BRIEF REPORT

Osong Public Health Res Perspect 2017;8(6):415–420
https://doi.org/10.24171/j.phrp.2017.8.6.09
eISSN 2233-6052

In Vitro Antiviral Activity of Sakuranetin against Human Rhinovirus 3

Hwa-Jung Choi

Department of Beauty Science, Kwangju Women's University, Gwangju, Korea

Objectives: Rhinoviruses (RVs) cause common cold and are associated with exacerbation of chronic inflammatory respiratory diseases. Until now, no clinically effective antiviral chemotherapeutic agents to treat diseases caused by human rhinoviruses (HRVs) have been reported. We assessed the anti-HRV3 activity of sakuranetin isolated from *Sorbus commixta* Hedl. in human epithelioid carcinoma cervix (HeLa) cells, to evaluate its anti-rhinoviral potential in the clinical setting.

Methods: Antiviral activity and cytotoxicity as well as the effect of sakuranetin on HRV3-induced cytopathic effects (CPEs) were evaluated using the sulforhodamine B (SRB) method using CPE reduction. The morphology of HRV3-infected cells was studied using a light microscope.

Results: Sakuranetin actively inhibited HRV3 replication and exhibited antiviral activity of more than 67% without cytotoxicity in HeLa cells, at 100 μg/mL. Ribavirin showed anti-HRV3 activity similar to that of sakuranetin. Treatment of HRV-infected HeLa cells with sakuranetin visibly reduced CPEs.

Conclusion: The inhibition of HRV production by sakuranetin is mainly due to its general antioxidant activity through inhibition of viral adsorption. Therefore, the antiviral activity of sakuranetin should be further investigated to elucidate its mode of action and prevent HRV3-mediated diseases in pathological conditions.

Key Words: sakuranetin, human rhinovirus, *Sorbus commixta*, antiviral

Corresponding author: Hwa-Jung Choi
E-mail: rerived@empal.com

Received May 13, 2017.
Revised September 22, 2017.
Accepted October 9, 2017.

INTRODUCTION

Healthcare costs and loss of productivity for non-influenza viral respiratory diseases in the US have been estimated to be US dollar 39.5 billion annually [1]. Rhinoviruses (RVs) are the most common cause of viral upper respiratory diseases resulting in rhinitis, sinusitis, pharyngitis, or otitis media, and can lead to the development of bacterial superinfections [2–7]. While most individuals with an RV infection only experience mild symptoms, children, elderly individuals, immunosuppressed individuals, and those with asthma, or cystic fibrosis are predisposed to lower respiratory tract symptoms including wheezing, asthma exacerbations, and respiratory distress, which can often result in hospitalization [8,9]. Owing to numerous different serotypes (currently about 150 identified), infections are recurrent and, so far, no antiviral drug is commercially available.

Sorbus commixta Hedl. (family Rosaceae) has been used to treat cough, asthma, and other bronchial disorders in East Asian countries, including Korea, China, and Japan [10]. It is reported to have promising antioxidant, anti-atherogenic, anti-inflammatory, anti-atherosclerotic, and

vascular relaxant effects [11,12].

Sakuranetin was first identified from the cortex of the cherry tree bark (*Prunus* spp.) as an aglycone of sakuranin [13]. It was recently shown to exhibit anti-inflammatory activity by inhibiting 5-lipoxygenase, antileishmanial, and antitrypanosomal activities [14,15]. In addition, sakuranetin was reported to enhance adipogenesis and insulin sensitivity of 3T3-L1 cells through upregulation of peroxisome proliferator-activated receptor γ2 (PPARγ2) [16]. Although several studies have reported the pharmacological properties of crude extracts and sakuranetin, antiviral effects of sakuranetin against human rhinoviruses (HRV) 3 have not yet been reported. This study includes the isolation of sakuranetin from the *S. commixta* and its antiviral activity against HRV3.

MATERIALS AND METHODS

1. Isolation of sakuranetin

S. commixta was obtained from Yellohip (Daejeon, Korea). The dried whole plant of *S. commixta* (1.2 kg) was extracted with 1 L of methanol twice at room temperature for 2 days and the extract filtered (Whatman No.2). The extract was dried by evaporation under vacuum, after which 18.84 g of solid material was obtained. The extract (18.84 g) was then suspended in distilled water and fractionated successively with *n*-hexane, ethylacetate, and *n*-butanol. The ethylacetate fraction (3 g) was subjected to silica column chromatography, and eluted with a gradient solvent system of ethylacetate: methylene chloride: methanol to obtain 18 fractions (1 Fr, 3.3 mg; 2 Fr, 30.2 mg; 3 Fr, 183.6 mg; 4 Fr, 316.0 mg; 5 Fr, 54.9 mg; 6 Fr, 54.6 mg; 7 Fr, 79.5 mg; 8 Fr, 1.7 mg; 9 Fr, 7.3 mg; 10 Fr, 76.0 mg; 11 Fr, 49.3 mg; 12 Fr, 39.8 mg; 13 Fr, 107.2 mg; 14 Fr, 515.0 mg; 15 Fr, 121.0 mg; 16 Fr, 63.2 mg; 17 Fr, 34.7 mg; 18 Fr, 35.7 mg). Fractions 1–3 contained pure sakuranetin, which was analyzed by nuclear magnetic resonance (^{1}H and ^{13}C) and low-resolution electron-impact mass spectrometry (Figure 1) [17].

2. Virus, cells, and reagents

HRV3 was obtained from ATCC (American Type Culture Collection, Manassas, VA, USA) and propagated in human epithelioid carcinoma cervix (HeLa) cells at 32°C. Hela cells were maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 0.01% antibiotic-antimycotic. Antibiotic-antimycotic, trypsin-EDTA, FBS, and MEM were obtained from Gibco BRL (Grand Island, NY, USA). The tissue culture plates were purchased from Falcon (BD Biosciences, Franklin Lakes, NJ, USA). Ribavirin and sulforhodamine B (SRB) were purchased Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of reagent grade.

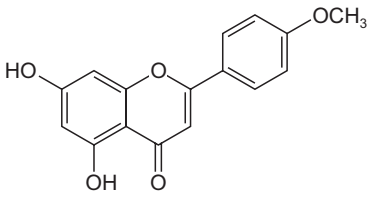

Figure 1. Structure of sakuranetin isolated from *Sorbus commixta*.

3. Assays of antiviral activity and cytotoxicity of sakuranetin and its effect on cytopathic effects

Antiviral activity and cytotoxicity, as well as the effect of sakuranetin on HRV3-induced cytopathic effects (CPEs) were evaluated using the SRB method using CPE reduction, as reported previously [18]. Briefly, 2×10^4 cells/well of Hela cells were seeded in MEM supplemented with 10% FBS and 0.01% antibiotic–antimycotic solution in a 96-well culture plate and incubated for 24 hours. Thereafter, medium was aspirated and cells were washed with phosphate buffered saline (PBS). Subsequently, the diluted virus suspension (0.09 mL), containing 30 mM $MgCl_2$, 1% FBS, and 50% tissue culture infective dose ($TCID_{50}$) of the virus, was added to Hela cells to produce appropriate CPEs within 48 hours after infection. Thereafter, MEM containing 50, 10, 2, and 0.4 μM of the gemcitabine (0.1 mL) were added. The culture plates were incubated at 37°C in 5% CO_2 for 2 days until 50% CPE was achieved. Subsequently, the 96-well plates were washed once with PBS (100 μL). Ice-cold 70% acetone in water (100 μL) was added to each well and incubated for 30 minutes at −20°C. After eliminating the 70% acetone, plates were dried in a drying oven at 55°C for 30 minutes. The 0.4% (w/v) SRB in 1% acetic acid solution (100 μL) was added to each well and left to stand for 30 minutes at room temperature. The SRB solution was then eliminated and the plates were washed 5 times with 1% acetic acid in water before oven-drying at 55°C. Bound SRB was then solubilized with 10 mM unbuffered Tris-base (Sigma-Aldrich) solution (100 μL). After 30 minutes, the absorbance was read at 524 nm with a VERSAmax microplate reader (Molecular Devices, Palo Alto, CA, USA) with a reference absorbance determined at 650 nm. The percent protection achieved by the test compound in the HRV3-infected cells was calculated using the following equation: Antiviral activity index = [(ODt)HRV3 – (ODc)HRV3]/[(ODt)mock – (ODc)HRV3] × 100%. Ribavirin was used as the positive control and DMSO as a negative control. The morphology of HRV3-induced cells was observed using a light microscope at 32×10 magnification (AXIOVERT10; ZEISS, Oberkochen, Germany), and images were recorded.

To evaluate cytotoxicity, Hela cells were seeded onto a 96-well

culture plate at a concentration of 2×10^4 cells per well. The next day, medium was replaced with media containing serially diluted compounds. After 2 days of incubation, cytotoxicity was evaluated using the SRB assay. The culture medium was aspirated and cells were washed with PBS. The next step was performed per the antiviral activity assay described above. Results are expressed as the percentage of the controls.

RESULTS

Sakuranetin exhibited excellent antiviral activity of approximately 67% against HRV3 at 100 μg/mL and of approximately 41% at 10 μg/mL (Figure 2A). Ribavirin also exhibited good antiviral activity of approximately 60% at 100 μg/mL and weak antiviral activity of less than 11% at less than 1 μg/mL (Figure 2A).

Cytotoxicity of each extract was assessed in parallel with antiviral activity. While sakuranetin exhibited antiviral activity, it was not toxic to Hela cells, yielding 100% cell viability at the tested concentrations (Figure 2B). Furthermore, ribavirin did not exhibit cytotoxicity in Hela cells with concentrations of 1–100 μg/mL (Figure 2B).

The morphology of HRV3-infected cells was observed in images obtained under a light microscope. There was no difference between the mock cells (Figure 3A) or those treated with 100 μg/mL sakuranetin (Figure 3C) or ribavirin (Figure 3E) in terms of typical spread-out shapes and normal morphology at 2 days after HRV3 infection. Only infection with HRV3 without sakuranetin or ribavirin resulted in a severe CPE (Figure 3B). Treatment of HRV3-infected HeLa cells with sakuranetin reduced the formation a visible CPE (Figure 3D). Furthermore, ribavirin decreased HRV3-induced CPE (Figure 3F). Therefore, sakuranetin isolated from *S. commixta* exhibited inhibitory effects against HRV3 in a HeLa cell line with HRV-induced CPE reduction.

DISCUSSION

Several drugs have been assessed for efficacy in treatment of HRV infections. Pleconaril is an orally absorbed viral capsid-function inhibitor that inhibits replication in 90% of RV serotypes [19]. However, the US Food and Drug Administration has not approved pleconaril because of concerns regarding the emergence of viral resistance and the reduced effectiveness of oral contraceptives among women using pleconaril [20]. Hence, the lack of effective therapy for HRV infections necessitates studies on new antiviral agents.

Many viruses can induce cell death, leading to lysis of infected cells [21]. In the late stages of HRV3 infection, morphological changes commonly known as CPE, can be observed microscopically. The morphology of HeLa cells after HRV3 infection was significantly different from that after treatment with sakuranetin.

Flavonoids constitute a large class of polyphenolic compounds and are integral components that are abundant in our daily diet, in vegetables, fruits, and plant-derived beverages. Numerous studies have suggested that flavonoids may protect against car-

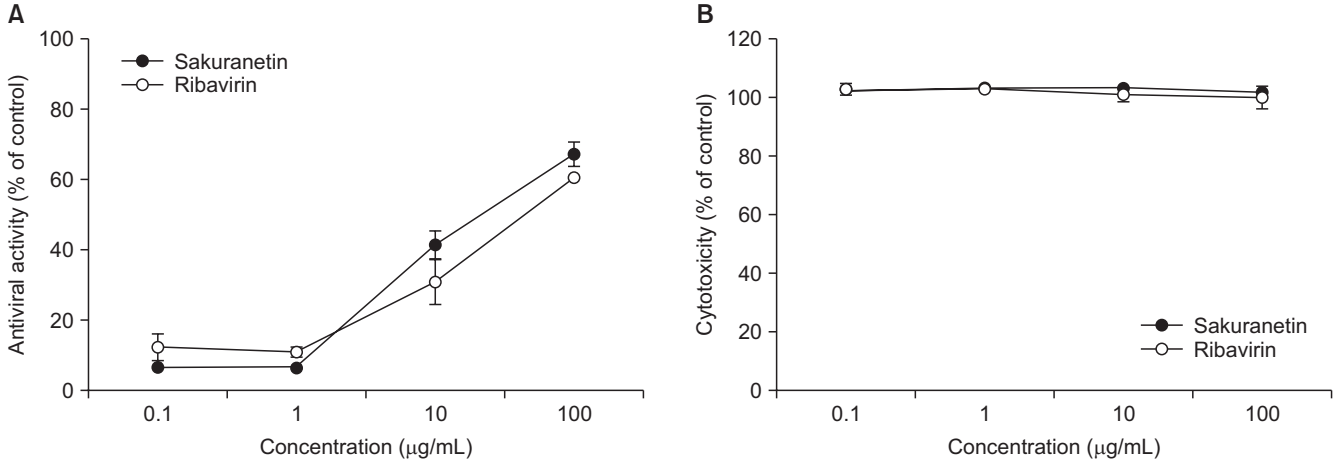

Figure 2. Antiviral activity of sakuranetin against human rhinovirus (HRV) 3 in human epithelioid carcinoma cervix (HeLa) cells. Antiviral activity of the three extracts against HRV3 in HeLa cells is shown; (A) Antiviral activity of sakuranetin against HRV3 in HeLa cells. (B) Cytotoxicity of sakuranetin in HeLa cells. The diluted virus suspension, containing 30 mM $MgCl_2$, 1% fetal bovine serum, and 50% tissue culture infective dose of the virus, was added to HeLa cells to produce the appropriate cytopathic effect (CPE) within 48 hours after infection. The antiviral activity and cytotoxicity of sakuranetin were investigated through the sulforhodamine B (SRB) assay with CPE reduction. Results are presented as the mean percentage values obtained from three independent experiments performed in triplicate ± standard deviation.

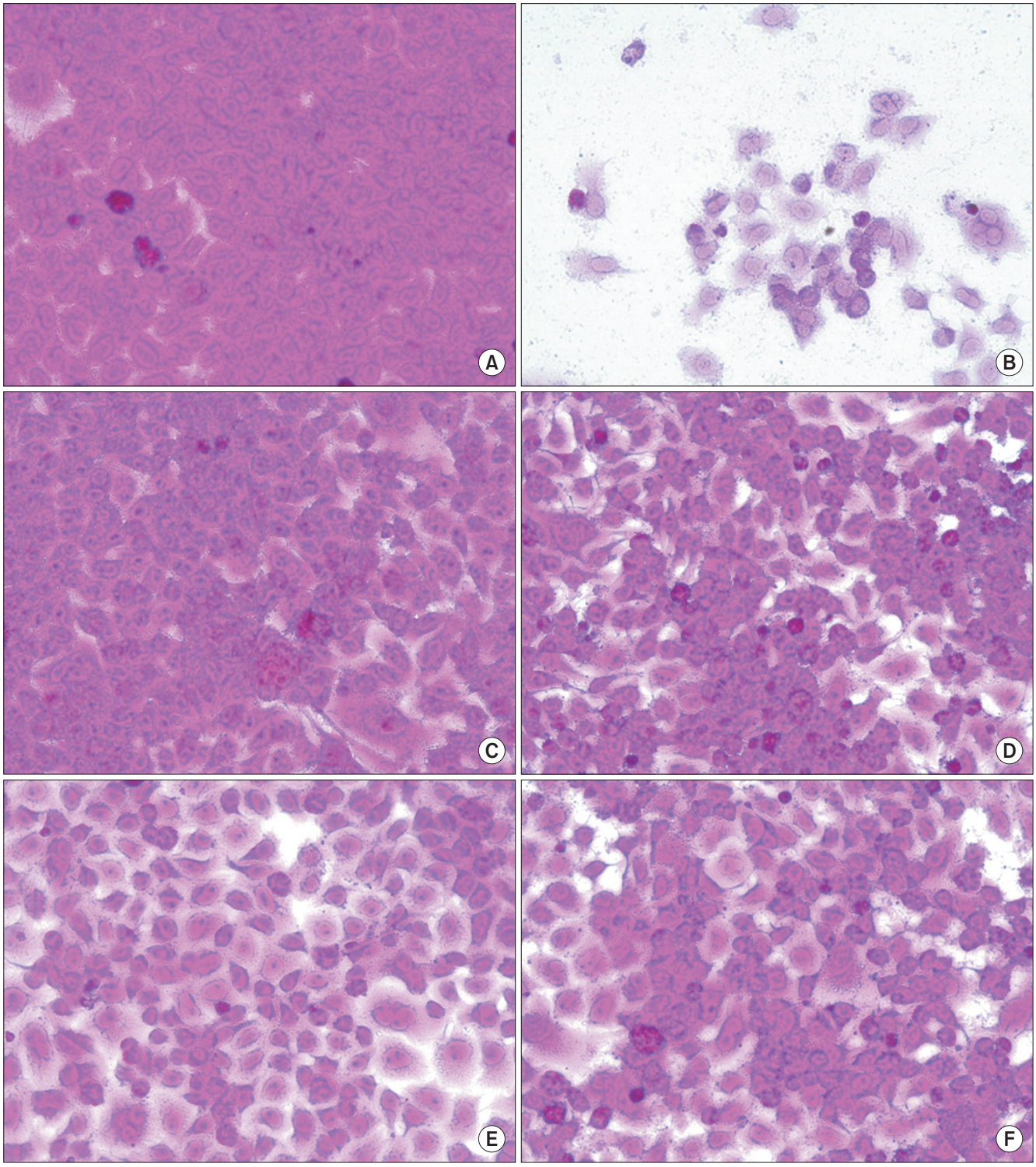

Figure 3. The effect of sakuranetin on human rhinovirus (HRV) 3-induced cytopathic effect (CPE). The effects of sakuranetin on HRV3-induced CPE are shown. Culture medium in 96-well tissue culture plates was aspirated and the cells were washed with phosphate buffered saline. Thereafter, 0.09 mL of the diluted virus suspension, containing 30 mM $MgCl_2$, 1% fetal bovine serum, and 50% tissue culture infective dose of the virus and 0.01 mL of medium were added to human epithelioid carcinoma cervix (HeLa) cells to produce the appropriate CPE within 48 hours after infection, and then sakuranetin or ribavirin (100 μg/mL) was added. After incubation at 32°C and 5% CO_2 for 2 days, the cells stained by SRB, and cellular morphology was studied using photographs taken under a light microscope (×400). (A) Non-infected cells; (B) HRV3-infected cells without sakuranetin or ribavirin treatment; (C) non-infected cells treated with sakuranetin; (D) virus-infected cells treated with sakuranetin; (E) non-infected cells with treated ribavirin; (F) virus-infected cells treated with ribavirin.

cinogens coronary heart disease, bone loss, and many other age-related diseases [22]. Several previous reports have documented that flavonoids possess anti-human immunodeficiency virus (HIV) [23]. Anti-hepatitis B virus activity and antiviral activities of flavonoids have also been observed against several other viruses [24]. Sakuranetin is a flavonoid phytoalexin that serves as a plant antibiotic and exists in *Prunus* and several other plant species [25]. In this study, the anti-HRV3 activity of sakuranetin was evaluated *in vitro*. Sakuranetin exhibited anti-HRV3 activity in the CPE reduction assay.

During the past few years, efforts have been made to increase the number of antiviral agents and a few belong to the class of nucleoside analogs such as ribavirin [26]. A previous study reported that ribavirin inhibited Lassa virus replication [27]. Ribavirin also inhibits Zika virus (ZIKV) replication *in vitro* and suppresses viremia in ZIKV-infected STAT1-deficient mice [28]. Although ribavirin has a high efficacy as an antiviral agent, certain viruses that acquired resistance to ribavirin have been isolated from various virus populations and detected in some patients [29]. In the present study, ribavirin showed antiviral activity in HRV3-infected HeLa cells.

In conclusion, sakuranetin was shown to be effective against HRV3. Further studies are required to understand its antiviral mechanism to develop a novel drug for treating HRV3 infections.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

ACKNOWLEDGMENTS

This study was supported (in part) by Research Funds of Kwangju Women's University in 2017 (KWUI17-033).

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