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Characterization and biological effects of two polysaccharides isolated from *Acanthopanax sciadophylloides*

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ABSTRACT

Two polysaccharides abbreviated ANP and AAP were isolated from the young buds of *Acanthopanax sciadophylloides*. ANP consisted of L-arabinose, D-mannose, D-glucose and D-galactose in a ratio of *ca* 1.0:2.6:2.5:1.4 and its weight average molecular weight (Mw) was 1.07×10^4. AAP consisted of L-arabinose, D-galactose and 4-*O*-methyl-D-glucuronic acid in a ratio of *ca* 5:10:1, and its Mw was estimated to be 8.40×10^4. ANP was suggested to be an acetylated heteropolysaccharide, whereas AAP was speculated to be a type II arabinogalactan on the basis of structural analysis data. Both polysaccharides were found to stimulate NO production and induce the expression of cytokine mRNAs including IL-1β, IL-6, IL-10 and TNF-α on RAW264.7 cells. They also induced NF-κB activation in RAW-Blue cells. NO production and NF-κB activation by both polysaccharides were decreased by pretreatment with neutralizing anti-TLR-4 and anti-CD14 antibodies but not with anti-TLR-2, anti-SR-A, anti-CD11c, and anti-Dectin-1 antibodies. Therefore, these immunostimulating effects of ANP and AAP were suggested to be promoted by the interaction through the membrane receptors, TLR-4 and CD14. In addition to immunomodulating effects, ANP showed anti-HSV-2 effects *in vitro*.

1. Introduction

Recently, virus infectious diseases become serious social problems in worldwide, *i.e.* there are increasing risks for spreading emerging and re-emerging viruses. In addition, herpes simplex virus type 2 (HSV-2) is a ubiquitous pathogen causing genital herpes. Immunocompetent people with genital HSV infection have frequent, painful, and recurrent genital lesions associated with much psychosocial distress. Over the past two decades, HSV-2 infection has also been linked to three times higher risk of sexually acquired human immunodeficiency virus (HIV) (Freeman et al., 2006). Acyclovir is the most commonly used chemotherapy as a very effective drug for HSV infection, however, it is not always tolerated and drug resistant mutants are rapidly emerging, particularly in immunocompromised patients (Whitley & Roizman, 2001).

In order to respond to these situations, immunomodulation, in particular against innate immune system, is thought to be one strategy for herpes infection (Vandevenne, Sadzot-Delvaux, & Piette, 2010). Under the point of view, botanical polysaccharides could be promising candidates for herpes infection because some of them have been found to possess immunomodulating effects (Schepetkin & Quinn, 2006) and anti-herpetic effects (Liu, Liu, Meng, Yang, & He, 2004; Oliveira et al., 2013).

For the purpose of searching for biologically active substances, we have been performing screening studies to evaluate potencies of edible and medicinal plants for virus infectious diseases. During the course of our studies, many polysaccharides from edible and/or medicinal resources have been found to possess desirable biological activities including antiviral and immunomodulating effects (Hayashi, Hayashi, & Kojima, 1996; Hoshino et al., 1998; Lee, Fukai, Hayashi, & Hayashi, 2011; Lee, Hayashi, Maeda, & Hayashi, 2004; Ohta, Lee, Hayashi, & Hayashi, 2009; Ohta et al., 2007). Therefore, our accumulated knowledge concerning biological activities of polysaccharides has prompted us to develop novel strategy to manage virus infectious diseases.

Recently, we have conducted a screening research of edible resources collected in Yamagata Prefecture in Japan to discover novel antiviral substances. The preliminary study indicated that hot water extract of young buds of *Acanthopanax sciadophylloides* Franch. et Savat. (Araliaceae) possessed antiviral effects against

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HSV-2. It is an endemic deciduous tree distributed in Japan, and its young buds have been traditionally used as an edible plant in Japan. The preliminary results prompted us to isolate active principle(s) from the hot water extract from *A. sciadophylloides*, and thus obtained results are reported in the present paper.

2. Materials and methods

2.1. Materials

Young buds of *A. sciadophylloides* were collected at Yamagata Prefecture in Japan in May 2010. A voucher specimen was deposited in the Laboratory of Medicinal Bioresources, Graduate School of Medicine and Pharmaceutical Sciences for Research, University of Toyama (Japan).

2.2. Isolation of polysaccharides

Fresh young buds (950 g) was cut into pieces, and extracted with 1 l of EtOH (95%) for 16 h at room temperature (three times). Extracts were combined and concentrated *in vacuo* to give EtOH extract (AE, 3.2%). The residue was extracted with 2.5 l of H_2O under reflux (three times). Combined extracts were concentrated and lyophilized to give H_2O extract (AW, 1.2%). Ten gram of AW was dissolved in H_2O and dialyzed against H_2O. Non-dialysate and dialysate were concentrated and lyophilized to give high molecular weight (AWH, 16.5%) and low molecular weight fractions (AWL, 61.0%), respectively. AWH was dissolved in H_2O and applied to a DEAE 650M anion exchange column (Cl^- form, 5 i.d. × 14 cm) followed by successive elution with H_2O, 0.5 M NaCl, and 1 M NaCl. Fractions of 15 ml were collected and monitored by the phenol–H_2SO_4 method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) and UV detection at 270 nm to monitor colored materials and proteins. The obtained three fractions were dialyzed and lyophilized to obtain AWH-1 (9.3%), -2 (38.2%) and -3 (4.8%). AWH-1 was applied to a Sepharose 6B gel filtration column (4.4 i.d. × 90 cm) and eluted with 0.1 M NaCl. Fractions of 15 ml were monitored by the phenol–H_2SO_4 method and UV detection at 270 nm, and gave AWH-1-1 (25.5%) and -2 (7.4%) on the basis of elution profile. AWH-1-1 was then chromatographed on a Sephacryl S-100HR column (2.2 i.d. × 90 cm) and eluted with H_2O. Carbohydrate positive fractions were combined and lyophilized to give a neutral polysaccharide (ANP, 39.8%). AWH-2 was dissolved in H_2O, applied to a DEAE 650M anion exchange column (Cl^- form, 5 i.d. × 15 cm), and eluted with a linear gradient system prepared by H_2O and 1 M NaCl. Fractions of 15 ml were collected and monitored by the phenol–H_2SO_4 method and UV detection at 270 nm. AWH-2-1 (26.2%), -2 (39.3%) and -3 (11.2%) were obtained on the basis of elution profile. AWH-2-1 was applied to a Sepharose 6B gel filtration column (4.4 i.d. × 90 cm) and eluted with 0.1 M NaCl. Fractions of 15 ml were monitored by the phenol–H_2SO_4 method and UV detection at 270 nm to give AWH-2-1-1 (40.8%), -2 (18.3%) and -3 (14.2%) on the basis of elution profile. AWH-2-1-1 was purified by a Sephacryl S-300HR column (2.2 i.d. × 90 cm), which was eluted with H_2O. Carbohydrate positive fractions were combined and lyophilized to give an acidic polysaccharide (AAP, 87.5%).

2.3. Estimation of molecular weight

The molecular weight of the isolated polysaccharides was estimated by HPLC analysis. The sample was applied on TSK GMPW$_{XL}$ gel filtration columns (7.6 × 300 mm × 2; Tosoh, Tokyo, Japan) and eluted with 0.1 M $NaNO_3$ at 0.6 ml/min. Commercially available pullulans (Shodex P-52; Showa Denko K.K., Tokyo, Japan) were used as standard molecular markers.

2.4. Chemical analyses of polysaccharides

Uronic acid content was determined by *m*-hydroxydiphenyl method (Blumenkrantz & Asboe-Hansen, 1973). Acetyl groups were quantified by HPLC after saponification. Briefly, sample was saponified in 50% isopropanol/0.4 M NaOH for 2 h at room temperature. After centrifugation, the supernatant was analyzed by HPLC equipped with an Aminex HPX-87H column (Bio-Rad Laboratories Inc., CA, USA) with 5 mM H_2SO_4 as mobile phase. Monosaccharide composition was analyzed by GC after methanolysis and trimethylsilylation (Bleton, Mejanelle, Sansoulet, Goursaud, & Tchapla, 1996). *myo*-Inositol was used as an internal standard. Absolute configuration of monosaccharides was analyzed as (+)-2-butyl-glycoside (Gerwig, Kamerling, & Vliegenthart, 1979). GC analyses were performed using DB-5 fused silica capillary column (30 m × 0.32 mm i.d.). Methylation of polysaccharides was performed by the Ciucanu's method (Ciucanu & Caprita, 2007). In the case of AAP, it was reduced with $LiBD_4$ in THF after methylation (Linnerborg, Wollin, & Widmalm, 1997). The methylated polysaccharides were hydrolyzed with 2 M TFA at 120 °C for 2 h, reduced with $NaBD_4$, and acetylated. The partially methylated alditol acetates were analyzed GC using an SP-2330 fused silica capillary column and GC-MS using an SPB-50 fused silica capillary column. Identification of partially methylated alditol acetates was carried out on the basis of relative retention time to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol and its mass fragmentation patterns (Carpita, Carpita, Shea, & Shea, 1989). Peak area of gas chromatogram in GC analysis was corrected using the published molar response factors (Sweet, Shapiro, & Albersheim, 1975).

2.5. Spectroscopic analyses of polysaccharides

IR spectra of polysaccharides were recorded with an FT/IR-460plus spectrophotometer using KBr method (JASCO, Tokyo, Japan). NMR spectra were recorded at 303 K on a Varian Unity 500 plus spectrophotometer, and sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) was used as internal reference.

2.6. Cells and viruses

Vero cells were grown in Eagle's minimum essential medium (MEM) supplemented with 5% fetal bovine serum (FBS) and kanamycin (60 mg/l). RAW 264.7 and RAW-Blue™ cells were grown in Dulbecco's modified MEM (DMEM) supplemented with 10% FBS. HSV-2 (UW264 strain) was propagated on Vero cells, and stored at −80 °C until use. An aliquot of the virus stock was titrated by a plaque assay on Vero cell monolayers.

2.7. Evaluation of immunostimulating effects of polysaccharides

Accumulated nitrite, which is a stable oxidized product of NO, in the culture media of RAW 264.7 cells was measured using a colorimetric assay based on the Griess reaction. Lipopolysaccharide (LPS) from *E. coli* O111:B4 (Sigma–Aldrich, St. Louis, MO, USA) was used as a TLR-4 agonist, and polymyxin B (Sigma–Aldrich, St. Luis, MO, USA) was used as an inhibitor of LPS. The cells (2.4×10^5) were seeded in a 96-well plate and incubated in the absence or presence of sample at 37 °C for 24 h. The culture supernatants were reacted with Griess reagent at room temperature for 10 min, and the nitrite concentration was determined by measuring the absorbance at 550 nm. The standard curve was obtained using the known concentrations of sodium nitrite.

From RAW 264.7 cells (2.4×10^5) incubated in the absence or presence of sample at 37 °C for 6 h, total RNA was extracted using an RNeasy Mini Kit (Qiagen, Hilden, Germany), and cDNA was reverse transcribed from total RNA using a Superscript III

system (Invitrogen, Carsbad, CA, USA). PCR amplification was carried out using GoTaq DNA polymerase (Qiagen) and specific primers described elsewhere (Lee, Ohta, Hayashi, & Hayashi, 2010).

RAW-Blue™ cells (RAW264.7 macrophages stably expressed a secreted embryonic alkaline phosphatase gene inducible by NF-κB and AP-1 and resistant to the selectable marker Zeocin) were seeded in 96-well plates at a density of 1×10^5 cells/well and grown overnight in a 5% CO_2 incubator at 37 °C. After treatment as indicated, the medium was harvested and mixed with QUANTI-Blue™ (InvivoGen) medium (160 μl) in 96-well plates at 37 °C for 30 min, and then the optical density at 620 nm was measured on an ELISA reader.

RAW264.7 cells were treated with neutralizing anti-TLR-2, anti-TLR-4, anti-CD11c, anti-CD14, anti-SR-A, or anti-Dectin 1 antibodies (InvivoGen) for 2 h before addition of the polysaccharides. Rat normal IgG_{2a} and IgG_{2b} (InvivoGen) were used as isotype-matched control antibodies. NO accumulation or NF-κB and AP-1 activation were quantified by using RAW 264.7 or RAW-Blue cells as describe above, respectively.

2.8. Evaluation of antiviral activities of polysaccharides

Vero cell monolayers were infected with HSV-2 at 0.1 plaque forming unit (PFU) per cell at room temperature as described elsewhere (Lee et al., 2011). After 1 h of viral infection, the monolayers were washed three times with phosphate-buffered saline (PBS) and incubated in a maintenance medium (MEM plus 2% FBS) at 37 °C. Sample was added either during infection and throughout the incubation thereafter (A) or immediately after the viral infection (B). Virus yields were determined by plaque assay at 1-day incubation point. The 50% inhibitory concentration (IC_{50}) was obtained from concentration-response curves. For cell growth inhibition study, Vero cells were incubated at an initial density of 1×10^4 cells/well in 96-well plates. After cells had been incubated for 1 day at 37 °C, sample was added and the incubation was continued for 3 days. Viable cell yield was determined by the trypan blue exclusion test. The 50% cytotoxic concentration (CC_{50}) was obtained from concentration-response curves. All data were expressed as mean ± SD from duplicate assays. To determine the effect of polysaccharides on direct inactivation of virus particles, HSV-2 (2×10^4 PFU/100 μl) was treated with an equal volume of polysaccharides at 37 °C. After 1.5 h, 100-fold dilutions of the mixture were added to Vero cell monolayers for 1 h at room temperature. The cell monolayers were overlaid with media containing 0.8% methylcellulose and 2% FBS to be plaque-assayed.

The study on *in vivo* anti-HSV-2 effects was performed as follows: Female BALB/c mice (5–6 weeks old) were obtained from Japan SLC, Shizuoka, Japan. All experiments were conducted in accordance with the animal experimentation guidelines of the University of Toyama and permitted by the Animal Care Committee at the University of Toyama. Mice were subcutaneously injected with 3 mg medroxyprogesterone 17-acetate at 6 days and 1 days before virus inoculation, and then were intravaginally infected with HSV-2 (1×10^5 PFU). Polysaccharides (1 mg/20 μl) or acyclovir (0.2 mg/20 μl) was administered intravaginally twice per day from 3 days before infection to 7 days post-infection. In the control group, mice were treated with 20 μl PBS. Clinical signs of infection were graded according to a five-point scale: 1, slight genital erythema and edema; 2, moderate genital inflammation; 3, severe exudative genital lesion; 4, hind limb paralysis; and 5, death. Viral shedding was determined by washing the vaginal cavity with 100 μl of PBS on day 3 post-infection and titrating the virus on Vero cell monolayers.

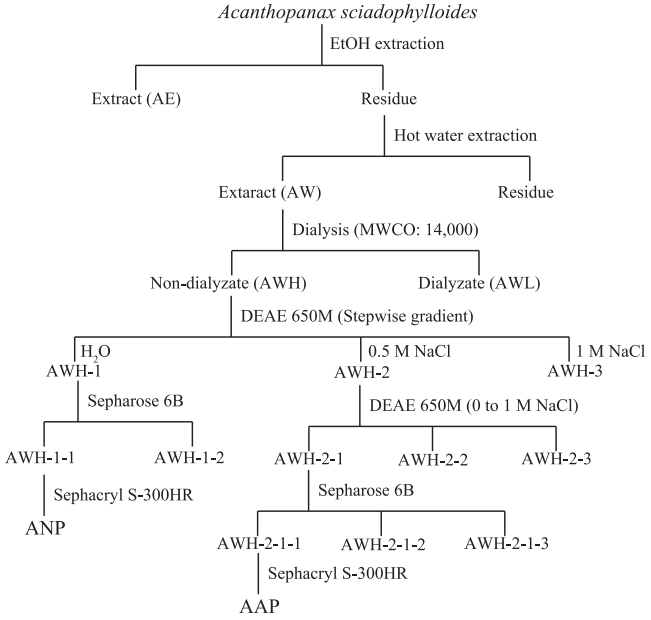

Fig. 1. Scheme for the fractionation and purification of polysaccharides from young buds of *Acanthopanax sciadophylloides*.

2.9. Statistical analyses

The data were presented as the mean ± SD. The differences between groups were analyzed using one-way analysis of variance (ANOVA), and correction for multiple comparisons was made through a Dunnett's multiple comparison test. In animal experiments, Kaplan–Meier survival curves were assessed by log-rank test.

3. Results

3.1. Isolation of polysaccharides

Extraction and isolation of polysaccharides from *A. sciadophylloides* was performed as shown in Fig. 1. After EtOH extraction, plant material was extracted with hot H_2O under reflux to obtain hot water extract (AW). Then, AW was dialyzed against H_2O to give low-(AWL) and high-molecular weight portion (AWH). AWH was fractionated by anion exchange column chromatography on DEAE 650M into three sub-fractions (AWH-1 to -3). AWH-1 was suggested to be neutral polysaccharide fraction because it was passed through the DEAE gel. AWH-1 was purified successively by gel filtration on Sepharose 6B and Sephacryl S-100 HR to yield a colorless polysaccharide (ANP). On the other hand, AWH-2 was further purified by anion exchange and gel filtration column chromatographies including DEAE 650M, Sepharose 6B and Sephacryl S-300 HR. The purified polysaccharide was regarded to be an acidic polysaccharide and it was named AAP.

3.2. Structural characterization of ANP and AAP

ANP and AAP were eluted as sharp and single peak on HPGPC and their molecular weights were estimated to be 1.07×10^4 ($M_w/M_n = 1.37$) and 8.40×10^4 ($M_w/M_n = 1.19$), respectively. Sugar composition analysis as trimethylsilyl derivatives of methyl- and (+)-2-butyl glycosides revealed that ANP consisted of D-Man, D-Glc, D-Gal, and L-Ara, whereas AAP consisted of L-Ara, D-Gal and 4-*O*-Me-GlcA as summarized in Table 1. IR spectra of ANP revealed that the polysaccharide contained acetyl groups because two absorption bands, the carbonyl stretching band around 1735 and the carboxyl

Table 1
Sugar composition of ANP and AAP from *A. sciadophylloides*.

Sugars	ANP	AAP
Ara	13.2	32.5
Gal	18.7	61.5
Man	34.4	–
Glc	33.7	–
4-*O*-Me-GlcA	–	6.0

Each value was expressed as mol%.

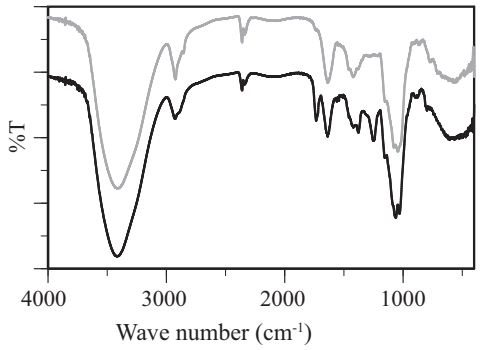

Fig. 2. IR spectra of isolated polysaccharides, ANP (black) and AAP (gray), from *A. sciadophylloides*.

stretching band around 1252 cm^{-1} (Fig. 2), were observed. In addition, ^1H NMR spectrum of ANP also indicated the presence of acetyl groups by the signals at 2.1–2.2 ppm (Supplemental Fig. 1). Their content was estimated to be 10.1% by chromatographic analysis after saponification. On the other hand, it was found that there are no acetyl groups in AAP (Fig. 2). In contrast to AAP, the IR spectrum of ANP showed weak bands between 1500 and 1600 cm^{-1} indicating the presence of impurities like phenolics and proteins. Results of methylation analyses of both polysaccharides were summarized in Table 2. ANP mainly consisted of 1,4-linked Man*p*, 1,4-linked Glc*p*, 1,5-linked and terminal Ara*f* residues. In addition to them, terminal and 2,4-linked Man*p* and 1,6-, 1,3,6- and terminal linked Gal*p* residues, and 4,6-linked Glc*p* residues were also detected. These results indicated that ANP might be a mixture of polysaccharides, type II arabinogalactan and (galacto)glucomannan

Table 2
Methylation analyses of ANP and AAP.

Methylated sugars	Deduced linkage	ANP (mol%)	AAP (mol%)
Arabinosyl			
2,3,5-Me_3-Ara[a]	Ara*f*-(1→	5.8	27.3
2,5-Me_2-Ara	→3)-Ara*f*-(1→	0.6	–
2,3-Me_2-Ara	→5)-Ara*f*-(1→	8.2	13.0
2-Me_3-Ara	→3,5)-Ara*f*-(1→	4.5	–
Galactosyl			
2,3,4,6-Me_4-Gal	Gal*p*-(1→	3.7	2.4
2,3,4-Me_3-Gal	→6)-Gal*p*-(1→	5.9	8.1
2,4,6-Me_3-Gal	→3)-Gal*p*-(1→	–	10.0
2,4-Me_2-Gal	→3,6)-Gal*p*-(1→	5.8	32.7
Mannosyl			
2,3,4,6-Me_4-Man	Man*p*-(1→	3.4	–
2,3,6-Me_3-Man	→4)-Man*p*-(1→	29.4	–
3,6-Me_2-Man	→2,4)-Man*p*-(1→	3.6	–
Glucosyl			
2,3,6-Me_3-Glc	→4)-Glc*p*-(1→	26.1	–
2,3-Me_2-Glc	→4,6)-Glc*p*-(1→	3.0	–
4-Me-Glucuronosyl			
2,3,4,-Me_3-Glc[b]	4-Me-GlcA*p*-(1→	–	6.5

[a] 1,4-Di-*O*-acetyl-2,3,5-tri-*O*-methyl-arabinitol.
[b] 6,6-Di-deuterio-1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-glucitol.

(Nunes, Reis, Silva, Domingues, & Coimbra, 2008; Teleman, Nordström, Tenkanen, Jacobs, & Dahlman, 2003). On the other hand, methylation analysis indicated that AAP consisted of terminal and 1,5-linked Ara*f* residues and terminal, 1,3-, 1,6-, and 1,3,6-linked Gal*p* residues. In addition, 1,6,6-tri-deuterio-1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-glucitol was also detected and it revealed that the 4-*O*-Me-GlcA*p* residue was present at non-reducing terminal.

^1H NMR spectrum of ANP gave multiple and broadened anomeric proton signals (Supplemental Fig. 1). Therefore, it was suggested that ANP possessed complicated structure. On the other hand, HSQC spectrum of AAP showed anomeric proton and carbon signals ranging from 4.45 to 5.24 and 105 to 112 ppm, respectively (Fig. 3), and chemical shifts of sugar residues in AAP were assigned as summarized in Table 3. Anomeric carbon signals, δ_C 111.6 and 109.7 ppm, were attributed to C-1 of arabinosyl residues, and were identical with 1,5-linked and non-reducing terminal α-L-Ara*f* residues (Bushneva et al., 2006; Nunes et al., 2008). On the other hand, anomeric carbon signals at δ_C 105–106 ppm were identical with those of terminal, 1,3-, 1,6-, and 1,3,6-linked β-D-Gal*p* residues. The other ring carbon signals were also consistent with those of reported values (Bushneva et al., 2006; Nunes et al., 2008). In addition, chemical shift of the 4-*O*-Me-GlcA residue was also identified, and its carboxyl carbon signal (δ_C 177.8 ppm) indicated that the residue was not methyl esterified. From the HMBC spectrum (Supplemental Fig. 2), the signal at δ 5.08 ppm (B) was correlated with the signal at δ 69.2 ppm (C-5 of 5-linked Ara*f* residue) and the signals at δ 5.23 (C) and 5.24 (A) ppm were correlated with the signal at δ 82.6 ppm (C-3 of 3-linked Gal*p* residue, E). These findings revealed that Ara*f* residue C and the disaccharide unit (B-A) were linked to *O*-3 position of Gal*p* residues (E) and/or (G). On the other hand, anomeric proton signals of all Gal*p* (δ 4.45–4.52 ppm) and 4-*O*-Me-GlcA residues (δ 4.48 ppm, H) showed inter-residual correlation between C-6 of Gal*p* residues (δ 71.9 ppm, F and G). From these results, it was suggested that AAP might be a type II arabinogalactan.

3.3. Immunomodulating effects of ANP and AAP

Since many botanical polysaccharides are well known to possess immunomodulating effects (Schepetkin & Quinn, 2006), we investigated the effect of both polysaccharides on the activation of macrophages. The RAW 264.7 murine macrophage cells were incubated along with the polysaccharides for 24 h, and nitric oxide (NO) concentrations in the culture supernatants were measured using the Griess reaction. As shown in Fig. 4A, ANP and AAP showed stimulatory effects on NO production in a dose-dependent manner. In addition, both polysaccharides induced iNOS mRNA expression in RAW264.7 cells in a dose-dependent manner.

We then investigated whether ANP and AAP could induce the expression of cytokine mRNAs from RAW264.7 cells. The cells were treated with the polysaccharides for 6 h, and then RNA was harvested from the cells and subjected to RT-PCR. As shown in Fig. 4B, ANP and AAP stimulated the induction of mRNAs of IL-1β, IL-6, IL-10, and TNF-α in a dose-dependent manner. Therefore, these polysaccharides might enhance the production of both pro-inflammatory (IL-1β, IL-6, and TNF-α) and anti-inflammatory (IL-10) cytokines.

Then, we confirmed whether ANP and AAP could activate NF-κB and/or AP-1, which is an important transcription factor for host immune responsive genes, by using RAW-Blue cells (Fig. 4C). The cells are derived from the murine RAW 264.7 macrophages with chromosomal integration of a secreted embryonic alkaline phosphatase (SEAP) reporter construct inducible by NF-κB and AP-1. As results, ANP and AAP induced NF-κB transcriptional activity in a dose-dependent manner.

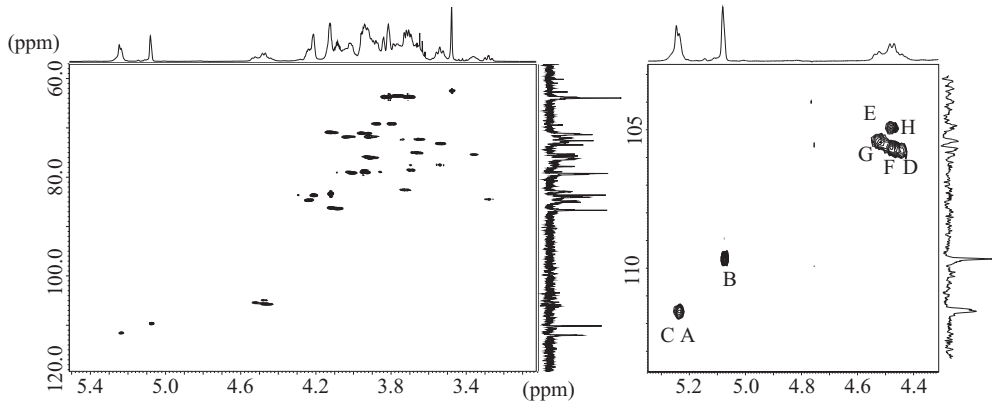

Fig. 3. HSQC spectrum of AAP from *A. sciadophylloides*. (A) Full spectrum, (B) enlarged anomeric region. Marks (A–H) indicated anomeric signals of sugar residues as shown in Table 3.

To elucidate the responsible receptors for the activation of macrophages by ANP and AAP, the cells were treated with various neutralizing antibodies against the candidate membrane receptors including TLR-2, TLR-4, CD14, CD11c, scavenger receptor A (SR-A), and dectin 1. As shown in Fig. 5, NO production and NF-κB activation were suppressed by the pretreatment with anti-TLR4 and anti-CD14 antibodies. The other neutralizing antibodies did not show the suppression of NO production and NF-κB activation by the polysaccharides. However, it may be possible that these effects of ANP and AAP were due to contamination of LPS. Thus, in an attempt to rule out the possibilities, both polysaccharides were pretreated with polymyxin B. As shown in Fig. 6, NO production by LPS was significantly suppressed by the pretreatment. In contrast, the polymyxin B-pretreated cells still possessed the activity for NO production in the presence of ANP and AAP.

3.4. Antiviral effects of ANP and AAP

ANP and AAP were evaluated for their *in vitro* antiviral potencies against HSV-2. In general, a sample was regarded as possessing antiviral activity when its selectivity index (SI, CC_{50}/IC_{50}) was higher than 10. As shown in Table 4, ANP showed anti-HSV-2 activity *in vitro* whereas AAP did not.

In order to investigate whether ANP and AAP could protect mice from lethal HSV-2 infection, the animals pretreated with medroxyprogesterone were given with the polysaccharides intravaginally. The mean titer of virus shed on day 3 were reduced from 89.0×10^4 PFU/mouse in untreated control mice to 37.2×10^4 (not significant), 23.6×10^4 ($p < 0.05$), and 0.54×10^4 PFU/mouse ($p < 0.01$) in mice that received ANP, AAP, and ACV, respectively. Therefore, virus titers in vaginal region were decreased by the administration of AAP. As predicted from virus shedding, AAP could elongate the survival period when compared with that of untreated control ($P = 0.0462$, log-rank test) in the lethal infection model.

4. Discussion

As described above, we have isolated two polysaccharides, ANP and AAP, from *A. sciadophylloides*. Our results revealed that ANP was suggested to be an acetylated heteropolysaccharide or mixture of two types of polysaccharides, whereas AAP was a type II arabinogalactan. AAP possessed unique structural feature, *i.e.* the presence of β-linked 4-*O*-methyl glucuronic acid residues at terminal ends. There are some reports concerning the presence of β-D-glucuronic acid and/or its 4-*O*-methyl derivatives in arabinogalactans (Cipriani et al., 2006; Cui, Eskin, Biliaderis, & Marat, 1996; Martínez et al., 2003; Tryfona et al., 2010). These reports support our findings about the presence of 4-*O*-methyl glucuronic acid in type II arabinogalactan. Chen et al. reported that antioxidant and immunological activities of a polysaccharide from *A. senticosus* (Chen et al., 2011), however, its sugar compositions are different from those of our polysaccharides. On the other hand, a polysaccharide from *Melia toosendan* was reported to be composed of Man, Glc, Gal, and Ara (He et al., 2011), and its sugar composition was resembled to that of ANP. In general, acetylated galactoglucomannans (GGMs) and glucomannans (GMs) are known to be common cell

Table 3
Assignments of ^1H and ^{13}C NMR Spectra of AAP.

Residue			1	2	3	4	5	6		—O—CH_3
L-Ara*f*-α-(1→	(A)	^{13}C	111.6	83.7	79.1	86.4	64.2			
		^1H	5.23	4.12	4.01	4.12	3.83	3.71		
L-Ara*f*-α-(1→	(B)	^{13}C	109.7	83.4	78.9	86.4	64.2			
		^1H	5.08	4.12	3.94	4.08	3.83	3.71		
→5)-L-Ara*f*-α-(1→	(C)	^{13}C	111.6	83.7	79.1	84.7	69.2			
		^1H	5.24	4.21	4.10	4.24	3.88	3.80		
D-Gal*p*-β-(1→	(D)	^{13}C	105.8	73.2	75.0	71.2	78.6	63.8		
		^1H	4.45	3.54	3.67	3.92	3.69	3.83	3.70	
→3)-D-Gal*p*-β-(1→	(E)	^{13}C	105.2	75.7	82.6	70.9	78.6	63.7		
		^1H	4.48	3.36	3.63	4.13	3.69	3.76		
→6)-D-Gal*p*-β-(1→	(F)	^{13}C	105.7	73.2	75.0	71.0	76.1	71.9		
		^1H	4.47	3.54	3.67	3.96	3.89	4.04	3.92	
→3,6)-D-Gal*p*-β-(1→	(G)	^{13}C	105.5	72.4	82.5	70.9	76.0	71.9		
		^1H	4.52	3.65	3.73	4.13	3.92	4.04	3.92	
D-4-Me-GlcA*p*-β-(1→	(H)	^{13}C	105.0	75.3	77.5	84.5	77.6	177.8		62.6
		^1H	4.48	3.36	3.54	3.28	3.70			3.48

Assignments were made from HSQC and HMBC.

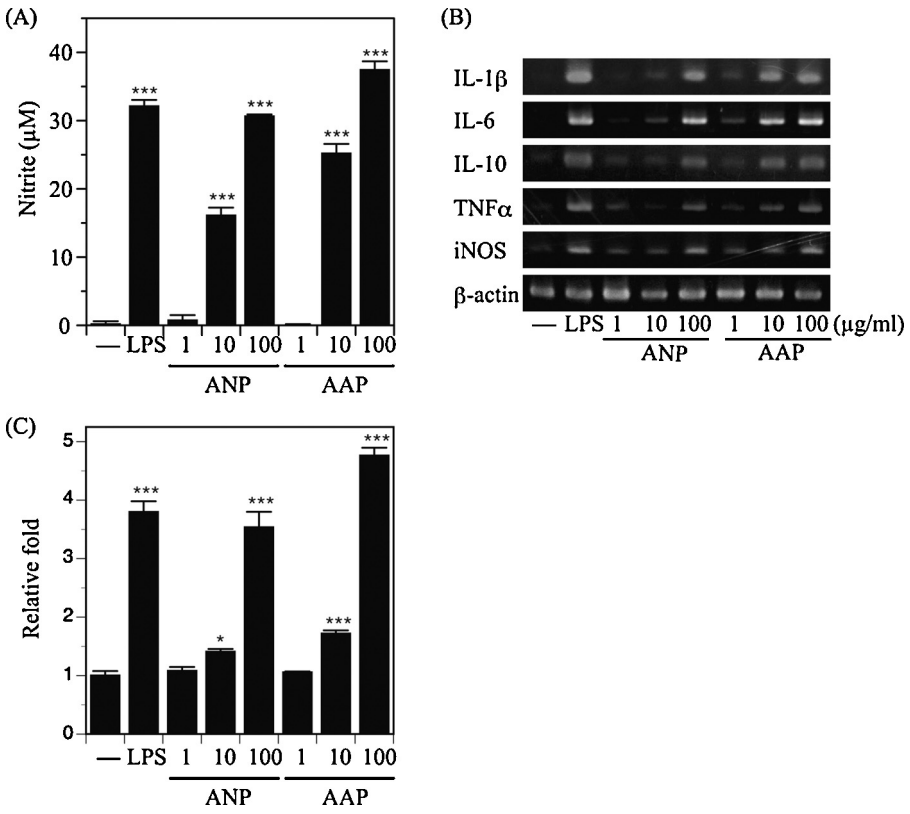

Fig. 4. Effect of ANP and AAP on (A) NO production, (B) mRNA expression in RAW 264.7 cells, and (C) NF-κB activation in RAW-Blue™ cells. (A) The cells were incubated with ANP or AAP for 24 h. The NO production was determined by measuring the accumulation of nitrite in the culture medium. No sample control and LPS (0.1 μg/ml) were also assayed. (B) The cells were cultured in the absence or presence of LPS (0.1 μg/ml) or polysaccharides (1, 10, or 100 μg/ml) for 6 h. mRNA expression was detected by RT-PCR. β-Actin was used as a control. (C) RAW-Blue™ cells were incubated for 24 h with LPS, ANP, or AAP. Secreted embryonic alkaline phosphatase activity was measured by QUANTI-Blue™. Any significant differences were determined using the Dunnett's test *versus* the control group. Data are expressed as mean ± S.D., $n = 3$, $^{*}p < 0.05$, $^{***}p < 0.001$.

wall constituents (Capek, Alföldi, & Lisková, 2002; Hannuksela & Hervé du Penhoat, 2004; Teleman et al., 2003; Xu et al., 2010). It has been proposed that their main backbone consists of 1,4-linked β-D-Man*p* and 1,4-linked β-D-Glc*p* residues. Since our polysaccharide also mainly consisted of 1,4-linked β-D-Man*p* and 1,4-linked β-D-Glc*p* residues, ANP might be one of acetylated galactoglucomannan type polysaccharides.

When two isolated polysaccharides were applied to antiviral tests against HSV-2 *in vitro*, ANP was confirmed to be an anti-HSV-2 polysaccharide. In addition, ANP and AAP were found to possess immunostimulatory effects such as enhancement of NO production and induction of cytokine mRNA expression on macrophages (Fig. 2). In the mucosal region, innate immune system is the important mechanism to proper the first line of host defense, and antigen-presenting cells like macrophages and dendritic cells possess a pivotal role in responding to infectious pathogens (Ellermann-Eriksen, 2005). NO may inhibit an early stage in viral replication, and thus prevent virus spread, promoting viral clearance and recovery of the hosts. ANP and AAP activated expression of pro-inflammatory cytokine mRNAs including IL-1β, IL-6, and TNF-α mRNAs. The release of pro-inflammatory cytokines is essential for host survival from infection and is also required for the repair of injured tissue. On the other hand, it was suggested that IL-10 induced by ANP and AAP might act in a negative feedback mechanism to prevent potential detrimental effects from excessive macrophage activation during inflammation (Choi, Kim, Kim, & Hwang, 2005). ANP and AAP potently activated transcriptional factor NF-κB in RAW-Blue™ cells. NF-κB is one of the most important transcription factors, and its activation plays a pivotal role in inflammation due to its ability to induce transcription of pro-inflammatory genes. Thus, it was suggested that induction of cytokine mRNA expression by both polysaccharides might be dependent on the activation of NF-κB.

When ANP and AAP were subjected to determine anti-HSV-2 effect *in vitro*, ANP solely showed the effect (Table 4). So far, we have experienced that a number of polysaccharides having no or weak antiviral activities *in vitro* suppressed virus replication in the animal models, and the effects were suggested to be derived from their immunostimulating effects (Lee et al., 2011, 2012; Ohta et al., 2007). In the mouse model of HSV-2 infection, AAP significantly reduced HSV-2 titer in vaginal washings at 3 days post-infection and elongated the survival period of HSV-2-infected mice (Fig. 7).

Table 4
Antiviral activities of ANP and AAP.

Sample	Cytotoxicity (CC_{50}, μg/ml)	Antiviral activity (IC_{50}, μg/ml)		Selectivity index (CC_{50}/IC_{50})	
		A	B	A	B
ANP	3300	52	67	63	49
AAP	2800	620	580	4.5	4.8

A: Sample was added to the medium during infection and throughout the incubation thereafter.
B: Sample was added to the medium immediately after viral infection.

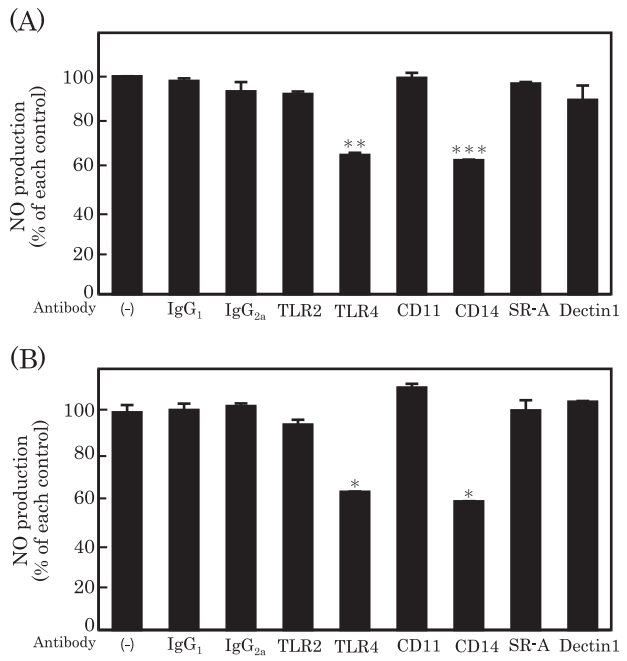

Fig. 5. Effects of ANP and AAP on NO production in the presence of neutralizing antibodies. RAW264.7 cells were pretreated with each antibody for 2 h before incubation with ANP (A) or APP (B) for 24 h. NO production in the culture medium was then determined using Griess reagent. Any significant differences were determined using the Tukey's test *versus* the control group. Data are expressed as mean ± S.D., $n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

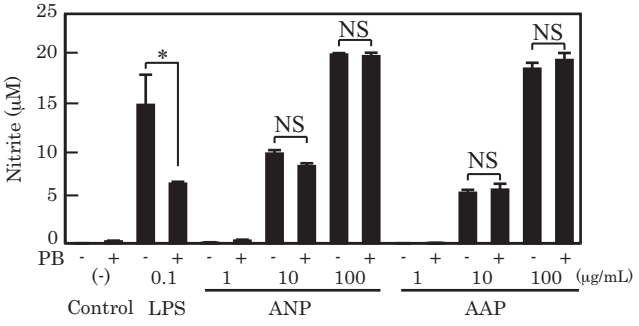

Fig. 6. Effect of polymyxin B on the NO production stimulated by polysaccharides. The cells were pretreated with polymyxin B for 2 h before incubation with ANP and AAP for 24 h. NO production in the culture medium was then determined using Griess reagent. Any significant differences between polymyxin B-treated and -untreated groups were determined using the Student's *t*-test. Data are expressed as mean ± S.D., $n = 3$, $^{*}p < 0.05$.

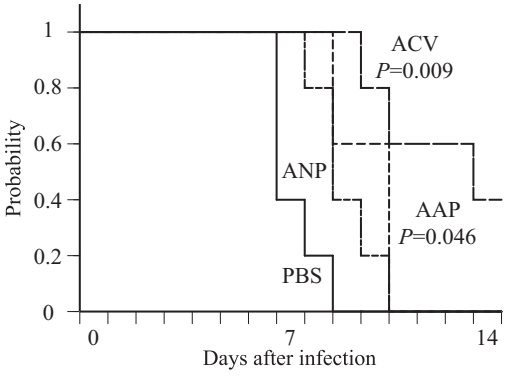

Fig. 7. Effects of ANP and AAP on experimental genital herpes infection. Mice ($n = 5$) were given PBS, ANP, AAP, or ACV intravaginally twice a day from 3 days before infection to 7 days after infection. The survived animals were recorded for 14 days after virus infection.

Since AAP did not possess direct antiviral effects, it has been suggested that its immunostimulating effects might contribute at least in part to the *in vivo* antiherpetic effect.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.04.013.

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