INTERNATIONAL JOURNAL OF VETERINARY SCIENCE AND MEDICINE
2024, VOL. 12, NO. 1, 134–147
https://doi.org/10.1080/23144599.2024.2408189

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Dual effects of ipecac alkaloids with potent antiviral activity against foot-and-mouth disease virus as replicase inhibitors and direct virucides

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ABSTRACT

Foot-and-Mouth Disease (FMD) is a contagious, blistering disease caused by the Foot-and-Mouth Disease virus (FMDV), which affects livestock globally. Currently, no commercial antiviral agent is available for effective disease control. This study investigated the antiviral potential of natural-derived alkaloids against FMDV in BHK-21 cells. Twelve alkaloids were assessed for their antiviral activities at various stages of FMDV infection, including pre-viral entry, post-viral entry, and prophylactic assays, as well as attachment and penetration assays by evaluating cytopathic effect reduction and directed-virucidal effects. The results showed that ipecac alkaloids, cephaeline (CPL) and emetine (EMT), exhibited dual effects with robust antiviral efficacy by reducing cytopathic effect and inhibiting FMDV replication in a dose-dependent manner. Evaluation through immunoperoxidase monolayer assay and RT-PCR indicated effectiveness at post-viral entry stage, with sub-micromolar EC_{50} values for CPL and EMT at 0.05 and 0.24 µM, respectively, and high selective indices. Prophylactic effects prevented infection with EC_{50} values of 0.23 and 0.64 µM, respectively. Directed-virucidal effects demonstrated significant reduction of extracellular FMDV, with CPL exhibiting a dose-dependent effect. Furthermore, the replicase (3Dpol) inhibition activity was identified using the FMDV minigenome assay, which revealed strong inhibition with IC_{50} values of 0.15 µM for CPL and 4.20 µM for EMT, consistent with the decreased negative-stranded RNA production. Molecular docking confirmed the interaction of CPL and EMT with residues in the active site of FMDV 3Dpol. In conclusion, CPL and EMT exhibited promising efficacy through their dual effects and provide an alternative approach for controlling FMD in livestock.

ARTICLE HISTORY
Received 13 June 2024
Revised 14 September 2024
Accepted 19 September 2024

KEYWORDS
Alkaloid; antiviral activity; foot-and-mouth disease virus; FMDV 3Dpol; virucidal effect

1. Introduction

Foot-and-mouth disease (FMD) poses a critical economic concern, significantly impacts on domestic cloven-hoofed livestock [1–4] as well as captive wild ungulates worldwide [5]. This transboundary animal disease manifests as independently circulating endemic pools, exerting a profound influence across multiple countries in the region [1–3]. Foot-and-mouth disease virus (FMDV), consisting of seven serotypes (A, O, C, Asia 1, SAT 1, SAT 2, and SAT 3), exhibits recurring cycles. Serotypes A, Asia 1, and O significantly impact a majority of countries in Asia [1,2,4]. Specifically, FMDV types A and O were predominantly present in outbreaks in Thailand, as reported in the Foot-and-mouth disease Quarterly Report for April – June 2023 [1,2]. The virus primarily infects susceptible cloven-hoofed animals, resulting in the development of vesicular lesions at mucocutaneous and coronary band junctions (e.g. nose, muzzle, tongue, teats, and feet). Clinical signs include anorexia, fever, excessive salivation, and lameness, leading to high morbidity and mortality in both adult and juvenile animals [6], and enhancing cytokine responses [7].

FMD is caused by the FMDV, a member of the family *Picornaviridae*, genus *Aphthovirus*. The FMDV genome, approximately 8,400 nucleotides, contains an open reading frame (ORF) with 5′ and 3′-untranslated regions (UTR) at its ends. The ORF encodes a polyprotein of over 2,300 amino acids, processed by viral proteases, leading to the formation of mature viral proteins and precursors. The viral polyprotein processing yield four structural proteins (VP1–VP4), forming a capsid covering the viral genome, and 10 nonstructural proteins (NSPs), encoded by genes L, 2A, 2B, 2C, 3A, 3B1, 3B2, 3B3, 3C, and 3D, respectively. These NSPs primarily contribute to the protein processing, replication processes and maturation of FMDV [3,8].

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Supplemental data for this article can be accessed online at https://doi.org/10.1080/23144599.2024.2408189

FMD presents a significant challenge in animal disease control due to its potential for transmission through physical contact, fomites, or ingestion of FMDV-contaminated foods. Additionally, the virus can spread over long distances via aerosols from FMD infected animals [9]. FMDV exhibits prolonged survival in the environment while retaining infectivity. The stamping-out or vaccination strategies during FMD outbreaks may not effectively eliminate the disease [1–3,6,9], permitting continual spread to susceptible animals. Therefore, considering the utilization of effective antiviral agents as a supportive strategy becomes crucial to mitigate FMD infection and enhances disease control measures. For example, T-1105, an RNA polymerase inhibitor, has shown some efficacy in decreasing clinical signs; however, it requires a high dosage of 200–400 mg/kg/day in pigs, making it impractical for field use. Currently, there are no effective antiviral treatments available for practical application in controlling FMD outbreaks [10,11].

Natural phytochemicals, particularly plant-derived products, have diverse applications, including potential use as antimicrobial and antiviral purposes [12]. These compounds are usually recognized for their relatively low toxicity. Alkaloids, a subgroup of natural phytochemicals characterized by organic compounds containing nitrogen rings, exhibit various biological and physiological functions and have shown potential in various therapeutic applications. [12,13]. Moreover, alkaloids from plants are naturally abundant in many countries, they present an opportunity for sustainable, locally-sourced antiviral agents, particularly advantageous for FMD control in the region.

Given the urgent need for effective antiviral treatments against FMDV, we aimed to explore a range bioactive alkaloids for potential antiviral activity against FMDV infection using a cell-based assay in BHK-21 cells. Our findings indicated that among the tested alkaloids, ipecac alkaloids derived from *Psychotria ipecacuanha* demonstrated particularly promising inhibitory effects against FMDV and also targeted viral polymerase. This approach could lead to the development of novel antiviral treatments for FMD.

2. Materials and methods

2.1. Alkaloids

In this study, we investigated bioactive compounds reported to exhibit activity against DNA and RNA viruses, as listed in Table 1. Alkaloids were obtained from a commercial source (ChemFaces, Wuhan, China), with a purity exceeding 98%. The compounds were dissolved in DMSO (Sigma-Aldrich, USA) to a concentration of 10 mm, and freshly prepared for use in all cell-based assays.

Table 1. Bioactive alkaloids and their antiviral activity against DNA and RNA viruses.

Alkaloids		Viruses	Inhibitory effects	References
Indole Alkaloid	Catharanthine (CTR)	HSV-1	EC_{50} of 0.014 to 0.106 mg/ml in Vero cells	[14]
	Indirubin (IDB)	JEV	EC_{50} of 0.47 to 23.50 µg/ml in BHK-21 cells	[15]
	Picrinine (PCN)	Influenza virus H1N1	Reduction of haemagglutination titres at 1/480; 12.5 mg/kg of *Alstonia scholaris* (L.) crude extract	[16]
	Reserpine (RSP)	SARS-CoV-2	Binding affinity of −7.4 kcal/mol to *in silico*	[17]
	Strictosamide (STM)	Influenza virus type ARSV	IC_{50} of 25.68 µg/ml in MDCK cells IC_{50} of 12.50 µg/ml in Hep-2 cells	[18]
	Tryptanthrin (TTT)	HCoV-NL63	IC_{50} of 1.52 µM in LLC-MK2 cells IC_{50} of 0.30 µM in Cula-3 cells IC_{50} of 0.06 µM (papain-like protease 2 protein inhibition)	[19]
Steroid Alkaloid	Conessine (CNS)	HCoV-OC43	EC_{50} of 2.34 µM in BHK-21 cells	[20]
		HCoV-NL63	EC_{50} of 10.74 µM in LLC-MK2 cells	
		MERS-CoV	EC_{50} of 4.98 µM in Vero E6 cells	
		MHV-A59	EC_{50} of 11.46 µM in DBT cells	
	Solamargine (SLG)	HBV	IC_{50} of 2.17 µM in HepG2 2.2.15 cell	[21]
	Solanine (SLN)	SARS-CoV-2	Binding affinity of −9.5 kcal/mol to spike protein and −10.3 kcal/mol to 3CL protease *in silico*	[22]
	Tomatidine (TMD)	CHIKV	EC_{50} of 1.3 µM in Huh7 cells	[23]
Ipecac alkaloid	Cephaeline (CPL)	SARS-CoV-2	EC_{50} of 0.0123 µM in Vero E6 cells Binding affinity of 8.9 µM (RdRp inhibition using surface plasmon resonance assay)	[24]
	Emetine (EMT)	HCoV-OC43	EC_{50} of 0.30 µM in BHK-21 cells	[20]
		HCoV-NL63	EC_{50} of 1.43 µM in LLC-MK2 cells	[24]
		MERS-CoV	EC_{50} of 0.34 µM in Vero E6 cells	[25]
		MHV-A59	EC_{50} of 0.12 µM in DBT cells	
		SARS-CoV-2	EC_{50} of 0.00771 µM in Vero E6 cells	
		EBOV	IC_{50} of 10.20 µM (viral-like particle entry)	
		ZIKV	IC_{50} of 121 nM (NS5 polymerase inhibition)	

Abbreviations: HSV-1, Herpes simplex virus 1; JEV, Japanese encephalitis virus; SARS-CoV-2, Severe Acute Respiratory Syndrome Coronavirus-2; RSV, Respiratory syncytial virus; HCoV, Human coronaviruses; MERS-CoV, Middle East respiratory syndrome coronavirus; MHV, Mouse hepatitis virus; HBV, Hepatitis B virus; CHIKV, Chikungunya virus; EBOV, Ebola virus; ZIKV, Zika virus.

2.2. Cells and viruses

The Baby Hamster Kidney (BHK-21) cell line (ATCC®, VA, USA) was employed for viral propagation and cell-based assays. BHK-21 cells were cultured in a complete media containing Minimum Essential Medium (MEM), supplemented with 2% L-glutamine (Invitrogen™, CA, USA), 10% foetal bovine serum (FBS; Gibco™ Thermo Fisher Scientific Inc., MA, USA), and 1% antibiotic – antimycotic (Invitrogen™, CA, USA).

For cytotoxicity and antiviral activity assays, the cells were seeded in 96-well plates at a density of 2×10^5 cells/ml (100 µl/well) and in 24-well plates at a density of 2×10^5 cells/ml/well (1 ml/well) for RT-qPCR. The cells were cultured in the same media and incubated at 37°C with 5% CO_2 overnight.

Foot-and-mouth disease virus (FMDV) serotype A (NP05) [26] was propagated in BHK-21 cells, and the viral titre was determined using the 50% Tissue Culture Infectious Dose ($TCID_{50}$) assay as described previously [27,28]. The viral stock had a titre of 10^7 $TCID_{50}$/ml and was kept in aliquots at −80°C until used in the experiments. The final DMSO concentration in all infection experiments was 0.1%, serving as the non-vehicle control.

2.3. Cytotoxicity assay

BHK-21 cells were seeded into each well of 96-well plate as described earlier and incubated at 37°C with 5% CO_2 overnight. Alkaloids were serially diluted in MEM with 2% FBS to the final concentrations of 0.01, 0.1, 1, 10, 25, 50, and 100 µM before incubating with the BHK-21 cells for an additional 24 h. On the subsequent day, the cells were washed with rinse saline, before adding a fresh complete media. Subsequently, 10 µl of Cell Counting Kit-8 (CCK-8; TargetMol®, MA, USA) were added to each well and the plate were then incubated at 37°C with 5% CO_2 for 2 h. The cell supernatant in each well was transferred to clean 96-well plates and the optical density (OD) was measured at 450 nm using a microplate reader (Synergy H1 hybrid Multi-Mode Reader, BioTek®, Winooski, VT, USA) to evaluate the formazan dye level produced by cellular dehydrogenases [27,28]. The OD intensity is directly correlated with the number of viable cells, and the percentage of cell death was expressed by the following equation.

$$\% \text{ Cytotoxicity} = \frac{(\text{OD alkaloid} - \text{OD blank})}{(\text{OD cell control} - \text{OD blank})} \times 100$$

The half-maximal cytotoxic concentration (CC_{50}) of the alkaloids was determined using dose-response curves generated from the cytotoxicity assay [27,28].

2.4. Antiviral activity assay

The antiviral activity of alkaloids against FMDV was assessed at each stage of infection, using methods modified from previous studies as illustrated in Figure 1 [29]. The evaluation initially involved pre-viral entry, post-viral entry, and prophylactic assays as first-line antiviral screening assay, which the initial concentrations of the tested alkaloids were ranged at least seven-point concentrations according to the cytotoxicity assay. For compounds showing promise in this initial pre-viral entry assay, additional assays––including attachment (binding to the receptor), penetration (during entry to the cells), and direct virucidal assays (direct viral inactivation)––were subsequently conducted to explore whether alkaloids could inhibit the virus at the early stage of infection, as detailed below. In all experiments, the cytopathic effect (CPE) reduction and FMDV-infection were observed using crystal violet staining and immunoperoxidase monolayer assay (IPMA) at 24 and 16 hours after inoculation, respectively.

2.4.1. Pre-viral entry assay

BHK-21 cells were seeded in a 96-well plate and incubated at 37°C with 5% CO_2 overnight. In the following day, a mixture of FMDV (10 $TCID_{50}$/well) and various concentrations of alkaloids were prepared

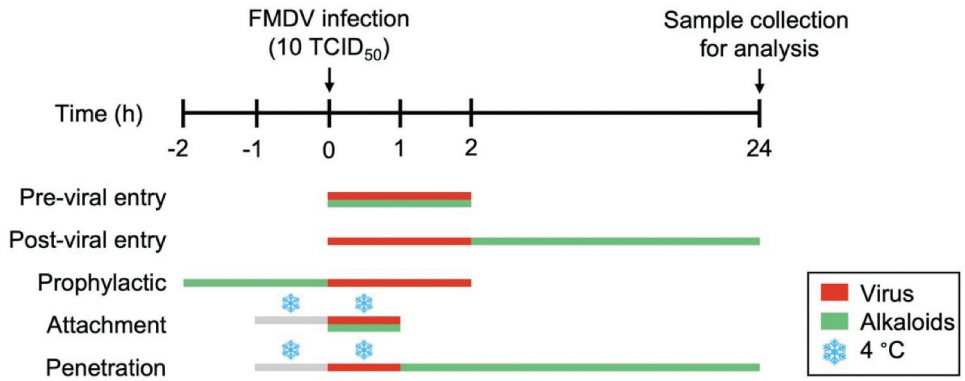

Figure 1. Schematic representation of the experimental design to evaluate the antiviral activity of alkaloids at different stages of FMDV infection in BHK-21 cells.

immediately before adding to the cells. This mixture was then added to the cells and incubated at 37°C for 2 h. This incubation period allows for potential interactions between the alkaloids and virus particles before examining infectivity. Subsequently, the cells were washed with rinse saline to remove unbound virus and alkaloids. Fresh MEM containing 2% FBS was added to each well, and the cells were incubated at 37°C for an additional 24 h to allow for viral replication of any internalized viruses. Compound showing antiviral potential in this assay was further evaluated in the attachment, penetration, and direct virucidal assays.

2.4.2. Post-viral entry assay

BHK-21 cells in a 96-well plate were incubated at 37°C with 5% CO_2 overnight. On the next day, FMDV at 10 $TCID_{50}$/well was incubated with the cells at 37°C for 2 h. Afterwards, the inoculum was removed, and the cells were washed with rinse saline to eliminate non-absorbed virus. Serially diluted concentrations of alkaloids were added to the culture media and the cells were further incubated at 37°C for an additional 24 h.

2.4.3. Prophylactic assay

BHK-21 cells in a 96-well plate were incubated at 37°C with 5% CO_2 overnight. On the next day, the spent media was replaced with the serially diluted concentrations of alkaloids in MEM with 2% FBS and the cells were further incubated at 37°C for 2 h. Subsequently, the alkaloids were removed, cells were washed, and FMDV at 10 $TCID_{50}$/well was added to the culture media, and the cells were incubated at 37°C for an additional 2 h. The cells were washed to remove the excess virus and incubated with MEM with 2% FBS at 37°C for 24 h.

2.4.4. Attachment assay

This assay evaluated whether alkaloids could inhibit the binding of virus to the cells. BHK-21 cells in a 96-well plate were incubated at 37°C with 5% CO_2 overnight. On the next day, the cell culture plates were prechilled at 4°C for 1 h. Mixtures of FMDV at 10 $TCID_{50}$ and serially diluted concentrations of alkaloids were added to the cells chilling on ice and then incubated at 4°C for 1 h. After incubation, the mixtures of non-absorbed virus were removed, and the cells were washed with rinse saline. Subsequently, fresh MEM with 2% FBS were then added to the cell wells and further incubated at 37°C for an additional 24 h.

2.4.5. Penetration assay

This assay evaluated whether alkaloids could inhibit virus penetration into the cell after virus binding. BHK-21 cells in a 96-well plate were incubated at 37°C with 5% CO_2 overnight. On the next day, the cell culture plates were prechilled at 4°C for 1 h. FMDV at 10 $TCID_{50}$/well was added onto the cells chilling on ice and further incubated at 4°C for an additional 1 h. After incubation, non-absorbed viruses were removed, and the cells were washed with rinse saline before incubating with serially diluted concentrations of alkaloids at 37°C for an additional 24 h.

2.4.6. Direct virucidal assay

This assay evaluated whether alkaloids could directly inactivate the virus. FMDV at 10 $TCID_{50}$ was incubated directly with alkaloids in a 1.5 ml-microtube at 37°C for 60 min. After incubation, the mixtures were 10-fold serially diluted in MEM with 2% FBS from 10^{-1} to 10^{-7}. Each dilution was used to infect 5 wells of BHK-21 cells grown in a 96-well plate for virus titration. The titres of the remaining post-treatment viruses were evaluated at 72 h post infection using $TCID_{50}$ assay as previously described [29,30]

2.5. Cytopathic effect (CPE) reduction assay

The CPE reduction assay was conducted to visually assess the protective effect of alkaloids against FMDV-induced cellular damage. Cells treated with alkaloids and infected with FMDV were fixed with cold methanol for 30 min and stained with a 0.5% crystal violet solution [28,29]. After washing with distilled water, the cell morphology was examined under a phase-contrast inverted microscope (Olympus IX73, Tokyo, Japan). FMDV infection typically induced CPE, most noticeably cell rounding and lysis. The degree of CPE was used as an indicator of viral infection and the protective effect of alkaloids. Images were recorded for further analysis using CellProfiler version 4.2.4 (Broad Institute, Cambridge, MA, USA).

2.6. Immunoperoxidase monolayer assay (IPMA)

To quantitatively evaluate the antiviral efficacy of alkaloids against FMDV, we performed the immunoperoxidase monolayer assay (IPMA). This assay allows for the specific detection of FMDV antigens in the infected cells through antigen-antibody interactions. The procedure was carried out as detailed in the previous study [27,28,31]. Briefly, FMDV infected and control BHK-21 cells from antiviral activity assays were fixed with cold methanol for 30 min. Following cell fixation, the cells were equilibrated with PBS containing 0.05% Tween 20 (PBST) for 5 min and subsequently incubated with BlockPRO™ 1 min Protein-Free Blocking Buffer (Energenesis Biomedical Co., Ltd., Taipei, Taiwan) for 1 min. After, washing with PBST for 5 min, the cells were incubated with a single-chain variable fragment with Fc fusion protein (scFv-Fc) specific to 3ABC of FMDV at 37°C for 1 h. Following a wash with PBS containing 0.05% Tween

20, the cells were treated with HRP-conjugated protein G (EMD Millipore Corporation, CA, USA) for 1 h at 37°C. The detection of FMDV-infected cells was visualized using the DAB+ Substrate Chromogen System (Dako Omnis, CA, USA), resulting in a dark-brown cytoplasmic staining. Image acquisition and recording were conducted using a phase-contrast inverted microscope (Olympus IX73, Tokyo, Japan). The antiviral effectiveness of the compounds was quantified by numbering the positively infected cells through analysis with CellProfiler version 4.2.4 (Broad Institute, Cambridge, MA, USA) following the previous study [27,28].

The effective concentrations that resulted in a 50% reduction (EC_{50}) in CPE reduction and IPMA assays were calculated from dose-response curves obtained from the antiviral activity assays using non-linear regression in GraphPad Prism version 10.0.1 (Prism, CA, USA). The selectivity index (SI) was determined by dividing CC_{50} by EC_{50}. This ratio indicated the alkaloid's potential therapeutic window between antiviral efficacy and cytotoxicity. A higher SI value suggests a more favourable safety profile for the alkaloids.

2.7. Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

To assess the antiviral efficacy of alkaloids against FMDV, all antiviral activity assays was performed in 24-well plates as mentioned above. The cells were harvested using Trizol™ reagent (Thermo Fisher Scientific Inc., USA) for total RNA extraction, followed by clarification using Direct-zol™ MiniPrep (Zymo Research Corporation, CA, USA). Subsequently, RNA concentration and purity were evaluated using the NanoDrop™ 2000c Spectrophotometer (Thermo Fisher Scientific, MA, USA) as described in the previous studies [27,28].

Briefly, 1 µg of total RNA was utilized as the template for cDNA synthesis employing RevertAid reverse transcriptase enzyme (Thermo Fisher Scientific Inc., MA, USA) and random hexamers (Invitrogen™, CA, USA). The primers used for amplification were as follows: FMDV-5'UTRF, 5'– CTGTTGCTTCGTAG CGGAGC –3' (forward), FMDV-5'UTRR, 5'– TCGCGTGTTACCTCGGGGTACC –3' (reverse) for viral load quantification, FMDV-3DF, 5'– TAGAG CAGTAGATGTTG –3' (forward), and FMDV-3DR, 5'–ATGAACATCATGTTTGAGG –3' (reverse) for negative-stranded RNA production according to the method of the previous study [27,28]. The qPCR assay utilized the FMDV primers and iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories, CA, USA). The PCR reactions were processed in the C1000 Touch thermal cycler (Bio-Rad Laboratories, CA, USA). The qPCR conditions included an initial DNA denaturation at 95°C for 30 sec, followed by 40 cycles of denaturation at 95°C for 5 sec, and annealing plus extension at 60°C for 30 sec for the first primer set (viral load quantification), and 55°C for 30 sec for the second primer set (negative-stranded RNA production) [27,28]. A melting curve analysis from 65 to 95°C with a 0.5°C increment was performed as per the manufacturer's instructions. To quantify viral loads, a plasmid carrying FMDV 5'UTR, ranging from 10^{-2} to 10^{-7} plasmid molecules/µl, was used to generate a standard curve. The viral copy numbers were calculated based on the cycle threshold (Ct) values obtained from qPCR. The percentage of viral reduction was calculated in relation to that of virus-infected cells treated with DMSO. The negative-stranded RNA-derived DNA was quantitated using delta Ct values by subtracting the Ct values of the alkaloid-treated, virus-infected cells with those of the virus samples as described previously [27,28].

2.8. Anti-FMDV 3Dpol assay using minigenome

The inhibitory effects of alkaloids on the RNA-dependent RNA polymerase (3Dpol) of FMDV were evaluated using an FMDV minigenome assay system. This assay employed three plasmids: pKLS3_GFP harbouring the green fluorescent protein (GFP) gene flanked by FMDV untranslated regions (UTRs) [32], pCAGGS_T7 expressing the T7 RNA polymerase, and pCAGGS_P3 encoding the FMDV P3 region essential for efficient viral RNA generation according to previous study [27,28,32].

Briefly, BHK-21 cells were seeded in 96-well plates at a density of 2×10^5 cells/ml (100 µl/well) and incubated at 37°C and 5% CO_2 overnight. On the day of transfection, the three plasmids (40 ng pKLS3_GFP, 120 ng pCAGGS_T7, and 40 ng pCAGGS_P3) with a total volume of 10 µl per well were combined with transfecting reagent (Fugene® HD, Promega, WI, USA) and Opti-MEM medium (Thermo Fisher Scientific, MA, USA) to prepare a transfection mixture. Then, the cells were treated with serially diluted concentrations of alkaloid compounds prepared in Opti-MEM medium.

Vehicle control wells containing 0.1% DMSO transfected with 3 plasmids served as positive controls for both transfection efficiency and 3Dpol activity, while transfection with pKLS3_GFP alone, without the helper plasmids, acted as a negative plasmid control. The cells were then incubated at 37°C with 5% CO_2 for 24–48 h [27,28,32]. GFP fluorescence expression in transfected cells, which is indicative of 3Dpol activity, was visualized using a fluorescent inverted microscope (Olympus IX73, Tokyo, Japan) and quantified using CellProfiler software version 4.2.4 by measuring fluorescent intensities after subtracting the background [27,28,32]. The half-maximal inhibitory concentration (IC_{50}) values, representing the alkaloid concentrations

that reduced 3Dpol activity by 50% compared to the DMSO control (set as 100% activity), were calculated using GraphPad Prism version 10.0.1 (Prism, CA, USA) [27,28,32].

2.9. Molecular docking

Cell-based assays were initially performed to evaluate the antiviral activity of alkaloid compounds against FMDV. The most promising alkaloid candidates from these assays were further evaluated using an FMDV minigenome assay to determine their specific inhibitory effects on FMDV 3Dpol. Molecular docking studies were then conducted as a final step to confirm the molecular interactions between these alkaloid candidates and the FMDV 3Dpol. The AutoDock Vina program implemented in PyRx 0.9.8 [33] was utilized for the docking simulations. The 3D structure of FMDV 3Dpol was modelled based on the PDB ID: 1wne.pdb according to a previous study [32] and prepared using Open Babel [34]. Water molecules were removed, polar hydrogens were added, and Gasteiger charges were assigned. The prepared protein was saved in.pdbqt format. Three-dimensional structures of the alkaloid ligands identified from the cell-based and minigenome assays were retrieved from PubChem and converted to.pdbqt format using Open Babel [34].

For the docking procedure, a grid box encompassing the entire FMDV 3Dpol structure was defined with dimensions of $65 \times 70 \times 65$ Å (x, y, z) and centred at the coordinates 19.34, 31.20, 23.25 (x, y, z) [32]. Docking simulations were carried out with an exhaustiveness of 20. The docked poses were then ranked based on their predicted binding affinities calculated by AutoDock Vina. The docked poses were visualized and analysed using Discovery Studio Visualizer 2021 (BIOVIA, Dassault Systèmes, San Diego, CA, USA) and ChimeraX (UCSF, San Francisco, CA, USA) to investigate potential interactions between the alkaloids and FMDV 3Dpol, including hydrogen bonds, hydrophobic contacts, and other non-covalent interactions. This *in silico* analysis provided confirmation of the promising alkaloid candidates' ability to bind and inhibit FMDV 3Dpol, corroborating the findings from the cell-based antiviral and minigenome assays.

2.10. Statistical analysis

All experiments in cell-based assays were performed in three independent experiments, and data are presented as mean ± standard deviation (SD). Error bars in both the tables and figures represent SD, which was calculated using GraphPad Prism version 10.0.1 (Prism, CA, USA). For comparisons between groups, we used one-way ANOVA followed by Tukey's post-hoc test to determine the significance of differences between experimental groups, with $p < 0.05$ considered statistically significant.

3. Results

3.1. Cytotoxicity of alkaloids

Twelve alkaloids were assessed for cytotoxicity. Among them, four compounds demonstrated non-toxicity to BHK-21 cells, with concentrations exceeding 300 µM, including catharanthine, indirubin, picrinine, and strictosamide. The remaining eight alkaloids exhibited mild toxicity to BHK-21 cells with the range of 12.54 to 154.40 µM. After determining the cytotoxicity levels, alkaloids were further tested for antiviral activity based on the CC_{50} values.

3.2. Antiviral activity against FMDV using CPE reduction and IPMA assays

The antiviral efficacy of the twelve alkaloids was assessed at various stages of FMDV infection through initial screening assays for specific FMDV antigen-antibody detection (IPMA), which enhanced our understanding on the antiviral effects of alkaloids as shown in Figure 2 and Supplementary Figure S1. This involved a seven-point dose-response confirmation based on their half-maximal effective concentration (EC_{50}) to determine their effectiveness in reducing the infected cells. Notable antiviral properties were observed in two alkaloids – cephaeline (CPE) and emetine (EMT) – during the initial antiviral assay which were comparable to the finding in CPE reduction as shown in Supplementary Table S1.

In the pre-viral entry assay, two out of twelve alkaloids exhibited significant inhibitory effects, with CPL and EMT demonstrating superior FMDV inhibition at sub-micromolar doses using IPMA assays. To calculate the SI, the CC_{50} value determined via the CCK-8 assay was divided by the EC_{50} value. The results showed that these alkaloids had high selective index values: CPL had an EC_{50} of 0.10 ± 0.10 µM (SI of 184.20), and EMT showed an EC_{50} of 0.13 ± 0.45 µM (SI of 96.46).

In the post-viral entry assay, CPL and EMT exhibited superior efficacy with EC_{50} values of 0.05 ± 0.13 µM (SI of 368.40) and 0.24 ± 0.63 µM (SI of 52.25), respectively. Moreover, these alkaloids demonstrated antiviral activity greater than that of rupintrivir, a drug control (EC_{50} of 3.43 ± 0.54 µM). In the prophylactic assays, CPL and EMT could inhibit FMDV at lower sub-micromolar levels as evidenced by IPMA with EC_{50} values of 0.23 ± 0.65 µM (SI of 80.09) and 0.64 ± 0.20 µM (SI of 19.59), respectively. Their efficacies were greater than rupintrivir, a drug control (Supplementary Figure S1B – C and E – F, and Table 2).

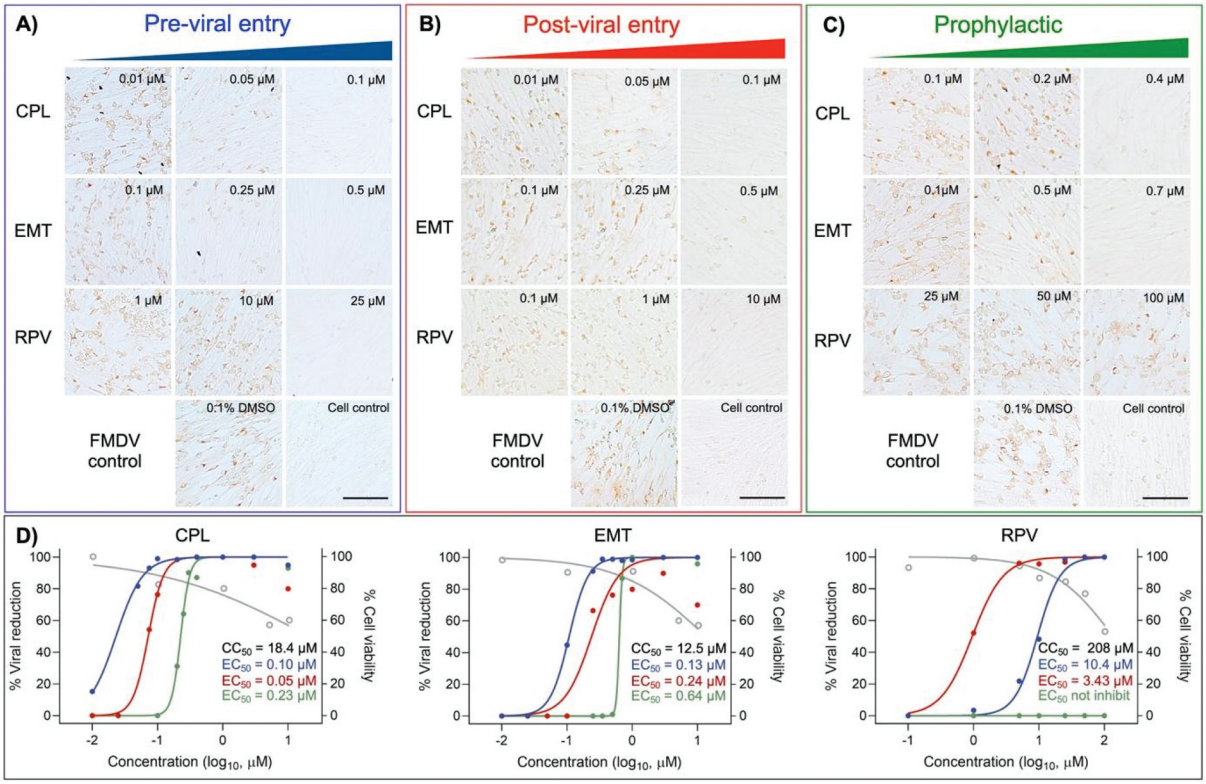

Figure 2. Reduction of FMDV-infected BHK-21 cells treated with potential alkaloids at three stages of infection: (A) pre-viral entry, (B) post-viral entry, and (C) prophylactic activity. Positive infected cells exhibit dark-brown cytoplasmic staining using the immunoperoxidase monolayer assay (IPMA) detection. Scale bars: 200 µm. (D) Graphs depict the cytotoxicity and antiviral activities of alkaloids, illustrating the percentage of viable cells (grey lines) and the percentage of viral inhibition (blue lines for post-viral entry, red lines for pre-viral entry, and green line for prophylactic) at various concentrations of alkaloids (CPL, cephaeline; EMT, emetine; RPV, rupintrivir).

Table 2. Antiviral activity screening of bioactive alkaloids against FMDV in BHK-21 cells.

	Inhibition of FMDV *in vitro*						
		Pre-viral entry		Post-viral entry		Prophylactic	
Alkaloids	CC_{50}, µM[a]	EC_{50}, µM[b]	SI[c]	EC_{50}, µM[b]	SI[c]	EC_{50}, µM[b]	SI[c]
Cephaeline (CPL)	18.42 ± 2.73	0.10 ± 0.10	184.20	0.05 ± 0.13	368.40	0.23 ± 0.65	80.09
Emetine (EMT)	12.54 ± 3.24	0.13 ± 0.45	96.46	0.24 ± 0.63	52.25	0.64 ± 0.20	19.59
Rupintrivir (RPV, drug control)	208.30 ± 1.72	10.45 ± 1.02	19.93	3.43 ± 0.54	60.73	NA	NA

NA: Not applicable.
ND: Not determined.
[a]CC_{50} values were determined with serially diluted concentrations of alkaloids in BHK-21 cells at 24 h post-incubation using the CCK-8 assay for cell viability.
[b]EC_{50} values, representing alkaloid concentrations that inhibited viral infection by 50%, were determined by assessing an antibody specific to the FMDV antigen in IPMA to detect FMDV 3ABC.
[c]The selective index (SI) values were calculated by dividing the CC_{50} (50% cytotoxic concentration) by the EC_{50} (50% effective concentration).

For further investigations on viral attachment and penetration stages, we explored the impact of alkaloids on virus attachment to BHK-21 cells and penetration into the cells (Table 3). The results indicated that none of them interfered with the viral attachment process. However, CPL (EC_{50} of 0.05 ± 0.38 µM; SI of 368.40) and EMT (EC_{50} of 0.12 ± 0.47 µM; SI of 104.50) demonstrated inhibitory effects on FMDV penetration, and they were greater than the efficacy of rupintrivir (EC_{50} of 2.06 ± 0.31 µM, Table 3).

3.3. Direct virucidal activity of CPL and EMT

The directed-virucidal assay evaluated the ability of the alkaloids to directly inactivate extracellular virus particles. In this assay, both CPL and EMT exhibited potent virucidal effects, leading to a significant reduction in extracellular virus titres. The virucidal activity was assessed at concentrations of 1× and 10× the EC_{50} values obtained from the pre-viral entry assay using IPMA (Table 2). CPL treatment resulted in a remarkable

Table 3. Antiviral activity of bioactive alkaloids against FMDV in BHK-21 cells during viral attachment, penetration and directed-virucidal effects.

	Inhibition of FMDV *in vitro*						
		Attachment (EC_{50}, μM)		Penetration (EC_{50}, μM)		% Virucidal effect (Virus titre reduction of log $TCID_{50}$/ml)	
Alkaloids	CC_{50}, μM[a]	EC_{50}, μM[b]	SI[c]	EC_{50}, μM[b]	SI[c]	Low dose[d]	High dose[d]
Cephaeline (CPL)	18.42 ± 2.73	NA	ND	0.05 ± 0.38	368.40	68.35	79.59
Emetine (EMT)	12.54 ± 3.24	NA	ND	0.12 ± 0.47	104.50	32.28	32.43
Rupintrivir (RPV, drug control)	208.30 ± 1.72	NA	ND	2.06 ± 0.31	101.12	ND	ND

NA: Not applicable.
ND: Not determined.
[a]CC_{50} values were determined with serially diluted concentrations of alkaloids in BHK-21 cells at 24 h post-incubation using the CCK-8 assay for cell viability.
[b]EC_{50} values, representing alkaloid concentrations that inhibited viral infection by 50%, were determined by assessing an antibody specific to the FMDV antigen in IPMA to detect FMDV 3ABC.
[c]The selective index (SI) values were calculated by dividing the CC_{50} (50% cytotoxic concentration) by the EC_{50} (50% effective concentration).
[d]Low and high doses for directed-virucidal effects on extracellular virus are at 1× and 10× of EC_{50} values from the pre-viral assay, respectively. The viral titre is evaluated and reported as the 50% Tissue Culture Infectious Dose ($TCID_{50}$).
Data present as mean and standard deviation (SD).

decrease in free virus particles. At 0.10 μM (1× EC_{50}), CPL reduced the log viral titre by 68.35% from 3.16 to 1.00. This virucidal effect was even more pronounced at 1 μM (10× EC_{50}), with CPL causing a 79.59% reduction in log viral titre from 4.90 to 1.00. EMT also displayed virucidal activity, but to a lesser extent compared to CPL. At 0.13 μM (1× EC_{50}), EMT reduced the log viral titre by 32.28%, decreasing it from 3.16 to 2.14. Interestingly, increasing the EMT concentration to 1.3 μM did not further enhance its virucidal effect, with the log viral titre remaining at 2.16 (Table 3). These results demonstrated the potent virucidal properties of CPL and EMT, highlighting their ability to directly inactivate extracellular virus particles.

3.4. Viral genome quantification

To complement these findings, RT-qPCR was employed to quantify the viral genome at each stage. The viral load reduction in cells treated with CPL and EMT, as determined by RT-qPCR, confirmed the patterns of CPL and EMT inhibited FMDV infection, including pre-viral and post-viral entry, prophylactic and penetration, in a dose-dependent manner, as observed in the viral infectivity reduction by CPE reduction and IPMA assay. The viral inhibition profiles of the potential alkaloids are illustrated in Figure 3.

3.5. Ipecac alkaloids interfered with FMDV 3Dpol activity in the FMDV minigenome assay and RT-qPCR

Among the tested alkaloids, we found that CPL and EMT – ipecac alkaloids – could preferentially inhibit FMDV following post-viral entry and penetration. Therefore, we further assessed their potential inhibitory effects on FMDV replication through targeting the FMDV 3Dpol function. The FMDV minigenome assay and quantification of negative-stranded RNA production were performed to evaluate the alkaloid inhibition of 3Dpol (Figure 4).

The FMDV minigenome assay revealed strong inhibition of replicase activity by both alkaloids CPL and EMT reduced GFP expression in a dose-dependent manner in 3Dpol-transfected cells, with IC_{50} values of 0.15 μM for CPL and 4.20 μM for EMT (Figure 4A). These results indicate that both alkaloids could inhibit the replicase activity in the lower micromolar concentrations in the 3Dpol-transfected cells. The inhibitory potency of these alkaloids was comparable to that of ribavirin, an established anti-RdRp inhibitor (IC_{50} of 1.45 μM). Additionally, CPL and EMT affected the production of negative-stranded RNA as depicted in Figure 4B.

3.6. Molecular docking analysis of ipecac alkaloids interacting with the active site of FMDV 3Dpol

We confirmed the potential antiviral activity of CPL and EMT through targeting FMDV 3Dpol using *in silico* molecular docking. The predicted complexes of these alkaloids demonstrated that the compounds preferentially occupied the FMDV 3Dpol active site, which the catalytic site was identified based on the study by Ferrer-Orta and colleagues [35]. The binding affinities of −7.8 kcal/mol for both CPL and EMT were demonstrated, respectively. EMT was positioned in the palm domain of 3Dpol and interacted with the catalytic residues Asp245 of motif A and Asp338 of motif C, which plays a critical function of 3Dpol, through π-anion bond and carbon-hydrogen bonds. CPL exhibited strong interactions with Asp245 through π-anion bond and with Asp338 through Van der Waals interaction as illustrated in Figure 5. These findings provided

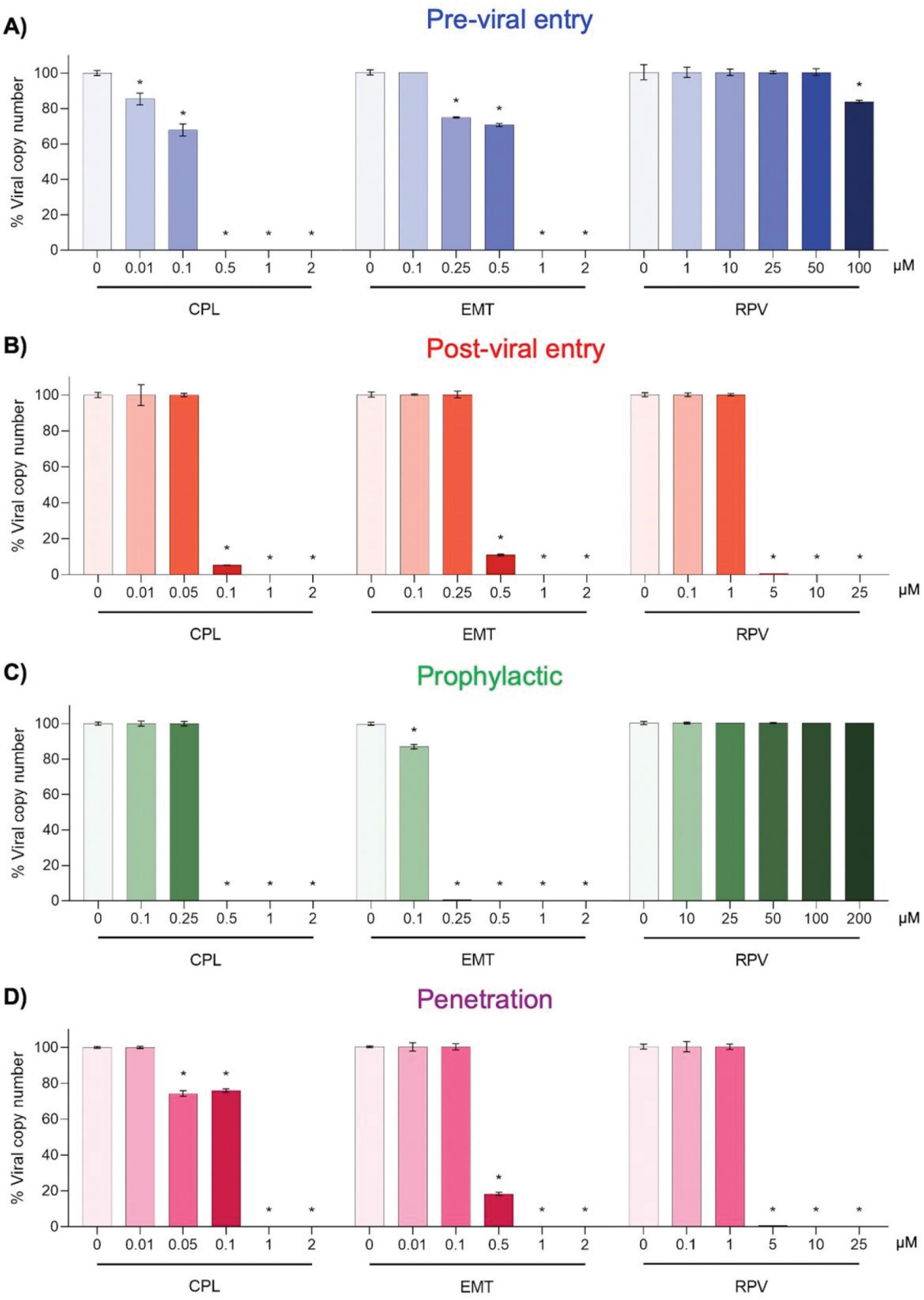

Figure 3. Percent viral copy numbers of FMDV-infected BHK-21 cells treated with serial concentrations of alkaloids, demonstrating a significant dose-dependent effect at four stages of infection: (A) pre-viral entry, (B) post-viral entry, (C) prophylactic activity, and (D) penetration. Data are represented as means with error bars showing standard of deviations (SD). (CPL, cephaeline; EMT, emetine; RPV, rupintrivir) *significant at $p < 0.001$.

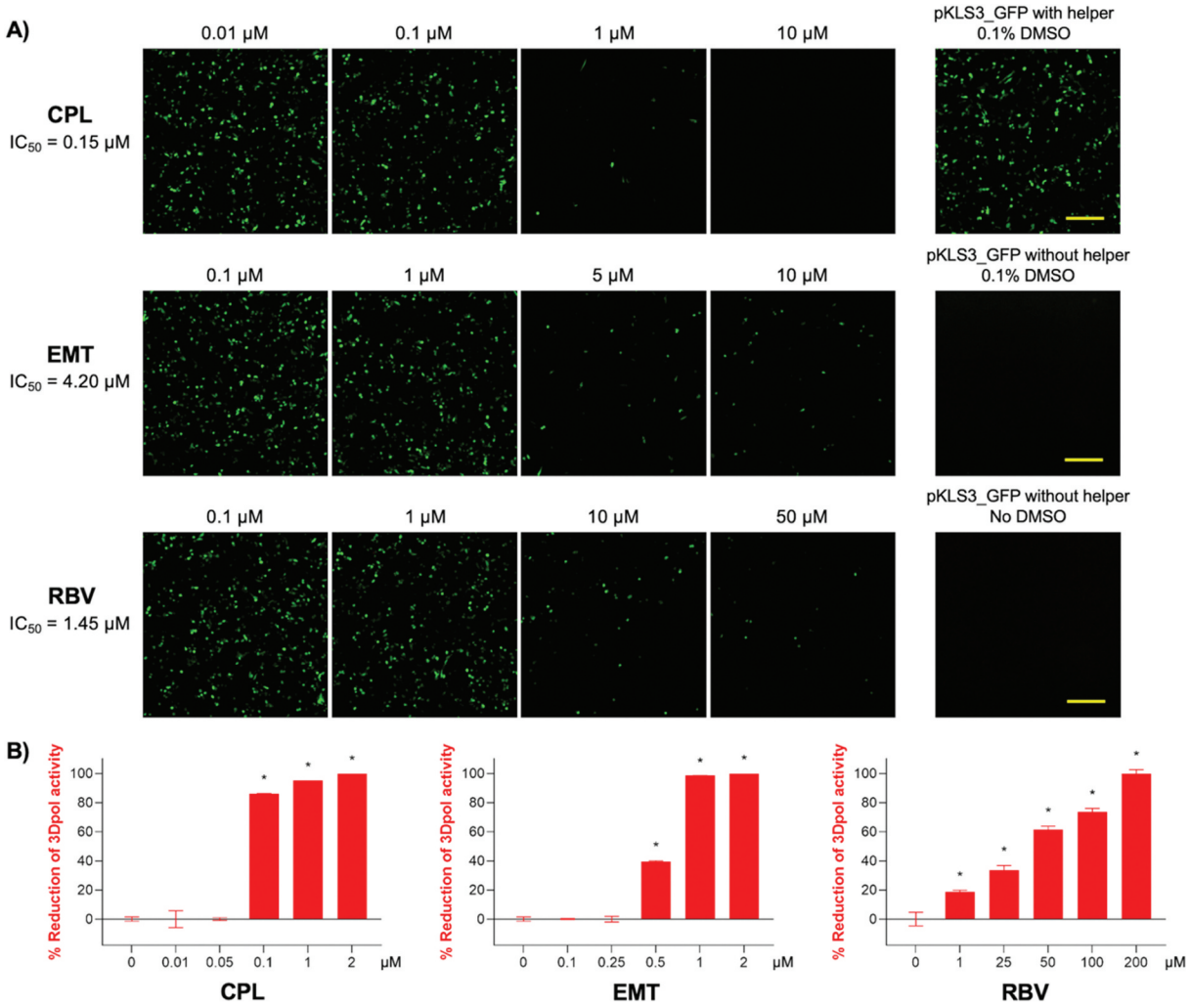

Figure 4. FMDV minigenome assay evaluating the inhibitory effects of cephaeline (CPL) and emetine (EMT) on FMDV 3Dpol activity. (A) CPL and EMT reduced the number of gfp-expressing BHK-21 transfected cells in a dose-dependent manner, compared to the control drug, ribavirin (RBV). (B) Negative-strand RNA production, reflecting FMDV 3Dpol function, was determined in alkaloid-treated BHK-21 cells using RT-qPCR. Data are represented as means with error bars showing standard of deviations (SD). * significant at $p < 0.001$.

suggestive evidence that these ipecac alkaloids targeted the FMDV 3Dpol active site and effectively inhibited FMDV RNA production.

4. Discussion

Foot-and-mouth disease virus (FMDV) poses a significant threat to cloven-hoofed animals, leading to substantial economic losses within the livestock industry [1,2]. Effective control measures are high-priority, and the exploration of antiviral agents capable of decreasing numbers of infective viruses from infected animals or contaminated fomites is essential [1–3,6,9]. This study focuses on investigating the potential of bioactive alkaloids as antiviral agents against FMDV, assessing their impact at various stages of the viral infection.

The utilization of bioactive or secondary metabolites from natural sources has garnered attention due to their potential to reduce virus infection and transmission. Within this context, we evaluated twelve alkaloids for their ability to inhibit FMDV infection, concurrently assessing their influence on each stage of the viral life cycle. Alkaloids, secondary metabolites found in plants, are recognized for their diverse biological activities, including antiviral properties against both DNA and RNA viruses [12]. These compounds demonstrate broad antiviral properties comparable to bioactive flavonoids [29,36,37]. All twelve alkaloids demonstrated non-toxic to mild-toxic levels in BHK-21 cells and the tested alkaloids have favourable safety profiles crucial for potential therapeutic applications in animals. Both cephaeline (CPL) and emetine (EMT) belong to the group of ipecac alkaloids, derived from the roots of *Psychotria ipecacuanha* in the *Rubiaceae* family. EMT, an FDA-approved anti-protozoal drug, serves dual role as an anti-protozoal agent effective against amebiasis and as an emetic, which was commonly used in poisoning management when administered as part of the ipecac plant [38]. While EMT has

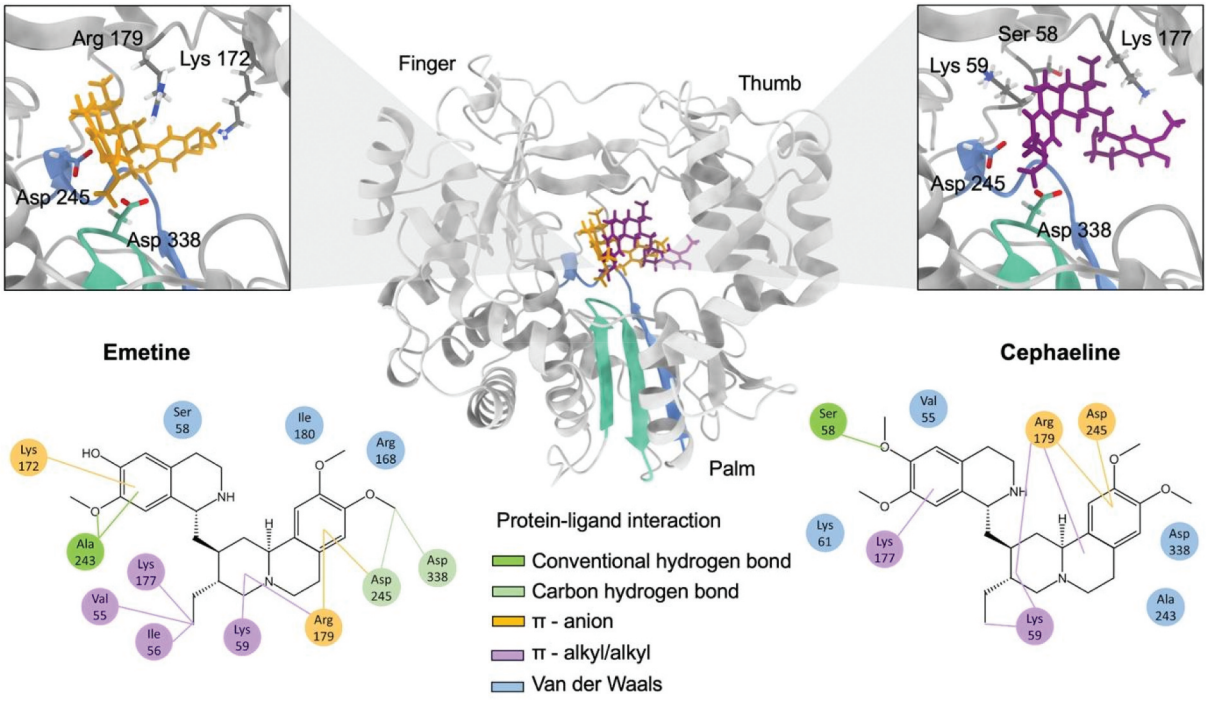

Figure 5. Protein-ligand interactions of emetine (EMT) and cephaeline (CPL) with foot-and-mouth disease virus (FMDV) 3D polymerase (3Dpol). EMT (orange; left panel) and CPL (purple; right panel) were docked into the active site of FMDV 3Dpol. The insets show the 2D and 3D interactions of EMT and CPL with amino acid residues in the active site. Both EMT and CPL could interact with the catalytic residues Asp245 (blue; motif A) and Asp338 (green; motif C).

shown cardiac toxicity at higher doses, as evidenced by ECG changes in humans [review in 38], it is important to note that our study demonstrates antiviral activity at significantly lower concentrations in the submicromolar range, as previously reported [20,24,25]. These lower doses are likely to result in reduced toxicity while maintaining antiviral effectiveness. Furthermore, CPL exhibits a structural difference in the isoquinoline core, featuring a hydroxyl functional group instead of a methoxyl group. This modification is hypothesized to reduce cytotoxicity and adverse effects [39]. Our results indicate that CPL is more potent against FMDV than emetine, suggesting that even lower doses might be effective for antiviral purposes. This improved potency could potentially lead to a more favourable safety profile. However, the investigation of efficacy and potential side effects, which is crucial for FMD therapeutic applications of these compounds, should be further required.

Our findings revealed that both ipecac alkaloids exhibited antiviral activity against FMDV with EC_{50} values in the sub-micromolar range, particularly evident in post-viral entry and penetration assays. This efficacy compares favourably to existing treatment and other compounds under this investigation. For instance, our results showed that these ipecac alkaloids demonstrated greater efficacy than ribavirin, a known antiviral drug that has been suggested to have good combination effect with vaccinations [40]. Furthermore, these two alkaloids showed better efficacy in our cell-based assays compared toT-1105, which has shown promise in similar cell-based assays. T-1105 demonstrated IC50 values of 11.50 μM (1.6 μg/mL) [10] and 5.39 μM (0.75 μg/mL) [11] against FMDV serotype O, and 7.97 μM (1.11 μg/mL) [11] against FMDV serotype A at post infection stage. Moreover, our results are consistent with previous observations of their antiviral efficacy against other viruses such as Zika Virus (ZIKV) and Ebola Virus (EBOV), where EMT demonstrated an IC_{50} of 15.2–516 nM, and CPL showed an IC_{50} of 5.26–200.6 nM [25]. Moreover, EMT has shown high potency against enterovirus (EV) A71, another picornavirus in the same family as FMDV, at the early stage of infection, with an EC50 of 0.04 μM in both RD cells and Vero cells [41].

Our investigation revealed that neither CPL nor EMT impeded the binding of FMDV in the attachment assay, consistent with previous findings in other viruses like buffalo poxvirus (BPXV), bovine herpesvirus 1 (BHV-1), and Newcastle disease virus (NDV) found in farm animals [42]. The different types of cell culture or virus strains used in testing may account for these variations.

The ability of CPL and EMT to target the viral 3Dpol is consistent with previous studies demonstrating their inhibitory effects on RNA polymerases and reverse transcriptases of other viruses, such as ZIKV and HIV [25,39,43,44]. These alkaloids have been shown to intercalate with nucleic acids, disrupt viral protein translation, and impair cellular processes essential for viral replication. For instance, EMT reacts with ZIKV NS5 RNA polymerase with an IC_{50} of 121

nM [25], and it reduces HIV reverse transcriptase activity by approximately 50% at a concentration of 10 μM [44]. Moreover, CPL and EMT can block RNA synthesis at the active site of RNA-dependent RNA polymerase (RdRp) in an *in vitro* studies [25,39], which could provide supportive evidence that these two alkaloids exhibited their inhibition in our mini-genome assay and an *in silico* prediction. Additionally, EMT disrupts cellular machinery, affecting translation of viral proteins, as observed in the inhibition of SARS-CoV-2 RNA synthesis, and the disruption of viral RNA binding with eukaryotic translation initiation factor 4E (eIF4E) [17]. CPL impairs cellular trafficking of cholesterol and lipids, it may hinder ZIKV infection [25]. While our study focused on the antiviral activity of alkaloids against FMDV based on cell-based assays and *in silico* prediction, we acknowledge the potential for more comprehensive analytical approaches in future investigations. Techniques such as Nuclear Magnetic Resonance (NMR) spectroscopy and metabolomics could offer deeper insights into the structural characteristics and mechanistic details of these alkaloids [45,46]. These advanced methods may elucidate the molecular interactions between the ipecac alkaloids and viral components, potentially revealing more detailed mechanisms underlying their antiviral effects as replicase inhibitors and direct virucides. Additionally, recent advancements in NMR sensitivity and resolution [47] could enable more precise identification and characterization of the bioactive compounds and their metabolites.

Besides inhibition to FMDV 3Dpol, these alkaloids demonstrated strong prophylactic and virucidal effects. The prevention effects showed great potential for inactivating the virus, with virucidal efficacy comparable to the finding in previous studies [48]. These results indicate the potential use of CPL and EMT in both treatment and prevention strategies for high-risk environments. However, it should be noted that our experiments were conducted *in vitro* using BHK-21 cells, which may not fully represent the complexity of FMDV infection in field situations.

The dual mechanism of action demonstrated by CPL and EMT, including both replicase inhibition and virucidal effects, warrants further investigation. This could potentially lead to the development of more potent and less toxic derivatives. Further studies should explore the efficacy of these alkaloids in animal models. Additionally, their effectiveness against other FMDV serotypes would be valuable for assessing their broad-spectrum potential and could inform strategies for comprehensive FMDV control.

5. Conclusion

Our findings demonstrate the promising antiviral potential of the ipecac alkaloids, cephaeline (CPL) and emetine (EMT), against foot-and-mouth disease virus (FMDV). These alkaloids exhibit potent dual antiviral effects: inhibiting the viral RNA-dependent RNA polymerase (3Dpol), a crucial enzyme for viral replication, and demonstrating significant virucidal activity. The ability to suppress 3Dpol activity likely underpins the observed reduction in FMDV replication, while the virucidal effects contribute to their prophylactic potential. Their high potency and favourable selectivity indices highlight their promise as candidates for further development into antiviral therapies against FMDV. These results provide a strong rationale for additional exploration, including *in vivo* studies. The natural origin of these alkaloids also suggests potential for topical application or environmental decontamination, offering an alternative approach to manage FMD outbreaks and potentially extending to related viruses affecting livestock.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This project is funded by the National Research Council of Thailand (NRCT) grant number: [N42A670624]; Agriculture Research and Development Agency grant number: [CRP6705031590] and the Faculty of Veterinary Medicine, Kasetsart University. Additionally, AP was supported by the Faculty of Veterinary Medicine, Kasetsart University (RA scholarship)

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Authors' contributions

ST and PL: Conceptualized and designed the experiments. AP, NM, PS, VL, and NP: Methodology. AP and ST: Data analysis. AP, NM and ST: Visualization. ST and PL: Supervision and project administration. AP, ST, and PL: Writing, reviewing, and contributing to revisions. All authors have read, reviewed, and approved the final manuscript.

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