Antiviral potential of a diterpenoid compound sugiol from *Metasequoia glyptostroboides*

Vivek K Bajpai[1$], Na-Hyung Kim[2$], Kangmin Kim[3*] and Sun Chul Kang[4]*

[1]Department of Applied Microbiology and Biotechnology, School of Biotechnology, Yeungnam University, Gyeongsan, Korea
[2]Department of Oriental Pharmacy, Wonkwang University, Iksan-city, Jeonbuk, Korea
[3]Division of Biotechnology, College of Environmental and Bioresource Sciences, Chonbuk National University, Gobong-ro, Iksan-si, Joellabuk-do (Jeonbuk), Korea
[4]Department of Biotechnology, College of Engineering, Daegu University, Gyeongsan, Gyeongbuk, Korea

Abstract: This research reports first time antiviral activity of sugiol, a diterpenoid isolated from *Metasequoia glyptostroboides* in terms of its ability to inhibit *in vitro* growth of H_1N_1 influenza virus. Antiviral potential of sugiol was evaluated through cytopathogenic reduction assay using Madin-Darby canine kidney (MDCK) cell line. Sugiol (500 µg/ml) was found to exhibit considerable anti-cytopathic effect on MDCK cell line confirming its antiviral efficacy against H_1N_1 influenza virus. These findings strongly reinforce the suggestion that sugiol could be a candidate of choice in combinational regimen with potential antiviral efficacy.

Keywords: Sugiol, diterpenoid, antiviral effect, H_1N_1 influenza virus

INTRODUCTION

The $H_1N_1$is the sub-type of influenza A virus that is known as the most common cause of human influenza. Influenza, commonly known as 'flu' is a contagious respiratory disease induced by influenza viruses. It has been confirmed that more than two million hospitalizations and approximately 40,000 annual deaths are reported by influenza viruses in the United States every year (Thompson *et al*., 2004; Rather *et al*., 2014). Current scenario on emergence of life threatening viruses has resulted in enormous attention on finding new effective class of antiviral drugs of natural origin due to the restricted efficacy of currently available vaccination program (Rather *et al*., 2014; Hancock *et al*., 2009), and limited use of chemical synthesized antiviral medicines (Beigel and Bray, 2008) against influenza viruses. This has created a dramatic need for the development of un-conventional measures against influenza viruses.

Metasequoia glyptostroboides is a deciduous coniferous tree of the red-wood family, Cupressaceae, propagated and distributed in several tropical and sub-tropical regions of Asia, America, and Europe. Previously we reported isolation of variety of terpenoid compounds from *M. glyptostroboides* and confirmed their various biological properties including antifungal, antibacterial, and antioxidant (Bajpai *et al*., 2010; Bajpai and Kang, 2011; Bajpai *et al*., 2014).

Literature survey confirms that there has been no report available in the literature on the antiviral effect of sugiol. There has been a growing interest for developing new antiviral candidates of natural origin having little or no side effects, especially from natural products for the preventive treatment of influenza viruses. Hence, in this study, we evaluated the antiviral efficacy of an abietane-type diterpenoid sugiol, against influenza virus H_1N_1.

MATERIALS AND METHODS

Plant material, extraction, isolation and purification of sugiol

M. glyptostroboides cones were obtained from a local market in Pohang, South Korea, and identification was made through morphological and anatomical database at the Department of Biotechnology, Daegu University, Korea (Bajpai *et al*., 2014) with a voucher specimen number. About 2 kilogram of powdered cones was macerated with ethyl acetate organic solvent at room temperature for 12 days. A reducing pressure evaporator (EYELA N1000, Japan) was used to dry the resulting ethyl acetate organic extract. About 7 g of organic extract was used for column chromatographic use over silica gel, and gravitationally eluted using a gradient solvent system including hexane, ethyl acetate and methanol, which resulted in the twenty fractions. Further purification of fraction # 14 over preparative TLC with a mobile phase of (hexane: ethyl acetate; 2:1) resulted in the isolation of a pure compound sugiol as confirmed by its NMR data interpretation (Bajpai and Kang, 2011; Bajpai *et al*., 2014).

Culture of MDCK cell

The MDCK monkey cells grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated standard fetal bovine serum (FBS) and 1% (v/v) 100U/mol penicillin and 100µg/ml streptomycin solution were used. During sub-culturing, 10ml of

**Corresponding authors:* e-mail: sckang@daegu.ac.kr/activase@jbnu.ac.kr
[$]Equally contributed

trypsin-versine solution in 90 ml of EDTA was mixed in a flask following one minute incubation for the detachment of cell monolayer (Lu *et al*., 2002). After that, a fresh DMEM was added to the flask so as to neutralize the activity of trypsin. Further, flask content was centrifuged (1,500rpm; 5min) and cells were harvested. The harvested cells were resuspended in fresh DMEM medium and again kept in the flask and observed routinely.

Virus culture and harvesting of H_1N_1 virus from embryonated egg

The influenza virus (IFV) H_1N_1procured from Korea Centers for Disease Control and Prevention, was grown in the allantoic cavity of 11 days old chicken embryos for 3 days. In brief, specific pathogen-free (SPF) chicken eggs were cleaned by 70% (v/v) ethanol and incubated for embrocation in rotating and static egg incubator for at 35ºC with humidified environment for 11 days. After 11 days, 100µl IFV H1N1 was inoculated in the allantoic cavity by using 1ml syringes with 27-gauge, 1-inch or 1.5-inch hypodermic or blunt-end needles and the punched holes were sealed with wax followed by the incubation at 35°C in a humidified static incubator for 2-3 days. Further, allantoic fluid was collected from eggs and stored as a stock viral solution at -80°C. Virus titers in the allantoic fluid stock solution were determined as embryo infection dose $10^{5.5}EID_{50}$/0.1ml by using 50% end point dilution assay described earlier (Reed and Muench, 1938).

Antiviral potential of sugiol in cytopathogenic reduction assay

For assessing the cytopathogenic reduction effect of the sugiol, the MDCK cell line was cultured in a 96 well microplate using Eagle minimal medium (MEM) for 24-36h at 37ºC, using a CO_2 cell-culture incubator. MDCK cells were seeded onto a 96-well culture plate at the concentration of 2×10^4cells per well. Sugiol was serially diluted two-fold with 2% FBS DMEM solution. IFV H_1N_1 was treated with two-fold diluted samples of sugiol at 37ºC, and 5% CO_2 in cell culture incubator for 1h. This mixture was inoculated into MDCK cells and incubated in DMEM solution with 2% FBS at 37ºC in a humidified chamber with 5% CO_2 for 72 h by using a multi-pipette. The cytopathic effect (CPE) was observed after 72 h, and percent reduction of CPE was regarded as antiviral activity (Rather *et al*., 2014).

RESULTS

Identification of sugiol

The ethyl acetate extract derived from the cones of *M. glyptostroboides* following chromatographic techniques resulted in the isolation of yellow colored crystal compound with a confirmed melting point of 282-284°C. The proton NMR data measured at 250 MHz showed the presence of two singlet protons at δ 8.39 and 7.16, one aliphatic methine signal at δ3.59, and five terminal methyl groups at δ 1.35, 1.33, 1.11, 0.83 and 0.79. Carbon NMR (5250 MHz) analysis further confirmed the presence of 20 carbons with the presence of a carbonyl group at δ 197.6. This interpretation confirmed its identification in the group of abietane-type diterpenoids as a sugiol (fig. 1) (Chang *et al*., 1990; Bajpai *et al*., 2011).

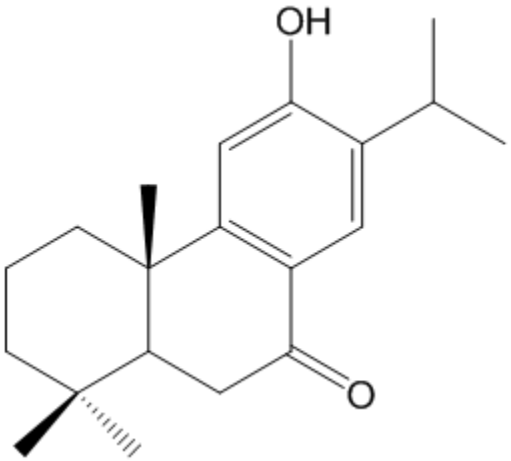

Fig. 1: Chemical structure of sugiol, an abietane-type diterpenoid isolated from *M. glyptostroboides*.

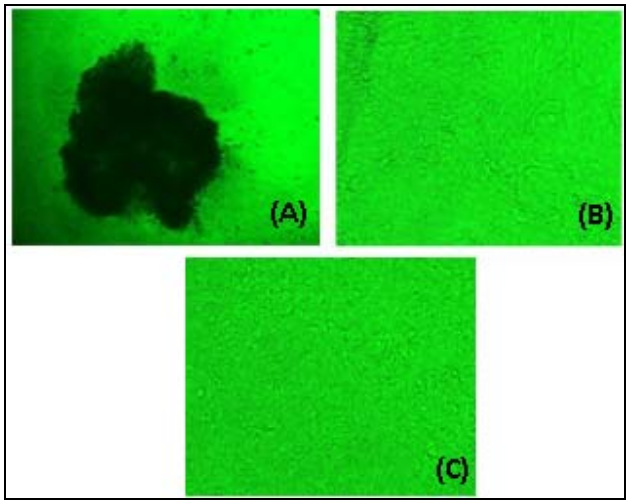

Fig. 2: Visualization of cytopathic inhibitory effects of sugiol (500 µg/ml) in MDCK cell line. Cells infected with H1N1 showing fusion of cells (A); control: cells without any treatment showing normal morphology (B); and cells treated with influenza virus H1N1 and sugiol showing normal cell morphology as a cytopathic inhibitory effect (C).

Inhibition of H_1N_1influenza virus

This study confirmed the therapeutic potential of sugiol as an antiviral candidate when tested on MDCK cell line against influenza virus H_1N_1. The results confirmed that when H_1N_1 inoculated with MDCK cell line, it induced severe cytopathic effect (CPE) (fig. 2A), whereas control cells without H1N1 did not cause CPE in MDCK cell line (fig. 2B). Further, results of microscopic observation confirmed that the cells when treated with sugiol (500

µg/ml) along with influenza virus H1N1) resulted in the demonstration of similar morphology of MDCK cells as observed in the control, and effect was consistent up to 72 h of incubation (fig. 2C). These findings suggested that sugiol isolated from *M. glyptostroboides* could be a potent antiviral candidate able to control CPE in MDCK cells caused by influenza virus H_1N_1.

DISCUSSION

The influenza virus is highly contagious in human populations around the world. Generally, influenza virus causes infections to the upper respiratory tract pertaining mucous membranes, however, it might also be invasive to the lungs (Brock *et al*., 1997). Such complications may further cause secondary viral infections to the populations having weakened immune system including elder people and infants (Brock *et al*., 1997; Kent *et al*., 1992). Among the influenza viruses, which are categorized in sub-type A, B and C, type-A viruses have shown alarming threats to the susceptible individuals (Park, 2003). Almost all the influenza viruses have shown distinct epidemiological symptoms due to the anti-genic and seasonal variations. The gradual anti-genic drift of influenza viruses including type-A and type-B in association with anti-genic shift make it very bothersome to prevent the infection (Park, 2003). Current therapeutic agents against influenza virus include amantadine and rimantadine, however, severe side effects and development of resistance have limited their practical applications, generating a huge need to develop new, effective and broad-spectrum antiviral agents to combat influenza virus (Morfin and Thouvenot, 2003).

A number of natural products have been found to possess multitude of therapeutic potential in terms of their biological properties including antiviral activity against various types of viruses. Niedermeyer *et al*. (2005) reported isolation of sterols and terpenoid constituents from the fruiting bodies of *Ganoderma pfeifferi*, among them, ganoderone A, lucialdehyde B, and ergosta-7, 22-dien-3beta-ol effectively inhibited *in vitro* growth of herpes simplex virus (HSV). Polsky *et al*. (1989) reported isolation of gossypol, a sesquiterpenoid from cotton seeds which exhibited potent antiviral activity inhibiting the *in vitro* growth of influenza virus. In addition, glycyrrhizin, a terpenoid isolated from *Glycyrrhiza glabra* has been found to display *in vitro* antiviral activity against influenza virus (Vanden Berghe *et al*., 1993).

Recently Park *et al*. (2013) reported antiviral activity of *Aronia melanocarpa*-based polyphenolic compounds against influenza virus H_1N_1. It was hypothesized that antiviral activity might be elicited possibly via anti-hem agglutination (HA) activity since HAs of different sub-types usually express distinct antigenic signature in the globular head domain (Park *et al*., 2013), suggesting this activity might be occurred by the non-specific masking of HA heads. Based on these findings, it can be hypothesized that in the present study, sugiol also exhibited antiviral activity against H_1N_1 in a similar manner since both the tested samples had similar chemical nature. In addition, inhibition of viral protein maturation via blocking of signaling pathways may also be proposed an antiviral action of sugiol as also observed by Park *et al*. (2013).

CONCLUSION

The importance of influenza virus H_1N_1 in causing respiratory diseases, and the potential for terpenoid compounds as natural inhibitors influenza virus may provide a viable modality for the prevention and treatment of viral diseases. This is the first report on the inhibitory effect of sugiol against influenza virus H1N1. The findings reported in the present study conclude that sugiol, a diterpenoid isolated from *M. glyptostroboides* appears to be a promising, and novel therapeutic agent, as confirmed by its antiviral potential against H1N1 influenza virus. Hence, it is suggested that using an innocuous natural products as monotherapy or in combination may lead to the development of a new and safer classes of potential antiviral agents. Future analysis of sugiol in a combinational regimen may elucidate increased effectiveness of this novel agent.

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