한국축산식품학회지
Korean J. Food Sci. Ani. Resour.
Vol. 28, No. 4, pp. 512~516(2008)

Screening of a Natural Feed Additive Having Anti-viral Activity against Influenza A/H5N1

Jang-Hyun Lee, Su Min Kwon, Sang Heui Seo[1], Young-Seo Park[2], Young-Bong Kim, Soo-Ki Kim, and Hyun-Dong Paik*

Division of Animal Life Science, Konkuk University, Seoul 143-701, Korea
[1]College of Veterinary Medicine, Chungnam National University, Daejeon 305-764, Korea
[2]Department of Food Science and Biotechnology, Kyungwon University, Seongnam, Gyonggi 461-701, Korea

안전한 닭고기 생산을 위한 고병원성 조류인플루엔자 A/H5N1에 항바이러스 효과를 가진 천연 사료첨가제의 탐색

이장현 · 권수민 · 서상희[1] · 박영서[2] · 김영봉 · 김수기 · 백현동*

건국대학교 동물생명과학부, [1]충남대학교 수의과대학, [2]경원대학교 식품생물공학과

Abstract

To search for anit-H5N1 influenza virus agent, the anti-viral activity of methanol and aqueous extracts from thirty medicinal plants were examined in this study. The plant material (30 g) was extracted with methanol (300 mL) for 24 hr at room temperature. Methanol extracts were filtered and evaporated, then freeze-dried. Aqueous extracts were prepared with dried plant material (30 g) and hot distilled water (300 mL). After 3 hr, the aqueous extracts were filtered and evaporated, then lyophilized. Extracts prepared from different plants were tested the antiviral activity against influenza viruses [A/Vietnam/1194/04 (H5N1)-NIBRG-14] using the hemagglutination (HA) assay. Among the test plants, *Asarum sieboldii* was found to be a potent inhibitor of H5N1 influenza virus in MDCK cell culture. Virus titers were 7 log, whereas with methanol extract of *Asarum sieboldii* for 48 hr titers were 3 log, indicating that methanol extract of *Asarum sieboldii* inhibited the H5N1 influenza viruses from the infected cells.

Key words : influenza A/H5N1, highly pathogenic avian influenza, feed additive, anti-viral activity, *Asarum sieboldii*

Introduction

Avian influenza A subtype H5N1 virus, with their ability to cause fatal infections and pathogenesis in humans has recently appeared in Asian countries (De Jong and Hien, 2006). The subsequent re-emergence of human H5N1 disease with high fatality rates has been reported in southern China (Shortridge *et al.*, 1998; Yan *et al.*, 2007), Thailand (Amonsin *et al.*, 2006), Indonesia, and Vietnam (Smith *et al.*, 2006). Currently, some drugs are available with anti-viral activity against influenza viruses: amantadine, remantadine, zanamivir, and oseltamivir (De Jong and Hien, 2006). But these synthetic drugs have side effects and limitations. Also, it has been reported that drug-resistant H5N1 influenza virus was isolated from Vietnam, and China (Le *et al.*, 2005; He *et al.*, 2008). The influenza virus (A/Hanoi/30408/2005 (H5N1)) isolated from a Vietnamese girl in 2005 was an oseltamivir-resistant virus (Le *et al.*, 2005), and 83.3% of influenza viruses isolated from chicken in Hebei Province of Northern China from 2001 to 2005 were resistant to amantadine (He *et al.*, 2008). Therefore, much attention has been placed into searching for natural substances having anti-influenza effects (Mukhtar *et al.*, 2008).

The efforts to search for influenza virus natural inhibitors are very ancient. In particular herbal drugs as anti-influenza agents have been mainly used in East Asia, China, Korea, and Japan (Wang *et al.*, 2006). The recent studies suggest that several natural substances isolated from several plants have anti-influenza activity both *in vitro* and *in vivo*.

*Corresponding author : Hyun-Dong Paik, Division of Animal Life Science, Konkuk University, Seoul 143-701, Korea. Tel.: 82-2-2049-6011, Fax: 82-2-455-1044, E-mail: hdpaik@konkuk.ac.kr

Polyphenolic compound catechins ((-)-epigallocatechin gallate, (-)-epicatechin gallate and (-)-epigallocatechin) from *Camellia sinensis* (green tea) inhibit influenza virus replication in MDCK cell culture and potentially direct virucidal effect (Song *et al.*, 2005). Methanol extracts from Korean medicinal plants, *Ephedra sinica* (Eupedraceae), *Areca catechu* (Palmae), *Paeonia lactiflora* (Ranunculaceae), and *Magnolia obovata* (Magnoliaceae), have inhibitory activity against the influenza virus (A/Taiwan/1/86 (H1N1)) (Park, 2003). A polyphenol rich extract from *Cistus incanus* PANDALIS has anti-influenza virus activity against the human influenza isolate (A/Thailand/1(KAN-1)/2004 (H5N1)) (Ehrhardt *et al.*, 2007). It also has reported that the extract of *Cochinchina momordica* seed as an adjuvant has potential to improve the efficacy of influenza vaccination (H5N1) in chickens (Rajput *et al.*, 2007).

In this study, we aimed to test thirty oriental medicinal plants with putative anti-viral activity against influenza A virus subtype H5N1 in MDCK cell cultures.

Materials and Methods

Viruses and cells

H5N1 (A/Vietnam/1194/04 (H5N1)-NIBRG-14) influenza viruses were propagated in the allantoic cavities of 11-d-old embryonated chicken eggs in approved biosafety level-3 (BL-3) containment facility. MDCK (Madin-Darby canine kidney) cells were cultivated in MEM supplemented with 10% FBS. The procedures for cell culture and virus titration were performed as described elsewhere (Seo *et al.*, 2004).

Preparation of plant extracts

Medicinal plants were selected from Korean medicine book describing traditional medicines. The scientific names of plants are shown in Table 1.

Extract of medicinal plants was prepared according to the standard methods with minor modification as previously reported (Kim *et al.*, 2007; Shin *et al.*, 2008). Briefly, the air-dried and finely ground samples were extracted by using the method as described below. Plants (30 g) were extracted

Table 1. Medicinal plant extracts used in this study

Botanical name (family)	Part used
Petasites japonicus (Asteraceae)	Flower
Trichosanthes kirilowii Maxim. (Cucurbitaceae)	Fruit
Platycodon grandiflorum (Campanulaceae)	Root
Ephedra sinica (Ephedraceae)	Aerial
Liriope platyphylla (Liliaceae)	Root
Brassica juncea Czern. et Coss (Cruciferae)	Seed
Ginkgo biloba L. (Ginkgoaceae)	Fruit
Stemona japonica Miq. (Stemonaceae)	Root
Eriobotrya japonca (Thumb.) Lindl. (Rosaceae)	Leaf
Adenophora triphylla var. *japonica* Hara (Campanulaceae)	Root
Morus alba L. (Moraceae)	Bark
Asarum sieboldii Miq. (Aristolchiaceae)	Root
Perilla frutescens var. *acuta* Kubo (Labiatae)	Leaf
Schizandra chinensis Baill. (Magnoliaceae)	Fruit
Polygala japonica Houtt. (Polygalaceae)	Root
Aster tataricus L. f. (Compositae)	Root
Peucedanum decursivum Maxim. (Umbelliferae)	Root
Fraxinus rhynchophylla Hance (Oleaceae)	Bark
Fritillaria thunbergii Miq. (Liliaceae)	Stem
Prunus armeniaca var. *ansu* Maxim. (Rosaceae)	Seed
Codonopsis pilosula (Franch) Nannf. (Campanulaceae)	Root
Polygonum multiflorum Thumb. ex Murray (Polygonaceae)	Root
Atractylodes macrocephala Koidzumi (Compositae)	Root
Astragalus complanatus R. Br. (Leguminosae)	Seed
Cornus officinalis Siebold & Zucc. (Cornaceae)	Fruit
Dioscorea japonica Thumb. (Dioscoreaceae)	Root
Nelumbo nucifera Gaertn. (Nympheaceae)	Seed
Acanthopanax sessiliflorus var. *parviceps* Rehder (Araliaceae)	Bark
Achyranthes japonica Nakai (Amarnthaceae)	Root
Cistanche deserticola Y. C. Ma (Orobanchaceae)	Root

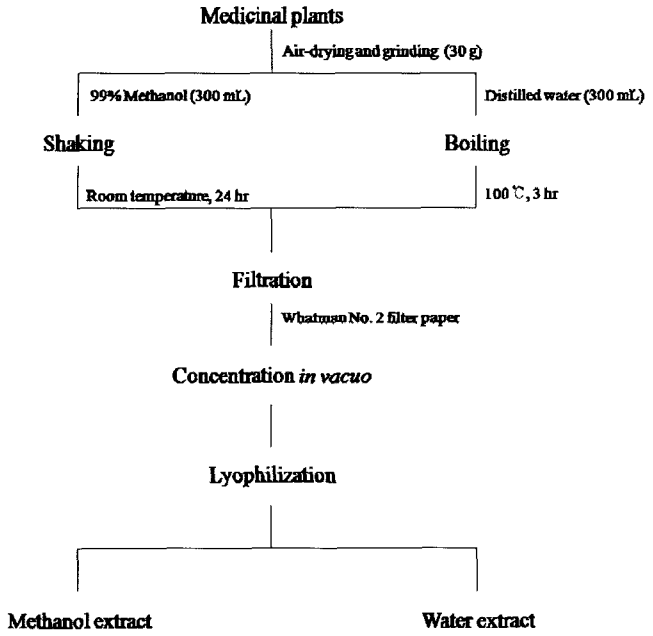

Fig. 1. Procedure to prepare extracts from medicinal plants in this study.

with 300 mL of methanol (99%) for 24 hr at room temperature on a rotating shaker (180 rpm). Crude aqueous extracts were also prepared with 30 g of powdered plant material. Each sample was added to 300 mL of distilled water in a glass flask, boiled for 3 hr. After filtration through Whatman No. 2 filter paper, the filtrate was concentrated under reduced pressure and lyophilized, then stored at 4°C (Fig. 1).

Anti-viral activity assay

The anti-viral activity of extracts was determined as described below. Briefly, confluent monolayers of MDCK cells on the 6-well plates were infected with 100 mL of 100 $TCID_{50}$/mL of H5N1 viruses. One hr later, plant extracts in MEM media (3 mL) at a non-toxic concentration were added to MDCK cells in well of 6-well plates, and cells were incubated for 48 hr. After this incubation period, virus titers were determined by hemagglutination (HA) assays. The anti-viral activity was defined as a 4 log reduction.

Table 2. Inhibitory activity of medicinal plants on influenza virus A/H5N1

Botanical name (family)	Methanol extract	Water extract
Petasites japonicus (Asteraceae)	-	-
Trichosanthes kirilowii Maxim. (Cucurbitaceae)	-	-
Platycodon grandiflorum (Campanulaceae)	-	-
Ephedra sinica (Ephedraceae)	-	-
Liriope platyphylla (Liliaceae)	-	-
Brassica juncea Czern. et Coss (Cruciferae)	-	-
Ginkgo biloba L. (Ginkgoaceae)	-	-
Stemona japonica Miq. (Stemonaceae)	-	-
Eriobotrya japonca (Thumb.) Lindl. (Rosaceae)	-	-
Adenophora triphylla var. *japonica* Hara (Campanulaceae)	-	-
Morus alba L. (Moraceae)	-	-
Asarum sieboldii Miq. (Aristolchiaceae)	+	-
Perilla frutescens var. *acuta* Kubo (Labiatae)	-	-
Schizandra chinensis Baill. (Magnoliaceae)	-	-
Polygala japonica Houtt. (Polygalaceae)	-	-
Aster tataricus L. f. (Compositae)	-	-
Peucedanum decursivum Maxim. (Umbelliferae)	-	-
Fraxinus rhynchophylla Hance (Oleaceae)	-	-
Fritillaria thunbergii Miq. (Liliaceae)	-	-
Prunus armeniaca var. *ansu* Maxim. (Rosaceae)	-	-
Codonopsis pilosula (Franch) Nannf. (Campanulaceae)	-	-
Polygonum multiflorum Thumb. ex Murray (Polygonaceae)	-	-
Atractylodes macrocephala Koidzumi (Compositae)	-	-
Astragalus complanatus R. Br. (Leguminosae)	-	-
Cornus officinalis Siebold & Zucc. (Cornaceae)	-	-
Dioscorea japonica Thumb. (Dioscoreaceae)	-	-
Nelumbo nucifera Gaertn. (Nympheaceae)	-	-
Acanthopanax sessiliflorus var. *parviceps* Rehder (Araliaceae)	-	-
Achyranthes japonica Nakai (Amarnthaceae)	-	-
Cistanche deserticola Y. C. Ma (Orobanchaceae)	-	-

+: detected, -: not detected.

Results and Discussion

The anti-viral activity of oriental medicinal plants against highly pathogenic avian influenza virus H5N1 was examined *in vitro*.

Out of the 30 samples tested, only the methanol extract of *Asarum sieboldii* was shown to inhibit the H5N1 (A/Vietnam/1194/04 (H5N1)-NIBRG-14) influenza virus (Table 2). Virus titers were 2^7 HA unit in case of sample-free, whereas with methanol extract of *Asarum sieboldii* virus titer was reduced to 3 log. *Asarum sieboldii* (Fig. 2), a perennial herb in the family Aristolochiaceae, is mainly distributed in Korea and China. It has been traditionally used as a medicine in cold and pains. It has been reported that its water extract have liver-protective effect (Cho and Yoon, 1999) and a propiophenone derivative from its rhizome inhibits harmful microorganisms (Lee *et al.*, 2005; Yu *et al.*, 2006). Kim *et al.* has also reported that *Asarum sieboldii* was shown to have a role of histamine pathways, and it has been used to treat histamine-related diseases traditionally (2003). This is the first report describing the anti-viral activity of *Asarum sieboldii* against high pathogenic avian influenza A H5N1.

Although further studies need to identify the bioactive substance of *Asarum sieboldii*, this early study suggests that an oriental traditional medicinal plant as a natural feed additive have a potential to be used in treatment of poultry diseases caused by high pathogenic avian influenza A H5N1.

Fig. 2. Root of *Asarum sieboldii* Miq. (Aristolchiaceae).

Acknowledgments

This work was supported by a grant (Code #200702157) from Biogreen 21 program, Rural Development Administration and the Brain Korea 21 Project, Korea.

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. Cho, E. and Yoon, S. (1999) Protective effect of *Asiasari Radix* on rat liver. *J. Korean Soc. Hyg. Sci.* **5**, 85-91.
3. De Jong, M. D., and Hien, T. T. (2006) Avian influenza A (H5N1). *J. Clin. Virol.* **35**, 2-13.
4. Ehrhardt, C., Hrincius, E. R., Korte, V., Mazur, I., Droebner, K., Poetter, A., Dreschers, S., Schmolke, M., Planz, O., and Ludwig, S. (2007) A polyphenol rich plant extract, CYSTUS052, exerts anti influenza virus activity in cell culture without toxic side effects or the tendency to induce viral resistance. *Antiviral Res.* **76**, 38-47.
5. He, G., Qiao, J., Dong, C., He, C., Zhao, L., and Tian, Y. (2008) Amantadine-resistance among H5N1 avian influenza viruses isolated in Northern China. *Antiviral Res.* **77**, 72-76.
6. Kim, H., Bang, C., Choi, Y., and Lee, J. (2007) Antioxidant and antiproliferative activities of methanol extracts from leafy vegetables consumed in Korea. *Food Sci. Biotechnol.* **16**, 802-806.
7. Kim, S., Zhang, C. G., and Lim, J. T. (2003) Mechanism of anti-nociceptive effects of *Asarum sieboldii* Miq. Radix: potential role of bradykinin, histamine and opioid receptor-mediated pathways. *J. Ethnopharmacol.* **88**, 5-9.
8. Le, Q. M., Kiso, M., Someya, K., Sakai, Y. T., Nguyen, T. H., Nguyen, K. H. L., Pham, N. D., Ngyen, H. H., Yamada S., Muramoto, Y., Horimoto, T., Takada, A., Goto, H., Suzuki, T., Suzuki, Y., and Kawaoka Y. (2005) Isolation of drug-resistant H5N1 virus. *Nature* **437**, 1108.
9. Lee, J. Y., Moon, S. S., and Hwang, B. K. (2005) Isolation and antifungal activity of kakuol, a propiophenone derivative from *Asarum sieboldii* rhizome. *Pest Manag. Sci.* **61**, 821-825.
10. 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.
11. Park, K. J. (2003) Evaluation of *in vitro* antiviral activity in methanol extracts against influenza virus type A from Korean medicinal plants. *Phytother. Res.* **17**, 1059-1063.
12. Rajput, Z. I., Xiao, C., Hu, S., Arijo, A. G., and Soomro, N. M. (2007) Improvement of the efficacy of influenza vaccination (H5N1) in chicken by using extract of *Cochinchina momordica* seed (ECMS). *J. Zhejiang Univ. Sci. B* **8**, 331-337.
13. Seo, S. H., Hoffmann, E., and Webster, R. G. (2004) The NS1 gene of H5N1 influenza viruses circumvents the host anti-viral cytokine responses. *Virus Res.* **103**, 107-113.
14. Shin, S. R., Hong, J. Y., and Yoon, K. Y. (2008) Antioxidant properties and total phenolic contents of cherry elaeagnus (*Elaeagnus multiflora* Thumb.) leaf extracts. *Food Sci. Bio-*

technol. **17**, 608-612.

15. 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.
16. 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.
17. Song, J., Lee, K., and Seong, B. (2005) Antiviral effect of catechins in green tea on influenza virus. *Antiviral Res.***68**, 66-74.
18. Wang, X., Jia, W., Zhao, A., and Wang, X. (2006) Anti-influenza agents from plants and traditional Chinese medicine. *Phytother. Res.* **20**, 335-341.
19. 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.
20. Yu, H., Seo, S., Hur, J., Lee, H., Lee, Y., and You, Y. (2006) *Asarum sieboldii* extracts attenuate growth, acid production, adhesion, and water-insoluble glucan synthesis of *Streptococcus mutans*. *J. Med. Food* **9,** 505-509.

(2008. 10. 1 접수/2008. 10. 19 채택)