science, engineering and health studies

Research Article

*Science, Engineering and Health Studies*
*https://lio1.tci-thaijo.org/index.php/sehs*
*ISSN (Online): 2630-0087*

# Activity of methanolic *Andrographis paniculate* crude extract and its purified andrographolide against Japanese encephalitis virus

*Jundee Rabablert[1*], Juree Charoenteeraboon[2], Supathra Tiewchareon[3], Natthapol Rabablert[2], Weesuda Makittithorn[2], Nantarat Chutipanyaporn[1], Supoth Rajchakam[4], Virach Junnu[3] and Sutee Yoksan[4]*

[1] Department of Biology, Faculty of Science, Silpakorn University, Nakhon Pathom 73000, Thailand
[2] Department of Biopharmacy, Faculty of Pharmacy, Silpakorn University, Nakhon Pathom 73000, Thailand
[3] Department of Parasitology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand
[4] Center for Vaccine Development, Institute of Molecular Science, Mahidol University, Nakhon Pathom 73170, Thailand

***Corresponding author**:
*Jundee Rabablert*
*rabablert_j@su.ac.th*

***Received**: 26 December 2020*
***Revised**: 25 February 2021*
***Accepted**: 1 March 2021*
***Published**: 31 August 2021*

***Citation**:
*Rabablert, J., Chareontheraboon, J., Tiewchareon, S., Rabablert, N., Makittithorn, W., Chutipanyaporn, N., Rajchakam, S., Junnu, V., and Yoksan, S. (2021). Activity of methanolic Andrographis paniculate crude extract and its purified andrographolide against Japanese encephalitis virus. Science, Engineering and Health Studies, 15, 21030007.*

## ABSTRACT

Infection of Japanese encephalitis virus (JEV) generates brain swelling, namely Japanese encephalitis (JE) in human. This virus is spread by *Culex spp.* mosquitoes and causes 30% case-fatality rate in Asia and Western Pacific regions annually. No specific cure for the disease is available. *Andrographis paniculate* methanolic extract and its purified andrographolide exhibit anti-inflammatory and antiviral properties. Our study is focused to antiviral activity of *A. paniculate* extracts and its elements against JEV *in vitro.* Half-maximal cytotoxicity ($CC_{50}$) and the maximum non-toxic doses (MNTD) of *A. paniculata* in SK-NMC and LLC-MK2 are less than the $CC_{50}$ and MNTD of andrographolide in SK-NMC and LLC-MK2 at 3 days-post treatment by MTT colorimetric assay. Both *A. paniculata* and andrographolide have antiviral effect by simultaneous treatment more than pre-treatment and post-treatment at 3 days-post treatment by time-of-addition assay. Antiviral action of these agents interfered virus attachment and had virucidal activity. The results indicated that *A. paniculata* and andrographolide have the potential antiviral activity against JEV.

**Keywords:** Antiviral; Flavivirus; human neuroblastoma; medicinal plant

## 1. INTRODUCTION

Japanese encephalitis virus (JEV) affects brain swelling and encephalitis in 50,000 confirmed cases and at least 15,000 death through many countries in Asia and Western Pacific zone, annually (Schuh et al., 2014). JEV causes ~30% case fatality and life-long neurological sequalae in survivors (Wang and Liang, 2015). There is no cure for the disease, treatment is restricted to relieve severe clinical signs of the patients.

Medicinal *Andrographis paniculata* (Burm. f) Nees, plant is used to treat an inflammation, sore throat, and infection of herpes and dengue viruses in many Asian countries including Thailand (Seubsasana et al., 2011; Wen et al., 2014; Ali-Seyed and Vijayaraghavan, 2020). Biological activities of andrographolide isolated from the leaves and stems of *A. paniculata* has anti-inflammation, anti-proliferation, neuro-and hepato-protection (Thakur et al., 2012a; Thakur et al., 2012b).

Andrographolide had antiviral activity against deoxyribonucleic acid (DNA) viruses, such as Epstein-Barr virus (EBV) (Lin et al., 2008), herpes simplex virus 1 (HSV-1) (Aromdee, et al., 2011; Priengprom et al., 2015) and ribonucleic acid (RNA) viruses, such as human immunodeficiency virus (HIV) (Reddy et al., 2005; Uttekar et al., 2012), influenza viruses A (IAV) (Yu et al., 2014; Cai et al., 2015), hepatitis C virus (HCV) (Lee et al., 2014) and dengue virus (DENV) (Tang et al., 2012; Ali-Seyed and Vijayaraghavan, 2020). However, antiviral activity of *A. paniculata* and its purified andrographolide against JEV have been not studied.

This study was aim to evaluate the 50% cytotoxicity concentration ($CC_{50}$) and maximum non-toxic dose (MNTD) of *A. paniculata* methanol extract and its purified andrographolide in SK-NMC and LLC-MK2 cell by MTT assay. Furthermore, antiviral activity of *A. paniculata* and andrographolide against JEV replication by time-of-addition assay were also investigated.

## 2. MATERIALS AND METHODS

### 2.1 Materials

Human neuroblastoma (SK-N-MC) cells were bought from Cell Line Service (CLS) (Germany) in 2006. Rhesus monkey kidney (LLC-MK2) and *Aedes albopictus* larvae (C6/36) cells, and JEV (SM-1 strain) were from Center for Vaccine Development, Institute of Molecular Bioscience, Mahidol University, Thailand. SK-N-MC was cultivated in Dulbecco's modified Eagle medium (DMEM): HAM's F-12/10% FBS (fetal bovine serum, Gibco-BRL, Gaithersburg, MD) whereas LLC-MK2 was cultivated in DMEM/10% FBS. Both cells were cultivated in a $CO_2$ incubator at 37°C (Luangboribun et al., 2014). C6/36 cells were grown in minimum essential medium (MEM, Gibco-BRL)/1% nonessential amino acids (NEAA)/10% FBS in an incubator at 28°C (Yoksan et al., 2013). JEV infection was propagated in C6/36 monolayer cells at 28°C for 5-7 days until 50% cytopathic effect occurred. The JEV-infected supernatant was harvested and centrifuged at 3,000×g in refrigerated centrifuge for 30 min, and frozen at −80°C as virus stock. The viral titer was evaluated by plaque assays in LLC-MK2 cells. The viral titers in this study were $7\times10^6$ plaques forming unit/ mL (PFU/mL). Andrographolide was purchased from Cayman Chemical Company (M.W. 350, Item: 11679) and kept at 4°C until use.

### 2.2 Medicinal plant extract and purified compounds

Medicinal *A. paniculata* plant was collected from the botanical garden and verified. The leaves were washed three times and dried at 60°C for 18 h. After grinding, the powder was filled to a Soxhlet apparatus and soaked in absolute methanol for 3 days. Crude extract was filtered and evaporated in a rotary evaporator until dry. Dried crude extract was collected in sterile glass bottles at −20°C. The stock solution was dissolved in Dimethyl sulfoxide (DMSO) as solvent control to be 50 mg/mL and then dissolved in DMEM to be 1 mg/mL and stored at −20°C until use. Andrographolide was also dissolved in DMSO to be 50 mg/mL and then dissolved in DMEM medium to be 1 mg/mL and kept at −20°C until use. The 2% DMSO dissolved in DMEM was used as solvent control.

### 2.3 MTT colorimetric assay

The $CC_{50}$ and the MNTD of *A. paniculata* and andrographolide on mammalian cells were determined by MTT colorimetric assay (Luangboribun et al., 2014; Roy et al., 2015). One hundred µL/well of each cell ($2\times10^5$ cells/mL) was cultured onto 96-well plates. After 24-h post-incubation, two-fold serial dilutions of *A. paniculata*, andrographolide or 2% DMSO were filled in each well, in triplicate, and grown at 37°C/5% $CO_2$ for 1, 2, 3 days. At indicated times, 100 µL of MTT solution (5 mg/mL) was filled in each well to and kept at 37°C for 2 h. The 100 µL of DMSO was filled to each well and kept at 37°C for 30 min. The absorbance at 550 nm was need for microplate reader interpretation (Wallac 1420 Multilabel Counter, Perkin). Media, 2% FBS and 2% DMSO, were used as negative control. The results were performed from three-independent experiments. The $CC_{50}$ and MNTD were determined by probit analysis software (Paswstat 18, LdP Line Software, USA)

### 2.4 Determination of virus titers by plaque assay

To study half-maximal inhibitory concentration ($IC_{50}$) of methanolic *A. paniculata* crude extract or andrographolide on JEV, the SK-N-MC cells were inoculated with JEV at multiplicity of infection (MOI) of 1.0 in the presence of. *A. paniculata* (0-100 µg/mL) or andrographolide (0-50 µg/mL) at 1 and 2 days. DMSO (0-2%) and DMEM-HAMF12 medium alone were tested as negative control. At indicated times, the supernatant from untreated or treated JEV-infected cells was collected and determined viral titers by plaque assay. Serial 10-fold dilution of cultured medium was filled into the well of LLC-MK2 cell monolayer and kept at 37°C with 5% $CO_2$ for 1 h. The cells were overlaid with agarose medium (2X Earl's balance salt solution containing 2% SeaKem® LE garose, Lonza, St. Louis, MO, USA), and kept at 37°C with 5% $CO_2$ for 3 days. Viral plaques were fixed and stained with 1.25% crystal violet staining. Plaque sizes and plaque numbers were recorded and then calculated as previous reported (Yoksan et al., 2013).

### 2.5 Plaque reduction and time-of-addition assay

To study inhibitory concentration presenting 50% JEV plaque reduction ($IC_{50}$) of *A. paniculata* and andrographolide on JEV replication by different times of addition (pre-, simultaneous and post-treatment). In pre-treatment assay, LLC-MK2 was pretreated with *A. paniculata* (0-100 µg/mL), andrographolide (0-50 µg/mL) or DMSO (0-2%) for 1 h, followed by 1-h incubation with JEV (100 PFU). In simultaneous treatment assay, *A. paniculata* (0-100 µg/mL), andrographolide (0-50 µg/mL) or 2% DMSO was mixed with JEV (100 PFU) at 4°C for 1 h. Then, the mixture was inoculated into the cells and kept at 37°C for 1 h. In post-treatment assay, LLC-MK2 was infected with JEV (100 PFU) for 1 h, followed by 1-h incubation with *A. paniculata* (0-100 µg/mL), andrographolide (0-50 µg/mL) or DMSO (0-2%). After indicated time, untreated or treated LLC-MK2 infected with JEV were overlaid with agarose and kept at 37°C

with 5% $CO_2$ for 3 days. Viral plaques were fixed and stained with 1.25% crystal violet staining. Data represent means ±SD of three independent experiments. The $IC_{50}$ was determined by probit analysis software.

### 2.6 Virus attachment assay

JEV (100 PFU) was mixed with *A. paniculate* (0-100 µg/mL), andrographolide (0-50 µg/mL), or DMSO (0-2%), inoculated to LLC-MK2 cell monolayer in 12-well plates, and kept at 4°C for 1 h to allow attachment. After 1 h post-exposure, the cell monolayer was washed with cold Phosphate buffered saline (PBS) and overlaid with agarose at 37°C, 5% $CO_2$ for 3 days. Viral plaques were fixed and stained with 1.25% crystal violet.

### 2.7 Virucidal activity assay

JEV ($10^5$ PFU) was mixed with *A. paniculata* (0-100 µg/mL), andrographolide (0-50 µg/mL) or DMSO (0-2%) and kept at 4°C for 1 h. A 1,000-fold dilution of the mixture was filled onto LLC-MK2 cell monolayer in 12-well plates and then kept at 37°C for 1 h. After 1-h post-exposure, the cell monolayer was overlaid with agarose at 37°C for 3 days. Viral plaques were fixed and stained with 1.25% crystal violet.

### 2.8 Statistical analysis

Data were analyzed by ANOVA using SPSS program (Paswstat 18, LdP Line Software, USA). *P*-value less than 0.05 was considered significantly.

## 3. RESULTS

### 3.1 Cytotoxicity of *A. paniculata* and andrographolide on mammalian cells

At 3 days post-incubation (Table 1), the $CC_{50}$ and MNTD of *A. paniculate* damaged to SK-N-MC were higher than LLC-MK2. Likewise, the $CC_{50}$ and MNTD of andrographolide damaged to SK-N-MC were higher than LLC-MK2. It is suggested that *A. paniculata* and andrographolide had a dose-dependence affected on both tested cells. *A. paniculata* and andrographolide were less toxic to LLC-MK2 than SK-N-MC. Hence, LLC-MK2 cells was suitable to be further used for measuring activity of these agents.

**Table 1.** $CC_{50}$ and MNTD of *A. paniculata* and andrographolide on mammalian cells

| Stimulator | $CC_{50}$ (µg/mL) | | MNTD (µg/mL) | |
|---|---|---|---|---|
| | SK-N-MC | LLC-MK2 | SK-N-MC | LLC-MK2 |
| *A. paniculata* | 183.68±0.93 | 280.78±1.02 | 15.63±1.39 | 31.25±1.78 |
| Andrographolide | 47.85±0.18 | 158.78±1.05 | 3.90 ± 1.23 | 15.63±1.55 |

### 3.2 Effect of *A. paniculata* and andrographolide on inhibition of JEV

The viral titers from JEV-infected supernatant in the absence and presence of *A. paniculata* and andrographolide were determined for 1 and 2 day(s) post-exposure using plaque assay. The yields of JEV treated with *A. paniculata* (50 µg/mL) on SK-N-MC cells were $18.75±1.77×10^4$ PFU/mL (31.92±3.98%) and $23.75±1.77×10^4$ PFU/mL (32.86± 4.04%) at 1 and 2 day(s) post-exposure (Figure 1a). The yields of JEV treated with andrographolide (25 µg/mL) were $20.00±3.54×10^4$ PFU/mL (34.15±7.04%) and $30.00± 3.54×10^4$ PFU/mL (41.55±6.86%) at 1 and 2 day(s) post-exposure (Figure 1b). In contrast, the yields of JEV treated with DMSO at 2% were $51.25±1.77×10^4$ PFU/mL (87.23± 0.38%) and $67.5±0.00×10^4$ PFU/mL (93.20±4.53%) (Figure 1c).

The $IC_{50}$ of *A. paniculata* on JEV-infected SK-N-MC was 45.26±3.19 and 44.40±1.98 µg/mL at 1 and 2 day(s) post-exposure, while $IC_{50}$ of andrographolide on JEV-infected SK-N-MC was 20.58±3.08 and 22.89±1.37 µg/mL at 1 and 2 day(s) post-exposure. The data showed that both agents had inhibitory effect on JEV in SK-N-MC in similar level.

### 3.3 Inhibitory effect on JEV replication by *A. paniculata* and andrographolide

To evaluate time-of-addition effect of *A. paniculata* and andrographolide on JEV replication, LLC-MK2 cells were pre-treated, simultaneously treated and post-treated with *A. paniculata* (0-50 µg/mL), andrographolide (0-50 µg/mL), or DMSO (0-2%). With simultaneous treatment plaque reduction assay, both *A. paniculata* and andrographolide showed concentration-dependent JEV plaques inhibition: $IC_{50}$ values of *A. paniculata* and andrographolide were 18.71±0.64 and 9.61 µg/mL, respectively (Figure 2 and Table 2).

Antiviral activity of *A. paniculata* and andrographolide on JEV replication was shown in pre- and post-treatment (Figure 2 and Table 2). The plaque reduction by 2% DMSO was not significantly observed. The results also demonstrated that simultaneous treatment of *A. paniculata* and andrographolide inhibited JEV replication.

### 3.4 Inhibitory effect on JEV attachment by *A. paniculata* and andrographolide

To evaluate inhibitory effect of *A. paniculata* and andrographolide on JEV attachment, JEV mixture (50 PFU) with *A. paniculata*, andrographolide, or DMSO was kept at 4°C with LLC-MK2 to allow attachment. After virus attachment for 1 h, each mixture was removed and the cells were washed with PBS. The $IC_{50}$ values of *A. paniculata* and andrographolide were less than simultaneous treatment (Figure 3 and Table 2). DMSO did not interfere virus attachment. The results also showed that *A. paniculata* and andrographolide had inhibitory effect on JEV attachment.

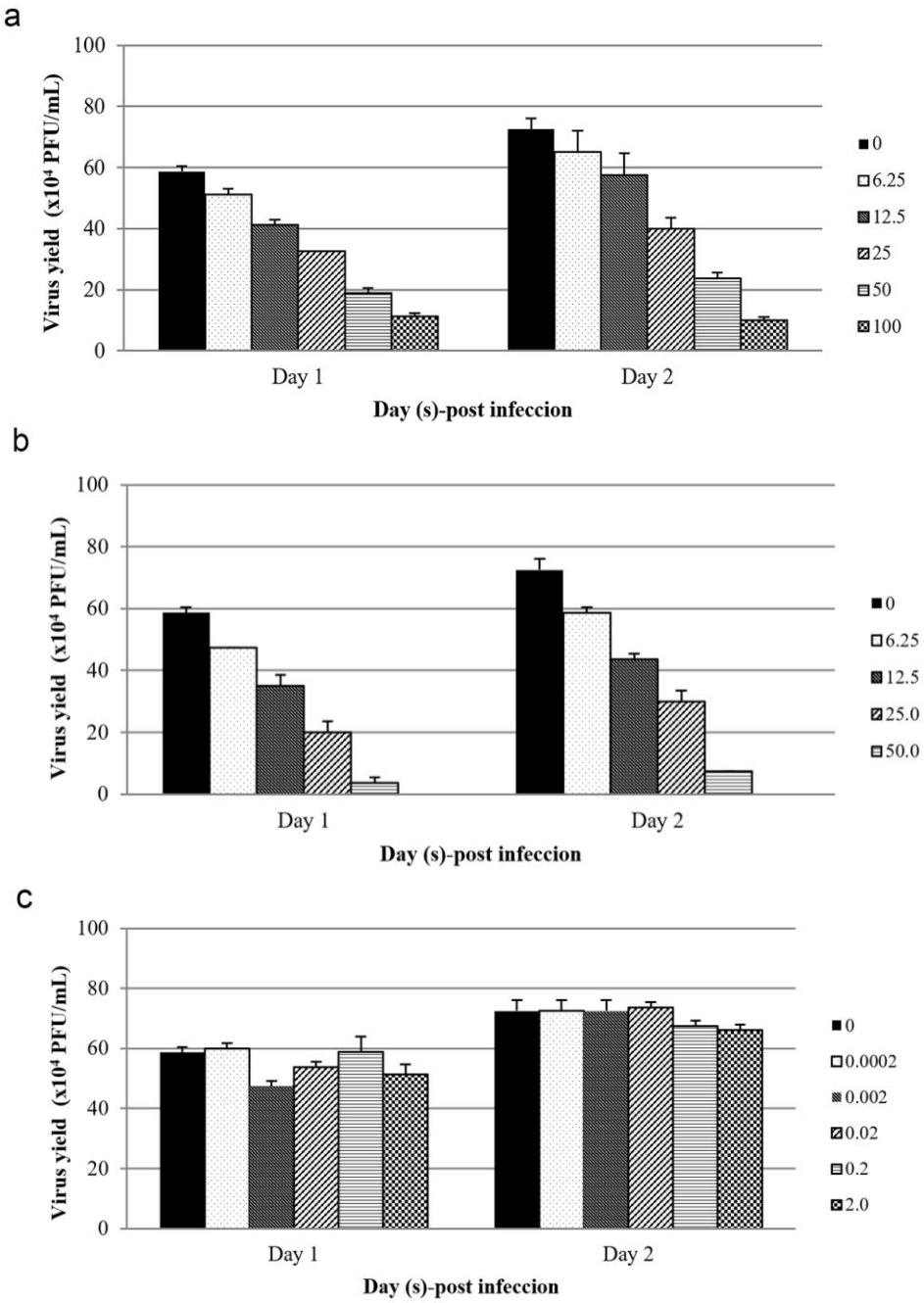


**Figure 1.** Inhibition of JEV on SK-NMC cells by (a) *A. paniculata* at 0-100 μg/mL, (b) andrographolide at 0-50 μg/mL, and (c) DMSO at 0-2% (n=3)

**Table 2.** Antiviral effects of *A. paniculata* and andrographolide against JEV

| Stimulator | $IC_{50}$ (μg/mL) | | | | |
|---|---|---|---|---|---|
| | Pre-treatment | Simultaneous treatment | Post-treatment | Virus attachment | Virucidal assay |
| *A. paniculata* | 30.37±0.56 | 18.71±0.64 | 38.79±1.36 | 16.46±0.08 | 12.80±0.23 |
| Andrographolide | 14.49±0.31 | 9.61±0.34 | 26.51±0.94 | 8.57±0.21 | 5.53±0.15 |

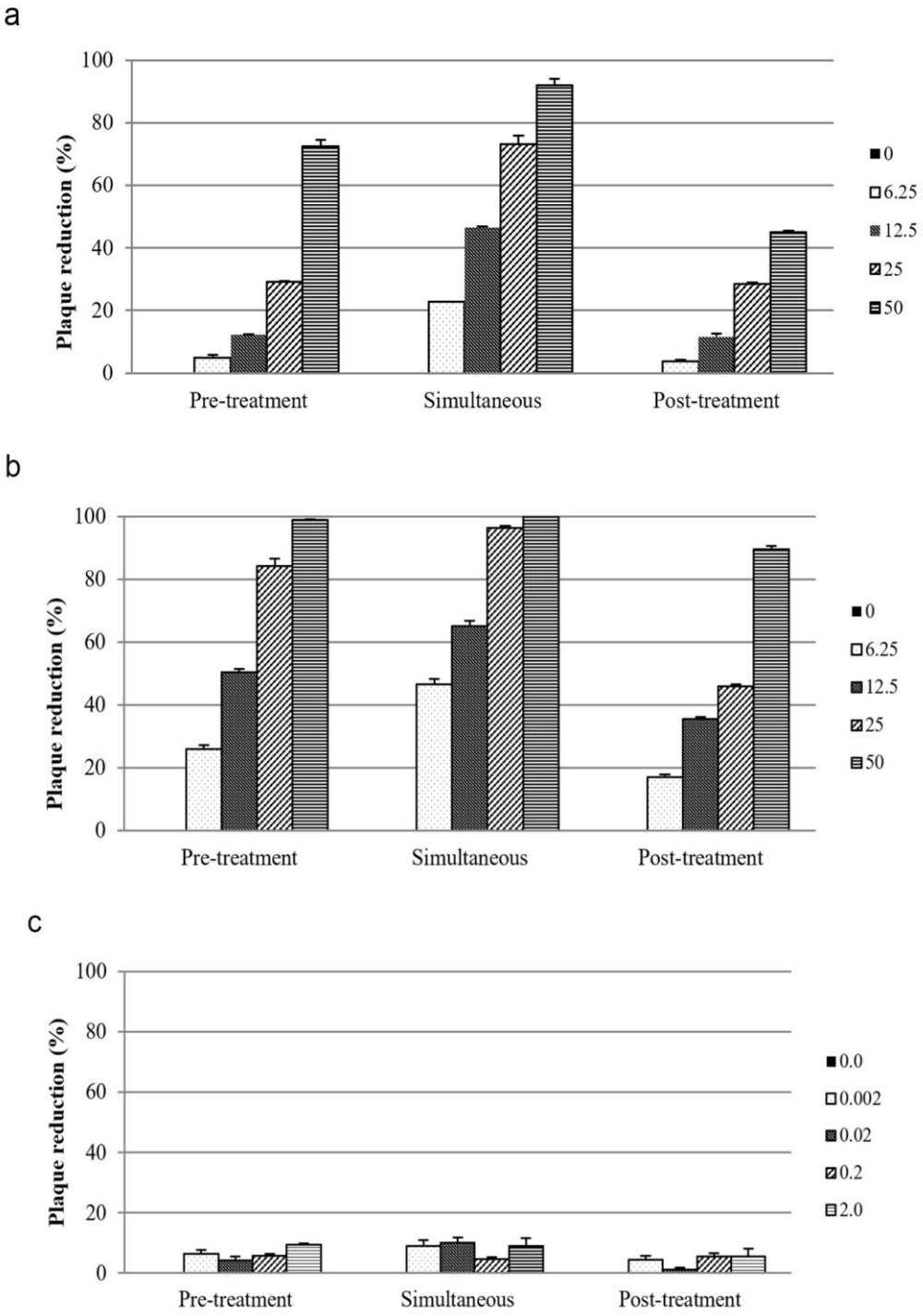


**Figure 2.** Plaque reduction of JEV by (a) *A. paniculata*, and (b) andrographolide and (c) DMSO in pre-treatment, simultaneous treatment, and post-treatment (n=3)

### 3.5 Virucidal activity of *A. paniculata* and andrographolide

To evaluate whether *A. paniculata* and andrographolide had a virucidal action by direct interfering with virus particles, JEV was pretreated with *A. paniculata*, andrographolide, or DMSO, and tested for residual infectivity by plaque assay (Figure 4 and Table 2). Both *A. paniculata* and andrographolide exhibited concentration-dependent virucidal activity as well as significant inhibitory effect on residual infectivity compared to controls. Virucidal $IC_{50}$ values against JEV were 12.80±0.23 μg/mL of *A. paniculata* and 5.53±0.15 μg/mL of andrographolide. Virucidal $IC_{50}$ values were less than virus attachments, simultaneous treatment, and pretreatment as well as post-treatment (Table 2), suggesting that *A. paniculata* and andrographolide directly inactivated JEV particles, exhibiting a potently virucidal action.

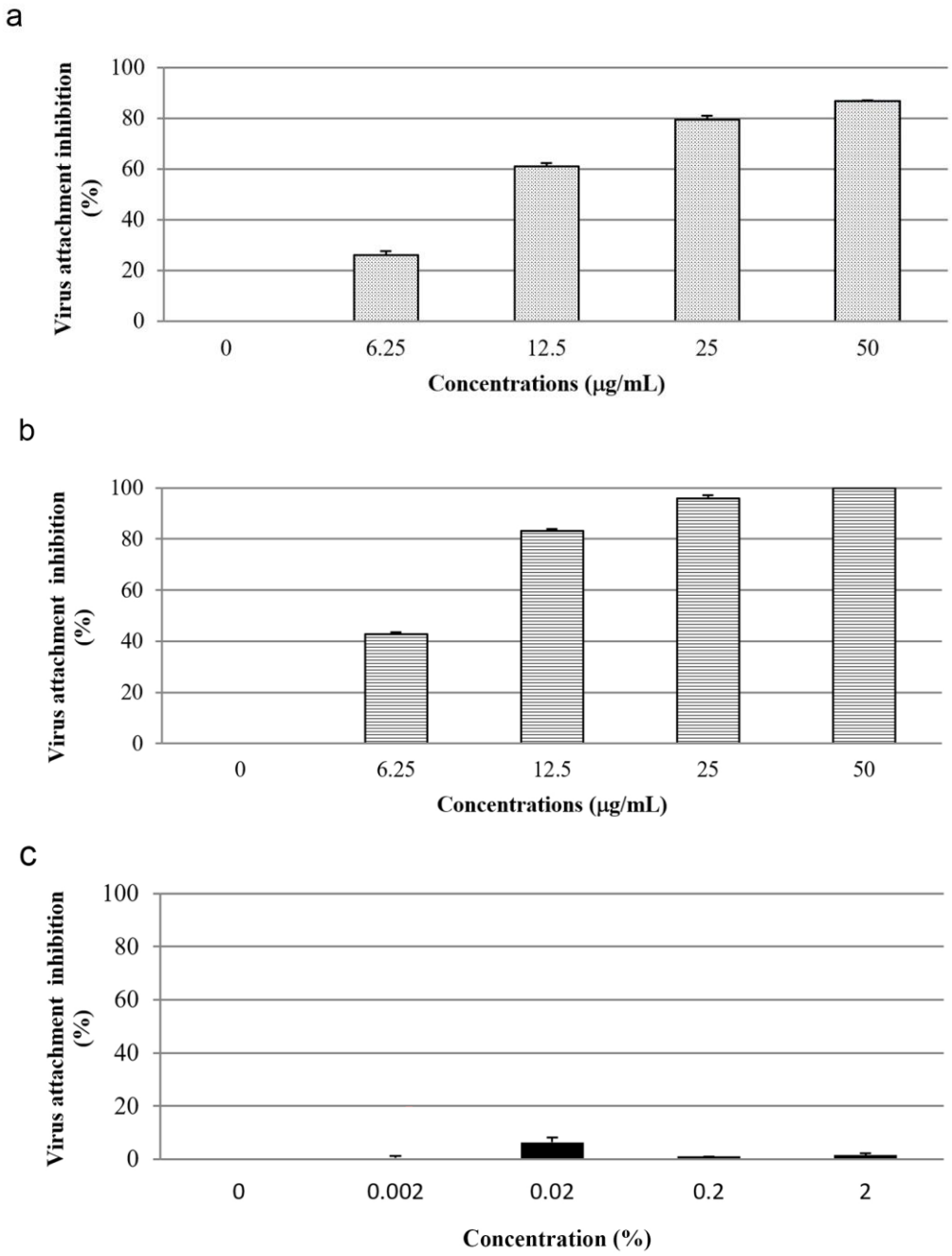


**Figure 3.** Inhibition of virus attachment by *A. paniculata* and andrographolide; (a) *A. paniculata*, (b) andrographolide, or (c) DMSO in serial dilution with JEV (100 PFU) (n=3)

## 4. DISCUSSION

Cytotoxicity and antiviral activity of methanolic *A. paniculata* crude extract and its purified andrographolide against JEV yields were evaluated using MTT colorimetric assay and plaque reduction neutralization test (PRNT) assay. The study demonstrated that methanolic *A. paniculata* crude extract and andrographolide had less cytotoxicity in LLC-MK2 than SK-N-MC. Both *A. paniculata* and andrographolide inhibited JEV yields and their mechanisms in virus yield reduction, blockage of virus attachment, and virucidal activation in LLC-MK2 cells (Table 1 and Table 2). Our data were consistent with several studies, which showed antiviral activity of *A. paniculata* crude extract against RNA viruses such as HIV, human influenza virus (H1N1) and avian influenza virus (H5N1) (Chen et al., 2009; Uttekar et al., 2012; Cai et al., 2015). Both *A. paniculata* and andrographolide had virucidal activity through direct inactivation of virus particles by simultaneous treatment, inhibition of virus by pretreatment, and significant blockage of virus attachment and yield *in vitro*. Our results were consistent with Tang et al., (2012) who proposed that *A. paniculata* inhibited DV1 in Vero cells.

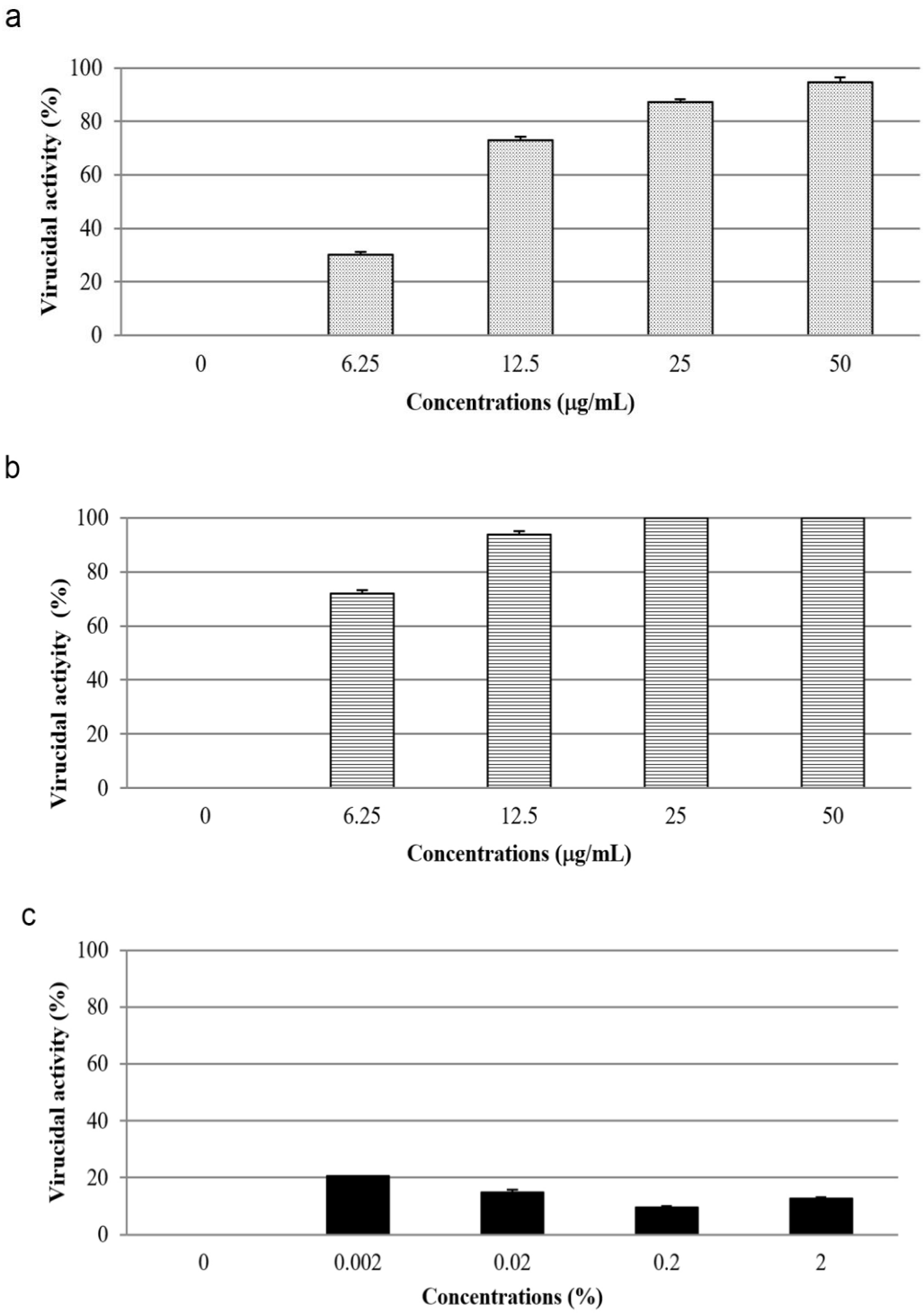


**Figure 4.** Virucidal activities of (a) *A. paniculata*, (b) andrographolide, or (c) DMSO (n=3)

Mode of antiviral action is blocking of viral adsorption and suppression of viral replication. Our study revealed that *A. paniculata* crude extract and andrographolide interfered JEV replication. Andrographolide had anti-Epstein-Barr virus activity by inhibition of lytic proteins in human B lymphocytes (P3HR1) (Lin et al., 2008). Andrographolide had anti-HCV activity by up-regulating haeme oxygenase-1 via the p38 MAPK/ Nrf2 pathway in human hepatoma cells. Increased interferon inhibited the activity of NS3/4A protease led to inhibition of HCV replication (Lee et al., 2014). The 14-deoxy-11, 12-dehydroandrographolide (DAP), a metabolite of andrographolide, inhibited H5N1 virus replication in human lung epithelial (A549) cell by suppression of viral mRNA, nucleoprotein and non structural protein 1 (NS1) protein. Andrographolide inhibited HIV gp120 at V3 loop region, which interfered virus attachment through binding of CD4 with co-receptor CCR5 & CXCR4 on surface of the human HeLa derivative TZM-bl cells, hence this virus could

not infect cells (Uttekar et al., 2012). The *A. paniculata* crude extract had anti-dengue activity on E protein target by blocking of cell membrane adhesion (Tang et al., 2012). JEV, like dengue virus, was classified as a member of the genus flavivirus within the Flaviviridae family, we therefore purposed that *A. paniculata* and andrographolide have anti-JEV activity by interfering E protein adhesion lead to inhibition of JE viral replication.

## 5. CONCLUSION

Methanolic *A. paniculata* crude extract and its purified andrographolide inhibit JEV by a means of PRNT *in vitro*. The action modes of *A. paniculata* and andrographolide are virucidal activity and blockage of viral adsorption.

## ACKNOWLEDGMENT

This study was supported by the Science Grants No. SRF-JRG-2557-3 from Department of Biology, Faculty of Science, Silpakorn University, Nakhon Pathom, Thailand.

## REFERENCES

Ali-Seyed, M., and Vijayaraghavan, K. (2020). Dengue virus infections and anti-dengue virus activities of *Andrographis paniculata*. *Asian Pacific Journal of Tropical Medicine*, 13(2), 49-55.

Aromdee, C., Suebsasana, S., Ekalaksananan, T., Pientong, C., and Thongchai, S. (2011). Stage of action of naturally occurring andrographolides and their semisynthetic analogues against herpes simplex virus type 1 in vitro. *Planta Medica*, 77(9), 915-921.

Cai, W., Li, Y., Chen, S., Wang, M., Zhang, A., Zhou, H., Chen, H., and Jin, M. (2015). 14-Deoxy-11,12-dehydroandrographolide exerts anti-influenza A virus activity and inhibits replication of H5N1 virus by restraining nuclear export of viral ribonucleoprotein complexes. *Antiviral Research*, 118, 82-92.

Chen, J. X., Xue, H. J., Ye, W. C., Fang, B. H., Liu, Y. H, Yuan, S. H., Yu, P., and Wang, Y. Q. (2009). Activity of andrographolide and its derivatives against influenza virus in vivo and in vitro. *Biological and Pharmaceutical Bulletin*, 32(8), 1385-1391.

Lee, J. C, Tseng, C. K, Young, K. C, Sun, H. Y., Wang, S. W., Chen, W. C, Lin, C. K., and Wu, Y. H. (2014). Andrographolide exerts anti-hepatitis C virus activity by up-regulating haeme oxygenase-1 via the p38 MAPK/Nrf2 pathway in human hepatoma cells. *British Journal of Pharmacology*, 171(1), 237-252.

Lin, T. P., Chen, S. Y., Duh, P. D., Chang, L. K., and Liu, Y. N. (2008). Inhibition of the Epstein-Barr virus lytic cycle by andrographolide. *Biological and Pharmaceutical Bulletin*, 31(11), 2018-2023.

Luangboribun, T., Tiewcharoen, S., Rabablert, J., Auewarakul, P., Lumlerdkii, N., Junnu, V., and Roytrakul, S. (2014). Screening of anti-amoebic activity on *Naegleria fowleri* in extracts of Thai medicinal plants. In *Proceedings of Joint International Tropical Medicine Meeting 2013*, pp. 16-22. Bangkok, Thailand.

Priengprom, T., Ekalaksananan, T., Kongyingyoes, B., Suebsasana, S., Aromdee, C., and Pientong, C. (2015). Synergistic effects of acyclovir and 3, 19-isopropylideneandrographolide on herpes simplex virus wild types and drug-resistant strains. *BMC Complementary and Alternative Medicine*, 15, 56.

Reddy, V. L. N, Reddy, S. M., Ravikanth, V., Krishnaiah, P., Goud, T. V., Rao, T. P., Ram, T. S., Gonnade, R. G., Bhadbhade, M., and Venkateswarlu, Y. (2005). A new bis-andrographolide ether from *Andrographis paniculata* nees and evaluation of anti-HIV activity. *Natural Product Research*, 19(3), 223-230.

Roy, S., Chaurvedi, P., and Chowdhary, A. (2015). Evaluation of antiviral activity of essential oil of *Trachyspermum ammi* against Japanese encephalitis virus. *Pharmacognosy Research*, 7(3), 263-267.

Schuh, A. J., Ward, M. J., Brown A. J. L., and Barret, A. D. T. (2014). Dynamics of the emergence and establishment of a newly dominant genotype of Japanese encephalitis virus throughout Asia. *Journal of Virology*, 88(8), 4522-4532.

Seubsasana, S., Pientong, C., Ekalaksananan, T., Thongchai, S., and Aromdee, C. (2011). A potential andrographolide analogue against the replication of herpes simplex virus type 1 in Vero cells. *Medicinal Chemistry*, 7(3), 237-244.

Tang, L. I., Ling, A. P., Koh, R. Y., Chye, S. M., and Voon, K. G. (2012). Screening of anti-dengue activity in methanolic extracts of medicinal plants. *BMC Complementary and Alternative Medicine*, 12(1), 3.

Thakur, A. K., Chatterjee, S. S., and Kumar, V. (2012a). General neuropharmacological screening of standardized extract of *Andrographis paniculata* in rodents. In *Proceedings of Annual Conference of Indian Academy of Neurosciences (IAN) with Annals of Neurosciences*, pp. 36-37. Amritsar, India.

Thakur, M., Weng, A., Fuchs, H., Sharma, V., Bhargava, C. S., Chauhan, N. S., Dixit, V. K., and Bhargava, S. (2012b). Rasayana properties of Ayurvedic herbs: are polysaccharides a major contributor. *Carbohydrate Polymers*, 87(1), 3-15.

Uttekar, M. M., Das, T., Pawar, R. S., Bhandari, B., Menon, V., Nutan, F., Gupta, S. K., and Bhat, S. V. (2012). Anti-HIV activity of semisynthetic derivatives of andrographolide and computational study of HIV-1 gp120 protein binding. *European Journal of Medicinal Chemistry*, 56, 368-374.

Wang, H., and Liang, G. (2015). Epidemiology of Japanese encephalitis: past, present, and future prospects. *Therapeutics and Clinical Risk Management,* 11, 435-448.

Wen, L., Xia, N., Chen, X., Li, Y., Hong, Y., Liu, Y. J., Wang, Z. Z., and Liu, Y. J. (2014). Activity of antibacterial, antiviral, anti-inflammatory in compounds andrographolide salt. *European Journal of Pharmacology*, 740(5), 421-427.

Yoksan, S., Rabablert, J., Chaiyo, K., Rajchakam, S., Tiewcharoen, S., Rabablert, N., Kerdkriangkrai, S., Samngamnim, N., Phurttikul, W., and Luangboribun, T. (2013). Cytokine gene expression in human hepatocytes infected with dengue virus serotype 3 (strain-16562). *Health*, 5(9), 1516-1525.

Yu, B., Dai, C. Q., Jiang, Z. Y., Li, E. Q., Chen, C., Wu, X. L., Chen, J., Liu, Q., Zhao, C. L., He, J. X., Ju, D. H., and Chen, X. Y. (2014). Andrographolide as an anti-H1N1 drug and the mechanism related to retinoic acid-inducible gene-I-like receptors signaling pathway. *Chinese Journal of Integrative Medicine*, 20(7), 540-545.