

In vitro Screening Antiviral Activity of Thai Medicinal Plants Against Porcine Reproductive and Respiratory Syndrome Virus

Chaiwat Arjin

Chiang Mai University Faculty of Agriculture Department of Animal and Aquatic Sciences

Kidsadagon Pringproa

Chiang Mai University Faculty of Veterinary Medicine

Surat Hongsibsong

Chiang Mai University Research Institute for Health Sciences

Warintorn Ruksiriwanich

Chiang Mai University Faculty of Pharmacy

Mintra Seel-audom

Chiang Mai University Faculty of Agriculture

Supamit Mekchay

Chiang Mai University Faculty of Agriculture

Korawan Sringarm (✉ kanok70@hotmail.com)

Chiang Mai University Faculty of Agriculture <https://orcid.org/0000-0003-0089-8183>

Research article

Keywords: porcine reproductive and respiratory syndrome, porcine reproductive and respiratory syndrome virus, antiviral activity, Thai medicinal plants

Posted Date: January 20th, 2020

DOI: <https://doi.org/10.21203/rs.2.21317/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Version of Record: A version of this preprint was published at BMC Veterinary Research on March 30th, 2020. See the published version at <https://doi.org/10.1186/s12917-020-02320-8>.

Abstract

Background

Porcine reproductive and respiratory syndrome (PRRS) caused by the PRRS virus (PRRSV) results in economic losses in the swine industry globally. Several studies have investigated the use of plant extracts for the prevention and control of PRRS outbreaks. Thai medicinal plants may be useful for treating PRRSV infection in pigs. Therefore, we investigated the *in vitro* anti-PRRSV and antioxidant properties of seven Thai medicinal plants: *Caesalpinia sappan* Linn., *Garcinia mangostana* Linn., *Houttuynia cordata*, *Perilla frutescens*, *Clinacanthus nutans*, *Phyllanthus emblica*, and *Tiliacora triandra*.

Results

In the antiviral screening test, we observed that the *T. triandra* extract strongly inhibited the infectivity of PRRSV into MARC-145 cells [virus titer 3.5 median tissue culture infectious doses (TCID₅₀)/ml (log₁₀)] at 24 h post-infection, whereas the *C. sappan* Linn. extract strongly inhibited PRRSV replication [virus titer 2.5 TCID₅₀/ml (log₁₀)] at 72 h postinfection. *C. sappan* Linn. extract had the highest total phenol content [220.52 mM gallic acid equivalents (GAE)/g] and the lowest half-maximal inhibitory concentration [IC₅₀; 1.17 mg/ml in 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2.58 mg/ml in 2,2-azino-bis (3-ethylbenzothiazole-6-sulfonic acid) diammonium salt (ABTS)].

Conclusion

Our study showed that *T. triandra* could inhibit the infectivity of PRRSV, whereas *C. sappan* Linn was the most effective for inhibiting PRRSV replication in MARC-145 cells. This study elucidates the antiviral activities of Thai medicinal plant extracts *in vivo*. The result promising that the extracts of Thai medicinal plants, especially that of *T. triandra* and *C. sappan* Linn, can be developed into pharmaceutical drugs for the prevention of PRRS in pigs.

Background

The porcine reproductive and respiratory syndrome virus (PRRSV) is endemic in most pig-producing countries, and it results in enormous economic losses to the swine industry globally [1]. This enveloped, positive-sense, single-stranded RNA virus belongs to the family Arteriviridae (order Nidovirales), which also includes the equine arteritis virus, mouse lactate dehydrogenase-elevating virus, and simian hemorrhagic fever virus [2]. In general, infection with PRRSV causes a disease that is characterized by reproductive failure in sows and respiratory infections in growing pigs [3] and this disease also predisposes the pigs to infection by bacterial and other viral pathogens [4, 5]. This disease is known as the porcine reproductive and respiratory syndrome (PRRS) and has become endemic in many countries throughout the world following an epidemic phase [6, 7]. The incidence of PRRS was first reported in Thailand in 1989, since then several outbreaks have been reported [8], and it has become a major

infectious disease that causes high mortality in swine and production losses in the swine industry in this country.

Although preventative measures such as the acclimatization of gilts, vigilant biosecurity, and vaccination have been shown to be useful in controlling the outbreaks of PRRS, and supportive treatments are available for alleviating its severity; no specific treatment for PRRS is currently available [9, 10]. Antiviral therapeutics are a critical tool for combating viral infections, particularly in cases wherein no vaccines are available against the circulating virus. Thus, pharmacological intervention may represent as an alternative approach in controlling PRRSV. A number of natural compounds and compositions have been shown to possess antiviral activities against PRRSV. Gao et al. [11] showed that the *Cryptoporus volvatus* extract exhibited antiviral activity against PRRSV infection and replication. Pringproa et al. [12] reported that the crude extract of *Cynodon dactylon* significantly inhibited the replication of PRRSV as early as 24 h postinfection (hpi). Therefore, the antiviral activities of other Thai medicinal plants against PRRSV should also be investigated. Thai medicinal plants such as *Caesalpinia sappan* Linn., *Garcinia mangostana* Linn., *Houttuynia cordata*, *Perilla frutescens*, *Clinacanthus nutans*, *Phyllanthus emblica*, and *Tiliacora triandra* are known to have antioxidant activities and antiviral activities. These plants have already been promoted for use in primary health care and have been classified according to their pharmacological actions [13–18]. Therefore, the aim of this study was to determine the antiviral activities of Thai medicinal plant extracts against PRRSV infection in vitro and to measure their phytochemical contents to develop an alternative anti-PRRSV therapy for use in veterinary medicine.

Results

Cytotoxic activities of the plant extracts

Prior to determining the antiviral activity, we evaluated the cytotoxicity of the seven Thai medicinal plant extracts on the viability of MARC-145 cells, and the viability was shown as 50% cytotoxic concentrations (CC_{50}). The results showed that the cytotoxic concentration CC_{50} of the seven Thai medicinal plant extracts ranged from 78 $\mu\text{g/ml}$ to 2,500 $\mu\text{g/ml}$, and the effect of the concentration of the Thai medicinal plant extracts on the tested cells increased in a dose-dependent manner (Fig. 1). *P. emblica* had the lowest CC_{50} concentration of 78 $\mu\text{g/ml}$. The CC_{50} of *G. mangostana* Linn. was the second lowest concentration (312.5 $\mu\text{g/ml}$) and that of *C. sappan* Linn. was 625 $\mu\text{g/ml}$. In this study, we found that *T. triandra* and *H. cordata* had CC_{50} of 1,250 $\mu\text{g/ml}$, whereas *C. nutans* and *P. frutescens* showed the highest CC_{50} concentration (2,500 $\mu\text{g/ml}$).

Inhibition of the PRRSSV infection by the extracts of the Thai medicinal plants

We treated the virus with different concentrations of Thai medicinal plant extracts by using a range of concentrations that were determined based on their CC_{50} values so that these plant extracts did not

affect the proliferative activity of MARC-145 cells. The screening results of the inhibition of virus infection activity showed the potential of the Thai medicinal plant extracts to inhibit the infectivity of PRRSV (Fig. 2). *T. triandra* significantly inhibited the infectivity of PRRSV into MARC-145 cells at 24 hpi when supplied at a concentration of 1,250 µg/ml ($P < 0.05$), and the observed virus titer at this concentration was 3.5 TCID₅₀/ml ($\log_{10}$). Interestingly, *P. emblica* at a low concentration of 78 µg/ml could inhibit the infectivity of PRRSV [virus titer = 4.5 TCID₅₀/ml($\log_{10}$)]. As shown in Fig. 3, the immunoperoxidase monolayer assay (IPMA) indicated that *T. triandra* and *P. emblica* blocked the infectivity of PRRSV in MARC-145 cells, as shown by the slight brown staining in the cells.

Thai medicinal plant extracts inhibit PRRSV replication

Different Thai medicinal plant extracts were tested in an in vitro inhibition screening assay to determine their inhibition of PRRSV replication at three time intervals (24, 48, and 72 hpi). At various time points after the infection, PRRSVs in the supernatants were quantified for determining the virus titer by IPMA. The screening results were the same as that of the inhibition test of virus infectivity, i.e., the PRRSV replication was inhibited in a dose-dependent manner (Fig. 4). Interestingly, as shown in Fig. 5, we found that *C. sappan* Linn. had significant potential to inhibit PRRSV replication in vitro. As shown in Fig. 5L, few cells that were stained brown showed the efficiency of *C. sappan* Linn. at a concentration of 625 µg/ml, and the inhibition of the replication activity of PRRSV by *C. sappan* Linn. was significantly stronger than that by the other plant extracts at 72 hpi [2.7 TCID₅₀/ml ($\log_{10}$)].

Phytochemical contents of Thai medicinal plant extracts

The total phenol contents of the seven Thai medicinal plant extracts were determined using the Folin–Ciocalteu assay by constructing a standard curve of gallic acid. The total phenol content was the highest in *C. sappan* Linn. [mean $\pm$ standard error: 220.52 $\pm$ 4.47 mM gallic acid equivalents (GAE)/g sample], followed by *G. mangostana* Linn. (91.16 $\pm$ 4.62 mM GAE/g sample), and the lowest phenol content was observed in *H. cordata* (8.51 $\pm$ 0.04 mM GAE/g sample) (Table 1).

Table 1

Total phenol contents and antioxidant activities of seven Thai medicinal plant extracts.

	Total phenol (mM GAE/g)	DPPH (IC ₅₀ , mg/ml)	ABTS (IC ₅₀ , mg/ml)	FRAP (mM Fe ²⁺ /g)
Caesalpinia sappan	220.52 ± 4.47	1.17 ± 0.06	2.57 ± 0.16	334.78 ± 13.15
Garcinia mangostana	91.16 ± 4.62	4.82 ± 0.58	4.98 ± 0.10	46.12 ± 1.27
Houttuynia cordata	14.25 ± 0.20	97.79 ± 4.14	72.02 ± 4.01	8.55 ± 0.18
Perilla frutescens	29.86 ± 0.41	11.68 ± 0.51	21.37 ± 1.28	43.32 ± 0.92
Clinacanthus nutans	25.52 ± 0.22	50.34 ± 5.60	37.82 ± 1.25	18.39 ± 0.54
Phyllanthus emblica	44.35 ± 0.24	3.49 ± 0.17	4.95 ± 0.11	94.17 ± 0.62
Tiliacora triandra	30.45 ± 1.51	17.77 ± 0.22	21.16 ± 1.06	30.58 ± 1.13
DPPH, 2,2-diphenyl-1-picrylhydrazyl; ABTS, 2,2-azino-bis (3-ethylbenzothiazole-6-sulfonic acid) diammonium salt; FRAP, ferric reducing antioxidant power; GAE, gallic acid equivalents; IC ₅₀ , half maximal inhibitory concentration.				

Antioxidant activities

The *C. sappan* Linn. extract had the highest antioxidant activity, with IC₅₀ values of 1.17 ± 0.06 mg/ml in 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2.57 ± 0.16 mg/ml IC₅₀ in 2,2-azino-bis (3-ethylbenzothiazole-6-sulfonic acid) diammonium salt (ABTS) and a reducing power of 334.78 ± 13.15 mM Fe²⁺/g in the ferric reducing antioxidant power (FRAP) assay (Table 1). The *P. emblica* extract had the second strongest antioxidant activity against free radicals, with IC₅₀ values of 3.49 ± 0.17 mg/ml in DPPH and 4.95 ± 0.11 mg/ml in ABTS and a reducing power of 94.17 ± 0.62 mM Fe²⁺/g sample in the FRAP assay.

Discussion

PRRS virus outbreaks causes significant economic loss in the pig production industry worldwide. The current commercial PRRSV vaccines are inadequate to protect pigs from virus infections [19]. Medicinal plants have progressively been explored as suitable alternative sources of antiviral agents [20]. Thai medicinal plants have widely been used as a source of herbal medicines because of their high bioactive compound contents that are effective against various diseases. In this study, seven Thai medicinal plant extracts were screened for their antiviral activity against PRRSV.

Before determining the antiviral properties of a compound, it is essential that a cytotoxicity assay is performed to determine the concentrations that can be used to avoid cell damage and ensure selectivity for the virus *in vitro*. In this study, we report the cytotoxicity as CC₅₀, which indicates the concentration of

a substance that can inhibit viral activity by 50%. We found that *P. emblica* showed the highest cell toxicity (78.1 µg/ml). The high potential plant extracts in this study were found to be *C. sappan* Linn. and *T. triandra*, with CC₅₀ concentrations of 625 and 1,250 µg/ml, respectively. Antiviral compounds should be highly effective while showing minimal toxicity to normal cells and tissues [21].

In this study, we investigated the antiviral activity of seven Thai medicinal plant extracts against PRRSV by checking the inhibition of virus infection and virus replication in MARC-145 cells. The range of concentrations of the plant extracts were determined based on their CC₅₀ values. The *P. emblica* extract inhibited PRRSV infection in MARC-145 cells, as well as *in vitro*. *P. emblica* at a concentration of 78 µg/ml inhibited the infectivity of PRRSV at a virus titer of 4.5 TCID₅₀/ml (log₁₀). In this study, *P. emblica* showed the highest cytotoxicity toward MARC-145 cells with CC₅₀ of <100 µg/ml. Therefore, the antiviral activity of other plant extracts were investigated in this study. We found that *T. triandra* at a concentration of 1,250 µg/ml significantly inhibited PRRSV infectivity at a virus titer 3.5 TCID₅₀ (log₁₀). While *T. triandra* has been used as an anti-inflammatory agent [22], anticancer agent [23], and antimicrobial agent against *Mycobacterium tuberculosis* [24], its antiviral activity, particularly against PRRSV, has not been investigated previously. Therefore, this is the first report to indicate that *T. triandra* could significantly prevent the entry of PRRSV into MARC-145 cells. However, *T. triandra* was not found to be effective in inhibiting PRRSV replication. All the plant extracts used in this study could inhibit PRRSV replication when applied at high concentrations, as shown by the linear regression model from 24 to 72 hpi after incubation with PRRSV. *C. sappan* Linn. at 625 µg/ml could inhibit PRRSV replication as 72 hpi [virus titer 2.7 TCID₅₀ (log₁₀)]. Although the antiviral activity of *C. sappan* Linn. against the influenza virus [13] and the antimicrobial properties of *C. sappan* Linn. [25] have previously been investigated, this is the first study to reveal the inhibitory activity of *C. sappan* Linn. on PRRSV replication in MARC-145 cells.

In terms of the phytochemical contents, *C. sappan* Linn. extract had the highest total phenol content (220.52 ± 4.47 mM GAE/g sample). The total phenol content of a plant is considered to be an indicator of its antioxidant capacity because the redox properties of the phenolic compounds allow them to act as reducing agents, hydrogen donors, and radical scavengers [20]. Previously, Lee et al. [21] reported that ethanolic *C. sappan* Linn. extract had a total phenol content of 723.67 µg GAE/mg. The values of the total phenol content in this study varied slightly compared with those reported previously in the literature. This may be because of the different durations, geographical variations, or methods of extraction, which may have altered the amounts of the phenol content. Ethanolic plant extracts can be used for the investigation of the antiviral activity in a cell line. Abu-Jafar and Huleihel [26] reported that ethanolic extracts of *Eucalyptus camaldulensis* leaves had strong antiviral activity against different members of the herpes virus family (HSV-1, HSV-2 and VZV). Ramalingam et al. [27] reported that the ethanolic extracts of *Andrographis paniculata* have the highest antiviral inhibitory effects against dengue virus in Vero cells.

The screening of plants as possible sources of antiviral agents has led to the discovery of potent inhibitors of *in vitro* viral replication, thereby increasing the probability of identifying new bioactive plant

compounds [28]. These findings suggest an appropriate species and the concentration of the plant extract that could effectively inhibit PRRSV replication with both *T. triandra* and *C. sappan* Linn. being highly effective in inhibiting PRRSV infection *in vitro* by interfering with viral attachment and inhibiting viral replication and/or virus release, respectively. The modes of action of *T. triandra* and *C. sappan* Linn. against PRRSV require further investigation but are likely to be related to the natural compounds they contain. Therefore, it was speculated that both *T. triandra* and *C. sappan* Linn. might have the potential to become candidates for preventing PRRSV infection in pigs. However, the plant extracts used for testing the antiviral activity was a crude extract. In future, we would like to purify the most effective Thai medicinal plant extract (*T. triandra* and *C. sappan* Linn.) for screening the active compound that is highly effective against PRRSV.

Conclusion

The findings of this study show that Thai medicinal plant extracts exhibit antiviral activity against PRRSV, and *T. triandra* effectively inhibiting PRRSV infection and *C. sappan* Linn. having the strongest antiviral activity against PRRSV replication. These activities can be presumably attributed to the total phenol contents and the antioxidant activities of these plant extracts. Although several previous studies have shown the antiviral activity of plant extracts against PRRSV, but there are no reports on the antiviral activities of *T. triandra* and *C. sappan* Linn. extracts against PRRSV. To the best of our knowledge, this study is the first to report the inhibitory activity of *T. triandra* and *C. sappan* Linn. against PRRSV activity *in vitro*. Further studies are required to elucidate the mechanisms of action of these plant extracts on PRRSV.

Methods

Chemicals

All of the chemicals that were used in this study were of analytical grade or higher. Ethanol and methanol were obtained from Merck (Darmstadt, Germany). 2,2-Azino-bis(3-ethylbenzothiazol-6-sulfonic acid) diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2-diphenyl-1-picryl hydrazyl (DPPH), Folin & Ciocalteu phenol reagent, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), sodium carbonate, and 2,4,6-tri-pyridyl-s-triazine (TPTZ) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ferric chloride hexahydrate and potassium persulfate were procured from LOBA CHEMIE PVT (Mumbai, India). Gallic acid was procured from Fluka Chemical Co. (Buchs, Switzerland). Dulbecco's modified Eagle's medium (DMEM) was procured from Gibco (Massachusetts, USA).

Plant extracts, cells, and viruses

Ethanollic extracts from the Thai medicinal plants *C. sappan* Linn., *G. mangostana* Linn., *H. cordata*, *P. frutescens*, *C. nutans*, *P. emblica*, and *T. triandra* were purchased from Specialty Natural Product Co. Ltd. (Thailand).

MARC-145 tissue culture cells were grown in Dulbecco's modified Engle medium (DMEM) containing 10% fetal bovine serum (Gibco) and 1% Penicillin/Streptomycin and incubated at 37°C in a 5% CO₂ atmosphere. To produce inoculated cells, PRRSV (VR2332 North American genotype) was propagated in the MARC-145 cells and the virus titer was quantified using the virus titer by immunoperoxidase monolayer assay (IPMA).

Cytotoxicity assay

The cytotoxicity of the seven Thai medicinal plant extracts was determined using the MTT assay. Briefly, MARC-145 cells were plated at a density of 5,000 cells/well in 96-well plates and incubated in a 5% CO₂ atmosphere at 37°C for 24 h. When the cells were at least 90% confluent, the medium was removed and replaced with medium containing two-fold serial dilutions of the plant extracts. In addition, medium without plant extract was used as a positive control. Incubation was then continued in a 5% CO₂ atmosphere at 37°C for 72 h. After this time, the medium was removed, 20 µl of freshly prepared MTT solution (5 mg/ml) was added to each well, and the plates were incubated at 37°C for 4 h. The medium was then replaced with 150 µl DMSO to dissolve the crystals and the plates were incubated at 37°C for 5 min to dissolve any air bubbles before measuring the MTT signal at an absorbance of 550 nm. The results were reported as 50% cytotoxic concentrations (CC₅₀).

Inhibition of virus infection assay

The inhibition of virus infection test was evaluated as previously described [12]. Briefly, the plant extracts at the concentration that was determined in the cytotoxicity test outlined above, as well as two lower level concentrations in two-fold dilution were mixed with PRRSV at 10⁸ TCID₅₀/ml in the ratio of 1:1 and incubated for 1 h at 37°C. DMSO (1%) containing medium mixed with PRRSV served as the control. Thereafter, the mixture of virus and plant extracts, as well as controls were inoculated into MARC-145 cells as 5,000 cells/well in a 96-well plate and incubated at 37°C for 1 h. Subsequently, the medium was removed and replaced with a fresh medium containing 10% FBS. The plates with MARC-145 cells were cultured under standard conditions for 24 h post-infection (hpi) and then the supernatants were collected to quantify the virus titer.

Inhibition of virus replication assay

The inhibition of virus replication test was performed as previously described [12]. Briefly, MARC-145 cells were plated at a density of 5,000 cells/well in 96-well plates and infected with PRRSV at a multiplicity of

infection (MOI) of 1 for 1 h at 37°C. The virus was then removed from each of the wells and replaced with the diluted plant extracts at the concentration that was determined in the cytotoxicity test, as well as two lower levels concentrations in two-fold dilution. Also, 1% DMSO was mixed to medium as a control. The plates were cultured under standard conditions, and the supernatants were collected at 24, 48, and 72 hpi and quantified the virus titer.

Virus Titer

The virus titer was further assessed by Immunoperoxidase Monolayer Assay (IPMA) was adapted from [29]. Briefly, cells were fixed with 100 µl cold 4% formalin for 15 min at room temperature (RT), washed once with 100 µl phosphate-buffered saline (PBS) and twice with 100 µl of 0.5% PBS Tween-20 (PBST), and then blocked with 100 µl of 1% BSA in 0.5% PBST for 30 min at RT. After blocking, the cells were stained with 70 µl anti-PRRSV NC protein monoclonal antibody (Median Diagnostics, Gangwon-do, Korea) diluted 1:400 for 60 min at RT and then washed and incubated with Peroxidase-conjugated AffiniPure Goat Anti-Mouse IgG (H+L) (Jackson ImmunoResearch, Pennsylvania, USA) diluted 1:1,200 for 60 min at RT. After washing three times in PBS, the cells were counter-stained with 1,5-diaminopentane (DAP) substrate and examined under a microscope. The virus titer was shown at the viral median tissue culture infectious dose (TCID₅₀) was then determined using the Reed–Muench method.

Phytochemical analysis

The total phenolic contents of the plant extracts were determined using the Folin–Ciocalteu method [30], and their free radical scavenging activities were determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging assay and 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) scavenging assay, as previously reported [31,32]. The antioxidant activities were determined using the ferric reducing antioxidant power (FRAP) assay, according to the Benzie and Strain method [33].

Statistical analyses

Differences in the antiviral activities among different concentrations of each plant extract were tested using one-way analysis of variance (ANOVA) with Tukey's post hoc test for a comparison of the means. The CC₅₀ was calculated through regression analysis of the dose-response curves for the MTT assay. All statistical analyzes were performed using the SPSS 23.0 software (SPSS Inc., Chicago, IL, USA) with a significance level of $P \leq 0.05$.

Declarations

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgment

The authors thank Dr. Wolfram Spreer of the University of Hohenheim for his critical comments on this article. This project was supported by research and researcher for industries projects (RRi), Thailand science research and innovation, under contract no. PHD61I0042 and partially supported by Chiang Mai University

References

1. Zhang Q, Yoo D. PRRS virus receptors and their role for pathogenesis. *Vet Microbiol* [Internet]. 2015;177(3–4):229–41. Available from: <http://dx.doi.org/10.1016/j.vetmic.2015.04.002>
2. Lunney JK, Fang Y, Ladinig A, Chen N, Li Y, Rowland B, et al. Porcine Reproductive and Respiratory Syndrome Virus (PRRSV): Pathogenesis and Interaction with the Immune System. *Annu Rev Anim Biosci* [Internet]. 2016;4(1):129–54. Available from: <http://www.annualreviews.org/doi/10.1146/annurev-animal-022114-111025>
3. Nilubol D, Tripipat T, Hoonsuwan T, Kortheerakul K. Porcine Reproductive and Respiratory Syndrome Virus, Thailand, 2010–2011. *Emerg Infect Dis* [Internet]. 2012;18(12):2039–43. Available from: http://wwwnc.cdc.gov/eid/article/18/12/11-1105_article.htm
4. Benfield DA, Nelson E, Collins JE, Harris L, Goyal SM, Robison D, et al. Characterization of Swine Infertility and Respiratory Syndrome (SIRS) Virus (Isolate ATCC VR-2332). *J Vet Diagnostic Investig* [Internet]. 1992 Apr 1;4(2):127–33. Available from: <https://doi.org/10.1177/104063879200400202>
5. PU X, LIANG J, SHANG R, WANG X, WANG Z, HUA L, et al. Influence of Hypericum perforatum Extract on Piglet Infected with Porcine Respiratory and Reproductive Syndrome Virus. *Agric Sci China* [Internet]. 2009;8(6):730–9. Available from: <http://www.sciencedirect.com/science/article/pii/S1671292708602722>
6. Albina E. Epidemiology of porcine reproductive and respiratory syndrome (PRRS): An overview. *Vet Microbiol* [Internet]. 1997;55(1):309–16. Available from: <http://www.sciencedirect.com/science/article/pii/S0378113596013223>
7. Thanapongtharm W, Linard C, Pamaranon N, Kawkalong S, Noimoh T, Chanachai K, et al. Spatial epidemiology of porcine reproductive and respiratory syndrome in Thailand. *BMC Vet Res* [Internet]. 2014 Aug 5;10:174. Available from: <https://www.ncbi.nlm.nih.gov/pubmed/25091559>
8. Damrongwatanapok S, Arsayuth K, Kongkrong C, Parchariyanon S, Pinyochon W TU. Serological studies and isolation of porcine reproductive and respiratory syndrome (PRRS) virus in Thailand. *J Thai Vet Med Assoc*. 1996;47:19–30.
9. Labarque G, Van Gucht S, Van Reeth K, Nauwynck H, Pensaert M. Respiratory tract protection upon challenge of pigs vaccinated with attenuated porcine reproductive and respiratory syndrome virus

- vaccines. Vet Microbiol [Internet]. 2003;95(3):187–97. Available from: <http://www.sciencedirect.com/science/article/pii/S0378113503001573>
10. Anantikulchai P, Emprom P, Pringproa K, Yamsakul P. In vitro Cytotoxicity Test and Antiviral Activity of Curcuminoids from Turmeric Extract Against PRRS Virus. 2017;15(November):199–205.
 11. Gao L, Zhang W, Sun Y, Yang Q, Ren J, Liu J, et al. Cryptoporus volvatus Extract Inhibits Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) In Vitro and In Vivo. PLoS One. 2013;8(5).
 12. Pringproa K, Khonghiran O, Kunanoppadol S. In Vitro Virucidal and Virustatic Properties of the Crude Extract of Cynodon dactylon against Porcine Reproductive and Respiratory Syndrome Virus In Vitro Virucidal and Virustatic Properties of the Crude Extract of Cynodon dactylon against Porcine Reprodu. 2014;(May).
 13. Liu A, Shu S, Qin H, Ming S, Lee Y, Wang Y, et al. In vitro Anti-Influenza Viral Activities of Constituents from Caesalpinia sappan. Planta Med [Internet]. 2009;75(4):337–9. Available from: doi: 10.1055/s-0028-1112208. Epub 2009 Jan 15.
 14. Shao-Xing Chen, Min Wan B-NL. Active Constituents Against HIV-1 Protease from Garcinia mangostana. Planta Med. 1996;62(4):381–2.
 15. Chiow KH, Phoon MC, Putti T, Tan BKH, Chow VT. Evaluation of antiviral activities of Houttuynia cordata Thunb. extract, quercetin, quercetrin and cinanserin on murine coronavirus and dengue virus infection. Asian Pac J Trop Med [Internet]. 2016;9(1):1–7. Available from: <http://www.sciencedirect.com/science/article/pii/S1995764515002230>
 16. Kawahata T, Otake T, Mori H, Kojima Y, Oishi I, Oka S, et al. A Novel Substance Purified from Perilla frutescens Britton Inhibits an Early Stage of HIV-1 Replication without Blocking Viral Adsorption. Antivir Chem Chemother [Internet]. 2002 Oct 1;13(5):283–8. Available from: <https://doi.org/10.1177/095632020201300503>
 17. Haetrakul T, Dunbar SG, Chansue N. Antiviral activities of Clinacanthus nutans (Burm.f.) Lindau extract against Cyprinid herpesvirus 3 in koi (Cyprinus carpio koi). J Fish Dis [Internet]. 2018 Apr 1;41(4):581–7. Available from: <https://doi.org/10.1111/jfd.12757>
 18. Xiang Y, Pei Y, Qu C, Lai Z, Ren Z, Yang K, et al. In vitro Anti-Herpes Simplex Virus Activity of 1,2,4,6-Tetra-O-galloyl- β -d-glucose from Phyllanthus emblica L. (Euphorbiaceae). Phyther Res [Internet]. 2011 Jul 1;25(7):975–82. Available from: <https://doi.org/10.1002/ptr.3368>
 19. Microbiol C, Feng J, Bai X, Cui T, Zhou H, Chen Y. In Vitro Antiviral Activity of Germacrone Against Porcine Reproductive and Respiratory Syndrome Virus. Curr Microbiol. 2016;
 20. Mehrbod P, Abdalla MA, Njoya EM, Ahmed AS, Fotouhi F, Farahmand B, et al. South African medicinal plant extracts active against influenza A virus. 2018;1–10.
 21. Adnan A, Allaudin ZN, Hani H, Loh H, Khoo T. Virucidal activity of Garcinia parvifolia leaf extracts in animal cell culture. 2019;5:1–10.
 22. Weerawatanakorn M, Rojsuntornkitti K, Pan M-H, Wongwaiwech D. Some Phytochemicals and Anti-inflammation Effect of Juice from *Tiliacora triandra* Leaves. J Food Nutr Res [Internet]. 2018 Jan 9;6(1):32–8. Available from: <http://pubs.sciepub.com/jfnr/6/1/6>

23. Rattana S, Cushnie B, Taepongsorat L, Phadungkit M. Chemical Constituents and in vitro anticancer activity of *Tiliacora triandra* leaves. *Pharmacogn J*. 2016;8(1).
24. Sureram S, Senadeera SPD, Hongmanee P, Mahidol C, Ruchirawat S, Kittakoop P. Antimycobacterial activity of bisbenzylisoquinoline alkaloids from *Tiliacora triandra* against multidrug-resistant isolates of *Mycobacterium tuberculosis*. *Bioorg Med Chem Lett [Internet]*. 2012;22(8):2902–5. Available from: <http://www.sciencedirect.com/science/article/pii/S0960894X12002454>
25. Srinivasan R, selvam GG, Karthik S, Mathivanan K, Baskaran R, Karthikeyan M, et al. In vitro antimicrobial activity of *Caesalpinia sappan* L. *Asian Pac J Trop Biomed [Internet]*. 2012;2(1, Supplement):S136–9. Available from: <http://www.sciencedirect.com/science/article/pii/S2221169112601440>
26. Abu-jafar A, Huleihel M. Antiviral activity of *Eucalyptus camaldulensis* leaves ethanolic extract on herpes viruses infection. 2017;1–9.
27. Ramalingam S, Karupannan S, Padmanaban P, Vijayan S, Sheriff K, Palani G. Anti - dengue activity of *Andrographis paniculata* extracts and quantification of dengue viral inhibition by SYBR green reverse transcription polymerase chain reaction. 2019;87–91.
28. Silveira VC, Products N. of The Brazilian Cerrado against the Avian. 2015;
29. Zhang J, Liu W, Chen W, Li C, Xie M, Bu Z. Development of an Immunoperoxidase Monolayer Assay for the Detection of Antibodies against Peste des Petits Ruminants Virus Based on BHK-21 Cell Line Stably Expressing the Goat Signaling Lymphocyte Activation Molecule. 2016;1–14.
30. Slinkard K, Singleton VL. Total Phenol Analysis: Automation and Comparison with Manual Methods. *Am J Enol Vitic [Internet]*. 1977;28(1):49–55. Available from: <http://www.ajevonline.org/content/28/1/49>
31. Brand-Williams W, Cuvelier ME, Berset C. Use of a free radical method to evaluate antioxidant activity. *LWT - Food Sci Technol [Internet]*. 1995;28(1):25–30. Available from: <http://www.sciencedirect.com/science/article/pii/S0023643895800085>
32. Binsan W, Benjakul S, Visessanguan W, Roytrakul S, Tanaka M, Kishimura H. Antioxidative activity of Mungoong, an extract paste, from the cephalothorax of white shrimp (*Litopenaeus vannamei*). *Food Chem [Internet]*. 2008;106(1):185–93. Available from: <http://www.sciencedirect.com/science/article/pii/S0308814607005250>
33. Benzie IFF, Strain JJBT-M in E. [2] Ferric reducing/antioxidant power assay: Direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. In: *Oxidants and Antioxidants Part A [Internet]*. Academic Press; 1999. p. 15–27. Available from: <http://www.sciencedirect.com/science/article/pii/S0076687999990055>

Figures

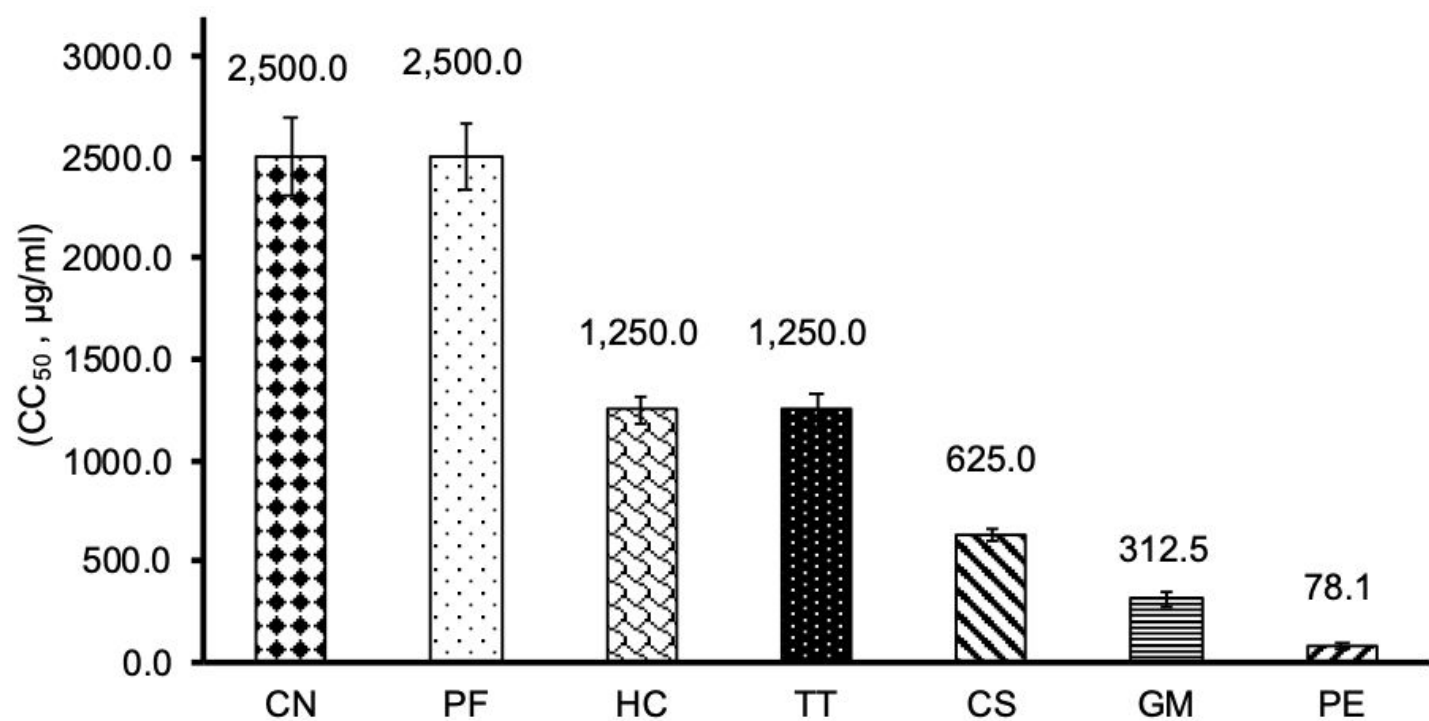


Figure 1

The cytotoxic activity of the seven Thai medicinal plant extracts in MARC-145 cells determined by the MTT assay. MARC-145 cells were incubated with various concentrations of the seven Thai medicinal plant extracts or the control without plant extract for 72 h prior to the MTT assay. Values are shown as means $\pm$ standard errors. CN, *C. nutans*; PF, *P. frutescens*; HC, *H. cordata*; TT, *T. triandra*; CS, *C. sappan* Linn.; GM, *G. mangostana* Linn. and PE, *P. emblica*; CC₅₀, 50% cytotoxic concentration.

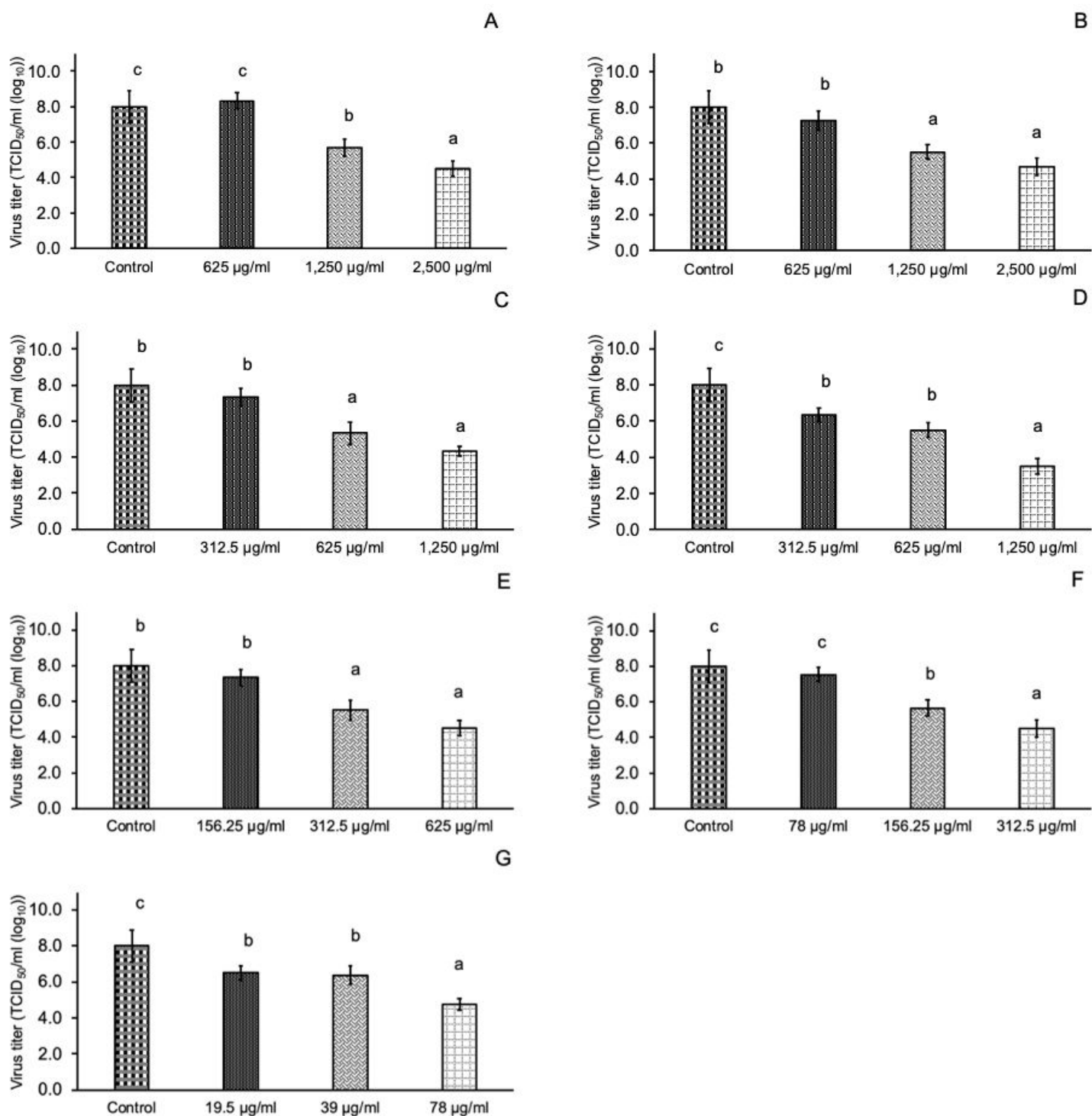


Figure 2

The virus titer of the inhibition of the viral infection activity of seven Thai medicinal plant extracts against PRRSV at 24-h postinfection (hpi). MARC-145 cells were infected with PRRSV at a multiplicity of infection of 1. Plant extracts were incubated with PRRSV for 1 h at 37°C before inoculation onto a monolayer of MARC-145 cells. At 24 hpi, the supernatants were collected to quantify the virus titer by the IPMA assay. The control was infected and virus titer was quantified as TCID₅₀ (log₁₀) = 8.0. The statistical analyses of the antiviral activity were performed using ANOVA. A, *C. nutans*; B, *P. frutescens*; C, *H. cordata*; D, T.

triandra; E, *C. sappan* Linn.; F, *G. mangostana* Linn. and G, *P. emblica*. a, b, and c; $P < 0.05$ compared with different concentrations of the plant extracts. All samples were analyzed in triplicate.

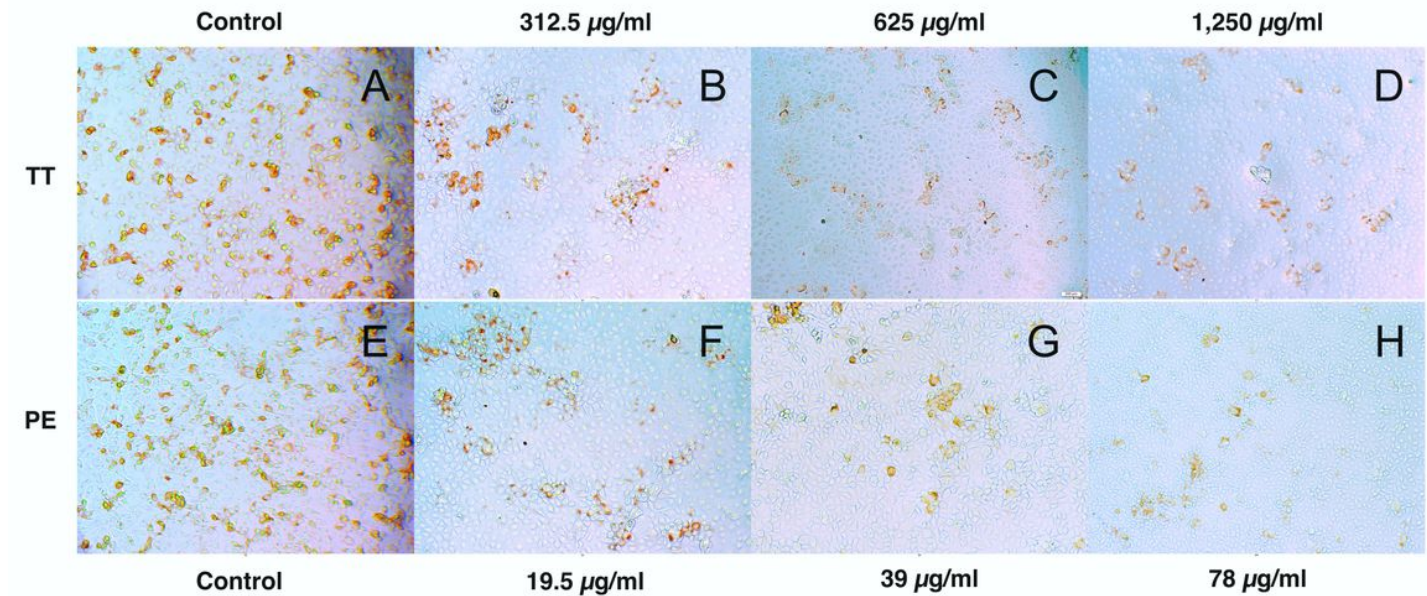


Figure 3

The immunoperoxidase monolayer assay (IPMA) showing the inhibition of PRRSV infection in MARC-145 cells by *T. triandra* (TT) at 312.5, 625, and 1,250 µg/ml (A-D) and *P. emblica* (PE) at 19.5, 39, and 78 µg/ml (E-H). *T. triandra* and *P. emblica* were incubated with PRRSV for 1 h at 37°C before inoculation onto a monolayer of MARC-145 cells. After 24 hpi, the supernatant was collected for quantification of the virus titer. The MARC-145 cells that were infected with PRRSV show brown staining. In the control, large numbers of PRRSV-infected cells were observed in the supernatants of the MARC-145 cells (A and E), while only few PRRSV-infected cells were observed in *T. triandra* and *P. emblica*.

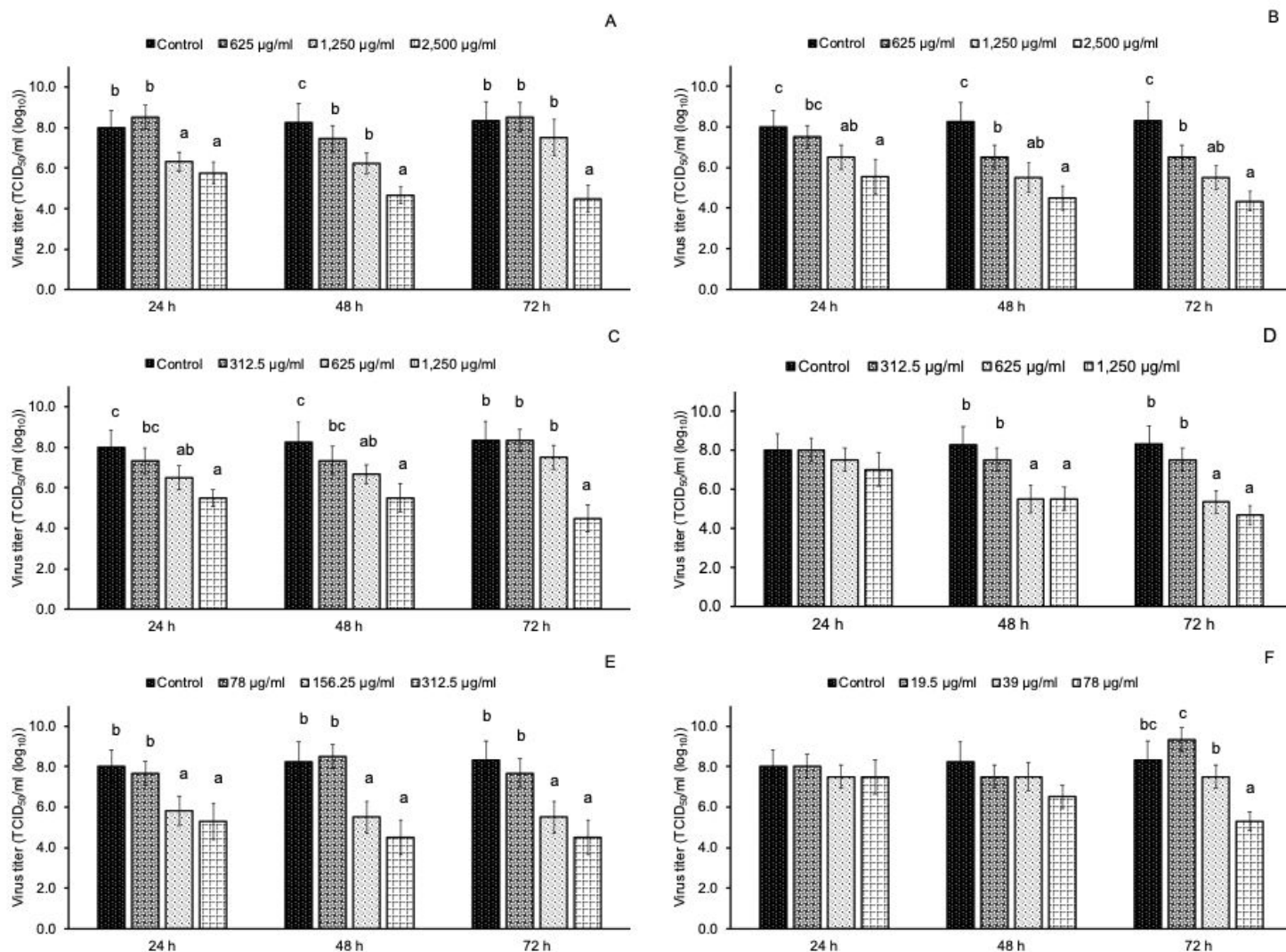


Figure 4

The virus titer in the inhibition of viral replication activity of Thai medicinal plant extracts against PRRSV at 24, 48, and 72-h post-infection (hpi). The MARC-145 cells were infected with PRRSV for 1 h and then placed in a medium containing Thai medicinal plant extracts at various concentrations according to CC50 and incubated at 24, 48, and 72 h, respectively. After various times of infection, the supernatant was collected for quantifying the virus titer by the IPMA assay. The control was infected and the virus titer was quantified as TCID₅₀ (log₁₀) = 8.0. The statistical analyses of the antiviral activity were performed using ANOVA. A, *C. nutans*; B, *P. frutescens*; C, *H. cordata*; D, *T. triandra*; E, *G. mangostana* Linn. and F, *P. emblica*. a, b, and c; $P < 0.05$ compared with the different concentrations of the plant extracts. All samples were analyzed in triplicate.

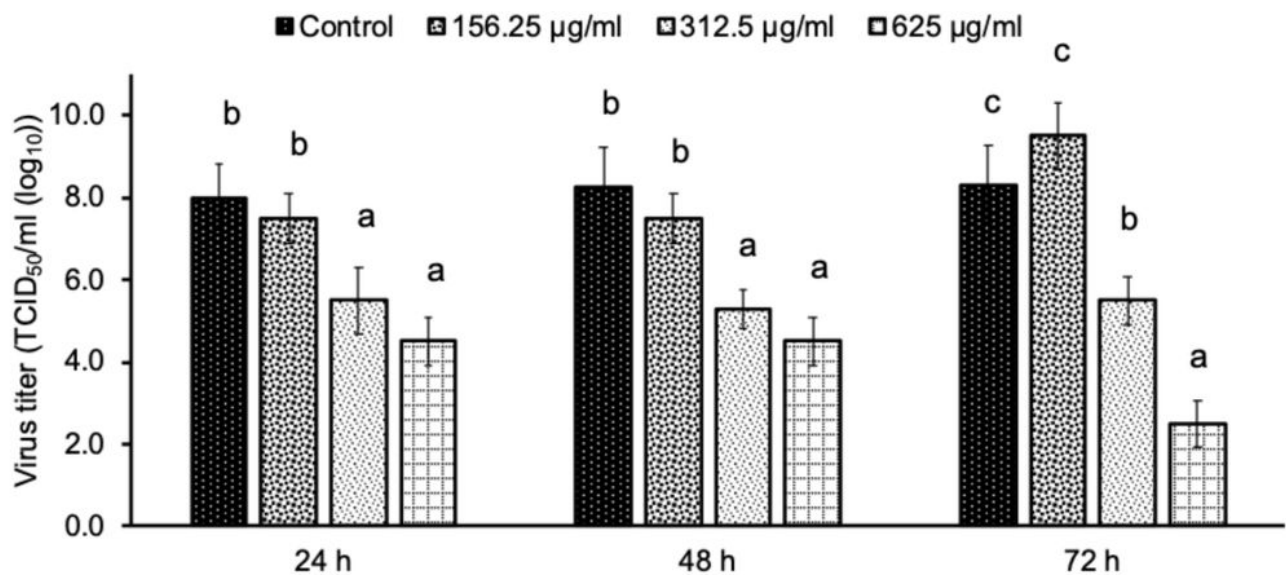
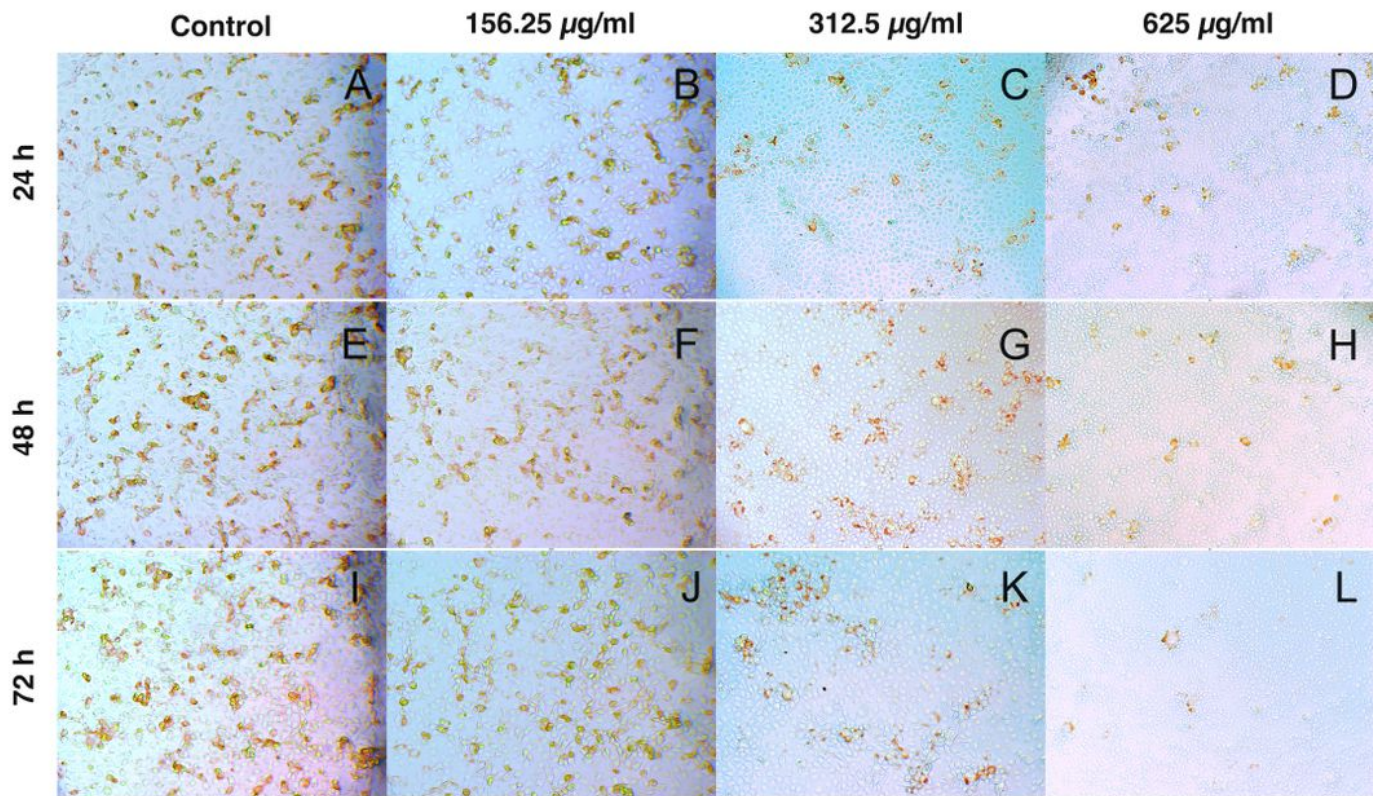


Figure 5

The IPMA assay of *Caesalpinia sappan* Linn. inhibiting the PRRSV replication of MARC-145 cells at 24 (A-D), 48 (E-F), and 72-h post-infection (hpi) (I-L). The MARC-145 cells were infected with PRRSV for 1 h and then placed in a medium containing *C. sappan* Linn. at various concentrations according to CC50 and incubated at 24, 48, and 72 h, respectively. After various times of infection, the supernatant was collected for quantifying the virus titer by IPMA that was used to detect the infectivity of PRRSV in MARC-145 cells.

The MARC-145 cells that were infected with PRRSV showed brown staining. In the control, large number of PRRSV-infected cells were observed in the supernatants, while only a few PRRSV-infected cells were observed in *C. sappan* Linn.-treated supernatants. The bar chart represents the virus titer of *C. sappan* Linn. inhibiting PRRSV replication at 24, 48, and 72 hpi. The control was infected and virus titer was quantified as TCID₅₀ (log₁₀) = 8.0. At *C. sappan* Linn. concentration of 625 µg/ml, the virus titer at 72 hpi was lower than that at other concentrations. Statistical analyses of the antiviral activity were performed using ANOVA. a, b, and c: $P < 0.05$ compared with different concentrations of *C. sappan* Linn. All samples were analyzed in triplicate.