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Research Article

Indonesian Herb Extracts Inhibit the Replication of Bovine Respiratory Syncytial Virus: *In Vitro* Study

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ABSTRACT

Bovine respiratory syncytial virus (BRSV) is highly prevalent in cattle. It is a major viral cause of bovine respiratory disease complex, which is associated with morbidity, mortality and substantial economic impact. Currently available treatments are only symptomatic, but no specific treatments are available for BRSV infection. This study aimed to identify new antiviral agents against BRSV, which could be used to control bovine respiratory disease complex in cattle with Indonesian herb extracts. Ethanol extracts prepared from Indonesian herbs including *Andrographis paniculata, Phyllanthus niruri, Curcuma aeruginosa*, and *Curcuma xanthorrhiza* were evaluated for anti-BRSV activity in A549 cells. The cytotoxicity of the herb extracts was evaluated using a CCK-8 cell viability assay. Antiviral activities of the herb extracts were examined using cell activity and cytopathic assays. The effect on virus production was evaluated by qRT-PCR and plaque-formation assays. Extracts of *Curcuma xanthorrhiza* (125 µg/ml), *Andrographis paniculata* (250 µg/ml), and *Phyllanthus niruri* (62.5 µg/ml) inhibited BRSV activity in A549 in pre-, simultaneously-, and post-infection treatment assays, respectively, as measured by the selective index. Reduction of BRSV activities by the herb extracts correlated with inhibition of viral gene expression and inhibition of plaque formation in a concentration- and time-dependent manner. Our findings suggest that these herb extracts have sufficient potency to be used not only as a therapeutic agent but also as a preventive agent to limit BRSV infection.

1. Introduction

Bovine respiratory syncytial virus (BRSV), a member of the genus *Orthopneumovirus* in the family Pneumoviridae, is highly prevalent in cattle. This viral infection economically impacts the cattle industry, with morbidity and mortality caused by respiratory disease (Larsen *et al.* 2001; Boxus *et al.* 2005; Gershwin *et al.* 2015). The most severe disease is observed in 1–3-month-old calves. Current understanding is that BRSV is transmitted by both direct and indirect routes, and possibly by aerosol over short distances (Van der Poel *et al.* 1993; Ohlson *et al.* 2013). BRSV replication in the upper and lower airways causes cellular damage and dysfunction, leads to abnormal immune responses, and facilitates secondary infection due to bacterial colonization in the lower respiratory tract (Larsen

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et al. 2001; Tjønehøj *et al.* 2003; Meyer *et al.* 2008; Sudaryatma *et al.* 2018a, 2019).

Bovine respiratory disease complex is usually initiated by BRSV infection. The effect of the BRSV on the host facilitates flow-through of bacteria into the lower respiratory tract to induce severe pneumonia (Sudaryatma *et al.* 2018a). There are no specific antiviral agents against BRSV at present. The major treatment protocol used in the management of bovine respiratory diseases complex is to treat the symptoms of secondary bacterial infection using antibiotics (Torres *et al.* 2013). However, antibiotic resistance in cattle populations is of growing public concern (Shahriar *et al.* 2002; Golkar *et al.* 2014; Wright 2014).

To address antibiotic resistance issues, several groups are investigating novel plant extracts that could be used for the development of new antimicrobial agents (Piras *et al.* 2012). Likewise, the most common prevention of disease transmission is through passive vaccination by forming maternal colostrum antibodies which are components of disease prevention and protection in newborn animals, including young calves (Kolb *et al.* 2020). However, maternal antibodies are often ineffective because they increase the potential for virus transmission in young calves. Furthermore, a number of traditional medicinal plants exhibit great therapeutic potential, thus botanical products are an important part of the health food market (Schilter *et al.* 2003). In Indonesia, several indigenous plants are used as traditional medicines. These plants have been examined for their chemical and biological properties (Wiradana *et al.* 2024). Because of the rich local phytocoenoses, Indonesian people have used herbal medicines known as "Jamu" for treating disease and maintaining health and wellness for centuries (Widhiantara *et al.* 2021; Widiastuti *et al.* 2023). In the present study, four Indonesian medicinal plants, *Curcuma xanthorrhiza, Curcuma aeruginosa, Phyllanthus niruri*, and *Andrographis paniculate*, were evaluated for antiviral activity. In addition to free-radical scavenging activity (Nurcholis *et al.* 2012), extracts from these plants prevent avian Influenza virus infection in chickens through their immune-stimulatory actions (Darusman *et al.* 2016; Priosoeryanto *et al.* 2016).

The rhizomes of *C. xanthorrhiza* and *C. aeruginosa* belong to the Zingiberaceae family and are known as "Temulawak" and "Temu ireng" in the Indonesian, respectively. The rhizomes of these plants have several biological activities including anti-inflammatory (Simamora *et al.* 2022), anti-mycobacterial (Ngadino *et al.* 2018b), anti-candida (Akarchariya *et al.* 2017), antiviral (Kim *et al.* 2021; Fazalul Rahiman *et al.* 2024), and anti-cancer activities (Hartati *et al.* 2024). *P. niruri* and *A. paniculata* are medicinal plants commonly known in Indonesia as "Meniran" and "Sambiloto", respectively. The leaves of these plants have several biological activities including anti-hepatotoxic (Coon & Ernst 2004), antiviral (Dahanukar *et al.* 2000; Wiart *et al.* 2005), antibacterial (Kīrtikara & Basu 2017), and anti-inflammatory activities (Singha *et al.* 2003), and they are used in the treatment of upper respiratory tract infections.

This study aimed to assess the *in vitro* antiviral activity of these four Indonesian plants (*C. xanthorrhiza, C. aeruginosa, P. niruri*, and *A. paniculata*) against BRSV; herb extracts that inhibit BRSV activity could be useful therapies for the control of bovine respiratory disease complex in cattle.

2. Materials and Methods

2.1. Ethical Approval

This study used virus isolates and cultured cells which were carried out on a laboratory scale *in vitro* and did not use ethical clearance in the process.

2.2. Plant Extract

We isolated ethanol extracts from four natural herbs, as previously described (Nurcholis *et al.* 2012). The herb extracts were derived from *A. paniculata* and *P. niruri, C. aeruginosa*, and *C. xanthorrhiza*. These herbs were obtained from The Conservation and Cultivation Unit of Tropical Biopharmaca Research Centre, IPB University, Indonesia. Voucher specimens of plant material were deposited in the herbarium of the Indonesia Institute of Sciences, Indonesia.

For further testing of biological activity, the extracts were dissolved in dimethyl sulfoxide (DMSO; Nacalai tesque, Japan) at a concentration of 1 mg/ml (stock solution). Serial 2-fold dilutions, ranging from 1,000 µg/ml to 7.81 µg/ml, of each extract were prepared in Roswell Park Memorial Institute 1640 medium (RPMI; Gibco, Japan) containing 100 U/ml penicillin, and 100 µg/ml streptomycin (1% Pe/St; Wako, Japan) without fetal bovine serum (Sudaryatma *et al.* 2018b).

2.3. Cells and Virus Sources

Adenocarcinoma human alveolar basal epithelial cell type II (A549) cells and Madin-Darby Bovine Kidney (MDBK) cells were used in this study. A549 cells were cultured in RPMI supplemented with 10%

heat-inactivated fetal bovine serum (FBS; Biowest, France) and 1% Pe/St. MDBK cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma, Japan) supplemented with 5% FBS and 1% Pe/St. Cells were maintained in a humidified atmosphere at 37°C and 5% CO_2.

We used a BRSV strain isolated from a nasal swab from Kyushu island, Japan. BRSV was propagated in Vero cells as previously described (Sudaryatma *et al.* 2019). The virus was diluted in serum-free RPMI to achieve a multiplicity of infection-1 (MOI-1).

2.4. Cytotoxicity Assay

A549 cells were seeded in a 96 well plate at 1 × 10^5 cells/ml to obtain 60-70% confluency. The culture media were replaced with RPMI media containing serially diluted herb extracts (7.81-1000 µg/ml). Cells were maintained for 2; 24; or 120 hours after treatment. Diluted DMSO was used as a negative control. The cytotoxic effect of each extract was tested using a Cell Counting Kit-8 (CCK-8; Dojindo, Japan) assay following the manufacturer's instruction. Absorbance at 450 nm was measured in a microplate reader (Bio-Rad, Japan). The concentration of herb extract that produced 50% reduction in cell viability (cytotoxic concentration, CC_{50}) and the concentration that maintained >95% cell viability, the safety concentration, were calculated by regression analysis as described previously (Ren *et al.* 2011). The calculated safety concentration of each herb extract was used in antiviral assays.

2.5. Antiviral Assay

Infection of A549 cells with BRSV was performed as previously described (Sudaryatma *et al.* 2018b). Herb extracts were used at 2-fold serial dilutions beginning with the calculated safety concentration. We used three types of assay to determine which step of the BRSV-induce cell damage is inhibited by treatment of herb extracts.

(1) Pre-infection assay: A549 cells at 1 × 10^5 cells/ml were seeded in a 96-well plate using culture medium containing serially diluted herb extracts. After 24 h, culture medium was removed and virus was inoculated. After adsorption time, inoculum was removed and replaced with fresh culture media without herb extracts.

(2) Simultaneously-infection assay: A mixture of virus and herb extracts was inoculated into a A549 cells. After 2 h, the inoculum was removed and replaced with fresh culture media without herb extracts.

(3) Post-infection assay: A549 cells were inoculated with BRSV. After adsorption, the inoculum was removed and replaced with fresh culture media containing serially diluted herb extracts for 120 h.

The antiviral effects of the herb extracts were measured using a CCK-8 kit and observation of cytopathic effects (CPE) under a microscope at 120 hours after virus infection. The concentration of extract that produced 50% inhibition (IC_{50}) of virus-induce cell damage was calculated as previously described (Kwon *et al.* 2015; Malik *et al.* 2016; Mounce *et al.* 2017; Reanmongkol *et al.* 2006).

2.6. Virus Yield Reduction Assay

A549 cells were seeded in a 12-well plate at 1 × 10^5 cells/ml. Herb extracts or 0.1% DMSO control were used for treatment in the 1) pre-, 2) simultaneously-, or 3) post-infection assays as described above. At 120 hours after infection, culture medium of each treatment was collected and inoculated (10-fold dilution) onto a monolayer of MDBK cells for quantification of viral plaque-forming as described previously (Sudaryatma *et al.* 2018b). Further, viral RNA was extracted from cell culture medium using a Nucleospin-virus purification kit (Takara Bio, Japan) or from cell lysate using a RNeasy Mini Kit (Qiagen, Germany). Quantitative real-time PCR (qRT-PCR) of BRSV RNA was performed as described previously (Sudaryatma *et al.* 2019).

2.7. Data Analysis

All experiments were performed in triplicate of independent culture. Data are expressed as the mean ± standard deviation (SD). Statistical analysis of data was performed using a one-way analysis of variance (ANOVA) and Tukey's Multiple Comparison Test, and a p value <0.05 was considered significant. Data were analyzed using RStudio version 1.0.143 (RStudio 2015).

3. Results

3.1. Determination of Cytotoxicity and Safety Concentration of Herb Extracts

The cytotoxicity of herb extracts (7.81-1000 µg/ml) on A549 cells was evaluated at 2, 24, and 120 hours after treatment. The cytotoxic activity of herb extracts was concentration- and time-dependent (Figures 1A-C) and used for calculating CC_{50} values (Table 1). CC_{50} of herb extracts over 250 µg/ml at 2 hours, over 125 µg/ml at 24 hours, and over 62.5 µg/ml

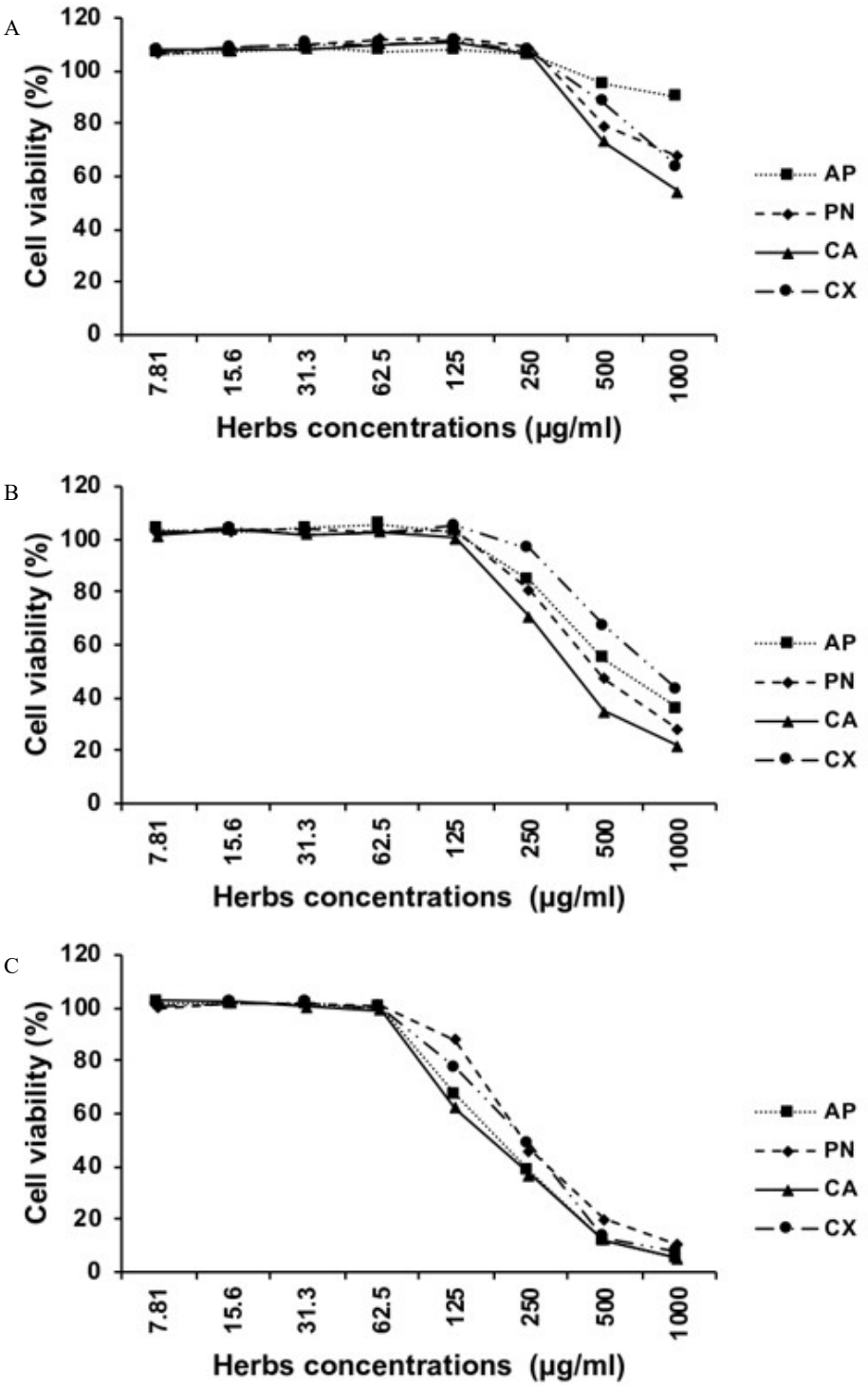

Figure 1. Cytotoxicity of herb extracts on A549 cells. A549 cells were treated with various concentrations (7.81-1,000 µg/ml) of herbs extract from *Andrographis paniculata* (AP) and *Phyllanthus niruri* (PN), *Curcuma aeruginosa* (CA), and *Curcuma xanthorrhiza* (CX). Treatment of cells with herb extracts for 2 h (A), 24 h (B), and 120 h (C) produced safety concentrations of 250, 125, and 62.5 µg/ml, respectively. Cell viability was measured using a CCK-8 assay

Table 1. Antiviral activities of herb extracts against bovine respiratory syncytial virus in pre-, simultaneously-, and post-infection treatment assays

Herbs	Pre-infection treatment			Co-infection treatment			Post-infection treatment		
	CC_{50} (µg/ml)	IC_{50} (µg/ml)	SI	CC_{50} (µg/ml)	IC_{50} (µg/ml)	SI	CC_{50} (µg/ml)	IC_{50} (µg/ml)	SI
AP	735.1	87.2	8.4	>1000	39.5	25.3	204.1	ND	ND
PN	649.3	ND	ND	>1000	ND	ND	306.9	9.8	31.4
CA	552.1	ND	ND	>1000	ND	ND	196.3	ND	ND
CX	853.0	37.1	23.0	>1000	ND	ND	241.3	ND	ND

CC_{50}: the concentration of herb extract producing 50% reduction in cell viability, IC_{50}: the concentration of herb extract producing 50% inhibition of virus-induce cell damages, SI: CC_{50}/IC_{50}, AP: *Andrographis paniculata,* PN: *Phyllanthus niruri,* CA: *Curcuma aeruginosa,* CX: *Curcuma xanthorrhiza,* ND: Not detected

at 120 hours. We determined the safety concentration of each herb extract that was the highest concentration that produced minimal toxicity on A549 cells (>95% cells viabilities). No cytotoxicity of 0.1% DMSO was observed in A549 cells during 120 hours of treatment.

3.2. Inhibitory Effect of Herb Extracts to BRSV-induced Cell Damage

We next evaluated the antiviral activity of herb extracts against BRSV. A549 cells were treated with herb extracts using three protocols (pre-, simultaneously-, or post-infection assay; as detailed in Materials and Methods). From cytopathic effects observation, *C. xanthorrhiza, A. paniculata,* and *P. niruri* treatments reduced the cell damages induce by BRSV infection in pre-, simultaneously-, or post-infection assay, respectively (Figure 2).

In the pre-infection assay, treatment with either *A. paniculata* or *C. xanthorrhiza* protected cells from BRSV infection, as measured using a CCK-8 assay (Figure 3A). In the simultaneously-infection assay, *A. paniculata* treatment (IC_{50}: 39.5 µg/ml) produced anti-BRSV effects, and in the post-infection assay, *P. niruri* treatment (IC_{50}: 9.8 µg/ml) showed anti-BRSV effects (Figures 3B and C).

In the pre-infection assay, the selective index (SI: ratio of CC_{50}/IC_{50} values) of AP treatment was 8.4, and for *C. xanthorrhiza* treatment, the SI was 23.0. In the simultaneously-infection assay, *A. paniculata* treatment showed the greatest SI value (25.3), and in the post-infection assay, *P. niruri* treatment generated the best SI value (31.4) (Table 1).

3.3. Inhibitory Effect of Herb Extracts to BRSV Replication and Plaque Formation

Antiviral activity of herb extracts against BRSV was quantified by qRT-PCR and assays measuring reductions in viral plaque formation. In the pre-infection assay, *C. xanthorrhiza* treatment decreased viral RNA load in culture supernatants and cellular lysates, and *A. paniculata* treatment decreased viral RNA levels in cellular lysates (Figure 4A; $p<0.05$). In simultaneously-infection assays, *A. paniculata* decreased viral RNA levels both in culture supernatants and in cell lysates (Figure 4B; $p<0.05$). In post-infection assays, *P. niruri* treatment produced the highest decrease in viral RNA levels in the cellular lysates (Figure 4C; $p<0.05$). These results are consistent with the reduction of plaque formation seen in A549 cells treated with herb extracts.

In the pre-infection assay, *C. xanthorrhiza* treatment inhibited plaque formation by 79.80% (Figure 5A; $p<0.05$). In the simultaneously-infection assay, *A. paniculata* treatment reduced plaque formation by 86.79%, and in the post-infection assay, *P. niruri* treatment reduced plaque formation by 70.21% (Figures 5B and C; $p<0.05$).

4. Discussion

In the present study, we demonstrated that Indonesian herb extracts have antiviral activity against BRSV, which occurs in a time- and concentration-dependent manner. *C. xanthorrhiza, A. paniculata*, and *P. niruri* inhibited BRSV activity via pre-, simultaneously-, and post-infection assays, respectively. These results suggest that the herb

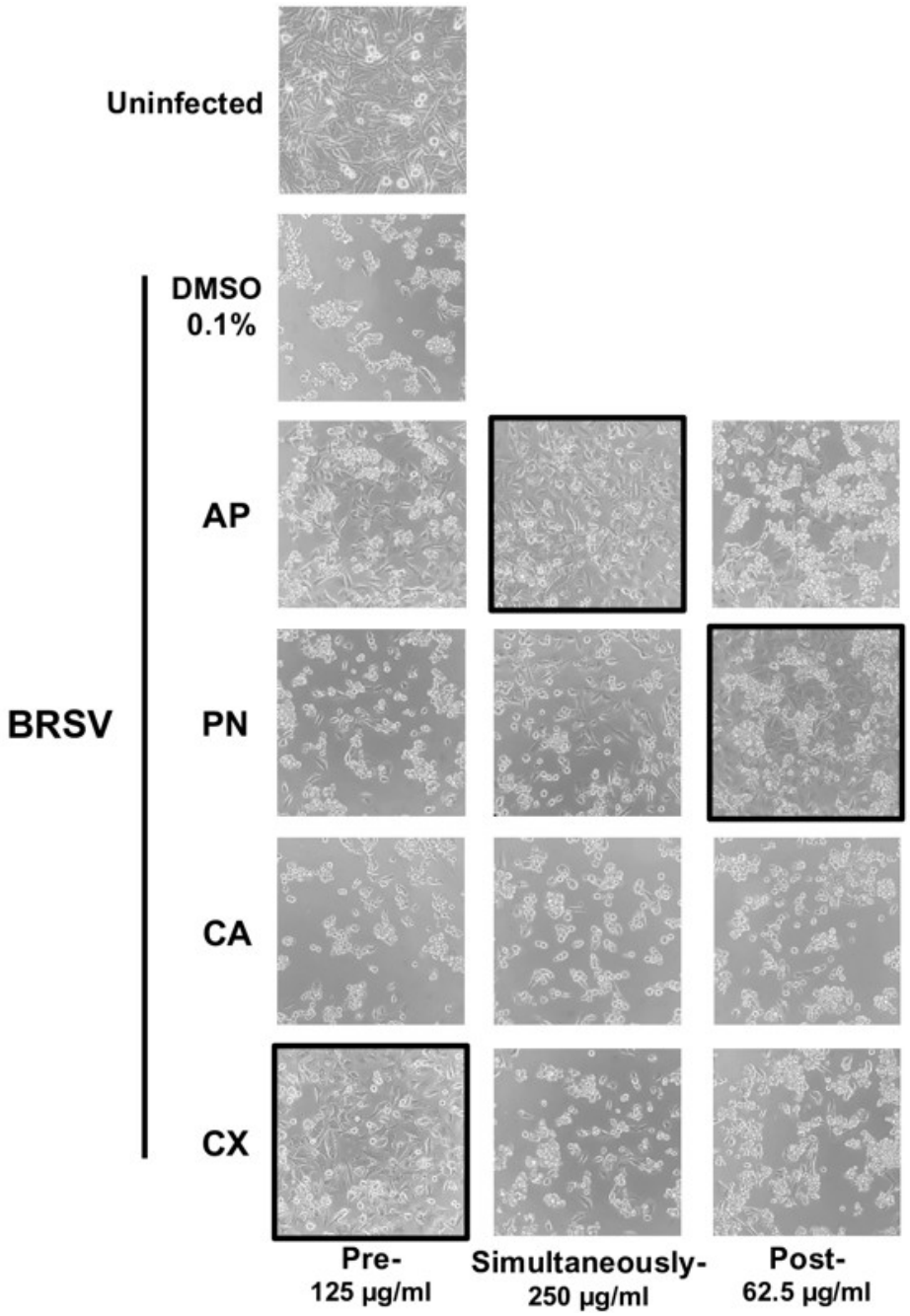

Figure 2. Herb extracts from *Andrographis paniculata* (AP) and *Phyllanthus niruri* (PN), *Curcuma aeruginosa* (CA), and *Curcuma xanthorrhiza* (CX) were inhibited BRSV-induces cytopathic effects (CPE) in A549 cells in a time- and concentration-dependent manner. Black-edged squares indicate images in which herb treatment inhibits the cytopathic effects by BRSV infection

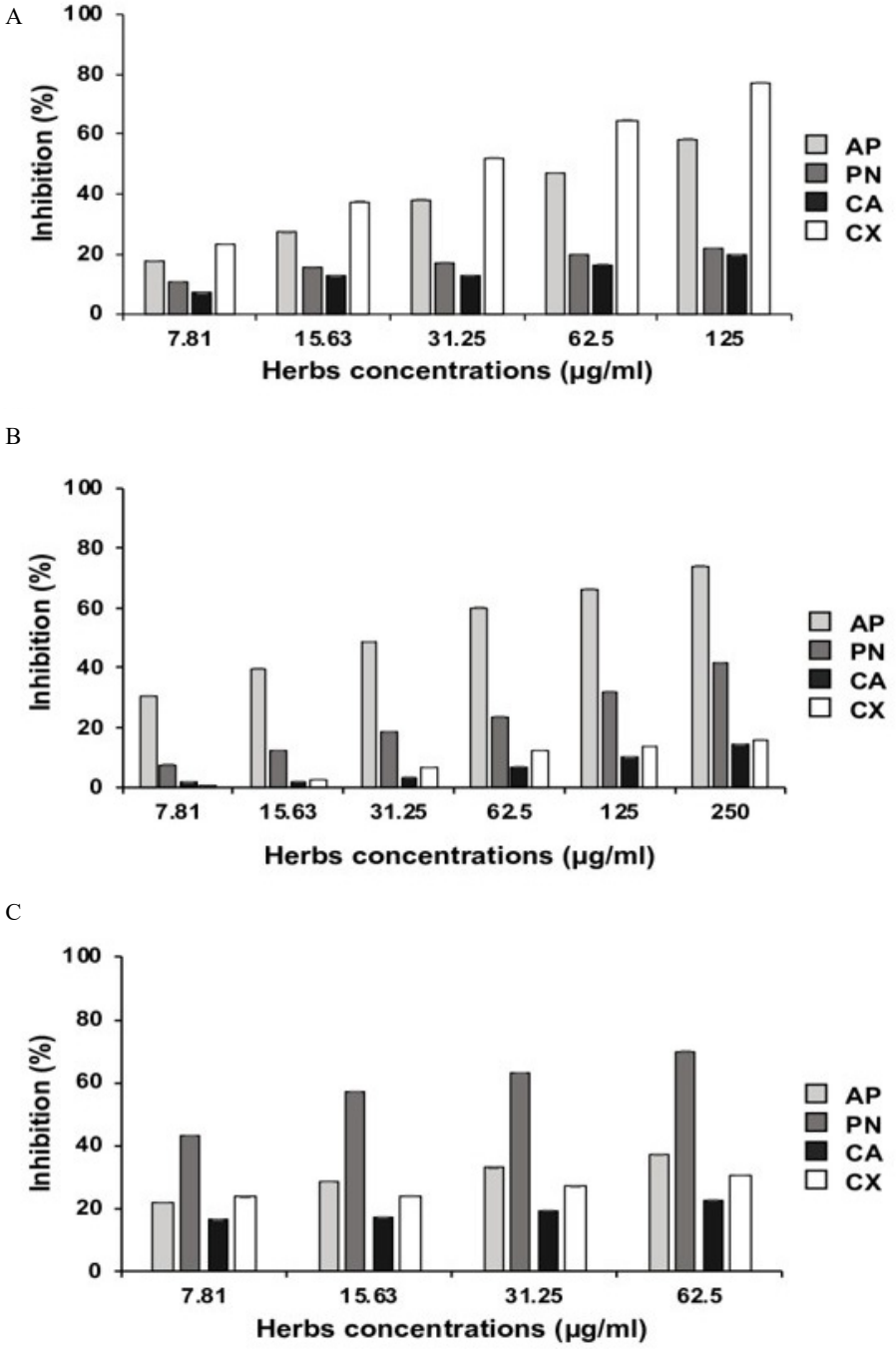

Figure 3. *In vitro* antiviral activities of the herb extracts from *Andrographis paniculata* (AP) and *Phyllanthus niruri* (PN), *Curcuma aeruginosa* (CA), and *Curcuma xanthorrhiza* (CX) against bovine respiratory syncytial virus (BRSV). (A) Pre-infection assay: A549 cells were treated with 2-fold serial dilutions of herb extracts, beginning with the safety concentration (7.81-125 µg/ml) 24 h prior to BRSV infection, and then following absorbance, cell culture conditions were maintained with normal medium culture. (B) Simultaneously-infection assay: A549 cells were treated with mixed of BRSV and 2-fold serial dilutions of herb extracts, beginning with the safety concentration (7.81-250 µg/ml), and then following adsorption, cell culture conditions were maintained with normal medium. (C) Post-infection treatment assay: BRSV-infected A549 cells were treated with culture medium containing 2-fold serial dilutions of herb extracts (7.81-62.5 µg/ml), beginning with the safety concentration. In each assay type, the *in vitro* antiviral effects of the herbs were evaluated using a CCK-8 assay after 120 h of BRSV infection

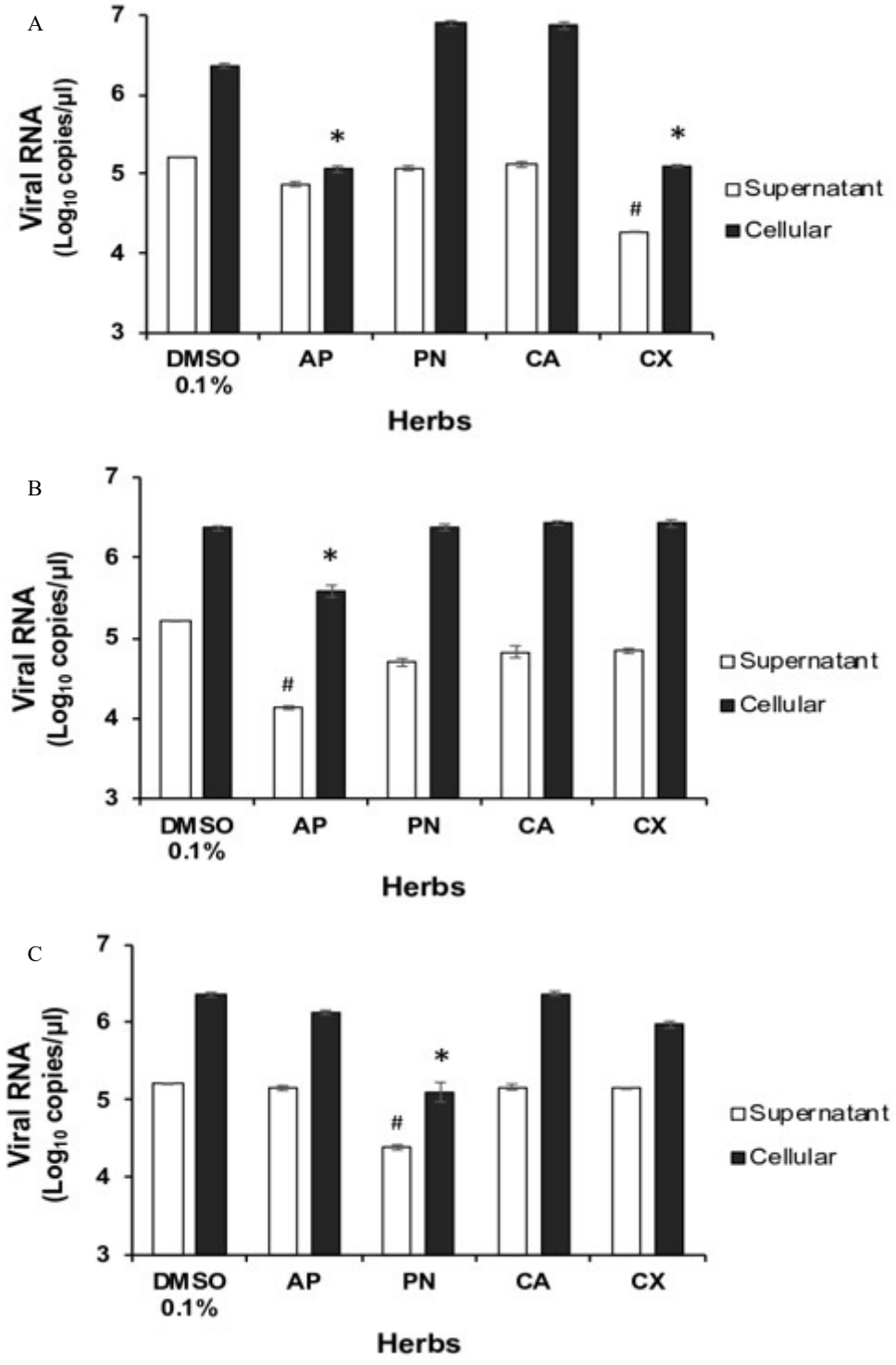

Figure 4. Quantitative real-time PCR of bovine respiratory syncytial virus (BRSV) RNA in supernatant and cellular lysates of A549 cells collected 120 h after (A) pre-infection, (B) simultaneously-infection, and (C) post-infection assays treatment with *Andrographis paniculata* (AP) and *Phyllanthus niruri* (PN), *Curcuma aeruginosa* (CA), and *Curcuma xanthorrhiza* (CX). Total virus RNA was extracted and the viral RNA load were measured. Data are expressed as the mean (±SD); n = 3; *$p<0.05$ for BRSV gene expression in supernatant of the cells treated with herbs compared with untreated cells; #$p<0.05$ for BRSV gene expression in cell lysates of the cells treated with herbs compared with untreated cells

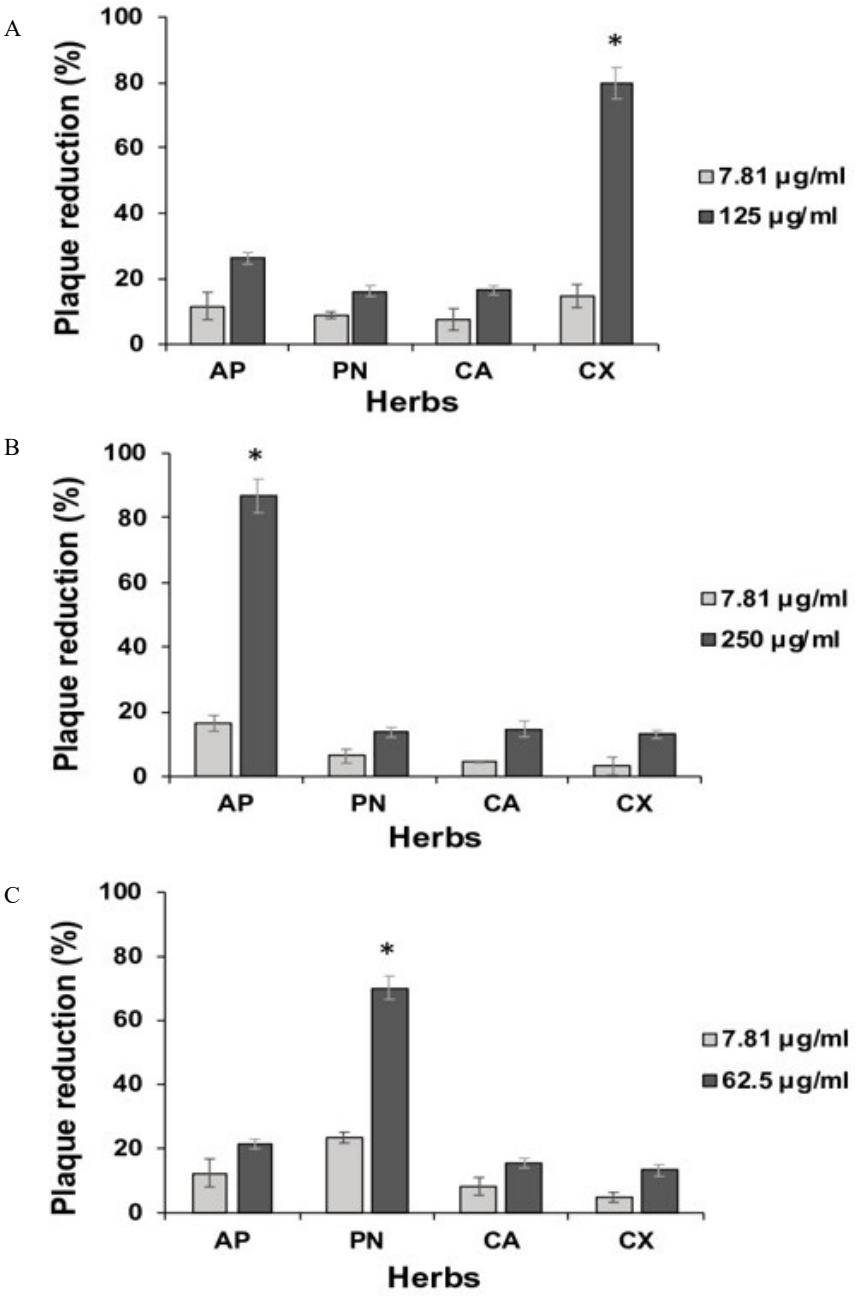

Figure 5. Herb extracts from *Andrographis paniculata* (AP) and *Phyllanthus niruri* (PN), *Curcuma aeruginosa* (CA), and *Curcuma xanthorrhiza* (CX) were reduced the number of viral plaques induced by bovine respiratory syncytial virus (BRSV) infection in the pre-infection (A), simultaneously-infection (B), and post-infection treatment assays (C). After 120 h incubation, culture medium from treated and untreated cells was removed and virus titers were determined based on the number of plaque-forming units in MDBK cells. Data are expressed as the mean (±SD); n = 3; *p<0.05, for percentage reduction in plaque formation for the highest concentration of herbs compared with the lowest concentration

extracts exert antiviral activity at several steps of the virus infection including 1) virus entry, 2) virus gene replication, and 3) virus release from infected cells.

C. xanthorrhiza is widely used as a traditional medicine for immune stimulation, especially for preventing or treating bacterial, fungi, and viral infections (Ozaki 1990; Stevensen 1999; Rukayadi *et al.* 2006; Sutha *et al.* 2010; Ngadino *et al.* 2018a). Our previous study showed that application of curcumin, a component of *C. xanthorrhiza* (Lechtenberg *et al.* 2004), prior to human respiratory syncytial virus (HRSV) infection upregulates of tight junction molecules and prevents virus entry into human nasal epithelial cells (Obata *et al.* 2013). Another group showed that pre-infection treatment of cells with curcumin inhibits replication of zika and chikungunya viruses (Mounce *et al.* 2017). Our previous study showed that BRSV infection was reduce intercellular adhesion molecule-1 from surface of the upper bovine respiratory epithelial cells (Sudaryatma *et al.* 2020). These results suggest the antiviral activity of *C. xanthorrhiza* and its derivatives prevent viral infection via down-regulation of cellular receptor. Alternatively, *C. xanthorrhiza* and its derivatives could activate interferon-stimulated genes in target cells, inducing an antiviral state. These potential mechanisms of action of *C. xanthorrhiza* warrant investigation in future studies. Addition of this herb to cattle diets would provide a simple means to prevent infection, either at the time of shipping calves, during the winter season or during other stressful conditions that induce respiratory viral infection in calves.

In the simultaneously-infection assay, *A. paniculata* strongly suppressed accumulation of viral RNA both in the culture medium and in cell lysates. Accumulation of viral RNA correlated with reduced plaque formation, suggesting that *A. paniculata* treatment together with BRSV suppressed production of infectious particles. This result suggests that *A. paniculata* modulates virus attachment and/or entry via direct binding with the virus and/or inactivation of viral proteins critical for infection. Attachment and penetration of BRSV into cells is mediated by activation of attachment binding protein to proteoglycans such as heparin (Larsen *et al.* 2001; Meyer *et al.* 2008). Recently, *Cinnamomum cassia* (Yeh *et al.* 2013) and Ge-Gen-Tang (Chang *et al.* 2012) have been shown to inhibit the activities of fusion protein and glycoprotein of HRSV in A549 cells. Andrographolide, as a compound isolated from *A. paniculata*, has been used to block the cellular entry of chikungunya virus (Wintachai *et al.* 2015). Our results suggest that *A. paniculata* could inhibit the binding and/or invasion of virus to cells. Alternatively, AP might regulate the expression of as-yet uncharacterized cellular receptor(s), as suggested from results of the pre-infection assay.

In the post-infection assay, we sought to inhibit viral replication and production of progeny viruses from infected cells. *P. niruri* significantly inhibited BRSV replication in A549 cells in a concentration-dependent manner. Results from our experiment showed that accumulation of viral RNA in the culture medium and cells was suppressed by *P. niruri*. This result correlated with reduced viral plaque formation in MDBK cells. A previous report showed that treatment of cells with Lophatherum gracile after HRSV infection decreases nitric oxide production in vitro, and reduces viral load and production of inflammatory factors *in vivo* (Chen *et al.* 2019). Another study reported that *Coptidis rhizoma* extract inhibits HRSV replication and reduces cell death of HRSV-infected HEp-2 cells (Lee *et al.* 2017). On the other hand, *Phyllanthus* species inhibits hepatitis C virus entry by binding to glycoprotein (Chung *et al.* 2016). Of these potential mechanisms, we suggest that PN activates of as-yet uncharacterized signaling pathway that inhibits virus RNA synthesis, so inhibiting BRSV replication. Alternatively, *P. niruri* might exert antiviral activity through suppression of a signaling pathway that supports BRSV replication.

Based on the results described in the above paragraph, we conclude that the natural products isolated from Indonesian herbs are promising antiviral candidates for the treatment or prevention of BRSV infection. Their advantages are strong antiviral activity against BRSV and low toxicity. The herb extracts suppressed BRSV activity by either preventing virus entry and/or replication, depending on the timing of the treatment. While the underlying antiviral mechanism remains unclear, these herb extracts could be safe and effective agents for preventing and treating bovine respiratory disease complex. Since we previously showed that BRSV infection enhances bacterial adherence to the respiratory epithelial cells (Sudaryatma *et al.* 2018b), these herbs could help minimize the use of antibiotics in cattle.

In conclusion, Our data showed that Indonesian herb extracts have antiviral activities; they inhibit virus entry, propagation, and/or release. These findings suggest that these low-toxic herb extracts could be used to prevent BRSV infection. Furthermore, these extracts could be used as therapeutic agents that reduce the use of antibiotics in bovine respiratory disease complex, which in turn will help prevent the global emergence of antimicrobial resistance.

Conflict of Interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

Akarchariya, N., Sirilun, S., Julsrigival, J., Chansakaowa, S., 2017. Chemical profiling and antimicrobial activity of essential oil from *Curcuma aeruginosa* Roxb., *Curcuma glans* K. Larsen & J. Mood and Curcuma cf. xanthorrhiza Roxb. collected in Thailand. *Asian Pacific Journal of Tropical Biomedicine*. 7, 881–885. https://doi.org/10.1016/j.apjtb.2017.09.009

Boxus, M., Letellier, C., Kerkhofs, P., 2005. Real time RT-PCR for the detection and quantitation of bovine respiratory syncytial virus. *Journal of Virological Methods*. 125, 125–130. https://doi.org/10.1016/j.jviromet.2005.01.008

Chang, J.S., Wang, K.C., Shieh, D.E., Hsu, F.F., Chiang, L.C., 2012. Ge-Gen-Tang has anti-viral activity against human respiratory syncytial virus in human respiratory tract cell lines. *Journal of Ethnopharmacology*. 139, 305–310. https://doi.org/10.1016/j.jep.2011.11.018

Chen, L.F., Zhong, Y.L., Luo, D., Liu, Z., Tang, W., Cheng, W., Xiong, S., Li, Y.L., Li, M.M., 2019. Antiviral activity of ethanol extract of *Lophatherum gracile* against respiratory syncytial virus infection. *Journal of Ethnopharmacology*. 242, 111575. https://doi.org/10.1016/j.jep.2018.10.036

Chung, C.Y., Liu, C.H., Burnouf, T., Wang, G.H., Chang, S.P., Jassey, A., Tai, C.J., Tai, C.J., Huang, C.J., Richardson, C.D., Yen, M.H., Lin, C.C., Lin, L.T., 2016. Activity-based and fraction-guided analysis of *Phyllanthus urinaria* identifies loliolide as a potent inhibitor of hepatitis C virus entry. *Antiviral Research*. 130, 58–68. https://doi.org/10.1016/j.antiviral.2016.03.012

Coon, J.T., Ernst, E., 2004. *Andrographis paniculata* in the treatment of upper respiratory tract infections: a systematic review of safety and efficacy. *Planta Medica*. 70, 293-298. https://doi.org/10.1055/s-2004-818938

Dahanukar, S.A., Kulkarni, R.A., Rege, N.N., 2000. Pharmacology of medicinal plants and natural products. *Indian Journal of Pharmacology*. 32, 81-118.

Darusman, L., Priosoeryanto, B., Purwakusumah, E., 2016. *Combination of Curcuma xanthorrhiza and Phylantus niruri for Control and Prevention of Avian Influenza Disease in Chicken. (Patent No. IDP000040513).* Directorate General of Intellectual Property the Ministry of Justice and Human Rights, The Republic of Indonesia.

Fazalul Rahiman, S.S., Al-Amin, M., Mohtar, N., Zakaria, I.I., Kahar, U.M., Khairuddean, M., Salhimi, S.M., 2024. Potential inhibitors isolated from *Curcuma aeruginosa* against dengue virus type 2 (DENV-2) NS2B-NS3 protease activity. *Journal of Biologically Active Products from Nature*. 14, 64–79. https://doi.org/10.1080/22311866.2024.2316637

Gershwin, L. J., Van Eenennaam, A. L., Anderson, M. L., McEligot, H. A., Shao, M. X., Toaff-Rosenstein, R., Taylor, J. F., Neibergs, H. L., Womack, J., 2015. Single pathogen challenge with agents of the bovine respiratory disease complex. *PLOS ONE*, 10, e0142479. https://doi.org/10.1371/journal.pone.0142479

Golkar, Z., Bagasra, O., Pace, D.G., 2014. Bacteriophage therapy: a potential solution for the antibiotic resistance crisis. *The Journal of Infection in Developing Countries*. 8, 129–136. https://doi.org/10.3855/jidc.3573

Hartati, F.K., Kurnia, D., Nafisah, W., Haryanto, I.B., 2024. Potential anticancer agents of *Curcuma aeruginosa*-based kombucha: *in vitro* and *in silico* study. *Food Chemistry Advances*. 4, 100606. https://doi.org/10.1016/j.focha.2024.100606

Kim, M., Cho, H., Ahn, D.G., Jung, H.G., Seo, H.Y., Kim, J.S., Lee, Y.J., Choi, J.Y., Park, I.H., Shin, J.S., Kim, S.J., Oh, J.W., 2021. *In vitro* replication inhibitory activity of Xanthorrhizol against severe acute respiratory syndrome coronavirus 2. *Biomedicines*. 9, 1725. https://doi.org/10.3390/biomedicines9111725

Kīrtikara, K.R., Basu, B.D., 2017. Indian medicinal plants. Sudhindra Nath Basu, Pâninî office, Bahadurganj. https://doi.org/10.5962/bhl.title.137025

Kolb, E.A., Buterbaugh, R.E., Rinehart, C.L., Ensley, D., Perry, G.A., Abdelsalam, K.W., Chase, C.C.L., 2020. Protection against bovine respiratory syncytial virus in calves vaccinated with adjuvanted modified live vaccine administered in the face of maternal antibody. *Vaccine*. 38, 298–308. https://doi.org/10.1016/j.vaccine.2019.10.015

Kwon, H.J., Jeong, J.H., Lee, S.W., Ryu, Y.B., Jeong, H.J., Jung, K., Lim, J.S., Cho, K.O., Lee, W.S., Rho, M.C., Park, S.J., 2015. *In vitro* anti-reovirus activity of kuraridin isolated from *Sophora flavescens* against viral replication and hemagglutination. *Journal of Pharmacological Sciences*. 128, 159–169. https://doi.org/10.1016/j.jphs.2015.04.007

Larsen, L.E., Tegtmeier, C., Pedersen, E., 2001. Bovine respiratory syncytial virus (BRSV) pneumonia in beef calf herds despite vaccination. *Acta Vet. Scand.* 42, 113-121. https://doi.org/10.1186/1751-0147-42-113

Lechtenberg, M., Quandt, B., Nahrstedt, A., 2004. Quantitative determination of curcuminoids in *Curcuma rhizomes* and rapid differentiation of *Curcuma domestica* Val. and *Curcuma xanthorrhiza* Roxb. by capillary electrophoresis. *Phytochemical Analysis*. 15, 152–158. https://doi.org/10.1002/pca.759

Lee, B.H., Chathuranga, K., Uddin, M.B., Weeratunga, P., Kim, M.S., Cho, W.K., Kim, H.I., Ma, J.Y., Lee, J.S., 2017. Coptidis Rhizoma extract inhibits replication of respiratory syncytial virus *in vitro* and *in vivo* by inducing antiviral state. *Journal of Microbiology.* 55, 488–498. https://doi.org/10.1007/s12275-017-7088-x

Malik, F.Z.A., Allaudin, Z.N., Loh, H.S., Nee, T.K., Hani, H., Abdullah, R., 2016. Antiviral and virucidal activities of *Duabanga grandiflora* leaf extract against *Pseudorabies* virus *in vitro. BMC Complementary and Alternative Medicine*. 16, 1–10. https://doi.org/10.1186/s12906-016-1120-2

Meyer, G., Deplanche, M., Schelcher, F., 2008. Human and bovine respiratory syncytial virus vaccine research and development. *Comparative Immunology, Microbiology and Infectious Diseases*. 31, 191–225. https://doi.org/10.1016/j.cimid.2007.07.008

Mounce, B.C., Cesaro, T., Carrau, L., Vallet, T., Vignuzzi, M., 2017. *Curcumin inhibits* Zika and chikungunya virus infection by inhibiting cell binding. *Antiviral Research*, 142, 148–157. https://doi.org/10.1016/j.antiviral.2017.03.014

Ngadino, Setiawan, Koerniasari, Ernawati, Sudjarwo, S.A., 2018a. Evaluation of antimycobacterial activity of *Curcuma xanthorrhiza* ethanolic extract against *Mycobacterium tuberculosis* H37Rv *in vitro. Veterinary World.* 11, 368–372. https://doi.org/10.14202/vetworld.2018.368-372

Ngadino, Setiawan, Koerniasari, Ernawati, Sudjarwo, S.A., 2018b. Evaluation of antimycobacterial activity of *Curcuma xanthorrhiza* ethanolic extract against *Mycobacterium tuberculosis* H37Rv *in vitro. Veterinary World*. 11, 368–372. https://doi.org/10.14202/vetworld.2018.368-372

Nurcholis, W., Priosoeryanto, B.P., Purwakusumah, E.D., Katayama, T., Suzuki, T., 2012. Antioxidant, cytotoxic activities and total phenolic content of four Indonesian medicinal plants. *Jurnal Kimia VALENSI*. 2, 501-510. https://doi.org/10.15408/jkv.v2i4.267

Obata, K., Kojima, T., Masaki, T., Okabayashi, T., Yokota, S., Hirakawa, S., Nomura, K., Takasawa, A., Murata, M., Tanaka, S., Fuchimoto, J., Fujii, N., Tsutsumi, H., Himi, T., Sawada, N., 2013. Curcumin prevents replication of respiratory syncytial virus and the epithelial responses to it in human nasal epithelial cells. *PLoS ONE*. 8, e70225. https://doi.org/10.1371/journal.pone.0070225

Ohlson, A., Alenius, S., Tråvén, M., Emanuelson, U., 2013. A longitudinal study of the dynamics of bovine corona virus and respiratory syncytial virus infections in dairy herds. *Veterinary Journal.* 197, 395-400. https://doi.org/10.1016/j.tvjl.2013.01.028

Ozaki, Y., 1990. Antiinflamatory effect os *Curcuma xabthoriza* ROXB. and its active principlkes. *Chem. Pharm. Bull*. 38, 1045–1048.

Piras, C., Soggiu, A., Bonizzi, L., Gaviraghi, A., Deriu, F., De Martino, L., Iovane, G., Amoresano, A., Roncada, P., 2012. Comparative proteomics to evaluate multi drug resistance in *Escherichia coli. Molecular BioSystems*. 8, 1060-1067. https://doi.org/10.1039/c1mb05385j

Priosoeryanto, B., Nurcholis, W., Purwakusumah, E., 2016. *Extract Composition of Curcuma xanthorriza, Phylantus niruri, Andrographis paniculata and Curcuma aeruginosa as an Immunostimulant for the Avian Influenza Disease*. (Patent No. IDP000040520). Directorate General of Intellectual Property the Ministry of Justice and Human Rights, The Republic of Indonesia.

Reanmongkol, W., Subhadhirasakul, S., Khaisombat, N., Fuengnawakit, P., Jantasila, S., Khamjun, A., 2006. Investigation the antinociceptive, antipyretic and anti-inflammatory activities of *Curcuma aeruginosa* Roxb. extracts in experimental animals. *Songklanakarin Journal of Science and Technology*. 28, 1-10.

Ren, X., Li, G., Sui, X., 2011. Antiviral activities of phosphonoformate sodium to pseudorabies herpesvirus infection *in vitro. Pharmaceutical Biology*. 49, 608–613. https://doi.org/10.3109/13880209.2010.538416

RStudio, Rs.T., 2015. *Integrated Development for R. RStudio*. RStudio, Inc., Boston, MA.

Rukayadi, Y., Yong, D., Hwang, J.K., 2006. *In vitro* anticandidal activity of xanthorrhizol isolated from *Curcuma xanthorrhiza* Roxb. *Journal of Antimicrobial Chemotherapy*. 57, 1231–1234. https://doi.org/10.1093/jac/dkl132

Schilter, B., Andersson, C., Anton, R., Constable, A., Kleiner, J., O'Brien, J., Renwick, A. Korver, O., Smit, F., Walker, R., 2003. Guidance for the safety assessment of botanicals and botanical preparations for use in food and food supplements. *Food and Chemical Toxicology*. 41, 1625–1649. https://doi.org/10.1016/S0278-6915(03)00221-7

Shahriar, F.M., Wobeser, G., Janzen, E., Clark, E.G., West, K., 2002. Coinfection with bovine viral diarrhea virus and mycoplasma bovis in feedlot cattle with chronic pneumonia. *Canadian Veterinary Journal*. 43, 863–868.

Simamora, A., Timotius, K.H., Yerer, M.B., Setiawan, H., Mun'im, A., 2022. Xanthorrhizol, a potential anticancer agent, from *Curcuma xanthorrhiza* Roxb. *Phytomedicine*. 105, 154359. https://doi.org/10.1016/j.phymed.2022.154359

Singha, P.K., Roy, S., Dey, S., 2003. Antimicrobial activity of *Andrographis paniculata. Fitoterapia*. 74, 692-694. https://doi.org/10.1016/S0367-326X(03)00159-X

Stevensen, C., 1999. JAMU: an Indonesian herbal tradition with a long past, a little known present and an uncertain future. *Complementary Therapies in Nursing & Midwifery*. 5, 1-3. https://doi.org/10.1016/S1353-6117(99)80062-6

Sudaryatma, P.E., Mekata, H., Kubo, M., Subangkit, M., Goto, Y., Okabayashi, T., 2019. Co-infection of epithelial cells established from the upper and lower bovine respiratory tract with bovine respiratory syncytial virus and bacteria. *Veterinary Microbiology*. 235, 80–85. https://doi.org/10.1016/j.vetmic.2019.06.010

Sudaryatma, P.E., Nakamura, K., Mekata, H., Sekiguchi, S., Kubo, M., Kobayashi, I., Subangkit, M., Goto, Y., Okabayashi, T., 2018a. Bovine respiratory syncytial virus infection enhances *Pasteurella multocida* adherence on respiratory epithelial cells. *Veterinary Microbiology*. 220, 33–38. https://doi.org/10.1016/j.vetmic.2018.04.031

Sudaryatma, P.E., Nakamura, K., Mekata, H., Sekiguchi, S., Kubo, M., Kobayashi, I., Subangkit, M., Goto, Y., Okabayashi, T., 2018b. Bovine respiratory syncytial virus infection enhances *Pasteurella multocida* adherence on respiratory epithelial cells. *Veterinary Microbiology*, 220, 33–38. https://doi.org/10.1016/j.vetmic.2018.04.031

Sudaryatma, P.E., Saito, A., Mekata, H., Kubo, M., Fahkrajang, W., Okabayashi, T., 2020. Bovine respiratory syncytial virus decreased *Pasteurella multocida* adherence by downregulating the expression of intercellular adhesion molecule-1 on the surface of upper respiratory epithelial cells. *Veterinary Microbiology*. 246, 108748.

Sutha, D., Sabariah, I., Surash, R., Santhini, M., Yam, M.F., 2010. Evaluation of the hepatoprotective activity of standardized ethanolic extract of *Curcuma xanthorrhiza* Roxb. *Journal of Medicinal Plants Research*. 4, 2512–2517. https://doi.org/10.5897/JMPR10.453

Tjønehøj, K., Uttenthal, Å., Viuff, B., Larsen, L. E., Røntved, C., Rønsholt, L., 2003. An experimental infection model for reproduction of calf pneumonia with bovine respiratory syncytial virus (BRSV) based on one combined exposure of calves. *Research in Veterinary Science* 74, 55-65. https://doi.org/10.1016/S0034-5288(02)00154-6

Torres, S., Thomson, D.U., Bello, N.M., Nosky, B.J., Reinhardt, C.D., 2013. Field study of the comparative efficacy of gamithromycin and tulathromycin for the treatment of undifferentiated bovine respiratory disease complex in beef feedlot calves. *American Journal of Veterinary Research*. 74, 847-853. https://doi.org/10.2460/ajvr.74.6.847

Van der Poel, W.H.M., Kramps, J.A., Middel, W.G.J., Van Oirschot, J.T., Brand, A., 1993. Dynamics of bovine respiratory syncytial virus infections: a longitudinal epidemiological study in dairy herds. *Archives of Virology*. 133, 309–321. https://doi.org/10.1007/BF01313771

Wiart, C., Kumar, K., Yusof, M.Y., Hamimah, H., Fauzi, Z.M., Sulaiman, M., 2005. Antiviral properties of ent-labdene diterpenes of *Andrographis paniculata* Nees, inhibitors of herpes simplex virus type 1. *Phytotherapy Research*. 19, 1069-1070. https://doi.org/10.1002/ptr.1765

Widhiantara, I.G., Permatasari, A.A.A.P., Rosiana, I.W., Wiradana, P.A., Widiastini, L.P., Jawi, I.M., 2021. Antihypercholesterolemic and Antioxidant Effects of *Blumea balsamifera* L. leaf extracts to maintain luteinizing hormone secretion in rats induced by high-cholesterol diets. *The Indonesian Biomedical Journal*. 13, 396–402. https://doi.org/10.18585/inabj.v13i4.1694

Widiastuti, N.K., Virginia, N.M., Fery Yastawan, I.M., Ayu Putri Permatasari, A.A., Wiradana, P.A., Widhiantara, I.G., Hari Sucipto, T., 2023. Cytotoxicity evaluation of *Erythrina lithosperma* Miq. leaf extract against vero cell lines: *in vitro* study. *Research Journal of Pharmacy and Technology*. 16, 153–158. https://doi.org/10.52711/0974-360X.2023.00028

Wintachai, P., Kaur, P., Lee, R.C.H., Ramphan, S., Kuadkitkan, A., Wikan, N., Ubol, S., Roytrakul, S., Chu, J.J.H., Smith, D.,R., 2015. Activity of andrographolide against chikungunya virus infection. *Scientific Reports*. 5, 14179. https://doi.org/10.1038/srep14179

Wiradana, P.A., Permatasari, A.A.A.P., Sari, N.K.Y., Widhiantara, I.G., Sandhika, I.M.G.S., Waruwu, E, Fimaputra, J.M., Panjaitan, N.S.D., Putri, F.R., Ansori, A.N.M., 2024. Isolation and molecular identification of endophytic fungi associated with brown algae for inhibiting *Escherichia coli* ESBL. *Journal Applied Veterinary Science and Technology*. 5, 31–41.

Wright, G.D., 2014. Something old, something new: revisiting natural products in antibiotic drug discovery. *Canadian Journal of Microbiology*. 60, 147–154. https://doi.org/10.1139/cjm-2014-0063

Yeh, C.F., Chang, J.S., Wang, K.C., Shieh, D.E., Chiang, L.C., 2013. Water extract of *Cinnamomum cassia* Blume inhibited human respiratory syncytial virus by preventing viral attachment, internalization, and syncytium formation. *Journal of Ethnopharmacology*. 147, 321–326. https://doi.org/10.1016/j.jep.2013.03.010