Antiviral Activity of *Paulownia tomentosa* against Enterovirus 71 of Hand, Foot, and Mouth Disease

Ping Ji, Changmai Chen, Yanan Hu, Zixuan Zhan, Wei Pan, Rongrong Li, Erguang Li, Hui-Ming Ge, and Guang Yang

*Jiangsu Key Laboratory of Molecular Medicine, Medical School, Nanjing University; 72 Guangzhou Road, Nanjing 210008, People's Republic of China. Nanjing Children's Hospital Affiliated with Nanjing Medical University; 72 Guangzhou Road, Nanjing 210008, People's Republic of China; and College of Life Sciences, Nanjing University; Nanjing 210093, People's Republic of China.*

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The bark, leaves, and flowers of *Paulownia* trees have been used in traditional Chinese medicine to treat infectious and inflammatory diseases. We investigated the antiviral effects of *Paulownia tomentosa* flowers, an herbal medicine used in some provinces of P.R. China for the treatment of skin rashes and blisters. Dried flowers of *P. tomentosa* were extracted with methanol and tested for antiviral activity against enterovirus 71 (EV71) and coxsackievirus A16 (CAV16), the predominant etiologic agents of hand, foot, and mouth disease in P.R. China. The extract inhibited EV71 infection, although no effect was detected against CAV16 infection. Bioactivity-guided fractionation was performed to identify apigenin as an active component of the flowers. The EC₅₀ value for apigenin to block EV71 infection was 11.0 µM, with a selectivity index of approximately 9.3. Although it is a common dietary flavonoid, only apigenin, and not similar compounds like naringenin and quercetin, were active against EV71 infection. As an RNA virus, the genome of EV71 has an internal ribosome entry site that interacts with heterogeneous nuclear ribonucleoproteins (hnRNP)s and regulates viral translation. Cross-linking followed by immunoprecipitation and reverse transcription-polymerase chain reaction (RT-PCR) analysis showed that EV71 RNA was associated with hnRNP A1 and A2. Apigenin treatment disrupted this association, indicating that apigenin suppressed EV71 replication through a novel mechanism by targeting the trans-acting factors. This study therefore validates the effects of *Paulownia* against EV71 infection. It also yielded mechanistic insights on apigenin as an active compound for the antiviral activity of *P. tomentosa* against EV71 infection.

**Key words** *Paulownia tomentosa*; enterovirus 71; apigenin; hand, foot, and mouth disease

Hand, foot, and mouth disease (HFMD) is an infectious disease that affects infants and young children. The pathogens that cause HFMD have been identified mainly as EV71 and CAV16. Symptoms of HFMD include fever, blister-like sores in the mouth, and skin rashes and blisters that are usually on the palms of the hands and soles of the feet or buttocks. Although it is a common dietary flavonoid, only apigenin, and not similar compounds like naringenin and quercetin, were active against EV71 infection. As an RNA virus, the genome of EV71 has an internal ribosome entry site that interacts with heterogeneous nuclear ribonucleoproteins (hnRNP)s and regulates viral translation. Cross-linking followed by immunoprecipitation and reverse transcription-polymerase chain reaction (RT-PCR) analysis showed that EV71 RNA was associated with hnRNP A1 and A2. Apigenin treatment disrupted this association, indicating that apigenin suppressed EV71 replication through a novel mechanism by targeting the trans-acting factors. This study therefore validates the effects of *Paulownia* against EV71 infection. It also yielded mechanistic insights on apigenin as an active compound for the antiviral activity of *P. tomentosa* against EV71 infection.

**Key words** *Paulownia tomentosa*; enterovirus 71; apigenin; hand, foot, and mouth disease

Pharmacological studies showed that extracts from *Paulownia* spp. with anti-viral, anti-bacterial, and anti-asthmatic activity. We found that a methanolic extract of *P. tomentosa* flowers was active against EV71, while no activity was detected against CAV16. An activity-directed isolation led to the identification of apigenin as an active component of *P. tomentosa* antiviral activity. The results are reported here.

**MATERIALS AND METHODS**

**Cells, Reagents, and Viruses** The rhabdomyosarcoma (RD) cells and green monkey kidney epithelial Vero cells were purchased from Cell Bank of China (Shanghai). The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (Life Technologies, Carlsbad, CA, U.S.A.). Antibodies were purchased from Abnova (Taiwan) and Bioworld (Minneapolis). Apigenin standard and ribavirin, naringenin, quercetin, dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). CAV16 was obtained from CCTCC (Wuhan). EV71 virus (Fuyang strain) was a clinical isolate provided by Jiangsu CDC and VP1 sequence analysis indicated it was prevalent EV71 strain in China. Viruses were propagated in RD cells as previously described.

**Preparation of Methanol Extract of *Paulownia tomentosa* Flowers (MEPTF)** Dried flowers were purchased from drug market of Bo Zhou, Anhui Province. The material was authenticated as *Paulownia tomentosa* (Thunb.) Steud. by
Yunxia Xu, and a voucher specimen was deposited at the Laboratory of Microbial Sciences, School of Medicine, Nanjing University, with the number of 2013001. The HPLC profiles of the major chemicals were consistent with those reported for plants from the *Paulownia* genus.\(^5\)\(^6\) To prepare a methanol extract, dried materials (200 g) were extracted twice with methanol for 2 h. The methanolic extract (8.0 g) was obtained after removal of methanol by evaporation, and was coded as MEPTF. For cytotoxicity and antiviral activity testing, the extract was dissolved in DMSO and freshly diluted at 1:1000 in DMEM before each experiment as we previously described.\(^9\)

**Cytotoxicity Assay** Monolayers of Vero cells in 96-well plates were incubated with plant extract or with apigenin at concentration as indicated or mock-treated with 0.1% DMSO since DMSO was used as a solvent. After 48 h incubation, the cells were rinsed with phosphate buffered saline (PBS) and cultured with DMEM containing 1 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 4 h. After removal of the medium, reduced MTT was dissolved in DMSO and the absorbance was then determined with a VersaMax plate reader (Molecular Devices). Optical density (OD) readings at 570 nm were used as a measure of cell viability. The percentages of cell survival were plotted using GraphPad Prism software and an EC\(_{50}\) value was extrapolated.

**Infection Assays** Virus infection was performed as we previously described.\(^9\) For quick assay of an antiviral effect, the viability of infected cells was determined by measuring their ability to reduce MTT\(^10\)\(^11\) or for the detection of EV71 infection was assessed using MTT method at 48 h PI. A percent value was extrapolated. The percentages of cell survival were plotted using GraphPad Prism software and an EC\(_{50}\) value was extrapolated.

**Time of Drug-Addition Assay** Vero cells were seeded in 96-well plates at 1×10\(^4\) cells per well. The cells were used for infection assays after overnight incubation. The test compound was added at 20 µM at 2 h prior to (−2 h), during (0 h), and varying times post inoculation, or remained untreated. The cells were infected with EV71 at an MOI of 0.1. The test compound was left in the culture during the infection process. In separate experiment, equal amount of EV71 in 10 µL complete DMEM was treated with apigenin at a final concentration of 20 µM for 1 h. The mixture was diluted with 90 µL complete DMEM (apigenin at a final concentration of 2 µM relative to Vero cells) and then used to infect Vero cells. The infection was assayed using MTT method at 48 h PI. A percentage of inhibition of each group was obtained and plotted.

**Activity-Directed Fractionation** Dried flowers of *P. tomentosa* (200 g) were extracted sequentially by refluxing with cyclohexane, chloroform, and acetyl acetate. After evaporation of the solvents, the extracts were redissolved in DMSO and tested at 100 µg/mL for antiviral activity against EV71 infection. Both CHCl\(_3\) (3.8 g) and EtOAc (1.1 g) extracts showed moderate activity against EV71 infection. The CHCl\(_3\) extract was then subjected to chromatography on silica gels and eluted using CHCl\(_3\) with increasing amount of methanol (19:1, 9:1, 8:1). Two major compounds were isolated and tested for their antiviral activity. Compound 1, 67 mg, identified as olea-nolic acid, showed no antiviral activity, while compound 2, isolated in 160 mg, showed anti-EV71 activity.

Compound 2 was identified as apigenin by NMR method. \(^1\)H- and \(^13\)C-NMR were performed on Bruker DPX-400 with DMSO-\(_d_6\), as a solvent. \(^1\)H-NMR (DMSO-\(_d_6\)) \(\delta\): 12.98 (s, 1H, OH), 10.84 (brs, 1H, OH), 10.39 (brs, 1H, OH), 7.93 (d, 2H, J=8.8 Hz), 6.93 (d, 2H, J=8.8 Hz), 6.79 (s, 1H), 6.49 (d, 1H, J=2.0 Hz), 6.20 (d, 1H, J=2.0 Hz). \(^13\)C-NMR (DMSO-\(_d_6\)) \(\delta\): 181.7 (C-4), 164.1 (C-2), 163.7 (C-7), 161.4 (C-9), 161.1 (C-4’), 157.3 (C-5), 128.4 (C-2’ and C-6’), 121.1 (C-1’), 115.9 (C-3’ and C-5’), 103.7 (C-3 or C-10), 102.8 (C-3 or C10), 98.8 (C-6), 93.9 (C-8).

**HPLC Analysis** We used a Hypersil Gold column (Thermo Fisher, 250 mm×4.6 mm, part number 25005–254630) for profiling of apigenin in the extracts. Acetonitrile–H\(_2\)O (30:70) containing 0.1% trifluoroacetic acid as a mobile phase was used. The components were monitored at 353 nm under a UV detector. Commercial apigenin from Sigma was used as a standard for HPLC studies.

**RNA–Protein Association Study** We used a RIP assay to demonstrate viral RNA association with host factors essentially as described.\(^12\) Briefly, monolayers of RD cells were treated with or without 30 µM apigenin for 2 h. The cells were then infected with EV71 for 6 h at an MOI of 40 as reported for the study of viral RNA association with host factors.\(^13\) The cells were harvested, washed with PBS, and then treated with formaldehyde to cross link ribonucleoproteins with associated nucleic materials.\(^12\) The cells were lysed with a buffer containing 150 mM NaCl, 50 mM Tris–HCl (pH 7.4), 1% NP-40, 0.1% sodium dodecyl sulfate (SDS), 1 mM ethylenediaminetetraacetic acid (EDTA) and a cocktail of protease inhibitors (Roche). The precleared lysates were immunoprecipitated with an antibody against human hnRNP A1 or A2. EV71 RNA in the immunocomplexes were detected using with reverse transcription-polymerase chain reaction (RT-PCR) using the following conditions for 40 cycles: 94°C for 30 s, 58°C for 30 s, and 72°C for 60 s. The primers used for the PCR study were as following: 5′-CCCATGGAATTAGAACG-3′ and 5′-CAGTTTAGCTGTGTTAAG-3′ (anneal to nt 97–745 of EV71 genome, GenBank accession number JX678878). PCR products were separated by agarose gel electrophoresis. The bands were stained with ethidium bromide and recorded on a Pei Qing gel imaging system (Shanghai).

**RESULTS**

**Cytotoxicity and Antiviral Activity Evaluation of Methyl-anol Extract of *Paulownia tomentosa* Flowers** The MEPTF was prepared by refluxing dried flowers with methanol. After removal of solvent, the dried extract was reconstituted in DMSO at 100 mg/mL as stock solution. The cytotoxicity and antiviral activity was tested using EV71 susceptible Vero cells. For cytotoxicity test, Vero cells were treated with the extract at varying concentrations. Cell viability was assayed...
48 h post the treatment using an MTT method. The extract showed no obvious toxicity to Vero cells at up to 250 µg/mL (Fig. 1A, dotted line). To test for antiviral effect, Vero cells were pretreated with MEPTF at concentrations that were not toxic to Vero cells. The cells were then infected with EV71 at a multiplicity of infection of 0.1 TCID₅₀ per cell (MOI=0.1) for another 48 h. Viral infection causes cell lysis. The antiviral effect was determined by measuring cell viability using an MTT assay (A, solid line). The plates were read at 570nm using a microtiter plate reader. Data are presented as mean±standard deviation (S.D.) of duplicate samples. In separated experiments, the production of infectious virion in MEPTF treated and untreated controls was titrated using a secondary infection assay. Data are presented as mean±S.D. of duplicate samples. Results are representative of three independent experiments.

The antiviral effect of MEPTF was confirmed using a secondary infection assay to determine infectious EV71 production. As shown in Fig. 1B, EV71 was detected in the untreated samples at 10⁶.⁵ TCID₅₀/mL, while those treated with MEPTF...
at 100 and 150 µg/mL suppressed infectious virion production by 1.0 and 2.1 logs, respectively, demonstrating that *P. tomentosa* flowers possessed antiviral activity against EV71 infection.

We attempted to identify the active components of *P. tomentosa* against EV71 infection. To this end, dried flowers were extracted with cyclohexane, CHCl₃, and then acetyl acetate (EtOAc). After removal of the solvents, the extracts were then tested against EV71 infection to trace the active component(s). Both CHCl₃ and EtOAc fractions contained activity against EV71 infection. After chromatographic separation followed by spectroscopic identification, apigenin was identified as an active compound against EV71 infection. Purified apigenin had ¹H- and ¹³C-NMR spectra characteristic of flavonoid (refer to ¹H- and ¹³C-NMR data in M&M).

**Antiviral Activity Evaluation of Purified Apigenin from *Paulownia tomentosa* Flowers** Apigenin was relative nontoxic to Vero cells. As shown in Fig. 2A, treatment of Vero cells with apigenin at 100 µM for 48 h reduced Vero cell viability by 60%, no significant reduction of cell viability was detected in samples treated with 60 µM or less of apigenin. CC₅₀ (50% cytotoxicity concentration) was extrapolated at 102.5 µM. We therefore used 30 µM as a maximal dose for antiviral studies by titration for virion production (Fig. 2B) and by immunoblotting for viral product expression (Fig. 2C). Vero cells that were treated with varying amount of apigenin were infected with EV71 at two different MOIs. Apigenin treatment significantly reduced infectious virion production at 10 and 30 µM concentrations (Fig. 2B). Concomitantly, reduced VP1 expression was also detected in apigenin treated samples (Fig. 2C). We then performed separated experiments to obtain a 50% effective concentration (EC₅₀) of apigenin against EV71 infection. The EC₅₀ value of apigenin against EV71 infection was estimated at approximately 11.0 µM (Fig. 2D) and selectivity index (SI, CC₅₀/EC₅₀) was calculated at 9.3.

**Apigenin Inhibits EV71 Infection by Targeting the Host, Not the Virus** We also performed experiments to determine whether apigenin possessed antiviral activity by directly targeting virus particles or indirectly by targeting the host cells. To this end, Vero cells were treated with 20 µM apigenin prior to, during or post inoculation. Alternatively, equal amount of EV71 inoculum in 10 µL volumes was pre-treated with apigenin at a concentration of 20 µM. The mixture was then diluted into a final volume of 100 µL with medium and used to infect Vero cells. As shown in Fig. 3A, treatment of host cells with apigenin, but not the virus, significantly inhibited EV71 infection. The result suggested that apigenin likely targeted the host cells, as compared to a virucidal effect, for its antiviral effect.

Apigenin is a polyphenolic compound which may exert its antiviral activity through its antioxidant property. We therefore tested whether similar compounds like naringenin, a dihydro-derivative of apigenin, or quercetin, a flavone with an extra hydroxyl group at the 3'-position had antiviral activity against EV71 infection (Fig. 3B). Unexpectedly, neither naringenin nor quercetin suppressed cell death caused by EV71 infection. This suggested to us that apigenin might employ a unique mechanism against EV71 infection.

**EV71 Infection Causes Viral RNA Association with hnRNP A1 and A2, Apigenin Treatment Disrupts RNA–Protein Association** EV71 infection requires viral RNA association with host factors like the hnRNP proteins for efficient translation. A recent report has demonstrated that apigenin can bind directly to hnRNP proteins and perturbs with their activity. We therefore investigated whether apigenin exhibited antiviral activity by targeting viral RNA association with hnRNP proteins. To this end, RD cells were infected with EV71 in the presence or absence of apigenin. hnRNP A1 or A2 was immunoprecipitated and EV71 RNA in the immunocomplexes was detected by RT-PCR analysis. As shown in Fig. 4, hnRNP A1 and hnRNP A2 were associated with EV71 RNA. Apigenin treatment resulted in absence of EV71 RNA in corresponding samples, indicating that apigenin exhibited antiviral activity by disrupting viral RNA association with hnRNP A1 and A2, important trans-acting factors for viral translation.

**Apigenin Is an Active Component of *Paulownia* Antiviral Activity** Apigenin, a dietary flavonoid, is abundantly distributed in plants and vegetables including basil, celery, parsley, and thyme. No previous studies have been reported on the antiviral activity of apigenin against EV71 infection. We therefore investigated whether apigenin was responsible for the anti-EV71 activity of *Paulownia* flowers by correlating apigenin content with the antiviral activity. First, we determined the...
that MEPTF at 150 µM possessed an antiviral activity comparable to that of apigenin and noticed a relative content of apigenin in MEPTF by HPLC analysis. As shown in Fig. 5, a standard sample of apigenin showed a retention time of 16.9 min under the separation condition. Under the same condition, a major peak at the same position was detected in MEPTF. Apigenin content in the MEPTF was determined at 11.1 ± 1.5 mg per gram, which translates to 4.1 µM and 6.2 µM apigenin in MEPTF of 100 µg/mL and 150 µg/mL, respectively. To extend this further, we compared the antiviral activity of MEPTF and purified apigenin and noticed that MEPTF at 150 µg/mL (apigenin at approximately 6.2 µM) possessed an antiviral activity comparable to that of apigenin at higher than 10 µM. Whether other factors are responsible for the inconsistency remains to be determined since apigenin was the only compound isolated with significant antiviral activity. Those results nonetheless demonstrated that apigenin was an active component of MEPTF antiviral activity.

In summary, we showed here that *P. tomentosa* flowers possessed activity against EV71 infection. In addition to the identification of apigenin as an active component, we also found that apigenin suppressed EV71 infection by disrupting the association of host factors with viral RNA, representing a novel mechanism for flavonoid antiviral activity.

**DISCUSSION**

Hand, foot, and mouth disease is a notifiable disease in China that affects over a million of children with hundreds of deaths each year. There is no vaccine or effective treatment approved for HFMD. The management includes those that relieving fever of children with mild symptoms or glucocorticoids and γ-globulin for patients with more severe symptoms. It is therefore very important to uncover antiviral agents for HFMD treatment. In a previous study, we evaluated a panel of herbs recommended by government agencies for the treatment of HFMD and found limited activity of those herbs when evaluated as individual ingredients. Although combined use of several herbs might enhance their antiviral activity, the study nonetheless underscores the need to expand the scope of screening of herbs for HFMD. In addition to the discovery of lead compounds with antiviral activity, this strategy will also provide virological evidence for folklorically used herbs for HFMD. We evaluated the effectiveness of *P. tomentosa* against EV71 and CAV16 infection and found that a methanolic extract of *P. tomentosa* flowers was active against EV71 infection, but not CAV16. Apigenin was identified as an active component of the flowers. Apigenin suppressed EV71 infection by blocking viral RNA association with hnRNP A1 and A2 proteins.

Flavonoids and terpenoids have been isolated from the leaves, barks or flowers of plants from *Paulownia* genus. Flavonoids such as fisetin and lutein have been reported with moderate activity against EV71 infection by blocking 3C protease activity at relatively high concentrations. Several geranyl-substituted flavanones that were isolated from *P. tomentosa* as minor components showed inhibitory activity against papain-like protease of coronavirus of severe acute respiratory syndrome (SARS). As polyphenolic compounds, the activity of flavonoids is commonly attributed to their antioxidant, anti-inflammatory activities. We showed that the antiviral effect of apigenin against EV71 infection was unlikely related to the antioxidant properties since similar compounds failed to inhibit EV71 infection. Consistent with a recent report on the molecular target of apigenin action, we found that apigenin blocked EV71 infection by targeting EV71 RNA association with host factors like hnRNP proteins, representing a novel mechanism for apigenin action.

Apigenin had an EC$_{50}$ of 11.0 µM against EV71 infection with a selective index of 9.3. It is noticed that an ideal drug should have a selective index of 1000 or better, but initial lead compounds may not have such indices. Apigenin is a dietary flavonoid with a relatively safe profile. Compared with the reported EC$_{50}$ (65 µg/mL) of ribavirin, apigenin is relatively potent agent against EV71 infection and deserves further con-
sideration of folkloric medicine as antiinfectives.

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Conflict of Interest The authors declare no conflict of interest.

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