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Procyanidins and butanol extract of Cinnamomi Cortex inhibit SARS-CoV infection

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ABSTRACT

We found that the butanol fraction of Cinnamomi Cortex (CC/Fr.2) showed moderate inhibitory activity in wild-type severe acute respiratory syndrome coronavirus (wtSARS-CoV) and HIV/SARS-CoV S pseudovirus infections. The inhibition on pseudovirus was also seen in cells pretreated with the CC and CC/Fr.2 (IC_{50} s, 283.4 ± 16.3 and 149.5 ± 13.5 μ g/ml, respectively), however the highest activities on wtSARS-CoV were observed when the viruses were treated by the extracts before challenging (IC_{50} s, 43.1 ± 2.8 and 7.8 ± 0.3 μ g/ml; SIs, 8.4 and 23.1, respectively). Among the compounds fractionated from CC, procyanidin A2 and procyanidin B1 showed moderate anti-wtSARS-CoV activity (IC_{50} s, 29.9 ± 3.3 and 41.3 ± 3.4 μ M; SIs, 37.35 and 15.69, respectively). We also sought to determine whether they could interfere with the clathrin-dependent endocytosis pathway using transferrin receptor (TfR) as an indicator. CC/Fr.2 inhibited the internalization of TfR but the procyanidins did not. Taken together, CC/Fr.2 contains unknown substances, that could inhibit the infection, probably by interfering with endocytosis, and it also contains procyanidins that did not inhibit the internalization but inhibited the infection. Therefore, CC extracts contain anti-virus activities that act through distinct mechanisms according to differences in the compounds or mixtures.

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1. Introduction

Severe acute respiratory syndrome (SARS) is an atypical type of contagious pneumonia with a high mortality rate, and is caused by SARS coronavirus (SARS-CoV) (Drosten et al., 2003; Ksiazek et al., 2003), which infected over 8000 patients of whom more than 700 died by July 2003 (Lingappa et al., 2004). Interferons (Haagmans et al., 2004; Paragas et al., 2005; Tan et al., 2004; Barnard et al., 2006), glycyrrhizin (Cinatl et al., 2003), 1,2,3,6-tetra-*O*-galloyl- β -D-

glucose (TGG), luteolin (Yi et al., 2004), Lycoris radiate (S.Y. Li et al., 2005), emodin (Ho et al., 2006), N4-hydroxycytidine (Barnard et al., 2004) and protease inhibitor (Chen et al., 2005) have been reported to have anti SARS-CoV activities in vitro. However, the selectivity indices of these materials are as high as 100 and the mechanisms of their actions are not clear (De Clercq, 2004, 2006). Therefore, more research is necessary to discover drugs and to study their mechanisms for the practical interference of SARS-CoV infection. It was reported that medicinal herbs combined with western medicines improved the symptoms and decreased the required dosage of corticosteroids for SARS patients (Liu et al., 2006). To identify potential inhibitor(s) of SARS-CoV, we screened seven different medicinal herbs that were reported to be used for SARS patients (Zhang et al., 2004). We also fractionated the extracts to characterize the inhibitory molecule(s).

The spike proteins (S) on the surface of the SARS-CoV particle mediate the binding of the virus to its cell surface receptor, angiotensin converting enzyme 2 (ACE2) (Simmons et al., 2004; Li et al., 2003). We used a pseudovirus that consisted of luciferase encoded human immunodeficiency virus (HIV) (Yang et al., 2004) and SARS-CoV S protein for screening inhibitors. Because a pseudotyped virus does not replicate, the luciferase activities specifically

Abbreviations: SARS-CoV, severe acute respiratory syndrome coronavirus; HIV, human immunodeficiency virus; VSV, vesicular stomatitis virus; Wild-type SARS-CoV, wtSARS-CoV; ACE2, angiotensin converting enzyme 2; IC_{50} , 50% inhibitory concentration; CC_{50} , 50% growth reduction of the cells; SI, selective index, CC_{50}/IC_{50} ; TCID₅₀, 50% tissue culture infectious dose; PBS, phosphate-buffered saline; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; FITC, fluorescein isothiocyanate; PE, phycoerythrin; CHX, cycloheximide; MFI, mean fluorescence intensity.

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reflect single-round infection. The inhibitory activities shown in the pseudotyped virus assay were also confirmed by the wild-type SARS-CoV (wtSARS-CoV) infection assay using SARS-CoV PUMC01 F5 (GenBank accession no. AY350750 B.J. Li et al., 2005).

Whether enveloped or nonenveloped, many viruses depend on the host cell endocytic pathways for entry. SARS-CoV S glycoprotein mediates viral entry through pH-dependent endocytosis, like vesicular stomatitis virus (VSV) (Sun et al., 2005). The endocytic pathways taken by viruses can be divided into clathrin-mediated and clathrin-independent pathways (Marsh and Helenius, 2006). Recently, we demonstrated that SARS-CoV mainly utilizes the clathrin-mediated endocytosis pathway for its entry to target cells (Inoue et al., 2007). Therefore, we examined whether effective herbs and their purified compounds may affect the endocytotic pathways.

2. Materials and methods

2.1. Cell lines

The following cell lines were obtained from Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University; HEK293T, HepG2, VeroE6 and Jurkat cells. The cells were cultured in either Dulbecco's modified Eagle's medium (DMEM) or RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin antibiotics.

2.2. Extracts of medicinal herbs and fractionated samples

The water extracts prepared from 7 kinds of dried medicinal herbs and four fractionated samples of Cinnamomi Cortex (CC) and Caryophylli Flos (CF) were provided by Tsumura & Co. (Tokyo, Japan). The dried medicinal herbs included Forsythiae Fructus, Scutellariae Radix, Astragali Radix, Bupleuri Radix, Glycyrrhizae Radix, CC and CF. Each dried medicinal herb (30 kg) was boiled under reflux in 360 l of distilled water for 1 h. The aqueous extract was separated from the residue by centrifugation followed by concentration under reduced pressure. The concentrated extracts were lyophilized. Four fractions of CC were obtained by the method shown in Fig. 1. CC (114.9 g) was extracted with 700 ml of 50% aqueous ethanol (aq. EtOH/H₂O under reflux for 2 h). The obtained extract was concentrated under reduced pressure to yield a 50% aq. EtOH/H₂O extract (13.5 g, fraction 1). Most of this (11.2 g) could be dissolved in distilled water, and it was then extracted with ethylac-

etate (AcOEt, 200 ml × 2) and *n*-butanol (*n*-BuOH, 200 ml × 2). The *n*-BuOH layer, aq. layer and AcOEt layer were concentrated under reduced pressure to successively yield fractions 2 (CC/Fr.2, 3.5 g), 3 (CC/Fr.3, 3.7 g) and 4 (CC/Fr.4, 2.8 g), respectively. The four fractions of CF were also obtained by an essentially similar method as described above. These fractions were suspended in dimethyl sulfoxide (DMSO) at a concentration of 100 mg/ml and then serially diluted with DMEM.

2.3. Chemicals

2-Phenylethanol (phenethyl alcohol), 2'-hydroxyacetophenone (*o*-hydroxyacetophenone), isoeugenol 2-hydroxycinnamic acid, 3,4-dimethoxycinnamic acid, 4-hydroxy-3-methoxycinnamic acid (ferulic acid), 4-hydroxycoumarin, 7-hydroxycoumarin, *p*-cymene, 4-allylanisole, ethylcinnamate and cinnamylacetate were purchased from Wako (Osaka, Japan). *Trans*-cinnamaldehyde, *trans*-cinnamic acid, licochalcone-A 4-hydroxy-3-methoxy cinnamaldehyde and cycloheximide (CHX) were purchased from Sigma (St. Louis, MO). The eight compounds procyanidin A2, procyanidin B1, procyanidin B2, procyanidin C1, procyanidin oligomer, (–)-epicatechin, (+)-catechin and cinnamtannin B1 were purified from CC as described previously (Nonaka et al., 1983). These chemicals were dissolved in DMSO at 100 mg/ml or 100 mM.

2.4. Infection of pseudoviruses

Pseudotyped lentiviruses were prepared as described previously (Yang et al., 2004). HEK293T cells were cotransfected with HIV-1-based lentiviral DNA and plasmids encoding full-length SARS-S (G.J. Nabel, National Institutes of Health, Bethesda, MD) or VSV-G (Naldini et al., 1996). The p24 Gag antigen levels were measured using an HIV-1 p24 Antigen Assay kit (ZeptoMetrix Corporation, New York). Five ng (p24) of the generated HIV/SARS-CoV S or HIV/VSVG were infected in HepG2 cells (1 × 10⁴ cells/well in 96-well plates). After 12 h of cultivation, the cells were washed twice with DMEM which was then replaced with complete medium. Forty-eight hours after infection, the luciferase activity was measured by Mithras LB940 (Berthold Technologies GmbH & Co KG, Germany) according to the Manufacturer's instructions.

2.5. Inhibition of the pseudovirus infection

The supernatants containing 5 ng (p24) of the HIV/SARS-CoV S or HIV/VSVG pseudoviruses were mixed with or without the reagents at different concentrations and were then added to HepG2 cells. The DMSO solvent was also used for a control. After 12 h infection the cells were washed twice with DMEM which was then replaced with complete medium without the reagents. The relative luciferase activities were calculated as percentages of the control culture-background. The 50% inhibition concentration (IC₅₀) of the reagents was determined from a curve relating the luciferase activities to the concentration of the reagents (Y. Xu et al., 2000).

To evaluate which step of virus replication was inhibited by the reagents, we examined the effects in an additional study in which the reagents were added on the cells at –1 or 12 h after HIV/SARS-CoV S infection because 12 h were required to obtain stable luciferase activities (Yang et al., 2004). Furthermore, the cells were pretreated with various concentrations of the reagents for 1 h followed by washing twice, and were challenged with pseudovirus to determine whether the reagents work on the cells or on the viruses.

2.6. Inhibition assay for wtSARS-CoV infection

The wtSARS-CoV strain PUMC01 F5 (GenBank accession no. AY350750) was used for the assay. We performed a plaque reduc-

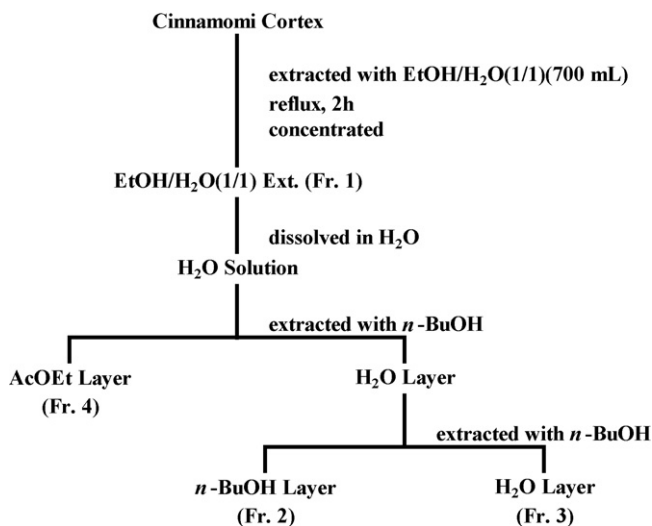


Fig. 1. Fractionation procedure of Cinnamomi Cortex (see text).

tion assay in which 100 times the 50% cell culture infective doses (CCID₅₀) of wtSARS-CoV was added with 100 μ l of each reagent for 0 or 60 min at 37 °C and then transferred onto 6-well plates (1 \times 10⁶ cells/well) with the VeroE6 cells. The DMSO solvent was also used as a control. After 1 h of infection, the cells were washed twice by DMEM, and then 2% agarose was added onto the cell surface. A 2% secondary agarose layer with 0.05% neutral red dye was added on the third day after incubation. The numbers of plaques were counted after adding neutral red dye. The inhibitory concentrations for a 50% plaque reduction (IC₅₀) were determined from a curve relating the plaque number to the concentrations of the reagents.

2.7. Cytotoxicity assay

In all experiments, we chose the optimal concentration of each reagent that yielded a cell viability of more than 90%. The cytotoxic analysis of the reagents for HepG2 was performed using a cell counting kit-8 (Dojindo Laboratories, Kumamoto, Japan) in the same conditions as described for the pseudovirus infection. For VeroE6 the extracts were incubated with the cells for 72 h and the viabilities were determined using Cell Titer 96 Aqueous Non-radioactive Cell Proliferation Assay Kits (Promega). The 50% growth reduction (CC₅₀) was calculated. The viabilities of Jurkat cells were determined by the trypan blue dye exclusion method. Selective index was calculated as CC₅₀/IC₅₀ as described elsewhere (Baba et al., 1987).

2.8. Flow cytometric analysis

To investigate whether the compounds affect the expression of ACE2 on the surface of HepG2 cells, the cells were either left untreated or were pretreated with CCE, CC/Fr.2, CFE (0.1 mg/ml) or three compounds (procyanidin A2, procyanidin B1 and cinnamtannin B1 at 100 μ M) in 37 °C for 60 min. The DMSO solvent was also used as a control. After washing with PBS, the cells were detached by treatment with 10 mM EDTA for 10 min. The detached cells were reacted with Biotinylated Anti-human ACE-2 ectodomain Antibody (R&D Systems). After reaction with phycoerythrin (PE) streptavidin (BD Pharmingen), the cells were washed and subjected to flow cytometry analysis.

For the TfR and CD59 staining, the Jurkat cells and HepG2 cells were used for the assay. Jurkat cells were incubated with the extracts at different concentrations with or without 10 μ g/ml of cycloheximide or at 100 μ g/ml of each extract for various peri-

ods (or 5–60 min) at 37 or 4 °C followed by washing twice with cold PBS containing 1% BSA. Ten μ l of PE-anti-TfR (BD Biosciences) and 10 μ l of FITC-anti-CD59 (BD Biosciences) were incubated with 5 \times 10⁵ cells for 30 min at 4 °C. After washing, the cells were analyzed by Cytomics (Beckman Coulter, Fullerton, CA). Data were analyzed by using CXP Analysis software version 2.0. The HepG2 cells were cultured in 12-well plates one day before treatment. After the extracts were added for 60 min, the cells were washed twice by PBS then detached by treatment with 10 mM EDTA for 10 min. The recovered cells were reacted with PE-anti-TfR (BD Biosciences) and of FITC-anti-CD59 (BD Biosciences). After washing, the cells were analyzed by Cytomics (Beckman Coulter, Fullerton, CA).

2.9. Internalization of fluorescent transferrin-A488

In order to observe the internalization of transferrin, the Jurkat cells were washed twice with cold PBS and 5 \times 10⁵ cells were incubated in 200 μ l PBS containing different concentrations of the extracts on ice for 30 min in polystyrene round-bottom tubes (Falcon 352058). Then, A488-labeled transferrin (Molecular Probes) was added at a final concentration of 50 μ g/ml and incubated for another 30 min on ice. The treated cells were washed twice with cold PBS, resuspended at 10⁷ cells/ml in cold PBS in the presence or absence of various concentrations of extracts and incubated at 37 °C for 15 min (Crotzer et al., 2004). Internalization was stopped by incubating the cells on ice and adding cold PBS. The cells were then fixed with 4% paraformaldehyde and plated on poly-L-lysine-treated cover slips (Sigma–Aldrich). Cells were viewed using a Zeiss LSM510 microscope, and a single section through the middle of the cell is shown.

3. Results

3.1. Inhibition of pseudovirus infection

First, we screened the extracts of the 7 medicinal herbs by HIV/SARS-CoV S pseudovirus infection and only two, CCE (Cinnamomi Cortex Extract) and CFE (Caryophylli Flos Extract), apparently showed low IC₅₀ (<100 μ g/ml) with slight and moderate SIs against HIV/SARS-CoV S pseudovirus (Table 1), and these inhibitory activities were dose-dependent (Fig. 2A). It is of note that both CCE and CFE also inhibited the infection of HIV/VSVG pseudovirus (Fig. 2B). To characterize the responsible substance(s), we separated the four fractions from CC and CF using aqueous ethanol (Fr.1), ethylacetate (Fr.4) and *n*-butanol (Fr.2) extraction

Table 1
Inhibitory effects of medicinal herbs on infection of HIV/SARS-CoV S pseudovirus.

Extracts	CC ₅₀ (μ g/ml)	IC ₅₀ (μ g/ml)	SI (CC ₅₀ /IC ₅₀)
Forsythiae Fructus	577.8 \pm 79.7	401.4 \pm 29.5	1.4
Scutellariae Radix	854.2 \pm 76.2	853.2 \pm 36.8	1.0
Astragali Radix	2726.5 \pm 125.2	1623.7 \pm 106.5	1.7
Bupleuri Radix	1213.6 \pm 54.5	1643.2 \pm 52.8	<1
Glycyrrhizae Radix	741.2 \pm 70.7	781.2 \pm 65.4	<1
Cinnamomi Cortex (CCE)	201.1 \pm 17.1	30.3 \pm 2.6	6.6
Ethanol extract of CC (Fr.1)	444.0 \pm 13.7	85.3 \pm 7.5	5.2
Butanol fraction of CC (Fr.2)	205.3 \pm 11.2	37.3 \pm 3.5	5.5
Aqueous fraction of CC (Fr.3)	267.7 \pm 23.6	68.6 \pm 5.7	3.9
Ethylacetate fraction of CC (Fr.4)	197.8 \pm 14.1	58.1 \pm 4.4	3.4
Caryophylli Flos (CFE)	757.2 \pm 59.3	58.8 \pm 5.6	12.9
Ethanol extract of CF (Fr.1)	553.4 \pm 35.7	102.0 \pm 7.3	5.4
Butanol fraction of CF (Fr.2)	1074.7 \pm 82.0	51.3 \pm 4.9	20.9
Aqueous fraction of CF (Fr.3)	1573.1 \pm 88.2	67.1 \pm 5.3	23.4
Ethylacetate fraction of CF (Fr.4)	441.5 \pm 14.5	60.4 \pm 5.5	7.3

Experiments were repeated three times, and the averages of IC₅₀ (the inhibitory concentration of extract to inhibit the luciferase activity to 50% control value) and CC₅₀ (the cytotoxic concentration of extract that reduced cell viability to 50%) are shown. The selectivity index (SI) corresponds to CC₅₀/IC₅₀.

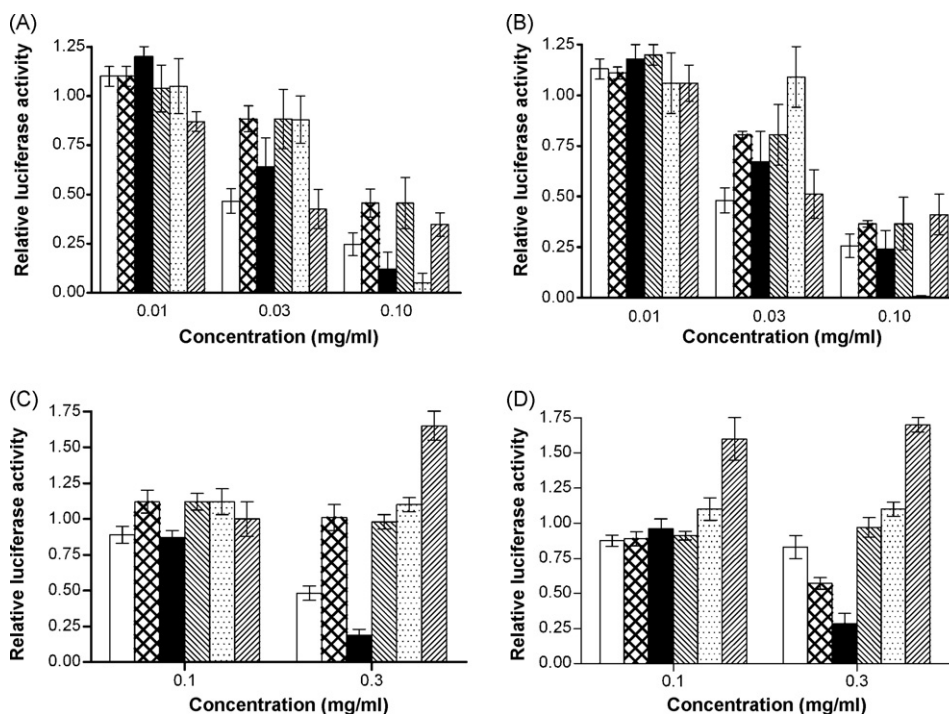


Fig. 2. Inhibition of pseudovirus infection by medicinal herbs. Pseudoviruses were treated with the following extracts and then incubated on the target cells. HIV/SARS-CoV S pseudovirus (A) or HIV/VSVG pseudovirus (B) was mixed with or without the reagents and was then added to HepG2 cells for infection. Target cells were treated by the extracts for 1 h followed by infection of HIV/SARS-CoV S pseudovirus (C) or HIV/VSVG pseudovirus (D) infection. The extracts used were as follows: (□) CCE; (▣) CC/Fr.1; (■) CC/Fr.2; (▤) CC/Fr.3; (▥) CC/Fr.4; (▧) CFE. The experiment was repeated three times and the average is shown. The error bar indicates standard deviation (SD).

methods (Fig. 1). The IC_{50} of CC/Fr.2 was the lowest among the four fractions of CC, although all fractions showed slight inhibitory activities (Table 1). The dose-dependent inhibition of CC/Fr.2 was also confirmed using both HIV/SARS-CoV S and HIV/VSVG pseudovirus infection (Fig. 2A and B). In the case of the CF fractions, CF/Fr.2, CF/Fr.3 and CF/Fr.4 showed moderate inhibitory activities (Table 1).

Pseudoviruses replicate in infected cells in a single cycle manner, and the luciferase activities are not solely dependent on the entry but also on transcription and protein synthesis. Therefore, the inhibitory activities of CCE and CFE were also examined at different times by adding them to the culture 1 h before infection or 12 h after infection by HIV/SARS-CoV S. The results showed that inhibition occurred only in the former case (data not shown) suggesting that they inhibited the HIV/SARS-CoV S infection only during the early steps of virus replication. Furthermore, pretreatment of HepG2 cells by CCE and CC/Fr.2 showed apparent inhibitory

effects on HIV/SARS-CoV S pseudovirus (IC_{50} s were 283.4 ± 16.3 and 149.5 ± 13.5 μ g/ml, respectively), however the other fractions of CC, CFE and its fractions (data not shown) did not show inhibition after pretreatment. Also they showed similar effects on HIV/VSVG pseudovirus by pretreatment of HepG2 cells. Based on the above results, we hypothesized that CC/Fr.2 and CCE may inhibit virus entry by acting on the cells. The inhibitory activity of CC/Fr.2 was always more prominent than that of CCE indicating that CC/Fr.2 contained the entry inhibitor that acted on the cells.

3.2. Inhibition of wtSARS-CoV infection

We examined the inhibitory effects of the selected extracts, CCE and CFE, using a quantitative assay (plaque reduction assay) for the infection of wtSARS-CoV. In this assay CCE inhibited wtSARS-CoV infection when the viruses were mixed with the herbs and added onto the target cells, but CFE showed only weak inhibitory activity (Table 2). The IC_{50} s counted by plaque numbers of the wtSARS-CoV, which were pretreated (1 h) with CCE, CC/Fr.2 or compounds (procyanidin A2, procyanidin B1 and cinnamtannin B1), were not different from those in which the viruses were added simultaneously with drugs (0 h) ($P > 0.05$). Therefore we could not prove virucidal effects of the compounds, and analyzed their inhibitory activities by adding the mixtures of compounds and the virus (0 h) to target cells. Among the four fractions of CC, CC/Fr.2 showed moderate inhibitory activities in the plaque reduction assay, suggesting that CC/Fr.2 contained more biologically active substances (Table 2). Unexpectedly, the four fractions of CF, as well as CFE, did not inhibit wtSARS-CoV infection (Table 2).

3.3. The effects of chemicals

Because CCE may contain specific compounds that inhibit virus infection through clathrin-dependent endocytosis, we tried to identify the compounds. Several compounds reported to be present

Table 2

Inhibitory effects of medicinal herbs and their fractionated samples on infection by wild-type SARS-CoV.

Extracts	CC_{50} (μ g/ml)	IC_{50} (μ g/ml)	SI
Cinnamomi Cortex extract (CCE)	360.2 ± 23.0	43.1 ± 2.8	8.4
Ethanol extract of CC (Fr.1)	180.5 ± 5.8	10.7 ± 0.4	16.9
Butanol fraction of CC (Fr.2)	180.0 ± 6.0	7.8 ± 0.3	23.1
Aqueous fraction of CC (Fr.3)	360.0 ± 19.7	39.7 ± 2.1	9.1
Ethylacetate fraction of CC (Fr.4)	90.1 ± 4.6	–	–
Caryophylli Flos extract (CFE)	180.0 ± 5.2	50.1 ± 3.5	3.6
Ethanol extract of CF (Fr.1)	50.5 ± 4.7	–	–
Butanol fraction of CF (Fr.2)	15.0 ± 0.8	–	–
Aqueous fraction of CF (Fr.3)	310.0 ± 16.3	–	–
Ethylacetate fraction of CF (Fr.4)	35.1 ± 2.8	–	–

Experiments were repeated three times, and the data show the average IC_{50} (the inhibitory concentration of extracts for a 50% plaque reduction of the virus). SI corresponds to CC_{50}/IC_{50} . '–' indicates that the extracts did not inhibit SARS-CoV infection at the highest optimal concentration.

Table 3
Inhibitory effects of chemical reagents on infection of HIV/SARS-CoV S pseudovirus.

No.	Extracts	CC ₅₀ (mM)	IC ₅₀ (mM)	SI (CC ₅₀ /IC ₅₀)
1	<i>Trans</i> -cinnamaldehyde	0.2 ± 0.014	0.2 ± 0.016	1
2	<i>Trans</i> -cinnamic acid	22.4 ± 1.230	3.0 ± 0.178	7.4
3	Licochalcone-A	0.03 ± 0.001	0.03 ± 0.003	1
4	2-Phenylethanol (phenethyl alcohol)	24.5 ± 1.533	4.1 ± 0.199	6.0
5	2'-Hydroxyacetophenone (<i>o</i> -hydroxyacetophenone)	1.8 ± 0.071	1.8 ± 0.102	1
6	Isoeugenol	3.9 ± 0.343	2.0 ± 0.142	2.0
7	2-Hydroxycinnamic acid	2.5 ± 0.122	0.3 ± 0.013	8.3
8	3,4-Dimethoxycinnamic acid	10.0 ± 0.620	5.0 ± 0.233	2
9	4-Hydroxy-3-methoxycinnamic acid (ferulic acid)	10.0 ± 0.555	5.0 ± 0.312	2
10	4-Hydroxy-3-methoxy cinnamaldehyde	0.7 ± 0.043	0.3 ± 0.013	2.3
11	4-Hydroxycoumarin	10.3 ± 0.660	10.3 ± 0.500	1
12	7-Hydroxycoumarin	10.7 ± 0.560	2.5 ± 0.172	4.3
13	<i>p</i> -Cymene	75.0 ± 4.350	75.0 ± 5.550	1
14	4-Allylanisole	8.0 ± 0.563	8.4 ± 0.650	<1
15	Ethylcinnamate	14.1 ± 1.220	7.1 ± 0.532	2.0
16	Cinnamylacetate	7.1 ± 0.420	7.1 ± 0.350	1

Experiments were repeated three times, and the averages of IC₅₀ (the inhibitory concentration of extract to inhibit the luciferase activity to 50% control value) and CC₅₀ (the cytotoxic concentration of extract that reduced cell viability to 50%) are shown. SI corresponds to CC₅₀/IC₅₀.

Table 4
The effects of compounds fractionated from CC on HIV/SARS-CoV S and SARS-CoV wild-type.

Compounds	MW	HIV/SARS-CoV S pseudovirus infection			Plaque reduction assay on SARS-CoV		
		CC ₅₀ (μM)	IC ₅₀ (μM)	SI	CC ₅₀ (μM)	IC ₅₀ (μM)	SI
Procyanidin A2	576.512	796.6 ± 63.7	120.7 ± 13.1	6.60	1116.7 ± 60.3	29.9 ± 3.3	37.35
Procyanidin B1	578.528	656.2 ± 36.7	161.1 ± 20.3	4.08	648.2 ± 43.4	41.3 ± 3.4	15.69
Cinnamtannin B1	864.769	242.3 ± 14.8	32.9 ± 2.8	7.36	184.7 ± 15.5	32.9 ± 3.9	5.61

Experiments were repeated three times, and the averages of IC₅₀ and CC₅₀ are shown. SI corresponds to CC₅₀/IC₅₀.

in CC and other derivatives have also been studied (Heide, 1972; Nonaka et al., 1983). Among these chemicals, *trans*-cinnamic acid, 2-phenylethanol (phenethyl alcohol) and 2-hydroxycinnamic acid showed slight suppressive effects [selectivity index (SI) > 5] on HIV/SARS-CoV S pseudovirus infection (Table 3). However, these chemicals did not show any anti-SARS-CoV activities in wtSARS-

CoV infection even at the highest optimal concentrations (data not shown). In the case of the eight compounds fractionated from CC, we examined all by pseudovirus and wtSARS-CoV infection. Among these compounds, procyanidin A2, procyanidin B1 and cinnamtannin B1 showed slight or moderate inhibitory effects in both assays (Table 4).

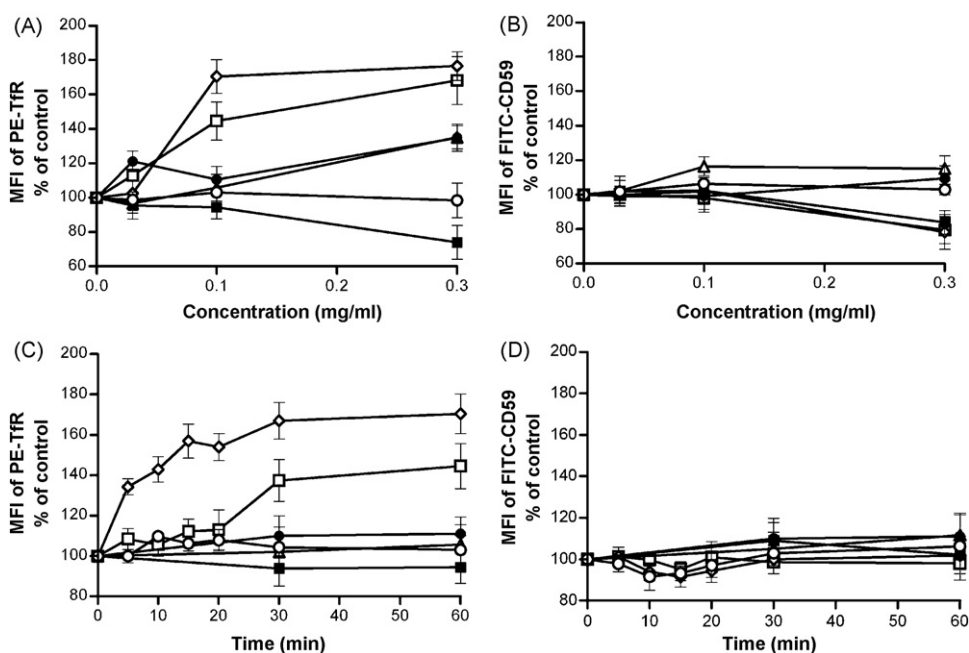


Fig. 3. Effect of medicinal herbs on Tfr or CD59 expression on Jurkat cells. A dose-dependent effect was observed (A and B). Jurkat cells were treated with various concentrations of the following extracts for 1 h followed by two-color staining with PE-anti-Tfr (A) and FITC-anti-CD59 (B). A time-dependent effect was also observed (C and D). Jurkat cells were treated with the various extracts for 0–60 min and were analyzed with PE-anti-Tfr (C) and FITC-anti-CD59 (D). (□) CCE; (△) CC/Fr.1; (◇) CC/Fr.2; (●) CC/Fr.3; (■) CC/Fr.4; (○) DMSO. Y-axis indicates MFI (mean fluorescence intensity) % of control. The experiment was repeated three times and the average is shown. The error bar indicates standard deviation (SD).

3.4. ACE2 expression after treatment by extracts and compounds

The HepG2 cells which were treated by CCE, CC/Fr.2, CFE (0.1 mg/ml) or three compounds (procyanidin A2, procyanidin B1 and cinnamtannin B1 at 100 μ M) in 37 °C for 60 min were used for flow cytometry analysis. Both the positive percentages and MFI of PE-anti-ACE2 were analyzed. There was no significant difference for both parameters between treated cells and untreated control. The percentages and MFI of non-treated cells were $35.5 \pm 2.6\%$, 635.7 ± 36.2 , and those of treated cells were $34.3 \pm 2.3\%$ to $39.9 \pm 2.8\%$, 601.3 ± 25.2 to 699.0 ± 40.4 , respectively.

3.5. Influence on the expression of TfR on Jurkat cell surface by the herbal treatment.

Because both CCE and CFE inhibited not only the SARS-CoV infection but also that of HIV/VSVG, we suspected that they may act through shared endocytic pathways of the viruses, and both viruses are known to enter into cells via clathrin-dependent endocytic pathways (Sun et al., 2005; Inoue et al., 2007). TfR is considered to be a marker of clathrin-mediated endocytosis and CD59 is a marker of clathrin-independent endocytosis (Mellman, 1996; Naslavsky et al., 2004). As expected, CC/Fr.2 and CCE up-regulated the TfR expression on Jurkat cell in dose- and time-dependent manners (Fig. 3A and C). Furthermore, neither CC/Fr.3, CC/Fr.4 (Fig. 3) nor CFE and its fractions (data not shown) influenced the TfR expression. The enhanced expressions of TfR on Jurkat cells by CC/Fr.2 could not be explained by enhanced protein synthesis, because the up-

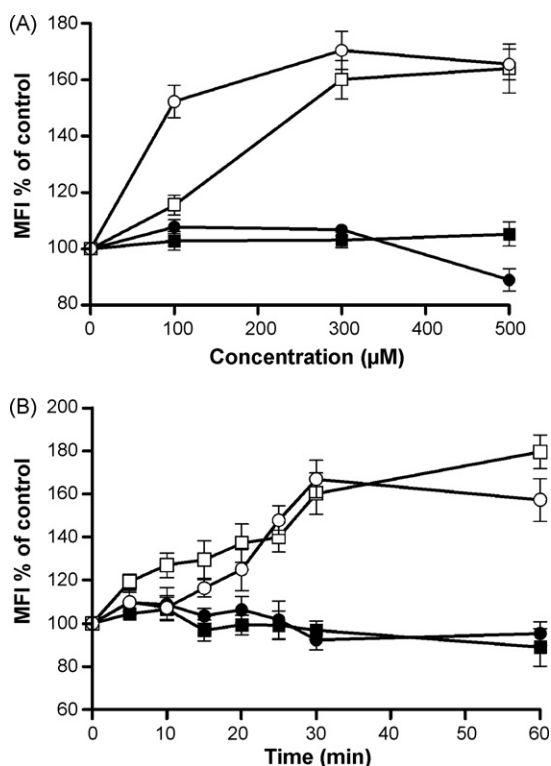


Fig. 4. The effects of procyanidin A2 and cinnamtannin B1 on TfR or CD59 expression on Jurkat cells. The dose- and time-dependent effects of procyanidin A2 and cinnamtannin B1 on Jurkat cells were examined. Jurkat cells were treated with various concentrations of procyanidin A2 and cinnamtannin B1 for 1 h (A) or were treated with 300 μ M of procyanidin A2 and 100 μ M of cinnamtannin B1 for various periods (B), following PE-anti-TfR and FITC-anti-CD59 staining flow cytometry was applied. (■) FITC-anti-CD59-procyanidin A2; (●) FITC-anti-CD59-cinnamtannin B1; (□) PE-anti-TfR-procyanidin A2; (○) PE-anti-TfR-cinnamtannin B1. The experiment was repeated three times and the average is shown. The error bar indicates standard deviation (SD).

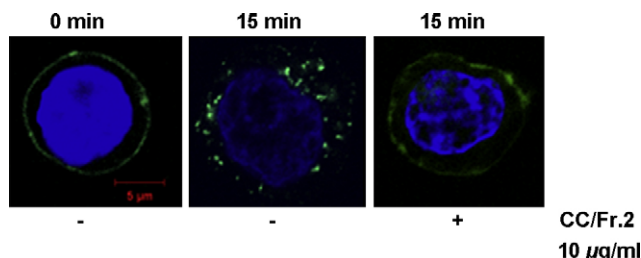


Fig. 5. CCE and CC/Fr.2 affect the internalization of TfR-A488 at the plasma membrane. Jurkat cells were treated with different concentration of CCE or CC/Fr.2 at 4 °C for 30 min. Then A488-conjugated transferrin was added and incubated at 4 °C for 30 min. After washing the Jurkat cells were incubated at 37 °C for 0 or 15 min in the presence or absence of the extracts. The nucleus is stained with DAPI. The results are representative of at least three independent experiments and numbers are provided in Section 3. Bar, 5 μ m for all panels.

regulation was also observed when the cells were incubated with CHX. No up-regulation of TfR was seen when the Jurkat cells were incubated with CCE at 4 °C. The up-regulation of only TfR was also found in treated HepG2 cells by CCE and CC/Fr.2 (MFI of untreated cells was 404.7 ± 25.5 , and 628.3 ± 37.1 and 759.0 ± 42.0 for CCE and CC/Fr.2, respectively). In the case of the eight compounds fractionated from CC, we examined all by pseudovirus and wtSARS-CoV infection. Because procyanidin A2, procyanidin B1 and cinnamtannin B1 showed inhibitory effects on SARS virus infection (Table 4), the effects on the expressions of CD59 and TfR on Jurkat cells were examined. Flow cytometric analysis showed that procyanidin A2 and cinnamtannin B1 up-regulated the expression of TfR in dose- and time-dependent manners (Fig. 4). Procyanidin B1 did not affect the expression of CD59 and TfR, even though it inhibited the infection of wtSARS-CoV with a SI of about 15.

3.6. Internalization of fluorescent transferrin-A488

Because TfR is a recycling molecule in cells, we considered that interference by CCE and CC/Fr.2 with the recycling of TfR resulted in the enhanced expression of TfR on Jurkat cells. The TfR-A488 was used for detection of the internalization, which is a step of endocytosis. At 37 °C incubation the TfR-A488 was internalized into Jurkat cells in the absence of the herbs after 15 min (Fig. 5, middle panel). We examined whether the extracts interfered with the internalization of TfR-A488 in Jurkat cells. CCE and all its fractions except for CC/Fr.4 blocked the internalization of TfR-A488 at 100 μ g/ml (Table 5), and no internalization was observed when CC/Fr.2 was added at a relatively low concentration (10 μ g/ml), which is close to the IC₅₀ for the wild-type infection (7.8 μ g/ml). CFE and the other

Table 5

The internalization of TfR-A488 in Jurkat cells after treatment with the extracts.

Extracts	Internalization of TfR-A488		
	100 μ g/ml	10 μ g/ml	6 μ g/ml
CCE	–	+	+
Ethanol extract of CC (Fr.1)	–	+	+
Butanol fraction of CC (Fr.2)	–	–	+
Aqueous fraction of CC (Fr.3)	–	+	+
Ethylacetate fraction of CC (Fr.4)	+	+	+
CFE	+	+	+
Ethanol extract of CF (Fr.1)	+	+	+
Butanol fraction of CF (Fr.2)	+	+	+
Aqueous fraction of CF (Fr.3)	+	+	+
Ethylacetate fraction of CF (Fr.4)	+	+	+

‘–’ indicates that no internalization of TfR-A488 was observed in 30 fields of observation. ‘+’ indicates that the internalization of TfR-A488 was observed in 30 fields of observation. The results are representative of at least three independent experiments.

fractions had no effect on the internalization (Table 5) even at a concentration (100 $\mu\text{g/ml}$) higher than the IC_{50} for HIV/SARS-CoV S pseudovirus infection (Table 5). Finally, no procyanidins inhibited the internalization at the optimal concentration.

3.7. Discussion

We used a pseudovirus to evaluate the anti-SARS-CoV activities of medicinal herbs using luciferase as a reporter. The 7 kinds of medicinal herbs which were recommended to be used for SARS patients (Liu et al., 2006) were examined for their effects on HIV/SARS-CoV S pseudovirus infection. Two herbal extracts (CCE and CFE) showed slightly or moderate inhibitory activities on both HIV/SARS-CoV S and HIV/VSVG pseudovirus infection.

We evaluated the anti-virus activities of the fractionated extracts and found that CC/Fr.1, CC/Fr.2 and all four fractions of CF showed slightly or moderate inhibitory activities against pseudovirus in the infection assay (Table 1). Because HIV-based pseudovirus RNA should be reverse transcribed followed by integration to make the reporter gene product, we examined whether these inhibitors could inhibit wild-type infection. In the plaque reduction assay of wtSARS-CoV infection, the SIs of CC/Fr.1 and CC/Fr.2 were found to be moderate; however, CFE and its fractions did not show significant inhibitory activities. We believe CCE may inhibit the early steps of SARS-CoV infection, based on the time of addition study and on the plaque reduction assay in the wild-type infection. Furthermore, the extract appeared to act on both cells and the virus because it inhibited infection of the cells by pretreatment of the cells or viruses. The $\text{IC}_{50\text{s}}$ (283.4 \pm 16.3 $\mu\text{g/ml}$ for CCE and 149.5 \pm 13.5 $\mu\text{g/ml}$ for CC/Fr.2) for pre-treatment of the cells were much higher than those obtained by the experiments in which the reagent was mixed with the virus first ($\text{IC}_{50\text{s}}$, 43.1 \pm 2.8 $\mu\text{g/ml}$ for CCE and 7.8 \pm 0.3 $\mu\text{g/ml}$ for CC/Fr.2), although we could not demonstrate virucidal activities in our assay. However, the mechanisms of the inhibitory activities by pre-treatment were studied. Because

CCE inhibited not only SARS-CoV but also HIV/VSVG pseudovirus infection, we suspected that they might interact with molecules that are important for the entry of both viruses. It is known that VSV and many enveloped viruses must be endocytosed for infection, and they are then transported to early endosomes, late endosomes, and lysosomes using the endocytic pathway. VSV is well-known to infect cells in a pH-dependent manner and was shown to utilize the clathrin-mediated endocytic pathway upon infection. We have also reported that SARS-CoV entry is clathrin-dependent. We therefore examined whether CCE or CFE could interfere with the recycling of TfR.

As we expected, CCE and CC/Fr.2 but not CFE up-regulated the TfR expression in time- and dose-dependent manners despite the presence of CHX (Fig. 3), and the TfR-inducing activity of CC/Fr.2 was higher than that of CCE. These results suggested that the anti-virus activity of CC/Fr.2, but not that of CFE, might be related to the endocytic pathway of viruses. TfR is a well-known clathrin-mediated recycling molecule, and interference with its recycling may enhance the TfR expression on the cell surface. Therefore, we hypothesized that CC/Fr.2 may have both biological activities via its effect on clathrin pathways. In fact, CC/Fr.2 inhibited the internalization of TfR at a relatively low concentration 10 $\mu\text{g/ml}$ (Table 5).

It is well-known that CC contains large amounts of proanthocyanidins (Prior and Gu, 2005), also known as condensed tannin, which has been accredited with a variety of beneficial effects on health (Beecher, 2004). Tannins and related compounds were reported to possess anti-HIV activity (Lee et al., 1992; H.X. Xu et al., 2000). Proanthocyanidins are reported to have anti-HIV and anti-HSV activities (De Bruyne et al., 1999). Therefore, we examined 8 compounds isolated from CC for various biological activities and found that three compounds that were structurally very similar showed inhibitory activities on SARS-CoV infection (Fig. 6) and two of them caused up-regulation of TfR expression. The structures of procyanidins B1 and B2 are closely related to each other except for the configuration at the C-3' position, but procyanidins B2 did not

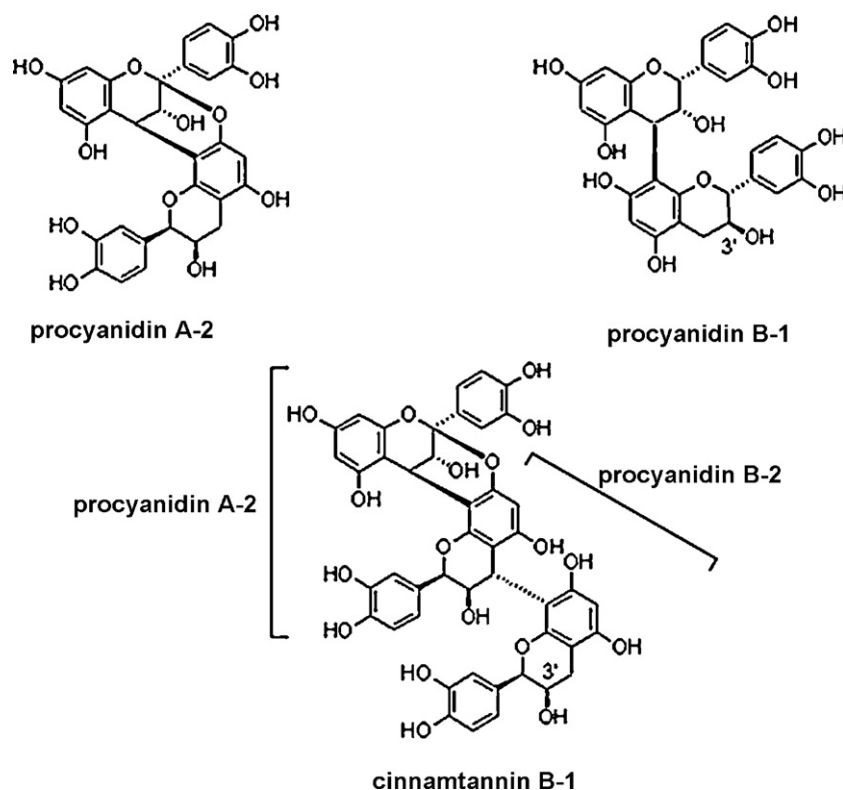


Fig. 6. Procyanidin A2, procyanidin B1 and cinnamtannin B1 are structurally very similar.

inhibit SARS-CoV infection nor up-regulate TfR expression (data not shown). These data strongly suggest that the anti-SARS-CoV activities in CC/Fr.1 and CC/Fr.2 could be explained by procyanidins. In fact, procyanidins were detected in Fr.1 and Fr.2 but not in Fr.3 (Tanaka, unpublished observation March 12, 2008). However, none of the procyanidins inhibited the internalization of TfR at the optimal concentration (data not shown), suggesting they may not act on the TfR recycling pathway directly. It was also demonstrated that the compounds did not affect ACE2 expression which is a SARS-CoV receptor. A previous study indicated that procyanidin A2 has the highest SI (24) against HIV suggesting that it has a virucidal effect (De Bruyne et al., 1999). Therefore it is possible that Fr.2 may contain both endocytosis inhibitor(s) and procyanidins, although we could not identify the substances which could inhibit endocytosis. The up-regulation of TfR by procyanidin is a puzzling phenomenon discovered in this experiment. It is known that proanthocyanidins and their related compounds have chelating activities (Scalbert et al., 2000) and depletion of Fe in cells or medium causes up-regulation of the TfR transcript. However, the up-regulation of mRNA was observed 24 h after the chelators were added (Xu et al., 2008). Iron chelation by clinically relevant anthracyclines causes an alteration in the expression of iron-regulated genes and atypical changes in intracellular iron distribution and trafficking (Xu et al., 2008). We could see the up-regulation within 15 min after the addition of procyanidins (Fig. 4) and it is also known that transferrin are the principal plasma proteins that bind wine catechins (Brunet et al., 2002), therefore, the swift up-regulation of TfR may be caused by depletion of Tfn in the medium. It is of note that α -interferon induces depletion of the intracellular iron content and up-regulation of functional TfR on human epidermoid cancer KB cells (Caraglia et al., 1994), and has also anti-SARS activities (Paragas et al., 2005; Tan et al., 2004; Barnard et al., 2006). Although, the relationships between these two activities are not clear, the higher SI of procyanidin A2 could be explained by its up-regulation of TfR.

In this study we found that CC/Fr.2 contains inhibitors of SARS virus infection. The fraction also inhibited the internalization of TfR indicating the interference of clathrin-dependent endocytosis. The fraction also contained procyanidins which could have a virucidal effect and some of them showed up-regulation of TfR, probably by depleting transferrin and/or iron from the culture. The novel and complex biological activities of CC could lead to the amelioration of symptoms of a number of virus-infected diseases, including SARS.

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