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Determination of Flavonoids from *Allium victorialis* var. *platyphyllum* and Their Effect on Gap Junctional Intercellular Communication

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Abstract This study was carried out to identify and quantify the flavonoids from 6 different plant parts of *Allium victorialis* var. *platyphyllum* (AVP), including the flower, leaf, root, stem, flower stalk, and flower seed, using liquid chromatography/mass spectrometry. Two major flavonoids were structurally identified as quercetin (3,5,7,3'4,'-pentahydroxyflavone) and kaempferol (3,5,7,4'-tetrahydroxyflavone) at contents of 11.8-25.8 and 6.0-64.4 μg/mL, respectively. In particular, the flower and root plant parts contained the highest amounts of quercetin and kaempferol compared to the other parts. We also assessed the recovery effects of each plant-part extract of AVP on gap junctional intercellular communication (GJIC) in WB-F344 cells by the scrape-loading and dye transfer (SL/DT) method. According to the results, GJIC was reduced by approximately 70.2% (62.3±12.5 cells) compared to the control (209±9.5 cells, 100%) when 12-*o*-tetradecanoylphorbol-13-acetate (TPA) was treated alone in the WB-F344 rat liver epithelial cells. However, the stem extract (0.2 mg/mL) restored GJIC to basal levels (92%, 204±2.3 cells, $p<0.01$) and the flower extract (0.2 mg/mL) stimulated GJIC to 82.5% (172.6±8.3 cells, $p<0.05$), when applied together with the TPA.

Keywords: *Allium victorialis* var. *platyphyllum*, gap junctional intercellular communication, kaempferol, quercetin

Introduction

Allium victorialis var. *platyphyllum* (AVP), which belongs to the Liliaceae family, is an herbaceous perennial plant grown in the Jirisan(Mt.), Odaesan(Mt.), and Ulleungdo (Island) regions of Korea. AVP has been used traditionally in Korea as an edible and medicinal wild herb for treating gastritis and heart disease (1, 2). It was previously established that AVP exerts pharmacological activities such as anti-hepatotoxic and anti-hyperlipemic activities in rats (3). This herbal plant is reported to contain 2-3% carbohydrate, and ascorbic acid in the leaf, as well as large quantities of sulfur-containing substances (4). Also, it was reported that two flavonoids (astragalin and kaempferol 3,4'-di-O-β-D-glucoside) were isolated from the bulbs of AVP (1). Flavonoids are found in nearly every plant, and recently there has been a resurgence of interest in flavonoids due to their potential health benefits as antioxidants, and as anti-inflammatory and cardioprotective agents (5-7).

Gap junctions are composed of a 6 transmembrane protein units called connexons (8). Gap junction channels connect neighboring cells and allow the intercellular transfer of ions as well as polarized and non-polarized molecules up to a Mw of 1 kDa (9). Gap junctional intercellular communication (GJIC) is an important mechanism controlling cellular homeostasis, proliferation, and differentiation (10, 11). Conversely, inhibition of GJIC between adjacent cells is postulated to be one of the important events that take place during the promotional stage of cancer (12, 13). It was demonstrated that GJIC is affected by various compounds, including retinoic acid, retinoid derivatives, and vitamin D, as well as tumor promoters such as phorbol esters (14). GJIC is also sensitive to oxidative and nitrosative stress, and hydrogen peroxide and peroxynitrite inhibit GJIC (15, 16).

In the present study, we identified and quantified the major flavonoids in AVP extracts by high-performance liquid chromatography/liquid chromatography-mass spectrometry (HPLC/LC-MS) analysis, and determined the anti-carcinogenic effects of the extracts during the promotional phase of cancer using 12-*o*-tetradecanoyl-phorbol-13-acetate (TPA) to inhibit GJIC, which is associated with tumor promotion.

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Materials and Methods

Chemicals and apparatus D-media (Formula No. 78-5407EF), fetal bovine serum (FBS), and penicillin streptomycin neomycin (PSN) were obtained from Life Technologies Inc. (Gibco BRL, Grand Island, NY, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), TPA, Lucifer yellow, quercetin, kaempferol, and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The analytical HPLC system was a Waters HPLC (Associates model 515; Milford, MA, USA) with a Phenyl-Hexyl (Phenomenex Co., Torrance, CA, USA) column (luna 5 μ, 150×4.6 mm i.d.) protected by an LC_{18} (10×4.0 mm i.d.) guard column, containing a UV detector. Mass spectra were obtained using LC-MS (HP-1100 HPLC and a QUATTRO LC Triple Quadrupole Tandem Mass Spectrometer; Waters Ltd.).

Sample preparation The AVP were obtained from Seorrim Farm in Gangwon Province, Korea, in June 2005 and divided into 6 parts (flower, flower stalk, seed, leaf, stem, and root). All the parts were storied at -60°C, and then lyophilized for 72 hr and pulverized with a blender.

To prepare extracts from the 6 dried plant parts of AVP, 10 g of each plant part was extracted 3 times with 70% ethanol under reflux for 2 hr at room temperature, and then filtered through Whatman No. 6 paper. The filtered supernatants were evaporated under vacuum at 50°C, and prepared by lyophilization in order to remove the residual solvents. Each extract was suspended in 0.1% DMSO and stored at 4°C until further use.

Determination of total phenolic content The total phenolic contents were measured by a colorimetric assay, based on the procedure described by Chung *et al.* (17). Briefly, 1 mL of sample was mixed with 1 mL of Folin-Ciocalteu's phenol reagent (Sigma Chemical Co.). After 3 min, 1 mL of saturated Na_2CO_3 solution was added to the mixture, where the volume was made up to 10 mL by adding distilled water. The reaction was kept in the dark for 90 min, after which the absorbance was read at 725 nm. Gallic acid was used to calculate the standard curve (0.01-0.1 mM). Estimation of the phenolic compounds was carried out in triplicate. The results are mean values ± standard deviations (SD), and expressed as mg of gallic acid equivalents (GAEs)/g of extract.

Determination of total flavonoid content The total flavonoid contents were measured according to the method of Choi *et al.* (18). Briefly, 1 mL of sample was mixed with 10 mL of diethylene glycol and 1 mL of 1 N NaOH. After the reaction mixture was kept at 37°C for 90 min, the absorbance was read at 420 nm. The absorbance of the yellow colored solution was recorded at 420 nm against a blank containing 0.05 mg/mL of 50% methanol. The total flavonoid contents were calculated as rutin equivalents using calibration curves prepared with rutin hydrate standard solutions covering a concentration range between 0 and 0.05 mg/mL (19). Estimation of the flavonoid compounds was carried out in triplicate. The results are mean values±SD, and expressed as mg of rutin equivalents (REs)/g of extract.

Identification of flavonoid compounds by LC-LC/MS Each sample of 500 mg was mixed with 40 mL of 62.5% aqueous methanol containing butylated hydroxyanisole (BHA, 2 g/L), and then 10 mL of 6 M HCl was carefully added to make 1.2 M HCl in 50% aqueous methanol. The extract was heated at 90°C in a water bath with refluxing for 2 hr, and then allowed to cool in the refrigerator (20). The hydrolysate was processed and subjected to qualitative and quantitative analyses using HPLC and LC-MS. Approximately 3 mL of the hydrolyzed samples were filtered through a 0.45 μm filter, prior to a 20 μL injection into the HPLC system. The mobile phase consisted of 1% formic acid in water (A) and 1% formic acid in acetonitrile (B). After LC separation, the analyses were performed by atmospheric pressure chemical ionization-tandem mass spectrometry in the positive ion mode. The chromatograph was operated in the gradient mode starting at a mobile phase of A:B (80:20, v/v), changing within 150 min to A:B (50:50, v/v). The eluent was delivered at a flow rate of 1 mL/min and the UV spectrum was monitored at 280 and 345 nm. Mass spectrometric analysis was performed on a mass spectrometer coupled with a syringe pump for continuous flow injection, consisting of an electo-spray ionization (ESI) module. The eluents were 1% formic acid in water (A) and 1% formic acid in acetonitrile (B), at a flow-rate of 1 mL/min, and the gradients were 20-55% (A) and 80-45% (B) in 16 min. The source block and desolvation temperatures were 200 and 70°C, respectively. The peaks were identified based on the comparisons of retention times, UV-Vis, and MS spectra of the standards (quercetin and kaempferol), and quantification was conducted by external standardization under HPLC conditions.

Cell culture The WB-F344 cells used in this study were kindly donated by Dr. J. Trosko at Michigan State University (East Lansing, MI, USA). The culture was maintained in a 5% CO_2 incubator at 37°C in D-media supplemented with 5% FBS and PSN mixture (3 mL/L), and continuously passaged at subconfluency. The cells were grown in 75 mm tissue culture plates and the culture medium was changed every other day.

Cell viability assay The cytotoxic effects of each sample on the WB-F344 cells were measured by MTT assay, based on the ability of the live cells to convert tetrazolium salt into purple formazan (21). Briefly, the cells were seeded into 24-well microplates and incubated overnight. The attached cells were treated with different concentrations of the test samples or its vehicle (0.1% DMSO) for 24 hr. During incubation, 50 μL of MTT stock solution (5 mg/mL) was added to each well and then the plates were further incubated for 4 hr at 37°C. The supernatant was removed and 500 μL of DMSO was added to each well to dissolve the insoluble purple formazan crystals; it was then transferred into 96-well microplates to read the absorbency at 570 nm with an EL800 microplate reader (Bio-Tek Instrument, Winooski, VT, USA). All the measurements were performed in triplicate. The results are expressed as percentages of proliferation with respect to the negative or vehicle control group.

GJIC assay GJIC was measured by the scrape loading/dye transfer assay (SL/DT) according to the method described by Jong *et al.* (22). Briefly, the WB-F344 cells were treated with or without various concentrations of the AVP extracts for 24 hr during the logarithmic phase of growth, followed by TPA (10 ng/mL) treatment for 1 hr. The GJIC assay was conducted at the non-cytotoxic dose levels of each chemical as determined by the MTT assay. Following incubation, the cells were washed twice with 2 mL of PBS. The fluorescent dye, Lucifer yellow, was injected into the washed cells and 3 scrapes were made with a surgical-steel-bladed scalpel at low light intensities. The scrapes were performed to ensure that the scrape traversed a large group of confluent cells. After 3 min of incubation, the cells were washed with 10 mL of PBS and then fixed with 2 mL of 4% formalin solution. The numbers of communicating cells visualized with the dye were counted under an inverted fluorescent microscope (Ix70; Olympus, Okaya, Japan).

Statistical analysis All results are expressed as the % mean ± standard deviation (SD), and the data were analyzed

using Student's *t*-tests for significant differences. Statistical significance was defined as $p<0.05$.

Results and Discussion

Total phenolic and flavonoid contents of AVP The total phenolic and flavonoid contents of all the plant parts are shown in Table 1. The results indicate that the total phenolic and flavonoid contents of the 6 plant-part extracts varied considerably and showed very similar patterns. The total phenolic contents of the different parts raged from 3.07 to 24.29 mg GAE/g of extract. The total phenolic contents in each plant-part extract were: leaf (24.29 mg GAE/g of extract) > seed (19.79 mg GAE/g of extract) > root (14.93 mg GAE/g of extract) > flower (12.50 mg GAE/g of extract) > flower stalk (11.29 mg GAE/g of extract) > stem (3.07 mg GAE/g of extract).

The total flavonoid contents of the different plant parts ranged from 2.53 to 20.00 mg GAE/g (Table 1). The total flavonoid contents of each plant-part extract were: leaf (20.00 mg RE/g of extract) > seed (16.29 mg RE/g of extract) > root (12.29 mg RE/g of extract) > flower (10.29 mg RE/g of extract) > flower stalk (9.29 mg RE/g of extract) > stem (2.53 mg RE/g of extract). The highest total phenolic and flavonoid content were observed in the leaf and the lowest levels were observed in the stem.

Identification and quantification of flavonoids by LC-MS analysis Food-derived flavonoids such as quercetin, kaempferol, and myricetin have various pharmacological activities, including antimutagenic, antioxidant, and anti-carcinogenic effects, both *in vitro* and *in vivo* (23, 24). The flavonoids from the 6 plant-part extracts of AVP were determined by HPLC analysis, and LC coupled with the MS technique was also applied for results confirmation. According to the LC/MS results, a mass spectrum found at peak 1 was quercetin ($[M]^+$ 303.2 *m/z*), and peak 2 was considered to be kaempferol, with an $[M]^+$ of 286.6 *m/z* (Fig. 1). Lim *et al.* (1) reported that the major flavonoids in AVP are astragalin and kaempferol 3,4'-*o*-β-D-glucopyranoside. For our study, the contents of quercetin and kaempferol in each extract were quantified and are listed in Table 2. The quercetin levels were highest in the flowers (25.8 μg/g), and kaempferol levels were highest in

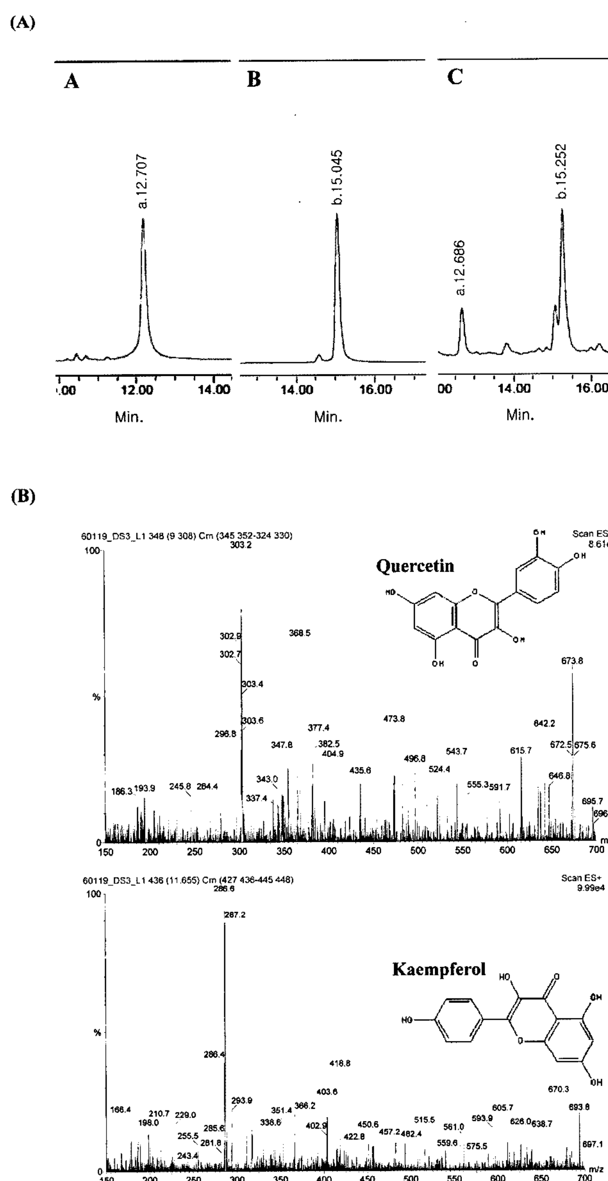

Fig. 1. HPLC-chromatogram (A) and MS spectra (B) of quercetin and kaempferol in AVP. (A) chromatogram of quercetin-A (a), kaempferol-B (b), and the flower portion of AVP extracts-C.

Table 1. Total phenolic and flavonoid contents of the different plant parts of AVP[1)]

	Total phenolic content (mg GAE/g extract)	Total flavonoid content (mg RE/g extract)	Yield (%)
Flower	12.50±0.01	10.29±0.08	47.7
Flower stalk	11.29±0.01	9.29±0.07	25.8
Seed	19.79±0.01	16.29±0.13	15.7
Leaf	24.29±0.02	20.00±0.15	32.8
Stem	3.07±0.00	2.53±0.02	45.6
Root	14.93±0.01	12.29±0.10	43.7

[1)]Results are mean values±SD of duplicate analyses of 3 replications.

Table 2. The quercetin and kaempferol contents of the different plant parts of AVP[1)]

	Quercetin content (μg/g powder)	Kaempferol content (μg/g power)
Flower	27.4±0.9	37.5±1.3
Flower stalk	12.9±0.0	8.4±0.1
Seed	12.1±0.1	7.9±0.4
Leaf	18.6±0.5	12.1±1.0
Stem	11.6±0.1	6.0±0.3
Root	-	67.4±0.4

[1)]Results are mean values±SD of duplicate analyses of 3 replications.

the roots (64.4 μg/g). Quercetin is one of the most abundant flavonoids present in fruits and vegetables, and it occurs primarily in the leaves and other plant parts of AVP. Quercetin's potential as a natural anticarcinogen has been reported (24). A previous study has offered data on the contents of quercetin and kaempferol in black tea (34.8 and 110 μg/L, respectively) and in linden flower (31.7 and 113 μg/L, respectively) in Turkey (25). Our result for the quercetin level of the flower part of AVP was higher than the level for linden flower, and the kaempferol level of the root part of AVP was lower than that of black tea or linden flower; however, it was higher than honey (24.2±9 μg/g).

Cytotoxic effects of the AVP extracts on WB-F344 rat liver epithelial cells The effects of the AVP extracts on toxicity and proliferation were measured in WB-F344 rat liver epithelial cells using an MTT assay. As shown in Fig. 2, at concentrations up to 1 mg/mL, the leaf, flower stem, seed, and stem extracts did not show significant effects on the morphology and viability of the cells. However, the flower and root extracts caused significant decreases of up to 70% (p<0.05) on cell viability at concentrations of more than 250 μg/mL (30%).

Effect of the AVP extracts on GJIC GJIC is a target of different modulators of carcinogenesis (tumor promoters and anti-promoters) (26). In the present study, we used non-tumorigenic WB-F344 rat liver epithelial cells as a model system to screen and verify the anticarcinogenic activity of AVP. In addition, TPA was used as a disruptor of GJIC and as a well-known cancer promoter that activates protein kinase C (PKC) (27). TPA almost immediately disrupts GJIC in several kinds of cell lines, including WB-F344 rat liver epithelial cells (28, 29). It has frequently been observed that most tumor promoting agents such as TPA, inhibit intercellular communications and reduced GJIC capacity during carcinogenesis (30, 31).

The GJIC of the WB-F344 cells was assessed by applying a cocktail of the fluorescent dye Lucifer yellow, which passes through the gap junction channel, after exposing the cells to TPA (10 ng/mL) for 1 hr. The Lucifer yellow only stayed at the incision sites or artificially damaged cells. According to the results, the treatments of the flower and stem AVP extracts (0.05-0.5 mg/mL) were effective at preventing TPA-induced down-regulation of GJIC (Fig. 3) in a dose-dependent manner. GJIC was reduced by approximately 70% (62.3±12.5 cells) compared to the control (209±9.5 cells, 100%) by TPA treatment alone in the WB-F344 rat liver epithelial cells. However, the stem extract (0.5 mg/mL) restored GJIC to basal levels (92%, 204±2.3 cells, p<0.01) and the flower extract (0.2 mg/mL) stimulated GJIC to 82.5% (172.6±8.3 cells, p<

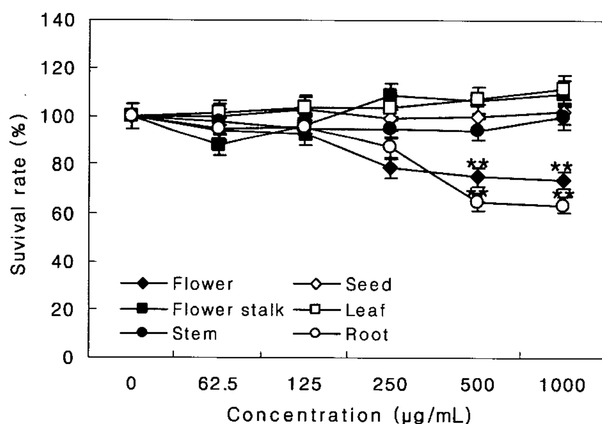

Fig. 2. Cell proliferative effects according to concentration of the different AVP plant-part extracts on the WB-F344 rat liver epithelial cells. Results represent the mean±SD (n=3). *p< 0.05 and **p<0.01, the control (0.1% DMSO treated) group vs. the treated groups.

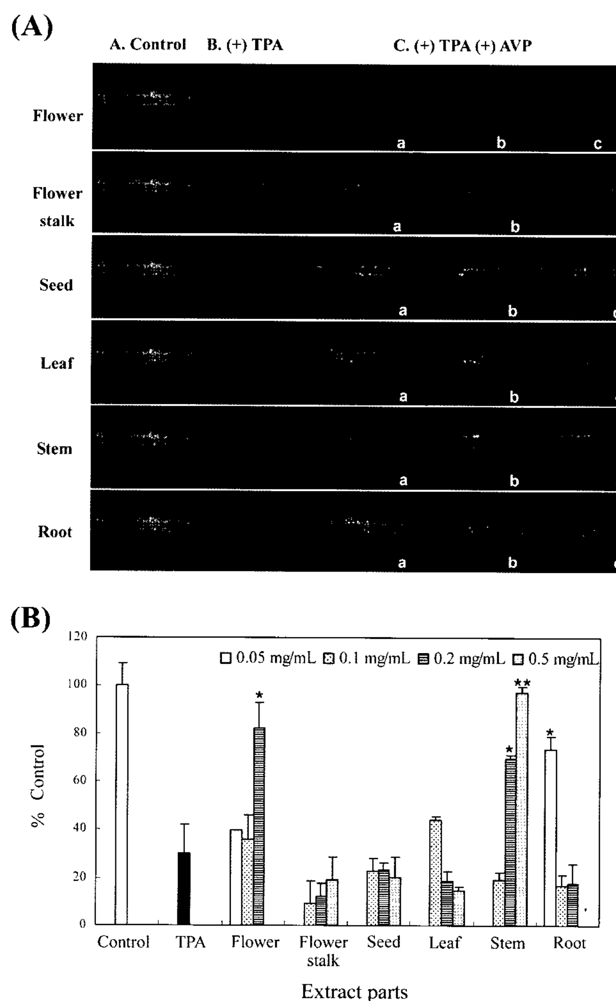

Fig. 3. Dose-response of the AVP extracts on TPA-induced down regulation of GJIC in WB-F344 rat liver epithelial cells. (A) GJIC in WB-F344 cells was measured by the Lucifer yellow dye transfer assay: A. Control (0.1% DMSO); B. TPA (10 ng/mL); C. TPA+each plant-part extract from AVP. The treated concentrations of the root and flower extracts were 0.05 (a), 0.1 (b), and 0.2 mg/mL (c), and the concentrations of the other plant-part extracts were 0.1 (a), 0.2 (b), and 0.5 mg/mL (c). (B) GJIC is presented as the percent of the control. Basal communication of the control was 209.3±9.5 cells, which was set to 100%. *p<0.01, significantly different from treatment with TPA. The data represent the mean±SD of 3 independent experiments.

0.05) when they were applied together with TPA. It was shown that the number of communicating cells was completely recovered from the loss observed in the TPA treated group. Although the root extract presented a recovery effect of about 40% at a low concentration (0.05 mg/mL) compared to the TPA alone, a stimulating effect of the root extract on GJIC at concentrations more than 0.05 mg/mL was not shown. We presumed the result was attributed to the extract's cell cytotoxicity. However, the other plant-part extracts did not show stimulating effects on GJIC either. Chaumontet *et al.* (32) demonstrated that two flavonoids (apigenin and tangeretin) enhanced GJIC in rat liver epithelial cells. Ale-Agha *et al.* (33) reported that GJIC is stimulated by several micronutrients such as genistein, retinoids, carotenoids, (-)-epicatechin (4-40 μM), and genistein (40 μM), and that they exhibited stimulatory effects on TPA-induced GJIC in WB-F344 rat liver epithelial cells.

In the present study, we identified structurally two major flavonoids, quercetin and kaempferol. In addition, we demonstrated that AVP extracts prevent the inhibition of GJIC induced by TPA in WB-F344 cells. Furthermore, the results seem to imply that the flower and stem portions of AVP could be used as anticarcinogenic agents. Further studies will be conducted to assess the mechanism of action of AVP on the signal transduction pathways related to carcinogenesis.

Acknowledgments

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