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NOTE

Screening of Antiviral Medicinal Plants against Avian Influenza Virus H1N1 for Food Safety

Jang-Hyun Lee, Nguyen Dinh Van, Jin Yeul Ma[1], Young Bong Kim, Soo-Ki Kim, and Hyun-Dong Paik*
Division of Animal Life Science and Bio/Molecular Informatics Center, Konkuk University, Seoul 143-701, Korea
[1]Center for Herbal Medicine Improvement Research, Korea Institute of Oriental Medicine, Daejeon 305-811, Korea

Abstract

Various extracts from 30 medicinal plants were evaluated for their antiviral activity against influenza virus A/Puerto Rico/8/34 (H1N1) and cytotoxicity in MDCK cell culture. The plant material (30 g) was extracted with methanol (300 mL) at room temperature for 24 h, after which the methanolic extracts were filtered, evaporated, and subsequently lyophilized. Evaluation of the potential antiviral activity was conducted by a viral replication inhibition test. Among these medicinal plants, *Tussilago farfara*, *Brassica juncea*, *Prunus armeniaca*, *Astragalus membranaceus*, *Patrinia villosa*, and *Citrus unshiu* showed marked antiviral activity against influenza virus A/H1N1 at concentrations ranging from 0.15625 mg/mL to 1.25 mg/mL, 0.3125 mg/mL to 10 mg/mL, 5 mg/mL to 10 mg/mL, 0.625 mg/mL to 10 mg/mL, 0.625 mg/mL to 10 mg/mL, and 0.3125 mg/mL to 5 mg/mL, respectively. The extracts of *Tussilago farfara* showed cytotoxicity at concentrations greater than 2.5 mg/mL, whereas the other five main extracts showed no cytotoxicity at concentrations of 10 mg/mL. Taken together, the present results indicated that methanolic extracts of the six main plants might be useful for the treatment of influenza virus H1N1.

Key words: influenza virus H1N1, avian influenza; antiviral activity, medicinal plant, *Tussilago farfara*, *Brassica juncea, Prunus armeniaca, Astragalus membranaceus, Patrinia villosa, Citrus unshiu*

Introduction

Human influenza viruses consist of the three subgroups (A, B, and C). They are enveloped viruses with single-stranded RNA that made up of segments (Charles, 2002). Three transmembrane proteins, the hemagglutinin (HA), the neuraminidase (NA) and the ion channel (M2) are embedded in the viral envelope (De Jong and Hien, 2006). Influenza virus infects the mucous membranes of the upper respiratory tract and occasionally invades the lungs and secondary bacterial infection may occur in individuals (Park and Lee, 2005). The respiratory disease caused by influenza A viruses is highly contagious for several animals including humans. Three influenza virus pandemics occurred in the last century, in 1918, 1957, and 1968 (Droebner *et al*., 2007). The first and most devastating of the 20th century pandemic strains emerged in 1918 and caused twenty million deaths worldwide (Basler and Aguilar, 2008; Droebner *et al*., 2007). Recently, highly pathogenic avian influenza H5N1 virus has appeared in Asian countries with high fatality rates in humans (Amonsin *et al*., 2006; Shortridge *et al*., 1998; Smith *et al*., 2006; Yan *et al*., 2007). Since March 2009, the outbreak of novel influenza A/H1N1 virus infection in humans has emerged in several countries and the human death toll continues to increase worldwide (Ginocchio *et al*., 2009; Naffakh and Werf, 2009; Rungrotmongkol *et al*., 2009). Currently, some drugs are available with antiviral activity against influenza viruses (Beigel and Bray, 2008). M2 ion channel inhibitors, amantadine and rimantadine, have been widely used in prophylaxis of influenza virus infections. Neuraminidase inhibitors, zanamivir and oseltamivir, are effective in both prophylaxis and treatment of influenza A and B viruses. However, their utilization in clinic is further limited by the rapid emergence of resistant virus mutants. The needs for an inhaler device and the risk of bronchospasm limit the use of zanamivir. Oseltamivir is being used although the gastrointestinal effects and emergence of resistant variants in some treated populations have limited the use of this drug (Hurt *et al*., 2009; Mossong *et al*., 2009). Therefore, much

*Corresponding author: Hyun-Dong Paik, Division of Animal Life Science and Bio/Molecular Informatics Center, Konkuk University, Seoul 143-701, Korea. Tel: 82-2-2049-6011, Fax: 82-2-455-3082, E-mail: hdpaik@konkuk.ac.kr

attention has been placed into searching for natural substances having anti-influenza effects (Mukhtar *et al*., 2008).

In East Asia, medicinal plants and traditional prescriptions have a long history of clinical application. Particularly, herbal drugs as prophylactic and treatment of anti-influenza agents have been used in East Asia, China, Japan, and Korea (Wang *et al*., 2006). Various natural compounds as anti-influenza agents have been reported. Polyphenolic compound catechins from green tea have anti-influenza virus in MDCK cell culture as inhibiting replication and potentially direct virucidal effect (Song *et al*., 2005). Pomegranate, purified polyphenol from *Punica granatum* has inhibitory activity against influenza virus and a synergistic effect with oseltamivir (Haidari *et al*., 2009). *Forsythia suspensa* is reported as a regulatory source with the effect on the production of regulated upon activation, normal T cell expressed and secreted (RANTES) and macrophage chemotactic protein-1 (MCP-1) in H1N1-infected A549 cells (Ko *et al*., 2005). Recently, we also reported that methanolic extract of *Asarum sieboldii* has antiviral activity against influenza (A/Vietnam/1194/04 (H5N1)-NIBRG-14) (Lee *et al*., 2008).

The aim of this study was to determine the antiviral activity of various medicinal plants against influenza A virus subtype H1N1.

Materials and Methods

Preparation of plant extracts

Medicinal plants were selected from Korean medicine book describing traditional medicines (Text Publishing Committee for Oriental Pharmacology, 2005). The list of the medicinal plants used in this study is shown in Table 1.

Methanolic extracts of the medicinal plants tested were prepared according to the procedures previously described with modification (Kim *et al*., 2007; Shin *et al*., 2008). Briefly, plant materials were extracted by using the method as described below. Dried and ground plant mate-

Table 1. Medicinal plant extracts used in this study

Botanical name	Part used	Uses
Tussilago farfara	Flower	Expectorant, antitussive
Ephedra sinica	Stem	Antitussive
Liriope platyphylla	Root	Expectorant, antipyretic, antitussive
Brassica juncea	Seed	Digestive tonic, antitussive
Stemona japonica	Root	Antitussive, insecticide
Eriobotrya japonca	Leaf	Expectorant, relieve nausea
Asarum sieboldii	Root	Analgesic, antipyretic, relieve headache
Polygala tenuifolia	Root	Restorative, expectorant, sedative
Prunus armeniaca	Seed	Antitussive
Forsythia suspensa	Fruit	Antipyretic, detoxicant,
Astragalus membranaceus	Root	Restorative
Angelica dahurica	Root	Analgesic, relieve headache
Lonicera japonica	Stem	Antibiotic, relieve cold, antipyretic
Cinnamomum cassia	Bark	Analgesic, relieve cold and diarrhea
Notopterygium incisum	Root	Analgesic, restorative, relieve muscle pain
Glycyrrhiza glabra	Root	Expectorant, detoxicant
Sinomenium acutum	Root	Uretic, antiphlogistic
Coptis japonica	Root	Antipyretic, relieve cold
Kalopanax pictus	Bark	Analgesic, relieve nausea
Asparagus cochinchinensis	Root	Restorative, uretic
Betula platyphylla	Bark	Detoxicant
Poncirus trifoliata	Fruit	Digestive tonic, relieve flatulence
Smilax china	Root	Antipyretic, detoxicant
Inula japonica	Flower	Expectorant, antitussive, relieve nausea
Schizonepeta tenuifolia	Aerial	Relieve cold and headache
Patrinia villosa	All	Detoxicant
Gentiana macrophylla	Root	Antipyretic
Citrus unshiu	Fruit	Digestive tonic, sedative
Scutellaria baicalensis	Root	Antipyretic, detoxicant, digestive tonic
Bupleurum falcatum	Root	Restorative

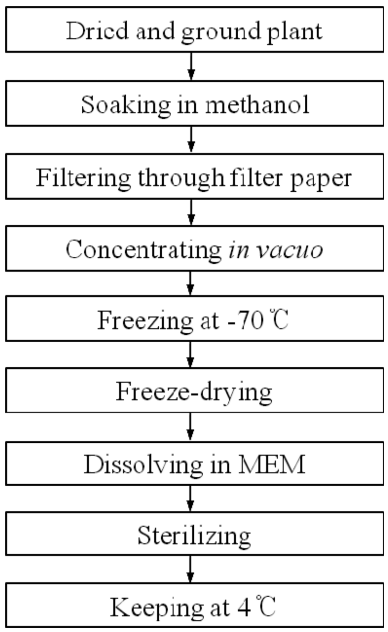

Fig. 1. Procedure to prepare extracts from medicinal plants in this study.

rial (30 g) was mixed with 300 mL of 99% methanol at room temperature and left overnight. After filtration through Whatman No. 2 filter paper, the filtrate was evaporated using rotary evaporator at 40°C. The concentrates were deep-frozen at -70°C overnight and the frozen mass was then subsequently subjected to lyophilization. Each compound was dissolved in serum-free Eagle's minimum essential medium (MEM) with concentration of 100 mg/mL. The solution was sterilized by filtration using membrane filter with 0.45 μm pore size and stored at 4°C (Fig. 1).

Viruses and cells

Influenza A/Puerto Rico/8/34 (H1N1) was propagated in 10-day-old embryonated chicken eggs in approved biosafety level-2 facility. Madin–Darby canine kidney (MDCK) cells were routinely grown in maintenance medium consisting Eagle's minimum essential medium (MEM) supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 0.1 mg/mL streptomycin. Virus growth medium for making virus pools and performing antiviral assays consisted of MEM with 0.3% Bovine Serum Albumin (BSA), 100 U/mL penicillin, 0.1 mg/mL streptomycin and 1 mg of *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin per mL.

Virus titration

Virus titration was performed by the end-point dilution method, using a 96-well microtitre plate with 6 wells per dilution and using 10-fold dilution method. The virus titer was estimated by cytopathic effect (CPE) of cells induced by viral infection and expressed as 50% tissue culture infectious doses ($TCID_{50}$). $TCID_{50}$ calculation was described by Reed and Muench method (Del Barrio and Parra, 2000).

Antiviral activity assay

In vitro antiviral ability evaluation was performed by cytopathic effect reduction assay (Li *et al*., 2007). MDCK cells were seeded on 96-well plate with 1.5×10^4 cells and incubated for 16 h in a 5% CO_2 incubator at 37°C. Confluent monolayer of MDCK cells were washed twice with 100 μL of PBS and infected with the influenza A/H1N1 virus at 100 $TCID_{50}$ for 2 h at 37°C. Following the incubation period, the unabsorbed portion of the viruses was washed out. The cell sheet was incubated with virus growth media containing serially diluted extract at 37°C for 48 h until viral CPE was visible. At each sheet, controls infected with 100 $TCID_{50}$ of virus and mock controls uninfected and untreated were performed in all experiments.

Cytotoxicity test

The maximum nontoxic concentration of each extract was determined based on cellular morphological alteration (Özçelik *et al*., 2009). MDCK cells were seeded at 1.5×10^4 cells in 96-well plate and incubated at 37°C for 16 h. After incubation, MDCK cell monolayers were washed twice with 100 μL of PBS, and then exposed to 100 μL of media containing serially diluted extract of each sample and incubated for 48 h in 5% CO_2 incubator at 37°C. At each sheet, controls without extracts were performed in all assays.

Results and Discussion

Different parts of 30 medicinal plants were tested for their antiviral activity and cytotoxicity. Of the extracts tested in this study, 6 major plants (Fig. 2), *Tussilago farfara*, *Brassica juncea*, *Prunus armeniaca*, *Astragalus membranaceus*, *Patrinia villosa*, and *Citrus unshiu*, were found to have antiviral activity against influenza A/Puerto Rico/8/34 (H1N1) at a non-toxic concentration to the MDCK cell lines. The results of 6 major plant extracts tested against viruses are shown in Table 2. The methanolic extract of *Tussilago farfara* was found to inhibit influenza virus at concentrations ranging from 1.25 mg/mL to 0.15625 mg/mL. However, the extract has cytotox-

Fig. 2. Picture of medicinal plants with antiviral activity against H1N1. a, *Tussilago farfara*; b, *Brassica juncea*; c, *Prunus armeniaca*; d, *Astragalus membranaceus*; e, *Patrinia villosa*; f, *Citrus unshiu*.

Table 2. Determination of maximum nontoxic concentration and antiviral activity of main plant extracts against influenza A/H1N1 virus in MDCK cell

Botanical name	Maximum nontoxic concentration (mg/mL)	CPE[1)] inhibitory concentration (mg/mL)	
		Maximum	Minimum
Tussilago farfara	2.5	1.25	0.15625
Brassica juncea	>10	>10	0.3125
Prunus armeniaca	>10	>10	5
Astragalus membranaceus	>10	>10	0.625
Patrinia villosa	>10	>10	0.625
Citrus unshiu	>10	5	0.3125

[1)]CPE, cytopathogenic effect.

icity at higher than 2.5 mg/mL. At a concentration lower than 10 mg/mL, the methanolic extracts of *Brassica juncea*, *Prunus armeniaca*, *Astragalus membranaceus*, *Patrinia villosa*, and *Citrus unshiu* showed no cytotoxicity to the MDCK cell. The extracts of *Brassica juncea* and *Citrus unshiu* presented antiviral effect at a concentration above 0.3125 mg/mL, respectively. *Patrinia villosa* extract was found to possess antiviral activity at the lowest concentration of 5 mg/mL. Examples were provided by the methanol extracts of *Astragalus membranaceus* and *Patrinia villosa*, which showed antiviral activity at the lowest concentration of 0.625 mg/mL, respectively. The oriental medicinal plants investigated here are used to treat respiratory ailment and to boost the body's immune system and digestion. In recent study, *Tussilago farfara* has found to have antioxidant activity (Kim *et al*., 2006) and anti-inflammatory activity (Hwangbo *et al*., 2009). *Brassica juncea*, *Prunus armeniaca*, *Astragalus membranaceus*, and *Patrinia villosa* have been reported to have anticarcinogenic properties (Cui *et al*., 2003; Munday *et al*., 2004; Peng *et al*., 2006; Toriyama-Baba *et al*., 2001). Lee *et al*. (2004) have also reported that *Citrus unshiu* was shown to have antiallergic activity of its hesperidin.

The antiviral activity of crude plant extract should be detectable in at least two subsequent dilutions of the maximum non-toxic concentration to ensure that the activity is not directly correlated with the toxicity of the extract. Six major active extracts in this study were found to have antiviral activity after two subsequent dilutions. It is possible that the elucidation of active constituents in these plants may provide useful leads in the development of antiviral therapeutics.

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References

1. Amonsin, A., Chutinimitkul, S., Pariyothorn, N., Songserm, T., Damrongwantanapokin, S., Puranaveja, S., Jam-on, R., Sae-Heng, N., Payungporn, S., Theamboonlers, A., Chaisingh, A. Tantilertcharoen, R., Suradhat, S., Thanawongnuwech, R., and Poovorawan, Y. (2006) Genetic characterization of influenza A viruses (H5N1) isolated from 3rd wave of Thailand AI outbreaks. *Virus Res.* **122,** 194-199.
2. Basler, C. F. and Aguilar, P. V. (2008) Progress in identifying virulence determinants of the 1918 H1N1 and the Southeast Asian H5N1 influenza A viruses. *Antiviral Res*. **79**, 166-178.
3. Beigel, J. and Bray, M. (2008) Current and future antiviral therapy of severe seasonal and avian influenza. *Antiviral Res*. **78**, 91-102.
4. Charles, G. P. (2002) Antiviral therapy for influenza virus infections. *Pediatr. Infect. Dis. J.* **13**, 31-39.
5. Cui, R., He, J., Wang, B., Zhang, F., Chen, G., Yin, S., and Shen, H. (2003) Suppressive effect of *Astragalus membranaceus* Bunge on chemical hepatocarcinogenesis in rats. *Cancer Chemother. Pharmacol*. **51**, 75-80.
6. De Jong, M. D. and Hien, T. T. (2006) Avian influenza A (H5N1). *J. Clin. Virol.* **35**, 2-13.
7. Del Barrio, G. and Parra, F. (2000) Evaluation of the antiviral activity of an aqueous extract from *Phyllanthus orbicularis*. *J. Ethnopharmacol*. **72**, 317-322.
8. Droebner, K., Ehrhardt, C., Poetter, A., Ludwig, S., and Planz, O. (2007) CYSTUS052, a polyphenol-rich plant extract, exerts anti-influenza virus activity in mice. *Antiviral Res*. **76**, 1-10.
9. Ginocchio, C. C., Zhang, F., Manji, R., Arora, S., Bornfreund, M., Falk, L., Lotlikar, M., Kowerska, M., Becker, G., Korologos, D., Geronimo, M., and Crawford, J. M. (2009) Evaluation of multiple test methods for the detection of the novel 2009 influenza A (H1N1) during the New York City outbreak. *J. Clin. Virol*. **45**, 191-195.
10. Haidari, M., Ali, M., Casscells, S. W., and Madjid, M. (2009) Pomegranate (*Punica granatum*) purified polyphenol extract inhibits influenza virus and has a synergistic effect with oseltamivir. *Phytomedicine* **16**, 1127-1136.
11. Hurt, A. C., Ernest, J., Deng, Y., Iannello, P., Besselaar, T. G., Birch, C., Buchy, P., Chittaganpitch, M., Chiu, S., Dwyer, D., Guigon, A., Harrower, B., Kei, I. P., Kok, T., Lin, C., McPhie, K., Mohd, A., Olveda, R., Panayotou, T., Rawlinson, W., Scott, L., Smith, D., D'Souza, H., Komadina, N., Shaw, R., Kelso, A., and Barr, I. G. (2009) Emergence and spread of oseltamivir-resistant A (H1N1) influenza viruses in Oceania, South East Asia and South Africa. *Antiviral Res*. **83**, 90-93.
12. Hwangbo, C., Lee, H. S., Park, J., Choe, J., and Lee, J. H. (2009) The anti-inflammatory effect of tussilagone, from *Tussilago farfara*, is mediated by the induction of heme oxygenase-1 in murine macrophages. *Int. Immunol*. **9**, 1578-1584.
13. Kim, H. K., Bang, C. S., Choi, Y. M., and Lee, J. S. (2007) Antioxidant and antiproliferative activities of methanol extracts from leafy vegetables consumed in Korea. *Food Sci. Biotechnol*. **16**, 802-806.
14. Kim, M. R., Lee, J. Y., Lee, H. H., Aryal, D. K., Kim, Y. G., Kim, S. K., Woo, E. R., and Kang, K. W. (2006) Antioxidative effects of quercetin-glycosides isolated from the flower buds of *Tussilago farfara* L. *Food Chem. Toxicol*. **44**, 1299-1307.
15. Ko, H., Wei, B., and Chiou, W. (2005) Dual regulatory effect of plant extracts of Forsythia suspense on RANTES and MCP-1 secretion in influenza A virus-infected human bronchial epithelial cells. *J. Ethnopharmacol*. **102**, 418-423.
16. Lee, J. H., Kwon, S. M., Seo, S. H., Park, Y. S., Kim, Y. B., Kim, S. K., and Paik, H. D. (2008) Screening of a natural feed additive having anti-viral activity against influenza A/H5N1. *Korean J. Food Sci. Ani. Resour*. **28**, 512-516.
17. Lee, N. K., Choi, S. H., Park, S. H., Park, E. K., and Kim, D. H. (2004) Antiallergic activity of hesperidin is activated by intestinal microflora. *Pharmacology* **71**, 174-180.
18. Li, Y., Jiang, R., Ooi, L. S. M., Butt, P. P. H., and Ooi, V. E. C. (2007) Antiviral triterpenoids from the medicinal plant *Schefflera heptaphylla*. *Phytother. Res*. **21**, 466-470.
19. Mossong, J., Opp, M., Gerloff, N., Hau, P., Kremer, J., Lackenby, A., Gregory, V., Even, J., Huberty-Krau, P., Muller, C. P., and Schneider, F. (2009) Emergence of oseltamivir-resistant influenza A H1N1 virus during the 2007-2008 winter season in Luxembourg: Clinical characteristics and epidemiology. *Antiviral Res*. **84**, 91-94.
20. Mukhtar, M., Arshad, M., Ahmad, M., Pomerantz, R. J., Wigdahl, B., and Parveen, Z. (2008) Antiviral potentials of medicinal plants. *Virus Res.* **131**, 111-120.
21. Munday, R. and Munday, C. M. (2004) Induction of phase II detoxication enzymes in rats by plant-derived isothiocyanates: comparison of allyl isothiocyanate with sulforaphane and related compounds. *J. Agric. Food Chem*. **25**, 1867-1871.
22. Naffakh, N. and Werf, S. (2009) April 2009: the outbreak of swine-origin influenza A(H1N1) virus with evidence for human-to-human transmission. *Microbes infect*. **11**, 725-728.
23. Özçelik, B., Gürbüz, I., Karaoglu, T., and Yeºilada, E. (2009) Antiviral and antimicrobial activities three sesquiterpene lactones from *Centaurea solstitialis* L. ssp. *solstitialis*. *Microbiol. Res*. **164**, 545-552.
24. Park, K. J. and Lee, H. H. (2005) *In vitro* antiviral activity of aqueous extracts from Korean medicinal plants against influenza virus type A. *J. Microbiol. Biotechnol*. **15**, 924-929.
25. Peng, J., Fan, G., and Wu, Y. (2006) Preparative isolation of four new and two known flavonoids from the leaf of *Patrinia villosa* Juss. by counter-current chromatography and evaluation of their anticancer activities *in vitro*. *J. Chromatogr. A* **1115**, 103-111.
26. Rungrotmongkol, T., Intharathep, P., Malaisree, M., Nunthaboot, N., Kaiyawet, N., Sompornpisut, P., Payungporn, S., Poovorawan, Y., and Hannongbua, S. (2009) Susceptibility of antiviral drugs against 2009 influenza A (H1N1) virus. *Biochem. Biophys. Res. Commun*. **385**, 390-394.

27. Shin, S. R., Hong, J. Y., and Yoon, K. Y. (2008) Antioxidant properties and total phenolic contents of cherry elaeagnus (*Elaeagnus multiflora* Thunb.) leaf extracts. *Food Sci. Biotechnol*. **17**, 608-612.
28. Shortridge, K. F., Zhou, N. N., Guan, Y., Gao, P., Ito, T., Kawaoka, Y., Kodihalli, S., Krauss, S., Markwell, D., Murti, K. G., Norwood, M., Senne, D., Sims, L., Takada, A., and Webster, R. G. (1998) Characterization of avian H5N1 influenza viruses from poultry in Hong Kong. *Virology* **252,** 331-342.
29. Smith, G. J. D., Naipospos, T. S. P., Nguyen, T. D., De Jong, M. D., Vijaykrishna, D., Usman, T. B., Hassan, S. S., Nguyen, T. V., Dao, T. V., Bui, N. A., Leung, Y. H. C., Cheung, C. L., Rayner, J. M., Zhang, J. X., Zhang, L. J., Poon, L. L. M., Li, K. S., Nguyen, V. C., Hien, T. T., Farrar, J., Webster, R. G., Chen, H., Peiris, J. S. M., and Guan, Y. (2006) Evolution and adaptation of H5N1 influenza virus in avian and human hosts in Indonesia and Vietnam. *Virology* **350**, 258-268.
30. Song, J. M., Lee, K. H., and Seong, B. L. (2005) Antiviral effect of catechins in green tea on influenza virus. *Antiviral Res.* **68**, 66-74.
31. Text Publishing Committee for Oriental Pharmacology (2005) Oriental Pharmacology. Sinil Co., Seoul, pp 101-758.
32. Toriyama-Baba, H., Logo, M., Asamoto, M., Iwahori, Y., Park, C. B., Han, B. S., Takasuka, N., Kakizoe, T., Ishikawa, C., Yazawa, K., Araki, E., and Tsuda, H. (2001) Organotropic chemopreventive effects of n-3 unsaturated fatty acids in a rat multi-organ carcinogenesis model. *Jpn. J. Cancer Res*. **92**, 1175-1183.
33. Wang, X., Jia, W., Zhao, A., and Wang, X. (2006) Anti-influenza agents from plants and traditional Chinese medicine. *Phytother. Res*. **20**, 335-341.
34. Yan, J., Lu, Y., Mao, H., Feng, Y., Xu, C., Shi, W., Weng, J., Li, M., Gong, L., Ge, Q., Zhou, M., Li, Z., and Chen, Y. (2007) Pathogenic and molecular characterization of the H5N1 avian influenza virus isolated from the first human case in Zhejiang province, China. *Diagn. Microbiol. Infect. Dis.* **58**, 399-405.

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