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Aronia melanocarpa and its components demonstrate antiviral activity against influenza viruses

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ABSTRACT

The influenza virus is highly contagious in human populations around the world and results in approximately 250,000–500,000 deaths annually. Vaccines and antiviral drugs are commonly used to protect susceptible individuals. However, the antigenic mismatch of vaccines and the emergence of resistant strains against the currently available antiviral drugs have generated an urgent necessity to develop a novel broad-spectrum anti-influenza agent. Here we report that *Aronia melanocarpa* (black chokeberry, Aronia), the fruit of a perennial shrub species that contains several polyphenolic constituents, possesses *in vitro* and *in vivo* efficacy against different subtypes of influenza viruses including an oseltamivir-resistant strain. These anti-influenza properties of Aronia were attributed to two constituents, ellagic acid and myricetin. In an *in vivo* therapeutic mouse model, Aronia, ellagic acid, and myricetin protected mice against lethal challenge. Based on these results, we suggest that Aronia is a valuable source for antiviral agents and that ellagic acid and myricetin have potential as influenza therapeutics.

1. Introduction

Influenza viruses cause acute respiratory distress in humans [1]. Once infection begins, these viruses produce respiratory symptoms before elimination by individual immune systems. In cases accompanied by various types of complications, such as secondary bacterial infections, influenza viruses cause severe clinical sequelae and are responsible for up to 250,000–500,000 human deaths annually worldwide [1]. Pandemic yields different mortality estimates. In 1918–19, more than 40 million people were victimized by an H1N1 influenza pandemic [2]. More recently, an estimated 570,000 deaths were found to be associated with the 2009 pandemic H1N1 (pH1N1) infections [3]. The recurring and intermittent influenza infections underline the necessity of organizing effective intervention methods against these respiratory pathogens.

Vaccines are considered the primary control measure of choice against influenza infections. However, disappointing vaccine efficacy has raised a concern for both very young and very old patients [4,5], and inactivated trivalent influenza vaccines (TIV) do not always yield the proper humoral responses, even in healthy recipients [6]. To overcome these technical hurdles, effective adjuvant supplementation [7] or an alternative route for vaccine administration [8] has been investigated along with other methodological breakthroughs [9]. Antiviral drugs are also available for the treatment of influenza patients [10]. However, the emergence of antiviral-resistant strains makes it difficult for physicians to select an appropriate agent in clinical situations [11]. In addition, the enhanced pathogenicity [12] and overperformance possibility [13] of oseltamivir-resistant strains against oseltamivir-sensitive counterparts highlight the urgent necessity to develop new anti-influenza agents. Polyphenolic agents may be an alternative. Various polyphenols have been investigated for their potential use as an anti-influenza remedy since the 1990s, and demonstrated the *in vitro* and/or *in vivo* efficacy by suppressing influenza infectivity [14,15].

Aronia melanocarpa (Aronia), a native black chokeberry in North America, is a polyphenol-rich perennial shrub and has been intensively studied for its medicinal potential [16]. Some of the chemical constituents of Aronia have been tested against viral diseases [17]. In particular, isoquercetin [18], kaempferol [19], ferulic acid [20], caffeic acid [21], and hydroxybenzoic acid [22] have been suggested for evaluation based on their anti-influenza activities. In the case of ellagic acid, synergistic efficacy against H3N2 viral replication was found in Madin–Darby canine kidney (MDCK) cells when applied simultaneously with oseltamivir [23]. However, the *in vivo* efficacy of Aronia polyphenols remains to be elucidated.

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In this study, we tested the *in vitro* and *in vivo* potential of Aronia and its chemical constituents against influenza viruses. Based on the results of virucidal tests, surface glycoprotein inhibition assays, replication inhibition assays, and *in vivo* therapeutic assessment, we conclude that Aronia, ellagic acid and myricetin have potentials to be developed as antiviral therapeutics.

2. Materials and methods

2.1. Ethics

This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Animal, Plant and Fisheries Quarantine and Inspection Agency, Republic of Korea. The protocols were approved by the Institutional Animal Care and Use Committee of Hallym University (Permit Number: Hallym 2012-22).

2.2. Viruses and cells

A/Korea/01/2009 (pH1N1 virus, H1/K09) and A/Korea/2785/2009 (pH1N1 virus possessing an H275Y mutation in the neuraminidase (NA) protein, H1/K2785) viruses were provided by the Korea Centers for Disease Control and Prevention (KCDC; Osong, Republic of Korea). A/Perth/16/2009 (H3N2, H3/PE16) and B/Brisbane/60/2008 (B/BR60) viruses were provided by the National Institute for Biological Standards and Control (NIBSC; Potters Bar, Hertfordshire, United Kingdom), and their NIBSC codes are 11/106 and 10/244, respectively. A/Puerto Rico/8/34 (H1N1, H1/PR8) and recombinant H1/PR8 expressing green fluorescent protein (rPR8-GFP) viruses were provided by Dr. Adolfo García-Sastre (Icahn School of Medicine at Mount Sinai, New York, NY). Using the avirulent HA (modified to retain a single basic amino acid in the cleavage site) and NA plasmids of highly pathogenic avian influenza (HPAI) H5N1 A/Chicken/Korea/IS/2006 (H5/IS06, provided by KCDC), a 6:2 recombinant H5N1 (rH5/IS06) virus was generated by reverse genetics in an H1/PR8 backbone.

MDCK and human lung epithelial (A549) cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA) and maintained in Eagle's Minimal Essential Medium and Dulbecco's Modified Eagle's Medium, respectively, with supplementations of 10% fetal bovine serum and antibiotics at 37 °C and 5% CO_2.

2.3. Aronia and chemical compounds

Aronia powder was prepared from crude *A. melanocarpa* plants by alcohol extraction. The chemical constituents of Aronia were purchased from Sigma–Aldrich (St. Louis, MO), and oseltamivir carboxylate and oseltamivir phosphate were purchased from Toronto Research Chemicals (Toronto, Ontario, Canada).

2.4. Cell-based assays

A virucidal assay was performed by modifying a protocol described previously [24]. Briefly, 100 plaque-forming units (PFU) of a virus were mixed with Aronia (0.0625–1 mg) in a total of 200 μl of PBS infection media and incubated at 37 °C for 1 h. A standard plaque assay in MDCK cells was used for the virus titration in this study. In a replication inhibition assay, MDCK cells were infected with each virus at a multiplicity of infection (MOI) of 0.01 and supplemented with Aronia or oseltamivir (0.125–1 mg/ml). At 24 h post-infection (hpi), the cell supernatants were collected for the virus titration. For the replication inhibition assay of each Aronia constituent (1 mM), MDCK cells were infected with the H1/K09 virus (MOI = 0.001), and cell supernatants at indicated time points were collected for the viral titration.

2.5. Hemagglutination inhibition (HI) assay

The viruses were titrated by a hemagglutination (HA) assay using 0.5% (v/v) turkey erythrocytes (tRBCs). 4 HA units of viruses were then incubated with 2.5 μg of Aronia or oseltamivir carboxylate in a total of 50 μl of PBS in 96-well plates at 37 °C for 1 h. After incubation, 50 μl of 0.5% tRBCs were added and HI titers were recorded after another 30 min.

2.6. Neuraminidase inhibition (NI) assay

Inhibition of NA enzymatic activity was assessed by modifying a protocol described previously [25]. First, 25 μl of Aronia or oseltamivir carboxylate (0.244–250 μg/ml) was two-fold diluted in reaction buffer (150 mM sodium acetate buffer, 1 mM calcium chloride in PBS). The same volume of virus was added into each well at 37 °C for 1 h. Then, 50 μl of 2′-(4-methylumbelliferyl)-a-D-N-acetylneuraminic acid (100 μM 4-MU-NANA; Sigma–Aldrich) was added as a substrate, and the resulting fluorescence was measured in triplicate. The NA inhibition rates of the testing agents were then calculated using GraphPad Prism software (version 5; GraphPad Software, La Jolla, CA).

2.7. Toxicity tests

To evaluate the cytotoxicity of Aronia in MDCK and A549 cells, a tetrazolium-based colorimetric (MTT) assay was performed using 48-well plates. After approximately 1.0×10^5 cells were treated with Aronia or oseltamivir carboxylate (0.03125–2 mg/ml) for 24 h, 100 μl of MTT (2 mg/ml) was added to each well at 37 °C for 2 h. All the media were then discarded, and 100 μl of dimethyl sulfoxide (DMSO) was added to dissolve the purple-colored formazan generated in viable cells after the addition of MTT. The results were read using an ELISA reader at a wavelength of 595 nm. The *in vivo* toxicity of Aronia, ellagic acid, and myricetin was assessed in BALB/c mice (five-week-old, female). Five mice per group were treated twice daily with 1 mg/kg of Aronia (per oral, p.o.), ellagic acid (intraperitoneal, i.p.), or myricetin (i.p.) for five days. Body weight changes of mice were measured daily for 14 days.

2.8. Mouse experiments

For *ex vivo* imaging of lungs, five-week-old female BALB/c mice were anesthetized and intranasally infected with 5 times the 50% mouse lethal dose (5 MLD_{50}) of the rPR8-GFP virus [26]. The infected mice were then treated twice daily for three days with the testing agents (1 mg/kg; oseltamivir phosphate and Aronia, p.o.; ellagic acid and myricetin, i.p.). The lungs of the infected mice were extracted at three days post-infection (dpi) and imaged using an IVIS-200 series imaging system (Perkin Elmer, Waltham, MA) and GFP excitation/emission filters with a 4-s exposure time. In addition, the extracted lungs of the separately infected mice were homogenized for the virus titration. To evaluate the therapeutic efficacy of the test agents, mice were infected with 5 MLD_{50} of the rPR8-GFP virus. The infected mice were then treated twice daily with the same dosages above for five days, and the body weight changes and survival rates of the mice were recorded for 14 days.

3. Results

3.1. Efficacy of Aronia against seasonal, oseltamivir-resistant, and H5N1 influenza viruses

The anti-influenza efficacy of Aronia was evaluated in the virucidal test against seasonal influenza viruses (H1/K09, H3/PE16, and B/BR60) including the oseltamivir-resistant H1/K2785 strain and the HPAI rH5/IS06 virus. In this assay, 0.0625–0.5 mg of Aronia exhibited cross-reactive efficacy to all tested viruses. Although relatively high concentrations were required for H5 and human B subtypes, 0.0625 mg of Aronia inhibited almost 70% viral plaques against H1 and H3 viruses including the oseltamivir-resistant H1/K2785 virus (Fig. 1). Increased concentrations of Aronia resulted in better reactivity, and 0.125 mg displayed more than 60% efficacy against all tested viruses (Fig. 1). These findings suggest that Aronia possesses wide antiviral efficacy against a variety of influenza virus strains.

3.2. In vitro effects of Aronia on the H1/K09 virus

As previously noted [24], a virucidal assay assesses whether an agent can destroy biochemical compositions in the viral tegument. Thus, we suspected that the surface glycoproteins of influenza viruses, mainly HA and/or NA, may be tentative target proteins of Aronia. To determine how Aronia restrained viral infectivity, we evaluated the inhibition efficacy of Aronia against H1/K09 HA and NA proteins.

First, we tested the HI reactivity of Aronia. In an HI assay, 2.5 μg of Aronia inhibited the binding interaction between the HAs and sialic acids of tRBCs, resulting in an 80 HI titer against the HA of the H1/K09 virus (Fig. 2A). In a subsequent fluorescent NA inhibition (NI) assay, approximately 1.95 μg/ml of oseltamivir carboxylate reduced the NA activity of the H1/K09 virus by more than 50% compared with a PBS-treated control, and 62.5 μg/ml completely abolished NA enzymatic activity (Fig. 2B). However, Aronia reduced H1/K09 NA activity by only approximately 10% at a 1.95 μg/ml concentration (Fig. 2B). No tested concentrations of Aronia achieved complete NA inhibition. We then compared the viral replication inhibition efficacy of Aronia with that of oseltamivir. Despite the lower effectiveness of Aronia compared with that of oseltamivir, we found that Aronia also inhibited H1/K09 virus replication in a dose-dependent manner (Fig. 2C). Based on the results of an MTT assay, we also found that Aronia appeared to be less cytotoxic to cultured cells (MDCK and A549 cells) than oseltamivir carboxylate (Fig. 2D). These results indicate that Aronia inhibits influenza virus replication mainly by targeting of the HA protein.

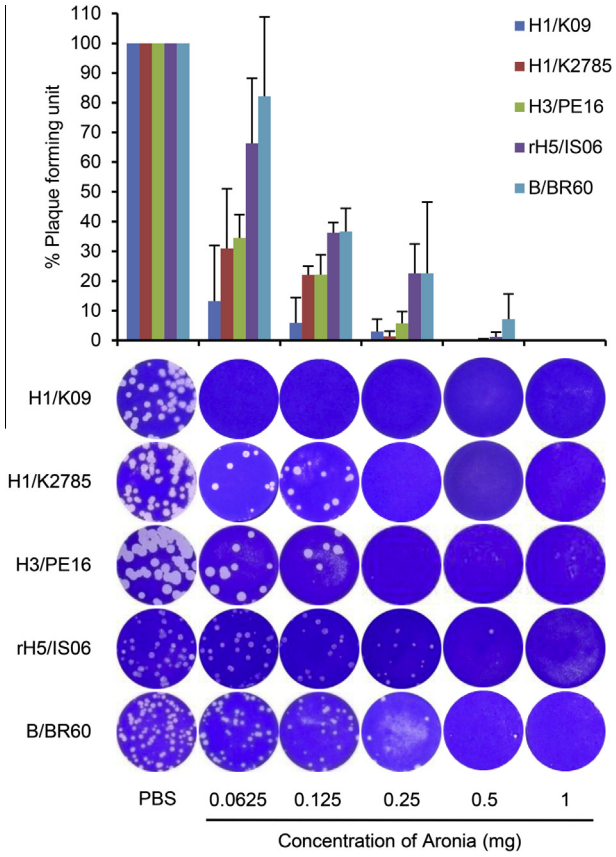

Fig. 1. Broad-spectrum efficacy of Aronia against various influenza viruses. The antiviral efficacy of Aronia (0.0625–1 mg/ml) was evaluated in the virucidal test against H1/K09, H3/PE16, and B/BR60 viruses, including oseltamivir-resistant H1/K2785 and rH5/IS06 viruses. Viruses incubated with PBS served as controls.

3.3. Chemical constituents of Aronia

In this study, we used Aronia prepared from crude *A. melanocarpa* fruits. The active ingredients of Aronia may have been mixed with non-specific chemicals. Therefore, we examined a previously reported composition index of Aronia [27] and evaluated the antiviral efficacy of seven selected polyphenolic constituents (caffeic acid, *p*-coumaric acid, ellagic acid, gallic acid, *p*-hydroxybenzoic acid, myricetin, and quercetin) of Aronia. In a replication inhibition assay in MDCK cells, ellagic acid, gallic acid, myricetin, and quercetin effectively restrained H1/K09 virus replication (Fig. 3). They demonstrated similar or better efficacies to that of oseltamivir carboxylate. However, gallic acid appeared be cytotoxic because most of the treated cells were detached from the cell plates, and quercetin and its derivatives were evaluated in many other studies. Hence, we selected only two Aronia constituents, ellagic acid and myricetin, for further *in vivo* investigation.

3.4. In vivo therapeutic efficacy of Aronia, ellagic acid, and myricetin in a mouse model

Aronia, ellagic acid, and myricetin were evaluated for their use as anti-influenza therapeutics. Using the rPR8-GFP virus [26,28], the severity of viral invasion and therapeutic recovery from viral pathogenicity were first determined in a mouse model. By detecting GFP signals from the extracted lungs of infected mice, we confirmed that the rPR8-GFP virus produced strong GFP signals on most of the infected lung surfaces (Fig. 4A). When treated with Aronia, ellagic acid, and myricetin, GFP expressions were reduced more than 50% in the lungs of the infected mice, compared with those of non-treated mice (Fig. 4A and B). Even if the GFP reduction rates did not reach those observed with oseltamivir phosphate treatment, Aronia and its two constituents successfully limited viral invasion and yielded 15–30% reductions of viral replication (Fig. 4C).

We then examined whether Aronia, ellagic acid, and myricetin could protect mice from a lethal challenge. The mice were challenged with the rPR8-GFP virus and treated with the same procedures above for five days. Without treatments, the rPR8-GFP virus was 100% lethal, as all the infected mice were dead at 10 dpi (Fig. 4E). However, oseltamivir phosphate treatment allowed infected mice to survive until 14 dpi (Fig. 4D and E). Aronia, ellagic acid, and myricetin also exhibited therapeutic efficacy. They reduced the levels of viral morbidity (Fig. 4D) and protected some mice (50, 50, and 37.5% survival rates, respectively) from lethal challenge with no signs of toxicity (Fig. 4E and F). Taken together,

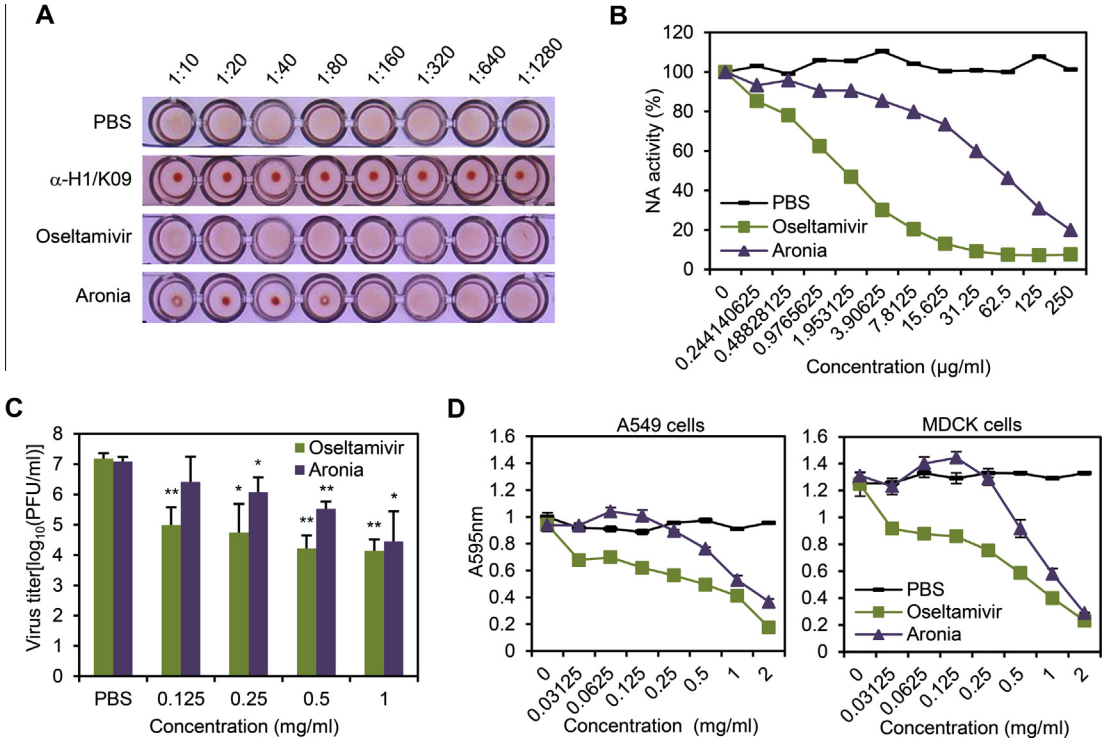

Fig. 2. Evaluation of the action mechanism and cytotoxicity of Aronia. (A, B) In the HI (A) and NI (B) assays, anti-influenza activity of Aronia was tested against the H1/K09 virus. Mouse anti-H1/K09 serum was used as an HI-positive control in HI assay. (C) The replication inhibition efficacy of Aronia was evaluated against the H1/K09 virus. The statistical significance of differences in viral reproduction was assessed by a Student's *t* test (two-tailed, unpaired), compared with that of PBS-treated controls ($^{*}P < 0.05$; $^{**}P < 0.01$). (D) The cytotoxicity of Aronia was evaluated in A549 and MDCK cells using MTT assay. PBS and oseltamivir carboxylate were used as controls.

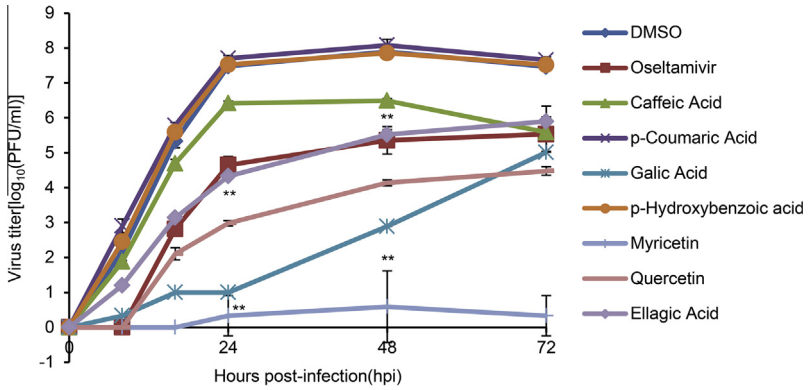

Fig. 3. Replication inhibition of Aronia constituents. Seven selected polyphenolic constituents of Aronia (caffeic acid, *p*-coumaric acid, ellagic acid, gallic acid, *p*-hydrobenzoic acid, myricetin, and quercetin) were evaluated for their replication inhibition efficacy against the H1/K09 virus (MOI = 0.001). The statistical significance of differences in ellagic acid- and myricetin-treated wells compared with that of DMSO-treated controls was assessed by a Student's *t* test (two-tailed, unpaired) ($^{**}P < 0.01$). Wells treated with DMSO and oseltamivir served as controls.

these results suggest that Aronia, ellagic acid, and myricetin have potentials as anti-influenza therapeutic agents.

4. Discussion

There are currently limited medical options available for humans against influenza infection. Vaccines and antiviral drugs are two main countermeasures. However, a vaccine mismatch and the occurrence of antiviral-resistant mutants are demanding the development of new medical intervention methods. As an alternative or natural remedy, polyphenolic compounds have been intensively investigated for the prevention of chronic diseases, such as cancers, cardiovascular diseases, and neurodegenerative disorders [29]. Moreover, the antimicrobial activity of polyphenols has been applied to the development of food preservatives [30] and new remedies for the treatment of many infectious diseases [31]. For influenza viruses, various polyphenols have been reported to have inhibitory efficacy. However, broad-spectrum efficacy has not been identified in suggested polyphenolic compounds.

In this study, Aronia exhibited cross-reactivity against influenza viruses including oseltamivir-resistant H1N1 and rH5/IS06 viruses (Fig. 1) possibly via anti-HA activity (Fig. 2A). The HAs of different subtypes usually express distinct antigenic signatures in the globular head domain [32]. Thus, the general efficacy of Aronia against various influenza viruses must be achieved by the non-specific masking of HA heads. The inhibitory function of Aronia may also be mediated by a mechanism other than the binding of viral surface proteins. Recently, myricetin has received attention for its efficacy as a viral enzyme inhibitor [33]. The inhibition of viral

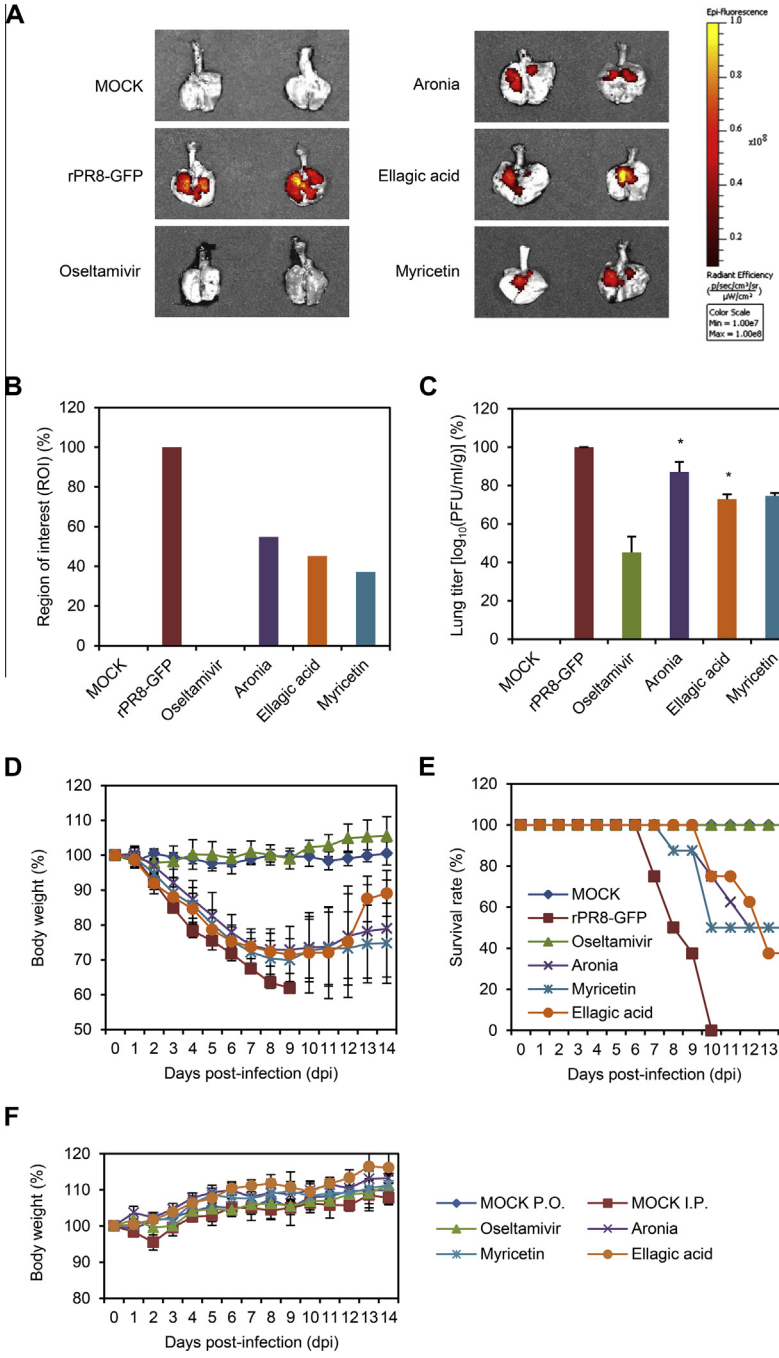

Fig. 4. Therapeutic efficacy of Aronia in a mouse model. To evaluate the therapeutic efficacy of Aronia, ellagic acid and myricetin, mice were intranasally infected with 5 MLD_{50} of the rPR8-GFP virus and treated with each agent. (A) The lungs of the mice were excised for *ex vivo* imaging at 3 dpi. (B) The viral invasion was determined as the value of the region of interest (ROI, %). (C, D, E, and F) The viral titers in the lungs of infected mice (C), body weight changes (D), survival rates (E), and *in vivo* toxicity (F) were measured to assess the therapeutic potentials of Aronia, ellagic acid, and myricetin. The statistical significance of differences in the viral titers compared with that of PBS-treated controls was assessed by a Student's *t* test (two-tailed, unpaired) (*$P < 0.05$). PBS (mock group) and oseltamivir phosphate were used as controls.

protein trafficking or maturation by the blocking of cellular signaling pathways has also been proposed for the action mechanism of the derivatives of polyphenolic curcumin or resveratrol [34]. Although viral infection was reduced by treatment with Aronia, ellagic acid, or myricetin in cell-based assays (Figs. 1–3), the exact mechanisms of action of these agents must be identified for the further development as broad-spectrum antiviral agents.

The reduced occurrence of resistant mutants is also needed for new antiviral candidates. Oseltamivir, which targets a catalytic site within the NA globular head of influenza viruses, is a highly potent and widely prescribed anti-influenza drug [35]. However, irrespective of the results of the oseltamivir treatment, the prevalence of oseltamivir-resistant H1N1 viruses in 2007–2009 has been the motivation for the development of new antivirals. To reduce the occurrence rate of resistant mutants, the development of antivirals that target cellular pathways may be a better method than directly targeting viral proteins because by blocking a universal cellular pathway, a virus itself can be spared from chemical pressures. In addition to the identified efficacy in this study, the unknown mechanisms of Aronia, ellagic acid, and myricetin must be elucidated, with a particular focus on the compound's effects on cellular signaling pathways.

Aronia is a rich repository of polyphenols. Along with the *in vitro* efficacy against tested influenza viruses, the results in this study suggest the *in vivo* therapeutic potential of Aronia, ellagic acid, and myricetin. Thus, revealing the responsible mechanisms underlying these candidates may facilitate the discovery of broad-spectrum antiviral agents and eventually enlighten our understanding of the etiologic progressions common in influenza viruses.

Acknowledgments

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