Identification of anti-HIV and anti-Reverse Transcriptase activity from *Tetracera scandens*

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We report here that an ethanol extract of *Tetracera scandens*, a Vietnamese medicinal plant, has anti-HIV activity and possesses strong inhibitory activity against HIV-1 reverse transcriptase (RTase). Using a MT-4 cell-based assay, we found that the *T. scandens* extract inhibited effectively HIV virus replication with an IC₅₀ value in the range of 2.0-2.5 μg/ml while the cellular toxicity value (CC₅₀) was more than 40-50 μg/ml concentration, thus yielding a minimum specificity index of 20-fold. Moreover, the anti-HIV efficacy of the *T. scandens* extract was determined to be due, in part, to its potent inhibitory activity against HIV-1 RTase activity in vitro. The inhibitory activity against the RTase was further confirmed by probing viral cDNA production, an intermediate of viral reverse transcription, in virus-infected cells using quantitative DNA-PCR analysis. Thus, these results suggest that *T. scandens* can be a useful source for the isolation and development of new anti-HIV-1 inhibitor(s). [BMB reports 2012; 45(3): 165-170]

INTRODUCTION

Human Immunodeficiency Virus type 1 (HIV-1) is the etiological agent of Acquired Immune Deficiency Syndrome (AIDS). Currently, therapeutic treatment of AIDS has mainly relied on the four types of anti-HIV/AIDS drugs: the viral reverse transcriptase (RTase) inhibitors that include nucleoside and non-nucleoside RTase inhibitors (1, 2), protease inhibitors (3), integrase inhibitors (4), and entry inhibitors (5). However, as the current drugs encounter problems such as the emergence of drug-resistant viruses and unexpected side effects, the development of new antiviral agents is being developed such as attachment inhibitors (6), a virion maturation inhibitor (7, 8), and CCR5 inhibitors (9). An alternative effort to solve these problems has also been to actively seek novel antiviral agents from various natural sources such as traditionally used medicinal herbs and plants (10). Some examples include betulinic acid from *Syzygium claviformum* (11, 12), various calanolides from *Calophyllum langerium* (13, 14), and geraniin from *Phyllanthus amarus* (15).

*Tetracera* is a genus of flowering plants of the Dilleniaceae family, which includes about 50 species. Among them, *Tetracera scandens* (*T. scandens*) is a traditional Vietnamese medicinal plant originating in the Quang Ninh province in Vietnam (16). *T. scandens* extract has been previously known to exhibit therapeutic activities against inflammation, hepatitis, and gout. A methanol extract of a branch of *T. scandens* stimulates glucose-uptake (17) and has anti-hyperglycemic activity, showing its potential for the treatment of type 2 diabetes mellitus (18). It has also been shown to have significant inhibitory activity against xanthine oxidase (19), an enzyme involved in purine metabolism, which has been a clinical target for the treatment of hyperuricemia and related medical conditions including gout. In addition, a ketone extract of *Tetracera boiviniana* was shown to exhibit DNA polymerase-β inhibitory activity (20).

Here, we report for the first time that an ethanol extract of *T. scandens* has anti-HIV activity and possesses strong inhibitory activity against HIV-1 RTase, as verified using a cell-based anti-viral assay as well as *in vitro* assays. These results suggest that an ethanol extract of *T. scandens* might be a new source for developing new types of anti-HIV-1 inhibitors and drugs for the treatment of HIV/AIDS.

RESULTS AND DISCUSSION

Identification of anti-HIV effects of *T. scandens* extract in a cell-based assay

To analyze the anti-HIV efficacy of a 70% ethanol extract of *T. scandens*, we first determined the cellular toxicity of the compound against MT-4 cells, which is the same cell-type used for the antiviral efficacy test described below. MT-4 cells were treated with serial 10-fold dilutions of the *T. scandens* extract from 400 μg/ml to 0.4 μg/ml and incubated at 37°C for 3 days. Following incubation, cell viability was then measured for each concentration using a cell titer GLO assay that de-
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Fig. 1. Determination of cellular toxicity of the T. scandens extract. Cellular toxicity of the T. scandens extract was determined using 1 x 10^5 MT-4 cells as described in Materials and Methods. All assays were performed in triplicate and a statistically significant reductions in MT-4 cell viability by the T. scandens extract is denoted as ***(Student’s t-test, P < 0.001) and ***(P < 0.0001).

Having established the CC50 value, we then proceeded to determine the anti-HIV efficacy of the T. scandens extract by employing various final concentrations at which no cellular toxicity was observed. To this end, MT-4 cells were infected with 20,000 pg of virus in the presence or absence of serially diluted T. scandens extract. 3'-Azido-3'-deoxy-thymidine (AZT) was employed as a positive control throughout the assay. The antiviral efficacy of the extract was determined by the following three ways: 1) First, we examined the level of EGFP expression using a fluorescence microscope, because the HIV-1 gene in place of the viral Nef gene, and the level of EGFP expression qualitatively indicates the degree of viral replication efficiency and easily reveals whether the compound inhibits viral replication within the cells. 2) Secondly, we measured quantitatively the number of virus particles produced in the cell medium in the presence or absence of the compound using an HIV-1 p24 antigen ELISA kit. 3) Thirdly, re-infecting fresh MT-4 cells with equal volumes of each medium from the above MT-4 cell cultures, which were either treated with the compound or left untreated, to further confirm the accuracy of the p24 antigen measurement for determining differences in the amounts of viruses produced in the presence or absence of the compound. Treatment of MT-4 cells with T. scandens extract during the course of HIV-1 virus infection resulted in a dose-dependent inhibition of virus production as shown in Fig. 2C. The half maximal inhibition concentration (IC50) of the T. scandens extract was found to be in the range of 2.0-2.5 μg/ml, yielding a therapeutic index of at least 20-fold, even with this crude form of the extract.

Fig. 2A shows a typical anti-HIV test result illustrating the strong anti-HIV activities of various concentrations of the compound. The results showed that while treatment with 1 nM AZT, a positive control, inhibited up to 50% of virus production compared to that of DMSO only as determined by the HIV-1 p24 antigen ELISA, treatments with 0.4 and 4.0 μg/ml of T. scandens extract resulted in 40% and 87% inhibition of virus production, respectively, as determined by the HIV-1 p24 antigen ELISA. The level of EGFP expression, another indicator of viral replication efficiency, was also well correlated with the level of inhibition
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Fig. 3. *T. scandens* extract inhibits strongly HIV-1 RTase activity in vitro (A-E). The effect of RTase activity was determined using HIV-1 RT as described in Materials and Methods. The levels of inhibition of virus production were measured and expressed as a percentage of the DMSO control. The compounds and the concentrations used are indicated. All assays were performed at least in triplicate and standard deviations are shown.
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**MATERIALS AND METHODS**

**Cell culture**

293FT and MT-4 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Hyclone, USA) and RPMI-1640 medium (Hyclone), respectively, with 10% FBS and 1% penicillin/streptomycin at 37°C in a humidified 5% CO2 atmosphere.

**Production of virus**

pNL4-3EGFP plasmid, a recombinant HIV-1 proviral molecular clone that expresses EGFP in place of the Nef protein, was used to produce HIV-1 virus. Typically, 293FT cells (2 x 10⁶) were seeded in a 6-well plate 1 day before transfection and then transfected with 2 μg pNL4-3EGFP plasmid using Lipofectamine2000™ (Invitrogen, USA) in 250 μl Opti-MEM (GIBCO, USA) according to the manufacturer’s protocol. After 48 h, the resulting supernatant containing the virus was harvested and filtered through a 0.45 μm pore size filter. To prepare a high concentration of stock virus, 1 ml of the final concentration (200,000 pg/ml) of the virus obtained from 293FT cells was infected into 1 ml of 1 x 10⁶ MT-4 cells in a 6-well plate and incubated for 72 h at 37°C in a humidified 5% CO2 incubator 311 Series (Thermo Scientific, USA). The resulting culture supernatants were harvested in a 50 ml conical tube by centrifugation at 1,500 rpm (Hanil Co., Korea) for 3 min, filtered through a 0.45 μm pore size filter, aliquoted into 1.5 ml Microcentrifuge tubes (SPL Co., Korea), and stored at −80°C until use.

**Compound preparation**

A powder form of the T. scandens extract as a concentrated and dried form of a 70% ethanol extracts of T. scandens leaves (total dry weight 200 g) was prepared and provided by Dr. Joo-Hwan Kim at Kyungwon University in Korea. The dried T. scandens extract compound (40 mg) was then dissolved in 500 μl DMSO (Sigma Chemical Co., USA), AZT (Sigma Chemical Co.), AZTTP (GeneCraft Co., USA), and Efavirenz and Etravirine (Toronto Research Chemicals, Canada) were prepared as 20 μM stocks in DMSO. 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was also purchased from Sigma Chemical Co.

**Cell cytotoxicity test**

Cell cytotoxicity was measured with a Cell Titer-Glo assay kit (Promega Co., USA). MT-4 cells (1 x 10⁵ cells) were seeded in a 96-well plate (Nunc Co., Denmark) with 50 μl of serial dilutions of the compound in each well and placed at 37°C in a 5% CO2 incubator for 3 days. At the end of incubation, the luminescence of each well was read with a Spectrofluorometer (Molecular Devices, USA) according to the manufacturer’s protocol.

In the antiviral assay, cell toxicity was determined with an MTT assay accordingly to the manufacturer’s protocol. The MT-4 cells used in the antiviral assay as described below were harvested by centrifugation at 6,000 rpm (Hanil Co.) for 3 min in 1.5 ml microcentrifuge tubes (SPL Co.), Cell pellets were re-suspended in 350 μl RPMI and then split into three aliquots (100 μl each) in a 96-well plate. MTT solution (5 mg/ml) was then added, and cells were incubated at 37°C in a humidified 5% CO2 incubator for 4 h. After incubation, 100 μl stop solution (0.04 N HCl) was added and the absorbance of each well was read in a Molecular Device ELISA reader at 570 nm according to the manufacturer’s instructions.

**Cell-based antiviral assay**

MT-4 cells (2 x 10⁵ cells) were seeded in a 48-well plate and infected with 20,000 pg of HIV-1 virus stock in the presence and absence of compounds in a total volume of 0.4 ml and in...
cubated at 37°C in a humidified 5% CO₂ incubator for 72 h. Cells were harvested by centrifugation at 6,000 rpm (Hanil Co.) for 3 min in 1.5 ml microcentrifuge tubes (SPL Co.) and re-suspended in 350 μl RPMI. Thirty microliters of the RPMI resuspension was then removed and analyzed for EGFP signals as well as cell morphology using a Fluorescence Inverted Microscope IX-71 (Olympus, Japan). Simultaneously, the resulting supernatants were used to measure virus production by each of the compounds using a p24 ELISA assay according to the manufacturer’s protocol described below. For the same volume infection assay, 50 μl of each virus supernatant was added to fresh MT-4 cells (1.5 x 10⁵ cells) and incubated at 37°C in a humidified 5% CO₂ incubator for 48 h. The resulting EGFP signals and cell morphologies were then analyzed using an inverted fluorescence microscope (Olympus Co.). The fluorescence from infected cell after 72 h was also detected in a 48-well plate with the fluorescence inverted microscope (exposure time: 10 ms).

**HIV-1 p24 antigen ELISA assay**

The number of viral particles in cell culture supernatants was measured as follows. Harvested viral supernatants as described above were serially diluted 10-fold with RPMI media. Diluted viral supernatants were added to the 96-well plate included in the HIV-1 p24 Antigen Capture Assay kit (Advanced BioScience Laboratories, USA) and the number of virus particles was analyzed according to the manufacturer’s instructions.

**Quantitative DNA-PCR**

The viral DNA level produced by infected MT-4 cells was determined using quantitative DNA-PCR. MT-4 cells (5 x 10⁵) were infected with 100,000 pg of virus particles in the presence and absence of each compound in a total volume of 1 ml in a 48-well plate. After 24 h incubation at 37°C in a humidified 5% CO₂ incubator, the total cellular DNA was extracted from the infected cells using a DNAeasy mini kit (Qiagen Co., USA). Quantitative DNA-PCR was then performed using a Light Cycler 480 (Roche Co., USA) and the SYBR Green I Master mix (Roche Co.) with the following HIV-1 specific LTR primers (Forward: 5’-GATCTGGAGCAGTTGCAGCTCTC-3’, Reverse: 5’-CCTTTAGCGTTCAAGTCCCTGTTC-3’) as described previously (21). For the PCR reaction, 1 μl of the extracted DNA was added to 5 μl of the SYBR Green I Master mix supplemented with 0.5 pmol of each LTR PCR primers. DNA copy numbers were calculated by interpolation from a standard curve of 10-fold serially diluted pNL4-3EGFP plasmid DNA determined using the Light Cycler 480 software provided by the manufacturer.

**In vitro Reverse transcriptase assay**

RTase activity was determined with an Enzchek® Reverse transcriptase-ase kit (Invitrogen Co.) with a purified recombinant HIV RTase (Ambion Co., USA) according to the manufacturer’s instructions. Briefly, 1 μl of serially diluted T. scandens extract was added to a reverse transcription reaction mixture containing HIV RTase (3 units) and long poly (A) templates primed with oligo-dT primers in a 25 μl reaction volume in a 96-well plate and incubated at room temperature for 1 h. The resulting DNA heteroduplexes generated in each well were then detected and quantitatively measured using the PicoGreen reagent (173 μl) provided in the kit using a spectrofluorometer GEMINI EM system (Molecular Devices) at an excitation wavelength of 480 nm and an emission wavelength of 520 nm.

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