.

18. **Cheung JY, Ong RC, Suen YK, *et al.*** Polyphyllin D is a potent apoptosis inducer in drug resistant HepG2 cells. Cancer Lett 2005; **217**: 203–11.

19. **Jin WD, Chen XP, Cai HJ.** *In vitro* cytotoxicity of Paridis-extract on Hep G2 cells. Acta Med Univ Sci Technol Huazhong 2006; **35**: 103–6.

20. **Lee MS, Yuet-Wa JC, Kong SK, *et al.*** Effects of polyphyllin D, a steroidal saponin in *Paris polyphylla*, in growth inhibition of human breast cancer cells and in xenograft. Cancer Biol Ther 2005; **4**: 1248–54.

21. **Gao D, Gao YL, Bai P.** Influence of *Rhizoma Paridis* on calcium messenger system in Hela cells. J Fujian College TCM 2003; **13**: 26-8 (In Chinese).

22. **Melzig MF, Bader G, Loose R.** Investigations of the mechanism of membrane activity of selected triterpenoid saponins. Planta Med 2001; **67**: 43–8.

23. **Menin L, Panchichkina M, Keriel C.** Macrocompartmentation of total creatine in cardiomyocytes revisited. Mol Cell Biochem 2001; **220**: 149–59.

24. **Plock A, Sokolowska-Kohler W, Presber W.** Application of flow cytometry and microscopical methods to characterize the effect of herbal drugs on Leishmania Spp. Exp Parasitol 2001; **97**: 141–53.

25. **Yoshihide T, Takashi N, Shigeomi S.** Mitochondrial membrane permeability transition and cell death. Biochim Biophys Acta 2006; **1757**: 1297–300.

26. **Jason EK, Katrina GW, Shawn EL, *et al.*** The ADP/ATP translocator is not essential for the mitochondrial permeability transition pore. Nature 2004; **427**: 461–5.

27. **Seeman P, Cheng D, Iles GH.** Structure of membrane holes in osmotic and saponin hemolysis. J Cell Biol 1973; **56**: 519–27.