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Original Article

Chemopreventive and Anticancer Activities of *Allium victorialis* var. *platyphyllum* Extracts

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Background: *Allium victorialis* var. *platyphyllum* is an edible perennial herb and has been used as a vegetable or as a Korean traditional medicine. *Allium* species have received much attention owing to their diverse pharmacological properties, including antioxidative, anti-inflammatory, and anticancer activities. However, *A. victorialis* var. *platyphyllum* needs more study.
Methods: The chemopreventive potential of *A. victorialis* var. *platyphyllum* methanol extracts was examined by measuring 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-induced superoxide anion production in the differentiated HL-60 cells, TPA-induced mouse ear edema, and Ames/*Salmonella* mutagenicity. The apoptosis-inducing capabilities of the extracts were evaluated by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay, 4',6-diamidino-2-phenylindole staining, and the DNA fragmentation assay in human colon cancer HT-29 cells. Antimetastatic activities of the extracts were also investigated in an experimental mouse lung metastasis model.
Results: The methanol extracts of *A. victorialis* var. *platyphyllum* rhizome (AVP-R) and *A. victorialis* var. *platyphyllum* stem (AVP-S) dose-dependently inhibited the TPA-induced generation of superoxide anion in HL-60 cells and TPA-induced ear edema in mice, as well as 7,12-dimethylbenz[*a*]anthracene (DMBA) and *tert*-butyl hydroperoxide (*t*-BOOH) -induced bacterial mutagenesis. AVP-R and AVP-S reduced cell viability in a dose-related manner and induced apoptotic morphological changes and internucleosomal DNA fragmentation in HT-29 cells. In the experimental mouse lung metastasis model, the formation of tumor nodules in lung tissue was significantly inhibited by the treatment of the extracts.
Conclusions: AVP-R and AVP-S possess antioxidative, anti-inflammatory, antimutagenic, proapoptotic, and antimetastatic activities. Therefore, these extracts can serve as a beneficial supplement for the prevention and treatment of cancer.
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Key Words: *Allium victorialis* var. *platyphyllum*, Anti-inflammation, Anti-mutagenicity, Induction of apoptosis, Anti-metastatic activity

INTRODUCTION

Chemoprevention is a way of controlling cancer in which the occurrence of the disease can be completely prevented, slowed down, or reversed by administering one or more naturally occurring compounds or synthetic agents.[1] All stages of carcinogenesis, termed initiation, promotion, and progression, can be targeted for chemopreventive intervention.[2] Recently, plant-derived phytochemicals have generated immense interest for their potential application in cancer chemoprevention and therapy because of their low toxicity and apparent benefit in other chronic diseases,[3] and in fact many of these compounds are currently under early phase clinical trials and available over the counter in most pharmacies.[4-7] Particularly, phytochemicals with antioxidative and anti-inflammatory activities have demonstrated potent chemopreventive and anti-cancer activity in multi-stage carcinogenesis.[8,9]

One of the attractive strategies considered in current cancer

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chemoprevention/chemotherapy is dietary or pharmaceutical manipulation to induce apoptotic cell death in preneoplastic or malignant cells with chromosomal aberration.[10] There is accumulated evidence that phytochemicals in medicinal herbs and dietary plants exert cancer chemopreventive or anticancer activity by inducing apoptosis in cancer cells.[11-15] Apoptotic cell death is regulated tightly by two major pathways, the mitochondrial and membrane death receptor pathways.[16,17] The mitochondrial-mediated pathway via caspase-9 and the death receptor-activated pathway via caspase-8 converge into caspase-3 and finally induce cell death via caspase-3-mediated cleavage of poly (ADP-ribose) polymerase.[18,19]

Allium victorialis var. *platyphyllum* (Liliaceae), better known as 'Myung-I' in Korea, is an edible perennial herb widely distributed in the northern part of Korea, such as Ulleung Island and Mt. Hambaek. Recently, *Allium victorialis* and *A. victorialis* var. *platyphyllum* have received much attention owing to their various pharmacological properties, including anti-arteriosclerotic, anti-cancer, antioxidant, anti-diabetic, anti-obesity, anti-neuroinflammatory, hepatoprotective, and nephroprotective effects.[20-22] The extract of *A. victorialis* var. *platyphyllum* contained 2-vinyl-4H-1,3-dithiin, gitogenin 3-O-lycotetroside, astragalin, and kaempferol 3, 4'-di-O-beta-D-glucoside as cytotoxic ingredients.[22]

In the present study to assess the cancer chemopreventive potential of the methanol extracts of *A. victorialis* var. *platyphyllum* rhizomes (AVP-R) and *A. victorialis* var. *platyphyllum* stems (AVP-S), we examined their inhibitory effects on 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-induced acute inflammation and carcinogen-induced bacterial mutagenesis and its apoptosis-inducing capability in human colon cancer HT-29 cells. We further evaluated antimetastatic activity of *A. victorialis* var. *platyphyllum* methanol extracts in a mouse lung metastasis model of colon cancer.

MATERIALS AND METHODS

1. Materials

AVP-R and AVP-S were provided by Professor Hee-Juhn Park, a co-author, and dissolved in dimethyl sulfoxide (DMSO). RPMI 1640 medium, Dulbecco's modified Eagle's medium (DMEM), phosphate-buffered saline (PBS), and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA). TPA, 7,12-dimethylbenz[*a*]anthracene (DMBA), *tert*-butyl hydroperoxide (*t*-BOOH), triphosphopyridine nucleotide, glucose 6-phosphate, cytochrome *c*, DMSO, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, 4',6-diamidino-2-phenylindole, sodium dodecyl sulfate, ribonuclease, Tritirachium alkaline proteinase, and rabbit anti-actin antibody were obtained from Sigma Chemical (St. Louis, MO, USA). The S9 extract from the livers of Arochlor 1254-treated male Sprague-Dawley rats was purchased from ICN Pharmaceuticals (Aurora, OH, USA). Anti-human procaspase-8, procaspase-9 and horseradish peroxidase-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-procaspase-3 and poly (ADP-ribose) polymerase antibodies were purchased from Transduction Laboratories (Lexington, KY, USA) and New England BioLab (Beverly, MA, USA), respectively. All other chemicals and reagents were of analytical grade.

2. Cell lines and cell culture

Human promyelocytic leukemia HL-60 cells, human colon cancer HT-29 cells, and murine colon cancer CT-26 cells were obtained from the Korea Cell Line Bank (Seoul, Korea). HT-29 cells and CT-26 cells were cultured in DMEM supplemented with 10% FBS and 1% antibiotic-antimycotic mixture, and HL-60 cells were maintained in RPMI 1640 medium containing 10% FBS and 1% antibiotic-antimycotic mixture in a humidified atmosphere of 5% CO_2 at 37°C.

3. Animals

Six-week-old female ICR mice and male BALB/c athymic nude mice were purchased from Central Laboratory Animals (Seoul, Korea) and were provided free access to a standard chow diet (Daejong, Seoul, Korea). All mice were allowed 1 week to acclimatize, and were maintained at 25 ± 2°C, with a relative humidity of 55 ± 5% and a 12-hour light-dark cycle. The animal studies were conducted in accordance with the experimental protocols of the animal ethics committee of the Yonsei University College of Dentistry.

4. The generation of superoxide anion

Inhibitory effects of extracts on the generation of TPA-induced superoxide anion in HL-60 cells were performed as reported previously.[23] Briefly, HL-60 cells (5×10^5 cells/mL) were stimulated with 1.25% DMSO for 6 days, harvested, and suspended with PBS (1×10^6 cells/mL). The differentiated cells were treated with the extracts at the indicated concentrations for 15 minutes and then exposed to TPA (8 μM) and cytochrome *c* (160 μM) for an additional 15 minutes. After centrifugation, the absorbance of supernatants at 550 nm was measured.

5. 12-*O*-tetradecanoylphorbol 13-acetate-induced mouse ear edema

The right ear of each female ICR mice (six per group) was topically treated with the indicated dose of the extracts or curcumin (5 mg/ear) as a positive control in 50 μL vehicle (DMSO: acetone = 15:85, v/v) 30 minutes prior to the application of 5 nmol TPA in 50 μL vehicle. Their left ears were treated with vehicle alone. The control group received only vehicle on both ears. Four hours later, the right and left ear punches of 6 mm diameter were taken from each mouse and weighed. Edema was represented as the increase in weight of the right ear punch over that of the left.

6. Ames/Salmonella mutagenicity assay

The bacterial mutagenicity assays were conducted with *Salmonella typhimurium* (*S. typhimurium*) TA100 and TA102 by the method developed by Maron and Ames.[24] Briefly, *S. typhimurium* TA100 and TA102 were grown in Oxoid nutrient broth medium for 11 hours, respectively. A culture (100 μL) of *S. typhimurium* TA100 was added to the mixture (600 μL) containing DMBA (100 nmol/plate), Aroclor 1254-induced rat liver microsomes (S9) mixture (1.95 mg protein), and the indicated dose of AVP-R or AVP-S extracts. An 11-hour culture of *S. typhimurium* TA102 was added to the mixture containing *t*-BOOH (400 μg/plate) and extracts. After pre-incubation for 30 minutes at 37°C in a rotary shaker, the mixture was transferred to 2 mL top agar containing 0.5 mM histidine-biotin and poured onto minimal glucose plates. The plates were incubated for 48 hours at 37°C and the number of his^+ revertant colonies was counted.

7. Cell viability

HT-29 cells (5×10^3 cells) were seeded into each well of a 96-well plate with 10% FBS-DMEM and cultured overnight. The cells were exposed to serum-free media with various concentrations of extracts for 24 hours. Then, 20 μL of 3-(4,5-dimethyl-2- thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide stock solution (5 mg/mL) was added to each well and the plates were incubated for 4 hours at 37°C. The cellular formazan product was dissolved with DMSO and the absorbance was measured at 570 nm using a microplate reader (BIO-RAD Laboratories, Hercules, CA, USA).

8. 4',6-Diamidino-2-phenylindole staining

HT-29 cells at 80% confluence were incubated in serum-free media with the indicated concentrations of the extracts for 24 hours. The cells were washed in ice-cold PBS, fixed with 4% formaldehyde for 10 minutes, and then permeabilized with 0.5% Triton X-100 for 30 minutes. After 20 minutes of staining with a 4',6-diamidino-2-phenylindole solution (1 μg/mL), the nuclear morphology of the cells was observed under a fluorescence microscope (Olympus IX52, Tokyo, Japan).

9. DNA fragmentation

HT-29 cells at 80% confluence were cultured in serum-free media with various concentrations of the extracts for 24 hours. The harvested cells were lysed in 50 mM Tris-HCl (pH 7.5) containing 200 mM NaCl, 5 mM EDTA, and 0.1% Triton X-100 for 2 hours on ice. Lysates were centrifuged at 10,000 × g for 10 minutes, and the supernatant was incubated with 50 μg/mL ribonuclease A, 120 μg/mL Tritirachium alkaline proteinase, and 0.5% sodium dodecyl sulfate for 4 hours at 55°C. DNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1) mixture and precipitated with ice-cold absolute ethanol. After the precipitate was resuspended with 30 μL of Tris-EDTA buffer (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA), each DNA sample was electrophoresed on 1.8% agarose gel with 0.5 μL/mL ethidium bromide and visualized under ultra-violet light.

10. Experimental mouse lung metastasis model

A pulmonary colonization assay was conducted as described by Fidler.[25] The BALB/c mice were divided into eight groups, each containing five mice. CT-26 mouse colon cancer cells (1×10^5 cells/0.2 mL) were injected into the tail veins of the mice. AVP-R or AVP-S extracts at the indicated doses were intraperitoneally administered 30 minutes prior to the injection of CT-26 cells, and then treated once a day for 2 weeks. The mice were sacrificed and the lungs were separated, washed, and fixed overnight in Bouin's solution. The number of surface tumor nodules was counted using an ocular micrometer.

11. Statistics

Data were expressed as the means ± standard error. Statistical analysis was performed with one-way analysis of variance and Student's *t*-test to express the differences between the groups. A value of $P < 0.05$ was considered statistically significant.

RESULTS

1. Antioxidative and anti-inflammatory activities of *Allium victorialis* var. *platyphyllum* rhizome and stem extracts

Oxidative stress and inflammation are implicated in multi-

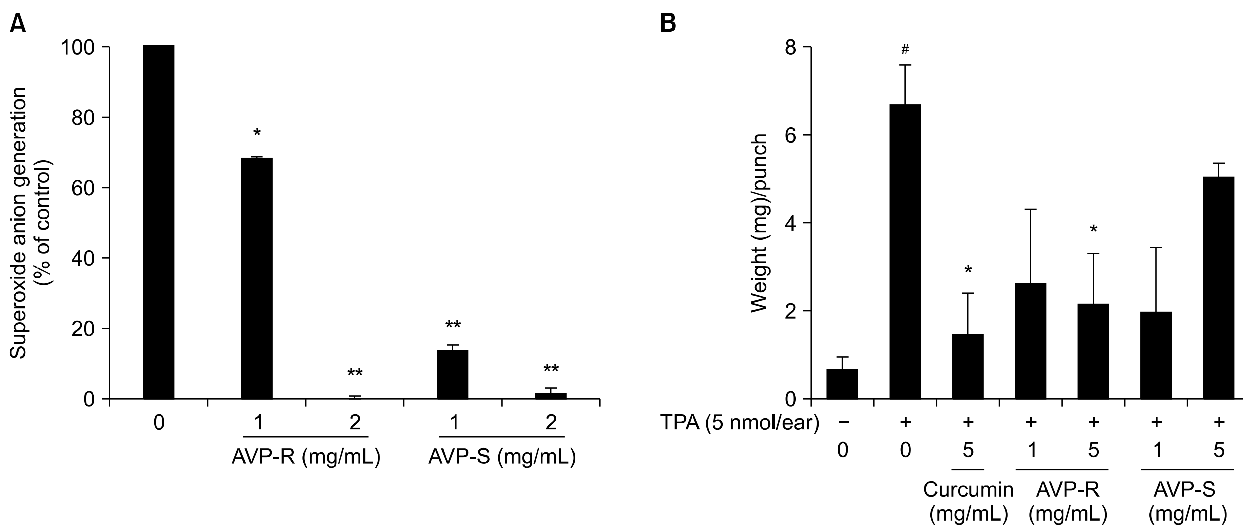

Figure 1. Antioxidative and anti-inflammatory activity of the methanol extract of *Allium victorialis* var. *platyphyllum* (*A. victorialis* var. *platyphyllum*). (A) The differentiated HL-60 cells were treated with the methanolic extracts of *A. victorialis* var. *platyphyllum* rhizomes (AVP-R) or *A. victorialis* var. *platyphyllum* stems (AVP-S) for 15 minutes at 37°C. Then, the cells were exposed to 12-*O*-tetradecanoylphorbol 13-acetate (TPA) (8 μM) and cytochrome *c* (60 μM) for 15 minutes. After centrifugation, absorbance at 550 nm was measured. Data are expressed as % of control. *$P < 0.05$, **$P < 0.001$ versus TPA alone-treated cells. (B) The right ear of each ICR mice was topically treated with the indicated dose of AVP-R, AVP-S, or curcumin in 50 μL vehicle (dimethyl sulfoxide:acetone = 15:85, v/v) 30 minutes prior to the application of 5 nmol TPA in 50 μL vehicle. Their left ears were treated with vehicle alone. Four hours later, edema was measured as the increase in weight of the right ear punch over that of the left. Data are expressed as mean ± standard error of 3 mice per group. $^{\#}P < 0.001$ versus vehicle-treated mice, *$P < 0.05$ versus TPA-treated mice.

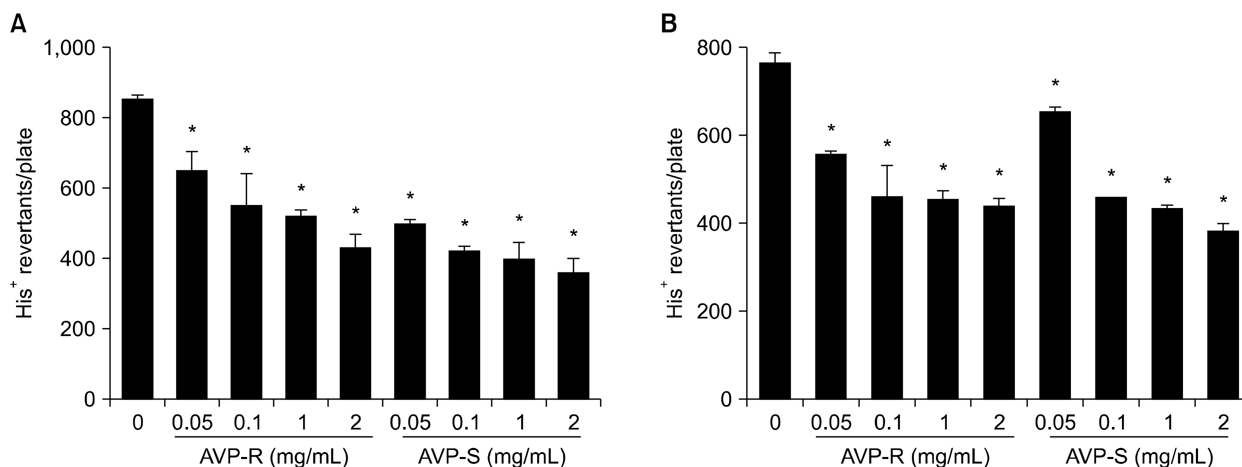

Figure 2. Antimutagenic activity of the methanol extract of *Allium victorialis* var. *platyphyllum* (*A. victorialis* var. *platyphyllum*). (A) An 11-hour culture of *Salmonella typhimurium* (*S. typhimurium*) TA100 was added to the mixture containing 7,12-dimethylbenz[*a*]anthracene (DMBA) (100 nmol/plate), the indicated concentration of *A. victorialis* var. *platyphyllum* rhizomes (AVP-R) or *A. victorialis* var. *platyphyllum* stems (AVP-S), and S9 mix. (B) An 11-hour culture of *S. typhimurium* TA102 was added to the mixture containing *tert*-butyl hydroperoxide (t-BOOH) (400 μg/plate) and AVP-R or AVP-S. After pre-incubation for 30 minutes at 37°C, the mixture was transferred to top agar containing histidine-biotin and poured onto minimal glucose plates. The plates were incubated for 48 hours at 37°C and the number of his^{+} revertant colonies was counted. Data are expressed as mean ± standard error from triplicate plates. *$P < 0.01$ versus the plates treated with DMBA (A) or *t*-BOOH alone (B).

stage carcinogenesis.[26-28] Reactive oxygen species (ROS), produced as typical by-products of eicosanoid metabolism during the inflammatory tissue damage, can alter the course of normal biochemical processes, leading to preneoplastic transformation of cells. Tumor promoters, such as TPA, generate superoxide anion in epithelial cells, leukocytes, and inflammatory cells.[29,30] The

methanolic extracts of AVP-R and AVP-S at 1 mg/mL inhibited TPA-induced generation of superoxide anion by 33% and 86% in the differentiated HL-60 cells, respectively. Both extracts at 2 mg/mL almost completely blocked the generation of superoxide anion induced by TPA stimulation (Fig. 1A) without affecting cell viability (data not shown).

The anti-inflammatory effects of AVP-R and AVP-S were evaluated by determining their inhibitory effects on TPA-induced ear edema in mice. The pretreatment with AVP-R at 5 mg/mL significantly suppressed ear edema induced by the topical application of TPA by 68% but AVP-S did not show significant inhibition (Fig. 1B). Topical application of AVP-R or AVP-S alone did not affect the induction of ear edema in mice (data not shown).

2. Antimutagenic activities of *Allium victorialis* var. *platyphyllum* rhizome and stem extracts

Tumor initiation is an irreversible event that begins when DNA in a cell or population of cells is damaged by exposure to exogenous or endogenous carcinogens. A chemical carcinogen causes a genetic error by modifying the molecular structure of DNA that can lead to a mutation during DNA synthesis.[31] AVP-R and AVP-S dose-dependently inhibited *his*$^+$ reversion in *S. typhimurium* TA100 treated with DMBA in the presence of S9 (half maximal inhibitory concentration = 2 mg/mL and 0.01 mg/mL, respectively) (Fig. 2A) and *t*-BOOH-treated *S. typhimurium* TA102 (half maximal inhibitory concentration > 2 mg/mL and 2 mg/mL, respectively) (Fig. 2B). AVP-R and AVP-S alone were not toxic at the concentrations tested.

3. Induction of apoptosis in HT-29 colon cancer cells

We assessed whether AVP-R and AVP-S could induce apoptosis in cancer cells. When HT-29 cells were treated with these extracts for 24 hours, cell viability was reduced in a dose-related manner (Fig. 3A). At 0.1 mg/mL and 0.2 mg/mL, AVP-R inhibited cell viability by 9% and 68% and AVP-S by 42% and 67%, respectively. Both extracts caused apoptotic morphological changes, including chromatin condensation and the formation of apoptotic bodies

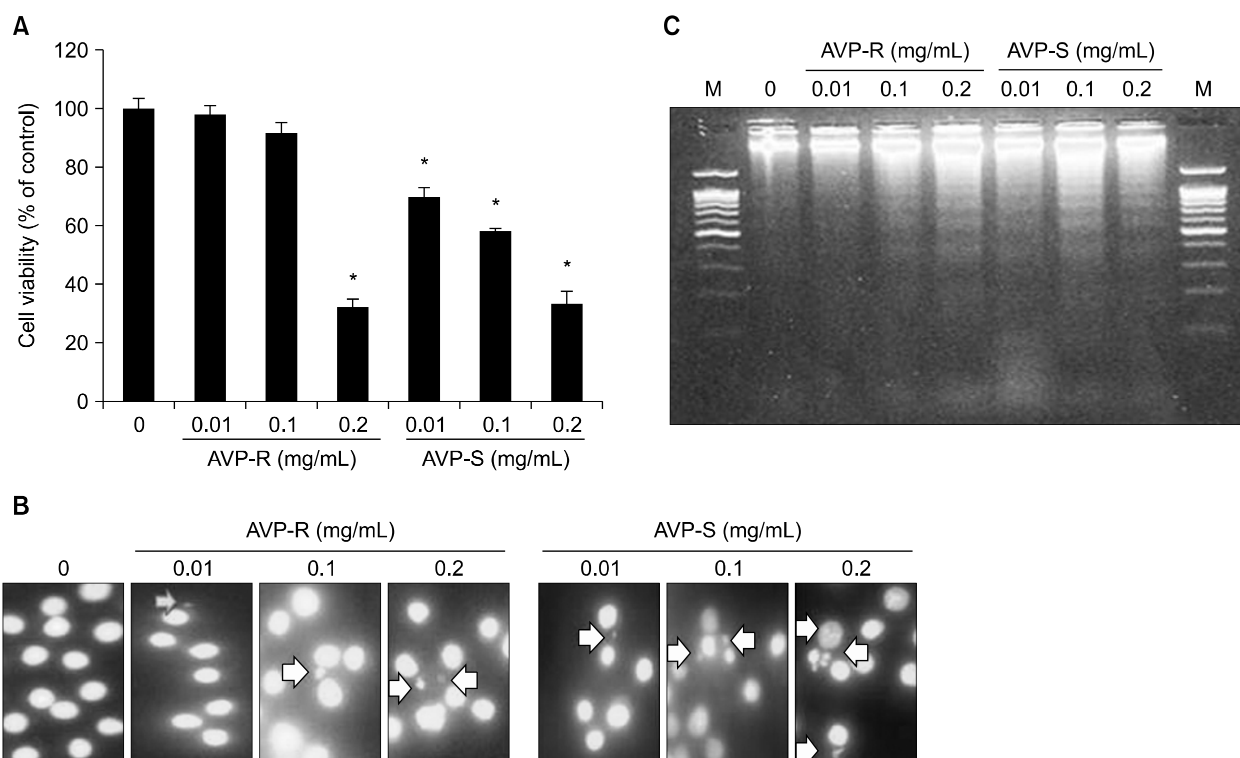

Figure 3. Induction of apoptosis in HT-29 colon cancer cells treated with the methanol extract of *Allium victorialis* var. *platyphyllum* (*A. victorialis* var. *platyphyllum*). HT-29 cells were treated with the indicated concentrations of *A. victorialis* var. *platyphyllum* rhizomes (AVP-R) and *A. victorialis* var. *platyphyllum* stems (AVP-S) for 24 hours. (A) Cell viability was evaluated by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay. *$P < 0.05$ versus untreated HT-29 cells. (B) The washed cells were fixed, permeabilized, and stained with a 4',6-diamidino-2-phenylindole solution. The nuclear morphology of the cells was observed under a fluorescence microscope (magnification, ×400). The arrows indicate DNA condensation in nuclei and apoptotic bodies. (C) HT-29 cells were treated with the indicated concentrations of extracts for 72 hours. Oligonucleosomal DNA was extracted and separated by gel electrophoresis. M, 100 base pair marker.

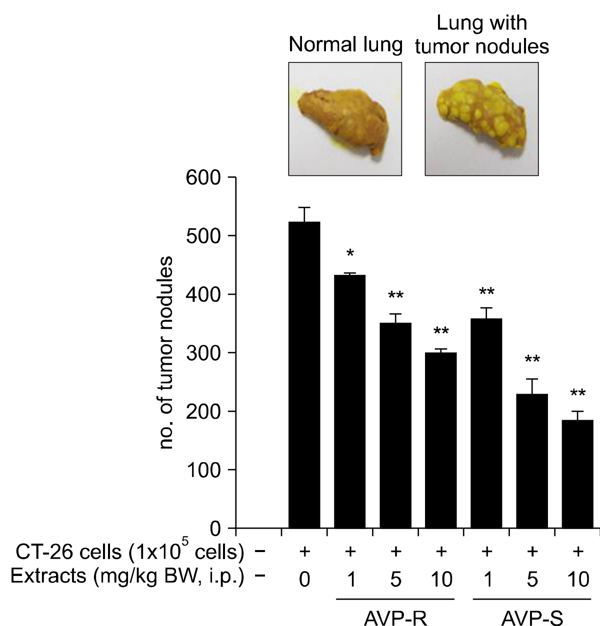

Figure 4. Antimetastatic activity of the methanol extract of *Allium victorialis* var. *platyphyllum* (*A. victorialis* var. *platyphyllum*). Murine colon cancer CT-26 cells (1 × 10^5 cells/0.2 mL) were injected into the tail veins of BALB/c mice (n = 5) 30 minutes after intraperitoneal administration of *A. victorialis* var. *platyphyllum* rhizomes (AVP-R) and *A. victorialis* var. *platyphyllum* stems (AVP-S). These extracts were administered once daily for 2 weeks, and the number of tumor nodules was counted. *$P < 0.05$, **$P < 0.01$ versus CT-26 cells alone-injected mice. BW, body weight; i.p., intraperitoneal injection.

(Fig. 3B), as well as internucleosomal DNA fragmentation in HT-29 cells (Fig. 3C).

4. Antimetastatic activity of *Allium victorialis* var. *platyphyllum* rhizome and stem extracts

We further determined the antimetastatic activity of AVP-R and AVP-S in a spontaneous lung metastasis animal model. The number of tumor nodules was dramatically increased in the lungs of mice inoculated with CT-26 cells but inhibited by intraperitoneal administration of AVP-R and AVP-S in a dose-related manner. AVP-R treatment at 1, 5, and 10 mg/kg BW reduced the formation of tumor nodules by 17%, 33%, and 43% and AVP-S treatment at the same doses showed decrease by 33, 46, and 65%, respectively (Fig. 4).

DISCUSSION

A. victorialis var. *platyphyllum*, one of the most popular *Allium* species, is widely distributed in the northern part of Korea. The leaves of *A. victorialis* var. *platyphyllum* are used as vegetable such as pickles in soy sauce, wrapped pock, and Kim-chi and also as a Korean traditional medicine for the treatment of gastritis and heart failures.[21] Previous phytochemical studies reported flavonoids, steroidal saponins, and sulfur compounds as its active principles.[32,33] *A. victorialis* var. *platyphyllum* has been known to possess anti-neuroinflammatory effects and significant cytotoxicities toward cancer cells.[17,20,34] The aim of this study is to evaluate the potential of *A. victorialis* var. *platyphyllum* as a promising chemopreventive/anticancer agents.

ROS induce membrane damage, DNA base oxidation, DNA strand breaks, chromosomal aberrations, and protein alterations, most of which would be involved in the carcinogenesis process. Therefore, agents with antioxidative activity can be candidates for chemopreventive agents. We found that the methanol extracts of AVP-R and AVP-S have potent antioxidative activity by significantly inhibiting the TPA-induced generation of superoxide anion in the differentiated HL-60 cells, which is a model system for the generation of ROS by various agents.[35]

Inflammation is causally linked to tumor promotion.[36] Mouse ear edema induced with topically applied TPA is an excellent acute inflammation animal model, closely related with the infiltration of neutrophil and macrophages, the induction of pro-inflammatory cytokines including tumor necrosis factor-α and interleukin-1, and the generation of ROS including superoxide anion.[37] Our data indicated that the methanol extracts of AVP-R and AVP-S exert anti-inflammatory activity by suppressing TPA-induced mouse ear edema in mice, thereby preventing inflammation-related stages of carcinogenesis.

The initiation stage of carcinogenesis involves changes at the genetic level after incorporation, distribution, and metabolism of carcinogenic agents within the body.[38] AVP-R and AVP-S dose-dependently inhibited DMBA-induced bacterial mutagenesis in *S. typhimurium* TA100 in the presence of S9 and *t*-BOOH-induced bacterial mutagenesis in *S. typhimurium* TA102. These results demonstrate antimutagenic activities of *A. victorialis* var. *platyphyllum* extracts.

In most cancer cells, abnormal proliferation is induced whereas cell death and differentiation are inhibited. Because apoptosis is considered as a defense strategy against tumorigenesis, apoptosis-inducing phytochemicals have high potential for the suppression of carcinogenesis and cancer progression.[10] In this study, the methanol extracts of AVP-R and AVP-S reduced cell viability and induced apoptotic morphological changes as well as internucleosomal DNA fragmentation in human colon cancer HT-29 cells.

One of the major causes of death in cancer patients is due to

the ability of tumor cells to metastasize. Colorectal cancer is the second most common cause of cancer deaths worldwide, and approximately 50% of the patients eventually develop distance metastasis.[39] The lung has been known as one of the most frequent sites of colorectal cancer metastases.[28,29] Our data showed that intraperitoneal administration of the methanol extracts of AVP-R and AVP-S remarkably reduced the number of tumor nodules in the lungs of mice inoculated with murine colon cancer CT-26 cells, suggesting antimetastatic activities of these extracts.

Taken together, the methanol extracts of AVP-R and AVP-S possess antioxidative, anti-inflammatory, antimutagenic, proapoptotic, and antimetastatic activities. Therefore, these extracts may serve as a beneficial supplement for preventing and treating cancer.

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CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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