ACTIVITY OF ANTHOCYANINS FROM FRUIT EXTRACT OF *RIBES NIGRUM* L. AGAINST INFLUENZA A AND B VIRUSES

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Summary. – Earlier, we have detected antiviral activity in an extract from *Ribes nigrum* L. fruits ("Kurokarin", name of the one species of black currant in Japanese) against influenza A and B viruses, and herpes simplex virus 1 (Knox *et al.*, *Food Processing* **33**, 21–23, 1998). In the present study, the antiviral activity of constituents of a Kurokarin extract and the mechanism of its antiviral action were examined. Kurokarin extracts were separated to fractions A to D by column chromatography. The major constituents of the fraction D were estimated as anthocyanins. The fraction D was further fractionated by thin-layer chromatography (TLC) to fractions A' to G'. The fraction E' consisted of 3-O-α-L- rhamnopyranosyl-ß-D-glucopyranosyl-cyanidin and 3-O-ß-D-glucopyranosyl-cyanidin, and the fraction F' consisted of 3-O-α-L-rhamnopyranosyl-ß-D-glucopyranosyl-delphinidin and 3-O-ß-D-glucopyranosyl-delphinidin, identified by high performance liquid chromatography (HPLC) with standards and by high resolution mass spectrometry. The fractions D' to G' showed potent antiviral activity against influenza viruses A and B. The additive antiviral effect of a combination of the fractions E' and F' was assessed. Anthocyanins in the fraction F' did not directly inactivate influenza viruses A and B, but they inhibited virus adsorption to cells and also virus release from infected cells.

Key words: Ribes nigrum L.; Kurokarin; anthocyanins; antiviral activity; influenza viruses A and B

Introduction

Many studies have shown that a certain group of flavonoids has several physiological activities, e.g. antioxidative activity (Tsuda *et al.*, 1994; Mathiesen *et al.*, 1996; Carrero *et al.*, 1998), reduction of serum cholesterol level (Igarashi *et al.*, 1990; Choi *et al.*, 1991; Jahromi and Ray, 1993; Monforte *et al.*, 1995), inhibition of platelet aggregation (Beretz *et al.*, 1986; Okada *et al.*, 1995; McGregor *et al.*, 1999) etc. Recent studies have also shown that several plant flavonoids have antiviral activity against influenza viruses (Nagai *et al.*, 1990, 1995a,b; Zakay-Rones *et al.*, 1995; Serkedjieva, 1995), parainfluenza viruses (Karagöz *et al.*, 1999), herpes simplex viruses (Amoros *et al.*, 1992) and human immunodeficiency viruses (Mahmood *et al.*, 1993).

Our previous studies have shown that a crude extract from fruits of *Ribes nigrum* L., (Kurokarin in Japanese) from a plant which grows wild in Hei Long Jiang Province of the People's Republic of China, had antiviral activity against influenza A and B viruses and herpes simplex viruses 1 and 2 (Knox *et al.*, 1998). These viruses were directly inactivated by the extract and consequently, their growth was inhibited.

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Abbreviations: HPLC = high performance liquid chromatography; MEM = Eagle's Minimum Essential Medium; p.i. = post infection; TLC = thin layer chromatography

Qualitative and quantitative analyses of the Kurokarin extract showed that it contains several anthocyanins (Knox *et al.*, 1998), i.e. glycosides of delphinidin, cyanidin and peonidin (the latter in trace amounts), which are a group of flavonoids representing vivid deep color components of several red, purple or black fruits or seeds.

The aim of this study was to fractionate the anthocyanins present in the Kurokarin extract, assess the inhibitory activity of individual components against influenza A and B viruses, and disclose the mechanism of their antiviral activity.

Materials and Methods

Viruses. The A/PR/8/34(H1N1) strain of influenza A virus, maintained at our laboratory, and the B/Gifu/2/73 strain of influenza B virus, supplied by Dr. H. Kida, Hokkaido University School of Veterinary Medicine, Sapporo, Japan), were propagated in 11-day-old embryonated eggs. The viruses were titrated by a plaque assay and stored at -80°C until use.

Cells. Mardin-Darby canine kidney (MDCK) cells, provided by Rikken Cell Bank, Tsukuba, Japan, were cultivated in Eagle's Minimum Essential Medium (MEM, Nissui Seiyaku Co. Ltd., Tokyo, Japan) supplemented with 10% of newborn calf serum.

Ion exchange column chromatography. Wild *Ribes nigrum* L. (Kurokarin) fruits, imported from the People's Republic of China, was ground down and filtrated through the filter paper No.3 from Toyo Roshi Co. Ltd., Tokyo, Japan. The filtrate represented the Kurokarin extract. A diluted Kurokarin extract was applied to an Amberlite XAD-7 resin column (Fluka Chemie). In this way, the non-adsorptive constituents (fraction A) were separated from the anthocyanin pigments. The constituents, which were eluted from the resin column by 99.9% ethanol and dried under a reduced pressure, represented the fraction B. The anthocyanin pigment fraction, eluted from the column by 70% ethanol, was filtrated and the filtrate was dried at 40°C under reduced pressure. The dry product was dissolved in 99.9% ethanol, and the soluble portion, separated from the insoluble fraction (fraction C) was dried again under reduced pressure to yield a dry powder. The latter was dissolved in 99.9% ethanol, precipitated with 10 volumes of diethylether, and finally freeze-dried (fraction D). Constituents of fractions A to D were identified by their characteristics of adsorption on resin column and solubility in ethanol.

TLC. Fraction D was further fractionated by TLC on a Kiesel Gel 60 (Merck). It was dissolved in 0.1% formic acid-50% ethanol and spotted on thin-layer. An ascending TLC was carried out with a water phase of n-butanol-acetic acid-distilled water (4:1:5) mixture for 18 hrs at 10°C, yielding the fractions A' to G'. These were carefully scraped off using a spatula, and eluted from the Kiesel Gel 60 with 1% formic acid-50% ethanol. The effluents were centrifuged at 15,000 rpm for 15 mins to remove insoluble components, then dried at 40°C under reduced pressure. The dried fractions were dissolved in 0.1% formic acid and applied to a Sep-Pak C18 column (Millipore) to remove organic acids. Adsorbed pigment components were eluted with 99.9% methanol and dried under reduced pressure. The dried fractions were redissolved in 20% ethanol and freeze-dried. For use in experiments with cells, the freeze-dried fractions were freshly dissolved in 0.1% formic acid to a concentration of 10 mg/ml, a stabilizer of anthocyanins.

HPLC was carried out using a TSK gel ODS-80 TM column (Toso Ind. Co., Tokyo, 4.6 mm x 25 cm) at 525 nm with Waters TM996 Photodiode-Array UV Detector (Waters Associates). Elution was carried out with acetonitril-0.1% trifluoroacetic acid (17.5%:82.5%, v/v) at 45°C and flow rate of 0.8 ml/min. The identification of peak constituents was done by comparison to standards and by high resolution mass spectrometry on a Mariner Biospectrometry Work Station (PerSeptive Biosystems Inc., USA) under the conditions of positive ion polarity, spray tip potential of 3600 V; mass range of 200–1000, and syringe pump rate of 5 µl/ml.

Assay of antiviral activity of samples against influenza viruses was carried out by the 50% plaque reduction method described by Hisaki *et al.* (1999). Briefly, confluent monolayers of MDCK cells in 24-well microplates were infected with approximately 50 PFU of the influenza virus A/PR/8/34 (H1N1) or B/Gifu/2/73 strain per well. After a 1.5 hr incubation at 37°C, the cell monolayers were washed three times with MEM and overlaid with MEM containing 1 µg/ml of trypsin (Sigma), a three-fold concentration of MEM-amino acids and vitamins (Nissui Seiyaku Co. Ltd., Tokyo, Japan), 1 mg/ml glucose, 100 µg/ml DEAE-dextran, 0.8% Agar Noble (Difco) and serial 10-fold dilutions of samples tested or serial 10-fold dilutions of 0.1% formic acid as control. The plates were incubated at 37°C for 2 days, fixed with formalin and stained with crystal violet after the removal of the agar overlay. The plaque counts were expressed as percentage values of controls and were plotted to give dose-response curves, from which the concentration required to inhibit the plaque number by 50% (IC_{50}, µg/ml) was calculated.

Experimental procedures for analysis of antiviral mechanisms. In order to analyze the mechanisms of antiviral activity of the anthocyanin fractions tested the following experimental procedures were designed.

Procedure 1. To analyze the direct effect of the anthocyanin fraction F' on the infectivity of viruses, it was serially diluted 10-fold with MEM, mixed with approximately 10^5 PFU of influenza A or B virus, incubated at 37°C for 30 mins, and the plaque titer was determined.

Procedure 2. To analyze the effect of the anthocyanin fraction F' on virus adsorption to cells, cell monolayers in 24-well plates were treated with the fraction diluted in MEM to a concentration of 1 mg/ml or with MEM (control). The plates were incubated at 37°C for 2 hrs, washed 3 times with MEM, and infected with influenza A or B virus at the multiplicity of 10 PFU/cell. After incubation at 4°C for 1 hr, the monolayers were washed 3 times with chilled MEM, scraped off in 1 ml of chilled MEM, freeze-thawed twice, and centrifuged at 1,500 rpm for 5 mins. The PFU titer of the virus released from cells into supernatant was assayed.

Procedures 3 and 4 were performed to analyze the effect of the anthocyanin fraction F' on the growth of influenza viruses in cells. Cell monolayers in 24-well plates were infected with influenza A or B virus at the multiplicity of 10 PFU/cell. The cell monolayers were incubated at 37°C for 1.5 hr to allow adsorption and penetration of the viruses into the cells and washed 3 times with MEM.

The monolayers were treated for 2 hrs with 1 mg/ml fraction F' at 0 to 2 hrs post infection (p.i.) (Procedure 3) or the monolayers were treated for 1 hr with 1 mg/ml fraction F' at 8 to 9 hrs p.i. (Procedure 4). The monolayers were washed 3 times with MEM. PFU titers of culture fluids were determined after harvesting the cultures at scheduled times.

Results

Chromatographic analyses

Chromatographic analyses of Kurokarin extract were conducted by ion exchange column chromatography, TLC and HPLC. Estimated constituents in fractions A to D obtained by column chromatography are summarized in Table 1. Comparative analysis was made between fraction D and standard anthocyanins using HPLC (Fig. 1). Fraction D consisted mainly of several anthocyanins; 3-O-α-L-rhamnopyranosyl-ß-D-glucopyranosyl-delphinidin, 3-O-ß-D-glucopyranosyl-delphinidin, 3-O-α-L-rhamnopyranosyl-ß-D-glucopyranosyl-cyanidin, and 3-O-ß-D-glucopyranosyl-cyanidin.

Next, further fractionation of fraction D by ascending TLC using a Kiesel Gel 60 thin layer (Fig. 2) was performed. We obtained 7 fractions (A' to G'). HPLC analysis of fractions D', E' and F' (Fig. 3) revealed that fraction D' consisted of cyanidinic constituents and phenol carboxylic acids while fraction E' was a mixture of 3-O-α-L-rhamnopyranosyl-ß-D-glucopyranosyl-cyanidin and 3-O-ß-D -glucopyranosyl-cyanidin. Fraction F' was a mixture of 3-O-α-L-rhamnopyranosyl-ß-D-glucopyranosyl-delphinidin and 3-O-ß-D-glucopyranosyl-delphinidin. These components were identified by HPLC with standards and also by high resolution mass spectrometry (Table 2). Flavonols, flavones, phenol carboxylic acids, and pro-anthocyanins were detected in fraction A' to C' (data not shown).

Antiviral activity of fractions

Experiments were carried out to assess the antiviral activity of the above anthocyanin fractions on influenza A and B viruses. Fractions D, D', E', and F' possessed conspicuous antiviral activity (Table 3). However, the antiviral activity of each fraction was slightly higher against influenza A virus compared to influenza B virus. In control experiments, 0.01% formic acid in MEM, the highest concentration used in these experiments, showed no antiviral or cytotoxic activity (data not shown).

Also the effect of a combination of fractions E' and F' (Table 4) was examined. It showed an additive type of antiviral effect.

Table 1. Estimated constituents in fractions obtained by column chromatography *Ribes nigrum* L. (Kurokarin) extract

Fractions	Constituents
A	Carbohydrates, organics acids, phytopigments
B	Tannic acids, chlorogenic acid
C	Aglycone of anthocyanin pigments
D	Anthocyanin pigments

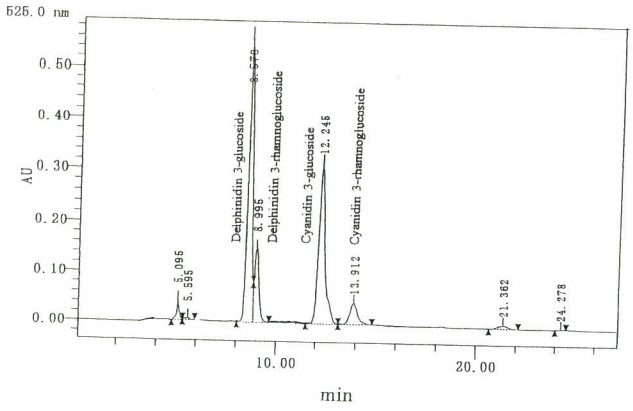

Fig. 1
HPLC analysis of fraction D
Abscissa: retention time (min); ordinate: UV absorbance.

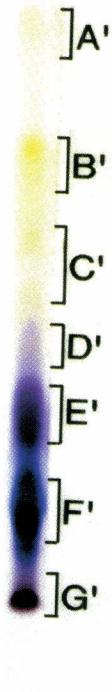

Fig. 2
TLC pattern of fraction D

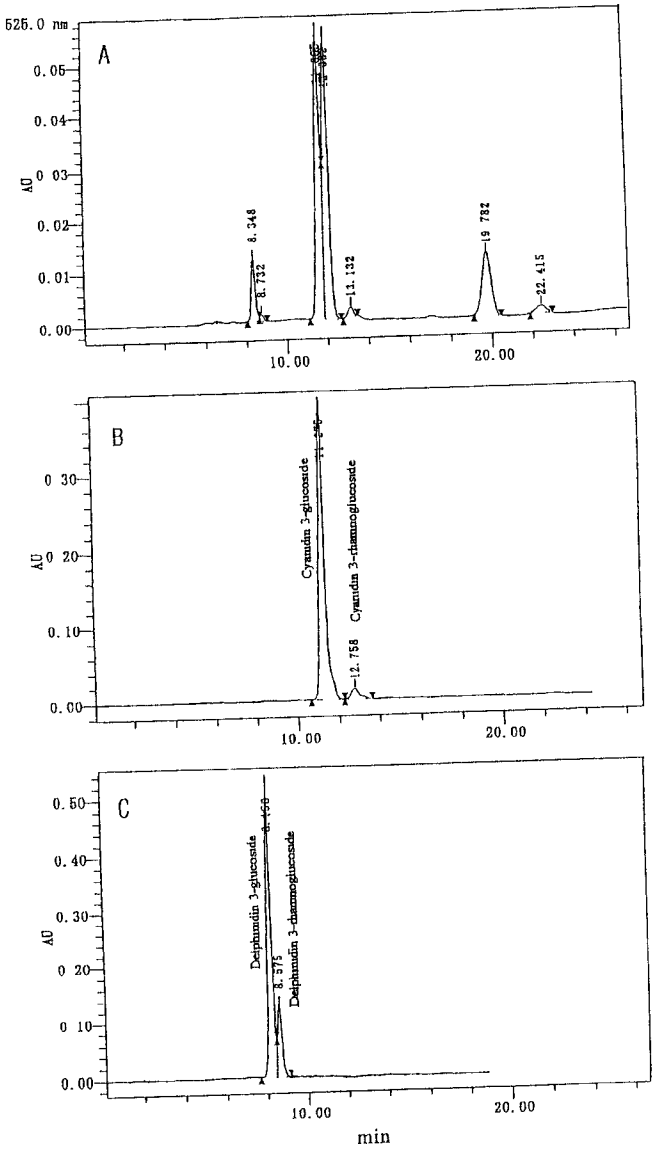

Fig. 3
HPLC analysis of fractions D' (panel A), E' (panel B) and F' (panel C)
For the legend see Fig. 1.

Table 2. Mass spectrometry of fractions separated by HPLC

Fractions[a]	m/z of $[M]^{+b}$	Mf/Mw[c]	Identification[d]
8.578	465	$C_{21}H_{21}O_{12}^{+}$/465.39	Dp-3-Glc
8.995	611	$C_{27}H_{31}O_{16}^{+}$/611.531	Dp-3-Rut
12.245	449	$C_{21}H_{21}O_{15}^{+}$/449.313	Cy-3-Glc
13.912	595	$C_{27}H_{31}O_{15}^{+}$/595.53	Cy-3-Rut

[a]Fractions are shown by retention time of peak in Fig. 1.
[b]m/z of $[M]^{+}$: mass number of molecular ion.
[c]Mf/Mw: molecular formula as flavylium ion/molecular weight.
[d]Dp = delphinidin; Cy = cyanidin; Glc = glucose; Rut = rhamnoglucoside.

Table 3. Anti-influenza virus activity of fraction D and fractions A' to F' from fraction D by thin-layer chromatography

Fractions	IC_{50} (μg/ml)	
	IVA[a]	IVB[b]
D	520~560	360~460
A'	1,500	980
B'	1,900	900
C'	1,170	660
D'	840	370
E'	550	370
F'	640	420

[a]IVA: influenza A virus.
[b]IVB: influenza B virus.

Table 4. Antiviral activity of the combination of fractions E' and F' against influenza A and B viruses

Fractions	IC_{50} (μg/ml)	
	IVA[a]	IVB[b]
Fraction E' alone (1 mg/ml)	580	190
Fractions F' alone (1 mg/ml)	550	170
Fractions E' + F' (1 mg/ml)[c]	550	210

[a]IVA: influenza A virus.
[b]IVB: influenza B virus.
[c]Fraction E' (500 μg/0.5 ml) and fraction F' (500 μg/0.5 ml) were mixed.

Effects of fraction F' on growth of influenza viruses

First the possible direct inactivating effect of fraction F' on influenza viruses was examined. The infectivity of influenza A and B viruses was reduced neither by the highly purified fraction F' in a concentration of 1 mg/ml nor by 0.01% formic acid in MEM (data not shown). Then the effect of fraction F' on adsorption of the viruses to cells was tested (Table 5). There was observed an inhibition by 80–90% at a concentration of 1 mg/ml for both influenza A and B viruses.

Next, the effect of fraction F' on the growth of the viruses in cells was analyzed (Fig. 4).

After a 2 hrs lasting treatment of cells with fraction F' at 0 to 2 hrs p.i. the growth of the viruses was inhibited. However, the inhibitory effect decreased with the time of harvesting the cultures. These findings indicate that the inhibition of virus growth in cells by anthocyanins is reversible.

Finally, we examined the virus release from the infected cells treated with fraction F' for 1 hr at 8 to 9 hrs p.i., at

Table 5. Effect of fraction F' on adsorption of influenza viruses to cells

Inoculated virus	Pretreatment of cell with fraction F' (μg/ml)	Virus titer (PFU/0.1 ml) recovered from cells	Reduction (%)
IVA[a]	0	2.5×10^2	0
	100	1.5×10^2	40
	1000	2.0×10^1	92
IVB[b]	0	3.8×10^2	0
	100	3.3×10^2	13
	1000	8.0×10^1	79

[a]IVA: influenza A virus.
[b]IVB: influenza B virus.

which time the virus growth was almost completed. As shown in Fig. 5, virus titers in culture fluids of the cells treated with fraction F' were markedly lower compared to controls at 16 hrs p.i., but they almost reached the control levels at 24 hrs p.i.

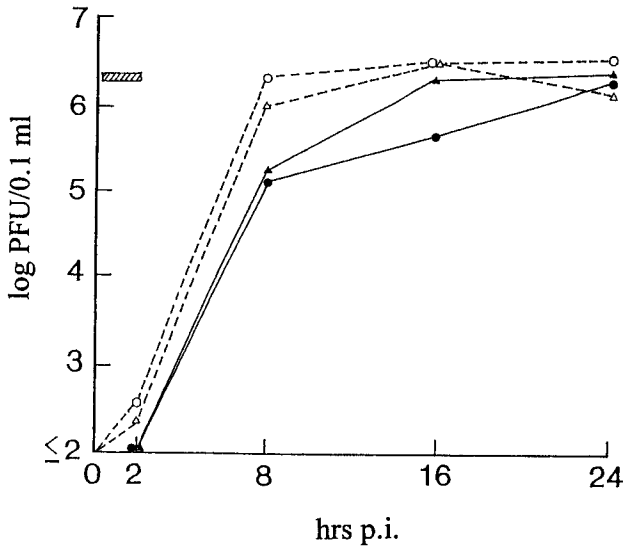

Fig. 4
Effect of fraction F' on growth of influenza viruses
Cells were treated with fraction F' or control medium for 2 hrs from 0 to 2 hrs p.i. For details see Materials and Methods, Experimental procedures for analysis of antiviral mechanisms, Procedure 3. Influenza A (○, ●) and B (△, ▲) viruses. Fraction F' (●, ▲). Control (○, △). Period of treatment of cells with fraction F' (striated rectangle).

Discussion

This study explored the constituents of an extract of fruits of *Ribes nigrum* L. (Kurokarin) and the anti-influenza virus activity of these constituents, especially anthocyanins which are color components of several red, purple or black fruits. HPLC and mass spectrum spectrometry of TLC fractions revealed that the anthocyanins in the Kurokarin extract consisted of 3-O-α-L-rhamnopyranosyl-ß-D-glucopyranosyl-cyanidin, 3-O-ß-D-glucopyranosyl-cyanidin (in TLC fraction E'), 3-O-α-L-rhamnopyranosyl-ß-D-glucopyranosyl-delphinidin, and 3-O-ß-D-glucopyranosyl- delphinidin (in TLC fraction F').

Recent reports have shown that many plant flavonoids have antiviral activity, for example, several flavones and flavonols against herpes simplex virus 1 (Amoros *et al.*, 1992), 5,7,4'-trihydroxy-8-methoxy-flavone against influenza A and B viruses (Nagai *et al.*, 1990, 1995a, 1995b), and several flavones and flavans against HIV 1 and 2 (Mahmood *et al.*, 1993). However, to date, there is no report on antiviral activity of anthocyanins which are one group of flavonoids. In our previous reports (Knox *et al.*, 1998, 2001), we have shown that a crude extract of Kurokarin directly inactivates influenza A and B viruses, and also inhibits their adsorption to cells and their release from infected cells. Here, fraction F' inhibited the adsorption and release of the viruses similarly to the crude extract, and these inhibitory effects were reversible. At present, the mechanism of the interaction between anthocyanins and the cell membrane is not yet

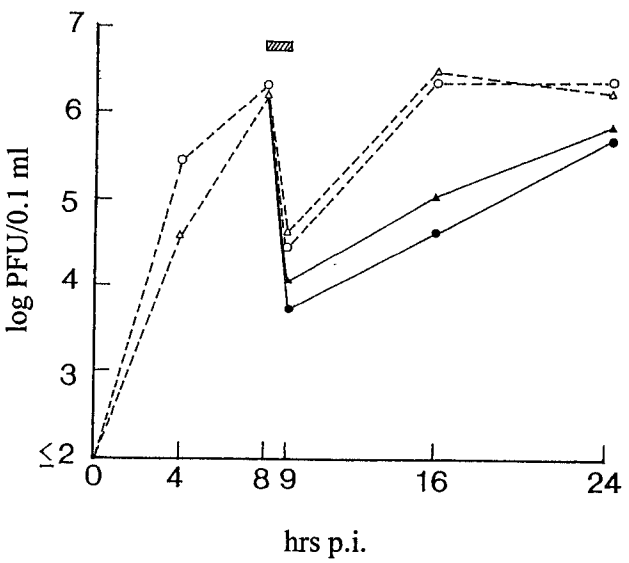

Fig. 5
Effect of fraction F' on virus release from infected cells
Cells were treated with fraction F' or control medium for 1 hr from 8 to 9 hr p.i. For details see Materials and Methods, Experimental procedures for analysis of antiviral mechanisms, Procedure 4. For the rest of the legend see Fig. 4

understood. Thus, further studies are needed to answer this question.

The data shown here did not demonstrate a direct inactivating activity of anthocyanins present in fraction F' against influenza viruses, suggesting that the direct inactivation of the viruses by crude extract described in previous report (Knox *et al.*, 2001) might be caused by components other than anthocyanins. Tannic acids have a special ability to combine with proteins so that tannic compounds in the crude extract investigated here probably combined with envelope proteins on the virus surface, resulting in direct inactivation of the viruses tested.

The experiment with the combination of fractions E' and F' showed an additive antiviral effect, indicating that there was no difference between antiviral mechanisms of anthocyanins tested, at least between those of cyanidin and delphinidin glycosides. Amoros *et al.* (1992) have reported a synergistic antiviral effect of flavones and flavonols against herpes simplex virus 1.

In fractions A' to C', several flavonoids other than anthocyanins, i.e. flavones, flavonols and phenolcarboxylic acids, and in fraction G', also tannic acids were found in our experiments. All these compounds may stabilize anthocyanin pigments as well as promote hydrophobic bonds. Because of the co-pigment effect (Goto and Kondo, 1988), the rigid structure of a hydrophobic bond and stabilization of pigments may work synergistically. From the nutraceutical point of view, functions of a mixture of phytochemicals are the key to our experiments. In our experiments, fractions A' to C' represented minor fractions, so that a sufficient amount of these constituents for combination experiments could not be obtained. A novel approach to fractionation and purification of the compounds of concern may help to pinpoint a single compound for the antiviral effects observed here.

Ballington *et al.* (1987) have reported a relationship between percentage content of anthocyanins and specific species of blueberries. They have also referred to the genetic possibility of future gene exchange or recombination based on genetic classification. Our future experiments should attempt to determine the antiviral activity of constituents of various currants against some other viruses, and also to determine the antiviral effect of cultured currants versus wild currants, and of oriental currants versus European currants.

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