INHIBITION OF INFLUENZA A VIRUS INFECTIVITY AND RNA-DEPENDENT RNA POLYMERASE ACTIVITY BY *ANDROGRAPHIS PANICULATA* ETHANOL EXTRACT

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Abstract. *Andrographis paniculata* extracts exhibit many pharmacological properties, such as antibacterial, antioxidant, anti-inflammation, antipyretic and antiviral activities, the latter against Epstein-Barr virus, flavivirus, herpes simplex virus, and human immunodeficiency virus. Anti-influenza A virus (IAV) properties of *A. paniculata* ethanol extract (APE) were investigated in IAV-infected Madin-Darby canine kidney cells. APE at >0.5 mg/ml exhibited anti-IAV effect in a dose-dependent manner using CCK-8 and cytopathic assays. APE pre-treatment demonstrated better antiviral activity than post-treatment. APE in the same dose range significantly inhibited viral RNA-dependent RNA polymerase activity. In conclusion, ethanol extract of *A. paniculata* inhibited influenza A virus infectivity and replication in Madin-Darby canine kidney cells, the latter effect through, in part, inhibition of viral RNA-dependent RNA polymerase activity. The study demonstrates chemical constituents in APE could have potential for discovery and development of novel anti-influenza agents.

Keywords: *Andrographis paniculata*, antiviral activity, ethanol extract, influenza A virus

INTRODUCTION

Control of influenza epidemic remains a challenge worldwide due to its fast spread via aerosol and high mutation rate (Liu *et al*, 2008). Common clinical antiviral drugs are amantadine and rimantidine, virus M2 ion channel blockers, thereby inhibiting viral replication or by changing conformation of cell lectin (Boltz *et al*, 2010). These drugs are also effective against IAV (Ge *et al*, 2010) as well as oseltamivir and zanamivir, neuraminidase inhibitors, which prevent the release of virus from infected cell (Samson *et al*, 2014), making them useful for treatment and prevention of influenza virus infection (Król *et al*, 2014). However, these drugs have side effects on the central nervous system and drug resistance has emerged in some viral strains (Ge *et al*, 2010), *eg* 2009 H1N1 strain (Ortigoza *et al*, 2012). Therefore, it is urgent to discover new drug targets and develop new anti-influenza virus drugs.

Influenza A virus (IAV) is a negative-strand RNA virus belonging to family *Orthomyxoviridae* (Lee *et al*, 2014), which contain three types, namely, IAV, IBV and ICV based on matrix and nucleoproteins (NPs). Both IAV and IBV cause respiratory

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illness, but IBV infection usually is associated with regional disease and IAV with pandemics and unpredictable antigenic changes (Paddock *et al*, 2012). Several subtypes of IAV hemagglutinins (HA) and neuraminidases (NA) were identified, *eg* 1918 H1N1, 1957 H2N2, 1968 H3N2, and 2009 H1N1 (Raza *et al*, 2011). Influenza virus Viral RNA (vRNA) genome binds NPs to form a complex responsible for virus genome replication and transcription initiated by viral RNA-dependent RNA polymerase (RdRP), consisting of three subunits, namely, PB1, PB2 and PA (Stubbs and Te Velthuis, 2014).

Traditional Chinese Medicine (TCM) has been used to treat colds and cold-like symptoms for thousands of years (Hailong *et al*, 2015). Compared to western medicine, TCM has several benefits, such as low toxicity, integrated regulation, and multiple targets, *etc* (Cheng *et al*, 2011). TCM herbs, such as *Forsythia suspense* and *Glycyrrhiza uralensis*, play an important role in the treatment of infectious diseases, especially respiratory viral infections (Ko *et al*, 2006). The anti-influenza activity of TCM herbs can be through (i) regulation of immune system and improvement of natural immunity, and (ii) direct action on one or more targets of the virus life cycle (Chen *et al*, 2006; Li and Peng, 2013). Importantly, TCM herbs provide relief of symptoms of virus infection without the adverse side effects of western antiviral drugs (Wang *et al*, 2006). Therefore, investigating and identifying safe and effective antiviral TCM herbal remedies are in high demand.

Andrographis paniculata, an annual herb, belonging to family Acanthacea, is found in subtropical regions and in China, it is distributed in Fujian, Guangdong, Guangxi, Hunan, Jiangxi, and Sichuan Provinces. *A. paniculata* aerial parts, containing diterpene glycosides, diterpenoids, flavonoid glycosides, flavonoids, and lactones, possess many medicinal functions, including fever reduction, detoxification and detumescence (Akbar, 2011). *A. paniculata* extracts have been reported to possess pharmacological activities, such as antibacterial, anti-inflammatory, antioxidant, antipyretic, and immunomodulatory functions (Coon and Ernst, 2004; Malahubban *et al*, 2013; Sunder *et al*, 2016). Interestingly, andrographolide, a major bioactive compound of *A. paniculata*, exhibits multiple anti-viral activities, *eg* against Epstein-Barr virus (EBV), flavivirus, hepatitis B virus (HBV), hepatitis C virus (HCV), herpes simplex virus (HSV), and human immunodeficiency virus (HIV) (Chen *et al*, 2014; Gupta *et al*, 2017).

Here, activity and mechanisms of action of *A. paniculata* ethanol extract against influenza A virus (IAV) were investigated to widen the anti-viral spectrum of this medicinal plant.

MATERIALS AND METHODS

Reagents

Transfection reagent (Lipofectamine™ 2000) was obtained from Invitrogen (New York, NY), substrate solution (including luciferin and ATP, but the recipe is unknown) for luciferase activity assay from Promega (Madison, WI), andrographolide from Shanghai Tauto Biotech Co Ltd (Shanghai, PR China), CCK-8 kit from Dojindo (Rockville, MD), and DMEM high glucose medium and fetal bovine serum (FBS) were from Gibco (Grand Island, NY). Madin Darby canine kidney (MDCK) cells, human embryonic kidney 293T cells, IAV 8-plasmid reverse genetic system (Li *et al*, 2010), plasmid pHH-Gluc, plasmid

pFlu-luc, and plasmid pcDNA3.1 were kindly provided by Dr Cen Shan (China Academy of Medical Sciences, Institute of Medicinal Biotechnology, Beijing, PR China). Amantadine was purchased from Sigma-Aldrich (St Louis, MO). Other reagents were of analytical grade. *A. paniculata* (Burm.f) Nees (Acanthaceae), commonly known as Kalmegh, was from Dongguan TCM Trading Center (Guangdong, PR China).

Cell cultures and virus preparation

MDCK and 293T cells were cultured in DMEM high glucose medium containing 10% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml). Human 293T cells and MDCK cells were co-cultured at a ratio of 2:1 at 37°C for 24 hours under a humidified atmosphere containing 5% CO_2, then seeded in a 6-well plate at 6×10^5 cells/well, transfected with an 8-plasmid influenza virus A (IAV) reverse genetics system (0.4 μg plasmid/well) using Lipofectamine™ 2000 and incubated for 12 hours as described above. A 20 μl aliquot of 1 mg/ml trypsin was added and incubation was continued for a further 48-hour period, then cells were sedimented at 3,000 *g* for 10 minutes and supernatants stored at -80 °C until used.

In addition, 50% tissue culture infective dose ($TCID_{50}$) of supernatant was determined as previously described (Reed and Muench, 1938). Briefly, 1.5×10^4 MDCK cells in 100 μl complete medium were seeded in each well of 96-well plate. The cells were cultured overnight to about 80% confluency. The original virus sample was diluted to make a serial 1:10 dilution of the virus. One hundred μl virus dilution per well was added into 4 wells. The cells were incubated at 37°C for 4 days under a humidified atmosphere containing 5% CO2. The cytopathic effect was observed after 4 days and $TCID_{50}$ was calculated using the Reed-Muench formula (Reed and Muench, 1938).

Preparation of *A. paniculata* ethanol extract (APE)

A. paniculata aerial parts were grounded and extracted with 20 ml of 95% ethanol/g for 30 minutes at 80°C using ultrasound cleaning bath (Crest Ultrasonics, Trenton, NJ) (40 KHz). The extract was filtered, concentrated and analyzed by HPLC (Agilent Technologies 1200 Series; Santa Clara, CA) equipped with a C18 column (250×4.6 mm, 5 μm). Solution (10 μl) was injected and eluted with a mobile phase (methanol:water at 52:48) at a flow rate of 1 ml/minute and $A_{225\ nm}$ monitored. Andrographolide was used as standard for quality control of APE (Sareer *et al*, 2014). Andrographolide constituted 9.5 mg/g of *A. paniculata* powder.

APE cell toxicity assay

MDCK cells were seeded in 96-well plate (3,000 cells/well) and cultured as described above for 24 hours, washed once with 154 mM phosphate-buffered saline pH 7.4 (PBS), and added with 100 μl aliquot of 0.1-30 mg/ml. APE in dimethyl sulfoxide (DMSO) was added to each well. Negative control wells were added with vehicle. After 72 hours of incubation, 10 μl aliquot CCK-8 solution was added to each well, incubated for two hours and $A_{450\ nm}$ measured with a Multi-Mode Microplate Reader (SpectraMax M5, Molecular Devices, San Jose, CA). A dose-response curve was constructed by plotting $A_{450\ nm}$ versus log APE concentration.

APE antiviral activity

MDCK cells were seeded in 24-well plate (1.5×10^4 cells/well) and cultured as described above for 24 hours. Cells were divided into two groups. In Group 1, cells

were infected with 100-fold $TCID_{50}$ ($10^{-2.12}$) A/WSN/33 (H1N1) influenza virus, cultured for three hours, washed twice with PBS, then 500 µl aliquot of 0.5-5.0 mg/ml APE in DMSO was added to each well and incubated for 36 hours. In Group 2, cells were incubated with 500 µl aliquot of 0.5-5.0 mg/ml APE in DMSO for three hours (negative control cells contained vehicle), then treated with H1N1 influenza as described above. Subsequently, in both Groups, 10 µl aliquot of CCK-8 solution was added to each well, and cells were incubated for two hours before survival of MDCK cells was measured as described above.

Plasmid pHH-Gluc transfection

MDCK cells were seeded in 6-well plate (6×10^4 cells/well), cultured as described above for 24 hours, then transfected with Gaussia luciferase-expressing pHH-Gluc (500 ng/well) using Lipofectamine™ 2000 and incubated for 12 hours. Transfected cells were seeded in 96-well plate (3,000 cells/well) and cultured for 24 hours. Cells were divided into two groups. In Group 1, cells were infected with 100-fold $TCID_{50}$ ($10^{-2.12}$) A/WSN/33 (H1N1) influenza virus, cultured for three hours, then incubated with 500 µl of 0.5-4.0 mg/ml APE in DMSO for 36 hours. In Group 2, cells were pretreated with APE solutions as described above for three hours, then infected with A/WSN/33 virus and cultured for 36 hours. Positive and negative control cells were treated with ribavirin (44.7 mM in DMSO) and vehicle respectively. Then in both groups, 20 μl aliquot of supernatant from each well was mixed with 1 μl of luciferase substrate solution (including luciferin and ATP, but the recipe is unknown) and fluorescence (excitation $\lambda_{480\ nm}$, emission $\lambda_{600\ nm}$) was measured by a Multi-Mode Microplate Reader (SpectraMax M5, Molecular Devices, San Jose, CA). A dose-response curve was constructed by plotting relative fluorescence versus APE concentration.

Cellular morphology determination

MDCK cells were seeded in 6-well plate, cultured as described above for 24 hours, infected with 100-fold $TCID_{50}$ ($10^{-2.12}$) A/WSN/33 virus, and treated with 0.5-4.0 mg/ml APE in DMSO for 36 hours. Positive control cells were treated with 5 μM amantadine and negative control cells were non-infected and treated with vehicle. Cells were observed under a light microscope (100X magnification).

Viral RdRP activity assay

Human 293T cells were seeded in 24-well plate (9×10^4 cells/well), cultured as described above for 24 hours, then a set of plasmids (pHW182-PB1, pHW181-PB2, pHW183-PA, and pHW185-NP; 100 ng each/well) to allow assembly of active viral RdRP were co-transfected with RdRP-driven luciferase reporter plasmid pFlu-luc using Lipofectamine™ 2000 and cultured for 24 hours before cells were treated with 0.5-4.0 mg/ml APE in DMSO for 36 hours. Then 20 μl aliquot of supernatant from each well was mixed with 1 μl of luciferase substrate solution (including luciferin and ATP, but the recipe is unknown) and fluorescence measured as described above. Ribavirin (44.7 mM) was used in positive control group, while DMSO and amantadine (5 μM) in negative control group.

Statistical analysis

Each experiment was carried out in three independent trials and data are expressed as mean ±SD. Statistical significance analysis was performed using one-way ANOVA and Newman-Keuls tests. A p-value <0.050 is considered significant.

RESULTS

Cytotoxicity and antiviral activity of APE on MDCK cells

Toxicity of APE on MDCK cells was determined using a CCK-8 method demonstrating a 50% cytotoxic concentration (CC_{50}) of 7.5 ± 0.4 mg/ml (Fig 1a). For determination of APE antiviral activity *in vitro*, MDCK cells were infected with 100-fold $TCID_{50}$ ($10^{-2.12}$) A/WSN/33 (H1N1) influenza virus, then treated with APE and cell viability measured using the CCK-8 method, which showed APE was able to prevent virus-infected MDCK viability with an IC_{50} value of 1.5 ± 0.06 mg/ml (Fig 1b), a concentration that was not toxic to MDCK cells. Pre-treatment with APE for three hours was significantly more effective at all concentrations tested (p-value < 0.050) (Fig 1b).

Anti-virus infectivity of APE on MDCK cells and morphology

MDCK cells transfected with pHH-Gluc, which expresses Gaussia luciferase when cells become infected with IAV (Lutz *et al*, 2005) were employed to determine APE anti-viral capability. APE demonstrated a dose-response inhibition of Gaussia luciferase activity in transfected MDCK cells infected with 100-fold $TCID_{50}$ ($10^{-2.12}$) A/WSN/33 (H1N1) influenza virus, both in pre- and post-treatment conditions, with pre-treatment being superior at all concentrations tested (Fig 2). Positive control employed 44.7 mM ribavirin, a broad spectrum guanosine analog antiviral active against both RNA and DNA viruses (Kirsi *et al*, 1983).

When examined under a light microscope, IAV-infected MDCK cells had a round appearance and became detached from bottom of well (Fig 3b),

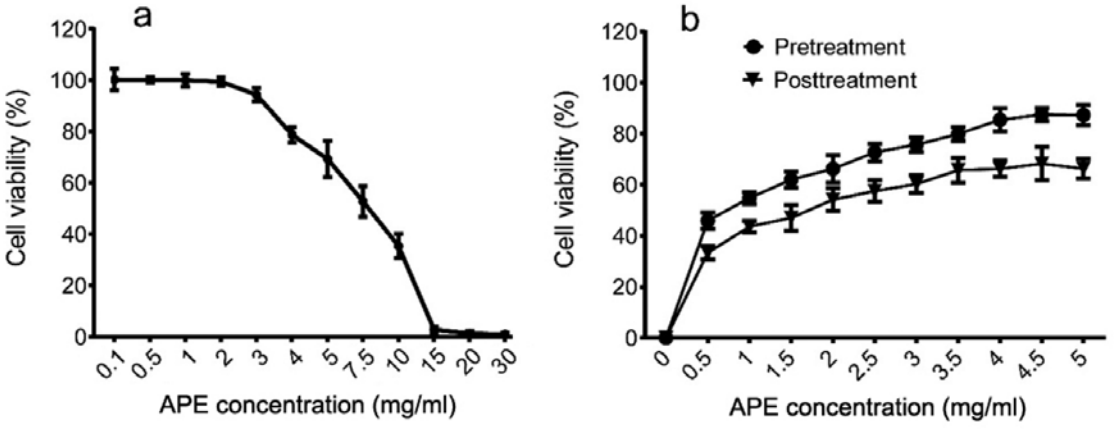

Fig 1-Cytotoxicity (a) and antiviral activity (b) of *Andrographis paniculata* ethanol extract (APE) on Madin Darby canine kidney (MDCK) cells.

(a) MDCK cells (3×10^3) were treated with APE (in DMSO) for 72 hours and cell viability determined using a CCK-8 assay; (b) MDCK cells (1.5×10^4) were infected with 100-fold $TCID_{50}$ ($10^{-2.12}$) A/WSN/33 (H1N1) influenza virus, treated with APE (in DMSO) for 36 hours (post-treatment) and then cell viability determined as in (a). In pretreatment, cells were treated with APE preparations prior to viral infection. Data are presented as mean ± SD of three independent experiments.

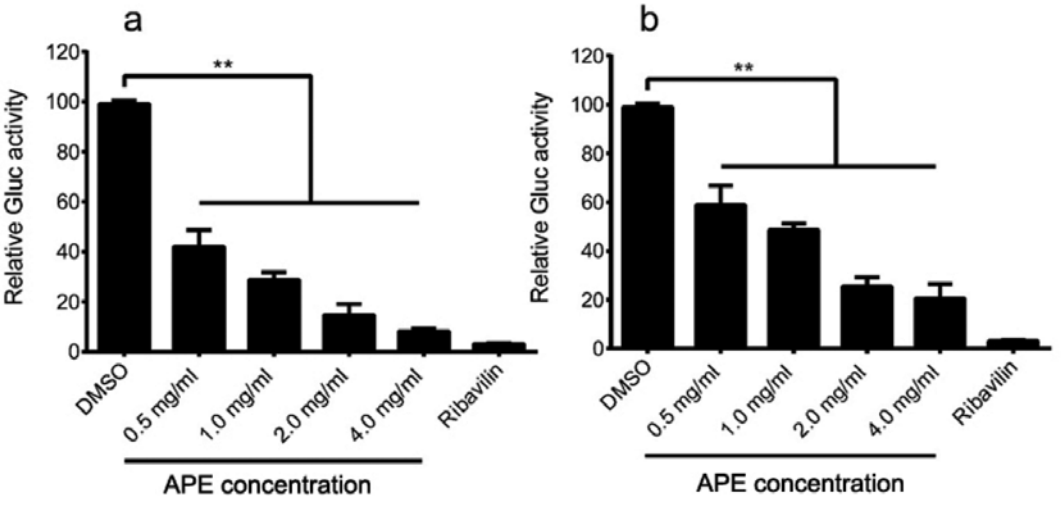

Fig 2-Pre-treatment (a) and post-treatment (b) with *Andrographis paniculata* ethanol extract (APE) on influenza virus A infectivity in Madin Darby canine kidney (MDCK) cells.

MDCK cells (6×10^4) were transfected with pHH-Gluc expressing influenza virus A-dependent Gaussia luciferase (Gluc) and then infected with A/WSN/33 (H1N1) influenza virus and treated with APE preparations as described in legend to Fig 1. An aliquot of supernatant was added to a solution of luciferase substrate (including luciferin and ATP, but the recipe is unknown) and fluorescence measured. Ribavilin (44.7 mM) was used as positive control. Data are presented as mean ± SD of three independent experiments. $^{**}p$-value <0.050.

but treatment with 0.5-4.0 mg/ml APE restored IAV-infected cells to their original morphology in a dose-dependent manner, with the highest concentration of APE restoring cell morphologies similar to uninfected cells and to those treated with 5 μM amantadine, an antagonist of IAV M2 proton channel and inhibitor of viral shedding (Davies *et al*, 1964**)** (Fig 3).

APE inhibition of viral RdRP activity in IAV-infected 293T cells

Test of APE ability to inhibit activity of IAV RdRP, four viral RdRP-expressing plasmids, *ie* pHW182-PB1, pHW181-PB2, pHW183-PA and pHW185-NP, which harbor expressing cassettes of PB1, PB2 and PA subunits of an RNA-dependent RNA polymerase and a single-strand RNA binding nucleoprotein (NP) (Li *et al*, 2010), and a reporter plasmid pFlu-luc were co-transfected into 293T cells. After treatment with 0.5-4.0 mg/ml APE for 36 hours, relative fluorescence intensity of transfected 293T cells (reflecting IAV RdRP activity) were measured, showing a significant decrease in IAV RdRP activity when cells were treated at threshold APE concentration of 2 mg/ml and remained unchanged above this concentration (Fig 4). Ribavilin (44.7 mM) inhibited 60% of IAV RdRP activity while amantadine (5 μM) was ineffective. These findings indicate APE could inhibit IAV infectivity in human cells, in part, through interfering with IAV ability to replicate.

DISCUSSION

Although previous evidences

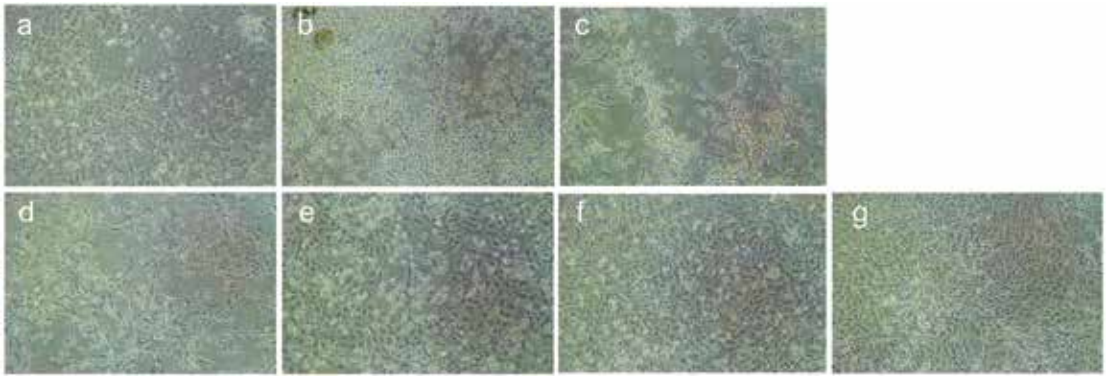

Fig 3-Morphology of influenza A virus-infected in Madin Darby canine kidney (MDCK) cells treated with *Andrographis paniculata* ethanol extract (APE).

MDCK cells were infected with A/WSN/33 (H1N1) influenza virus and treated with APE preparations as described in legend to Fig 1, then examined under a light microscope (100x magnification). a: Uninfected; b: Treated with vehicle (DMSO); c: Treated with 0.5 mg/ml APE; d: Treated with 1 mg/ml APE; e: Treated with 2 mg/ml APE; f: Treated with 4 mg/ml APE; g: Treated with 5 μM amantadine.

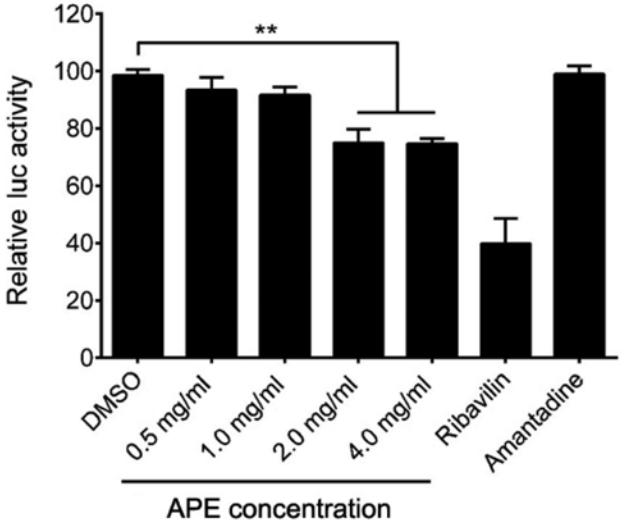

Fig 4-Inhibition of influenza virus A RNA-dependent RNA polymerase (vRdRP) by *Andrographis paniculata* ethanol extract (APE).

Human 293T cells were co-transfected with a set of plasmids (pHW182-PB1, pHW181-PB2, pHW183-PA, and pHW185-NP) allowing assembly of vRdRP, and pFlu-luc, expressing vRdRP-dependent luciferase (luc), and then treated with APE preparations as described in legend to Fig 1. An aliquot of cell lysate was added to a solution of luciferase substrate (including luciferin and ATP, but the recipe is unknown) and fluorescence measured. Ribavilin (44.7 mM) and amantadine (5 μM) was used as positive and negative drug control respectively. Data are presented as mean ± SD of three independent experiments. **p-value <0.050.

indicated *A. paniculata* extracts exhibit multiple antivirus activities (Jayakumar *et al*, 2013), its anti-influenza virus activity and underlying mechanism remains to be elucidated. The study, using a reverse genetic 8-plasmid system to recapitulate the life cycle of IAV in vitro, reveals APE at non-cytotoxic concentrations had a significant anti-IAV activity in a dose-dependent manner with an IC_{50} of 1.5 mg/ml. APE treatment of IAV-infected cells at 3-fold IC_{50} value almost completely reversed morphology changes induced by virus infection, similar to that observed with antiviral amantadine (5 μM). The study adds IAV to the list of both DNA (*eg* EBV, HBV and HSV) and RNA (flavivirus, HIV) viruses (Gupta *et al*, 2017) affected by *A. paniculata* extracts.

Anti-virus activity of an agent can be through inhibition of virus replication (*eg* rimantidine), egress (*eg* zanamivir) or ingress (*eg* neutralizing antibodies) (Paintsil and Cheng, 2009). APE inhibited both IAV ingress and RdRP activity, which play a central role in RNA virus replication. Influenza virus RdRP is highly conserved among all strains and subtypes, making it a promising target for influenza drugs with low potential of developing drug resistance (Furuta *et al*, 2013). Since IAV has a high mutation rate and manifests frequent genetic recombination under selective pressure to obtain expanded tropism and enhanced pathogenicity leading to antiviral drug resistance and even pandemic (Hussain *et al*, 2017), an agent with multiple targets is also advantageous. *A. paniculata*-derived andrographolide shows strong anti-dengue viral property through reducing virus production and infectivity (Panraksa *et al*, 2017). In addition, dehydroandrographolide from *A. paniculata* exerts anti-IAV activity through inhibiting nuclear export of viral ribonucleoprotein complexes (Cai *et al*, 2015).

In conclusion, the study demonstrates ethanol extract of *A. paniculata* (containing mainly andrographolide) exhibited *in vitro* anti-influenza A viral activity through inhibiting viral infectivity and RNA dependent RNA polymerase activity of IAV, suggesting chemical components of *A. paniculata* ethanol extract as potential candidates for development of anti-influenza agents.

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