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RESEARCH NOTE

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Pharmacological Effects of Asaronaldehyde Isolated from *Acorus gramineus* Rhizome

Hyo-Gyung Kim, Ju-Hyun Jeon, Moo-Key Kim* and Hoi-Seon Lee*

Faculty of Applied Biotechnology and Research Center for Industrial Development of Biofood Materials, College of Agriculture, Chonbuk National University, Chonju, Jeonbuk 561-756, Korea

Abstract Antibacterial and antiplatelet activities of *Acorus gramineus* rhizome-derived asaronaldehyde and asaron were analyzed using platelet aggregometer and six human intestinal bacteria. Active constituent of *A. gramineus* rhizome was isolated and characterized as asaronaldehyde by spectral analyses. At 2 and 1 mg/disk, asaronaldehyde exhibited strong inhibition of *Clostridium perfringens* and *C. difficile* without adverse effects on growth of beneficial bacteria such as *Bifidobacterium bifidum*, *Lactobacillus acidophilus*, and *L. casei*. Asaron also revealed moderate growth inhibition against *C. perfringens* and *C. difficile* at 2 mg/disk, no growth-inhibiting activity was observed on *B. bifidum*, *L. acidophilus*, *L. casei*, and *E. coli*. At 50% inhibitory concentration (IC_{50}) value, asaronaldehyde was effective in inhibiting platelet aggregation induced by collagen (IC_{50}, 27.6 μM) and arachidonic acid (IC_{50}, 53.7 μM). These results suggest asaronaldehyde may be useful as lead compound for inhibiting platelet aggregation induced by collagen and arachidonic acid.

Keywords: *Acorus gramineus*, asaronaldehyde, asaron, intestinal bacteria, *Clostridium perfringens*

Introduction

Acorus (*Acorus gramineus* L., Araceae) is an aromatic herb, indigenous to East Asia (1), and is often regarded as a member or a close relative of the Arum family (Araceae). Dried rhizomes of *A. gramineus* are widely used in the Chinese and Ayurvedic traditional medicine systems for a variety of indications (2, 3). The rhizome contains active ingredients possessing anthelmintic (2, 4), insecticidal (5), antifungal (6), anticholinesterase (7), antihistaminic (8), neuroprotectic (9), and therapeutic activities (10-12). *A. gramineus*, in combination with other herbal drugs, is also one of the major components in oriental medical prescriptions for treatment of stroke (13, 14). The dry rhizome of *A. gramineus* Soland contains various compounds such as (*Z*)-asarone (63-81%), (*E*)-asarone (8-14%), caryophyllene (1-4%), isoasarone (0.8-3.4%), (*Z*)-methyl isoeugenol (0.3-6.8%), and safrol (0.1-1.2%) (15, 16). Although various inhibitory effects of *A. gramineus* have been reported, relatively little work has been done on the inhibitory activities of the harmful intestinal bacteria and platelet aggregation by *A. gramineus*. In this study, to develop new and safer types of antibacterial and antiplatelet agents, the growth-inhibitory effect of an active compound isolated from the rhizome of *A. gramineus* was assessed against intestinal bacteria and platelet aggregation. Additionally, the antibacterial and antiplatelet activities of commercially available compounds derived from the rhizome of *A. gramineus* were assessed for comparison.

Materials and Methods

Chemicals The rhizome of *A. gramineus* was purchased from Boeun medicinal herb shop (Kyungdong Market, Seoul, Korea). Collagen, arachidonic acid (AA), and thrombin were obtained from Chrono-Log Co. (Havertown, PA, USA). Other chemicals were of analytical grade.

Isolation and identification The dried rhizome (6 kg) from *A. gramineus* was finely powdered, extracted twice with methanol (25 L) at room temperature, and filtered. The combined filtrate was concentrated under vacuum at 40°C, resulting in 14.5% yield (dry weight basis). The extract was sequentially partitioned into hexane, chloroform, ethyl acetate, butanol, and water portions for subsequent bioassay. The organic solvent portions were concentrated to dryness by rotary evaporation at 40°C, while the water portion was freeze-dried. The hexane fraction (13 g) was chromatographed on a silica gel column (Merck 230-400 mesh, 650 g, 7 cm i.d. × 85 cm), and successively eluted with a stepwise gradient of chloroform/methanol (100:0, 90:10, 70:30, 50:50, 0:100, v/v). Column fractions were analyzed by TLC (silica gel G), and fractions (P1-P4) showing similar TLC patterns were pooled. The bioactive fraction (P2, 5.9 g) was successively re-chromatographed on a silica gel column using hexane/ethyl acetate (120:1, v/v). The resulting six fractions (P31-P36) were bioassayed as described below. The active P33 fraction was purified by Prep HPLC (Spectra System P2000, Thermo Separation Products, Madison, WI, USA) to separate the antibacterial and antiplatelet constituents. The column was a μBondapak C_{18} (39 mm i.d. × 300 mm, Waters, MA, USA). Four fractions (P41-P44) were eluted by methanol/water (4:6, v/v) at a flow rate of 2.4 mL/min, and one potent active compound (asaronaldehyde, 28 mg) was isolated.

Structural determination of the active isolates was made by spectroscopic analysis. ^{1}H-NMR and ^{13}C-NMR spectra were recorded in deuterochloroform with a BRUKER AM-500 spectrometer (Rheinspettem, Germany) at 400

*Corresponding author: Tel: +82-63-270-2544; Fax: +82-63-270-2550
E-mail: mookey@chonbuk.ac.kr, hoiseon@chonbuk.ac.kr
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and 100 MHz (TMS as an internal standard), respectively, and chemical shifts were given in δ (ppm). UV spectra were obtained in methanol with a JASCO V-550 spectrometer (Tokyo, Japan) and EI-MS spectra on a JEOL GSX 400 spectrometer (Tokyo, Japan).

Bacteria strains and culture conditions The bacterial strains used in this study were *Bifidobacterium bifidum* ATCC 29521, *Clostridium perfringens* ATCC 13124, *C. difficile* ATCC 9689, *Escherichia coli* ATCC 11775, *Lactobacillus acidophilus* KCTC 3145, and *L. casei* ATCC 27216 isolated from human feces. These strains were routinely grown on bacteria culture [Brain Heart Infusion broth (pH 7.6), deMan Rogosa Sharpe broth (pH 5.7), and 25% glycerol] at -80°C and subcultured on EG agar (Eiken Chemical, Tokyo, Japan) when required. The plates were incubated at 37°C for 2 days in an anaerobic chamber with an atmosphere of 80% N_2, 15% CO_2, and 5 % H_2. The bacteria were then grown in EG broth (pH 6.8).

Growth-inhibiting assay One loopful of bacteria was suspended in 1 mL sterilized physiological saline. An aliquot (0.1 mL) of the bacterial suspensions was seeded on an EG agar. A sample in 100 μL methanol solution was applied using a Drummond glass microcapillary to a paper disk (Advantec 8 mm-diameter and 1-mm thickness). After evaporation of the solvent, the disks were placed on the agar surface inoculated with the test bacteria. All plates were incubated anaerobically at 37°C for 2 days. The control disks received 100 μL methanol, the concen-tration at which no adverse effect against the micro-organisms used was observed. All tests were performed in triplicates. The inhibitory responses were classified as previously described (17): potent response, ++++, zone diameter >30 mm; strong response, +++, zone diameter 21-30 mm; moderate response, ++, zone diameter 16-20 mm; weak response, +, zone diameter 10-15 mm; and little or no response, -, zone diameter <10 mm.

Preparation of washed rabbit platelets Platelet-rich plasma (PRP) obtained from the blood of a healthy male white rabbit was anticoagulated with one-tenth volume of 1% EDTA by centrifugation at 230 × *g* for 10 min at room temperature. Platelets were sedimented by centrifugation of the PRP at 800 × *g* for 15 min and washed twice 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% bovine serum albumin and 0.4 mM EDTA. The washed platelets were resuspended in Hepes buffer (pH 7.4). The platelet number was counted by Coulter Counter (Coulter Electronics, Hialeah, FL, USA) and adjusted to a concentration of 3×10^8 platelets/mL.

Aggregation of washed rabbit platelets Platelet aggregation was measured using an aggregometer (470-vs, Chrono-log Co., City, PA, USA) as previously described (18). Briefly, washed platelets (3×10^8 platelets/mL) were incubated at 37°C for 3 min in the aggregometer with various concentrations of samples for 3 min in the presence of 1 mM $CaCl_2$. Platelet aggregation was then induced by addition of collagen (2 μg/mL), AA (100 μM) or thrombin (0.1 unit/mL). The resulting aggregation, measured as the change in light transmission, was recorded for 10 min. Each inhibition rate was obtained from the maximal aggregation induced by respective agonists. The extents of inhibition of platelet aggregation are expressed as % inhibition (*X*) using the following equation: $X = [(A-B)/A] \times 100$; A: maximal aggregation of control, B: maximal aggregation of sample treated washed platelets.

Results and Discussion

Biological activity of hexane fraction obtained from *A. gramineus* rhizome-derived extracts Antibacterial and antiplatelet constituents from the hexane fraction of *A. gramineus* rhizomes were purified using silica gel column. Four subfractions (P1-P4) obtained from the hexane fraction were analyzed for their inhibitory effects against human intestinal bacteria and collagen-induced platelet aggregation, and the bioactive P2 fraction showed high inhibitory effects against harmful intestinal bacteria such as *C. perfringens* and *C. difficile*, and collagen-induced platelet aggregation, whereas P1, P3, and P4 fractions exhibited weak activities (data not shown).

Identification of active constituent Purification of the antibacterial and antiplatelet components of P2 fraction by prep. HPLCgave one bioactive compound (P42). Bioassay-guided fractionation of the *A. gramineus* rhizomes afforded an active constituent identified by spectroscopic analyses, including EI-MS, ^{1}H-NMR, and ^{13}C-NMR and by direct comparison with an authentic reference compound. The active constituent was characterized as asaronaldehyde. The compound was identified based on the following evidence: asaronaldehyde ($C_{10}H_{12}O_4$, MW: 196.2); EI-MS (70 eV) m/z (% relative intensity): M^+ 196 (100), 181 (54), 150 (25), 139 (9), 125 (23), 110 (12), 95 (7), 69 (8), 53 (6); ^{1}H-NMR (CD_3OD); δ 10.18 (1H, *d*, *J*= 2.44 Hz), 7.23 (1H, *d*, *J*= 2.44 Hz), 6.68 (1H, *d*, *J*= 2.16 Hz), 4.87 (1H, *d*, *J* = 2.44 Hz), 3.93 (1H, *m*, *J* = 6.6 Hz), 3.78 (1H, *d*, *J*= 2.44 Hz); ^{13}C-NMR (CD_3OD); 189.47, 160.77, 158.17, 144.92, 118.03, 110.25, 97.59, 56.84, 56.72, 56.70.

Growth-Inhibiting Activity of Asaronaldehyde The growth-inhibiting activity of asaronaldehyde against the six intestinal bacteria was examined using the impregnated paper disk method (Table 1). The responses varied according to the dose and the bacterial strain tested. In the test with *C. perfringens* and *C. difficile*, asaronaldehyde exhibited strong inhibition at 2 mg/disk and moderate at 1 and 0.5 mg/disk, whereas little or no inhibition toward *B. bifidum*, *E. coli*, *L. acidophilus*, and *L. casei* at 2 mg/disk (Table 1). This result indicated that the growth-inhibiting activity of asaronaldehyde was more pronounced in *C. perfringens* and *C. difficile*, as compared to bifidobacteria, *E. coli*, and lactobacilli, confirming the usefulness of asaronaldehyde as a lead agent. The growth-inhibiting activity of asarone identified in the *A. gramineus* rhizomes was examined against the six intestinal bacteria (Table 1). Asarone exhibited moderate and weak inhibitions against *C. perfringens* and *C. difficile* at 2 and 1 mg/disk, respectively, whereas little or no activity was observed

Table 1. Growth-inhibiting responses of asaron and asaronaldehyde derived from the rhizomes of *A. gramineus* against human intestinal bacteria

Compound	Dose (mg/disk)	Bacterial Strain[1)]					
		B. bifidum	*C. perfringens*	*C. difficile*	*L. acidophilus*	*L. casei*	*E. coli*
Asaronaldehyde	2.0	-[2)]	+++	+++	-	-	-
	1.0	-	++	++	-	-	-
	0.5	-	+	+	-	-	-
Asarone	2.0	-	++	++	-	-	-
	1.0	-	+	+	-	-	-

[1)]They were cultured on Eggerth-Gagnon agar at 37°C for 2 days in an atmosphere of 80% N_2, 15% CO_2 and 5% H_2.
[2)]Inhibitory zone diameter >30 mm, ++++; 21-30 mm, +++; 16-20 mm, ++; 10-15 mm, +; and <10 mm, -.

against *B. bifidum*, *E. coli*, *L. acidophilus*, and *L. casei* at 2 and 1 mg/disk. This result indicated that the growth-inhibiting activity of the *A. gramineus* rhizomes against *C. perfringens* and *C. difficile* can be mostly attributed to asarone and asaronaldehyde.

The infectious diseases caused by clostridia have a broad spectrum of clinical severity that ranges from mild outpatient illness to sudden death. Among clostridia, *C. perfringens* and *C. difficile* have been associated with sudden death, toxicity, and gastrointestinal disease (17, 19, 20). In contrast, bifidobacteria are taken as useful indicators of human health under most environmental conditions, based on the fact that they play important roles in metabolism such as amino acid production, defense against infections, immunopotentiation, and pathogen inhibition (19, 20). Accordingly, it is desirable to inhibit the growth of potential pathogens such as clostridia and/or increase the number of bifidobacteria in the human digestive system. The inhibitors against harmful bacteria are especially important for human health, because intake of these materials can normalize disturbed physiological functions, resulting in the prevention and treatment of various diseases caused by pathogens in the gastrointestinal tract. In recent years, much attention has been focused on selective plant-derived growth modulators in the intestine, because most plant-derived materials are relatively nontoxic to humans. For example, a-cedrene isolated from *Juniperus virginiana* leaves and various potato varieties have been shown to not only enhance the growth of bifidobacteria, but selectively inhibit various clostridia (17, 19). In the current study, the growth inhibitory constituent of *A. gramineus* rhizomes was identified as asaronaldehyde against *C. perfringens* and *C. difficile*, without any adverse effects on the growth of bifidobacteria and lactobacilli.

IC_{50} of asaronaldehyde The inhibitory activities of asaronaldehyde against platelet aggregation induced by collagen (2 μg/mL), AA (100 μM), and thrombin (0.1 unit/mL) were compared with that of aspirin as a standard antiplatelet agent (Table 2). Asaronaldehyde was most effective in inhibiting platelet aggregation induced by collagen (IC_{50}, 27.6 μM) and AA (IC_{50}, 53.7 μM), whereas showed no effect on thrombin. Aspirin inhibited platelet aggregation induced by AA with IC_{50} value of 35.9 μM, whereas had no or weak inhibitory effect on collagen and thrombin, showing asaronaldehyde is a significantly more potent platelet inhibitor than aspirin.

Table 2. IC_{50} (μM) of asaron, asaronaldehyde, and aspirin on platelet aggregation induced by various agonists in washed rabbit platelets

Agonists[1)]	Asaronaldehyde	Asaron	Aspirin
Collagen	27.6 ± 2.8[2)]	> 200	> 200
AA	53.7 ± 3.4	> 200	35.9 ± 2.7
Thrombin	> 200	> 200	> 200

[1)]Washed rabbit platelets were preincubated with asaron, asaronaldehyde, DMSO (0.5% control), and aspirin at 37°C for 3 min in the presence of 1 mM $CaCl_2$, then platelet aggregation was induced by addition of collagen (2 μg/mL), AA (100 μM/mL), or thrombin (0.1 unit/mL).
[2)]The 50% inhibitory concentration (IC_{50}) values were calculated from at least three separate experiments. Values are presented as means ± S.D.

When blood vessels are damaged, platelet aggregation occurs rapidly to form haemostatic plugs or arterial thrombi at the sites of vessel injury or in regions where blood flow is disturbed. These thrombi are the sources of thromboembolic complications of atherosclerosis, heart attacks, and peripheral vascular disease (21). The inhibition of platelet aggregation represents a promising approach for the prevention of thrombosis. Recent studies revealed the active components derived from plants display numerous biological activities (14-16, 22); in human blood *in vitro*, gallic acid and methyl gallate (Galla Rhois) inhibited platelet aggregation induced by collagen and arachidonic acid with IC_{50} values of 5 and 94 μM, and that induced by collagen and AA with IC_{50} values of 33 and 11 μM, respectively (15). Eugenol was effective in inhibiting platelet aggregation induced by AA (IC_{50}, 0.05 μM) and collagen (IC_{50}, 0.7 μM), and isoeugenol was most effective in inhibiting that induced by AA (IC_{50}, 0.3 μM), collagen (IC_{50}, 0.9 μM), and platelet-activating factor (IC_{50}, 12.2 μM) (22). Results of this study indicate *A. gramineus* rhizome-derived asaronaldehyde has at least one of the pharmacological actions for inhibiting platelet aggregation induced by collagen and AA.

In conclusion, these results indicate that *A. gramineus* rhizome-derived asaronaldehyde has antibacterial and antiplatelet effects *in vitro*. Based upon our limited data and some earlier findings, *A. gramineus* rhizome-derived asaronaldehyde may be useful as a lead compound for an antibacterial agent, an antiplatelet agent, and a medicinal foodstuff, although *in vivo* efficacy and clinical utility remain to be evaluated.

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