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BRIEF REPORT

Evaluation of Antiviral Activity of *Zanthoxylum* Species Against Picornaviruses

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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

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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