J. Microbiol. Biotechnol. (2005), **15**(5), 924–929

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

In Vitro Antiviral Activity of Aqueous Extracts from Korean Medicinal Plants Against Influenza Virus Type A

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Received: September 21, 2004
Accepted: February 2, 2005

Abstract Boiled-water extracts from 101 Korean medicinal plants were tested *in vitro* for their inhibitory activity against influenza virus type A by means of a modified hemagglutination inhibition test. Thirteen of the 101 extracts exhibited strong anti-influenza virus type A activity at concentrations of less than 780 μg/ml. Out of the above 13 extracts, MW-40 (*Chaenomeles speciosa*), MW-88 (*Citrus junos*), and MW-100 (*Zingiber officinale*) exhibited marked antiviral activity in the concentration range of 0.195 μg/ml to 100 mg/ml, 0.0487 μg/ml to 100 mg/ml, and 0.0487 μg/ml to 100 mg/ml, respectively. The extracts MW-88 and MW-100 were not cytotoxic to red blood cells, whereas MW-40 showed very weak cytotoxicity in the concentration range of 50 mg/ml to 100 mg/ml. Therefore, the present results demonstrate that boiled water extracts of 2 Korean medicinal plants, MW-88 and MW-100, have strong anti-influenza virus type A activity and no cytotoxic effects, and they may inhibit attachment of the virus to the cell and may be used for prophylaxis.

Key words: Influenza virus, hemagglutination inhibition test, Korean medicinal plants, boiled water extracts

Influenza virus infects the mucous membranes of the upper respiratory tract and occasionally invades the lungs. As a consequence, secondary bacterial infection may occur in susceptible individuals such as infants and the elderly [3, 7]. Influenza viruses are classified into types A, B, and C, with type A appearing to be the most clinically important. The epidemiology of influenza virus types A and B is distinctive because of their seasonality and antigenic variation. The gradual antigenic drift of influenza virus types A and B coupled with occasional antigenic shifts make it difficult to control the infection [15]. The influenza virus is a myxovirus and possesses two external glycosylated proteins, hemagglutinin (HA) and neuraminidase (NA). HA is the major component of the viral envelope and accounts for approximately 30% of total virus protein [14, 9], and plays an essential role in the entry of the virus into the cell and is responsible for the attachment of the virus to neuramic acid-containing receptors on the cell surface [8]. These characteristics of the influenza virus make it possible to screen antiviral agents by means of hemagglutination inhibition (HI) tests.

Amantadine and rimantadine are currently used as therapeutic agents against influenza type A virus. However, side effects, limited utility, and resistance to these compounds necessitate the development of new antiviral agents to effectively combat this virus [5]. Two agents in a new antiviral class, the neuraminidase inhibitors, have recently been licensed for the treatment and prophylaxis of influenza in the USA: Oseltamivir and zanamivir are well tolerated with safety, and are available in capsules or a liquid suspension for children [2]. In Korea, many medicinal plants and traditional prescriptions have a long history of clinical application, and indeed, they are utilized as safe anti-influenza agents. We, therefore, focused on the extracts of these plants as candidate anti-influenza virus agents [1, 2, 6]. In this study, the major finding is that 2 boiled water extracts of medicinal plants with significant anti-influenza virus type A activity were detected by an HI assay. These candidates may be useful as prophylactic agents in the future.

MATERIALS AND METHODS

Viruses, Cells, and Reagents

Influenza virus [A/Taiwan/1/86 (H_1N_1)] was obtained from the Korean National Institute of Health. The virus was propagated in 10-day-old embryonated eggs by administration of 0.1 ml (0.5 HA unit) inoculum into the allantoic fluid. Following incubation at 34°C for 48 h, the eggs were chilled, and the allantoic fluid was carefully harvested with a syringe. The preparations were centrifuged at 1,500 ×*g*

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for 20 min to remove insoluble material. Chicken blood was collected in Alsever's solution (pH 6.1). Cells were washed three times in phosphate-buffered saline (PBS, pH 7.2) by centrifugation at 1,000 ×*g* for 10 min, 4°C, and resuspended in PBS. The buffer was removed by aspiration after each wash. The red blood cells (RBC) were diluted to 0.5% concentration with PBS prior to use [9]. Amantadine·HCl was purchased from Sigma Co. (St Louis, MO, U.S.A.).

Extracts from Korean Medicinal Plants

Korean medicinal plants were selected from Korean traditional medicine books [17, 4]. One-hundred-and-one medicinal plants were used in this study. Plants were identified and deposited in the Herbarium of Konkuk University. Boiled-water extracts were prepared by the following methods [10, 11]. A sample of each herb was cut to the size of 10-mesh using a cutting mill machine, and 60 g of each herb was added to 1,100 ml of sterilized water and boiled for 150 min. The aqueous extracts were filtered through 3 MM paper (Whatman, Maidstone, England), lyophilized, re-dissolved in 0.8% methanol, and filtered through a membrane of 0.2 μm pore size (Millipore, St. Quentin-Yuelines, France). These samples were stored at 4°C prior to testing. A partial list of the plants (13 of 101 medicinal plants) evaluated is shown in Table 1.

Hemagglutination (HA) Assay

Serial two-fold dilutions of the virus (50 ml) were made with PBS in a microtiter plate (Nunc, Roskilde, Denmark), and 50 μl of 0.5% (v/v) RBC suspension was added to the plate and gently mixed. The cells were allowed to settle and then incubated for 60 min at room temperature. Titration endpoints were determined as described previously [12, 16].

Hemagglutination Inhibition (HI) Test

For initial screening of crude extracts, serial two-fold dilutions of each extract, starting with a concentration of 100 mg/ml, were made with PBS in 96-well microtiter plates. A viral suspension, diluted to contain 4 HA (hemagglutination) units/25 μl, was added to each well, and the plate was incubated at room temperature for 30 min. Fifty microliters of 0.5% RBC suspension was then added to each well, and the plate was incubated at room temperature for a further 60 min [12]. The results of HI tests were expressed as strong positive (++), positive (+), or negative (−).

Estimation of Cytotoxic Effect

Serial two-fold dilutions of each extract, starting with a concentration of 100 mg/ml, were made with PBS in 96-well microtiter plates, and 25 μl of PBS was added to each well. After gentle mixing, 50 μl of 0.5% RBC suspension

Table 1. A partial list of Korean medicinal plants used in this experiment.

Exp. number	Family and sp.	Vernacular name	Part used	Medical utilization
1	Megascolecidae *Pheretima communisima*	Kuin	Whole body	Used for the treatment of convulsions due to high fever and antiasthmatic for bronchitis
7	Portulacaceae *Portulacea herba*	Mach'ihyun	Aerial part	Used for anti-inflammation
9	Meliacease *Melia japonica*	Cheollyounja	Fruits	Used as carminative
12	Umbelliferae *Angelicae dahuricae Radix*	Paekchi	Roots	Used as diaphoretic
40	Malaceae *Chaenomeles sinensis*	Mokkwa	Fruits	Used as antispasmodic agent and fever treatment of the body
63	Asteraceae *Arctium lappa*	Ubangja	Fruits	Used for affection due to wind and heat with cough and sore throat, also for mumps, and erysipelas
72	Palmae *Arecae semen*	Pillang	Seeds	Diuretic for edema
88	Rutaceae *Citrus junos* Tanaka	Yuja	Fruits	Used as expectorant for cough
95	Umbelliferae *Ledebouriellae radix*	Pangpung	Roots	Used as diaphoretic agent
97	Umbelliferae *Angelica koreana* Max.	Kanghwal	Rhizomes	Fever treatment of the upper part of body
100	Zingiberaceae *Zingiber officinale* Roscoe	Saeng-gang	Rhizomes	Used as diaphoretic for affection due to wind and cold
109	Alismataceae *Alisma canaliculatum* All. Braun *et* Bouche	Taeksa	Rhizomes	Used as diuretic for oliguria and edema
110	Magnoliaceae *Magnolia officinalis* Rehd.	Hubak	Barks	Used for antibacterial agent and to invigorate the function of the spleen

was added to each well, and the wells were incubated at room temperature for 60 min. The result was expressed as strongly positive (++), positive (+), weak positive (△), or negative (−).

Contents of Control Drugs

The contents of each control drug [4] were as follows: K-1 [Ssanghwatang, *Paeonia lactiflora* Pall (3.13 g), *Cnidium officinale* Makino (1.25 g), *Angelica gigas* Nakai (1.25 g), *Rehmannia glutinosa* Libosch (1.25 g), *Astragalus membranaceus* Bunge (1.25 g), *Cinnamomum cassia* Presl (0.94 g), *Glycyrrhiza uralensis* Fisch (0.94 g), *Zizyphus jujuba* Mill (0.67 g), *Zingiber officinale* Rosc (0.5 g), sodium benzoate (100 mg), methylparaoxybenzoate (10 mg), propyl paraoxybenzoate (5 mg) in 75 ml water]; K-2 [Kalgeuntang, *Pueraria lobata* Ohwi (3.56 g), *Ephedra sinica* Stapf (1.78 g), *Ziziphus jujuba* Mill (1.78 g), *Cinnamomum cassia* Presl (1.33 g), *Paeonia lactiflora* Pall (33 g), *Glycyrrhiza uralensis* Fisch (0.89 g), *Zingiber officinale* Rosc (0.44 g), sodium benzoate (0.1 g) in 75 ml water]; K-3 [Ssanggamtang, kungjihyangsosanyeoncho (3.7 g), *Cyperus rotundus* L (2.5 g), var. acuta Kudo (2.5 g), *AtraPeriia fructescensctylodes japonica* Koidz (1.875 g), *Citrus unshiu* Markovich (1.25 g), *Cnidium officinale* Makino (1.25 g), *Angelica dahurica* Benth. et Hooker f (1.25 g), *Glycyrrhiza uralensis* Fisch (0.625 g), *Zingiber officinale* Rosc (0.5 g), *Ziziphus jujuba* Mill (0.67 g), dehydroacetic acid (75 mg), sodium benzoate (75 mg) in 60 ml water]; K-4 [Insamsamultang, *Angelica gigas* Nakai (450 mg), *Paeonia lactiflora* Pall (2 g), *Rehmannia glutinosa* Libosch (2 g), *Cnidium officinale* Makino (2 g), *Angelica gigas* Nakai (2 g), sodium benzoate (80 mg), butylparaoxybenzoate (20 mg) in 75 ml water]; J-1 [Kalgeuntang kanebo, *Pueraria lobata* Ohwi (8 g), *Ephedra sirica* Stapf (4 g), *Ziziphus jujuba* Mill (4 g), *Cinnamomum cassia* Presl (3 g), *Paeonia lactiflora* Pall (3 g), *Glycyrrhiza uralensis* Fisher (2 g), *Zingiber officinale* Rosc (1 g) in 75 ml water]; J-2 [Kakonaru, *Pueraria lobata* Ohwi (8 g), *Ephedra sinica* Stapf (4 g), *Ziziphus jujuba* Mill (4 g), *Cinnamomum cassia* Presl (3 g), *Paeonia lactiflora* Pall (3 g), *Glycyrrhiza uralensis* Fisch (2 g), *Zingiber officinale* Rosc (1 g) in 75 ml water]; C-1 [Chongshihoumch-ungje, *Bupleurum falcatum* L (4 g), *Citrus unshiu* Markovich. (2 g), *Ledebouriella seseloides* Wolf (3 g), *Paeonia lactiflora* Pall (8 g) in 75 ml water]; C-2 [Pallamgeunchung-je, *Isatis indigotica* Fort (16 g) in 60 ml water]; and C-3 [Kammochong-yolchunje, *Schizonepeta tenuifolica* Briq (4 g), *Platycodon grandiflorum* A. DC (2 g), *Bupleurum falcatum* L (4 g), *Angelica dahurica* Benth. et Hooker f (0.725 g), *Prunus armeniaca* L. var. ansu Maxim (450 mg), *Taraxacum mongolium* Hand.-Mazz (0.5 g) in 75 ml water]. Extracts of each control drug were prepared by the following method [11]: The aqueous extracts of each control drug were filtered through 3 MM paper (Whatman, England), lyophilized, re-dissolved in 0.8% methanol, and filtered through a membrane of 0.2 μm pore size. These samples were stored at 4°C prior to test.

RESULTS

Anti-Influenza Virus Type A Activity of Control Drugs

Amantadine·HCl, an antiviral chemotherapeutic agent, was used as a positive control. Oriental traditional drugs extracted from herb complexes and in clinical use for prophylaxis and therapy of influenza in Korea (K-1, K-2, K-3, and K-4), Japan (J-1 and J-2), and China (C-1, C-2,

Table 2. Inhibitory effects of control drugs on influenza virus type A.

No/Con (μg/ml)	-1 (200)	-2 (100)	-3 (50)	-4 (25)	-5 (12.5)	-6 (6.25)	-7 (3.13)	-8 (1.56)	-9 (0.78)	-10 (0.39)	-11 (0.195)	-12 (0.0975)
A	++	++	++	++	++	−	−	−	−	−	−	−
No/Con (μg/ml)	**-1 (100)**	**-2 (50)**	**-3 (25)**	**-4 (12.5)**	**-5 (6.25)**	**-6 (3.13)**	**-7 (1.56)**	**-8 (0.78)**	**-9 (0.39)**	**-10 (0.195)**	**-11 (0.0975)**	**-12 (0.0487)**
K-1	++	++	++	++	+	+	−	−	−	−	−	−
K-2	+	++	++	+	−	−	−	−	−	−	−	−
K-3	++	++	++	+	−	−	−	−	−	−	−	−
K-4	+	+	+	−	−	−	−	−	−	−	−	−
J-1	++	++	++	++	++	+	+	−	−	−	−	−
J-2	++	++	++	++	++	+	−	−	−	−	−	−
C-1	++	+	+	+	+	−	−	−	−	−	−	−
C-2	+	−	−	−	−	−	−	−	−	−	−	−
C-3	++	++	+	+	+	−	−	−	−	−	−	−

Anti-influenza virus effects of each drug determined by hemagglutination inhibition test.

A: Amantadine·HCl; K-1, K-2, K-3, and K-4: Oriental traditional drugs extracted from herb complexes in clinical use for prophylaxis and therapy of influenza in Korea; J-1 and J-2: Oriental traditional drugs extracted from herb complexes in clinical use for prophylaxis and therapy of influenza in Japan; C-1, C-2, and C-3: Oriental traditional drugs extracted from herb complexes in clinical use for prophylaxis and therapy of influenza in China. Con: concentration of each extract; ++, +, and −: strong positive, positive, and negative, respectively.

Table 3. Cytotoxic effects of control drugs on red blood cells.

No/Con (mg/ml)	-1 (200)	-2 (100)	-3 (50)	-4 (25)	-5 (12.5)	-6 (6.25)	-7 (3.13)	-8 (1.56)	-9 (0.78)	-10 (0.39)	-11 (0.195)	-12 (0.0975)
A	+	+	+	+	–	–	–	–	–	–	–	–
No/Con (mg/ml)	-1 (100)	-2 (50)	-3 (25)	-4 (12.5)	-5 (6.25)	-6 (3.13)	-7 (1.56)	-8 (0.78)	-9 (0.39)	-10 (0.195)	-11 (0.0975)	-12 (0.0487)
K-1	–	–	–	–	–	–	–	–	–	–	–	–
K-2	–	–	–	–	–	–	–	–	–	–	–	–
K-3	–	–	–	–	–	–	–	–	–	–	–	–
K-4	–	–	–	–	–	–	–	–	–	–	–	–
J-1	–	–	–	–	–	–	–	–	–	–	–	–
J-2	–	–	–	–	–	–	–	–	–	–	–	–
C-1	–	–	–	–	–	–	–	–	–	–	–	–
C-2	–	–	–	–	–	–	–	–	–	–	–	–
C-3	–	–	–	–	–	–	–	–	–	–	–	–

Cytotoxicity to red blood cells with each control drug.
A: Amantadine·HCl; K-1, K-2, K-3, and K-4: Oriental traditional drugs extracted from herb complexes in clinical use for prophylaxis and therapy of influenza in Korea; J-1 and J-2: Oriental traditional drugs extracted from herb complexes in clinical use for prophylaxis and therapy of influenza in Japan; C-1, C-2, and C-3: Oriental traditional drugs extracted from herb complexes in clinical use for prophylaxis and therapy of influenza in China. Con: concentration of each extract; +, △, and –: positive, weak positive, and negative, respectively.

and C-3) were tested for their inhibitory activity against influenza virus type A (Table 2). Amantadine·HCl exhibited antiviral activity at a concentration of 12.5 μg/ml with cytopathic effects appearing in the range of 25 and 200 μg/ml. It is notable that K-1, J-1, and J-2 exhibited strong antiviral activity at 3.13 to 100 mg/ml, 1.56 to 100 mg/ml, and 3.13 to 100 mg/ml, respectively (Table 2).

Estimation of Cytopathic Effect of Control Drugs

We used the red blood cell cytotoxicity assay in order to determine the cytopathic effects of various drugs. Amantadine· HCl exhibited cytopathic effects in the range of 25 μg/ml and 200 μg/ml, whereas the oriental traditional medicines, including K-1, K-2, K-3, K-4, J-1, J-2, C-1, C-2, and C-3, were not cytotoxic (Table 3).

Anti-Influenza Virus Type A Activity of Boiled-Water Extracts from Korean Medicinal Plants

Boiled-water extracts from Korean medicinal plants were tested for inhibitory effects on influenza virus type A using the modified HI test. Thirteen of the 101 single medicinal plants evaluated in this study exhibited strong antiviral activity at concentrations less than 780 μg/ml (Table 4). These included MW-1 (body extract of *Pheretina aspergillium*), MW-7 (aerial parts of *Portulaca oleracea*), MW-9 (fruit extract of *Melia toosendan* Sieb. et Zucc.), MW-12 (root extract of *Angelica dahurica* Benth et Hook.), MW-40 (fruit extract of *Chaenomeles speciosa* Narcci), MW-63 (fruit extract of *Arctium lappa* L.), MW-72 (seed extract of *Areca catechu* L.), MW-88 (unripe fruit extract of *Citrus junos*), MW-95 (root extract of *Ledebouriella seseloides* Wolff), MW-97 (rhizome extract of *Notopterygium incisum* Ting ex H.T), MW-100 (rhizome extract of *Zingiber officinale* Rosc), MW-109 (tuber extract of *Alisma orientale*), and MW-110 (stem bark extract of *Magnolia obovata*). It is notable that three of the most potent extracts, MW-40, MW-88, and MW-100, exhibited significantly strong antiviral activity at 195 μg/ml to 100 mg/ml, 48.7 μg/ml to 100 mg/ml, and 48.7 μg/ml to 100 mg/ml, respectively.

Estimation of Cytopathic Effect of Korean Medicinal Plants

To determine the cytopathic effect of boiled-water extracts from Korean medicinal plants, we examined their cytopathic effect on red blood cells. Among the 13 boiled-water extracts with strong anti-influenza virus activity, MW-1, MW-7, MW-9, MW-12, MW-63, MW-88, MW-95, MW-97, MW-100, and MW-109 exhibited no cytopathic effect at the concentrations tested. On the other hand, MW-40, MW-72, and MW-110 did exhibit cytopathic effect in the range of 50 to 100 mg/ml, 3.13 to 100 mg/ml, and 780 μg/ml to 100 mg/ml, respectively (Table 4).

Discussion

The hemagglutinin of influenza virus binds to a neuramic acid-containing receptor on a target cell [8]. The interaction between virus and cell, therefore, makes it possible to detect anti-influenza virus agents on the basis of a modified hemagglutination inhibition (HI) test. When serial dilutions of boiled-water extracts are added before adsorption of virus on the cell surface, extracts with antiviral activity inhibit the hemagglutinin of the influenza virus. The

Table 4. Inhibitory and cytotoxic effects of Korean medicinal plants whose boiled water extracts showed strong antiviral activity on influenza virus type A.

No/Con (mg/ml)	-1 (100)	-2 (50)	-3 (25)	-4 (12.5)	-5 (6.25)	-6 (3.13)	-7 (1.56)	-8 (0.78)	-9 (0.39)	-10 (0.195)	-11 (0.0975)	-12 (0.0487)
MW-1	++	++	++	++	++	+	+	+	+	–	–	–
7	++	++	+	+	+	+	+	+	+	–	–	–
9	++	++	++	++	++	++	++	+	-	–	–	–
12	++	++	++	++	++	+	+	+	-	–	–	–
40	++	++	++	++	++	++	++	++	++	+	–	–
63	++	++	++	++	++	++	++	+	–	–	–	–
72	–	–	–	–	–	++	++	++	–	–	–	–
88	++	++	++	++	++	+	+	+	+	+	+	+
95	++	++	++	++	+	+	+	+	+	–	–	–
97	++	++	++	++	++	++	++	+	+	–	–	–
100	+	+	+	+	+	++	++	++	++	++	+	+
109	++	++	++	++	++	++	++	+	–	–	–	–
110	–	–	–	–	–	–	–	++	+	–	–	–

Anti-influenza virus effects of boiled water extracts (MW) of Korean medicinal plants determined by haemagglutination inhibition test. Con.: concentration of each extract, ++, + and –: strong positive, positive and negative, respectively.

No/Con (mg/ml)	-1 (100)	-2 (50)	-3 (25)	-4 (12.5)	-5 (6.25)	-6 (3.13)	-7 (1.56)	-8 (0.78)	-9 (0.39)	-10 (0.195)	-11 (0.0975)	-12 (0.0487)
MW-1	–	–	–	–	–	–	–	–	–	–	–	–
7	–	–	–	–	–	–	–	–	–	–	–	–
9	–	–	–	–	–	–	–	–	–	–	–	–
12	–	–	–	–	–	–	–	–	–	–	–	–
40	△	△	–	–	–	–	–	–	–	–	–	–
63	–	–	–	–	–	–	–	–	–	–	–	–
72	+	+	+	+	+	△	–	–	–	–	–	–
88	–	–	–	–	–	–	–	–	–	–	–	–
95	–	–	–	–	–	–	–	–	–	–	–	–
97	–	–	–	–	–	–	–	–	–	–	–	–
100	–	–	–	–	–	–	–	–	–	–	–	–
109	–	–	–	–	–	–	–	–	–	–	–	–
110	+	+	+	+	+	+	+	+	–	–	–	–

Cytotoxicity to red blood cells with boiled water extracts of Korean medicinal plants (MW). Con.: concentration of each extract, +, △ and –: positive, weak positive and negative, respectively.

results of the present study indicated that the extracts with antiviral activity interfered with virus-cell binding, thus demonstrating that the HI test can be used to detect antiviral agents that may be useful prophylactic agents. MW-40 (*Chaenomeles speciosa*), MW-88 (*Citrus junos*), and MW-100 (*Zingiber officinale*) were found to be promising antiviral agents for influenza virus type A: Boiled-water extracts of MW-40, MW-88, and MW-100 had strong antiviral activity over a wide range of concentrations with very little cytotoxicity association (Table 4), and they should be considered for use as prophylaxis for influenza virus infection. The active boiled-water extracts containing MW-40, MW-88, and MW-100 would be expected to exert synergistic effects if specifically combined with other active extracts. The extracts MW-88 and MW-100 were not cytotoxic to red blood cells, whereas MW-40 showed very weak cytotoxicity in the concentration range of 50 mg/ml to 100 mg/ml (Table 4), showing that boiled-water extracts of 2 Korean medicinal plants, MW-88 and MW-100, exhibit strong anti-influenza virus type A activity and no cytotoxic effects. In the course of developing oriental medicine, the activity of mixture of compounds often lose their efficacy, and this phenomenon is a bottleneck for the development of drug discovery from oriental plant sources [13]. Using embryonated eggs, we recently carried out a primary *in vivo* chick embryo assay. Five were active in 6 embryonated eggs (data not shown). The detailed *in vivo* data will be published elsewhere. In summary, boiled-water extracts of 2 Korean medicinal plants, MW-88 and MW-100, showed strong anti-influenza virus type A activity and no cytotoxic effects. It is highly likely that they may inhibit attachment of the virus to the cells, and therefore, may be used for prophylaxis in the future.

Acknowledgment

This work was supported by Korea Research Foundation Grant (KRF-2002-075-E00005).

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