J. Dairy Sci. 97:5383–5386
http://dx.doi.org/10.3168/jds.2014-8016

Short communication: Antiviral activity of subcritical water extract of *Brassica juncea* against influenza virus A/H1N1 in nonfat milk

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ABSTRACT

Subcritical water extract (SWE) of *Brassica juncea* was studied for antiviral effects against influenza virus A/H1N1 and for the possibility of application as a nonfat milk supplement for use as an "antiviral food." At maximum nontoxic concentrations, SWE had higher antiviral activity against influenza virus A/H1N1 than *n*-hexane, ethanol, or hot water (80°C) extracts. Addition of 0.5 mg/mL of *B. juncea* SWE to culture medium led to 50.35% cell viability (% antiviral activity) for Madin-Darby canine kidney cells infected with influenza virus A/H1N1. Nonfat milk supplemented with 0.28 mg/mL of *B. juncea* SWE showed 39.62% antiviral activity against influenza virus A/H1N1. Thus, the use of *B. juncea* SWE as a food supplement might aid in protection from influenza viral infection.
Key words: *Brassica juncea*, influenza virus, antiviral activity, subcritical water extraction

Short Communication

Medicinal plants have been studied for various properties, including antioxidant, antibacterial, and antiviral activities (Park, 2003; Shan et al., 2007; Veskoukis et al., 2012). Most of the antiviral effects possessed by medicinal plants are related to the presence of a variety of phytochemicals, such as alkaloids, flavonoids, glucosides, polyphenols, and saponins (Mukhtar et al., 2008). Several different antiviral mechanisms have been described for plant extracts, including immune modulation, prevention of host cell penetration, as well as inhibition of neuraminidase, uncoating, and nucleic acid synthesis (Strasfeld and Chou, 2010). *Brassica juncea*, commonly called brown mustard, has traditionally been used as an herbal drug with anticarcinogenic and antimicrobial properties (Rhee et al., 2003; Lee et al., 2010). *Brassica juncea* extract is composed of glucosinolates (sinigrin, gluconapin, and glucobrassicin), phenolic compounds, flavonoids, phytic acid, and brassinosteroids (Fabre et al., 1996; Munday and Munday, 2004). Among these components, brassinosteroids are naturally occurring polyhydroxy steroids and are reported to have antiviral activity against pathogenic viruses (Kumar and Andy, 2012).

Influenza A viruses are negative-strand RNA viruses that belong to the family *Orthomyxoviridae*; they have a segmented genome and possess 2 external glycosylated proteins—hemagglutinin (HA) and neuraminidase (NA; Hennet et al., 1992; Beigel and Bray, 2008). The influenza virus has been shown to be a major viral respiratory tract pathogen with worldwide prevalence. In the United States, zanamivir (Relenza, Glaxo Smith Kline, Research Triangle Park, NC) and oseltamivir (Tamiflu, Gilead Sciences, Foster City, CA) are US Food and Drug Administration-approved antiviral drugs for influenza (Vergara-Jaque et al., 2012). However, resistance to these therapies was recently reported during the 2009 pandemic of influenza virus (A/H1N1). Therefore, it is necessary to develop novel antiviral agents without any side effects.

Subcritical water extraction is based on the unique solvent properties of water. The advantages of this method compared with traditional extraction methods include shorter time for extraction, higher quality of the extract, and an environmentally compatible technique (Herrero et al., 2006). The potential antiviral activity of methanol extracts of *B. juncea* against influenza virus A/H1N1 has been shown previously (Lee et al., 2010). However, *B. juncea* SWE has not been studied for this activity. Therefore, we investigated the antiviral effects of *B. juncea* SWE against influenza virus and as a supplement to nonfat milk.

Madin-Darby canine kidney (**MDCK**) cells were obtained from the Korean Cell Line Bank (KCLB, Seoul, Korea). The MDCK cells were cultivated with Eagle's minimum essential medium (**EMEM**; Invitrogen, Carlsbad, CA) containing 10% heat-inactivated fetal

Received February 4, 2014.
Accepted April 29, 2014.
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Table 1. Cytotoxicity and antiviral activity of various extracts of *Brassica juncea* against influenza virus A/H1N1

Solvent	Yield (g/g)	MNTC[1] (mg/mL)	Antiviral activity at MNTC (%)
Subcritical water (110°C)	0.14	0.5	50.35
n-Hexane	0.25	0.041	NI[2]
Ethanol	0.13	1.5	40
Hot water (80°C)	0.16	>25	NI

[1]MNTC = maximum nontoxic concentration.

[2]NI = no inhibition.

bovine serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. The A/Puerto Rico/8/34 strain of influenza virus type A was propagated in 10-d-old embryonated eggs in an approved biosafety level-2 facility. The viral growth medium for generating virus pools and performing antiviral assays consisted of EMEM with 10% BSA (Invitrogen, Paisley, UK), antibiotics, and *N*-tosyl-L-phenylalanine chloromethyl ketone-treated trypsin per milliliter.

To perform extraction from *B. juncea* seeds, a subcritical extractor system (Dionex ASE 100; Dionex Corp., Sunnyvale, CA) was utilized. The extractions were performed using only distilled water as a solvent. Powdered *B. juncea* seeds and diatomaceous earth (DE) were mixed in stainless steel cells at a sample to DE ratio of 1:3. The cell volume was 34 mL and the cells were equipped with filters. The extraction temperature and static time were set to 110°C and 10 min, respectively. The collected extracts were stored at 4°C until further study.

Extractions using *n*-hexane, ethanol, and hot water were performed. For each of the *n*-hexane and ethanol extracts, 100 g of *B. juncea* seeds was extracted for 24 h at room temperature using 1 L of each solvent. For hot water extraction, 100 g of *B. juncea* seeds was extracted with 1 L of water for 3 h in an 80°C water bath. After filtration through Whatman No. 1 filter paper, the filtrate was concentrated by using a rotary evaporator. The concentrates were lyophilized and then stored at 4°C until used.

Brassica juncea SWE was added to nonfat sterilized milk at a concentration of 2.5 mg/mL and stored at 4°C for 8 d. After the storage period, the SWE was serially diluted 3-fold and mixed with the medium for use in antiviral evaluation.

The maximum nontoxic concentration of the SWE sample was determined based on the measurement of cell viability. First, MDCK cells (1.5×10^4 cells/well) were seeded in 96-well plates and incubated in 5% CO_2 at 37°C for 16 h. When cell growth reached confluence, the medium was removed and replaced with 100 μL of the medium containing the serially diluted sample. After incubation for 48 h, the medium was aspirated and 50 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (**MTT**; Sigma-Aldrich, St. Louis, MO) solution was added to each well and the plates were incubated for 4 h. The MTT solution was removed without disturbing the cells, and 100 μL of dimethyl sulfoxide was added to each well to dissolve the formazan crystals. After gently shaking the plates to dissolve the crystals completely, the absorbance was read using a microplate reader (Molecular Devices, Seoul, Korea) at 540 nm. The percentage survival was calculated using the optical density of treated versus control samples.

The MDCK cells were seeded in 96-well plates at 1.5×10^4 cells/well and incubated for 16 h in a 5% CO_2 incubator at 37°C. When cell monolayers were confluent, the medium was removed from the wells and washed twice with 100 μL of PBS, and then infected with influenza virus at 100 × 50% tissue culture infectious dose ($\mathbf{TCID_{50}}$) for 2 h at 37°C. After the unabsorbed viruses were removed, the cell sheet was incubated with the virus growth medium containing serially diluted samples at 37°C. After 48 h, all wells were observed and scored for viral cytopathic effects under the light microscope, and the MTT assay was conducted as described above. For each sheet, controls infected with 100 $TCID_{50}$ of virus and mock controls uninfected and untreated were included in all experiments. The percentage protection were calculated by using the formula $[(A - B)/(C - B)] \times 100$, where A, B, and C indicate the absorbance values of the sample, virus, and cell controls, respectively. Each experiment was conducted as 3 replicates.

Herbal remedies are generally perceived as harmless. However, several studies have suggested that cytotoxicity may be associated with herbal medications (Pak et al., 2004). Therefore, the cytotoxicity of *B. juncea* extracts was assessed using MDCK cells. Supercritical water extract and ethanol extract of *B. juncea* were found to be nontoxic to MDCK cells at 0.5 and 1.5 mg/mL, respectively. The antiviral activity was then evaluated by MTT assay in MDCK cells inoculated with influenza virus. At maximum nontoxic concentrations, SWE and ethanol extract were shown to have 50.35%