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# Polyphenol rich sugarcane extract (PRSE) has potential antiviral activity against influenza A virus *in vitro*

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ABSTRACT

Influenza A virus (IAV) is one of the major global public health concerns but the emerging resistance of IAV to currently available antivirals requires the need to identify potential alternatives. Polyphenol rich sugarcane extract (PRSE) is an extract prepared from the sugarcane plant *Saccharum Officinarum*. Herein we aimed to determine if PRSE had antiviral activity against IAV. We showed that treatment of IAV-infected cells with PRSE results in a dose-dependent inhibition of virus infection at concentrations that were non-cytotoxic. PRSE treatment limited the early stages of infection, reducing viral genome replication, mRNA transcription and viral protein expression. PRSE did not affect the ability of IAV to bind sialic acid or change the morphology of viral particles. Additionally, PRSE treatment attenuated the replication of multiple IAV strains of the H3N2 and H1N1 subtype. In conclusion, we show that PRSE displays antiviral activity against a broad range of IAV strains, *in vitro*.

## 1. Introduction

Influenza A virus (IAV) is an enveloped, negative-sense single-stranded RNA virus with a segmented genome, belonging to the *Orthomyxoviridae* family (Arbeitskreis Blut, 2009). It is classified into different subtypes according to the antigenic variations in hemagglutinin (HA) and neuraminidase (NA) proteins present on the surface of the virus particle (Javanian et al., 2021). Specifically, the accumulation of mutations in viral HA and NA gives rise to new IAV strains within a particular subtype, causing seasonal epidemics of disease. Occasionally, the emergence of an IAV with novel HA and/or NA subtypes, to which the human population has no pre-existing immunity, results in a pandemic associated with significant morbidity and mortality (Nypaver et al., 2021; Vousden and Knight, 2021). Therefore, continued seasonal epidemics of IAV disease (Ryu and Cowling, 2021), and the increasing emergence of highly pathogenic avian IAV strains with pandemic potential (Liu et al., 2021; Yamaji et al., 2020) mean that IAV continues to be a major public health concern globally.

Currently, the most effective way to prevent severe influenza disease is through vaccination to avoid infection. However, influenza vaccines need to be re-evaluated every year as new seasonal strains arise, and additional factors including age can also influence overall vaccine efficacy in particular individuals (Kini et al., 2022; McLean and Belongia, 2021; Nypaver et al., 2021). Furthermore, seasonal vaccines are unlikely to provide protection against a newly emerging strain of IAV during a pandemic (Doyon-Plourde et al., 2023).

In conjunction with vaccination, antiviral drugs have been used to treat influenza virus infection to prevent severe disease, particularly in patients at high-risk of severe complications. Currently, the three categories of influenza antivirals used clinically are the M2 ion channel inhibitors (e.g. Amantadine, Rimantadine, adamantane derivates), NA inhibitors (including Oseltamivir, Laninamivir, Peramivir and Zanamivir) and the cap-dependent endonuclease inhibitor targeting the viral polymerase acidic protein (PA) (the FDA licensed antiviral, Baloxavir) (Swierczynska et al., 2022). However, newly emerging IAV strains continue to show mutations in the M2 ion channel, NA and PA proteins associated with antiviral drug resistance. For example, circulating H1N1 subtypes are completely replaced with strains carrying the M2-S31 N substitution (Duwe, 2017). Moreover, resistance to NA inhibitors through the NA H274Y mutation has been widely documented

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(Davidson, 2018; Gubareva and Fry, 2020; Yadav et al., 2021; Yusuf et al., 2016) and low frequency of resistance to Baloxovir has been observed and is associated with specific mutations in the viral PA protein (Gubareva and Fry, 2020; Hickerson et al., 2023). It is also possible that the currently used antivirals will not be effective against newly emerging IAV strains during a pandemic (Chan and Hui, 2023; Li et al., 2023). Therefore, it is imperative to identify alternative antiviral drugs that are highly effective against a broad range of influenza virus strains.

Plants have been used for medical purposes to enhance the general health and wellbeing of humans for centuries (Sen and Samanta, 2015). It was estimated that 80% of the world population has used herbal medicine and plant extracts in their treatment of disease and clinical conditions (Sen and Samanta, 2015). Polyphenol rich sugarcane extract (PRSE) is an extract prepared from the molasses of a sugarcane plant, *Saccharum Officinarum*, based on a patented hydrophobic resin procedure by The Product Makers Pty Ltd (Ji et al., 2019). *Saccharum Officinarum* is thought to be of great value to study as an herbal medicine and there are multiple studies showing that the derivatives of *Saccharum Officinarum* are physiologically beneficial to both humans and animals. (Ahtesh et al., 2020; Awad et al., 2016; Dewi et al., 2021; Ogunwole et al., 2020; Prakash et al., 2021; Zhao et al., 2015).

Polyphenols, including flavonoids and phenolic acids, are a group of compounds found in plants that are beneficial to human health. Resveratrol (3,4′,5-trihydroxystilbene, 3,4',5-stilbenetriol), as an example of polyphenols, which can be found in plants, has been shown to have antioxidant and anti-inflammatory properties (Luca et al., 2020). Another example of polyphenol can be curcumin (diferuloylmethane; 1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione), which is derived from turmeric. It is known to have antimicrobial and antitumor activities (Luca et al., 2020). PRSE, the product of the patented hydrophobic resin extraction procedure with enriched contents of polyphenols, thus is likely to possesses similar benefits to human (Deseo et al., 2020). PRSE contains a 221 mg gallic acid equivalency (GAE)/g polyphenol, which is much higher than natural products which are known for their polyphenol contents such as ground turmeric spice, dry cocoa powder and sumac bran (Ji et al., 2019). A previous study showed that PRSE displayed anti-inflammatory properties which was suggested to be achieved via inhibiting pro-inflammatory cytokines and initiating the nuclear factor erythroid 2-related factor 2 - antioxidant response element (Nrf2-ARE) pathway (Ji et al., 2020). In addition, PRSE demonstrated antioxidant activity which corelated with the raised polyphenol composition achieved in the purification process (Deseo et al., 2020).

Liquid chromatography – mass spectrometry (LC-MS) analysis on PRSE revealed the polyphenol composition to contain chlorogenic acid, tricin and luteolin (Deseo et al., 2020). Although PRSE has not been previously investigated for its antiviral activity against IAV, it was observed that chlorogenic acid has antiviral activity against IAV (both H1N1 and H3N2) potentially by the inhibition of neuraminidase activity (Ding et al., 2017). Tricin was found to inhibit human cytomegalovirus (HCMV) replication by depressing CC-motif ligand 2 (CCL2) (Akai et al., 2017). In addition, luteolin was reported to inhibit Japanese encephalitis virus (JEV) by both extracellular virucidal activity and stages after entry (Fan et al., 2016). Herein, we investigated the antiviral potential of PRSE against IAV *in vitro* and established that there was broad spectrum antiviral activity against a range of different IAV strains, modulating the early stages of viral replication.

## 2. Materials and methods

### 2.1. Cells

Madin-Darby canine kidney (MDCK) cells (American type culture collection (ATCC), Manassas, VA) were maintained and passaged in RPMI 1640 Medium (Gibco, Thermo Fisher Scientific, MA, USA) supplemented with 10% (v/v) heat-inactivated foetal bovine serum (FBS) (GE Healthcare Life Sciences, Utah, USA), 1% sodium pyruvate (Gibco), 1% Glutamax (Gibco) and 100 units/mL penicillin and 100 μg/mL streptomycin (Gibco) at 37 °*C* in 5% $CO_2$. A549 human lung epithelial cells (ATCC, Manassas, VA) were maintained and passaged in Kaighn's modification of Ham's F-12 medium (Gibco, Thermo Fisher Scientific, MA, USA) supplemented with 10% (v/v) FBS, 1% sodium pyruvate, 1% Glutamax and 100 units/mL penicillin and 100 μg/mL streptomycin at 37 °*C* in 5% $CO_2$.

### 2.2. Virus strains

The influenza viruses used in this study were provided as allantoic fluid by Prof Patrick Reading from the World Health Organisation Collaborating Centre for Reference and Research on Influenza, VIDRL at the Doherty Institute). A/Beijing/353/89 (H3N2), A/X-31 (X31) (H3N2): PB1, PB2, NP, PA, M and NS gene segments of A/Puerto Rico/8/34 (PR8) virus and the HA and NA gene segments of A/Aichi/2/68 (H3N2) virus, A/Udorn/307/1972 (H3N2), A/Brazil/11/1978 (H1N1), A/Solomon Islands/3/2006 (H1N1), A/Auckland/1/2009 (pdmH1N1) and A/Fiji/2/2016 (pdmH1N1) were used as the representative IAV strains in this study.

### 2.3. PRSE

PRSE is extracted from sugarcane molasses using a patented hydrophobic extraction process by The Product Makers Pty Ltd (Ji et al., 2019, 2020). PRSE powder was stored at room temperature. The PRSE compound was reconstituted at a stock solution of 10 mg/mL in serum-free medium (RPMI) and filtered through a Millex 0.22μm PVDF syringe filter (Merck Millipore, Germany). Filtered PRSE was aliquoted and stored at -80 °C before dilution in serum-free medium to obtain the required concentration indicated in the experiments.

### 2.4. Virus infection

MDCK cell monolayer was cultured in 12-well tissue culture plates (Corning, New York, USA) overnight to reach 70% confluency. Cells were infected with IAV in serum-free media for an hour at 37 °*C* in the presence of 5% $CO_2$. The virus inoculum was then removed, and monolayers washed with phosphate buffered saline (PBS) to remove residual input virus and maintained in serum-free medium with a final concentration of 0.5 μg/ml TPCK treated trypsin (Worthington Biochemical Corporation NJ, USA) (to allow for multicycle replication) at 37 °*C* in 5% $CO_2$. Equal volume of 2 mg/ml PRSE was added to the wells at 2 h.p.i. (to make the final concentration 1 mg/mL). Cell-free supernatants were harvested at 24 h.p.i. for further titration by plaque assay. Whole cell lysates or infected cells were as well harvested at 24 h.p.i. for western blotting, immunofluorescence assay (IFA) or flow cytometry analysis.

### 2.5. Plaque assay

MDCK cell monolayer was cultured in 6-well tissue culture plates (Corning, New York, USA) overnight to reach 100% confluency. Media was replaced with serum-free media before the addition of virus inoculum. Virus inoculum was removed after 45min incubation at 37 °*C* in the presence of 5% $CO_2$. 3 ml overlay containing Leibovitz's L-15 Medium (Gibco, Thermo Fisher Scientific, MA, USA), 0.9% agarose (Sigma-Aldrich, St. Louis, Missouri, United States) and 0.5ug/ml TPCK treated trypsin (Worthington Biochemical Corporation NJ, USA) were added and incubated for 72h before identification of plaques.

### 2.6. Quantitative real-time polymerase chain reaction (qRT-PCR) to detect IAV viral messenger RNA (mRNA) and viral RNA (vRNA)

Total RNA was isolated from the lysates of infected cells by TRIzol RNA

extraction. cDNA was prepared using the Tetro cDNA synthesis kit according to manufacturers instructions (Bioline, TN, USA). Oligo DT primer was used for cDNA synthesis of mRNA and Uni12 primer (Fwd: CTGATCTAGACCTGCAGGCTCAGCAAAAGCAGG) was used for cDNA synthesis of vRNA. qPCR was performed using the SensiFAST 2x SYBR Lo-Rox kit (Bioline) with primer pairs specific for IAV M gene (Fwd: GACCRATCCTGTCACCTCTGAC; Rev: GGGCATTYTGGACAAAKCGTCTACG) and NA gene (Fwd: CAACCAAGTAATGCCGTGTG; Rev: TTGTCACCCAAATGTCTCCA). Standard curves were generated for quantification of expression. Data acquisition and analysis was performed using the QuantStudio 7 Flex Real-Time PCR System and Design and analysis software (Applied Biosystems).

### 2.7. Detection of IAV protein expression by Western blot

Whole cell lysates were prepared using 1% NP40 cell lysis buffer (Invitrogen, Waltham, Massachusetts, United States). Samples were heated to 90 °C for 5 min before separation by SDS-PAGE using precast 4-15% gradient gels (Thermo Fisher Scientific, MA, USA), followed by transfer to polyvinylidene difluoride (PVDF) membrane (Thermo Fisher Scientific, MA, USA). Membranes were blocked in PBS with 5% (w/v) bovine serum albumin (Sigma-Aldrich, St. Louis, Missouri, United States) (BSA) and 0.1% (v/v) Tween20 (Sigma-Aldrich, St. Louis, Missouri, United States). All subsequent washes and antibody binding steps were performed in PBS containing 0.1% (v/v) Tween 20. The cellular protein calnexin (approximately 67 kDa in size) was also visualised to ensure equivalent amount of sample loading using a rabbit polyclonal antibody to calnexin (Ab22595, Abcam, Cambridge, United Kingdom) in conjunction with donkey anti-rabbit Ig-Horseradish peroxidase (HRP) (A16035, Invitrogen, Waltham, Massachusetts, United States). IAV viral proteins were detected using a mouse monoclonal antibody to IAV nucleoprotein (NP) (OBT1555, BioRad, Hercules, California, United States) or a mouse monoclonal antibody to IAV M1 protein (MCA401, BioRad, Hercules, California, United States) in conjunction with goat anti-mouse Ig-HRP (G21040, Invitrogen, Waltham, Massachusetts, United States) and detected by enhanced chemiluminescence (ECL) after adding Western Lightning Ultra (Perkin Elmer, VIC, Australia) on an Amersham Imager 600 Series (GE Healthcare Life Sciences, Utah, USA).

### 2.8. Visualisation of IAV protein expression by immunofluorescence assay (IFA)

PRSE treated (24h), infected MDCK cells, mock-treated, IAV-infected MDCK cells and mock-treated, uninfected MDCK cells on coverslips were fixed with 4% (v/v) Paraformaldehyde (PFA) in PBS and blocked with 0.1 M glycine and 5% (w/v) BSA with 5% FBS (v/v) in PBS. Cells were then permeabilised with 5% (v/v) Triton-X-100 in PBS and stained with Hoechst33342 (H1399, Life technologies, Carlsbad, California, United States) and mouse anti-IAV NP antibody (OBT1555, BioRad, Hercules, California, United States) in conjunction with donkey anti-mouse-Alexa flour 647 (A-31571, Life technologies, Carlsbad, California, United States). Coverslips were then mounted on glass-slides by Prolong Diamond antifade mountant (P369665, Thermo Fisher Scientific, MA, USA) and left dry overnight at RT. Slides were then stored at 4 °*C* and cells were analysed using a Zeiss 780 Confocal Microscope and Zen™ Zeiss ® software.

### 2.9. Determination of cytotoxicity and detection of IAV protein expression by flow cytometry

PRSE-treated (24h) MDCK cells or IAV-infected MDCK cells were detached and stained with fixable viability dye eFluor 780 (Invitrogen, Waltham, Massachusetts, United States) before fixation with 4% (v/v) PFA in PBS. After fixation, IAV-infected cells were permeabilised with 0.5% (v/v) Triton-X-100 in PBS and stained with mouse anti-M1 antibody (MCA401, BioRad, Hercules, California, United States) in conjunction with donkey anti-mouse Alexa flour 647 (A-31571, Life technologies, Carlsbad, California, United States). Samples were analysed on a FACSCanto II (BD Biosciences, CA, USA) or a LSRFortessa flow cytometer (BD Bioscience) before data analysis using FlowJo software (version 10.4). The CC50 of PRSE in MDCK cells was determined as 4.47 mg/mL and the IC50 of PRSE was determined to be 0.45 mg/mL.

### 2.10. Electron microscopy

Purified preparations of IAV BJx109 (H3N2), a high-yielding reassortant of PR8 with A/Beijing/353/89 (Beij/89; H3N2) bearing the H3N2 surface glycoproteins, were fixed with 4% (v/v) PFA in PBS and placed onto glow discharged 100 mesh, formvar treated copper grids before staining with 1% Uranyl acetate in H2O. Stained samples were analysed on a Thermo Scientific™ Talos™ L120C transmission electron microscope (TEM) at the Ian Holmes Imaging Centre, University of Melbourne. Images were collected on a digital camera and processed in Adobe photoshop.

### 2.11. Haemagglutination (HA) and haemagglutination inhibition assay (HIA)

Hemagglutinating units (HAU) of A/Beijing/353/89 virus stock was first determined by hemagglutination assay (HA). Two-fold dilution was performed on IAV stock followed by addition of 1% turkey red blood cells (RBCs) kindly supplied by the WHO Collaborating Centre for Reference and Research on Influenza. The results were read by the presence of hemagglutination post incubation. To determine the impact of PRSE on RBCs, RBCs were incubated with PRSE for 2h before centrifugation and washes with PBS to remove remaining PRSE. 1% Treated (and untreated) RBCs were added and incubated with diluted viruses before read-out. To determine the impact of PRSE on virus particles, haemagglutination inhibition assay (HIA) was performed. IAV was diluted to 4HAU and incubated with different concentrations of PRSE for an hour before the addition and incubation with 1% RBCs. Results were read and recorded after incubation.

### 2.12. Statistical analysis

Graph presentation and statistical analysis of data was performed using GraphPad Prism (GraphPad Software, San Diego, CA). P values were calculated by one-way ANOVA with Dunnett's multiple comparison test or unpaired, two-tailed *t*-test as indicated.

## 3. Results

### 3.1. PRSE inhibits IAV infection in vitro

PRSE has been assessed previously for its potential biological benefits (Ahtesh et al., 2020; Ji et al., 2019, 2020). However, a role as an antiviral has received little attention. Thus, we aimed to evaluate the antiviral potential of the PRSE against IAV (A/Beijing/353/89 (BJ89); H3N2). We initially tested the cytotoxicity effect on MDCK cells after 24h at concentrations ranging from 10 mg/mL to 1 ng/mL and observed that concentrations of up to 2 mg/mL did not induce severe cytotoxic effects in these cells (Fig. S1). To assess the antiviral effect of PRSE against IAV *in vitro* we infected MDCK cells with IAV at an MOI of 0.01 and the cells were subsequently treated at 2 h.p.i. with PRSE at 1 mg/mL, 0.5 mg/mL, 0.1 mg/mL or left untreated. At 24 h.p.i. cell-free supernatants and whole cell lysates were collected and the effect of PRSE on the production of infectious virus and viral protein production was evaluated by plaque assay and western blotting, respectively (Fig. 1).

We observed that PRSE had a dose-dependent inhibitory effect on IAV replication, with the addition of 1 mg/mL PRSE demonstrating the most significant antiviral activity (Fig. 1). We observed that treatment

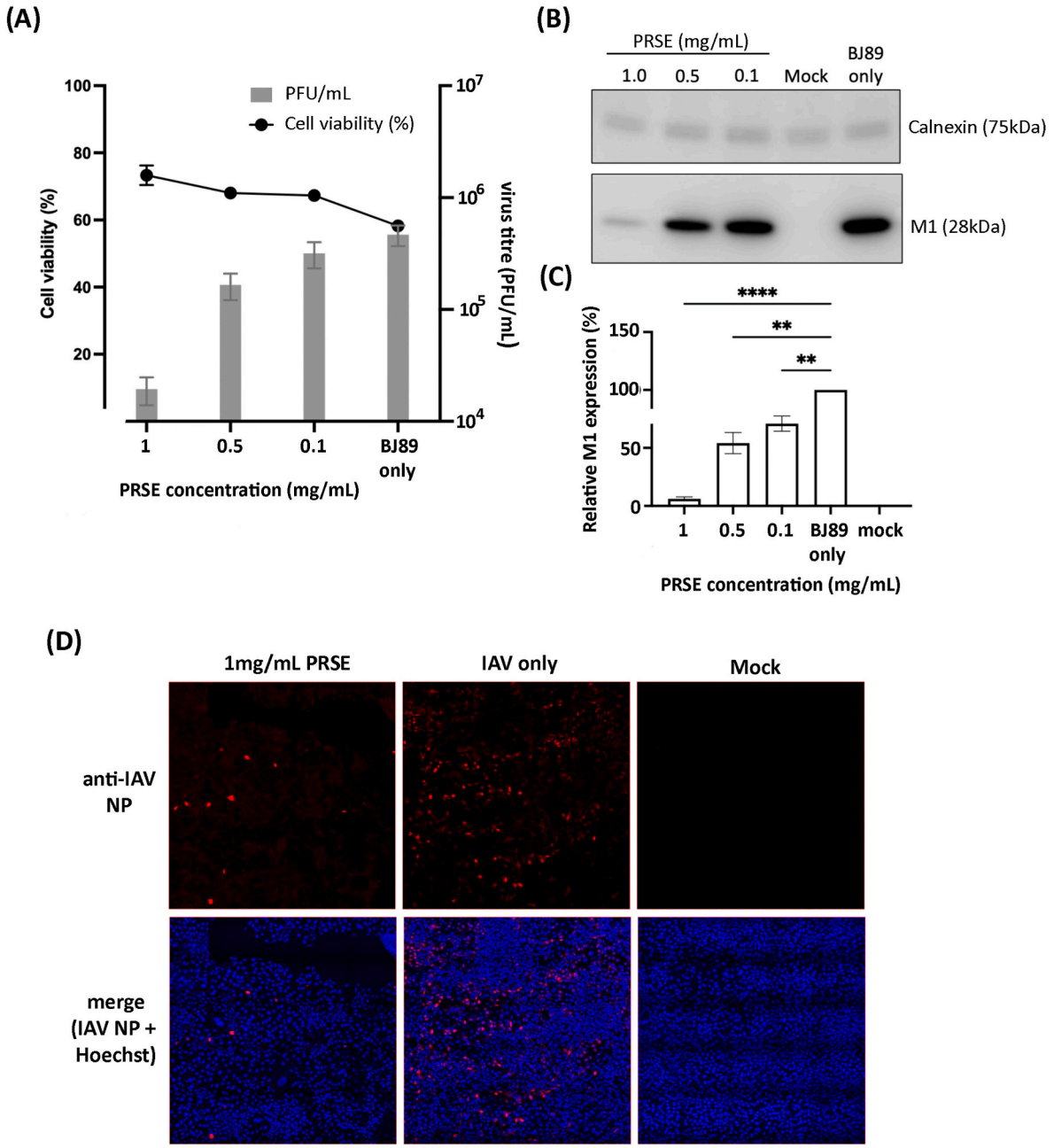



**Fig. 1. PRSE has antiviral activity against influenza A virus (IAV) (A/Beijing/353/89) replication *in vitro*.** MDCK cells were infected with IAV (A/Beijing/353/89) (BJ89, H3N2) at an MOI of 0.01 and PRSE (1 mg/mL, 0.5 mg/mL, or 0.1 mg/mL) was added at 2 h.p.i. and maintained in culture media for duration of infection. Cell free supernatants and whole cell lysates were harvested at 24 h.p.i. for subsequent analyses. **(A)** Infectious virus in cell free supernatant was quantitated by plaque assay, and virus titres are expressed as PFU/ml. Cell viability and cytotoxicity of MDCK cells following PRSE treatments was assessed by flow cytometry and expressed as percentage of viable cells. Data is generated from 3 independent experiments performed in triplicate. **(B)** Viral protein expression in whole cell lysates were determined by Western blot. IAV M1 protein (28 kDa) was probed by anti-M1 antibodies in conjunction with HRP-conjugated secondary antibodies and detected by ECL. Calnexin (75 kDa) was used as a protein loading control. A representative Western blot from three independent experiments is shown as above. **(C)** Quantitation of M1 proteins in western blots is expressed as relative M1 expression normalised against calnexin expression (N = 3 independent experiments). Error bars = SEM, **p<0.01, ****p<0.0001, by one-way ANOVA with Dunnett's multiple comparison test, where comparisons are made to the virus only control. **(D)** IAV Infection in MDCK cells with and without PRSE treatment at 24 h.p.i were visualised by immunofluorescence assay. IAV infection was visualised with anti-NP antibodies conjugated with species-specific anti-IgG AF647 secondary antibodies, and the nuclei were counterstained by Hoechst 33342. Images were captured by 5x5 tile scanning and processed for publication in Adobe Photoshop.

with 1 mg/mL reduced infectious virus production by ~95% compared to virus only, while addition of 0.5 mg/mL or 0.1 mg/mL PRSE reduced the viral yield by ~40% and 30%, respectively (Fig. 1A). All three concentrations exhibited minimal cytotoxicity effects on MDCK cells at 24h compared to medium only control (Fig. 1A). In addition to the reduction in infectious viral yield, the antiviral effect of PRSE was also confirmed by analysis of IAV protein expression using western blotting. Again, we observed a significant inhibitory effect of 1 mg/mL PRSE on IAV viral protein (M1) expression. The expression of IAV M1 protein was observed to correlate with virus production as treatment with 1, 0.5 or 0.1 mg/mL PRSE reduced the protein production by 95%, 45% and 30%, respectively (Fig. 1B and C). Moreover, the antiviral effects of PRSE were

also visualised by microscopy on MDCK cells at 24h.p.i (Fig. 1D). The number of IAV NP-expressing cells were observed to be significantly lower in 1 mg/mL PRSE-treated, IAV-infected cells compared to mock-treated, IAV-infected MDCK cells, which indicated less infection as a result of PRSE treatment. We also observed a similar reduction in A549 cells infected with IAV (Fig. S2), indicating this wasn't a cell specific effect.

Overall, these observations suggest that PRSE treatment can attenuate the replication of IAV *in vitro* and restricts the production of IAV proteins and the release of infectious virus particles.

### 3.2. PRSE inhibits IAV replication by modulating IAV viral protein expression

As we had observed that PRSE treatment resulted in a reduction in both infectious IAV yield and IAV M1 protein expression (Fig. 1), we aimed to determine if this was due to an impact on the production of viral mRNA and/or vRNA. To assess this, MDCK cells were infected with IAV at an MOI of 0.01 and PRSE was added at 1 mg/mL at 2 h.p.i. At 2, 8 and 24 h.p.i., cells were harvested, and RNA was extracted for qRT-PCR analysis to determine the production of mRNA (viral M and NA genes) and vRNA (viral M and NA genes). At 8 h.p.i., both mRNA and vRNA were observed to increase in expression compared to the 2 h.p.i. input control in the infected only samples, indicative of viral replication following infection (Fig. 2A-D). However, we observed that the PRSE treated, and IAV-infected cells expressed significantly lower levels of vRNA and slightly lower levels of mRNA for both genes when compared to untreated and IAV-infected cells at 8 and 24 h.p.i. (Fig. 2A-D). These results indicate that PRSE treatment may impact on early viral transcription events.

After observing the effect of PRSE on IAV replication at the viral genome and mRNA level, we utilised flow cytometric analysis to evaluate IAV infection and viral protein expression in cells after PRSE treatment. We used the IAV M1 protein as the marker for infection and replication. From the results displayed in Fig. 2E and F, we observed a significant reduction in the expression of M1 protein in IAV-infected MDCK cells treated with PRSE (8.7% cells positive for M1 protein) compared to untreated cells (36.7% of cells positive for M1 protein).

These results confirm our previous observations and indicate that PRSE treatment impacts on the ability of IAV to replicate *in vitro* and to produce both viral protein and infectious virus during infection.

### 3.3. PRSE acts at early stages of IAV replication

A complete IAV replication cycle includes virus attachment, entry, uncoating, genome transcription, protein synthesis, virion assembly and release (Dou et al., 2018). To determine if the antiviral effect of PRSE against IAV is solely dependent on its modulation on viral genome transcription and viral protein expression, a time-of-addition (TOA) assay (Kim et al., 2021; Wu et al., 2015) was designed and performed as illustrated in the scheme in Fig. 3A. MDCK cells were infected with IAV at an MOI of 0.01. 1 mg/mL PRSE was added at different time points pre- and post-infection (-2 h to 0 h, -2 h to 24 h, -1 h to 0 h, -1 h to 24 h, 0 h to 24 h, 2 h to 24 h and 8 h to 24 h) and the virus inoculum was also treated with 1 mg/mL PRSE prior to infection, to evaluate its impact on different stages of the IAV replication cycle. Cell-free supernatants and whole cell lysates were collected at 24 h.p.i. and examined to assess the antiviral activity of PRSE on both infectious virus production and viral protein expression.

As shown in Fig. 3B-D, the inhibitory effect of PRSE against IAV displayed a time of addition dependency during IAV replication. We observed that addition of PRSE 2 h prior to infection and maintained in the medium during the course of infection significantly reduced the production of infectious virus and IAV NP protein production (Fig. 2B-D). However, pre-treatment of the virus inoculum for 1 h prior to infection and maintained within the infection medium had the most significant impact over the course of infection (Fig. 2B-D). In contrast to this, pre-treatment of the virus inoculum only had no impact on IAV replication at all (Fig. 2B-D). Subsequent treatment of the infected cells with PRSE after initial attachment (*i.e.* from 0 h.p.i.) showed a time dependency of inhibitory activity with addition at 0 h.p.i. having a significant effect up to the mildest, non-significant effect observed when PRSE was added at 8 h.p.i. (Fig. 2B-D).

These results suggest that PRSE might act not only at viral genome transcription and protein translation, but also at additional early IAV replication stages such as virus attachment, entry or uncoating. The above results also suggest that PRSE is more likely to inhibit IAV replication rather than affecting the interaction of the virus with the host cells directly.

### 3.4. PRSE does not alter virion morphology or interfere with virus attachment during IAV replication

We had observed in Fig. 3 that early addition of PRSE exhibited the most potent antiviral effect against IAV replication. In addition, the pre-treatment of virus with PRSE prior to infection enhanced this inhibitory effect. Therefore, to determine if PRSE induces any morphological changes in virion morphology which might contribute to the above observations we performed electron microscopy on PRSE-treated purified IAV BJx109 (H3N2), a high-yielding reassortant of PR8 with A/Beijing/353/89 (BJ89; H3N2) bearing the H3N2 surface glycoproteins (Fig. 3A-D). We observed IAV particles of ~120 nm in size with a pleomorphic morphology with prominent "spikes" protruding from the virus structure in the untreated samples (Fig. 4C and D). The PRSE-treated virions had a very similar morphology (with respect to shape and presence of the surface HA and NA proteins) to the mock-treated virions (Fig. 4A and B). These observations revealed that PRSE does not have direct virucidal effect that could induce significant morphological changes to IAV virions.

An additional step that PRSE could potentially influence during the IAV replication cycle is virus attachment. It is well known that IAV attachment requires the binding between the viral HA protein and sialic acid receptors expressed on the cell surface (Samji, 2009). Thus, we assessed PRSE-mediated inhibition of IAV HA binding to sialic acid for potential contribution to the observed antiviral activity. The results showed that PRSE-treatment of virus did not inhibit the ability of IAV HA to bind to sialic acid on turkey RBCs, therefore resulting in no inhibition of hemagglutination (Fig. 4E). In addition, PRSE-treatment of the RBCs themselves did not affect the ability of the virus to hemagglutinate turkey RBCs (Fig. 4F).

The results from these two assays confirmed that PRSE does not affect the morphology or structural integrity of the IAV particle itself nor does it inhibit IAV replication by blocking the ability of the IAV HA protein to bind to sialic acid.

### 3.5. PRSE is antiviral against multiple IAV strains in vitro

Our studies so far had indicated that treatment with PRSE had antiviral activity against IAV A/Beijing/353/89 (BJ89), an H3N2 influenza virus strain. We aimed to extend these observations and determine if the inhibitory effect of PRSE against IAV was strain specific. Thus, we extended the study to include six additional IAV strains, including H3N2 (A/Aichi/68 X-31 and A/Udorn/307/1972), pre-2009H1N1 (A/Brazil/11/1978 and A/Solomon Islands/3/2006) and post-2009H1N1pdm (A/Auckland/1/2009 and A/Fiji/2/2016). The treatment type that demonstrated most antiviral effect in our TOA assay (Fig. 3) was adopted in this analysis. Viruses were treated with 1 mg/mL PRSE 1 h prior to infection and then used to infect MDCK cells at 0.01 MOI. PRSE was also included within the infection medium at the time of infection until the supernatants were collected at 24 h.p.i. for analysis via plaque assay (Fig. 5).

We observed that PRSE exhibited significant antiviral activity

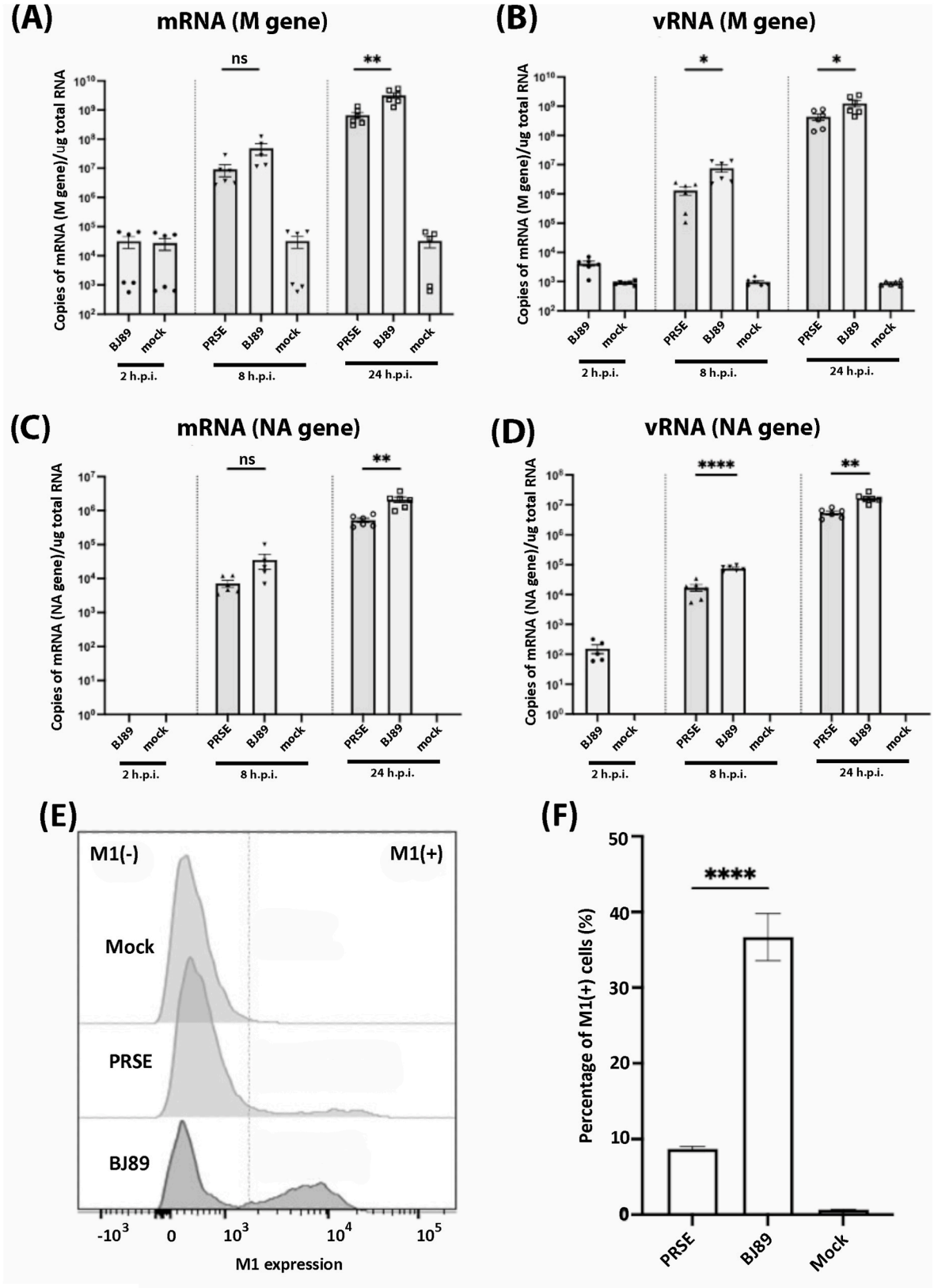



**Fig. 2. PRSE treatment reduces IAV viral mRNA, vRNA and protein expression.** MDCK cells were infected with IAV (A/Beijing/353/89) (BJ89, H3N2) at an MOI of 0.01 and PRSE (1 mg/mL) was added at 2 h.p.i. maintained in culture media for duration of infection. Infected cells were harvested at 24 h.p.i. to extract RNA to quantitate viral mRNA for vRNA or to be stained for IAV proteins which were quantitated by FACS analysis. **(A and B)** IAV M gene and **(C and D)** NA gene mRNA (**A and C)** and vRNA **(B and D)** expression was assessed by qRT-PCR (n = 2 independent experiments performed in triplicate, error bars = SEM, *Unpaired two -tailed *t*-test., **** p< 0.0001 ** p< 0.01, * p< 0.05, ns p> 0.05). **(E and F)** IAV infected and PRSE treated cells were stained with anti-M1 antibodies and protein expression analysed by flow cytometric analysis. PRSE (n = 2, error bars = SEM, *Unpaired two -tailed *t*-test., **** $p \leq 0.0001$.

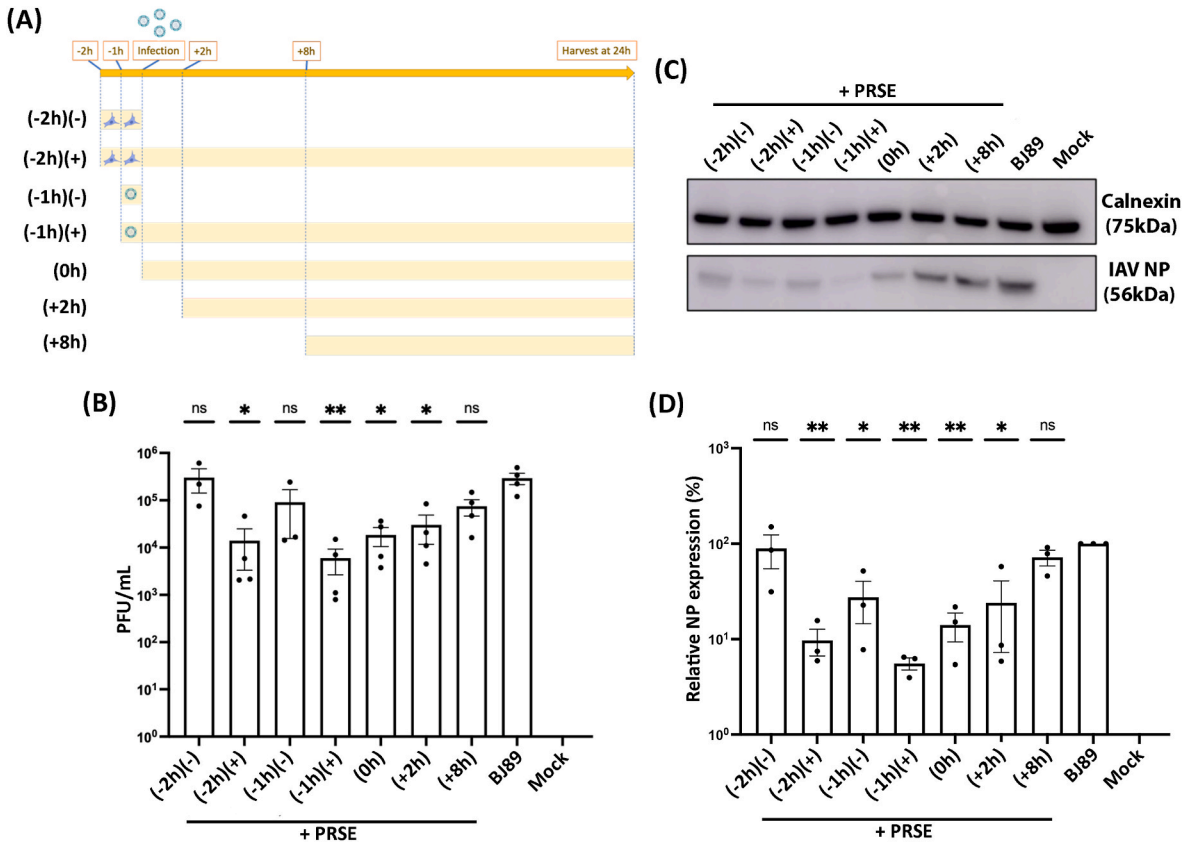



**Fig. 3. Addition of PRSE early in the IAV lifecycle restricts viral protein and infectious virus production. (A)** Schematic of the timing for PRSE treatment of MDCK cells and/or IAV (A/Beijing/353/89) (BJ89, H3N2) at various times pre- and post- IAV infection. MDCK cells were infected with IAV (A/Beijing/353/89) (BJ89, H3N2) at MOI 0.01 with the addition of PRSE at different time points. **(B)** Infectious virus released in cell-free supernatants was quantitated by plaque titration at 24 h.p.i. Data is generated from 3 independent experiments performed in triplicate. Error bars = SEM. *p<0.05, **p<0.01, ns = not significant, by one-way ANOVA with Dunnett's multiple comparison test, where comparisons are made to the virus only control. **(C) and (D)** Whole cell lysates were retrieved and analysed for viral protein expression by western blotting. IAV NP protein (56 kDa) was probed to determine IAV viral protein expression. Calnexin was used as a protein loading control. Quantitation of the western blots from all experiments were performed. NP protein expression was normalised against calnexin. Data is generated from 3 independent experiments performed in triplicate. Error bars = SEM. *p<0.05, **p<0.01, ns = not significant, by one-way ANOVA with Dunnett's multiple comparison test, where comparisons are made to the virus only control.

against all six additional H3N2 and H1N1 IAV strains. The most significant reduction of viral yield was observed in the A/X-31 (H3N2)- and A/Fiji/2/2016 (H1N1pdm)-infected and treated cells with a reduction of 2-2.5 logs observed compared to the virus only (Fig. 5A and D). Treatment of Udorn (H3N2)-, Brazil (H1N1)- and Auckland (H1N1pdm)-infected cells resulted in a reduction of 1.5-2 logs in virus titre (Fig. 5B, C and E). The most modest antiviral effect was observed in the Solomon Islands (H1N1)-infected cells, but the infectious virus production was still reduced by ~1 log (18.68%) after pre-treatment with PRSE (Fig. 5F).

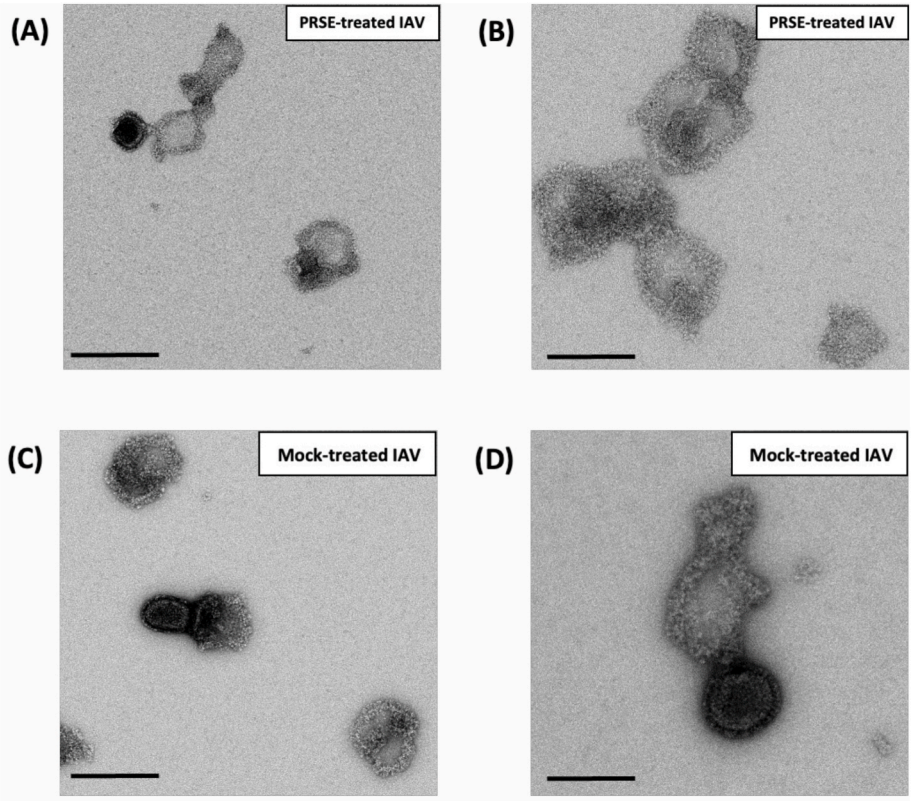
Overall, these observations indicate that the antiviral effect of PRSE against IAV replication *in vitro* is effective against a broad range of IAV strains, including H3N2 and H1N1 strains both pre- and post-2009 pandemic.

## 4. Discussion

Different classes of antiviral drugs have been clinically used as an important intervention to combat severe IAV disease. There are three categories of antivirals that have been previously used or are currently used against IAV infection, which are M2 ion channel inhibitors, neuraminidase inhibitors and PA endonuclease inhibitors. However, the emerging resistance of IAV to these three categories of antivirals urges the development of alternative IAV antivirals with different targets (Caceres et al., 2022; Chan and Hui, 2023; Duwe, 2017; Gubareva and Fry, 2020; Luo et al., 2023).

In this study, we showed that PRSE displayed potent inhibitory activity against multiple IAV strains *in vitro*. PRSE treatment inhibited up to 90% of viral replication of all selected strains in both MDCK cells (Fig. 5) and A549 cells (Fig. S2), indicating broad-spectrum anti-IAV activity of this compound. Dose-dependent reduction in both infectious virus production and viral protein expression was observed upon PRSE treatment. To determine whether this effect was due to an impact on the transcription of viral RNA, we performed qRT-PCR to determine the abundance of viral mRNA and vRNA during viral infection post PRSE treatment. We observed that at 8 h.p.i. and 24 h.p.i., PRSE treatment reduced the amount of viral mRNA and vRNA by 80% (Fig. 2A-D), which is similar to some known PB2 inhibitor such as D715-2441, was observed to bind to the PB2 cap protein and reduce viral mRNA expression by 80% (Liu et al., 2018). This reduction in viral genome transcription could impact on the reduction in viral protein translation we observed (Fig. 1B, C, and 2E and F), which would in turn result in reduced production of infectious viruses (Fig. 1A). However, whether the inhibitory effect of PRSE is solely dependent on the modulation of viral genome expression remains unknown and needs to be further investigated.

Based on the above results, time of addition assays were performed to assess if PRSE can act on any other stages of IAV replication. The results showed that early addition of PRSE enhanced the inhibitory effect against IAV (Fig. 3). These observations have indicated that apart




**(E) Pre-treat IAV:**

| PRSE | 2mg/ml | 1mg/ml | 500ug/ml | 250ug/ml | 125ug/ml | 63ug/ml | 31ug/ml | 16ug/ml |
|---|---|---|---|---|---|---|---|---|
| Replicate #1 | + | + | + | + | + | + | + | + |
| Replicate #2 | + | + | + | + | + | + | + | + |
| Replicate #3 | + | + | + | + | + | + | + | + |
| HAI control | 1:10 | $1:10^2$ | $1:10^3$ | $1:10^4$ | $1:10^5$ | $1:10^6$ | $1:10^7$ | $1:10^8$ |
| | - | +/- | + | + | + | + | + | + |

**(F) Pre-treat RBC:**

| Virus dilution | 1:1 | 1:2 | 1:4 | 1:8 | 1:16 | 1:32 | 1:64 | 1:128 | 1:256 | 1:512 | 1:1024 | 1:2048 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Untreated RBC | + | + | + | + | +/- | - | - | - | - | - | - | - |
| PRSE treated RBC #1 | + | + | + | + | +/- | - | - | - | - | - | - | - |
| PRSE treated RBC #2 | + | + | + | + | +/- | - | - | - | - | - | - | - |
| PRSE treated RBC #3 | + | + | + | + | +/- | - | - | - | - | - | - | - |

Key: '+' = haemagglutination, '+/-" = partial haemaglutination, '-' = no haemaglutination

**Fig. 4. PRSE does not alter virion morphology nor interfere with virus attachment during IAV replication.** Negative staining of purified IAV (A/Beijing/89 X-109) treated with 1 mg/mL PRSE for 1hr **(A) and (B)** or mock-treated **(C) and (D)**. Magnification bars represent 250 nm in **(A)** and **(C)** and 100 nm in **(B)** and **(D)**. **(E)** Hemagglutination inhibition assay was performed on IAV (A/Beijing/353/89) (BJ89, H3N2) pre-treated 1h with differing concentrations of PRSE. '+' = Hemagglutination, '+/-' = Partial hemagglutination, '-' = No hemagglutination. **(F)** RBCs were treated with 1 mg/mL PRSE for 2h treated RBCs before addition of IAV. '+' = Hemagglutination, '+/-' = Partial hemagglutination, '-' = No hemagglutination.

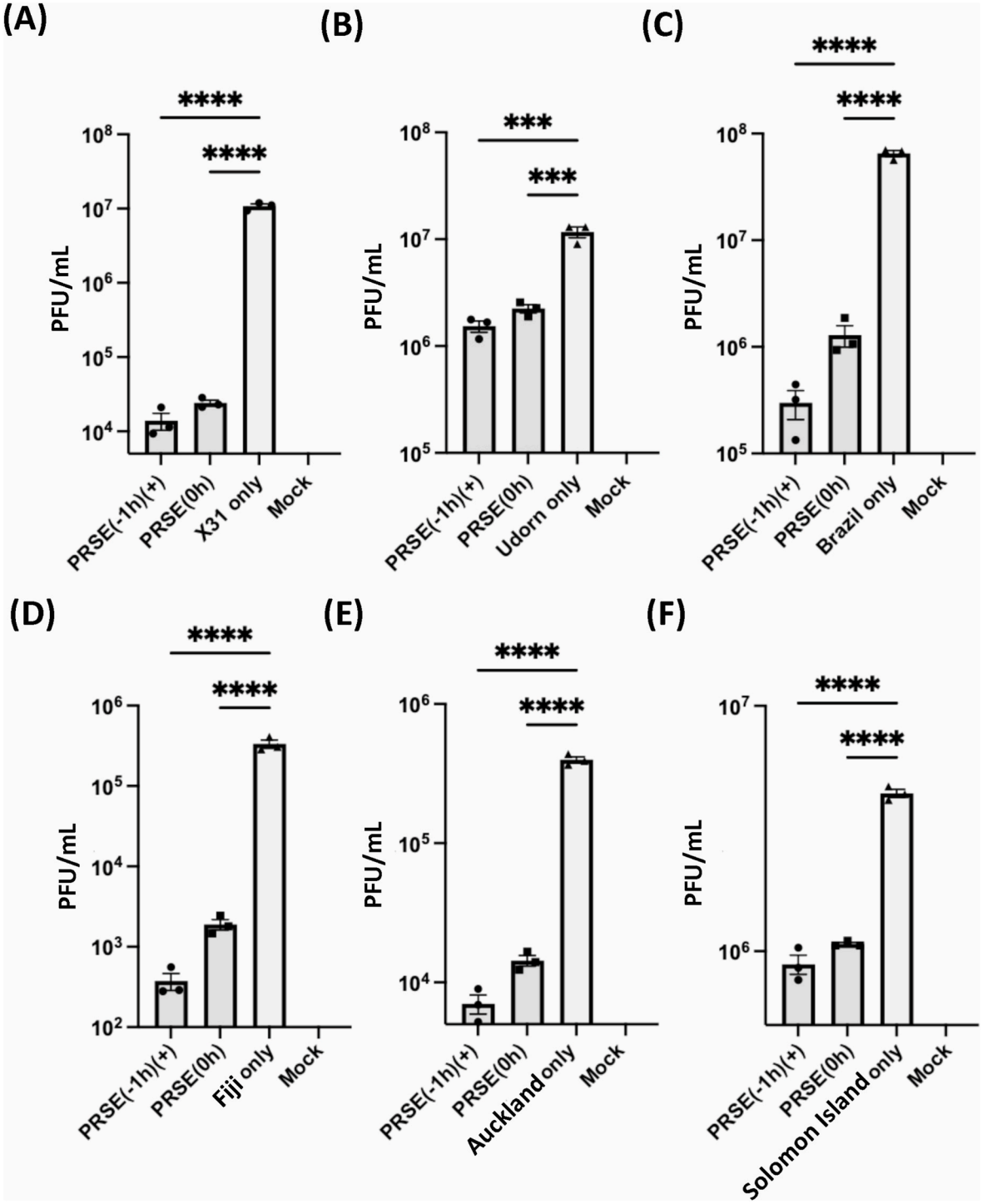



**Fig. 5. PRSE has an inhibitory effect against multiple IAV strains *in vitro*.** MDCK cells were infected with different IAV strains (MOI = 0.01) pre-treated for 1h with 1 mg/mL PRSE or mock-treated. Cell-free supernatants were harvested at 24 h.p.i. and infectious virus quantitated by plaque titration, expressed as PFU/ml. Representatives of replicate experiments (n = 2, performed in triplicate) are shown; **(A)** X31 (H3N2), **(B)** A/Udorn/307/1972 (Udorn72, H3N2), **(C)** A/Brazil/11/1978 (Brazil78, H1N1), **(D)** A/Fiji/2/2016 (Fiji16, H1N1), **(E)** A/Auckland/1/2009 (Auckland09, H1N1) and **(F)** A/Solomon Islands/3/2006 (Solomon06, H1N1). Error bars = SEM, ***p<0.001, ****p<0.0001, by one-way ANOVA with Dunnett's multiple comparison test, where comparisons are made to the virus only control.

from viral genome transcription and viral protein translation, PRSE may influence other stages of the IAV replication cycle such as the entry process itself or an additional step or aspect related to early viral transcription in the nucleus. These aspects are part of our on-going research studies to determine if there are multiple mechanisms of action (MOA).

To evaluate any potential direct PRSE virucidal activity we employed electron microscopy to visualise any potential and significant morphological changes imposed on the virion itself. To this end, we observed no obvious morphological changes nor effects on the structural integrity of the virus or presentation of the viral structural proteins HA and NA after PRSE treatment (Fig. 4A-D). This finding also supports our observation that pre-treatment of the virus alone for 1 h prior to infection had no impact on IAV to infect cells and produce infectious virus (Fig. 3). This was further corroborated by our observations that PRSE

does not affect the hemagglutination ability of IAV through binding to sialic acid. (Fig. 4E and F). Thus, virus attachment is likely not to be impacted by PRSE treatment.

In conjunction with the results from TOA assay which showed that PRSE acts at early stages of IAV replication, virus entry (including endocytosis, fusion and uncoating) and/or viral genome transcription are likely to be the main target of PRSE. However, the possibility that PRSE attenuates IAV activity by acting at multiple stages of viral replication via multiple mechanisms cannot be excluded. Further experiments are still needed to elucidate the detailed MOA of PRSE. Currently, apart from the approved antivirals against IAV, there are several potential drug candidates with different mechanisms of action under research and development including FA-6005, Arbidol and Favipiravir (Kang et al., 2023; Shiraki and Daikoku, 2020; Yang et al., 2021). However, the MOA of these potential antivirals are unitary, which is not ideal in combating the rapid development of IAV antiviral resistance due to the high mutation rate of IAV. Therefore, antivirals with different MOA are needed, and PRSE might act as a competitive candidate.

Previous LC-MS analyses of PRSE revealed a very complex composition (Deseo et al., 2020), suggesting PRSE contains not only polyphenols that were previously associated with antiviral activity against different viruses (e.g. IAV, Japanese encephalitis virus (JEV) and human cytomegalovirus (hCMV)), but also components that have not been tested for antiviral activity (Ding et al., 2017; Fan et al., 2016; Itoh et al., 2018). Therefore, it is possible that PRSE contains multiple antiviral components with different MOAs. Moreover, the antiviral activity of PRSE may not be restricted to IAV, where our ongoing studies will explore the antiviral activity and MOA against a broad range of viruses.

## 5. Conclusions

In conclusion, we have shown that PRSE exhibited potent inhibitory effect against a broad range of historical and contemporary IAV strains of the H1N1 and H3N2 subtypes *in vitro*. Our experiments showed that PRSE potentially acts at an early stage of replication cycle to attenuate IAV replication. Our results would also indicate that PRSE has the potential to be a broad-spectrum IAV antiviral candidate, but further experiments are still required to elucidate the mechanism of action and the target of PRSE. In addition, further identification of other viruses that can potentially be inhibited by PRSE would also be advantageous given the restricted number of pathways and cellular processes shared by the replication of different virus families.

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## Data availability

Data will be made available on request.

## CRediT authorship contribution statement

**Caolingzhi Tang:** Formal analysis, Investigation, Validation, Writing – original draft. **Julio Carrera Montoya:** Formal analysis, Investigation, Methodology, Validation, Writing – review & editing. **Svenja Fritzlar:** Investigation, Methodology, Validation. **Matthew Flavel:** Funding acquisition, Resources, Writing – review & editing. **Sarah L. Londrigan:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Supervision, Writing – review & editing. **Jason M. Mackenzie:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Supervision, Writing – review & editing.

## Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: This research was supported by funding from The Product Makers. M.F. is employed by The Product Makers. All other co-authors have no competing interests.

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## Appendix A. Supplementary data

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