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Pulsatilla Saponin D: the Antitumor Principle from *Pulsatilla koreana*

Yong Kim, Seong-Cheol Bang, Ji-Hyun Lee, and Byung-Zun Ahn

SB Pharmaceutical Co. LTD, 533-2 Bonggok-ri, Banpo-myeon, Gongju-shi 314-923, Chungnam, Korea

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By bioassay-guided separation, an already known saponin, *Pulsatilla* saponin D was isolated from the root of *Pulsatilla koreana* Nakai as a antitumor component when evaluated by *in vivo* antitumor activity as well as *in vitro* cytotoxic activity test. It showed potent inhibition rate of tumor growth (IR, 82 %) at the dose of 6.4 mg/kg on the BDF1 mice bearing LLC cells.

Key words: *Pulsatilla* saponin D, Antitumor activity

INTRODUCTION

The *Pulsatilla* genus, *Pulsatilla koreana* Nakai (Ranuculaceae) in Korea is an important herb in traditional medicine that has been used to treat amoebic dysentery and malaria (Bae, 1999). It had been reported that plants from the *Pulsatilla* genus contain ranunculin, anemonin, protoanemonin, triterpenes, and saponins (9 %), mainly the oleanane and lupane-type saponins. However, the pharmacological effects of these ingredients have not been studied extensively but protoanemonin was previously reported to exhibit antifungal and antibiotic properties (Martin *et al.*, 1990; Campbell *et al.*, 1979). Li, *et al*. also reported that ranunculin has cytotoxicity against KB cells by inhibiting DNA polymerase (Li *et al*., 1993). Recently, deoxypodophyllotoxin, which was isolated from the roots of *P. koreana*, was found to inhibit the tube-like formation of HUVECs (human umbilical venous endothelial cells) and have a potent antitumor effect (Kim *et al*., 2002).

Previously, it was reported that the anticancer preparation called SB31® (Kim *et al.*, 1994), which was produced from the roots of *Pulsatilla koreana*, the roots of *Panax ginseng*, and the rhizome of *Glycyrrhiza glabra*, exhibited potent antitumor activity on nude mice bearing human tumor cell lines. Therefore, its major ingredients, the roots of *P. koreana*, might be an important component that is responsible for its potent antitumor activity (Kim *et al.*, 2004).

In this study, the active ingredients were isolated from the roots of *P. koreana* by activity-guided fractionation and their antitumor activity on BDF-1 mice bearing Lewis lung carcinoma (LLC) cells as well as its cytotoxic activity against some cancer cell lines were evaluated.

MATERIALS AND METHODS

Plant materials

The roots of *P. koreana* Nakai were collected in April, 2000 at Keryong mountain near Daejeon city, Korea. This plant was identified by Professor Ki-Hwan Bae in the College of Pharmacy, Chungnam National University. A voucher specimen was deposited at the R&D center, SB Pharmaceutical Co. LTD., Korea.

Extraction and isolation

The powdered roots of *P. koreana* (50 g) were extracted three times with 50 % aqueous EtOH (500 mL), and the resulting extracts were combined and concentrated *in vacuo* to yield a light brown residue (22 g). The residue was suspended in acetone (300 mL), and successively centrifuged (3,000 rpm), and the resulting supernatant was removed to give a brown precipitate. The precipitate was poured into water (100 mL) and filtered to remove the insoluble part. The filtrate was concentrated to give a brown mass (17.8 g, WT fraction). The WT fraction (560 mg) was chromatographed over a Sephadex LH-20 column (200 g, 60×4 cm) with MeOH-H_2O (80:20) to give 4 fractions, SPX1 (139 mg, 24.8 %), SPX2 (344 mg, 61.4 %), SPX3 (61 mg, 10.9%), and SPX4 (15.7 mg, 2.8%).

Correspondence to: SB Pharmaceutical Co. LTD, 533-2 Bonggok-ri, Banpo-myeon, Gongju-shi 314-923, Chungnam, Korea
Tel: 82-41-857-3686, Fax: 82-41-857-7854
E-mail: ahnbj@cnu.ac.kr

The eluents were detected by TLC (BuOH-AcOH-H_2O = 4:1:1, Si gel, 0.25 mm). After heating, the chromatogram was sprayed with 10 % H_2SO_4. The third fraction, which exhibited the most potent activity, was again chromatographed by HPLC (solid phase; RP-C_{18}, 250×10 mm, mobile phase; MeOH-H_2O (80:20) as the mobile phase, 210 nm, 1 mL/min) to give major three fractions. Among them, the third fraction, which exhibited the most potent activity, was purified by HPLC to give *Pulsatilla* saponin D (2.8 mg).

Cell lines and cell culture

The four cancer cell lines used in this study, A-549, SK-MEL-2, MCF-7, and Lewis lung carcinoma (LLC) cells were obtained from the Korea Research Institute of Bioscience and Biotechnology (KRIBB). The cell lines were maintained as a monolayer in RPMI1640 media supplemented with 10% fetal bovine serum (GIBCO, Grand Island, NY), sodium bicarbonate, penicillin G, and streptomycin at 37 °C under a humidified atmosphere of 5% CO_2.

In vitro cytotoxic assay

The level of cytotoxicity was measured using the sulforhodamine B (SRB) method (Skehan *et al.*, 1990). Viable cells were seeded in the growth medium (180 μL) into 96 well microtiter plates (3-4×10^4 cells per each well) and allowed to attach overnight. The test sample (*Pulsatilla* saponin D) was dissolved in DMSO and adjusted to a final sample concentration ranging from 0.3 ng/mL to 10 μg/mL by a dilution with the growth medium. Each sample was prepared in triplicate. The final DMSO concentration was then adjusted to <0.1%.

After 72 h incubation, the medium was removed and the remaining cells were fixed with 10% trichloroacetic acid (TCA) for 1 h at 4°C. The TCA-treated cells were washed extensively with water and dried in air. Subsequently, 50 μL of the SRB solution (0.4% in AcOH) was added to each well at room temperature. After standing for 1 h, the wells were washed 3-4 times with 1 % AcOH and dried in air. The bound dye was dissolved in Tris base (100 μL of 10 mM). The absorbance of the Tris solution was measured using a micro-plate reader at 520 nm. The ED_{50} value was defined as the *Pulsatilla* saponin D concentration needed to reduce the absorbance relative to the vehicle-treated controls by 50%.

In vivo anti-tumor activity assay

The antitumor activity was measured according to Teruhiros method (Teruhiro *et al.*, 1996). 2×2×2 mm^3 tumor fragments of a Lewis lung carcinoma (LLC) were transplanted *s.c.* into the auxiliary region of the BDF1 mice.

Twenty-four hours after tumor transplantation, the mice were divided into several groups consisting of 5 mice each. The samples, WT, SPX fractions, and *Pulsatilla* saponin D were then dissolved in saline, and injected intraperitoneally on the 1st to 7th and 9th to 14th day at a dose of 280 mg/kg of WT, 70 mg/kg of SPX1, 171 mg/kg of SPX2, 30.5 mg/kg of SPX3, 8.1 mg/kg of SPX4, and 6.4 mg/kg of *Pulsatilla* saponin D, respectively.

The experimental mice were weighed twice a week in order to evaluate the toxicity of *Pulsatilla* saponin D. The tumor volume (TV) was measured on the 15th and 16th day, and calculated according to the following formula:

$$TV = L\ (mm) \times W^2\ (mm^2) / 2$$

where L and W represent the length and the width of the tumor mass, respectively.

The inhibition ratio (IR) was calculated according to the following formula:

$$IR\ (\%) = [(\text{Mean TV of control group} - \text{Mean TV of treated group}) / \text{Mean TV of control group}] \times 100$$

RESULTS AND DISCUSSION

The 50% aqueous EtOH fraction (WT fraction) of the roots of *P. koreana* was evaluated in a BDF1/LLC animal model. The WT fraction was injected at the maximum tolerated dose of 280 mg/kg/day (Table I) and Adriamycin, which was used as the positive control, was administered at 0.5 mg/kg/day to the right groin of the BDF1 mice bearing the LLC cells. The results, which are expressed as the inhibition ratio (IR, %), are summarized in Table 1. The WT fraction exhibited significant antitumor activity with an IR of 56 % (day 14) and 55 % (day 15), although it was less than that of adriamycin (60% and 64%).

The WT fraction was further fractionated by Sephadex (LH-20) chromatography to give four fractions. The fractions

Table I. Antitumor Activity of the Sephadex LH-20 fractions on the BDF1 mice bearing the LLC cells

Treatments (Dose)[b]	Inhibition ratio (%)[c]	
	14 day[a]	15 day
WT (280)	56	55
SPX (170)	10	12
SPX2 (171)	25	30
SPX3 (30.5)	57	60
SPX4 (8.1)	8	10
Adriamycin	60	64

[a] Days after tumor transplantation.
[b] Numbers in parenthesis represents the maximum tolerated doses in mg/kg/day.
[c] Determined as described in Materials and Methods. LLC; Lewis Lung Carcinoma.

were tested for their antitumor activity using the same animal tumor model. Each fraction was injected at the maximum tolerated dose (Table I). The IR values are summarized in Table I. Of the fractions, the 3rd fraction (SPX3) exhibited the most potent antitumor activity with IR values of 57% (day 14) and 60% (day 15), while the activity of the other fractions was negligible. A chromatographic purification of the SPX3 fraction by semi-preparative HPLC provided a pure compound **1**, which exhibited the most potent activity (IR, 82%).

The FABMS of compound **1** showed a quasi-molecular ion at m/z 935 $[M+Na]^+$, which is consistent with a quasi-molecular formula of $C_{47}H_{76}O_{17}$. Acid-hydrolysis of compound **1** produced hederagenin, arabinose, rhamnose and glucose. According to its ^{1}H-NMR there were three anomeric protons at δ 5.11 (β-glucose, H-1‴, d, *J* = 7.8 Hz), 6.25 (α-rhamnose, H-1″, s, br) and 4.97 (α-arabinose, H-1′, d, *J* = 6.66 Hz). The corresponding anomeric carbons appeared at δ 106.7 (glycopyranosyl, C-1‴), 104.2 (arabinopyranosyl, C-1′) and 101.7 (rhamnopyranosyl, C-1″). The C-3 of aglycon was observed in a lower field (d 81.0) than that of authentic hederagenin (δ 73.7, Ye *et al*., 1995), corresponding to a glycosylation shift of 7.3 ppm. The chemical shift of C-28 was shown at δ 180.0, which is coincident with that of a hederagenin carboxylic carbon. From these NMR observations, it was concluded that C-3 was linked with the trisaccharide, while the carboxylic group at C-28 was not esterified. When comparing the spectral data of compound **1** with those of the literature values (Kang, 1989; Viqar, 2000; Yoshihiro *et al*., 1999), compound **1** was found to be *Pulsatilla* saponin D [hederagenin 3-*O*-α-L-rhamnopyranosyl(1→2)-[β-D-glucopyranosyl(1→4)]-α-L-arabinopyranoside].

The *Pulsatilla* saponin D (**1**) was further evaluated for their antitumor activity in the same animal tumor model, and for their cytotoxic activity against three cancer cell lines (A-549, human lung cancer; SK-MEL-2, human melanoma, MCF-7, human breast cancer). The results are summarized in Table II. The *Pulsatilla* saponin D showed moderate cytotoxic activity (ED_{50}, 6.3 μg/mL to >10 μg/mL) against the cancer cell lines. Considering its

Table II. Cytotoxicity of *Pulsatilla* saponin D against the cancer cell lines[a], and its antitumor activity on the BDF1 mice bearing LLC cells

Compound	Cytotoxicity (ED_{50}, μg/mL)[b]			Dose (mg/kg/day)	Antitumor activity (IR, %)
	A-549	SK-MEL-2	MCF-7		
Pulsatilla saponin D	6.3	>10	>10	6.4	82
Adriamycin	0.017	0.094	NT	0.5	60

[a] Cell lines: A-549; human lung cancer, SK-MEL-2; human melanoma, MCF-7; human breast cancer.

[b] The sample concentration produces a 50% reduction in cell growth. LLC; Lewis Lung Carcinoma.

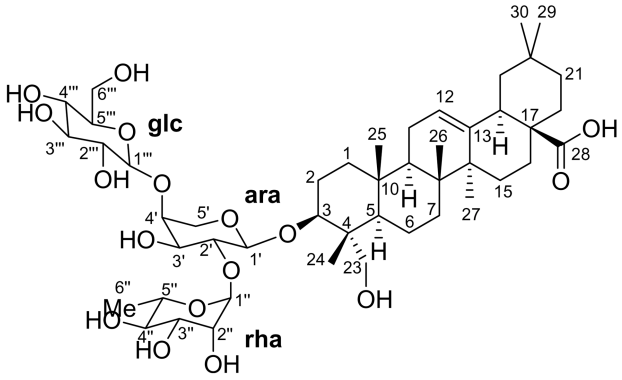

Fig. 1. The structure of *Pulsatilla* saponin D (1, hederagenin 3-*O*-α-L-rhamnopyranosyl(1→2)-[β-D-glucopyranosyl(1→4)]-α-L-arabinopyranoside)

low cytotoxic activity, it is remarkable that the *Pulsatilla* saponin D has such a potent antitumor effect (IR, 82 %) on a solid tumor, which is higher than Adriamycin (IR, 64%).

In conclusion, the *Pulsatilla* saponin D was found to be a main antitumor component of the roots of *P. koreana*, and therefore the most active component of SB31®.

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