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Antineoplastic Activity of *Solidago virgaurea* on Prostatic Tumor Cells in an SCID Mouse Model

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Abstract: Solidago virgaurea *(goldenrod) has traditionally been used as an anti-inflammatory herbal medicine for the treatment of various symptoms, including prostatic diseases. The plant has also been reported to have antibacterial, spasmolytic, and carminative properties. During the course of our screening for antineoplastic activities in various herbal plants, we found that the extract of* S. virgaurea *exhibits strong cytotoxic activities on various tumor cell lines. The active component mostly resides in the leaves of the plant and is soluble in water. When the extract was fractionated by a Sephadex G-100 column, the active fraction corresponded to a molecular weight of ~40,000. This cytotoxic activity is effective on various tumor cell lines, including human prostate (PC3), breast (MDA435), melanoma (C8161), and small cell lung carcinoma (H520). To examine the effect of the cytotoxic activity on tumor cells in vivo, we used the rat prostate cell line (AT6.1) and an SCID mouse model. AT6.1 cells were injected into the flank of SCID mice, and then the G-100 fraction of* S. virgaurea *was administered intraperitoneally or subcutaneously every 3 days. The size of the tumor was measured for up to 25 days. The growth of the tumor was significantly suppressed by the G-100 fraction at 5 mg/kg without any apparent side effects. Therefore,* S. virgaurea *is considered to be promising as an antineoplastic medicine with minimal toxicities.*

Introduction

Prostate cancer is the most frequently diagnosed cancer among men in the United States, exceeding lung cancer by a narrow margin (1). It represents ~7% (40,000) of all cancer deaths, and it ranks as the second leading cause of cancer death in men. Most prostate cancer patients initially respond well to hormone therapy; however, they eventually develop nonresponsive metastatic disease. A variety of chemotherapeutic agents have been tested to treat patients with prostate cancer at different stages. The results of these trials have not been promising, and the active search for better therapeutic remedies continues (2). Because of the high incidence of prostate cancer, significant attention has recently been focused on preventive remedies for this disease, such as traditional herbal medicines as chemopreventive agents because of their low toxicity.

Solidago virgaurea is a perennial herb, and the whole plant has been used as a food as well as a traditional medicine in many geographical locations, including the United States (3). Various species of this plant have been reported to possess antibacterial, anti-inflammatory, spasmolytic, and carminative properties (4,5). *S. virgaurea* is particularly known to be effective for urological diseases, and it is commercially available under the trade name Urol mono (6,7). It has also recently been reported that the methanol extract of *S. virgaurea* possesses cytotoxic activity against a human cancer cell line (4). The active compounds were later identified as α-tocopherol quinone, *trans*-phytol, and 2-methoxybenzyl-2,6-dimethoxybenzoate. These low-molecular-weight components showed various degrees of cytotoxicity on different cancer cell lines in vitro. Plohmann et al. (8) found that saponins prepared from *S. virgaurea* showed a stimulatory effect on the activity of murine bone marrow macrophages and, further, an induction of cytotoxic macrophage and tumor necrosis factor-α release from macrophages. They also showed that saponin E of this plant has antitumoral effects on an allogeneic sarcoma 180 tumor model (8). These results suggest that *S. virgaurea* may be a good chemopreventive or therapeutic remedy for prostate cancer, in addition to its known effectiveness in various urological diseases. In this report, we describe a high-molecular-weight protein factor that possesses a strong cytotoxic activity against prostate cancer cells in vitro and in vivo.

Materials and Methods

Preparation of Extracts From *S. virgaurea*

S. virgaurea was obtained from Frontier Natural Products (Norway, IA). To extract active components from the plant,

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we initially tested various solvents and extraction methods on a small scale. The plant was ground to a fine powder, and 1 g of the sample was suspended with 10 ml of H_2O, ethanol, or chloroform. The samples were incubated at 90°C for 15 min (H_2O) or at room temperature for 24 h (H_2O, ethanol, and chloroform) and then centrifuged at 3,000 *g* for 10 min, and the supernatants were lyophilized. The dried powder was resuspended with the corresponding solvent at 1 mg/ml and tested for cytotoxic activities. Each part of the plant, i.e., leaves, stems, and flowers, was also tested separately; they were ground to a powder and extracted with H_2O at room temperature. For a large-scale preparation of the active fraction, 100 g of the plant were cut and ground to a fine powder using a homogenizer. The sample was suspended in 1 liter of distilled water and extracted for 24 h at room temperature. The suspension was then filtered through sterile gauze to yield a light-brown filtrate that was then centrifuged at 3,000 *g* for 10 min. The supernatant was lyophilized and yielded ~10 g of dry powder. The sample was reconstituted with phosphate-buffered saline (PBS), pH 7.4, at 3 g/ml. The solution was then applied to a Sephadex G-100 column (0.7 cm × 30 cm) and fractionated with PBS. Each fraction was assayed for cytotoxic activity and for protein concentration using the Bradford method. In parallel, molecular weight marker proteins (Bio-Rad) were run in the same column to determine the molecular weight of the active component. The active fractions resulting from 12 column chromatographies were combined and lyophilized. The sample was reconstituted with PBS at 1 mg/ml and used for subsequent experiments.

Cytotoxic Activity Assay

Cytotoxic activities of protein fractions were measured by 1) colorimetric assay by crystal violet staining or 2) colony formation assay as follows.

Colorimetric assay by crystal violet staining: To measure the cytotoxic activity, samples were added to each well of a 12-well plate that contained 0.5 ml of RPMI medium and 5×10^5 human prostate cancer PC3 cells. The plate was incubated for 24 h at 37°C in the presence of 5% CO_2. Cells in the wells were stained with 0.5% crystal violet in methanol for 3 min and then rinsed with water. The crystal violet was eluted from the cells by addition of methanol to each well and incubated for 1 h. The absorbance at 595 nm was measured for each well by a spectrophotometer (model UV20, Beckman). In parallel, the number of surviving cells was measured by the trypan blue exclusion assay (9).

Colony formation assay: For colony formation assay, PC3 cells were grown in RPMI medium, trypsinized, harvested, and resuspended in the same medium. The fractions of *S. virgaurea* were mixed with 5×10^5 cells in 1 ml of RPMI medium. After the mixture was incubated at 37°C for various time periods, 1-µl samples were withdrawn and plated in 10-cm tissue culture plates containing 10 ml of RPMI medium. Plates were incubated at 37°C for 7 days, the resultant colonies were stained with crystal violet, and the number of cells was counted.

Antitumor Effects in Mice

The effect of the G-100 fraction on tumor growth in vivo was examined using a rat prostatic tumor AT6.1 cell line. After injection of 0.5×10^6 cells in 0.2 ml of PBS into the dorsal flank of 5-wk-old SCID mice (Harlan Sprague Dawley, Indianapolis, IN), the G-100 active fraction was administered. Five mice were used for each experimental group. Mice were monitored daily, and the tumor volume was measured as an index of the growth rate. Tumor volume was calculated as follows: volume = $(W + L)/2 \times W \times L \times 0.5236$, where W is width and L is length. The doubling time of the tumor during the fastest-growing period was calculated by measuring the tumor volume every 3 days. Mice were weighed and sacrificed 25 days after the inoculation of the cells.

Flow Cytometric Analysis

Analysis of the cell cycle by a flow cytometer was done as previously described (10). About 10^6 exponentially growing cells were treated with or without 10 µg/ml of the G-100 fraction in RPMI medium containing 10% fetal bovine serum for 12 h at 37°C. Cells were then harvested, resuspended in 0.2 ml of residual supernatant, and mixed with 2 ml of propidium iodide solution (50 µg/ml propidium iodide, 0.6% NP-40, and 0.2% bovine serum albumin). After 2 min, the suspension was passed through a 26-gauge needle, mixed with 20 µl of 38% formaldehyde, and analyzed using a Becton-Dickinson flow cytometer. Each fraction of G_1, S, and G_2 was calculated by the Modfit software (Verity).

Caspase-3 Activity Assay

For assaying apoptosis, we used the ApoAlert caspase colorimetric assay kit (Clontech). Briefly, PC3 cells were cultured in RPMI medium and treated with G-100 fraction for various time periods. Cells were then harvested, resuspended in 50 µl of cell lysate buffer, and centrifuged. The supernatant was in 50 µl of a reaction mixture containing DEVD-pNA as a substrate at 37°C. After the mixture was incubated for 2 h, the enzymatic activity of caspase-3 was measured by reading absorbance at 400 nm.

Results

Cytotoxic Activity of *S. virgaurea*

To examine the effects of *S. virgaurea* on tumor cells, the powdered plant was extracted with H_2O with or without heat treatment at 90°C, ethanol, or chloroform. The filtered and

Table 1. Cytotoxic Activity of *Solidago virgaurea*[a]

Extracts	Cytotoxic Activity, %
No extract	0
H_2O	
−heat	86.0 ± 1.3
+heat	81.7 ± 4.0
Ethanol	89.5 ± 4.2
Chloroform	6.1 ± 15.1
Leaf	85.3 ± 3.2
Flower	81.2 ± 3.2
Stem	12.2 ± 0.7
Protease treatment	1.2 ± 0.1

a: Values are means ± SD measured by colorimetric assay.

lyophilized samples were then reconstituted with the corresponding solvent at 1 mg/ml. The samples were tested for their cytotoxic activities on human prostatic tumor PC3 cells. The extracts prepared with H_2O or ethanol showed strong cytotoxic activities on PC3 cells, regardless of the temperature during the extraction, while the chloroform extract had little effect on PC3 cells (Table 1). When the extracts prepared from the different parts of the plant, i.e., leaves, flowers, and stems, were tested, we found that the leaves and flowers had significantly higher activities than the stems. These results suggest that the leaves of *S. virgaurea* have cytotoxic activity on prostatic tumor cells and that this activity is water soluble and heat resistant. When the extract was treated with protease, the activity was almost completely lost, suggesting that the activity is due to a protein factor (Table 1).

Fractionation and Characterization of the Activity

To further characterize the activity, we fractionated the extracts with a Sephadex G-100 column. The results of the column chromatography indicated that the activity was recovered as a single peak that corresponds to a molecular weight of ~40,000 (Fig. 1). The results of the colony-forming assay for the cytotoxic activity of the peak fraction revealed that >97% of the cells were killed after 29 h of incubation (Fig. 2A). This cytotoxic activity followed first-order kinetics and was concentration dependent up to 12 μg/ml in culture medium, suggesting that a single molecule is responsible for the activity (Fig. 2B).

We also tested the G-100 fraction for the activity on various human tumor cell lines, including human prostate (PC3 and ALVA) (11), breast (MDA435) (12), melanoma (C8161) (13), small cell lung carcinoma (H520) (14), and lung adenocarcinoma (A549) (15). The growth of all cell lines was significantly suppressed by the G-100 fraction at 5 μg/ml, although the degree of the inhibition varied 40–93% depending on the cell line (Fig. 2C).

To understand the molecular mechanism of the cytotoxic activity, we performed the caspase assay. After incubation of PC3 cells with the G-100 fraction for 9 and 20 h, the cell lysates were prepared and caspase-3 activity was assayed by the ApoAlert kit (Clontech). Cells treated with the G-100 fraction exhibited significant caspase-3 enzyme production compared with untreated cells (Fig. 3). To further analyze the cytotoxic activity, we performed flow cytometric analysis. After PC3 cells were mixed with the G-100 fraction, incubated for 12 h, and treated with propidium iodine, they

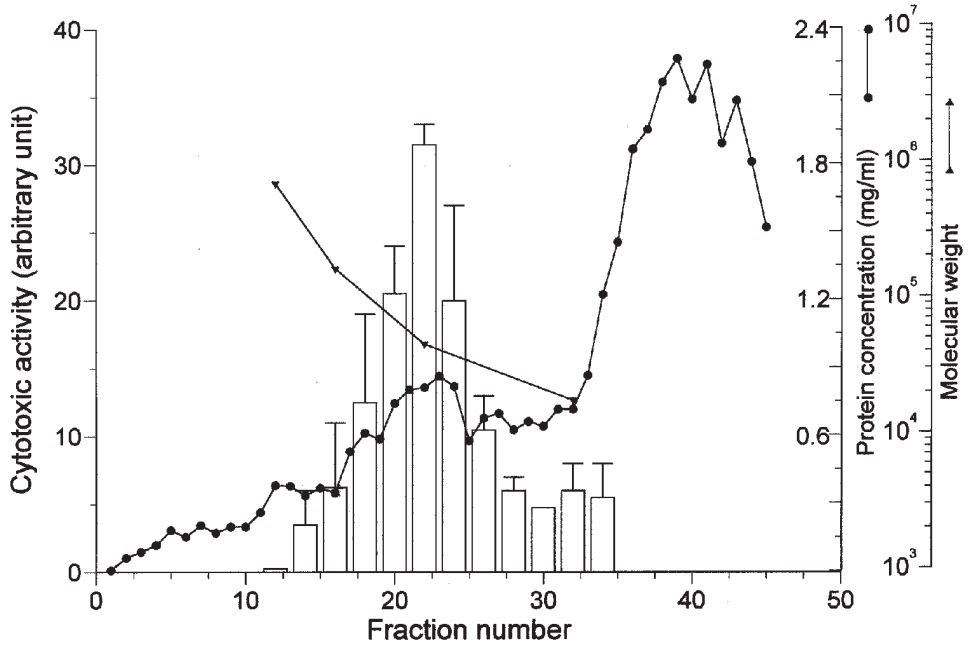

Figure 1. Fractionation of *Solidago virgaurea* extracts by a Sephadex G-100 column. Crude extracts of *S. virgaurea* were applied to a Sephadex G-100 column and fractionated with phosphate-buffered saline. Each fraction was assayed for cytotoxic activity (*open bars*) by colorimetric assay and protein concentration (*filled circles*). Molecular weight marker proteins were also fractionated in parallel (*filled triangles*).

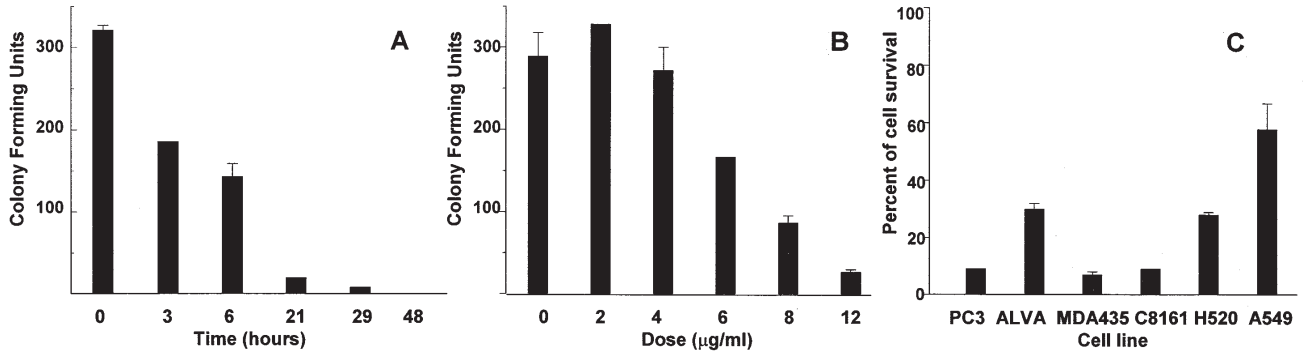

Figure 2. Characteristics of cytotoxic activity of G-100 fraction. A: kinetics of cytotoxic activity. PC3 cells (5×10^5) were incubated with 10 μg of G-100 fraction in 1 ml of RPMI medium at 37°C. Samples (1 μl) were withdrawn and plated in 10-cm culture plates containing 10 ml of RPMI medium. After 7 days of incubation at 37°C, colonies were counted. Values are means ± SD of triplicate measurements. B: dose effect. PC3 cells were incubated with G-100 fraction in RPMI medium for 24 h at 37°C, and cytotoxic activity was measured by colony formation assay as described in A. Values are means ± SD of triplicate measurements. C: effect of G-100 fraction on growth of various tumor cell lines. G-100 fraction was added at 5 μg/ml to 10^5 tumor cells from various origins that were then incubated for 24 h at 37°C, and cytotoxic activity was measured by colorimetric assay. Value of control well was set at 100%, and each sample value was normalized accordingly. Values are means ± SD of triplicate measurements.

were subjected to a flow cytometric analysis. When the cells were untreated, the number of cells at G_0/G_1, S, and G_2/M phases was 58, 34, and 7, respectively (Table 2). On the other hand, when cells were treated with *S. virgaurea*, a significant fraction of the cells shifted from the S to the G_0/G_1 phase. These results of caspase assay and flow cytometric analysis suggest that the cytotoxic effect of *S. virgaurea* is due to cell cycle arrest at the G_0/G_1 phase followed by apoptosis.

Antitumor Activity of *S. virgaurea*

To examine the effect of *S. virgaurea* on tumor growth in mice, we used the rat prostatic tumor model (AT6.1) in SCID mice (16). After injection of AT6.1 into the flank of the mice, the G-100 fraction (5 mg/kg) was administered intraperitoneally or intraperitoneally and subcutaneously. The G-100 fraction was injected every 3 days for 25 days, and the tumor size was measured. The growth of the tumor was significantly suppressed in both groups of mice injected with *S. virgaurea* by the intraperitoneal route and the intraperitoneal-subcutaneous route without apparent side effects (Fig. 4). The average weight of mice between the groups did not change significantly (data not shown). Intraperitoneal-subcutaneous injection of *S. virgaurea* appears to be more effective than intraperitoneal injection in inhibiting tumor growth. However, this difference is not statistically significant. These results of the animal study are consistent with our in vitro results and suggest that *S. virgaurea* can be an effective antineoplastic agent.

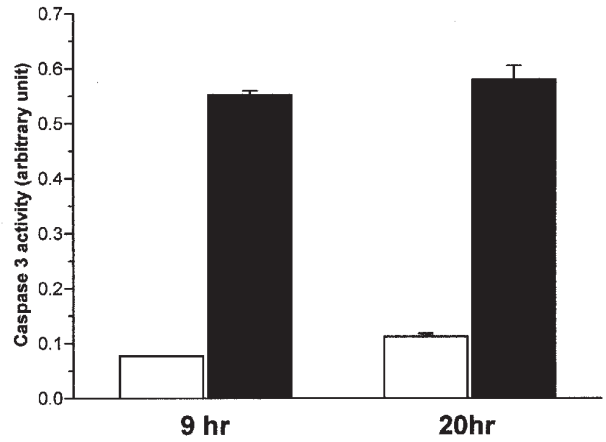

Figure 3. Caspase assay. PC3 cells (5×10^6) were mixed with (*filled bars*) or without (*open bars*) G-100 fraction in 1 ml of RPMI medium for 9 and 20 h at 37°C. Cells were then harvested, and cell lysates were tested for caspase-3 activity by ApoAlert kit (Clontech). Values are means ± SD of triplicate measurements.

Discussion

The anti-inflammatory effect of *S. virgaurea* extract has been relatively well studied (4,17,18). However, only a few published reports have described the antitumor activity of this plant. Saponin E prepared from *S. virgaurea* has been shown to have a stimulatory effect on murine bone marrow macrophages and also to induce tumor necrosis factor-α (8). The same saponin preparation was also shown to suppress tumor growth in mice (8). Therefore, saponin appears to work as an immunomodulant to suppress tumor growth.

Table 2. Flow Cytometric Analysis of Effect of *S. virgaurea* on Cell Cycle[a,b]

	% of Cell Population	
	−Solidago	+Solidago
G_0/G_1	58.0 ± 6.2	80.8 ± 1.5*
S	34.4 ± 3.1	14.4 ± 3.4*
G_2/M	7.7 ± 2.8	4.6 ± 1.9

a: Values are means ± SD.
b: Statistical significance is as follows: *, $P < 0.05$.

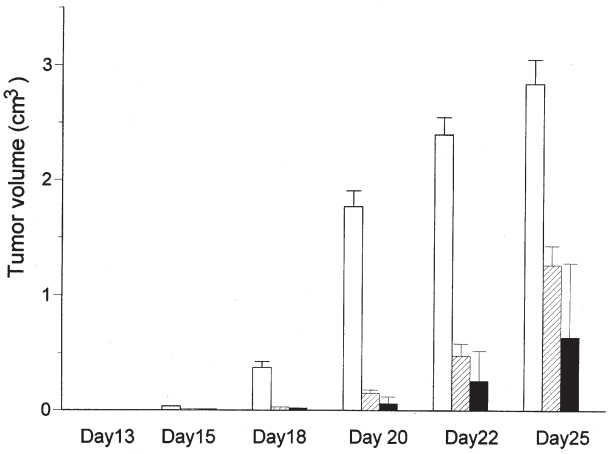

Figure 4. Inhibitory effect of *S. virgaurea* on tumor growth in mice. Rat prostate cancer cell, AT6.1, was injected subcutaneously into SCID mice. G-100 fraction (5 mg/kg) was injected intraperitoneally (*hatched bars*) or subcutaneously and intraperitoneally (*filled bars*) into 5 mice for each group every 3 days. Tumor volume was measured for each mouse until Day 25. *Open bar*, no treatment.

More recently, Sung et al. (4) identified three different compounds in the methanol extracts of *S. virgaurea*: erythrodiol-3-acetate, α-tocopherol quinone, and *trans*-phytol. These compounds have small molecular weights, i.e., 250–500, and are water insoluble (4). When tested in various human cancer cell lines, all these compounds showed significant cytotoxic activity in vitro; the in vivo effect is still unknown. On the other hand, the cytotoxic activity described in this report has characteristics clearly distinct from those of the previously identified compounds. The results of the Sephadex column chromatography indicate that the molecular weight of the active component is ~40,000. The activity is heat stable, but it is sensitive to protease treatment, suggesting that it is a protein factor. Therefore, the cytotoxic activity described here is clearly distinct from the previously reported antitumor components in *S. virgaurea*. The cytotoxic effect of *S. virgaurea* appears to be due to cell cycle arrest at the G_0/G_1 phase followed by apoptosis, as suggested by the flow cytometric analysis and results of the caspase assay.

When the G-100 fraction prepared from *S. virgaurea* was administered to mice every 3 days, the growth of tumors was significantly suppressed without apparent side effects (Fig. 4). Because we used SCID mice, this growth suppression is considered to be the direct effect of the G-100 fraction on tumor cells, rather than augmentation of the immune system of animals, which is consistent with the results of our in vitro experiments. The low toxicity appears to be due to its specific cytotoxic effect on rapidly growing tumor cells. These results are particularly encouraging, because the animals appear to be able to tolerate higher doses of *S. virgaurea*, which would result in a greater degree of suppressive activity. *S. virgaurea* has been used as an anti-inflammatory medicine and has been taken as a food in many areas (3–7). Therefore, *S. virgaurea* seems to be a promising antineoplastic medicine, especially when used with other cytotoxic chemotherapeutic agents. Further purification and characterization of the suppressor activity are underway.

Acknowledgments and Notes

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