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| Citation | Fukushima Journal of Medical Science. 59(1): 35-38 |
| Issue Date | 2013 |
| URL | http://ir.fmu.ac.jp/dspace/handle/123456789/362 |
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| DOI | 10.5387/fms.59.35 |
| Text Version | publisher |

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[Original Article]

ANTI-INFLUENZA VIRUS ACTIVITY OF TWO EXTRACTS OF
THE BLACKCURRANT (*RIBES NIGRUM* L.)
FROM NEW ZEALAND AND POLAND

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(Received October 26, 2012, accepted January 15, 2013)

Abstract : We investigated the inhibitory effect of extracts of blackcurrant (*Ribes nigrum* L.) from New Zealand and Poland on 4 strains of influenza virus (IFV) by the inhibition of virus adsorption ; pandemic flu from 2009-2010 (IFV-AH1pdm), Hong Kong flu (IFV-AH3), oseltamivir phosphate-resistant Russian flu (IFV-AH1tam^r) and influenza virus type B (IFV-B). The inhibitory effect of the extracts of blackcurrant or blueberry on the infectivity of the virion were evaluated by the inhibition of virus adsorption on the cell surface (adsorption-inhibitory assay). Three percent solutions of the blackcurrant extracts from New Zealand and Poland were enough to disinfect more than half of IFV-AH1pdm and IFV-B, and 10 % solutions from both regions disinfect all IFV strains completely. Our previous study showed that the antiviral effect of the blackcurrant differed according to viral species. Here we showed that although the antiviral effect of Blackcurrant was slightly different within viral strains from one species, the extract of Blackcurrant could disinfect all of 4 IFV strains we examined. The extracts of blackcurrant showed definite potential for use as a disinfectant and antiseptic agent to prevent IFV infection.

Key words : antiviral agents, Blackcurrant, disinfectant, functional food

INTRODUCTION

Polyphenols in cranberries (*Vaccinium macrocarpon* Aiton) and elderberries (*Sambucus nigra* L.) are known to have the potential to prevent infectious diseases. For instance, cranberries can prevent gastrointestinal disorders associated with *Helicobacter pylori*¹⁻³⁾, reovirus⁴⁾, rotavirus⁵⁾ and influenza virus⁶⁾ infections, and elderberry inhibits the replication of influenza virus⁷⁾ and ease its symptoms⁸⁾. Blackcurrant (*Ribes nigrum* L.) is also known to inhibit the growth of herpes simplex virus type1⁹⁾, the growth and release of influenza virus type A and B^{10,11)}. Here we focused on the blackcurrant's preventive effect against influenza virus infection by investigating the inhibitory effect against viral adsorption. However, our previous study showed that the antiviral effect (inhibition of viral adsorption and replication) of the blackcurrant

differed according to viral species¹²⁾. In order to clarify whether this difference in effect is also observed between strains from one species, the inhibitory effects against pandemic flu from 2009-2010 (IFV-AH1 pdm) and oseltamivir phosphate-resistant Russian flu strain (IFV-AH1tam^r), a newly emergent strain, were investigated through a comparison with the IFV strains studied previously.

MATERIALS AND METHODS

Preparation of the fruit extracts

Blackcurrant and blueberry fruit products were ground down and centrifuged at 1,600×g for 10 min to remove insoluble contents and then stored at -20°C until use. The pH of the extract was adjusted from 2.8 to 7.2 using a sodium hydroxide solution. On neutralization, some factors were

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observed as insoluble particles. These particles were precipitated as a pellet by centrifugation at $1,600\times g$ for 10 min and the supernatant was used as the extract.

Cell and viruses

MDCK (Madin-Darby canine kidney) cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4% fetal calf serum, antibiotics and antifungal agent (300 $\mu\text{g/ml}$ of streptomycin, 300 U/ml of penicillin and 1 $\mu\text{g/ml}$ of Amphotericin B). Four strains of influenza virus, pandemic flu from 2009/2010 (IFV-AH1pdm; A/Yamagata/165/2009pdm), Hong Kong flu (IFV-AH3; A/Yamagata/72/2009), oseltamivir phosphate-resistant Russian flu (IFV-AH1tam^r; A/Yamagata/5/2009) and influenza virus type B (IFV-B; B/Yamagata/89/2009, Victoria lineage), were used in this study. All of the strains were isolated in the Yamagata Prefectural Institute of Public Health in Yamagata, Japan, in 2009. All of the viruses were made by growing in the MDCK cells. The cells were frozen and thawed twice to harvest as much of the viruses as possible. Viral titers were measured prior to the assays.

Antiviral assays

The inhibitory effect of the extracts of blackcurrant or blueberry on the infectivity of the virion were evaluated by the inhibition of virus adsorption

on the cell surface (adsorption-inhibitory assay) as previously described⁹. Confluent monolayers of MDCK cells in 12-well microplates were inoculated with 200, 2,000 or 20,000 pfu/well ($\text{Moi}=4\times 10^{-4}$, 4×10^{-3} or 4×10^{-2}) of the viruses and 0, 0.3, 1, 3 or 10% solutions of the blackcurrant or the blueberry at the same time. After adsorption for 5 min at 37°C, the inocula containing the virus and the solution were removed, and the cells were cultured in DMEM containing 1% Ultra Pure Agarose (Invitrogen, Carlsbad, CA) with 1 $\mu\text{g/ml}$ of trypsin (Sigma-Aldrich, Co., St. Louis, MO) in a 5% CO₂ incubator. After 3 days of incubation, the cultures were fixed with 10% formaldehyde in phosphate-buffered saline, stained with 0.05% crystal violet, and the number of plaques was counted. The inhibitory effect was expressed as a percentage relative to that of the blackcurrant-negative control.

RESULTS

The 10% blackcurrant solution from both New Zealand (Figure 1A) and Poland (Figure 1B) completely inhibited adsorption of IFV-AH3 (closed circles), -AH1tam^r (open squares) and -B (closed squares). The 10% blueberry solution (Figure 1C) did not inhibit the adsorption of IFV-AH3 or IFV-AH1tam^r but did effectively inhibit IFV-AH1pdm (open circles) and IFV-B. The inhibition ratios were $0.0\pm 0.0\%$, $39.0\pm 5.9\%$, $97.8\pm 2.2\%$ and

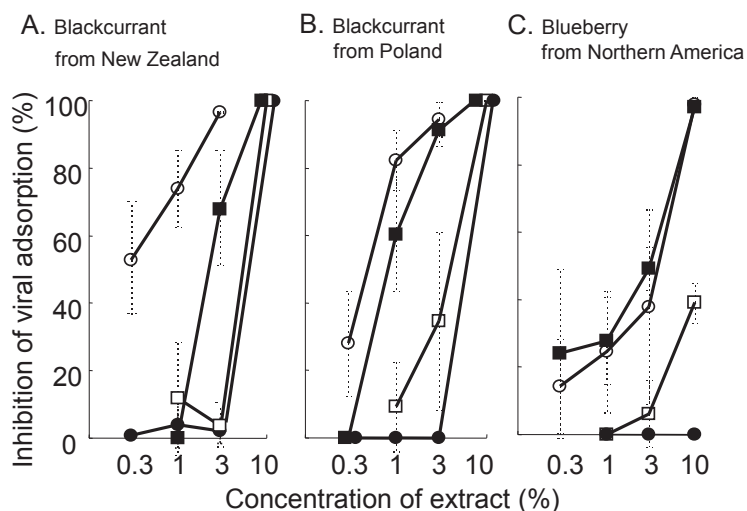


Fig. 1. Inhibitory effect of the blackcurrant extract on viral adsorption to cells. The adsorption-inhibitory assay was performed against IFV-AH1pdm (open circles), -AH3 (closed circles), -AH1 tam^r (open squares) and -B (closed squares). The blackcurrants were imported from (A) New Zealand or (B) Poland. (C) The blueberries were imported from Northern America, and used as control to assess the anti-IFV effect. The blackcurrants and blueberries inhibited the adsorption of IFA-AH1pdm and IFV-B effectively but not of IFV-AH3 and IFV-AH1tam^r. The 10% extracts of blackcurrants disinfect all of 4 IFV strains completely. Each data was the average from 3 independent assays.

97.1±0.0%, respectively. Similar tendencies were obtained with the 3% blackcurrant solution, which effectively inhibited the adsorption of IFV-AH1pdm and IFV-B, but not IFV-AH3 or IFV-AH1tam^r.

DISCUSSION

Our previous study showed that the blackcurrant has antiviral effects against several viral species, such as herpes simplex virus type1⁹⁾, respiratory syncytial virus, IFV-A and IFV-B¹²⁾, but the antiviral effects of the blackcurrant differ between virus species, the 50% inhibitory concentrations (%) were 0.74±0.06, 0.18±0.06, 0.28±0.07 and 0.64±0.18, respectively. Therefore, in this study, we set out to determine whether the extracts of the blackcurrant have antiviral effects against several strains of IFV. Anthocyanins from blackcurrant are known to inhibit influenza virus adsorption to cells and also virus release from infected cells¹⁰⁾. Here we focused on the preventive effect against influenza virus infection. Our particular focus was on whether the extract of blackcurrant can disinfect pandemic or oseltamivir phosphate-resistant IFV, both of which have been the cause of severe social unrest either through their spread across the globe due to the lacking specific immunities or difficulties in their treatment due to their oseltamivir phosphate-resistance. Although we could employ limited 4 strains of IFV as representatives, our results showed that a 10% blackcurrant solution had an antiseptic effect against multiple strains of IFV. Thus the blackcurrant extract has the potential to prevent infection with pandemic and drug-resistant IFV at least the strains that we employed.

We speculate two possible mechanisms for its antiviral effects. One possibility is that some component(s) in the blackcurrant affects the viral receptors on the cell surface, and the other possibility is that the component(s) affects IFV hemagglutinin (HA). Differences in the effects on IFV-AH1pdm and IFV-AH1tam^r, which possess similar HA1, indicate the former possibility. A previous report also showed that the pretreatment of cell cultures with cranberry juice could reduce reovirus infectivity⁴⁾. However, the different effects of blackcurrant on the adsorption of the 4 IFV strains to the same MDCK cell line, support latter possibility as well. We already described that the blackcurrant has different antiviral effects against several viral species¹²⁾. Another group also reported non-specific antiviral effects towards unrelated viral species such as T2 and T4 bacteriophages and the sim-

ian rotavirus SA-11⁵⁾. Taken together, these results suggest that the blackcurrant acts against both the viral and cellular surfaces. Although one of the promising candidates for the antiviral effects is anthocyanin, it is thought that there are many more candidates for the effective ingredient in the blackcurrant¹⁰⁾.

Moreover, the benefits obtained from natural products might vary according to place of origin, and this may reduce the scientific reliability of the effects. We investigated blackcurrant extracts from different two regions, New Zealand and Poland. The blackcurrant extracts were from several farms in each region. Although we cannot exclude the possibilities that there are any other strains of blackcurrant with higher/lower antiviral effects in both regions, so far there were no significant differences in antiviral effects between the extracts from New Zealand and Poland, and the efficiency of both was better than the blueberry extract from North America used as the control.

In conclusion, the blackcurrant extract has the potential to prevent the adsorption of IFV, both of the strain from the pandemic in 2009-2010 to which very few people had immunity and of oseltamivir phosphate-resistant IFV, and could be used as a component in health care goods such as gargle products, candies and juices for the prevention of IFV infection.

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