Anti-Influenza Virus Activity of Myrica rubra Leaf Ethanol Extract Evaluated Using Madino-Darby Canine Kidney (MDCK) Cells

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Myrica rubra leaf ethanol extract was added to culture medium of Madino-Darby canine kidney (MDCK) cells inoculated with influenza virus, and the inhibition of influenza virus replication was measured. Myrica rubra leaf ethanol extract showed anti-influenza virus activity irrespective of the hemagglutinin antigen type in the influenza virus type A (H1N1), its subtype (H3N2), and type B.

Key words: anti-influenza virus; Myrica rubra leaves; ethanol extract; hemagglutinin antigen type; MDCK cells

Myrica rubra is a tall, dioecious evergreen tree belonging to Myricaceae Myrica. Its fruits are round, contain polyphenol,1) and are eaten by many people. Although the melanin synthesis-inhibitory 2) and antitumor3) actions of Myrica rubra have been confirmed and are attracting attention due to functions, no anti-influenza virus activity of Myrica rubra leaves has been reported.

Influenza, which has a considerable influence on global health, is increasingly common. It is caused by influenza virus infection. There are many subtypes of influenza virus type A, and the viruses alter with hemagglutinin (HA) antigen variation caused by gene crossing and point mutation, for which no effective prophylactic or therapeutic drugs are available, and pandemics occur periodically. In the treatment of influenza, protein inhibitor (amantadine) and neuraminidase inhibitors (oseltamivir and zanamivir) are used; the former is effective only for type A, and the latter is effective for both type A and type B, but problems regarding these chemotherapeutic drugs have been reported: adverse effects, risk of emergence of resistant viruses, and loss of efficacy due to serotype variation, due to which the development of a safe and effective anti-influenza virus drug showing no adverse effects is awaited. In this study, marked anti-influenza virus activity was identified in extracts of Myrica rubra leaves.

Myrica rubra leaves cultivated in Matsue, Japan, were harvested in August 2005. The leaves were immediately dried in the sun, and 2 g of the dried leaves was mixed with 40 ml of 70% ethanol solution and kept at room temperature for 1 d. The mixture was then centrifuged (2,000 rpm, 10 min), and the supernatant was filtered through filter paper (5A). This supernatant was used as an ethanol extract (dry weight, 80 mg) of Myrica rubra leaves.

The influenza viruses used were strains that caused outbreaks during the influenza season in 1977/78 and 2001/2002 in Shimane Prefecture, Japan: A/shimane/32/78 and A/shimane/37/78 and A/shimane/48/2002 strains of the A/H1N1 type, A/shimane/31/77 and A/shimane/122/2002 strains of A/H3N2, and B/shimane/3/77 and B/shimane/2/2002 strains of type B, sit 6 strains in total. The evaluation of anti-influenza virus activity, maintenance medium I was used for virus type A and maintenance medium II for type B. Maintenance medium I was a liquid medium prepared by adding 5 ml of 7% sodium hydrogen carbonate, 8 ml of 5% bovine serum albumin, 1 ml of trypsin (2,000 units), and 2 ml of 30% glucose to Dulbecco’s modified Eagles minimal essential medium (DMEM) to a total of 200 ml. Maintenance medium II was a liquid medium prepared identically, excluding 1 ml of trypsin (2,000 units).

Madino-Darby canine kidney (MDCK) cells provided by the National Institute of Public Health of Japan (Tokyo) were used. The cell growth medium employed was 10% calf serum (CS)-supplemented Eagle’s minimal essential medium (MEM). The cells were cultured at 37 °C in a 5% CO₂ incubator.

To assess the cytotoxicity of the test samples, MDCK cells were cultured in 10% CS-supplemented MEM in a 12-well tissue culture plate at 37 °C in a 5% CO₂ incubator. After 3 d of culture, the medium was changed to 2 ml of maintenance medium containing 10 μl of extract. After the cells were cultured for 3 d, the viable cell rate was measured by WST-1 assay.4) The dry weight of the leaf extract in the assay was 20 μg/well (DMEM medium, 2 ml).
To evaluate anti-influenza virus activity, MDCK cells were cultured in 10% CS-supplemented MEM in a 12-well tissue culture plate at 37°C in a 5% CO₂ incubator. After 3 d of culture, the cells were inoculated with influenza virus (20 PFU/well). The cells were maintained at 35°C for 60 min to enable the viruses to adsorb to them. They were then washed twice with phosphate buffered saline (PBS, pH 7.4) to remove non-adsorbed viruses on the cell surface. Maintenance media I and II were added to influenza virus type A- and B-infected cells respectively at 2 ml/well, followed by the addition of extracts of the Myrica rubra leaf regions at 10 μl/well. The dry weight of the leaf extract in this assay was 20 μg/well (DMEM medium, 2 ml). These cells were cultured at 35°C in a 5% CO₂ incubator. After 3 d of culture, 12-well tissue culture plates were frozen and thawed twice and centrifuged for cell fragment sedimentation, and the supernatant containing the influenza virus was collected. The virus titer was determined by plaque assay, briefly described as follows: MDCK cells (2 × 10⁵ cells) were cultured in 10% CS-supplemented MEM in a 30-mm tissue culture dish at 37°C in a 5% CO₂ incubator. After 4 d of culture, the medium was removed and the cells were washed twice with PBS. Influenza viruses were adsorbed to the cells by maintaining them at 35°C for 60 min. After adsorption, the cells were washed twice with PBS, followed by overlaying of 2 ml of maintenance medium containing 1.2% agar (Bact-Agar). To the influenza virus type B maintenance medium, 1% DEAE-dextran aqueous solution was added at 1 ml/100 ml of medium. After 96 h of culture, 1 ml of maintenance medium containing neural red was overlaid, and plaques were counted to calculate the virus titer (PFU/ml). The percentage of plaque formation inhibition was calculated.

Substances with anti-influenza activity are required to inhibit influenza virus replication while showing no cytotoxicity against influenza virus host cells. Under the experimental conditions in this study, the viability of MDCK cells, serving as the hosts of influenza viruses, was not affected at the concentration of Myrica rubra leaf extract tested in the maintenance medium, confirming that the extract concentration tested exhibited no cytotoxicity against MDCK cells. Hence anti-influenza virus activity experiments were performed as outlined below.

Table 1 shows the inhibition of plaque formation by Myrica rubra leaf extract on the influenza virus in MDCK cells. The male-plant extract inhibited plaque formation in the A/shimane/32/78, A/shimane/31/77, and B/shimane/3/77 strains, by 88.3%, 81.4%, and 82.6% respectively, showing high plaque formation inhibition. As shown in Table 2, inhibition of plaque formation in the 2001/2002 epidemic strains was also higher than 80%. The female-plant extract also showed similarly high plaque formation inhibition rates for all strains (Tables 1 and 2). These findings suggest that there was no marked difference between the influenza virus replication-inhibitory activities of the male and female plants.

The inhibition of influenza virus replication in vitro and the remission of symptoms in patients during influenza virus type B outbreaks due to elderberry (Sambucus nigra L.) leaf extract,9) and complete inhibition of influenza virus type A (A/PR/8/34) replication, but not type B (B/Lee/40), due to Sanicula europaea L. (Apiaceae) leaf extract9) has been reported. The Myrica rubra leaf extract tested (Tables 1 and 2) inhibited the replication of not only influenza virus type A but also its subtypes (AH1N1 and AH3N2) and type B, regardless of the HA antigen type.

Influenza virus causes outbreaks every year. Since the surface HA antigen structure mutates, antibodies acquired via vaccination or infections by strains causing previous seasonal outbreaks might not prevent infections by current strains. We compared the replication-inhibitory effects of Myrica rubra leaf extract on strains with different HA antigen structures, strains that caused outbreaks in 1977/78 (Table 1) and 2001/2002 (Table 2). The rates of replication inhibition were higher than 80% in the epidemic strains for both seasons, revealing that Myrica rubra leaf extract inhibits influenza virus replication regardless of HA antigen mutation.

In 1949, Green10) observed inhibition of influenza virus proliferation due to tea extracts in embryonated chicken eggs, and reported that its active component is an alcohol-insoluble component (a non-polyphenol fraction). However, Nakayama et al.7,11) and Song et al.12) confirmed that polyphenol (catechin) contained in black/green and green teas respectively, inhibited influenza virus infection in an in vitro system using MDCK cells. On the other hand, Mantani et al.13)
observed inhibition of influenza virus infection due to catechin as an extract component of Ephedraceae. Since *Myrica rubra* contains polyphenols such as catechins and tannin, there is a possibility that the active component exhibiting anti-influenza virus activity is polyphenol. Currently, we are working to identify the active anti-influenza virus component contained in this extract.

References


