Bioscience, Biotechnology, and Biochemistry

ISSN: 0916-8451 (Print) 1347-6947 (Online) Journal homepage: https://www.tandfonline.com/loi/tbbb20

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To cite this article: Emiko KINOSHITA, Kyoko HAYASHI, Hiroshi KATAYAMA, Toshimitsu HAYASHI & Akio OBATA (2012) Anti-Influenza Virus Effects of Elderberry Juice and Its Fractions, Bioscience, Biotechnology, and Biochemistry, 76:9, 1633-1638, DOI: 10.1271/bbb.120112

To link to this article: https://doi.org/10.1271/bbb.120112

Published online: 22 May 2014.

Biosci. Biotechnol. Biochem., **76** (9), 1633–1638, 2012

Anti-Influenza Virus Effects of Elderberry Juice and Its Fractions

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Received February 16, 2012; Accepted May 26, 2012; Online Publication, September 7, 2012
[doi:10.1271/bbb.120112]

Elderberry (*Sambucus nigra* L.) has traditionally been used for treating influenza and colds. We evaluated the antiviral effect of concentrated juice of elderberry (CJ-E) on the human influenza A virus (IFV). CJ-E had a relatively strong effect on IFV-infected mice, although its anti-IFV activity was weak in a cell culture system. The *in vivo* anti-IFV activities of the fractions were determined after separating CJ-E by ultrafiltration and anion-exchange chromatography. Oral administration of the high-molecular-weight fractions of CJ-E to IFV-infected mice suppressed viral replication in the bronchoalveolar lavage fluids (BALFs), and increased the level of the IFV-specific neutralizing antibody in the serum, as well as the level of secretory IgA in BALFs and feces. Fr. II from high-molecular-weight fraction HM, which contained acidic polysaccharides, showed relatively strong defense against IFV infection. We conclude that CJ-E had a beneficial effect by the stimulating immune response and preventing viral infection.

Key words: elderberry; influenza virus; immune response

The elder (*Sambucus nigra* L.) is widely distributed throughout the world. Its berries, elderberries, are dark violet-black drupes that grow in a cluster and are only edible when fully ripened. The elderberry has been traditionally used in the treatment of influenza, common cold infections, diabetes, and other ailments.[1–3] Although several clinical trials have shown that such symptoms as fever and sneezing derived from influenza were rapidly recovered from by treating with elderberry juice or its concentrated syrup,[4,5] its main components and the mechanism for its anti-IFV action have been not fully understood. Elderberry is known to be rich in phenolic compounds, including phenolic acid, flavonoids, catechins, and proanthocyanidins.[6,7] These phenolic compounds have been reported to enhance the immune response.[8,9] The main anthocyanins in elderberry have been identified as cyanidin-3-glucoside and cyanidin-3-sambubioside.[6] Elderberry possesses potent antioxidative activity because it is rich in these compounds.[6]

Influenza, a respiratory disease caused by the influenza virus, remains a worldwide threat with high potential to cause a pandemic. A critical condition often results in both old and young people. However, only a few medications that act directly and specifically against this virus have been approved for use in the treatment of influenza. A serious problem with these medications is that drug resistance can develop relatively quickly.[10] Drug development often faces difficulties because the influenza A virus (IFV) changes in its characteristics by antigenic mutation; there is therefore a strong need for novel classes of anti-IFV agents.

The anti-IFV effects of various food materials have been examined.[11] Such flavonoids in tea as theaflavin digallate and (−)-epigallocatechin digallate have been reported to act by blocking IFV binding to host cells *in vitro*.[12,13] Several flavonoids in elderberry have been reported to bind to host cells and prevent IFV infection *in vitro*.[14] The anti-IFV activity of phenolic compounds has been suggested to be due to their direct action on IFV. It is currently considered that indirect action, including the activation of immune responses, would be important for treating IFV infection. For example, the acidic polysaccharide isolated from *Cordyceps militaris* grown on germinated soybeans,[15] fucoidan from the *Undaria pinnatida* brown alga,[16] artiin and its aglycone from the fruits of *Articum lappa* L.[17] and *Lactobacillus plantarum* strains[18] have all shown antiviral effects *in vivo*. These findings show the possibility that other natural products could also exert protective effects not only by directly inhibiting virus replication but also by stimulating the adaptive immune defense functions of the host.

We investigated in the present study the effects of concentrated elderberry juice (CJ-E) and its fractions on IFV replication and the specific defense functions involved in IFV infection.

Materials and Methods

Materials. CJ-E as concentrated juice of elderberry supplied by Bayernwald (Hengersberg, Germany) was purchased from Oyama Company (Kobe, Japan). CJ-E was prepared by vacuum concentration from squeezed juice of the berries of elder harvested in Hungary in 2008.

Oseltamivir phosphate was purchased from Hoffman-La Roche (Basel, Switzerland). SEP-1013 and SPL-1053 ultrafiltration modules were obtained from Asahi Kasei Chemicals Corporation (Kanagawa, Japan). Toyopearl DEAE 650M was obtained from Tosoh Corporation

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Abbreviations: IFV, influenza A virus; CJ-E, concentrated juice of elderberry; PFU, plaque-forming unit

(Tokyo, Japan). The Folin-Ciocalteau phenol reagent was obtained from Sigma Chemicals Co. (St. Louis, MO, USA), and Eagle's minimum essential medium (MEM) was obtained from Nissui Pharmaceutical Co. (Tokyo, Japan). All other chemicals were from Wako Pure Chemical Industries (Osaka, Japan).

Cells and virus. The Madin-Darby canine kidney (MDCK) cell line and influenza A virus (A/NWS/33, H1N1) were obtained from Denka Seiken Co. (Niigata, Japan).

Preparation and fractionation of the elderberry samples. Lyophilized powder samples of CJ-E and its fractions were prepared for chemical analyses and for *in vitro* and *in vivo* assays. The fractions were prepared from CJ-E (1,600 g) by ultrafiltration with SEP-1013 (3,000 molecular weight cutoff) and SPL-1053 (10,000 molecular weight cutoff), and three fractions with a molecular weight higher than 10,000 (HM, 27 g), lower than 3,000 (LM, 1,560 g), and intermediate between 3,000 and 10,000 (MM, 19 g) were obtained. The HM fraction was further fractionated by ion-exchange chromatography in a Toyopearl DEAE 650M column (50 mm i.d. × 100 mm) that had been equilibrated with water. HM applied to the column was first eluted with 4 volumes of distilled water, and then with a successive stepwise gradient of 0.5, 1.0 and 2.0 M NaCl to give respective fractions I, II, III and IV. Each fraction was collected, dialyzed and lyophilized.

Analyses of the fractions. The total sugar and reducing sugar contents were respectively determined by the phenol-H_2SO_4[19] and DNS methods.[20] Total polyphenol was determined by the Folin & Ciocalteau method,[21] and the anthocyanin content was determined by the vanillin-HCl method.[22]

Evaluation of the in vitro *antiviral activity and cytotoxicity.* The cytotoxic assay used MDCK cells in MEM containing 5% fetal bovine serum (FBS) that had been seeded into each well of 96-well plates and cultured for 3 d at 37 °C in the presence of a sample (0.1–50,000 μg/mL). The viable cells were counted by the trypan blue exclusion method, and the 50% cytotoxic concentration (CC_{50}) was obtained from the concentration-response curve. The anti-IFV assay used MDCK cell monolayers in 48-well plates that had been infected with the virus at 0.1 plaque-forming units (PFU) per cell for 1 h at room temperature, washed twice with phosphate-buffered saline (PBS), and incubated in a maintenance medium (MEM plus 2% FBS) at 37 °C. A sample (0.1–5,000 μg/mL) was added during virus infection and thereafter throughout incubation (A) or immediately after infection (B). The cells were harvested 24 h post-infection and then stored at −80 °C. The virus yield was determined by a plaque assay after 2 d of incubation on MDCK cell monolayers in 35-mm dishes. The concentration for 50% inhibition of viral replication (IC_{50}) was obtained from a concentration-response curve, and the selectivity index (CC_{50}/IC_{50}) was calculated from the CC_{50} and IC_{50} values.

Animal experiments. Female BALB/c mice (6 weeks old) were purchased from Japan SLC (Shizuoka, Japan). All animal 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. No side effects due to drug administration were apparent throughout the experiments. The mice were intranasally infected with IFV at 2×10^4 PFU/50 μL/mouse on day 0. A test compound was orally administered twice a day (9.00 a.m. and 6.00 p.m.) from 3 d prior to virus infection until 7 d post-infection. The control mice were orally treated with the vehicle alone (distilled water). Half of the mice in each group were observed for 14 d to monitor the body weight change, and their feces, blood, bronchoalveolar lavage fluids (BALFs) and lungs were collected on day 14 post-infection. The remaining mice in each group were sacrificed to collect BALFs and lungs on day 3 post-infection. Blood samples were centrifuged at 3,000 rpm for 10 min, and sera were stored at −20 °C. Each lung sample was sonicated for 10s, after adding PBS at 10 μL per mg of lung tissue, and centrifuged at 10,000 rpm for 30 min to separate the supernatant which was then stored at −80 °C. BALFs were collected by washing four times with 0.8 mL of ice-cold PBS *via* a tracheal cannula, and centrifuged at 1,500 rpm for 10 min to obtain the supernatant which was then stored at −80 °C. Fecal extracts were prepared by adding PBS at 10 μL per mg of feces. The virus yield in each lung and BALF sample was determined by the plaque assay on MDCK cell monolayers.

The levels of IFV-specific IgA in BALFs and IgG in the serum were determined by an enzyme-linked immunosorbent assay (ELISA). A 96-well plate coated with purified IFV (1 μg/mL) was blocked with 2% bovine serum albumin, BALFs or serum was added, and the contents reacted with the HRP-conjugated anti-mouse IgA or HRP-conjugated anti-mouse IgG antibody. The titer of the IFV-specific neutralizing antibody was determined by a 50% plaque reduction assay: After 2–30,000 times dilution of a serum or BALF sample with PBS, each dilution was mixed with an equal volume of approximately 200 PFU of the virus and then incubated for 1 h at 37 °C. The mixture was added to MDCK cell monolayers to measure the remaining infectious virus by a plaque assay. The titer of the neutralizing antibody is defined as the highest dilution of serum or BALF that reduced the plaque number by 50% when compared with the control IFV-infected mice without the sample.[23] The titer of the neutralizing antibody was less than 20 when the serum from an uninfected mouse was subjected to this assay.

Statistical analysis. All data are expressed as the mean ± SD. A one-way analysis of variance (ANOVA) was conducted, and correction was done by *Tukey's* multiple-comparison test.

Results

In vitro *anti-IFV activity of CJ-E*

CJ-E was evaluated for its antiviral potency against IFV. In general, when a CC_{50}/IC_{50} value is higher than 10, a sample is regarded as possessing antiviral activity. The values for CC_{50}, IC_{50}, and CC_{50}/IC_{50} of CJ-E are summarized in Table 1. The selectivity index of CJ-E for IFV was more than 10 when added during infection (A), and lower than 10 when added immediately after virus infection (B). These results suggest that CJ-E would interact with the early stages of viral replication, including viral attachment to and penetration into the host cells.

Effect of CJ-E on IFV infection in mice

The effect of CJ-E on the change in body weight of mice infected with IFV at 2×10^4 PFU/50 μL/mouse were examined (Fig. 1). Mice in the control group showed approximately 26% loss of body weight on day 7 post-infection, and mice treated with oseltamivir showed only 3.3% loss on day 5. Those treated with CJ-E at a dose of 1 mg/d and 5 mg/d respectively showed only 19.7% and 15.4% loss on day 7 post-infection.

The virus yields in the BALFs and lungs of IFV-infected mice are shown in Fig. 2. Administration of CJ-E at a dose of 1 mg or 5 mg/d on day 3 post-infection caused a significant reduction of virus yield in the BALF samples when compared with the control group. The virus yield in the lung samples of the CJ-E groups, however, showed no significance reduction when compared with the control group.

Table 1. Anti-Influenza A Virus Activity and Cytotoxicity of CJ-E

Cytotoxicity (CC_{50}, μg/mL)	Antiviral acivity (IC_{50}, μg/mL)		Selectivity index (CC_{50}/IC_{50})	
	A	B	A	B
26,000 ± 3,000	720 ± 79	3,600 ± 590	36 ± 6.7	7.3 ± 1.6

Each value in mean ± SD from triplicate assays.
A, Sample is added during virus infection and throughout the incubation thereafter. B, Sample is added immediately after infection.

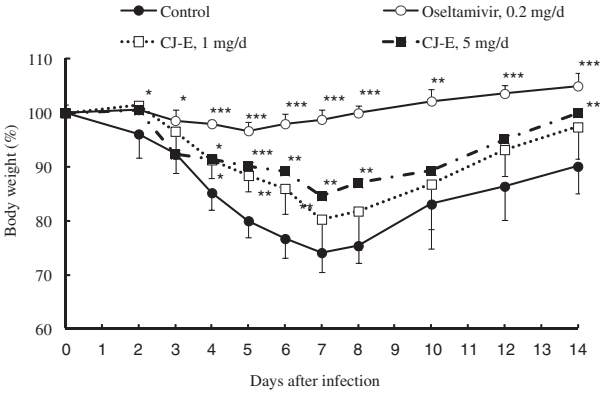

Fig. 1. Effect of CJ-E on the Change in Body Weight of the Mice.

IFV-infected mice were orally administered with distilled water (control), 0.2 mg/d of oseltamivir, or 1 mg or 5 mg/d of CJ-E from 3 d prior to virus infection to 7 d post-infection. The body weight on the day of viral infection (0 d) was taken as 100%. Each value is presented as the mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs*. the control group.

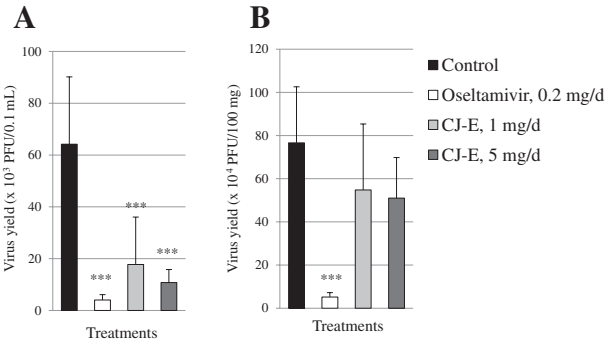

Fig. 2. Effect of CJ-E on the Virus Yield in the Mice.

IFV-infected mice were treated as described for Fig. 1. The virus yields in the BALF (A) and lung samples (B) were measured by a plaque assay on day 3 post-infection. Each value is presented as the mean ± SD. $^{***}p < 0.001$ *vs*. the control group.

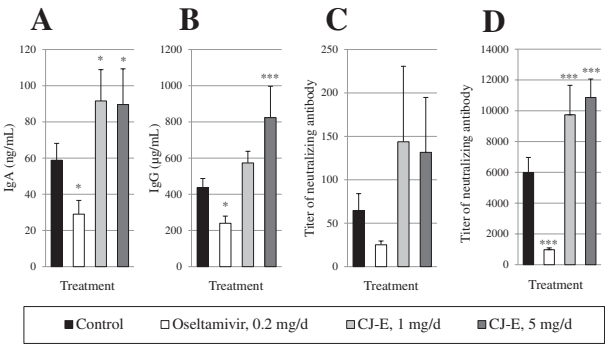

Fig. 3. Effect of CJ-E on the Production of IFV-Specific Antibodies and Neutralizing Antibodies in the Mice.

IFV-infected mice were treated as described for Fig. 1. BALFs and sera were obtained on day 14 post-infection. The IFV-specific IgA and IgG levels in BALFs (A) and sera (B) were determined by an ELISA assay. The titer of the virus-neutralizing antibody is presented as the reciprocal of the dilution of BALF (C) or sera (D) that reduced the plaque number to a level below 50% of that seen in the virus control. Each value is presented as the mean ± SD. $^{*}p < 0.05$, $^{***}p < 0.001$ *vs*. the control group.

Table 2. Chemical Compositions of CJ-E and Its Fractions

	Recovery (%)	Total sugar (mg/g)	Reducing sugar (mg/g)	Total polyphenol (mg/g)	Anthocyanin (mg/g)
CJ-E	100	610	620	42	6.6
Fractions of CJ-E by ultrafiltration modules					
LM	97.1	670	660	37	6.4
MM	1.2	610	590	288	34.8
HM	1.7	470	240	88	13.0
Fractions of HM by Toyopearl DEAE 650M					
Fr. I	17.0	240	<50	22	0.1
Fr. II	80.3	550	<50	9	0.5
Fr. III	1.4	220	120	39	1.8
Fr. IV	1.3	n.d.	n.d.	2	n.d.

n.d., not detected.

To elucidate whether CJ-E could stimulate the systemic and local immunoresponses in mice, the levels of virus-specific antibodies in the BALFs and sera were determined at 14 d post-infection (Fig. 3). The production of the secretory IgA antibody in the BALFs (Fig. 3A) and of the systemic IgG antibody in the sera (Fig. 3B) was increased in the CJ-E groups. The titers of the neutralizing antibody were significantly higher in the sera (Fig. 3D) of mice treated with CJ-E when compared with the control group, in spite of the lower virus yield of the CJ-E groups than that of the control group; the administration of 1 mg and 5 mg/d of CJ-E respectively caused a 1.6 and 1.8 times higher titer in the sera than that in the control group. In contrast, oseltamivir significantly suppressed the production of antibodies in both BALFs and sera when compared with the control group.

Chemical composition of CJ-E and its fractions

CJ-E was fractionated in order to characterize the main components of CJ-E responsible for the anti-IFV activity. Three fractions of low (LM), medium (MM) and high molecular weight (HM) were separated by ultrafiltration according to their molecular weights. The HM fraction was further separated into four fractions by anion-exchange chromatography on Toyopearl DEAE 650M, fractions I and II being respectively eluted with H_2O and 0.5 M NaCl. The chemical composition of each of these fractions is summarized in Table 2. LM showed the highest recovery of 97.2% from CJ-E and contained reducing sugars and polyphenols. Although their recovery was much less than that in LM, MM was relatively rich in polyphenols and reducing sugars, and HM was rich in sugars with less reducing sugar. Fr. II, the main fraction separated from HM by anion-exchange chromatography, was also rich in polysaccharides.

Effects of the fractions of CJ-E on IFV infection in mice

Figure 4 shows the virus yield in the BALFs and lungs of IFV-infected mice on day 3 post-infection. While CJ-E and its high-molecular-weight fractions (HM, Fr. I and Fr. II) significantly reduced the virus yield in BALFs without dose dependency when compared with the control group, a dose-dependent reduction in virus yield was apparent in the lungs of these groups. The suppressive effects were more potent in BALFs than in the lungs, HM showing the most potent activity in the lung samples among the LM, MM and HM fractions comparable to that of CJ-E. Fr. II derived

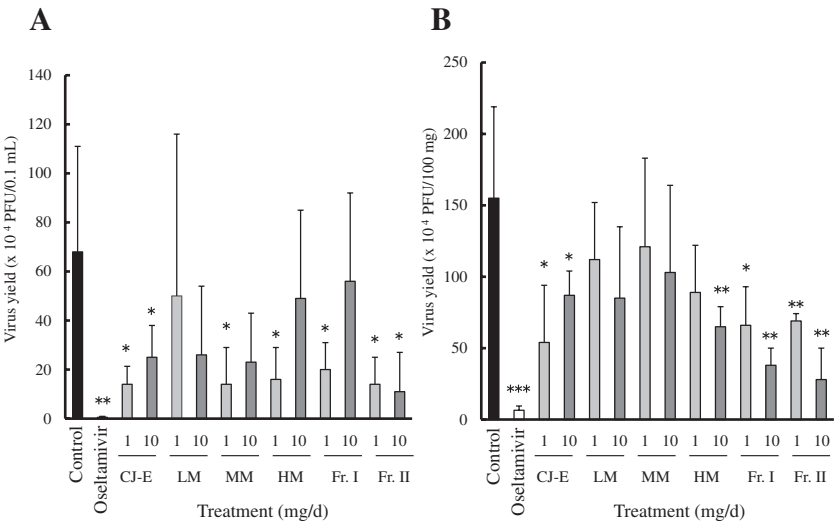

Fig. 4. Effect of Each CJ-E Fraction of on the Virus Yield in the Mice.

IFV-infected mice were orally administered with distilled water (control), 0.2 mg/d of oseltamivir, or 1 mg or 5 mg/d of a CJ-E fraction as described for Fig. 1. The virus yields in the BALF and lung samples were measured by a plaque assay on day 3 post-infection. Each value is presented as the mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* control group.

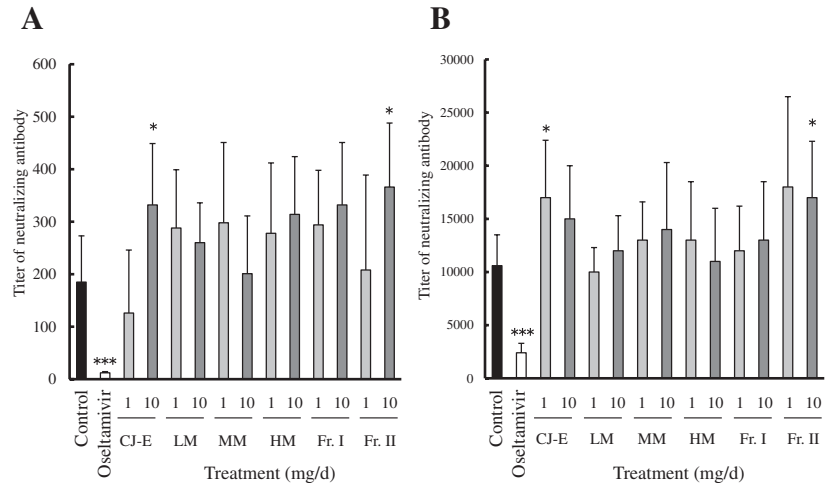

Fig. 5. Effect of Each CJ-E Fraction on the Production of Neutralizing Antibodies in the Mice.

IFV-infected mice were treated as described for Fig. 4. BALFs and sera were obtained on day 14 post-infection. The neutralizing antibody titer is presented as described for Fig. 3. Each value is expressed by the mean ± SD. $^{*}p < 0.05$, $^{***}p < 0.001$ *vs.* the control group.

from HM showed the most potent activity among the fractions tested. LM caused no marked inhibition of viral replication in both the BALF and lung samples.

The effects of these fractions on the immune response was evaluated in IFV-infected mice at 14 d post-infection. Figure 5 shows the titers of the neutralizing antibody in BALFs and sera. Only the antibody titers of the CJ-E and Fr. II groups were significantly higher than that in the control group. Unlike these fractions, oseltamivir produced a significantly lower titer than that in the control group.

Since the secretory IgA antibody in the mucosa plays a crucial role in suppressing IFV replication, the levels of the virus-specific IgA antibody in the respiratory organs (BALFs) and feces were determined by an ELISA assay. Ten mg/d of CJ-E significantly increased the IgA level by 1.3-fold of the control value in both BALF and feces (Fig. 6). MM, HM, Fr. I and Fr. II significantly increased the IgA production in BALFs by 1.2–1.4 times the control value, and HM, Fr. I and Fr. II also significantly increased the IgA level in the feces by 1.2–1.3 times. LM showed, however, no stimulation of IgA production in either BALFs or feces.

Discussion

Elderberry has been traditionally used for treating influenza and colds. Syrups and supplements of an elderberry extract have been developed as a traditional remedy for various diseases in Europe. Judging from the pharmacological and clinical effects of elderberry reported by Vlachojyannis *et al.*,[3] almost all the effects of elderberry warrant further investigation. Several *in vitro* studies on the anti-IFV activity of elderberry have found the inhibition of both haemagglutination[4] and viral proliferation,[24] and prevention of the adhesion of the virus to the host cell receptors.[14] In addition, elderberry has been reported to stimulate the immune system by enhancing the production of cytokines by monocytes.[8] These results suggest that flavonoids,

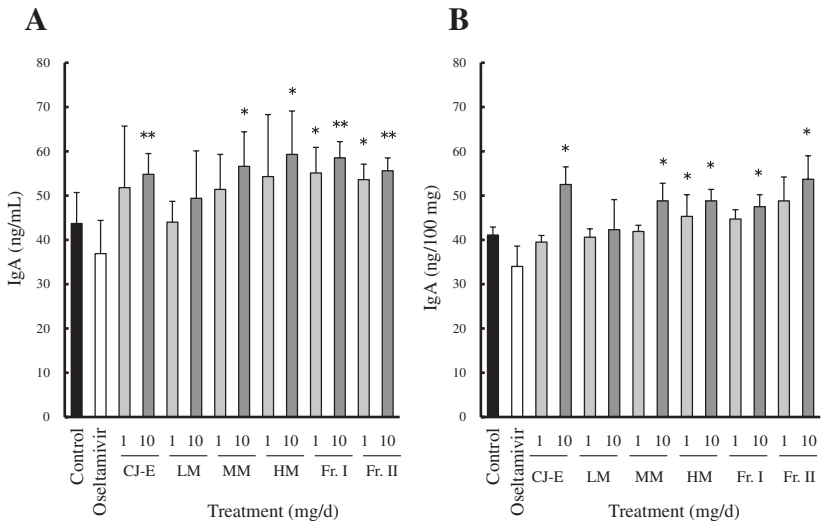

Fig. 6. Effect of Each CJ-E Fraction on the Production of Mucosal IgA in the Mice.
IFV-infected mice were treated as described for Fig. 4. The IFV-specific IgA levels in BALFs and feces were determined by an ELISA assay. Each value is presented as the mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$ *vs.* the control group.

which are responsible for the powerful antioxidative activity of elderberry, might have contributed to the anti-IFV activity.

Our results show that the *in vitro* anti-IFV activity of CJ-E was weak, unlike the results in the literature just mentioned, while in contrast, its *in vivo* activity was found to be relatively strong. The direct effect on IFV replication might therefore not play an important role in the treatment of IFV infection with elderberry. The difference in the *in vitro* results between our study and the other studies reported in the literature might have been due to different preparation of the samples: we used the concentrated juice of elderberry, while most researchers performed their experiments by using various types of commercial syrup whose production processes were not clearly elucidated and which frequently included such other materials as Echiacea, propolis and so on. This present work can therefore be considered as the first report on the bioactivity of elderberry itself.

The results of the present study clearly show the *in vivo* efficacy of elderberry in suppressing viral replication and stimulating the immune response (Figs. 2 and 3). The animal experiments show that CJ-E did not significantly increase the titer of the neutralizing antibody in the BALF samples of mice when it was administered at doses of 1 and 5 mg/d (Figs. 3 and 5). At a dose of 10 mg/d, however, the juice caused a significant increase in the antibody titer of the BALF samples as shown in Fig. 5. These results might have been due to a dose-dependent effect of CJ-E on stimulating the immune functions.

CJ-E was fractionated in order to characterize the main components of CJ-E involving its anti-IFV activity. After separating by ultrafiltration and anion-exchange chromatography, the obtained fractions were evaluated for their *in vivo* efficacy toward mice infected with IFV. Oral administration of the high-molecular-weight fractions (HM, Fr. I and Fr. II) suppressed the viral yield in the BALFs and lungs (Fig. 4). The levels of the IFV-specific neutralizing antibody in the BALFs and sera were significantly higher in the mice treated with Fr. II, while less influence on the antibody production was apparent in the mice treated with the low-molecular-weight fraction (LM) (Fig. 5). Fr. II, an acidic polysaccharide-rich fraction, exerted the most potent suppressive effect on virus replication (Fig. 4), and also had a potent stimulating effect on neutralizing the antibody production (Fig. 5). These results suggest the contribution of Fr. II to the anti-IFV activity of CJ-E. The levels of IFV-specific secretory IgA in the BALFs and feces were higher in the mice treated with CJ-E and its fractions, except for LM (Fig. 6). The high-molecular-weight fractions (HM and Fr. II) revealed a more potent *in vivo* anti-IFV effect than the low-molecular-weight fractions (LM and MM) (Figs. 4–6). LM was assumed to contain antocyanins and proantocyanidins, these being lower polymers than 6-mers,[6] so that these low-molecular-weight compounds seemed not to be essential to the anti-IFV activity of elderberry, although Halliwell *et al.*[25] and Droebner *et al.*[13] have suggested that phenols might exert a direct effect on the virus. Although the contribution of high-molecular-weight compounds to anti-IFV, including the acid polysaccharides derived from elderberry, might be considered as one possibility, further experiments will be required to confirm this notion.

It was suggested that stimulating the immune response would contribute mainly to the anti-IFV activity of elderberry toward mice, while oseltamivir, an inhibitor of neuraminidase, did not stimulate the immune response. These results mean that the mechanism for the anti-IFV action differed between elderberry and oseltamivir. It has recently been recognized that drug-resistant viruses frequently emerged, especially in an immunosuppressed state.[26,27] In relation to this, the immunostimulating effect of CJ-E might be useful to control viral infection and to avoid prolonged therapy.

In conclusion, elderberry juice (CJ-E) had a favorable effect on the control of influenza virus infection, the fractions containing high-molecular-weight compounds seeming to play an important role in the anti-influenza effect.

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