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Anti-tumor Activity of *Saussurea laniceps* against Pancreas Adenocarcinoma

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Abstract – The purpose of this study was to confirm the anti-tumor activity of an ethanol extract of *Saussurea laniceps* against pancreatic tumor and to isolate the active compound from *S. laniceps* extract. Treatment with *S. laniceps* extract and hispidulin inhibited proliferation of pancreatic cell lines, such as Capan-1, Capan-2, Panc-1 and S2-013 in a dose-dependent manner using the hollow fiber assay. Hispidulin showed typical hallmarks of apoptotic cell death a significant anti-tumor activity on Capan-2 cells at a dose of 100 mg/kg and 200 mg/kg. *S. laniceps* has potential cytotoxic and apoptotic effects on human pancreatic carcinoma cells. Its mechanism of action might be associated with the apoptotic cell death through DNA fragmentation.
Keywords – *Saussurea laniceps*, Pancreatic carcinoma, Hispidulin, Hollow fiber assay

Introduction

Herbal plant-derived compounds have played an important role in the development of over 60% of clinically useful anti-tumor agents. Lots of natural products are in clinical and pre-clinical stage, even though most products and analogues failed testing during clinical stages. Many researchers are still screening anti-tumor compounds from various herbal plants. We wanted to screen the antitumor activity of unique herbal plants from alpine district, starting from the hypothesis that more extreme environmental conditions resulted in stronger biological activity of these herbal plants. The genus *Saussurea* (Compositae) contains more than 300 species distributed in north-temperate zone of the world, of which over 200 species are found in mainland of cold countries such as Mongolia and China.[1] *S. laniceps* is one origin of "snow lotus flower", a well-known Tibetan medicine for the treatment of rheumatic arthritis and menstrual disorders.[2] From this herb, cynaropicrin, acacetin, coumarin, syringin, benzyl glucopyranoside rutin, hispidulin and apigenin have been known as biological compounds.[1,3] Resent researches showed that this herb has anti-inflammatory and analgesic, anti-metastasis and anti-cancer activities *in vitro*.[5-6] We have been carried out *in vivo* study to screen anti-tumor activity using a hollow fiber assay for developing lead compounds of the pancreatic cancer drug, because pancreatic cancer is quite advanced, complete surgical removal is not possible and there is no optimum chemotherapy. The hollow fiber assay, which has a high correlation with xenograft assay, is used by the National Cancer Institute (NCI) of the National Institute of Health (NIH) for screening compounds as anti-cancer agents.[8] In this study, we examined the anti-cancer effects of ethanol extracts from *S. laniceps* and further elucidated the molecular mechanism of action in pancreas tumor cell lines.

Experimental

Sample extraction and isolation – *S. laniceps* collected in Mongolia was used in this study. 1 kg of air-dried herbs were kept in 8,000 mL of 95% ethanol for 48 h and then filtered. The ethanol was evaporated with a rotary vacuum evaporator and freeze-dried to give a powder (yield 47 g). About 74 g of the crude extract was dissolved in a minimum amount of warm dichloromethane and chromatographed over silica gel (80 - 100 mesh) eluting successively with petroleum ether/ethyl acetate gradient mixtures (from 1:0 to 1:1). The eluate was collected in 50 mL portions and combined upon monitoring by TLC (Silica gel plates F254) using developing solvents mixtures (chloroform-methanol (75:5, v/v)), to a total of 10 fractions (A–J). 18 mg of cynaropicrin was obtained from Fraction

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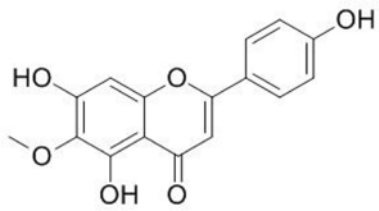

Fig. 1. Structure of hispidulin.

A, 5 mg of 3α, 8α-dihydroxy-11βH-11,13-dihydrodehydrocostuslactone, 2 mg of mokko lactone, 2 mg of 3α-hydroxy-11βH-11,13-dihydrodehydrocostuslactone 8-*O*-β-*D*-glucoside from Fraction B and C, 40 mg of acacetin, 1.2 mg of coumarin and 84 mg of hispidulin (Fig. 1) from Fraction F, G and H, respectively. All isolated compounds were characterized by comparison of the physical and spectral data with the literature.[3] The origin of *S. laniceps* was identified by Prof. Ki-Hyeong Lee, Kongju National University, and voucher specimens were deposited in Kongju University, Korea.

Cell lines – Four human pancreatic tumor cell lines used in this study were Capan-1, Capan-2, Panc-1 and S2-013 that were maintained in a minimum essential medium (MEM), supplemented with 10% fetal bovine serum (FBS, JRH Bioscience).

In Vitro Anti-tumor activity – Anti-tumor activity was quantified by CCK-8 assay. Briefly, the cells were plated in 96-well culture plates at a density of 1×10^5 cells/mL and allowed to adhere at 37 ºC for 24 h. Then cells were exposed to various doses of *S. laniceps* ethanol extract (SLE) and fractions. After 48 hours of exposure, 10 µl of CCK-8 solution for forming the formazan was added to each well of the plate. Then after incubation of 1 h at 37 ºC, the absorbance at 450 nm was determined using an ELISA plate reader (Tecan, Switzerland).[8] Percent growth inhibition of cells exposed to treatments was calculated as follows; % Inhibition = 100 – (OD of treatment / OD of control) × 100.

Animals – Five weeks old BALB/C nu/nu mice (male), purchased from Japan SLC, Inc., were acclimatized under controlled standard conditions (temperature of 23 ± 2 ºC, relative humidity of 50 ± 5% and illumination cycle of 12/12 h light/darkness, respectively), and housed in polycarbonate cages for a week prior to the experiment. Mice were maintained according to accredited procedures in our facility, and fed irradiated Orient Bio (Korea) chow and UV sterilized water *ad libitum*. This animal study was approved by the local ethics committee of the Department of Biology Kongju National University on the human use of animals for scientific research.

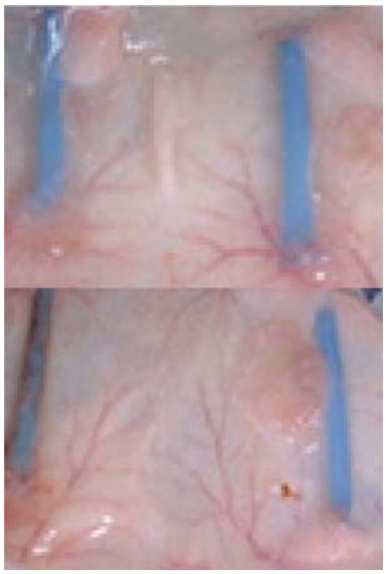

Fig. 2. Extirpated hollow fibers from animals.

Hollow fiber assay – For the assay, capsules were prepared following the procedures outlined in previous research.[9] Prior to filling with cells, each fiber was individually rinsed with ice-cold fresh MEM, containing 20% fetal bovine serum (FBS). The cell suspension was drawn into a 5 mL syringe, and the fibers were filled with the cell suspension via a 20-gauge needle. After filling, the ends of the fibers were heat-sealed, with individual fibers filled with cells prepared by heat-sealing the fibers at 2 cm intervals. Heat sealing was accomplished by clamping the fibers with hot smooth-jawed needle holders. Prior to implantation of capsules into the mice, the capsules were incubated overnight at 37 ºC in a 5% CO_2 atmosphere. For subcutaneous implantation, a small skin incision was made at the nape of the neck to allow insertion of an 11-gauge tumor implant trocar. The trocar containing the hollow fiber capsules was inserted through the subcutaneous tissue on posteria region of back site. Generally, each mouse received four hollow fiber capsules, each containing one of the four different cell lines. Sample treatment by intraperitoneal route was carried out for two weeks with a double injection per week, and then the experiment was terminated in a further two weeks. Therefore, the total evaluation term was four weeks. The anti-tumor activity was evaluated using the CCK-8 assay. For CCK-8 assay, the fibers are collected from the mice and examined any side effect on the fiber implanted position (Fig. 2). The fibers were placed into 2 mL of fresh, pre-warmed (37 ºC) culture medium (MEM)/35 mm dish, and allowed to equilibrate for 30 min at 37 ºC. The fibers were then stained with a tetrazolium salt solution

(CCK-8 solution) and washed twice with phosphate buffered saline (PBS) containing 2.5% protamine sulfate. The formazan extracted from the fiber was dissolved with DMSO, transferred to individual wells in 96-well plates, and then accessed for optical density at a 450 nm.

DNA fragmentation assay – 5×10^5 cells/mL were treated with various doses of sample for 24 h, and then cells were collected by centrifugation. Pellets were lysed by DNA lysis buffer (10 mM TrisHCl, 10 mM EDTA, 0.5% TritonX-100) and then centrifuged. The supernatant obtained was incubated overnight with proteinase K (0.1 mg/mL) and then with RNase (0.2 mg/mL) for 2 h at 37 ºC. After extraction with phenol: chloroform: isoamylalcohol (25:24:1), the DNA was separated in 1.8% agarose gel and visualized by UV after staining with ethidium bromide.

Statistical analysis – Statistical analysis of data was performed using one-way analysis of variance (ANOVA). The results are presented as mean and standard deviation (mean ± SD). Multiple comparisons were made by using post-hoc tests (Tukey's method) to determine which groups significantly differed from each other. Significance was accepted at a family error rate of 0.05.

Result and Discussion

As shown in Table 1, the inhibition rate showed that SLE, which was extracted with ethanol from *S. laniceps*, had dose-dependent growth inhibitory effects on Capan-1, Capan-2, Panc-1 and S2-013. All cell lines used in this study were significantly sensitive to 500 μg/mL of SLE; 42 ± 4%, 43 ± 5%, 39 ± 5% and 28 ± 4% against Capan-1, Capan-2, Panc-1 and S2-013, respectively. In 100 μg/mL of SLE, Capan-1 and Capan-2 showed significantly more sensitivity to inhibition of cell proliferation than Panc-1 and S2-013. Among chromatographic fractions isolated from the ethanol extract of *S. laniceps*, 10 μg/mL of Fraction H only that showed highly sensitive activity to the used cell lines; 37 ± 3%, 49 ± 5%, 30 ± 4% and 32 ± 3% against Capan-1, Capan-2, Panc-1 and S2-013, respectively. Hispidulin, which was isolated from Fraction H, exhibited significant inhibitory activity against all cell lines used at the concentration of 10 μg/mL. The strange thing is that even if hispidulin exhibited significant activities against four tumor cell lines at the dose of 10 μg/mL, the inhibitory activity was not increased compared to the activity of Fraction H at the same dose, excepting Capan-2. IC_{50} values of hispidulin on each cell line were 44.3, 10.2, 26.5 and 17.9 μg/mL on Capan-1, Capan-2, Panc-1 and S2-013, respectively. As a positive control, IC_{50} (μg/mL) of Gemicitabine, a well-known inhibitory agent of pancreatic cancer, were 0.029, 0.025, 0.053 and 0.1 μg/mL on Capan-1, Capan-2, Panc-1 and S2-013, respectively. The anti-tumor activities of Fraction H and hispidulin that was isolated from Fraction H as a major compound were tested against various pancreatic tumor cell lines using a hollow fiber assay (Table 2). Fraction H exerted inhibitory activities against four tumor cell lines in a dose-dependent manner. There were significant activities at 500 mg/kg of Fraction H against three cell

Table 1. Anti-tumor activity of the extract and chromatographic fractions isolated from *S. laniceps* using CCK-8 assay

Sample	Dose (μg/mL)	Capan-1	Capan-2	Panc-1	S2-013
SLE	10	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	100	27 ± 3*	31 ± 4*[b]	21 ± 5	14 ± 5
	500	42 ± 4*	43 ± 5*	39 ± 5*	28 ± 4*
Fraction A	10	5 ± 1	6 ± 2	10 ± 3	4 ± 1
Fraction B	10	12 ± 5	5 ± 3	10 ± 3	19 ± 6
Fraction C	10	4 ± 1	3 ± 1	3 ± 1	5 ± 1
Fraction D	10	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Fraction E	10	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Fraction F	10	21 ± 7	0 ± 0	0 ± 0	0 ± 0
Fraction G	10	18 ± 5	20 ± 8	30 ± 8	20 ± 7
Fraction H	10	47 ± 3*	59 ± 5*	40 ± 4*	32 ± 3*
Fraction I	10	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Fraction J	10	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Hispidulin	10	24 ± 3*	52 ± 7*	24 ± 8	39 ± 7*

SLE: *S. laniceps* ethanol extract, Values are expressed Mean ± SD, ($n = 6$), *$p < 0.05$ compared to the control group. One-way ANOVA followed by Tukey's Multiple Comparison Test.

Table 2. Anti-pancreatic effect of Hispidulin and Fraction H isolated from *S. laniceps* against pancreatic tumor cell lines

Sample	Dose (μg/kg)	Capan-1	Capan-2	Panc-1	S2-013
Fraction H	20	0 ± 0	4 ± 1	4 ± 2	5 ± 2
	200	14 ± 6	22 ± 5*[a]	19 ± 6	20 ± 5
	500	35 ± 4*	55 ± 5*	30 ± 4[a]	20 ± 7
Hispidulin	50	5 ± 2	17 ± 6	14 ± 5	20 ± 8
	100	17 ± 6	44 ± 3*	31 ± 3*	29 ± 3*[a]
	200	52 ± 5*	73 ± 4*	40 ± 5*	45 ± 7*

Anti-pancreatic effect of 200 mg/kg of Gemicitabine as positive control was 57 ± 5%, 87 ± 8%, 23 ± 7% and 40 ± 9% against Capan-1, Capan-2, Panc-1 and S2-013, respectively.
Values are expressed Mean ± SD, (n = 6), *$p < 0.05$ compared to the control group. One way ANOVA followed by Tukey's Multiple Comparison Test.

lines; the inhibition rate was 35 ± 4%, 55 ± 5% and 30 ± 4% against Capan-1, Capan-2 and Panc-1, respectively. At 500 mg/kg of Fraction H, only Capan-2 showed a significant inhibition of 22 ± 5%. Hispidulin also showed inhibitory activities against four tumor cell lines in a dose-dependent manner. In case of treatment with 200 mg/kg of hispidulin, there were significant sensitivities against four tumor cell lines. More specifically, three tumor cell lines, Capan-2, Panc-1 and S2-013, showed significant activities at the dose of 100 mg/kg, excepting Capan-1. According to this result, Fraction H and hispidulin was found more sensitive to Capan-2, which showed a significant inhibitory rate of 55 ± 5% in Fraction H and 73 ± 4% in hispidulin, among four pancreatic tumor cell lines.

One of the well-known mechanisms by which cell growth is suppressed is apoptotic cell death. Therefore, the effect of hispidulin on DNA fragmentation was examined in Capan-2 cells that were shown as more sensitive to hispidulin than other cell lines in CCK-8 assay and hollow fiber assay. The DNA fragmentation was observed when cells were treated with 2 μg/mL and 10 μg/mL of hispidulin for 24 h. The profile for hispidulin induced cell death closely correlated with its growth suppressive effects. Thus, the growth suppression induced by hispidulin in Capan-2 cells may be related to the induction of cell death (Fig. 3).

Pancreatic cancer is the most malignant cancer with a high mortality and there is no optimum cure treatment with a cancer agent, for which current therapeutic approaches are still very limited. Although chemotherapy is unique for pancreatic cancer, the toxic effects are difficult to tolerate,[10] so there is an urgent need for development of better cancer agents which are more effective and simultaneously cause fewer side effects. Natural products, such as an herbal extract and a traditional Chinese medicine in cancer treatment, may reduce adverse

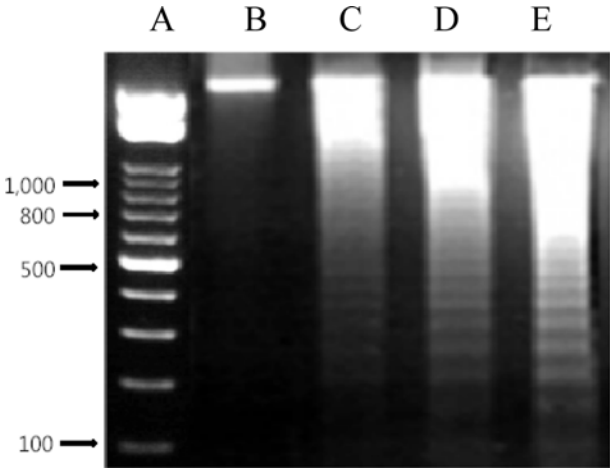

Fig. 3. Effect of Hispidulin on DNA fragmentation of Capan-2 cells. (A) Ladder marker; (B) Control; (C) 2 μg/mL of Hispidulin; (D) 10 μg/mL of Hispidulin; (E) 2 μg/mL of Gemicitabine.

side effects against synthetic chemotherapeutic agents. Natural products of plant origin are some potential candidates for cancer chemotherapy drugs because they have low or almost no toxicity to normal tissues, hence, more attention is being paid to searching for new antitumor agents from natural products nowadays.[11] *S. laniceps* and *S. involucrata* belong to the *Asteraceae* family, are a rare and beneficial traditional medicinal herb in Mongolia and China. The dried aerial parts of theses herbs have been used for the treatment of common diseases for very long period. Recent studies have shown that ethanol extracts of *S. laniceps* exerted some potent effects against experimental edema and pain in animal models[12] and *S. involucrata* shows anti-metastatic activity against hepatic tumor cells. However, information on its potential mechanism remains limited. In this study, *S. laniceps*'s anti-proliferative activities on pancreatic carcinomas such as Capan-1, Capan-2, Panc-1 and S2-013 cell lines were elucidated. An extract demonstrated significant cytotoxicity on Capan-1, Capan-2, Panc-1 and S2-013 as 42 ± 4%, 43

± 5%, 39 ± 5% and 28 ± 4% at the dose of 500 μg/mL, respectively. At the dose of 100 μg/mL of SLE, Capan-1 and Capan-2 showed significantly more sensitive activity than Panc-1 and S2-013. Hispidulin isolated from ethanol extract of *S. laniceps* showed improved anti-tumor activity against a pancreatic carcinoma through the CCK-8 and hollow fiber assay. 10 μg/mL of hispidulin on Capan-1, Capan-2, Panc-1 and S2-013 showed cytotoxicity of 24 ± 3%, 52 ± 7%, 24 ± 8% and 39 ± 7%, respectively, using CCK-8 assay. 200 mg/kg of hispidulin on Capan-1, Capan-2, Panc-1 and S2-013 showed anti-tumor activity of 52 ± 5%, 73 ± 4%, 40 ± 5% and 45 ± 7%, respectively, using the hollow fiber assay. The growth inhibition activity in CCK-8 and hollow fiber assay was associated with a characteristic type of cell death evidenced by DNA fragmentations, which are an important hallmark of apoptosis. Recent studies showed that hispidulin efficiently arrests G1/S-phase cell cycle.[13]

Taken together, the potential anti-tumor activity of the component and *S. laniceps* extract against pancreatic cancer *in vitro* and its partial molecular mechanism of action was investigated in this experimental study. The results demonstrated that the *S. laniceps* extract has strong anti-tumor activity against pancreatic cancer such as Capan-1, Capan-2, Panc-1 and S2-013, and the mechanism of its action might be associated with the apoptotic cell death by fragmentation of their own DNA. *S. laniceps* is a strong candidate for use as an anti-tumor therapeutic agent for the treatment of pancreatic carcinoma, and deserves to be investigated further. In our study, we found that *S. laniceps* has potential cytotoxic and apoptotic effects on human pancreatic carcinoma cells. The action of hispidulin, which is isolated from *S. laniceps*, was identified as pro-apoptotic through DNA fragmentation.

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