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Original Article

Probing the impact of quercetin-7-O-glucoside on influenza virus replication influence

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ABSTRACT

Background: Influenza virus is still at large and seriously affects social welfare and health. *Dianthus superbus* is a well-known medicinal plant widely used in Mongolian and Chinese traditional medicine for anti-inflammatory purposes.

Purpose: To investigate the influence of this novel herbal medicinal product over virus infection and virus-induced symptoms

Method: Quercetin-7-O-glucoside was isolated by bioassay (anti-influenza)-guided fractionation. The structural elucidation was made with 1H-NMR and 13C-NMR. Influenza A/Vic/3/75 (H3N2), A/PR/8/34 (H1N1), B/Maryland/1/59 and B/Lee/40 viruses were used for the evaluation of the antiviral activity. Virus-induced reactive oxygen species and autophagy formation levels were studied. The antiviral mechanism was elucidated via time-dependent, pre-, post-incubation assay methods. The viral RNA replication inhibition of Q7G was analyzed using quantitative RT-PCR method. The blocking of polymerase basic protein subunits of influenza viral RNA polymerase by Q7G was detected by *in silico* molecular docking assays using AutoDock Vina program with m^7GTP. Additionally, Q7G was tested against M-MuLV RNA polymerase.

Results: Q7G was not cytotoxic (CC_{50}>100 μg/ml) in MDCK cells and it showed 3.1 μg/ml, 6.61 μg/ml, 8.19 μg/ml and 5.17 μg/ml IC_{50} values against influenza A/PR/8/34, A/Vic/3/75, B/Lee/40 and B/Maryland/1/59 virus strains, respectively. Treatment of Q7G highly reduced ROS and autophagy formation induced by influenza virus infection. Q7G did not reduce NA activity and did not directly interact with the virus particles. Since viral RNA synthesis was blocked by treatment of Q7G. We targeted viral RNA polymerase for further probing. Interestingly, the binding energy of Q7G on viral PB2 protein was −9.1 kcal/mol and was higher than m^7GTP recorded as −7.5 kcal/mol. It also was observe to block M-MuLV RNA polymerase.

Conclusion: Isolated compound Q7G showed strong inhibition activity against influenza A and B viruses. It also reduced virus-induced ROS and autophagy formation. Q7G does not directly bind to the virus particles and did not affect NA activity. These results indicated that Q7G inhibits viral RNA polymerase, and that it occupies the binding site of m^7GTP on viral PB2 protein.

1. Introduction

20th century has seen an increase in influenza outbreaks leaving thousands either infected or dead. The vaccines are used as a primary prophylaxis against influenza virus. However, because of influenza virus genetic shift and drift, there is a limitation to control influenza pandemics (Kaiser et al., 2003). For example, 105,000–395,000 deaths were recorded owing to H1N1 influenza pandemic during 2009–2010. Neuraminidase inhibitors such as oseltamivir and zanamivir are commonly used for stopping influenza virus infections. Unfortunately, the 2007–2008 seasonal influenza crisis revealed that some isolates of H1N1 were resistant towards the commercial drug oseltamivir (Deyde et al., 2009; Sheu et al., 2008). Because of lack of scientific knowledge on aspects of anti-influenza drug development, more safe, inexpensive and low

Abbreviations: AVO, Acidic vesicular organelles; Bu, Butanol; DMEM, Dulbecco's Modified Eagle's Medium; DS, *Dianthus superbus*var.*longicalysinus*; DW, Distilled water; EA, Ethyl acetate; MDCK, Madin-Darby Canine Kidney; MeOH, Methanol; NA, Neuraminidase; PBS, Phosphate buffered saline; PB2, Polymerase basic protein 2; Q7G, Quercetin-7-O-glucoside; ROS, Reactive oxygen species; RT-PCR, Reverse-transcription polymerase chain reaction; SRB, Sulforhodamine B.

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resistance drugs are needed for the protection of our society (Ozawa et al., 2011).

Flavonoids are one of the large classes of polyphenolic compounds and are also abundant in our daily food, such as fruits, vegetables, and plant-derived foods. Flavonoids are already known for protection and prevention against heart diseases, cancer, inflammation, oxidation and bone loss (Havsteen, 2002; Hertog et al., 1993; Potter et al., 1998). Moreover, the antiviral activity of flavonoids has been known for their anti-influenza activity (Liu et al., 2008; Mori et al., 2008).

Dianthus superbus var. *longicalysinus* (DS) (Caryophyllaceae) is widely distributed in European and Asian countries and well-known in Mongolian and Chinese traditional medicine. This has long been used in herbal medicine as an anti-inflammatory agent (Lopez-Exposito et al., 2011; Shin et al., 2012). Anticancer activity of ethyl acetate extracts of *D. superbus*, which contained some cyclic peptides, was reported in various cancer cells (Ding et al., 2013; Hsieh et al., 2004). The proteins isolated from leaves of *D. cary-ophyllus* capable of depurinating HIV-1 RNA and inhibiting HIV-1 replication in human peripheral blood mononuclear cells (Korinov et al., 2004). Additionally, they reduced viral yield, plaque-forming efficiency and protein synthesis of herpes simplex virus-1 (HSV-1) or with polio-virus I (Foà-Tomasi et al., 1982). Recently, crude leaf extract of DS has been proved to exhibit antiviral activity against tobacco mosaic virus (Cho et al., 2000).

In this study, we investigated the possibility that some flavonoids from DS exhibit antiviral activity against influenza A and B viruses. Moreover, we investigated the action of the same flavonoids on influenza virus multiplication and inhibition of virus-induced factors on host cells (Fig. 9).

2. Materials and methods

2.1. Extraction and isolation

Dry DS leaves were purchased from a pharmacy located in Ulaanbaatar, Mongolia in July 2014. Plant materials (**dried leaves)** were minced using an automatic mixer (Hanil Co. Seoul, South Korea). The ground materials (1 kg) were extracted two times with pure MeOH (2×9 l) and once with 70% of MeOH (1×10 L) for 14 days at room temperature. Then the evaporated MeOH extract (221 g) was partitioned using ethyl acetate (EA), butanol (Bu) and water (DW). Then the Bu fraction (47 g) was loaded onto silica gel column (Merck, 70–230 mesh, 60×9 cm) and eluted with a gradient of $CHCl_3$:MeOH (100:0, 98:2, 96:4, 92:8, 85:15, 80:20, 75:25, 65:35, 50:50, 100:0and each 1.5 l) allowing to collect ten fractions (Fr.1-Fr.10). Moreover, fraction Fr.7 (1.7 g) was separated into 12 sub-fractions (Fr.7-1-12) using reverse phase column (LiChrprep RP-18, 40–63 μm, 60×3 cm) and eluted with MeOH-H_2O (gradient from 20% to 100% MeOH). Further purification was carried out using HPLC with gradients from 30% to 100% acetonitrile in water. The structures of the active compounds were identified via spectroscopic analysis, including electron spray ionization mass spectrometry (EI-MS), ^{1}H NMR, and ^{13}C NMR. Spectral data of purified compound was validated to be Quercetin-7-O-glucoside (Fig. 1) (Legault et al., 2011; Morina et al., 2015).

2.2. Cells, viruses, and reagents

Madin-Darby Canine Kidney (MDCK) cells were obtained from American Type Tissue Culture Collection (CCL-34, ATCC, USA) and cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco BRL, Grand Island, NY, USA), supplemented with 10% of fetal bovine serum (FBS, Gibco BRL, Grand Island, NY, USA) and 0.01% of penicillin (Gibco BRL, Grand Island, NY, USA) at 37 °C with 5% CO_2 incubator.

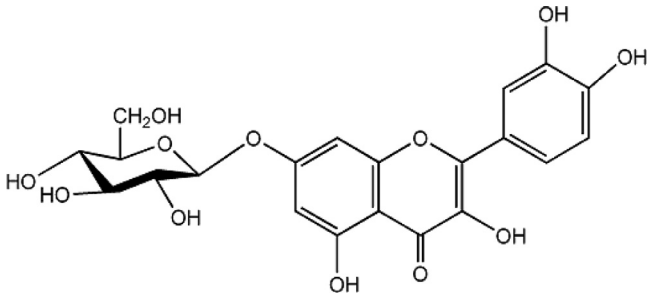

Fig. 1. Structure of quercetin-7-O-glucoside.

Influenza viruses as A/Vic/3/75 (H3N2, VR-822), A/PR/8/34 (H1N1, VR-1469), B/Maryland/1/59 (VR-296) and B/Lee/40 (VR-1535D) were obtained from ATCC. The 400x trypsin-EDTA (1x Trypsin-EDTA solution, Sigma-Aldrich, USA) and 0.01% of penicillin supplemented DMEM without FBS were used during antiviral assays. 50% of the tissue culture infective dose ($TCID_{50}$) of each influenza viral strain was utilized in all antiviral studies. Oseltamivir (Tamiflu®, Roche-Korea), purchased from a pharmacy in Korea as prescribed by a medical doctor was used as positive control.

2.3. Cytotoxicity and antiviral activity assays

Cytotoxicity and antiviral activity assays were conducted using SRB assay via the cytopathic effect (CPE) reduction method as previously reported (Enkhtaivan et al., 2015). Cytotoxic effect of Q7G was tested on non-infected MDCK cells. Briefly, 1.5×10^4 concentration of MDCK cells *per* well were cultured in a 96-well plate and incubated for 24 h in a humidified incubator at 37 °C with 5% of CO_2. After 24 h, cell culture medium was removed and washed twice with phosphate buffered saline (PBS) replenished with new medium. Different concentration (0.1 μg/ml, 1 μg/ml, 10 μg/ml, 100 μg/ml) of Q7G and oseltamivir were treated on cultured MDCK cells, and cells were incubated for 48 h. Further cytotoxicity of Q7G was studied by SRB assay as previously described (Choi et al., 2009).

Using SRB assay antiviral activity of Q7G was evaluated by time-dependent, pre-incubation and post-incubation methods. The time-dependent method was performedfollowing the recently described procedure with minor modifications (Choi et al., 2009). The MDCK cellwas cultured in 96-well plates at a density of 1.5×10^4 cells *per* well and incubated for 24 h. Then cultured cells were washed with PBS, medium containing Q7G (10 μg/ml) and oseltamivir (10 μg/ml) were added to the cells either before (–2 h, –1 h), during (0 h) or after (1 h, 2 h, 4 h, 7 h, 10 h, 15 h and 24 h) the period of influenza A/PR/8/34 virus infection. After 48 h of incubation, the antiviral activity of Q7G was carried out following the SRB assay.

For determining the Q7G effect on influenza A/PR/8/34 virus particles, they were incubated with Q7G for 2 h at 4 °C and then MDCK cells were infected by Q7G (10 μg/ml) treated influenza A/PR/8/34 virus particles at 37 °C for 1 h. Then unbound virus particles were removed by washing with PBS twice and subsequently MDCK cells were incubated with infected medium with or without Q7G treatment. Using SRB assay we estimated antiviral activity of Q7G after 48 h. Oseltamivir (0.1 μg/ml, 1 μg/ml, 10 μg/ml and 100 μg/ml) was used as positive control.

2.4. Quantitative RT-PCR analysis

Quantitative RT-PCR analysis was performed by a method described previously (Muthuraman et al., 2014). Briefly, the MDCK cells (1.5×10^5 cells/ml) were cultured in 6-well plates and