Osong Public Health Res Perspect 2016 7(6), 400–403
http://dx.doi.org/10.1016/j.phrp.2016.11.003
pISSN 2210-9099 eISSN 2233-6052

Centers for Disease Control & Prevention
Republic of Korea

BRIEF REPORT

Evaluation of Antiviral Activity of *Zanthoxylum* Species Against Picornaviruses

Hwa-Jung Choi*

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

Received: September 6, 2016
Revised: November 3, 2016
Accepted: November 8, 2016

KEYWORDS:
antiviral,
enterovirus,
human rhinovirus,
picornavirus,
Zanthoxylum

Abstract
Human rhinoviruses and enteroviruses (family Picornaviridae) infect millions of people worldwide each year, but little is known about effective therapeutical treatment for the infection caused by these viruses. We sought to determine whether or not *Zanthoxylum* (Rutaceae) species can exhibit antiviral activity against picornaviruses. The leaf parts of four *Zanthoxylum* species were extracted with methanol, and the extracts were investigated for their antiviral activity against picornaviruses using cytopathic effects by cytopathic effect reduction. Leaf extracts of *Zanthoxylum piperitum* among four *Zanthoxylum* species were found to possess only broad-spectrum antipicornavirus activity against human rhninovirus 2 with a 50% inhibitory concentration (IC_{50}) value of 59.48 μg/mL, human rhinovirus 3 with an IC_{50} value of 39.94 μg/mL, coxsackie A16 virus with an IC_{50} value of 45.80 μg/mL, coxsackie B3 virus with an IC_{50} value of 68.53 μg/mL, coxsackie B4 virus with an IC_{50} value of 93.58 μg/mL, and enterovirus 71 virus with an IC_{50} value of 4.48 μg/mL. However, ribavirin did not possess antiviral activity against human rhinovirus 3 and four enteroviruses. Therefore, leaves of *Z. piperitum* showed broad-spectrum antipicornavirus activity, and may be useful as a candidate for studying picornavirus agents and development of pharmaceuticals.

1. Introduction

Human rhinoviruses (HRVs) belong, together with enteroviruses, to the family Picornaviridae, and cause a wide variety of diseases in humans and animals [1]. Infections with HRVs lead to the common cold with symptoms such as sore throat, rhinitis, nasal congestion, and cough [2]. HRVs also lead to severe respiratory tract illnesses in children, immunosuppressed patients, and the elderly [3,4]. Most enterovirus infections are asymptomatic or result in only mild illness, but enteroviruses can also cause a wide variety of clinical illnesses, including acute hemorrhagic conjunctivitis, aseptic meningitis, undifferentiated rash, acute flaccid paralysis, myocarditis, and neonatal sepsis-like disease [5]. Curing virus infections harbors an enormous economic potential, and the search for new antiviral substances is of great interest for worldwide health. Despite significant efforts, no antiviral agent is approved for the prevention or treatment of HRV or enterovirus infection.

Zanthoxylum (Rutaceae) species has been used for centuries as a source of spices in Asian cuisine and traditional Asian medicine [6–8]. In a previous study, leaf extracts of *Zanthoxylum piperitum* were shown to possess antiviral activities against influenza A/WS/33, A/PR/8, and B/Lee/40 viruses [9]. In this study, we aimed to identify the

*Corresponding author.
E-mail: rerived@empal.com

antiviral activity of *Zanthoxylum* species against two HRVs (HRV2 and HRV3) or four enteroviruses (coxsackie A16, B3, and B4 viruses, and human enterovirus 71).

2. Materials and methods

Leaf parts from two *Zanthoxylum* species (*Z. piperitum* and *Zanthoxylum schinifolium*) were collected from Mt. Gwanggyo (Suwon, Korea), and another two *Zanthoxylum* species (*Zanthoxylum coreanum* and *Zanthoxylum planispinum*) were collected from National Institute of Forest Science, Seoul. Voucher specimens have been identified by Soon-Il Lee (School of Agricultural Biotechnology, Seoul National University, Seoul) and deposited in the herbarium of the School of Agricultural Biotechnology, Seoul National University [*Z. piperitum* (ZP) leaves: ZP3; *Z. schinifolium* (ZS) leaves: ZS2; *Z. coreanum* (ZC) leaves: ZC1; *Z. planispinum* (ZPS) ZP leaves: ZP4]. They were air dried at room temperature and pulverized. Each 100-g sample of the specimen plants was extracted twice with 600 mL of methanol at room temperature for 3 days and filtered (Whatman No. 2). The combined filtrate was concentrated to dryness by rotary evaporation at 40°C. Each extract was solubilized in dimethyl sulfoxide at a concentration of 100 μg/mL and stored at −20°C.

HRV2 and HRV3 were provided by American Type Culture Collection (Manassas, VA, USA) and were propagated in human epitheloid carcinoma cervix (HeLa) cells at 32°C. Coxsackie A16, coxsackie B3, and coxsackie B4 viruses, and human enterovirus 71 (EV71) were obtained from Chungcheongnam-Do Health and Environment Research Institute in Korea, and were propagated in African green monkey kidney (Vero) cells at 37°C. HeLa or Vero cells were maintained in minimal essential medium supplemented with 10% fetal bovine serum and 0.01% antibiotic−antimycotic. Antibiotic−antimycotic, trypsin−EDTA, fetal bovine serum, and minimal essential medium were supplied by 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 from Sigma-Aldrich (St. Louis, MO, USA). Oseltamivir (F. Hofmann-La Roche Ltd, Basel, Switzerland) was purchased from a pharmacy in Korea as prescribed by a medical doctor. All other chemicals were of reagent grade.

Assays of antiviral activity and cytotoxicity were evaluated by the SRB method using cytopathic effect reduction, already reported [10]. Briefly, 1 day prior to infection, Vero or HeLa cells were seeded onto a 96-well culture plate at a concentration of 2×10^4 cells/well. The following day, the culture medium was removed and cells were washed with phosphate-buffered saline. The infectivity of each virus was determined by the SRB method monitoring the cytopathic effect, allowing for the percentage of cell viability to be determined. Based on the mammalian cell viability determined for each virus, 0.09 mL of diluted virus suspension containing 50% cell culture infective dose of virus stock was added to mammalian cells. This dose was selected to produce the appropriate cytopathic effects 48 hours after infection. For compound treatments, 0.01 mL of the medium containing the selected concentration of the compound was added to the cells. The antiviral activity of each test material was determined using a 10-fold diluted concentration range of 0.1−100 μg/mL. Four wells were used as virus controls (virus-infected, nondrug-treated cells), and four wells were used as cell controls (noninfected, nondrug-treated cells). Culture plates were incubated at 37°C in 5% CO_2 for 48 hours. After washing once with phosphate-buffered saline, 100 mL of cold (−20°C) 70% (v/v) acetone was added to each well and left for 30 minutes at −20°C. The acetone was removed from cells, after which 96-well plates were left to dry in an oven at 60°C for 30 minutes. Then, 100 mL of 0.4% (w/v) SRB in 1% acetic acid (v/v) was added to each well and incubated at room temperature for 30 minutes. Unbound SRB was removed by washing the plates five times with 1% acetic acid (v/v), and the plates were then left to dry in an oven. After drying for 1 day, fixed SRB in wells was solubilized with 100 mL of unbuffered Tris-base solution (10mM), and plates were incubated at room temperature for 30 minutes. Absorbance in each well was read at 540 nm using a VERSAmax microplate reader (Molecular Devices, Palo Alto, CA, USA) and a reference absorbance of 620 nm. Ribavirin was used as a positive and dimethyl sulfoxide as a negative control. To calculate the 50% inhibitory concentration (IC_{50}) values, the results were transformed to percentage of controls and the IC_{50} values were graphically obtained from the dose−response curves. The percent protection achieved by the test compound in virus-infected cells was calculated by the following formula:

$$[(\mathrm{ODt})\mathrm{virus} - (\mathrm{ODc})\mathrm{virus}]/[(\mathrm{ODc})\mathrm{mock} - (\mathrm{ODc})\mathrm{virus}] \times 100(\mathrm{expressedin\%})$$

where (ODt)virus is the optical density measured with a given concentration of the test compound in virus-infected cells, (ODc)virus is the optical density measured for the control untreated virus infected cells, and (ODc)mock is the optical density measured for the control untreated mock-infected cells. The concentration achieving 50% protection according to the above formula was defined as the IC_{50}. The therapeutic index was defined as CC_{50}/IC_{50}.

3. Results

Leaf parts of four *Zanthoxylum* species were investigated for its antiviral activity against picornaviruses

(HRV2, HRV3, coxsackie A16, coxsackie B3, coxsackie B4, and EV71). *Z. schinifolium* and *Z. planispinum*, from the tested crude extracts, were active against HRV2. Their IC_{50} values were 47.05 μg/mL and 66.55 μg/mL, respectively, and their therapeutic index values were 4−5. In addition, *Z. planispinum* showed strong antiviral activity against HRV3, with an IC_{50} value of 29.58 μg/mL. *Z. piperitum* showed moderate anti-HRV2 and anti-HRV3 activities. *Z. piperitum* showed broad anti-coxsackie A16, anti-coxsackie B3, anti-coxsackie B4, and anti-EV71 activities, with IC_{50} values of 4.48−93.58 μg/mL. Anti-coxsackie B3 activity with an IC_{50} value of 6.20 μg/mL was exhibited by *Z. coreanum*. Furthermore, *Z. piperitum*, *Z. schinifolium*, *Z. planispinum*, and *Z. coreanum* possessed strong antiviral activity against EV71, with IC_{50} values ranging from <0.1 μg/mL to 56.05 μg/mL (Tables 1 and 2).

Cytotoxicity of each extract was evaluated in parallel with antiviral activity evaluation. As a result, *Z. planispinum* among the above extracts showing antiviral activity was slightly toxic to HeLa cells, with a CC_{50} value of 224.70 μg/mL. *Z. planispinum* among them was also slightly toxic to Vero cells, with a CC_{50} value of 345.35 μg/mL. Its therapeutic index is shown in Table 1.

4. Discussion

In this study, ribavirin showed weak antiviral activity in HeLa cells infected with HRV2, but did not possess antiviral activity against HRV3 and four enteroviruses. Therefore, we were able to ascertain that ribavirin possesses some antiviral properties, although it is strongly influenced by the strain of virus tested.

Previous studies of rhinovirus capsid-binding compounds tested against serotype HRVs revealed the existence of another serotype HRVs, based on differential susceptibility to antiviral compounds [11]. In 2002, the U.S. Food and Drug Administration did not approve pleconaril for the treatment of the common cold, as the panel remained unconvinced about the drug's safety profile [12]. Medicinal plants are increasingly being pursued as suitable alternative sources for discovery of antiviral agents [13,14]. It has been reported that *Z. piperitum* (leaves) possesses strong antiviral activity against human influenza virus [15]. *Z. schinifolium* (barks and stems) has been reported to exhibit anti-hepatitis B virus activity [16]. However, until now, no effective treatment has shown broad-spectrum antiviral activity. In this study, *Z. piperitum* showed broad-spectrum antiviral activity against two HRVs and four

Table 1. Antiviral activity of *Zanthoxylum* species against HRV2 and HRV3.

		HRV2		HRV3	
Plant species	CC_{50}*	IC_{50}†	TI‡	IC_{50}†	TI‡
Z. piperitum	> 100	59.48 ± 5.01	> 1.68	39.94 ± 0.27	> 2.5
Z. schinifolium	202.30	47.05 ± 18.02	4.3	47.48 ± 7.18	4.26
Z. coreanum	> 100	ND§	—	ND§	—
Z. planispinum	345.35	66.55 ± 8.52	5.19	29.58 ± 7.58	11.68
Ribavirin	> 100	21.74 ± 1.53	> 4.6	42.21 ± 9.21	> 2.37

*The 50% cytotoxic concentration for Hela cells in μg/mL; †Concentration of compound in μg/mL producing 50% inhibition of virus-induced cytopathic effects; ‡Therapeutic index = CC_{50}/IC_{50}; §IC_{50} value within the concentration of the compound to be tested not determined due to maximum inhibition rate under 50%. Results are presented as the mean IC_{50} values obtained from three independent experiments carried out in triplicate ± SD. HRV = human rhinovirus; IC_{50} = 50% inhibition concentration; ND = not determined; SD = standard deviation; TI = therapeutic index.

Table 2. Antiviral activity of *Zanthoxylum* species against enteroviruses.

		CA16		CB3		CB4		EV71	
Plant species	CC_{50}*	IC_{50}†	TI‡	IC_{50}†	TI‡	IC_{50}†	TI‡	IC_{50}†	TI‡
Z. piperitum	> 100	45.80 ± 2.45	> 2.18	68.53 ± 4.72	> 1.46	93.58 ± 2.74	> 1.07	4.48 ± 0.90	> 22.35
Z. schinifolium	> 100	ND§	—	ND§	—	70.02 ± 4.74	> 1.43	< 0.1	> 1,000
Z. coreanum	> 100	ND§	—	6.20 ± 0.70	> 16.13	ND§	—	< 0.1	> 1,000
Z. planispinum	224.70	ND§	—	39.87 ± 8.73	5.64	75.70 ± 6.34	2.97	56.05 ± 4.50	4.01
Ribavirin	191.64	ND§	—	ND§	—	ND§	—	ND§	—

*The 50% cytotoxic concentration for Vero cells in μg/mL; †Concetration of compound in μg/mL producing 50% inhibiton of virus-induced cytopathic effects; ‡Therapeutic index = CC_{50}/IC_{50}; §IC_{50} value within the concentration of the compound to be tested not determined due to maximum inhibition rate under 50%. Results are presented as the mean IC_{50} values obtained from three independent experiments carried out in triplicate ± SD. CA16 = coxsackie A16; CB3 = coxsackie B3; CB4 = coxsackie B4; EV71 = human enterovirus 71; HRV = human rhinovirus; IC_{50} = 50% inhibition concentration; ND = not determined; SD = standard deviation; TI = therapeutic index.

enteroviruses, with differences in inhibitory efficacy among the strains. Therefore, further studies on the isolation of antiviral compounds from *Z. piperitum* are necessary.

Conflicts of interest

The authors report no conflicts of interest.

Acknowledgments

This study was supported (in part) by Research Funds of Kwangju Women's University (Gwangju, Korea) in 2016 (KWUI16-078).

References

1. Makela MJ, Puhakka T, Ruuskanen O, et al. Viruses and bacteria in the etiology of the common cold. J Clin Microbiol 1998 Feb; 36(2):539–42.
2. Whitton JL, Cornell CT, Feuer R. Host and virus determinants of picornavirus pathogenesis and tropism. Nat Rev Microbiol 2005 Oct;3(10):765–76.
3. Imakita M, Shiraki K, Yutani C, et al. Pneumonia caused by rhinovirus. Clin Infect Dis 2000 Mar;30(3):611–2.
4. Gutman JA, Peck AJ, Kuypers J, et al. Rhinovirus as a cause of fatal lower respiratory tract infection in adult stem cell transplantation patients: a report of two cases. Bone Marrow Transplant 2007 Oct;40(8):809–11.
5. Renwick N, Schweiger B, Kapoor V, et al. A recently identified rhinovirus genotype is associated with severe respiratory-tract infection in children in Germany. J Infect Dis 2007 Dec; 196(12):1754–60.
6. Bryant BP, Mezine I. Alkylamides that produce tingling paresthesia activate tactile and thermal trigeminal neurons. Brain Res 1999 Sep;842(2):452–60.
7. Shibata CI, Sasaki H, Naito TU, et al. The herbal medicine Dai-Kenchu-Tou stimulates upper gut motility though cholinergic and 5-hydroxytryptamine 3 receptors in conscious dogs. Surgery 1999 Nov;126(5):918–24.
8. Cho MG, Chang CS, Chae YA. Variation of volatile composition in the leaf of *Zanthoxylum schinofolium* siebold et zucc. and *Zanthoxylum piperitum* DC. Korean J Med Crop Sci 2002 Sep; 10(3):162–6.
9. Choi HJ, Song JH, Kwon DH, et al. Antiviral activity of *Zanthoxylum* species against influenza virus. Korean J Med Crop Sci 2008 Jul;16(4):273–8.
10. Choi HJ, Kim JH, Lee CH, et al. Antiviral activity of quercetin 7-rhamnoside against porcine epidemic diarrhea virus. Antiviral Res 2009 Jan;81(1):77–81.
11. Andries K, Dewindt B, Snoeks J, et al. A comparative test of fifteen compounds against all known human rhinovirus serotypes as a basis for a more rational screening program. Antiviral Res 1991 Oct;16(3):213–25.
12. Senior K. FDA panel rejects common cold treatment. Lancet Infect Dis 2002 May;2(5):264.
13. Cowan MM. Plant products as antimicrobial agents. Clin Microbiol Rev 1999 Oct;12(4):564–82.
14. Jassim SA, Naji MA. Novel antiviral agents: a medicinal plant perspective. J Appl Microbiol 2003 Sep;95(3):412–27.
15. Ha SY, Youn H, Song CS, et al. Antiviral effect of flavonol glycosides isolated from the leaf of *Zanthoxylum piperitum* on influenza virus. J Microbiol 2014 Apr;52(4):340–4.
16. Chen IS, Lin YC, Tsai IL, et al. Coumarins and anti-HBV constituents from *Zanthoxylum schinifolium*. Phytochemistry 1997 Aug;45(7):1419–22.