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Antiviral and Immunostimulating Effects of Lignin-Carbohydrate-Protein Complexes from *Pimpinella anisum*

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Three antiviral and immunostimulating substances (LC1, LC2 and LC3) were isolated from a hot water extract of seeds of *Pimpinella anisum* by combination of anion-exchange, gel filtration and hydrophobic interaction column chromatographies. Chemical and spectroscopic analyses revealed them to be lignin-carbohydrate-protein complexes. These lignin-carbohydrate complexes (LCs) showed antiviral activities against herpes simplex virus types 1 and 2 (HSV-1 and -2), human cytomegalovirus (HCMV) and measles virus. LCs were also found to interfere with virus adsorption to the host cell surface and directly inactivate viruses. Furthermore, they enhanced nitric oxide (NO) production by inducing iNOS mRNA and protein expression in RAW 264.7 murine macrophage cells. The induced mRNA expression of cytokines including IL-1β and IL-10 was also apparent. These results suggest that the lignin-carbohydrate-protein complexes from *P. anisum* possessed potency as functional food ingredients against infectious diseases.

Key words: *Pimpinella anisum*; lignin-carbohydrate-protein complex; antiviral activity; macrophage; nitric oxide

The herpes simplex viruses (HSVs) and human cytomegalovirus (HCMV) are ubiquitous infectious pathogens with worldwide distribution and associated with opportunistic diseases. HSV-associated diseases are among the most widespread infections, affecting nearly 60% to 95% of human adults.[1] On the other hand, the seroprevalence rate of HCMV varies widely from 40% to almost 100% depending on the socio-economic and geographic locations. Patients compromised by immunosuppressive therapy, underlying disease or malnutrition are also at increasing risk of severe HSV and HCMV infections. In particular, HCMV is the major cause of morbidity and mortality in transplant recipients by a combination of the direct cytopathic effect of viral replication and the indirect host-dependent immunopathological mechanism.[2] Although several effective antiviral agents are available for treating these infections, they are associated with toxicity and the emergence of drug resistance. Hence, novel antiviral drugs with different targets from available agents are urgently required.

Although the development of effective vaccines is the most effective strategy to combat viruses, there remains no effective vaccine against HSV or HCMV. It has been suggested from another perspective that stimulating the host innate immune response might provide prophylactic protection against infectious diseases.[3,4] The macrophage function has attracted particular attention as a target for immunomodulation, since macrophages play a central role in the innate immune system.[5,6] In respect of this concept, we have recently reported that a polysaccharide from *Cordyceps militaris* grown on soybeans showed *in vivo* antiviral activity through stimulation of the host immune function.[7] We have thus continued to discover and evaluate novel antiviral candidates from food resources.

Herbs and spices are familiar food components, and their functions are of interest for human health. Among them, anise (*Pimpinella anisum*) has been used as a popular aromatic herb and spice from ancient times, and its seeds are used as a folk medicine and food material for cooking. A concoction of the powder of anise in hot water is used for medicinal purposes as a carminative, antiseptic, digestive and aphrodisiaiac, and as a remedy for insomnia and constipation. Our preliminary study has revealed that the hot water extract from anise seeds possessed antiviral activities against HSVs, and this observation prompted us to characterize the antiviral principles from this extract. We describe in the present paper the isolation, characterization of the chemical nature and evaluation of the biological activities, including the antiviral and immunostimulating effects, of the active substances from *P. anisum*.

Materials and Methods

Materials. Seeds from *P. anisum* were purchased from Tochimoto Tenkaido (Osaka, Japan). A voucher specimen of the seeds has been deposited in the pharmacognosy laboratory of the Graduate School of Medicine and Pharmaceutical Sciences for Research at University of Toyama. Eagle's minimal essential medium (MEM) and Dulbecco's MEM (DMEM) were obtained from Nissui Pharmaceuticals (Tokyo, Japan). Toyopearl DEAE 650 M, Phenyl 650 M, and TSKgel GMPW$_{XL}$ columns were purchased from Tosoh (Tokyo, Japan). Sepharose 6B, Sephadex G-10, and Sephacryl S-300 HR were from GE Healthcare (Piscataway, NJ, USA), and all other chemicals were obtained from Wako Pure Chemicals (Osaka, Japan).

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Abbreviations: HSV, herpes simplex virus; HCMV, human cytomegalovirus; LC, lignin-carbohydrate complex; FBS, fetal bovine serum; PBS, phosphate-buffered saline; MEM, minimum essential medium; SI, selectivity index; PFU, plaque-forming unit; MOI, multiplicity of infection; p.i., post-infection

Extraction and fractionation of the lignin-carbohydrate-protein complexes. Seeds of anise (500 g) were extracted with boiling H_2O (3 L) for 1 h under reflux. The obtained extract was filtered, and the residue was extracted again with boiling H_2O (3 L) for 30 min under reflux. The extracts were combined, concentrated *in vacuo* and lyophilized to give a brown residue (PA, 104.3 g). PA was dialyzed through a seamless cellulose tube (14,000 MWCO; Wako Pure Chemicals, Osaka, Japan) against H_2O to obtain a non-dialysate (PAH, 17% yield) and dialysate (PAL, 67% yield). PAH (2 g) was applied to a Toyopearl DEAE 650 M anion-exchange column (5 i.d. × 14 cm), which was eluted with a linear gradient system prepared from H_2O and 2 M NaCl. Fractions of 15 mL were collected and monitored by the phenol–H_2SO_4 method[8] and UV absorbance at 275 nm. The respective yields of fractions PAH-1, -2 and -3 were 18%, 35% and 8%. PAH-3 was re-chromatographed in the same manner to obtain PAH3a (4%) and PAH3b (60%). PAH3b was applied to a Sepharose 6B gel filtration column (2.2 i.d. × 95 cm) and eluted with a 10 mM citrate-phosphate buffer (pH 7) containing 0.1 M NaCl. Fractions of 4 mL were collected and monitored by the phenol–H_2SO_4 method and UV absorbance at 275 nm to give two fractions, PAH3b-1 (16%) and PAH3b-2 (65%). PAH3b-2 was applied to a Sephacryl S-300 HR gel filtration column (2.2 i.d. × 97 cm) and eluted with a 10 mM citrate-phosphate buffer (pH 7) containing 0.1 M NaCl. Fractions of 4 mL were collected and monitored by the phenol–H_2SO_4 method and UV absorbance at 275 nm to give two fractions, PAH3b-2a (71%) and PAH3b-2b (22%). PAH3b-2a was subjected to Phenyl 650M chromatography (2.5 i.d. × 7 cm column), and fractions of 10 mL were collected and monitored by the phenol–H_2SO_4 method and UV absorbance at 275 nm to give three fractions, LC1 (25%), LC2 (36%) and LC3 (13%), which were obtained on the basis of their elution patterns.

HPLC analyses. Each sample was applied to TSK GMPW$_{XL}$ gel filtration columns (7.6 i.d. × 300 mm × 2) and eluted with 0.1 M $NaNO_3$ at 0.6 mL/min. The sample was subjected to UV and refractive index detection, standard pullulans (Showa Denko, Japan) being used as molecular markers.

Spectroscopic and chemical analyses. NMR spectra were recorded by a Varian Unity500 Plus spectrophotometer in D_2O, and IR spectra were measured by an FT/IR-460 spectrophotometer (Jasco, Kyoto, Japan), using the KBr method. Elemental analyses were performed by a 2400 Series II CHNS/O system (Perkin Elmer, Wellesley, MA, USA). The total sugar content and uronic acid content were respectively determined by the phenol–H_2SO_4 method and *m*-hydroxydiphenyl method.[9] The sugar composition was analyzed by GC-MS after methanolysis and trimethylsilylation. Briefly, a sample was dissolved in MeOH containing acetyl chloride, and then heated at 80 °C for 16 h. After removing the solvent under N_2 gas, the residue was trimethylsilylated with a TMSI-H reagent (GL Science, Tokyo, Japan) and then analyzed by GC-MS, using a DB-5MS fused silica capillary column (30 m × 0.32 mm i.d.; J&W Scientific) with an oven temperature of 110–260 °C (5 °C/min). The protein content was calculated from the nitrogen content by multiplying the content by 6.25 (protein contains 16.5% nitrogen). The amino acid composition was determined as PTC-amino acid derivatives.[10] Briefly, a sample (100 μg) was hydrolyzed with 6 N HCl at 110 °C for 24 h. After removing HCl, the hydrolysate was reacted with 200 μL of an EtOH/H_2O/triethylamine/phenylisothiocyanate (7:1:1:1) solution for 20 min. The resulting mixture was dried, dissolved in a 150 mM ammonium acetate buffer (pH 6) containing 5% acetonitrile, and then analyzed by HPLC. The sample was applied to a Luna 5C18-II column (4.6 × 150 mm; Phenomenox, Torrance, CA, USA) eluted with a linear gradient system between a 150 mM ammonium acetate buffer containing 5% acetonitrile and 60% acetonitrile at 0.8 mL/min and 40 °C. The sample was subjected to UV detection at 254 nm, and the lignin content was estimated by the acetyl bromide method.[11] Briefly, 25% acetyl bromide in glacial acetic acid (0.5 mL) was added to the sample (1 mg), and the solution then heated at 50 °C for 30 min. After cooling, acetic acid (2.5 mL), 0.3 M NaOH (1.5 mL) and a 0.5 M hydroxylamine hydrochloride solution (0.5 mL) were added, and the mixture was shaken. After adding acetic acid to give a final volume of 10 mL, the UV absorbance of the solution was measured at 275 nm. Alkaline nitrobenzene oxidation was performed to analyze the lignin monomers as described by Meyer *et al.*[12] Briefly, a sample (5 mg) was mixed with 2 M NaOH (0.7 mL) and nitrobenzene (40 μL), and the mixture was incubated at 170 °C for 4 h. The reaction mixture was cooled to room temperature and extracted with $CHCl_3$ (1 mL). The aqueous phase was acidified with 4 N HCl and extracted twice with $CHCl_3$ (0.9 mL). The combined $CHCl_3$ phase was dried with anhydrous sodium sulfate, and $CHCl_3$ was evaporated under N_2 gas. After the dried residue had been resuspended in pyridine (50 μL), 50 μL of MSTFA (*N*-methyl-*N*-trifluoroacetamide) was added, and the silylated products were analyzed by GC-MS, using a J&W DB-1MS column (30 m × 0.32 mm i.d.) with an oven temperature of 70–250 °C (5 °C/min). The identity of each peak was confirmed with GC-EI-MS by comparing to an authentic sample.

Cells and viruses. Vero and MRC-5 cells were respectively obtained from Denka Seiken Co. (Japan) and American Type Culture Collection. They were grown in MEM supplemented with 5% fetal bovine serum (FBS). RAW 264.7 cells were grown in DMEM supplemented with 10% FBS. HSV-1 (HF strain) and HSV-2 (UW 268 strain), measles virus (Toyoshima strain), mumps virus (EY strain), poliovirus type 3 (Sabin strain), and coxsackie virus type B-1 (Conn-5 strain) were grown on Vero cells, while human cytomegalovirus (Towne strain) and human coronavirus (229E strain) were grown on MRC-5 cells.

In vitro *antiviral activity and cytotoxicity.* Vero and MRC-5 cells were cultured for 72 h in the presence of an increasing amount of a sample for cell growth inhibition studies. The viable cell yield was determined by the trypan blue exclusion test. The inhibition data were plotted as a dose–response curve, from which the 50% cell growth inhibitory concentration (CC_{50}) was obtained. Cell monolayers in 48-well plates were infected with the virus at 0.2 plaque-forming unit (PFU) per cell at room temperature, except for human coronavirus. 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. A sample was added during infection and thereafter throughout incubation, or immediately after virus infection. The cell culture was harvested 24 h post-infection (p.i.) for each virus except HCMV, which was harvested 5 d p.i. Each virus yield was determined by a plaque assay after 2 d of incubation, except for HCMV. In the case of HCMV, the virus yields were determined by a plaque assay after 5 d of incubation. The anti-human coronavirus assay was conducted on MRC-5 cells that had been infected with the virus at 100 $TCID_{50}$ (50% tissue culture infectious dose) and incubated in the medium for 3 d. Titers of this virus were determined by the cytopathic effects, and $TCID_{50}$ values were calculated by the Reed-Muench method.[13] The antiviral activities are expressed as the 50% inhibitory concentration (IC_{50}), this being the concentration of a sample that reduced virus production by 50% in the treated culture as compared with the no-drug control.

Time-of-addition experiments. Vero cell monolayers were infected with HSV-2 at 10 PFU per cell. Each sample was added before 1 h of viral infection, immediately after infection, and 1 h, 3 h, 6 h or 8 h p.i. The cell cultures were harvested 24 h p.i. and subjected to a plaque assay. MRC-5 cell monolayers were infected with HCMV at 1 PFU per cell in another experiment. Each sample was added before 1 h of viral infection, immediately after infection, and 1 h, 3 h, 6 h, 24 h, 48 h or 76 h p.i. The cell cultures were harvested 120 h p.i. and subjected to a plaque assay.

Virus adsorption assay. The effect of each sample on HSV-2 or HCMV adsorption to the host cells was evaluated by an infectious center assay.[14] Briefly, a Vero cell suspension, HSV-2 (1 PFU/cell) and lignin-carbohydrate complex were pre-cooled on ice for 3 h before mixing at 4 °C. After 1 h incubation at 4 °C, the cell suspension was washed three times with ice-cold PBS to remove the unbound virus and free compound. The cell pellet was diluted 10-fold with ice-cold PBS and immediately added to Vero cell monolayers in a 35-mm dish to be plaque-assayed. The assay was performed in the same manner for HCMV as that for HSV-2 by using an MRC-5 cell suspension.

Virus penetration assay. Virus penetration into the host cells was evaluated according to the method reported by Huang and Wagner[15] with some modifications. Vero or MRC-5 cell monolayers in 12-well plates pre-cooled for 3 h on ice were respectively infected with HSV-2 or HCMV (approximately 100 PFU/well) at 4 °C for 1 h in the absence of the sample compound. After washing three times with ice-cold PBS, cell monolayers were incubated at 37 °C in the medium containing a sample. The cell monolayers were treated for 1 min with a 40 mM citrate buffer (pH 3.0) 0, 0.5, 1, 2, 3 and 6 h after the temperature shift to 37 °C to inactivate any unpenetrating virus, and overlaid with the medium containing 0.8% methylcellulose and 2% FBS to be plaque-assayed.

Virucidal assay. To determine the effect of each lignin-carbohydrate complex on direct inactivation of the virus particles, HSV-2 or HCMV (2×10^4 PFU/100 μL) was treated with an equal volume of a sample at 37 °C. After 1.5 or 24 h, a 100-fold dilution of the mixture was added to Vero or MRC-5 cell monolayers for 1 h at room temperature. The cell monolayers were overlaid with the medium containing 0.8% methylcellulose and 2% FBS to be plaque-assayed.

NO production. Accumulated nitrite, which is a stable oxidized product of NO, in the culture medium of RAW 264.7 cells was measured by using a colorimetric assay based on the Griess reaction.[16] Briefly, the cells (2.0×10^5) were seeded in 96-well plates and incubated in the absence or presence of a sample with or without 100 U/mL of polymyxin B (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C for 20 h. The culture supernatant was reacted with the Griess reagent at room temperature for 10 min, and then the nitrite concentration was determined by measuring the absorbance at 550 nm. A standard curve was obtained by using known concentrations of sodium nitrite.

RT-PCR. RAW 264.7 cells (2.4×10^5) were incubated in the absence or presence of a sample at 37 °C for 4, 12, or 20 h. Total RNA was extracted by using an RNeasy mini kit (Qiagen, Hilden, Germany), and cDNA was reverse-transcribed from total RNA by using a Superscript III system (Invitrogen, Carlsbad, CA, USA). PCR amplification was carried out with GoTaq DNA polymerase (Promega, Madison, WI, USA) and specific primers as reported elsewhere.[7] PCR products were run on 2% agarose gel and visualized by ethidium bromide staining.

Western blotting. RAW 264.7 cells (3.2×10^5) were seeded in 24-well plates and incubated at 37 °C for 24 h in the absence or presence of a sample. The cells were washed three times with PBS, lysed with an RIPA buffer (PBS containing 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS), and then centrifuged. The supernatant was subjected to 10% SDS–PAGE and transferred on to a PVDF membrane. The membrane was washed with Tris-buffered saline (TBS) and blocked with 5% skim milk in TBS. After incubating with the antibody against iNOS or β-actin, the membrane was incubated with an appropriate HRP-conjugated antibody. The bands were developed by using the Luminol chemiluminescence reagent (Santa Cruz Biotechnology, CA, USA) according to the manufacturer's instructions.

Statistical analysis. All data are presented as the mean ± SD. Student's *t*-test, an analysis of variance (ANOVA), and then Dunnett's multiple-comparison test were performed to assess differences between the relevant control and each experimental group.

Results

Isolation of antiviral substances from P. anisum

The preliminary screening of hot water extracts from herbs and spices showed the extract from seeds of anise to have the most potent antiviral activity against HSV-1. We thus undertook fractionation of the antiviral substances from *P. anisum*. The seeds of *P. anisum* were extracted twice with H_2O under reflux to give a brown residue (PA), this then being fractionated by dialysis into high-molecular-weight (PAH) and low-molecular-weight portions. Since PAH showed anti-HSV-1 activity, this was fractionated by anion-exchange column chromatography to give three fractions, the last-eluted fraction (PAH-3) showing an antiviral effect. Following gel filtration on Sepharose 6B, PAH-3 was separated into PAH-3a and PAH-3b, and the latter was applied to the same column to give PAH-3b-2. PAH-3b-2 was fractionated by gel filtration on Sephacryl S-300 HR, and thus obtained PAH-3b-2a was applied to a Phenyl 650M hydrophobic chromatography. Three compounds, LC1, LC2 and LC3, were isolated as a result.

Characterization of LCs

LC1, LC2 and LC3 analyzed by HPLC on TSK $GMPW_{XL}$ enabled these compounds to be regarded as homogeneous substances on the basis of the molecular weight distribution, their apparent molecular weights being estimated to range from 7,000 to 8,000. The chemical properties of these compounds are listed in Table 1. LC1, LC2 and LC3 were found to contain neutral sugars and uronic acids, because they showed a positive reaction by the phenol–H_2SO_4 and *m*-hydroxydiphenyl methods. An analysis of the sugar composition as methyl glycosides showed that LC1 consisted of glucose, arabinose, galacturonic acid, galactose, rhamnose and xylose in a ratio of 22:18:21:18:15:6. On the other hand, the major component sugars of LC2 and LC3 were glucose and arabinose. The acetyl bromide method estimated the respective lignin contents of LC1, LC2 and LC3 as 12.1%, 37.7% and 30.8%. We applied these substances to alkaline nitrobenzene oxidation to qualitatively estimate the lignin monomer components of those substances, and such lignin monomer derivatives as vanillin, syringaldehyde, *p*-hydroxybenzaldehyde, vanillic acid, *p*-hydroxybenzoic

Table 1. Chemical Composition of the Lignin-Carbohydrate Complexes from *P. anisum*

	LC1	LC2	LC3
Lignin (%)[a]	12.1	37.7	30.8
Total sugar (%)[a]	60.2	19.0	21.1
Uronic acid (%)[a]	15.4	2.3	2.0
Protein (%)[a]	13.8	51.8	52.1
Lignin monomer			
Vanillin	+	+	+
Syringaldehyde	−	−	+
p-Hydroxybenzaldehyde	+	+	+
Vanillic acid	−	+	+
p-Hydroxybenzoic acid	−	+	+
p-Hydroxyacetophenone	+	+	+
Cinnamic acid	+	+	+
Component sugars (mol%)			
Arabinose	18.2	42.8	24.2
Xylose	6.3	5.9	9.7
Rhamnose	15.2	n.d.[b]	n.d.
Galactose	17.9	5.0	n.d.
Glucose	21.7	46.5	66.0
Galacturonic acid	18.2	42.8	24.2

[a] The total sugar, uronic acid, protein, and lignin contents were respectively determined by the acetyl bromide method, phenol-H_2SO_4 method, *m*-hydroxydiphenyl method and elemental analysis.
[b] n.d., not detected

Table 2. Amino Acid Composition of the Lignin-Carbohydrate Complexes from *P. anisum*

Amino acid	LC1	LC2	LC3
Asx[a)]	6.5	2.9	2.4
Glx[a)]	19.2	8.6	9.1
Ser	10.7	6.7	8.2
Gly	18.5	19.7	22.3
Arg	4.9	4.3	3.4
Thr	5.9	5.7	6.1
Ala	7.4	9.6	9.8
Pro	4.5	9.2	8.0
Tyr	0.0	2.5	2.8
Val	3.4	6.7	7.1
Ile	5.9	6.7	5.8
Leu	6.3	8.5	6.4
Phe	1.8	3.7	3.5
Lys	5.0	5.4	5.2

[a)]Gln and Asn were quantitatively converted to Glu and Asp, respectively, during acid hydrolysis.
Trp, Met and Cys were not evaluated.

acid and cinnamic acid were detected by GC-MS analyses as trimethylsilylated compounds. These data indicated the substances to contain a lignin portion. Furthermore, their IR spectra indicated the presence of protein or peptide, since amide-I (1654 cm^{-1}) and amide-II (around 1540 cm^{-1}) absorptions were observed. The ^{1}H-NMR spectrum indicated exchangeable amide proton signals (7–8 ppm) in all substances (data not shown). They therefore seemed to contain a proteinaceous portion. The respective protein contents of LC1, LC2 and LC3 were estimated to be 13.8%, 51.8% and 52.1% by elemental analyses. Amino acid compositions of these substances were also determined as PITC derivatives. As shown in Table 2, LC1, LC2 and LC3 had high contents of Gln/Glu and Gly. In addition, LC2 and LC3 contained a relatively high amount of Pro. We concluded from these data that the substances were lignin-carbohydrate-protein complexes.

Antiviral activities of LCs from P. anisum

The antiviral potency of LC1, LC2 and LC3 was evaluated against eight viruses as shown in Table 3. All samples were assayed for their cytotoxicity to host cells as well as for their inhibition of viral replication in the host cells. The antiviral potency of each was estimated by the selectivity index (SI) expressed as the ratio of CC_{50} to IC_{50}. In experiment A, a sample was added to the medium during viral infection and thereafter throughout the incubation, while in experiment B, a sample was added immediately after viral infection. No marked cytotoxic effect was apparent in any of the substances since their CC_{50} values were higher than 10,000 μg/mL. It was found that they all had antiviral activity against four viruses (HSV-1, HSV-2, HCMV and measles virus), and that LC1 and LC3 also had respective selective toxicity against HCoV and coxsackie virus on the basis of their SI values of more than 10. Although LC1 showed no marked difference in antiviral potency between experiments A and B, the SI values for LC2 and LC3 were higher in experiment A than those in experiment B. Therefore, one of antiviral targets of LC2 and LC3 seemed to be the early stage(s) of virus replication such as virus adsorption to and penetration into host cells. We subsequently studied the modes of action of LCs in the replication of the two most sensitive viruses, HSV-2 and HCMV.

Table 3. Antiviral Activities of the Lignin-Carbohydrate Complexes from *P. anisum*

Virus	Sample	Cytotoxicity (CC_{50}, μg/mL)	Antiviral activity (IC_{50}, μg/mL)		Selectivity index (CC_{50}/IC_{50})	
			A[a)]	B[b)]	A	B
HSV-1	LC1	>10000	660	710	>15	>14
	LC2	>10000	210	230	>48	>43
	LC3	>10000	70	270	>140	>37
HSV-2	LC1	>10000	920	>1000	>11	≧10
	LC2	>10000	170	600	>59	>17
	LC3	>10000	47	260	>210	>38
HCMV	LC1	>10000	18	20	>560	>500
	LC2	>10000	6.0	17	>1700	>590
	LC3	>10000	3.2	11	>3100	>910
Measles	LC1	>10000	1000	>1000	>10	≧10
	LC2	>10000	160	370	>63	>27
	LC3	>10000	200	420	>50	>24
Mumps	LC1	>10000	>1000	>1000	≧10[c)]	≧10
	LC2	>10000	>1000	>1000	≧10	≧10
	LC3	>10000	>1000	>1000	≧10	≧10
Poliovirus	LC1	>10000	>1000	>1000	≧10	≧10
	LC2	>10000	>1000	>1000	≧10	≧10
	LC3	>10000	>1000	>1000	≧10	≧10
Coxsackie	LC1	>10000	>1000	>1000	≧10	≧10
	LC2	>10000	>1000	>1000	≧10	≧10
	LC3	>10000	670	>1000	>15	≧10
HCoV	LC1	>10000	430	>1000	>23	≧10
	LC2	>10000	>1000	>1000	≧10	≧10
	LC3	>10000	>1000	>1000	≧10	≧10

[a)]The sample was added to the medium during infection and throughout incubation.
[b)]The sample was added to the medium immediately after viral infection.
[c)]≧means not clear whether more or less than the value.

LCs were applied to time-of-addition experiments in order to delineate the most drug-sensitive phase of HSV-2 and HCMV replication. Vero or MRC-5 cells were respectively infected with HSV-2 or HCMV in these experiments at a high multiplicity of infection of 10 or 1, and each sample was added to the medium at the indicated time and throughout the incubation thereafter. The respective concentrations tested were 2 mg/mL and 100 μg/mL for HSV-2 and HCMV, as judged from the IC_{50} values of the samples. As shown in Fig. 1A, there were no striking time-of-addition-dependent differences in HSV-2 production for LC1 and LC2, whereas LC3 strongly suppressed virus production when added before 1 h of virus infection. Due to their relatively high molecular weights (7,000–8,000), LCs might not be internalized into the host cells. These substances therefore seemed to interfere with virus adsorption and/or penetration, or to inactivate virus particles in the replication cycle of HSV-2. LCs also showed potent suppression of HCMV production when added before or at the same time as virus infection and throughout incubation thereafter (Fig. 1B). These data reveal that LCs might strongly inhibit the adsorption and penetration steps of HCMV.

The effects of LCs on virus adsorption to the host cells were evaluated by infectious center assays which determined the number of cells binding the virus particles at low temperature (4 °C) in the presence of

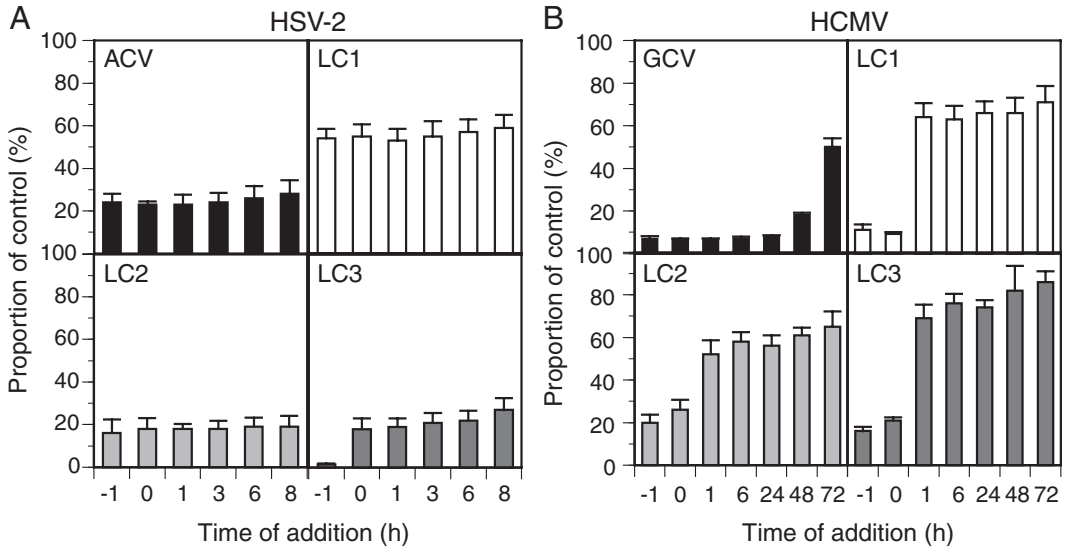

Fig. 1. Antiviral Effects of LCs on HSV-2 (A) and HCMV (B) at Different Times of Viral Infection.
Each sample was added to the medium at the indicated time and throughout the incubation thereafter. ACV (positive control) and LCs were respectively administered at 25 μM and 2 mg/mL for an HSV-2 assay. GCV (positive control) and LCs were respectively administered at 5 μM and 100 μg/mL for an HCMV assay. Each value is the mean ± SD from triplicate assays, and asterisks indicate statistical difference from a control.

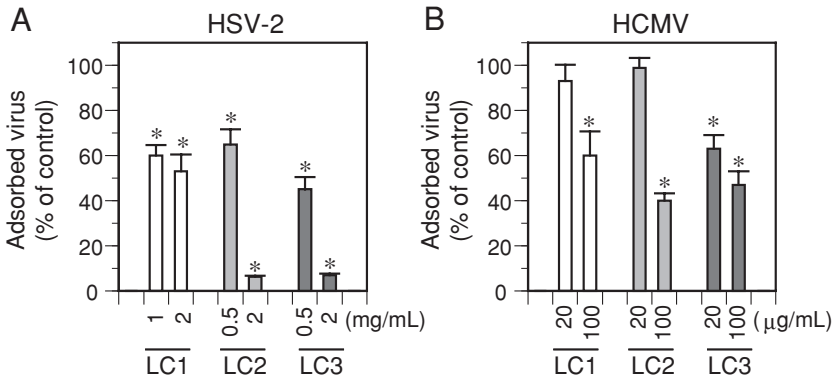

Fig. 2. Effects of LCs on HSV-2 (A) and HCMV (B) Adsorption.
A cell suspension was infected for 1 h with the virus at 4 °C in the presence of LC1, LC2 or LC3. A ten-fold dilution of the cell suspension was subjected to a plaque assay. The plaque numbers with no drug control are taken as 100%. Each value is the mean ± SD from triplicate assays, and asterisks indicate statistical difference from a control: $^{*}p < 0.001$.

different concentrations of the samples. As shown in Fig. 2, LCs significantly suppressed the adsorption of both HSV-2 and HCMV in a dose-dependent manner. After being adsorbed to the host cell surface, these viruses started to penetrate the cells. We then tested whether these substances could inhibit virus penetration or not. After the virus particles had been adsorbed to the host cell surface at 4 °C, each sample was added to the culture, and the temperature was raised to 37 °C. The viruses that had penetrated during an incubation period of 0.5 to 6 h were quantified by plaque assays. As shown in Fig. 3, LC3 had a moderate inhibitory effect on the penetration of HSV-2 and HCMV, whereas the others did not. It therefore seemed that the inhibition of viral penetration might play a less important role in their antiviral actions than that of viral binding.

We have already described that one of the antiviral targets of LCs was preventing virus adsorption to the host cell surface. However, there was another possible antiviral target, since they showed a considerably high anti-HSV-2 effect by their addition to the medium even after 6 h post infection, when the virus binding and penetration step(s) had been completed (Fig. 1A). If LCs could exert a virucidal effect, this might contribute to the antiviral activity by delayed addition of these substances. We therefore attempted to elucidate whether or not they had an effect on virus infectivity. As shown in Table 4, pre-incubation of the viruses with LCs resulted in time- and dose-dependent reduction of the remaining infectivity; for example, LC2 and LC3 completely abolished the infectivity of HSV-2 by a 24-h treatment at the concentrations tested. The remaining infectivity of HCMV was also reduced to 74% and 63% of the no-drug control by respective 24-h incubation with LC2 and LC3 at 100 μg/mL. As predicted from the results of the time-of-addition experiments, LC2 and LC3 exerted a much less virucidal effect on HCMV than that on HSV-2.

Effects of LCs on RAW264.7 cells

Although the host defense mechanism is complex, macrophages are key participants in the innate immune

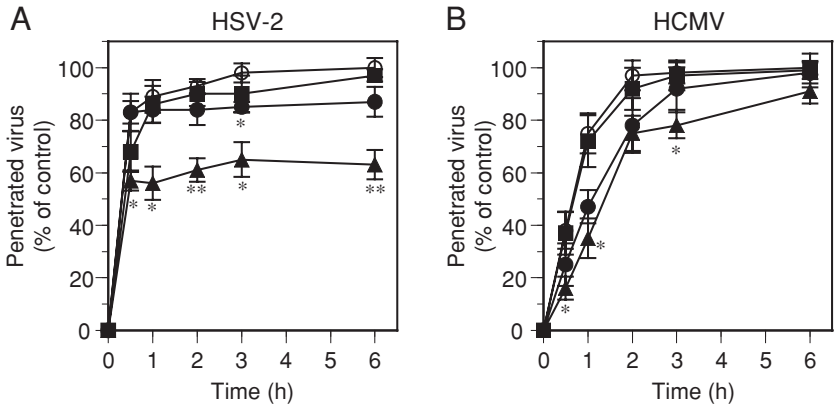

Fig. 3. Effects of LCs on HSV-2 (A) and HCMV (B) Penetration.

Cell monolayers were infected for 1 h with the virus at 4 °C in the absence of LCs and then at 37 °C in the presence of an LC. The cell monolayers were treated with a citrate buffer (pH 3.0) after 0.5, 1, 2, and 6 h, and then subjected to a plaque assay. The plaque numbers with no drug control after 6 h at 37 °C are taken as 100%. Each value is the mean ± SD from triplicate assays, and asterisks indicate statistical difference from a control: $^{*}p < 0.01$; $^{**}p < 0.001$. LC1 (filled squares), LC2 (filled circles), or LC3 (filled triangles) was added at 2 mg/mL for HSV-2 and at 100 μg/mL for HCMV. Unfilled circles indicate no drug control.

Table 4. Virucidal Activities of LCs towards HSV-2 and HCMV

Sample	Concentration (μg/mL)	Residual infectivity (% of control)	
		1.5 (h)	24 (h)
HSV-2			
LC1	1000	97	92
	2000	95	80
LC2	1000	87	0
	2000	41	0
LC3	1000	73	0
	2000	34	0
HCMV			
LC1	20	100	96
	100	95	83
LC2	20	93	96
	100	89	74
LC3	20	89	95
	100	84	63

system to respond to the invasion of pathogenic organisms. We therefore investigated the effects of LC1, LC2 and LC3 on the activation of macrophages. After RAW 264.7 murine macrophage cells had been incubated with those compounds for 20 h, the NO concentration in each culture supernatant was measured. As shown in Fig. 4A, these substances each had a stimulatory effect on NO production in a dose-dependent manner. In addition, polymyxin B, which is well known to form an inactive complex of LPS *in vitro*, did not influence their stimulating effects on NO production (data not shown). Their effects on the mRNA and protein expression of iNOS in RAW 264.7 cells showed that LC1, LC2 and LC3 induced iNOS mRNA expression in a time-dependent manner (Fig. 4B). The iNOS protein level in RAW 264.7 cells also increased in the presence of these substances (Fig. 4C). Since many immunomodulatory substances are known to modulate cytokine production, we analyzed the immunomodulatory properties of the lignin-carbohydrate complexes by detecting cytokine mRNA with RT-PCR. The result shows that the LCs induced the expression of both IL-1β and IL-10 mRNAs (Fig. 5).

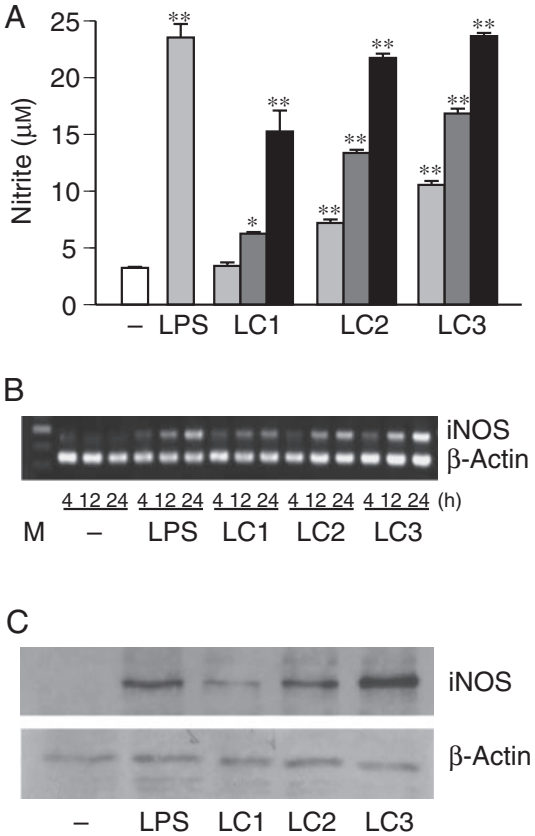

Fig. 4. Effect of LCs on NO Production and iNOS Expression in RAW 264.7 Cells.

A, The cells were incubated with different concentrations of an LC for 20 h. NO production was determined by measuring the accumulation of nitrite in the culture medium. A no-sample control (−) and lipopolysaccharide (LPS, 1 μg/mL) were also assayed. Data are expressed as the mean ± SD of triplicate cultures, and asterisks indicate statistical difference from a control: $^{*}p < 0.01$; $^{**}p < 0.001$. B, The cells were incubated with 100 μg/mL of an LC for 4, 12 or 24 h. A no-sample control (−) and lipopolysaccharide (LPS, 1 μg/mL) were also assayed. iNOS mRNA expression was detected by RT-PCR. C, The cells were incubated with 100 μg/mL of an LC for 24 h. A no-sample control (−) and lipopolysaccharide (LPS, 1 μg/mL) were also assayed. The iNOS protein was detected by western blotting.

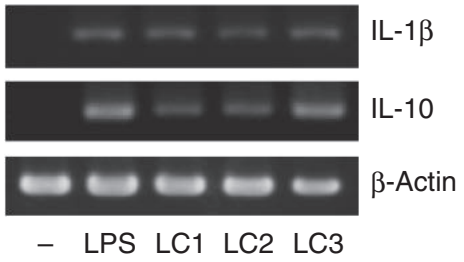

Fig. 5. Effect of LCs on Cytokine mRNA Expression in RAW 264.7 Cells.

The cells were cultured in the absence or presence of LPS (1 μg/mL) or an LC (100 μg/mL) for 4 h. Cytokine mRNA expression was detected by RT-PCR.

Discussion

Studies on the chemical constituents of *P. anisum* have so far been focused on lipophilic compounds, especially essential oils; for example, trans-anethole is well known to be a major constituent of anise and has been reported to possess a characteristic odor. On the other hand, limited reports have shown that an aqueous extract from *P. anisum* demonstrated such biological effects as antioxidative, antimicrobial and selective estrogen receptor modulator-like activities.[17,18] However, it was uncertain which class of compounds contributed to those activities. We have shown in the present paper that the lignin-carbohydrate complexes from anise seemed to be the active principles for exhibiting antiviral and immunostimulatory effects.

There are several reports on lignin-carbohydrate complexes from other plants possessing antiviral and immunomodulatory effects. The lignin-carbohydrate complex from *Prunella vulgaris* with a molecular weight of 8500 has been reported to show anti-herpes activity.[19] The authors revealed that the active principle appeared to exert its anti-HSV effect by inhibiting viral binding and penetration. Our results are consistent with those, although it is noteworthy that the virucidal effects of lignin-carbohydrate complexes have not been shown except for our present data. Pine cone lignin-carbohydrate complexes have also been reported to show antiviral effects against HIV-1, influenza virus and HSV.[20] In addition, the lignin-carbohydrate complexes from pine cone possess various biological activities including immunopotentiation. Kiyohara *et al.* have also reported that lignin-carbohydrate complexes in the kampo (Japanese herbal) medicine, juzen-taiho-to, modulated the intestinal immune system.[21] Lignin-carbohydrate complexes therefore seem to be an important chemical class showing such biological activities as antiviral and immunomodulatory effects. It has been reported that standard lignin did not have any effect on the intestinal immune system.[21] An investigation of the mechanisms of action and structural requirements of lignin-carbohydrate complexes will be needed to understand their biological effects and for the future applications. In conclusion, it is suggested that the lignin-carbohydrate complexes from *P. anisum* might contribute to the prevention of viral infection.

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