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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 IC_{50} 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 type 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, new types of antiviral inhibitors are 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 claviflorum* (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 antiviral 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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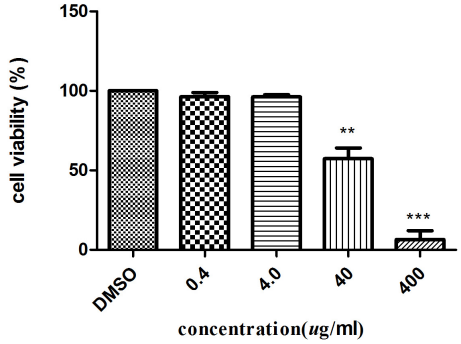

Fig. 1. Determination of cellular toxicity of the *T. scandens* extract. Cellular toxicity of the *T. scandens* extract was determined using 1 × 10^4 MT-4 cells as described in Materials and Methods. All assays were performed in triplicate and a statistically significant reductions in MT-4 cell viability by the *T. scandens* extract is denoted as **(Student's *t*-test, P < 0.01) and ***(P < 0.001).

termines the number of viable cells in culture based on the quantification of ATP produced compared to cells incubated in the absence of the compound (in this case, 0.5% dimethyl sulfoxide, DMSO alone). The assay result showed that *T. scandens* extract showed no cellular toxicity at concentrations up to 4 μg/ml, whereas higher concentrations produced cellular toxicity in a dose-dependent manner, generating nearly 90% cell killing at a final concentration 400 μg/ml. From this dose-response curve, the 50% cell cytotoxicity concentration (CC_{50}) of the *T. scandens* extract was estimated to be around 40 μg/ml (Fig. 1).

Having established the CC_{50} 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-thyminine (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 virus used in this assay harbors a *EGFP* 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

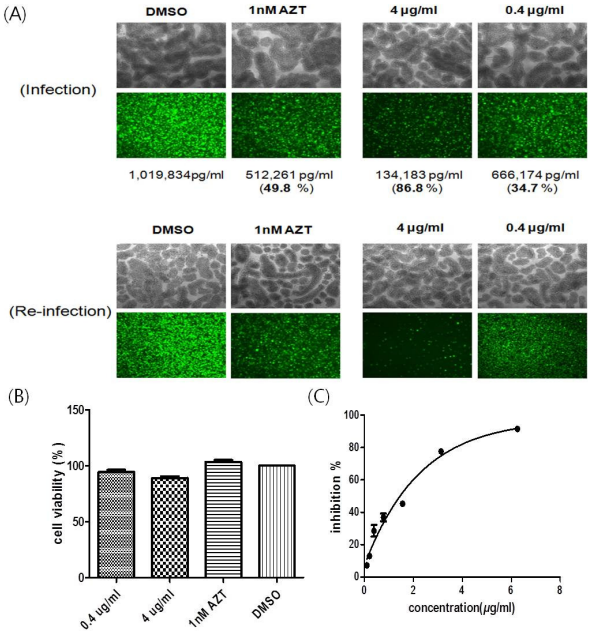

Fig. 2. Determination of the anti-HIV effect of the *T. scandens* extract in the MT-4 cell-based assay. (A) The anti-HIV efficacy of the *T. scandens* extract was determined as described in Materials and Methods. Phase-contrast microscopy and fluorescence microscopy images of infection (upper panel) and reinfection (lower panel) are shown. The values of HIV-1 p24 measurement are also shown in the upper panel, with percent inhibition values, compared to that of DMSO only, indicated in parentheses. An equal volume of each supernatant from the infection assay was used to reinfect fresh MT-4 cells for the reinfection assay as described in the text. (B) Cell viability during the anti-viral assay from above was measured using the MTT assay. (C) A dose-response curve for the anti-HIV efficacy of the *T. scandens* extract. The concentrations of the *T. scandens* extract used and the percent inhibition of virus production compared to that of DMSO only as determined by the HIV-1 p24 antigen ELISA are shown. The experiments were performed in triplicate and statistical significance was determined using the Student's *t*-test (P < 0.001).

inhibition of virus production as shown in Fig. 2C. The half maximal inhibition concentration (IC_{50}) 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 treatment with DMSO alone (5 nM AZT inhibited nearly 90% of virus production (data not shown)), 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

of virus production (see fluorescence data in the upper panel, designated as "infection", in Fig. 2A). Moreover, we also independently measured the viability of cells in this assay using another MTT assay to further verify that the inhibition of virus production in the presence of the compound during the infection period was not a result of cell death. No quantitative difference in cellular viability of MT-4 cells was observed in all cases as shown in Fig. 1B and a similar observation was also observed qualitatively with phase-contrast microscopy (first row of the upper panel in Fig. 2A). Finally, to further confirm the accuracy of the measurement of HIV-1 p24 viral antigen, an equal volume (50 μl) of each of cell medium containing different amounts of viral particles was used to reinfect into fresh MT-4 cells for 48 h, in the expectation that a lower number of viruses in the cell culture medium would result in lowered EGFP signals in the second round re-infection experiment. As shown in Fig. 2A (see fluorescence data in the lower panel, designated as "re-infection"), the level of EGFP signal was strongly correlated with the measured p24 values in the viral supernatant samples from cells treated with 1 nM AZT as well as from cells treated with 0.4 and 4.0 μg/ml *T. scandens* extract by which a nearly 90% reduction of EGFP signals was observed when compared to control samples treated with DMSO, thus confirming the accuracy of the viral particle p24 antigen measurements. Thus, these results clearly demonstrate that *T. scandens* has strong anti-HIV activity as determined by this MT-4 cell-based assay.

Determination of anti-RTase activity of the *T. scandens* extract *in vitro*

In an effort to examine the molecular mechanism of the anti-HIV effect by the *T. scandens* extract, we examined if it could inhibit viral RTase activity. Thus, the inhibitory effect of *T. scandens* extract against the activity of HIV-1 RTase was determined using a reverse transcriptase assay kit *in vitro*. For this assay, we employed various types of known RTase inhibitors such as the nucleoside inhibitor (AZTTP), as well as the non-nucleoside inhibitors, Etravirine and Efavirenz, as positive controls. As shown in Fig. 3, the tri-phosphated form of AZT, AZTTP, inhibited HIV-1 RTase in a dose-dependent manner, while nonphosphorylated AZT did not, demonstrating the specificity of this *in vitro* assay system. A similar level of anti-RTase activity was also observed with Etravirine and Efavirenz, respectively. We also observed that HIV-1 RTase was inhibited by *T. scandens* extract in a dose-dependent manner, with an estimated IC_{50} level of 0.7 μg/ml against the HIV-1 RTase *in vitro*. Thus, these results not only confirm that *T. scandens* has anti-HIV efficacy as seen in the cell-based assay, but also indicates that *T. scandens* extract has strong inhibitory activity against the HIV-1 RTase, and it is as potent as known HIV-1 RTase inhibitors.

Determination of anti-RTase activity of the *T. scandens* extract within cells

To further confirm the result of the *in vitro* RTase assay, we further analyzed relative viral cDNA production in the presence or absence of *T. scandens* extract in cells infected with the virus, which is a replication intermediate generated by the viral RTase during viral infection. Thus, 1×10^6 MT-4 cells were infected with HIV-1 virus with or without the extract for 24 h and total cellular DNAs were extracted and subjected to real-time DNA qPCR analysis using HIV-1 long terminal repeat

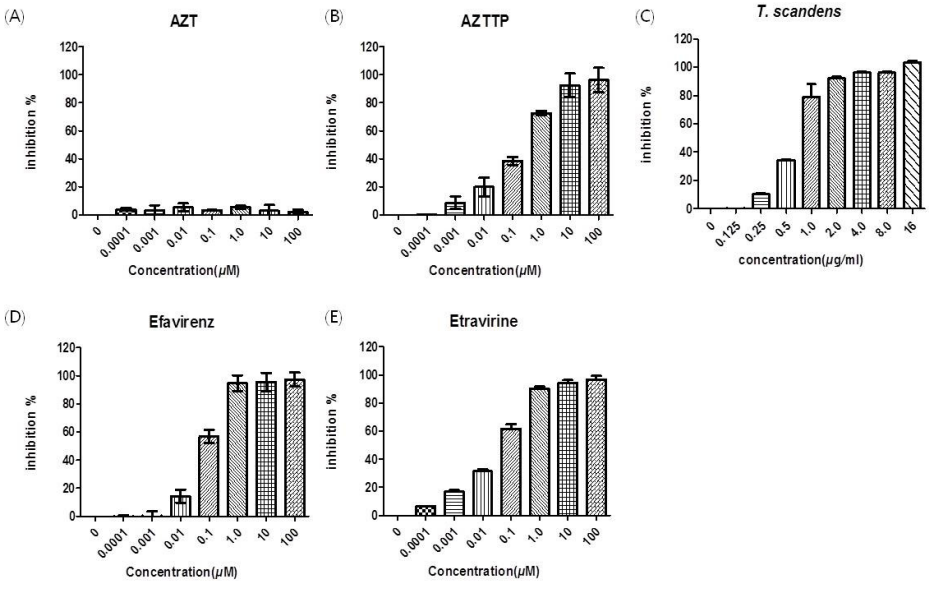

Fig. 3. *T. scandens* extract inhibits strongly HIV-1 RTase activity *in vitro* (A-E). The effect of RTase activity was determined using HIV-1 RT as described in Materials and Methods. The levels of inhibition of virus production were measured and expressed as a percentage of the DMSO control. The compounds and the concentrations used are indicated. All assays were performed at least in triplicate and standard deviations are shown.

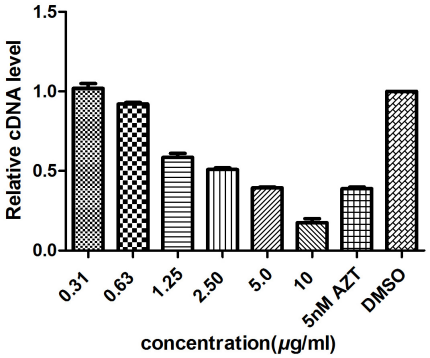

Fig. 4. The efficacy of *T. scandens* extract in inhibiting of HIV-1 viral cDNA production. Inhibition of HIV-1 RTase activity in cells by the *T. scandens* extract was determined by measuring viral cDNA production during viral infection. The relative levels of HIV-1 cDNA production in comparison to DMSO only are shown. AZT (5 nM) was used as a positive control in the assay. The experiments were performed in triplicate and standard deviations are shown.

(LTR)-specific primers. The results showed that viral cDNA production was inhibited in a dose-dependent manner, and that 5 μg/ml *T. scandens* extract reduced viral cDNA production by a little more than 50% in virus-infected cells in comparison to cells treated with DMSO alone. A similar level of reduction was also observed with 5 nM AZT, which served as a positive control in this cell-based antiviral assay (Fig. 4). These results correlated nicely with the *in vitro* anti-RTase activity observed with the extract and further verified that the *T. scandens* extract harbors potent anti-HIV-1 activity, which is most likely due to its specific and effective anti-HIV-1 RTase inhibitory activity. Thus, although the active ingredient(s) responsible for the activity remains to be determined, these results clearly indicate that the natural *T. scandens* extract might be another useful source for isolating and developing new anti-HIV and anti-HIV RTase inhibitor(s) that may have a therapeutic value in the treatment of anti-HIV/AIDS.

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% CO_2 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 (2×10^6) were seeded in a 6-well plate 1 day before transfection and then transfected with 2 μg pNL4-3EGFP plasmid using Lipofectamine2000^{TM} (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 1×10^6 MT-4 cells in a 6-well plate and incubated for 72 h at 37°C in a humidified 5% CO_2 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 (1×10^4 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% CO_2 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% CO_2 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 (2×10^5 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% CO_2 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 (1.5×10^5 cells) and incubated at 37°C in a humidified 5% CO_2 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 (5×10^5) 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% CO_2 incubator, the total cellular DNA was extracted from the infected cells using a DNeasy 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'-GATCTGAGCCTGGGAGCTCTC-3', Reverse: 5'-CCTTTCGC TTTCAAGTCCCTGTTC-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 assay 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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