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Inhibitory Effects of *Allium victorialis* var. *platyphyllum* Extracts on Platelet Aggregation and Vascular Smooth Muscle Cell Proliferation

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Abstract The $CHCl_3$, EtOAc, and *n*-BuOH fractions showed a marked inhibition of 5% fatal bovine serum (FBS)-induced cell proliferation. The IC_{50} values of the chloroform fractions from leaf, stem, and root as well as the *n*-BuOH and EtOAc fraction from root on cell proliferation were 1.2±0.4, 17.2±6.4, 81.8±33.2, 40.8±8.0, and 237.1±85.6 μg/mL, respectively. On the other hand, the EtOAc fractions, and the $CHCl_3$ fraction significantly inhibited collagen-, arachidonic acid-, U46619-, and thrombin-induced platelet aggregations. The IC_{50} values of EtOAc fraction from leaf, and the $CHCl_3$ and EtOAc fraction from stem were 214.1±12.2, 134.3±2.5, and 42.6±7.0 μg/mL with collagen, 312.4±7.5, 158.9±1.7, and 82.2±2.7 μg/mL with arachidonic acid, 31.1±2.4, 48.7±0.3, and 29.7±1.1 μg/mL with U46619, and 36.7±2.4, 69.1±11.3, and 34.2±0.1 μg/mL with thrombin, respectively. Taken together, these data provide new evidence that fractions from *Allium victorialis* var. *platyphyllum* (AVP) are able to inhibit vascular smooth muscle cell (VSMC) proliferation and platelet aggregation, which may be a novel resource for the development of anti-atherothrombotic agents.

Key words: *Allium victorialis* var. *platyphyllum* (AVP), antiproliferation, antiplatelet, atherothrombotic agent

Introduction

Platelet aggregation and vascular smooth muscle cell (VSMC) proliferation are essential events in the pathogenesis of atherothrombotic diseases such as stroke, myocardial infarction, and restenosis after angioplasty (1,2). A number of platelet active substances such as collagen, thromboxane A_2, and thrombin, as well as peptide growth factors and cytokines such as platelet derived growth factor (PDGF), epidermal growth factor, and tumor necrosis factor alpha, etc, released from platelets and VSMCs in response to vascular injury are known to play important roles in stimulating platelet activation and VSMC proliferative responses (3). Therefore, the inhibition of both platelet activation and VSMC proliferation represents a promising approach for the prevention of cardiovascular diseases such as thrombosis and atherosclerosis (2,4).

Natural products have been the starting point for the discovery of many important modern drugs. *Allium victorialis* var. *platyphyllum* (AVP) is a perennial herb widely distributed in Odaesan(Mt), Ullungdo(Island) and northeast Asia (5,6). The leaves and bulbs have been used not only as edible herbs, but also as functional foods for improving gastritis and heart failures (7). It has been reported that the ethanol fraction of AVP has antitumor and antiatherogenic effects. AVP fractions have also been reported to have cholesterol lowering, liver protective and anti-diabetes effects (8). However, the effects of AVP fractions on platelet aggregation and VSMC proliferation remain unknown. Therefore, this study was conducted to investigate antiplatelet and antiproliferative activities of AVP fractions on platelet aggregation induced by various agonists and 5% FBS-stimulated rat aortic VSMC proliferation.

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Materials and Methods

Materials Collagen, arachidonic acid, ADP, and thrombin were purchased from Chrono-Log Co. (Havertown, PA, USA). U46619 and bovine serum albumin (BSA) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). 2-(2-Methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H* tetrazolium monosodium salt (CCK-8) (Dojindo, Kumamoto, Japan). All other chemicals were of analytical grade.

Preparation of AVP fractions AVP was collected in June 2006 on Seorrim, Gangwon, Korea, and divided into leaf, stem, and root portions. Each portion was freeze dried and pulverized. Dried plant material was stored in a freezer at −60°C. The dried leaf, stem, and root parts of AVP were extracted with EtOH. The EtOH extract was filtered on a rotary evaporator under reduced pressure to remove the solvent. The EtOH extract was fractionated with $CHCl_3$, EtOAc, *n*-BuOH, and H_2O, and each fraction was dried by evaporation (9,10).

Animals New Zealand white rabbits were purchased from Sam-Tako Animal Co. (Osan, Korea) and acclimatized for 1 week at 24°C and 55% humidity. The animals had free access to a commercial pellet diet obtained from Samyang Co. (Wonju, Korea) and drinking water before experiments. The animal studies have been carried out in accordance

with the Guide for the Care and Use of Laboratory Animals, Chungbuk National University, Korea.

Washed rabbit platelet preparation and platelet aggregation assay Blood was withdrawn from the ear aorta of male New Zealand white rabbits and collected directly into 0.15%(v/v) anticoagulant citrate dextrose (ACD) solution that contained 0.8% citric acid, 2.2% trisodium citrate, and 2%(w/v) dextrose. Washed platelet were prepared as previously described (11). Briefly, platelet rich plasma (PRP) was obtained by the centrifugation of rabbit blood at 230×g for 10 min. Platelets were sedimented by centrifugation of the PRP at 800×g for 15 min and washed with Hepes buffer (137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 5.6 mM glucose, and 3.8 mM Hepes, pH 6.5) containing 0.35% BSA, and 0.4 mM EGTA [ethylene glycol bis (β-aminoethyl ether) N,N,N'N' (N')-tetraacetic acid]. The washed platelets were resuspended in Hepes buffer (pH 7.4) and adjusted to 4×10^8 platelets/mL.

Platelet aggregation was measured by using an aggregometer (Chrono-Log Co.) according to the turbidimetry method of Born and Cross (12) as previously described (11). Briefly, washed platelet suspension was incubated at 37°C in the aggregometer with stirring at 1,000 rpm, and then distilled water or various concentrations (12.5-400 μM) of AVP fractions were added. After a 3 min preincubation, platelet aggregation was induced by the addition of collagen (10 μg/mL), arachidonic acid (100 μM), U46619 (1 μM), or thrombin (0.05 U/mL). The resulting aggregation measured as the change in light transmission was recorded for 8 min. The results were expressed a percentage the control.

Vascular smooth muscle cell (VSMC) proliferation assay VSMC proliferation was assessed using the CCK-8-assay kit according to the manufacturer's instructions. In brief, cells were seeded at a concentration of 50,000-100,000 cells/mL in 96-well plates and grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) for 48 hr. The cells were then cultured with serum-free medium containing various AVP fractions for 24 hr. Cells were then stimulated with 5% FBS for another 24 hr. Ten μL of CCK-8 reagent was added to each well at 22 hr, and the plate was further incubated the plate at 37°C for 2 hr. The optical density was then read at 450 nm in an enzyme-linked immunosorbent assay (ELISA) reader (Molecular Devices, Sunnyvale, CA, USA).

Statistical analysis The experimental results were expressed as the mean±SEM. One-way analysis of variance (ANOVA) was used for multiple comparisons followed by Dunnett's test. The data were considered a significantly different with a probability less than 0.05.

Results and Discussion

Effect of AVP fractions on washed rabbit platelet aggregation *in vitro* As shown in Fig. 1, the EtOAc fractions from leaf and stem, and the $CHCl_3$ fraction from stem inhibited collagen-, arachidonic acid-, U46619-, and thrombin-induced platelet aggregation *in vitro*. The IC_{50} values of the EtOAc fraction from leaf, the $CHCl_3$ and EtOAc fractions from stem were 214.1±12.2, 134.3±2.5, and 42.6±7.0 μg/mL with collagen, 312.4±7.5, 158.9±1.7, and 82.2±2.7 μg/mL with arachidonic acid, 31.1±2.4, 48.7±0.3, and 29.7±1.1 μg/mL with U46619, and 36.7±2.4, 69.1±11.3 and 34.2±0.1 μg/mL with thrombin, respectively.

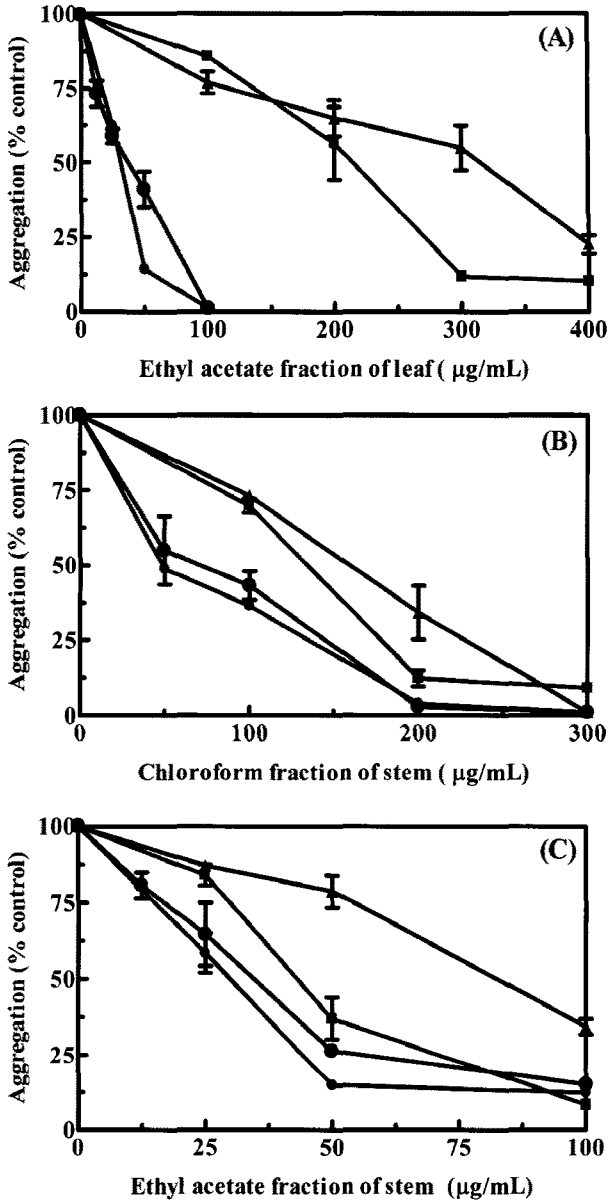

Fig. 1. Effect of various fractions from *Allium victorialis* var. *platyphyllum* on washed rabbit platelet aggregation *in vitro*. -■-: collagen (10 μg/mL), -▲-: arachidonic acid (100 μM), -●-: U46619 (1 μM), -●-: thrombin (0.05 U/mL). Data are expressed as the mean±SEM (n=4).

Effect of AVP fractions on the proliferation of VSMCs As shown in Fig. 2, cell proliferation induced by 5% FBS was significantly inhibited by the $CHCl_3$ fraction from leaf, stem and root (3, 20 and 100 μg/mL) as well as the *n*-BuOH fraction from root (10 and 50 μg/mL). However, the EtOAc fraction from stem had only a slight inhibitory effect (100 μg/mL). The IC_{50} values of the $CHCl_3$ fraction from leaf, stem, and root as well as the *n*-BuOH fraction from root, and the EtOAc fraction from stem on 5% FBS-induced VSMC proliferation were estimated 1.2±0.4,

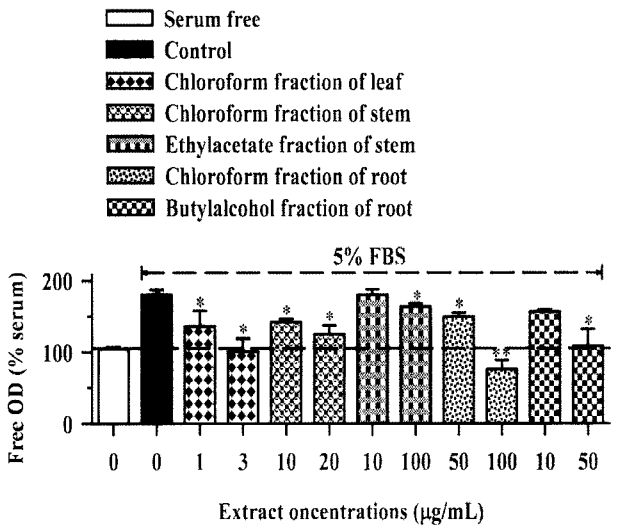

Fig. 2. Effect of various fractions from *Allium victorialis* var. *platyphyllum* on 5% FBS-stimulated VSMC proliferation. Data are expressed as the mean±SEM (n=3). $*p<0.05$, $**p<0.01$ compared with 5% FBS alone.

Table 1. IC_{50} values of various fractions on 5% FBS-induced VSMC proliferation[1)]

Fraction of *Allium victorialis* var. *platyphyllum*	IC_{50} value (μg/mL)
$CHCl_3$ fraction from leaf	1.2±0.4
$CHCl_3$ fraction from stem	17.1±6.4
EtOAc fraction from stem	237.0±85.6
$CHCl_3$ fraction from root	81.8±33.2
n-BuOH fraction from root	40.8±8.0

[1)]Inhibitory activity was expressed as the mean of the 50% inhibitory concentration (IC_{50}) of triplicate experiments obtained by the interpolation of concentration-inhibition curves.

17.2±6.4, 81.8±33.2, 40.8±8.0, and 237.1±85.6 μg/mL, respectively (Table 1).

It has been reported that antiplatelet agents are effective in the prevention and treatment of thrombotic disorders such as acute coronary syndromes and myocardial infarction (4). In addition, inhibition of the abnormal proliferation of vascular smooth muscle cells in arterial walls has also been demonstrated to be effective in disorders such as atherosclerosis and restenosis after angioplasty (3). Considering that platelet aggregation and VSMC proliferation occur together in patients with the risk of atherothrombosis, the inhibition of both platelet aggregation and VSMC proliferation provides a promising approach to the treatment of such diseases. As part of our ongoing search for inhibitors of platelet aggregation and VSMC proliferation derived from natural products, we clearly demonstrate here that various fractions of AVP display potent inhibitions of platelet aggregation and VSMC proliferation.

Washed rabbit platelets were preincubated with various concentrations of each fraction derived from AVP, and then exposed to 4 different agonists to elucidate the inhibitory effects of each fraction on platelet aggregation. As shown in Fig. 1, the EtOAc fractions from leaf and stem, and the $CHCl_3$ fraction from stem inhibited collagen-, arachidonic acid-, U46619-, and thrombin-induced platelet aggregation *in vitro*. The EtOAc fraction from stem exhibited the most potent inhibition of platelet aggregation, and the relative order of potency of the various fractions was EtOAc fraction from stem>$CHCl_3$ fraction from stem>EtOAc fraction from leaf, while the water and *n*-BuOH fractions were ineffective at all concentrations tested. The inhibitory potency of the EtOAc fractions from leaf on platelet aggregation varied with the different agonists used. It was the most effective at inhibiting platelet aggregation induced by U46619 and thrombin, yet was weak at inhibiting stimulation by collagen and arachidonic acid. Though U46619 and thrombin activate platelets via different receptors, both receptors belong to the G protein super family, which indicates that the EtOAc fraction from leaf may be especifically effective at inhibiting signals transduced via G protein-coupled receptors in platelets. The $CHCl_3$ and EtOAc fractions from stem, exhibited a consistent degree of inhibition with each agonist (Fig. 1B and 1C), and the EtOAc fraction was more potent than the $CHCl_3$ fraction.

The inhibitory effects of various fractions of AVP on the proliferation of rat aortic VSMCs induced by 5% FBS were determined by CCK-8 assay. The $CHCl_3$ fraction from leaf exhibited the most potent inhibition of VSMC proliferation, and the order of antiproliferative potency of the various fractions was $CHCl_3$ fraction from leaf>$CHCl_3$ fraction from stem>*n*-BuOH fraction from root>$CHCl_3$ fraction from root>EtOAc fraction from stem, while the water fraction was ineffective at all concentrations tested.

Since FBS consistes of various growth factors and cytokines, the inhibition of these factors may be due to the inhibition of intracellular signals involved in cell proliferation rather than specific receptors. Further studies addressing this possibility are currently in preparation.

The different biological activities of AVP fractions may be due to the different chemical constituents contained in the fractions. It has been reported that allicin and steroidal saponin were found in AVP, and that these components may contribute to the antibiotic, cholesterol lowering, anti-atherosclerotic, anti-tumor, and antioxidative activities (5, 13-15). Though the molecular targets of AVP fractions on platelet aggregation and VSMC proliferation remain unknown, it seems reasonable to speculate that the above described constituents of AVP have such pharmacological activities.

In conclusion, our results demonstrate that specific fractions of AVP have a potent inhibitory effect on platelet aggregation and rat aortic VSMC proliferation, thus AVP may be a natural resource in the development of novel therapeutic agent for the treatment and prevention of thrombosis and atherosclerosis diseases.

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