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Byproduct of crocodile liver extract: Potential in vitro viral inhibition in a flu model

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Abstract

Crocodile organs have been used in traditional medicines for the purpose of disease reduction. However, these claims have not been rigorously studied. This study aimed to explore the potential of crude extract from the liver of *Crocodylus siamensis* (CLE) against influenza virus. The toxicity concentration (CC50) of CLE was assayed by a colorimetric assay for cellular metabolic activity (MTT) and was found to be 32.0 mg/mL, and the concentration reducing replication (EC50) of influenza A (H3N2/Vic) evaluated by a viral titration technique for A549 cells was 0.039 mg/mL. The selectivity index (SI), which is the ratio between cytotoxicity and antiviral activity, was 818. A time-of-addition assay was used to identify which step(s) of the viral propagation chain was blocked by CLE. The expression of immune-related genes was tested by RT-PCR to observe the possible mechanisms of antiviral activity. The antiviral activity of CLE on multiple cycles of viral replication was assessed by the viral titration technique. CLE pretreatment of cells resulted in higher viral inhibition. The mRNA expression levels of the immune-related genes MxA, IL-2, CCL5/RANTES, and CXCL10/IP-10 were significantly different in the CLE-treated group. We found that CLE inhibited the propagation of influenza A (H3N2/Vic). This study is a starting point to investigate the scientific analysis of crocodile byproducts.

Keywords: Animal Byproduct, Antiviral Agents, Crocodile, Influenza A Virus, Liver Compounds

1. Introduction

Crocodiles are large and ancient aquatic reptiles that share the same archosaur lineage with dinosaurs. These animals have evolved an active immune system to defend against pathogens that is a part of the evolutionary adaptation of tAhese species. Presently, crocodile farms are widespread in Thailand and Southeast Asia. [1]. Therefore, captive breeding is implemented on these farms to serve as a conservation strategy for this endangered species [2].

Animal-based medicines or zootherapy are widely used as first lines of defense for healing disease. Natural bioactive molecules are increasingly valued as substances in modern medicine. Records of utilization and conservation of animal parts have been described [3,4]. The crocodile immune system has evolved for more than 200 million years. In traditional medicines, crocodile meat is used as a drug for the treatment of asthma [5], and the oil is used for wound healing [6]. The skin of *C. niloticus* has been used to treat tuberculosis [7]. More recently, crocodile blood, serum, and leukocytes were found to exhibit antibacterial, anticancer, and antiviral activity [8-10]. Fresh serum shows a strong inhibitory effect on human immunodeficiency virus (HIV) type I, West Nile virus (WNV), and herpes simplex virus (HSV) type I [11].

While the liver plays a central role in metabolism and protein production in every vertebrate animal, no scientific study on the antiviral properties of liver compounds has been reported to date. In the study of active ingredients derived from animal sources, the liver is an interesting organ, as it plays a key role in body

metabolism. Crocodiles live in ecosystems with high pathogenic pressure but are observed to be resistant to microbial infection. The innate immunity of crocodiles against microbes is more robust than that of mammals [12]. Promising reports regarding the antibacterial activities of crocodile tissues [13] and crocodile serum [8,14–16] have been published. Furthermore, the antiviral activity of crocodile serum against human immunodeficiency virus (HIV-1), West Nile virus (WNV), and herpes simplex virus (HSV-1) [17] has been reported. To the best of our knowledge, this is the first study to determine the anti-influenza A virus (IAV) activity of an animal-sourced compound, namely, an extract from the liver of *C. siamensis*.

The seasonal flu is a contagious respiratory disease, often resulting in a severe illness, high mortality rate, and economic problems due to loss of human productivity. Flu is a major public health issue in the 20th century [18], and nature-based anti-influenza drugs have become an active area of research. IAV is one of the major viral strains affecting the population at present. H3N2-type human IAV attacks people of any age and causes death in high-risk groups (children, elderly individuals, and immunocompromised individuals). In August 2020, of almost 20,000 reported cases of influenza, the H3N2 type accounted for 32.6% of all cases [19]. Antigenic drift and shift result in genetic changes in the virus, allowing reinfection and drug resistance of this strain. Alveolar epithelial cells (AECs) are the main target site of IAV infection [20]. Induced by an infection, AECs secrete type I and III interferons, cytokines, and chemokines, resulting in the promotion and recruitment of immune cells to control the infection [21].

This study aimed to evaluate the potential use of *C. siamensis* crude liver extract against influenza infection. Furthermore, this study aimed to assess the potential of the compound mixture as a medicinal supplement. The identification of the pharmacologically active compounds within the mixture will continue in further study.

2. Materials and methods

2.1 Cell lines and viral strain

Human lung carcinoma (A549) cells and Madin-Darby canine kidney epithelial (MDCK-II) cells were maintained in Dulbecco's modified Eagle medium (DMEM) (Gibco, USA) supplemented with 10% fetal calf serum (FCS, PAA, Austria), 100 U/mL penicillin and 0.1 mg/mL streptomycin (P/S) (Gibco, USA) in a tissue culture flask (Corning, USA) at 37 °C in a 5% CO_2 atmosphere. Human IAV/Victoria/3/1975 (H3N2/Vic) was propagated in MDCK-II cells at 37 °C in a 5% CO_2 atmosphere, and the virus-containing supernatant was stored at -80 °C. Viral titration was performed by focus assay and hemagglutination assay (HA).

2.2 Preparation of C. siamensis liver extract

C. siamensis liver was collected from Sriracha Moda Farm Co., Ltd. (Chon Buri, Thailand). The liver was removed and maintained at -80 °C until the extraction process. Crocodile liver was aseptically homogenized with cold 0.9% NaCl at a ratio of 1:1 (*w/v*) and centrifuged at 12,000 x *g* for 40 min at 4 °C, where the aqueous layer was collected and centrifuged again at 14,000 x *g* for 40 min at 4 °C. The supernatant was then removed and passed through a 0.2 µM syringe filter (PALL Life Sciences Acrodisc®). The lipid phase was removed, and a saline soluble compound mixture was collected. This sterile crude crocodile liver extract (CLE) was dried in a freeze dryer and kept at -80 °C for further experiments. The physical appearance of CLE is a brown powder, dissolve well in water. The characteristic of CLE in this study was selected to use protein/peptide of the extract, perform by a Bradford assay (Sigma, USA), was used to determine the total protein concentration present in CLE. The absorbance at 465-595 nm was measured using bovine serum albumin (BSA) for a standard curve setting. The peptide fragment analysis by gel electrophoresis.

2.3 Cytotoxicity concentration 50% (CC_{50})

CC_{50} is the concentration at which 50% of the cells are still viable. In this study, it was a concentration of CLE that was toxic to the cells by 50%. A549 monolayers were continuously grown for 24 h on 24-well plates at a density of approximately 3×10^4 cells per well. Culture media (DMEM with 10% FCS, PAA, 100 U/mL penicillin, and 0.1 mg/mL streptomycin) was removed and washed with PBS twice. Cells were incubated with different CLE concentrations (0, 10, 20, 40, 80, 100 mg/mL) in culture media at 37 °C in a 5% CO_2 atmosphere for 24 h. The freeze-dried CLE was dissolved in cell culture medium (RPMI-1640). The negative control was RPMI-1640 alone. After washing with PBS containing Ca++ and Mg++ (PBS++), an MTT assay was performed (n = 6 per CLE concentration) by adding 200 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, USA) in DMEM for 90 min. After this period, 200 µl of isopropanol was dissolved after removing the supernatant and fixing the cells with 4% paraformaldehyde (PFA), and the absorbance was analyzed at 490 nm. The CC_{50} was calculated from 100% cell viability determined in culture media without

CLE-treated cells. The OD represents the viability of the cells. The CC_{50} was calculated by regression curve analysis using the GraphPad Prism software package (GraphPad Prism, USA).

$$\text{Percentage of cell viability} = \frac{\text{OD(490) Test}}{\text{OD(490) Control}} \times 100 \quad (1)$$

2.4 Effective concentration 50% (EC_{50})

EC_{50} is the (CLE) concentration that reduces the viral load by 50%. A549 cells were grown for 24 h to a confluent monolayer in a 24-well plate. The culture medium was removed, and the cells were washed twice with 1x PBS followed by infection with 200 µl of IAV (H3N2/Vic) at a multiplicity of infection (MOI) of 0.1 suspended in PBS/BA (PBS++ containing 0.21% BSA and 1% P/S) for 1 h at room temperature (RT). Infected cells were washed again with PBS++ to remove the excess virus. Cells were incubated in different concentrations of CLE (0.0, 0.001, 0.01, 0.1, 1, 10, 100, 1000 µg/mL) (n = 3) in DMEM/BA (DMEM containing 0.21% BSA, 1% P/S, and 1 mg/mL TPCK-treated trypsin (Sigma-Aldrich)) at 37 °C, 5% CO_2 humidified atmosphere for 24 h. The viral yields in the supernatant were titered via focus assay on MDCK-II cells.

2.5 Hemagglutination inhibition (HAI) assay

HAI assay was used to determine possible interference of CLE in binding of viral hemagglutinin (HA) to the sialic acid receptor of red blood cells (RBCs). The HA titer of the viral suspension used was determined via chicken RBC agglutination. A titer of 5129 HAU/mL, which allows complete aggregation, is called the HA unit. To determine a possible effect of CLE on the binding site of H3N2/Vic HA to the RBC sialic acid receptor, 25 µl of 0.35 mg/mL (EC_{90}) CLE was incubated with an equal volume of the fourth back titer of HA units of H3N2/Vic in a microtiter V-shaped plate for 30 min at RT. This procedure allowed CLE to react with viral HA before adding RBCs. One percent chicken erythrocytes suspended in PBS was added to each well. Virus that had not been incubated with CLE was used as a positive control, and PBS without virus was used as a negative control. The reaction was mixed on a plate shaker and then incubated at 4 °C until the negative control showed dot formation.

2.6 Viral yield reduction assay

2.6.1 Time-dependent viral yield reduction

A549 cells were infected with IAV (H3N2/Vic) (MOI=0.1) for 1 h at RT. After infection, the cells were incubated with DMEM/BA containing 0.35 mg/ml (EC_{90}) CLE for 8 hours post infection (h p.i.) allowing a single viral replication cycle or for 24 h p.i. allowing multicycle replication. The viral titers were determined via focus assay on MDCK-II cells.

2.6.2 Pretreatment with CLE

A549 cells were preincubated with 0.35 mg/mL (EC_{90}) CLE for 2 h before infection with IAV (H3N2/Vic) or were left untreated before infection. Viral infection (MOI = 0.1) was performed for 1 h at RT. After infection, the cells were incubated with DMEM/BA containing 0.35 mg/mL (EC_{90}) CLE until 24 h p.i. allowing multicycle replication. The viral titers were determined via focus assay on MDCK-II cells.

2.7 Viral titration assay

Focus assay was used to titer viral yield. MDCK-II cells (3 x 10^4 cells per well) were grown for 24 h on 96-well plates. Twenty microliters of cell culture medium from A549 cells infected with IAV (H3N2/Vic) treated with or without CLE was serially diluted 10-fold with 180 µl PBS/BA. Fifty microliters of each viral dilution was incubated on MDCK-II cells for 1 h at RT. After washing the cells with PBS three times, titration media (DMEM/PS/BA containing 1 mg/mL TPCK-treated trypsin and 2.5% Avicel CL-611 (FMC Corporation)) was added. Cells were then incubated at 37 °C in a 5% CO_2 humidified atmosphere for 27 h. Then, the titration media was removed, and the cells were washed three times with PBS++. Then, 100 µl fixing solution (PBS containing 1 mM $MgCl_2$, 0.9 mM $CaCl_2$, 4% PFA, 1% Triton X-100) was added, and the cells were incubated for 60 min at RT. The fixing solution was then removed, and the cells were washed three times with washing solution (PBS containing 0.05% TWEEN 20). Staining solution (Santa Cruz, USA), 50 µl of anti-NSI influenza was added, incubated at RT for 60 min. Anti-mouse IgG-HRP was used as secondary antibody. Following incubation for 40 min at 37 °C by 40 µl AEC (3-amino-9-ethylcarbazole), the substrate solution was removed,

and the cells were washed 2x with dH2O to remove salts. To detect and quantify foci, 96-well plates were scanned with a resolution of 1200 dpi using an Epson Perfection V500 Photo scanner (Epson, Japan) and analyzed using Photoshop software (Adobe, USA). The results represent the mean (± SEM) from three independent experiments.

2.8 Primers and quantitative RT-PCR

Quantitative mRNA expression was analyzed on 100 ng of total RNA from (a) control (uninfected nontreated) A549 cells, (b) virus-infected A549 cells, and (c) virus-infected A549 cells treated with CLE. RT-qPCR analysis was performed using a QuantiFast SYBR Green RT-PCR kit (Qiagen, Germany). For each condition, 1.2106 A549 cells were cultured to confluency at 37 °C in 5% CO_2 for 24 h. Then, the culture media were removed and washed with PBS containing 0.9 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS++). The cells in (b) and (c) were infected with IAV (H3N2/Vic) at an MOI of 1 for 1 h at RT, and then the cells were washed once with PBS++. In (c), the cells were incubated with 0.35 mg/mL (EC_{90}) CLE. At 24 h p.i., total RNA for every condition was extracted by using TRIzol reagent (Invitrogen, USA) and purified following the protocol of the RNeasy kit (Qiagen, Germany). The purified RNA concentration was cleaned with DNase I (Invitrogen, USA) and measured by a UV/Vis photometer (Eppendorf BioPhotometer plus) at 260 nm absorption. Primer extension is described in Table 1.

Table 1 Primers used in RT-qPCR amplification.

Gene	Forward Primer (5' - 3')	Reverse Primer (5' - 3')	References
β-actin	CATGAAGTGTGACGTGGACATCC	GCTGATCCACATCTGCTGGAAGG	[22]
IFN-α	CTTGTGCCTGGGAGGTTGTC	TAGCAGGGGTGAGAGTCTTTG	[23]
IFN-β	CAGCAATTTTCAGTGTCAGAAGC	TCATCCTGTCCTTGAGGCAGT	[22]
IL-2	CAACTGGAGCATTTACTGCTGGA	TCAGTTCTGTGGCCTTCTTGG	[24]
IL-6	ACCTGAACCTTCCAAAGATG	GCTTGTTCCTCACTACTCTC	[23]
IL-8	TGCCAAGGAGTGCTAAAG	CTCCACAACCCTCTGCAC	[12]
FasL	GTTCTGGTTGCCTTGGTAGG	TGTGCATCTGGCTGGTAGAC	[25]
MxA	ACAGGACCATCGGAATCTTG	CCCTTCTTCAGGTGGAACAC	[26]
CCL5/RANTES	TACCATGAAGGTCTCCGC	GACAAAGACGACTGCTGG	[27]
CXCL10/IP10	CTGCTTTGGGGTTTATCAGA	CCACTGAAAGAATTTGGGC	[28]

RT-qPCR was performed using a Light Cycler 480 Multiwell Plate 96 (Roche Diagnostics, Switzerland). Reaction cycling data were analyzed with a comparative threshold (CT) cycle by normalization to the ß-actin housekeeping gene, and housekeeping gene, and the fold change was calculated using $2^{-\Delta\Delta CT}$.

2.9 Statistical analysis

Data were analyzed with Microsoft Excel 2010 (Microsoft Corporation, USA) and GraphPad Prism 7 (GraphPad Software, Inc., USA). All experiments were performed (n = 3 to 6), and the results are presented as the mean ± SEM. Statistical analysis was performed by GraphPad Prism using transformed data, *t*-tests, and multiple *t*-tests.

3. Results and discussion

3.1 CLE reduces H3N2/Vic propagation in A549 cells but does not bind to viral HA receptor

The cellular viability (CC_{50}) and viral inhibition (EC_{50}) after treatment with CLE were assayed in A549 cells. The CC_{50} was determined following the procedure outlined in Section 2.3. As shown in Figure 1A, the CC_{50} for CLE on A549 cells was 32.01 mg/mL. The EC_{50} is determined from the method in Section 2.4. The percent viral titers at different CLE concentrations were plotted as described in Figure 1B, perform by focus assay explained in section 2.8. The focus forming unit shows in figure 1C. The value was 0.039 mg/mL. The EC_{90} was calculated as the most effective concentration that inhibited viral yield by one $\log_{10}$, and the equation is shown below.

$$EC_F = ((F/100\text{-}F)^{1/H}) \times EC_{50} \quad (1)$$

with H as the Hill slope. The EC_{90} is 0.35 mg/mL. The selectivity index (SI) expressing the ability of antiviral compounds shows a high score of 818. The *SI* was calculated by the following equation.

$$\text{Selectivity index } (SI) = \frac{CC50}{EC50} \quad (2)$$

The impact of CLE on viral adsorption and HAI was assayed as described in Section 2.6 and 2.7. CLE was not found to inhibit RBC agglutination, i.e., CLE was incapable of interfering with H3N2/Vic HA binding to its sialic acid (SA) receptor on RBCs, resulting in HAI negativity. This indicates that the antiviral activity of CLE is not based on blocking H3N2/Vic entry via HA/SA binding. The reduction in virus may have been caused by further steps of intracellular target cells. The study was designed to allow viruses to propagate and observe intracellular cytokine/chemokine gene expression.

In this study, it was hypothesized that bioactive compounds in crocodile liver would demonstrate potential as an anti-IAV (H3N2/Vic), agent on human lung carcinoma (A549) cells. These cells are used in lieu of AECs [29]. This knowledge can be used to investigate the innate immune responses triggered after IAV infection. CLE efficacy on H3N2/Vic reduction was found in the following observations: (i) CLE can strongly inhibit the replication of H3N2/Vic in A549 cells, with a high selective index (*SI*) of 818. This strong *SI* is considered to indicate a high potential anti-IAV property compared to other natural compounds. (ii) CLE exerts an effect within the infected cell. However, the HAI results showed that CLE does not inhibit H3N2/Vic by blocking the viral adsorption step.

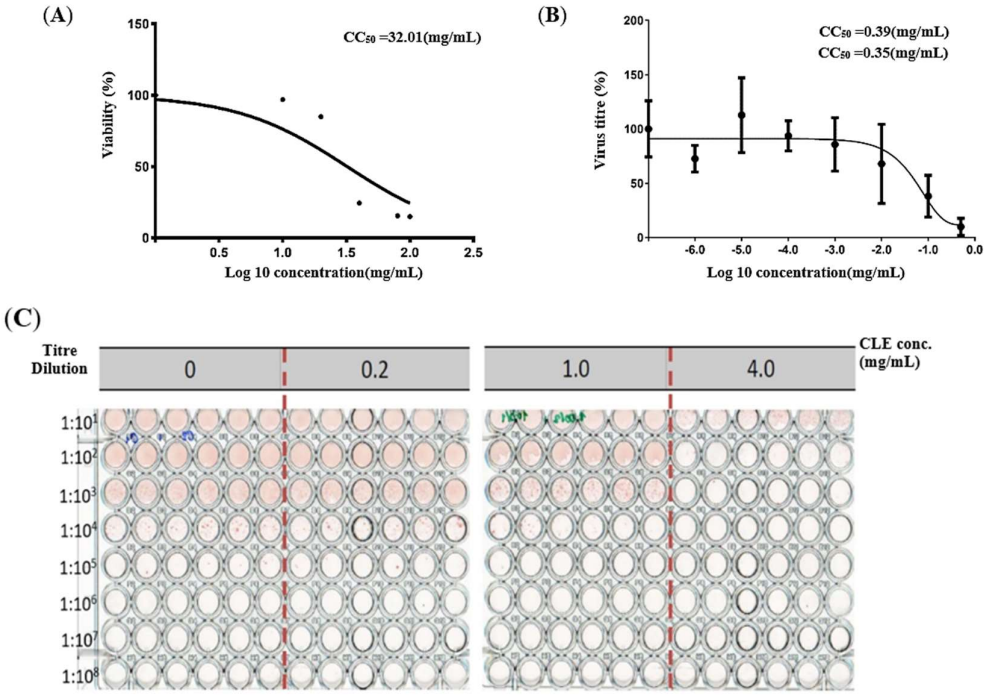

Figure 1 Cytotoxic concentration 50% (CC_{50}) and effective concentration 50% (EC_{50}) of CLE in A549 cells; (A) Cell viability was tested at different CLE concentrations and measured by MTT assay, (B) EC_{50} was calculated by incubating IAV (H3N2/Vic)-infected (MOI=0.1) A549 cells with different CLE concentrations for 24 h and titration of the viral yields in the supernatants via focus assay on MDCK cells and (C) Focus formation assay; the infected cells stained with anti-NS-1 antibody are shown in red. Each assay was performed three to six data with three times repeated.

3.2 Time-of-addition approach to viral inhibition

To characterize the mechanism of viral inhibition, the time of compound addition approach to viral yield reduction was determined. The impact of CLE compounds pretreated and post-treated with H3N2/Vic on A549 propagation was determined. For this, viral yield after single- or multicycle replication of the flu virus was analyzed at 8 or 24 h p.i. via the focus assay on MDCK-II cells. As shown in Figure 2A, CLE exhibited a significant viral titer reduction compared to the control without CLE treatment at 24 h p.i. This is a multicycle replication time, indicating that an antiviral compound significantly impacts multiple cycles of viral propagation. CLE may require time to promote viral infection reduction by intracellular absorption.

The potential of CLE against IAV (H3N2/Vic) propagation in non-pretreatment and pretreatment with CLE before viral infection was evaluated in A549 cells, and titration was observed by focus assay on MDCK-II cells, as described in Section 2.6.2. The result indicated a significant increase in viral reduction in cells pretreated with CLE compounds compared to those in the non-pretreatment group, as shown Figure 2B. Pretreatment with CLE

and infection at 8 h p.i. did not exhibit significant reduction (data not shown). CLE compounds did not inhibit the initial virus infection. It can be assumed that CLE might exert intracellular antiviral activity. The purpose of the time of the compound addition study was to observe the effect of only CLE compounds on A549 cells before viral infection, and CLE was found to increase viral reduction in a time-dependent manner.

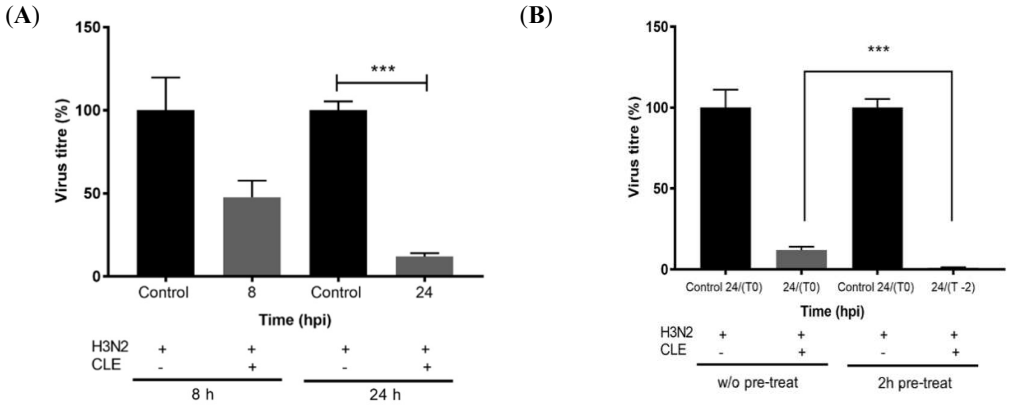

Figure 2 Time-dependent inhibition of IAV replication and the effect of CLE preincubation on IAV replication: (A) A549 cells were infected with H3N2/Vic and treated with 0.35 mg/mL (EC_{90}) CLE, and viral titers were determined 8 h p.i., allowing a single viral replication cycle, and 24 h p.i., allowing multicycle replication. Virus was titered by focus assay on MDCK cells. At 24 h p.i., CLE exhibits a significant viral reduction (***) $p<$ 0.001, and (B) A549 cells were preincubated with 0.35 mg/mL (EC_{90}) CLE for 2 h before infection. After infection, cells were incubated with 0.35 mg/mL (EC_{90}) CLE, and viral titers were determined 24 h p.i. allowing multicycle replication. (***) $p < 0.001$. Each assay was performed three to six data with three times repeated.

3.5 CLE promotes cytokine, ISG, and chemokine mRNA expression

To determine the antiviral mode of action (MoA) of CLE, we aimed to evaluate whether CLE might affect the cellular innate immune reaction against viral infection by determining the promotion of innate immune gene expression. Therefore, A549 cells were incubated with 0.35 mg/mL (EC_{90}) CLE for 24 h, and the transcription rates for several genes of the innate immune system were analyzed via RT-PCR and compared with the untreated control. The results show that CLE alone induces mRNA expression of interleukin (IL) genes, interferon-stimulated genes (ISGs), and chemokine genes. Compared with the control, the levels of IL2, MxA, CCL5/RANTES, and CXCL10/IP10 were significantly upregulated as shown in Figure 3. The expression level of the housekeeping gene β-actin in the CLE-treated group was not reduced compared with that in the control group.

The intracellular effect resulting in a viral load reduction might be the viral defense mechanism of the target cells. The indirect effect of promoting the mRNA expression of virus-related genes is possibly one of the mechanisms of CLE that helps reduce viral infection.

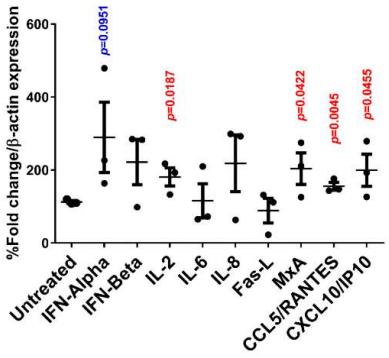

Figure 3 Expression levels of mRNA in CLE-treated A549 cells.

A549 cells were treated with 0.35 mg/ml (EC_{90}) CLE for 24 h, and the mRNA levels of IL-2, MxA, CCL5/RANTES and CXCL10/IP-10 were determined. The cellular actin mRNA level was used for

normalization. The fold change in the mRNA amount showed increased transcription of cytokine, ISG, and chemokine genes in CLE-treated A549 cells compared to nontreated cells (set to 100%). Each assay was performed three to six data with three times repeated.

CLE incubated with virus in A549 cells strongly exhibited a reduction in multicycle viral replication (24 h p.i.). This indicates that infected cells require CLE to be absorbed to interfere with intracellular viral replication. A549 cells pretreated with CLE 2 h before viral infection demonstrated increased viral reduction, suggesting that CLE reduces H3N2/Vic by time-dependent viral reduction. It is speculated that CLE must be absorbed into the target cell to improve its viral defense mechanism but does not directly show a virucidal effect. Time-dependent CLE incubation was designed to answer the question of whether CLE can promote the intracellular environment of target cells before viral infection. This investigation was intended as a pilot study to examine the potential for supplemented action by CLE.

3.4 CLE induces IL-2 and IL-6 gene upregulation after IAV (H3N2/Vic) infection

The impact of CLE compounds on innate immune genes helps to narrow the possible mechanism of the inhibition of IAV (H3N2/Vic) infection. In this study, A549 cells were directly incubated with 0.35 mg/ml (EC_{90}) CLE for 24 h and then infected with IAV (H3N2/Vic) for 1 h at RT. The results showed that IL-2 and IL-6 mRNA expression was upregulated in the CLE treatment group compared with the control group without CLE treatment and that both groups were infected with the virus as shown in Figure 4. This figure demonstrates that the antiviral properties of CLE compounds are related to the promotion of IL genes.

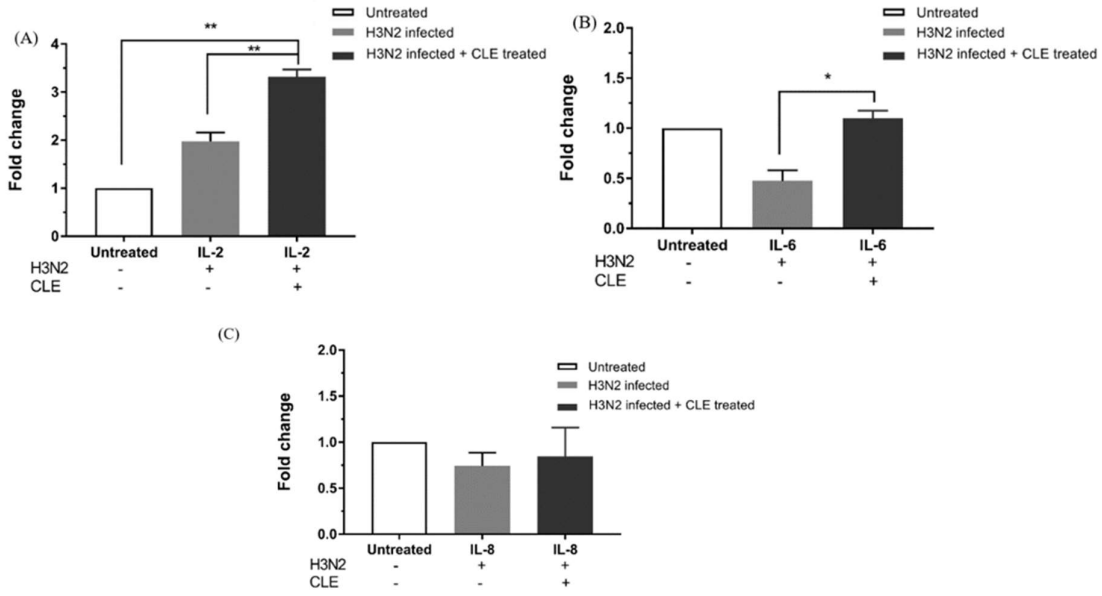

Figure 4 Comparison of the expression levels of mRNA encoding interleukin genes in A549 cells infected with H3N2, Vic (MOI=1) vs. infection plus treatment with CLE 0.35 mg/ml (EC_{90}) at 24 h p.i. The mRNA expression levels of (A) IL-2, (B) IL-6, and (C) IL-8 showed that the IL-2 and IL-6 genes were significantly upregulated in the CLE-treated cells after viral infection. (*) $p < 0.05$. (**) $p < 0.01$. Each assay was performed three to six data with three times repeated.

Compared with those in the control group, the levels of CCL5/RANTES were upregulated in A549 cells infected with H3N2/Vic, whereas the levels of IL-2, CCL5/RANTES, and CXCL-10 were upregulated in the CLE-treated A549 infection group (Figure 5).

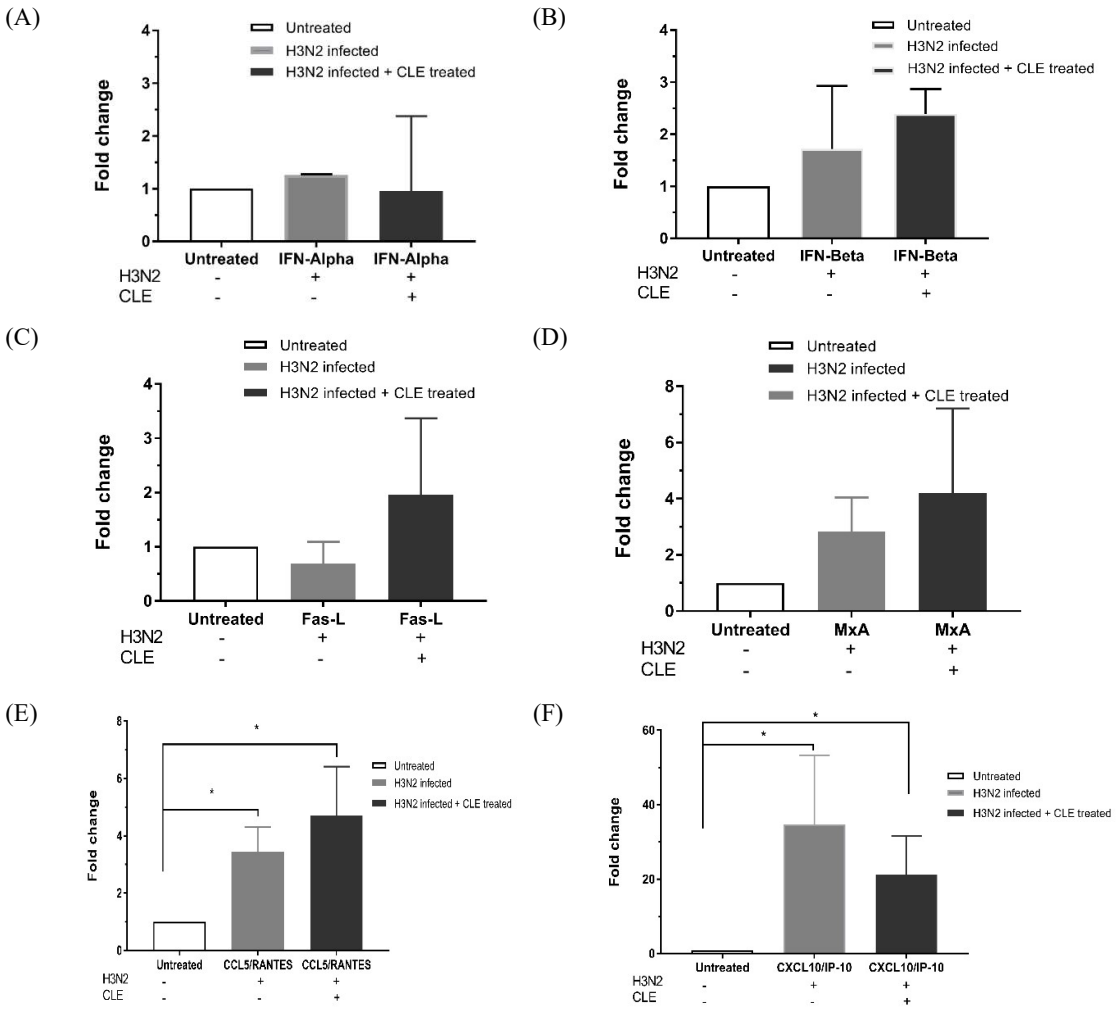

Figure 5 Expression levels of mRNA encoding antiviral factors in IAV-infected and CLE-treated A549 cells compared with the control group. mRNA expression levels of (A) interferon-alpha and (B) interferon-beta (C, D) ISGs (Fas-L, MxA) and (E,F) chemokines (CCL5/RANTES, CXCL10/IP-10) in A549 cells infected with H3N2/Vic (MOI 1) at 24 h p.i. vs. infection plus treatment with CLE 0.35 mg/ml (EC90). The fold change in the mRNA amount showed specific upregulation of IL-2, CCL5/RANTES, and CXCL10/IP-10. (*) $p < 0.05$. Each assay was performed three to six data with three times repeated.

The mRNA expression of immune-related genes found in A549 cells demonstrated a possible mechanism of CLE in its role as an immunomodulator. AEC type II cells, which are lung epithelial cells, are well known as amateur antigen-presenting cells that play roles in the innate immune system [30]. In this study, CLE promoted the upregulation of genes, including IL-2, MxA, CCL5/RANTES, and CXCL10/IP10, in agreement with the current understanding of these genes. IL-2 does not directly possess an antiviral effect but plays a crucial role in promoting antiviral activity by reducing oxidant injury in rat lung epithelial cells [31]. Myxovirus resistance A (MxA), an important ISG gene that encodes a broad antiviral intracellular protein, was identified as a potent antiviral response that inhibits the primary steps of the viral replication cycle [32,33]. CCL5/RANTES, a chemokine, provides strong evidence of *in vitro* and *in vivo* production from IFV infection. A study in a mouse model with CCR5 knocked out demonstrated delayed viral clearance [34]. Lung epithelial cells themselves have an antiviral role, inducing chemokine and cytokine production. Similar to CCL5/RANTES, IFN-γ inducible protein-10 (CXCL10/IP-10) has a key role in chemokine responses to influenza infection. This chemokine recruits innate immune cells [35].

The mRNA upregulated genes after treatment with CLE for 24 h and after IAV (H3N2/Vic) infection were IL-2 and IL-6. As previously mentioned, IL-2 is closely related to innate immune cells and T-cell-dependent IFN-γ production. Therefore, it is unclear whether IL-2 directly promotes viral reduction or costimulation with other cytokines/chemokines. IL-6 plays an essential role in both pro- and anti-inflammatory responses [35].

Supporting the clearance of virus from infected (mouse) lungs results in protection against IAV infection [36]. IL-6 plays crucial roles during viral infection by preventing virus-induced apoptosis in lung epithelial cells [37] The results of this study indicate the possible role of CLE compounds in the inhibition of IAV (H3N2/Vic) infection through the induction of cytokine mRNA. CLE is absorbed into target cells and interferes with viral propagation. The bioactive compounds in CLE indirectly reduce viral propagation by promoting chemokines and cytokines in the target cells. Virus-host interactions are dual controlled by pro- and antiviral signaling molecules over multiple rounds of viral replication.

3.5 Characterization of CLE compounds

The identification of active compounds of CLE with potential as anti-influenza agents is compulsory. However, the property found in this study might be a synergistic effect. A group of proteins/peptides were classified to be lower than 200 kDa and screened for anti-influenza properties (Figure 6,7). Pro-viral properties were found in some fractions. The peptide fragment alignment shows group of hemoglobin in the most virus inhibitory fraction (data not shown). An antiviral peptide from natural source is potent to be bioactive compound. To purification and identification of the compound are compulsory in further study.

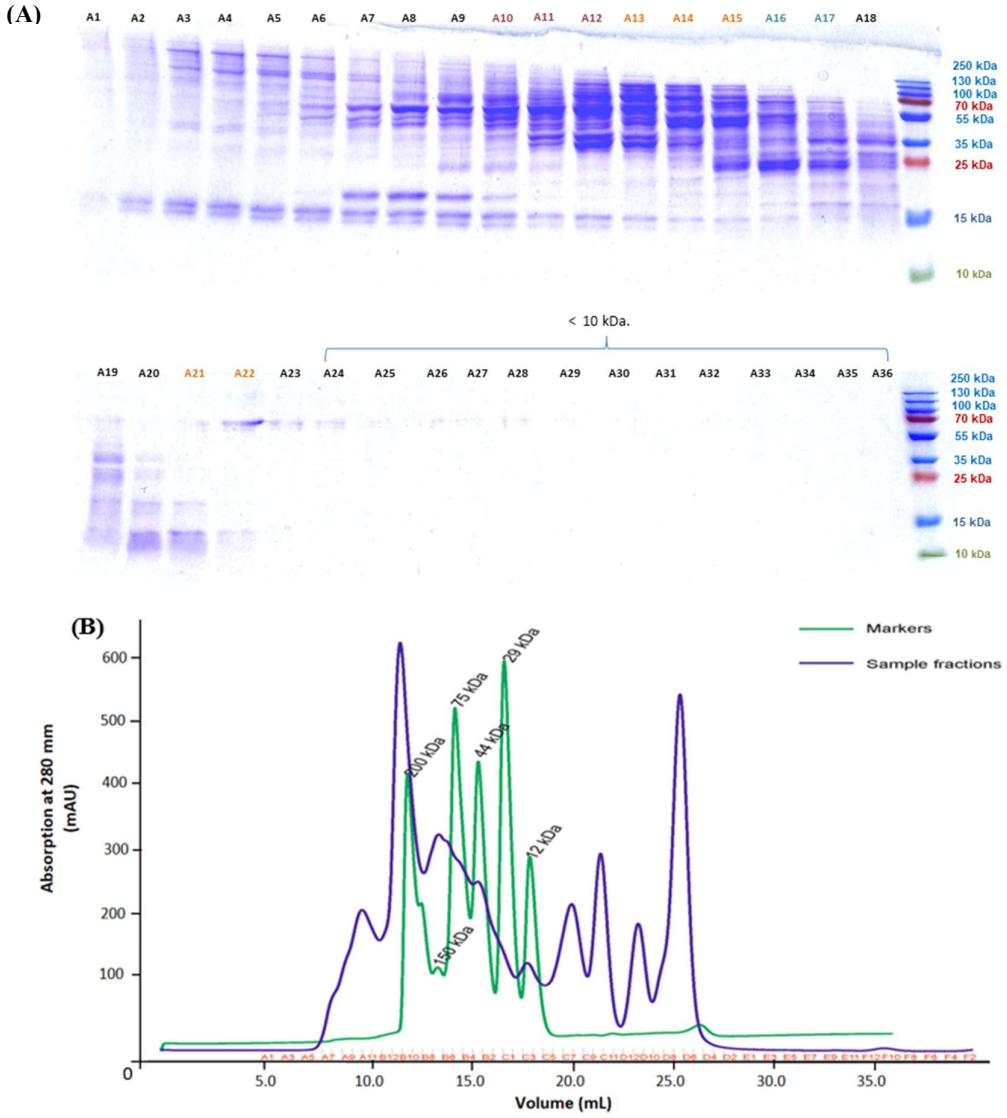

Figure 6 Partial purification of CLE; fractions tested on H3N2/Vic. infection. (A) CLE < 200 kDa on 10% SDS-PAGE, (B) crude liver extract was run through size exclusion chromatography, 200 kDa. Standard protein marker (green line) was run together.
The data show the mean ± SEM of %virus from triplicated tests, 3 times repeated.

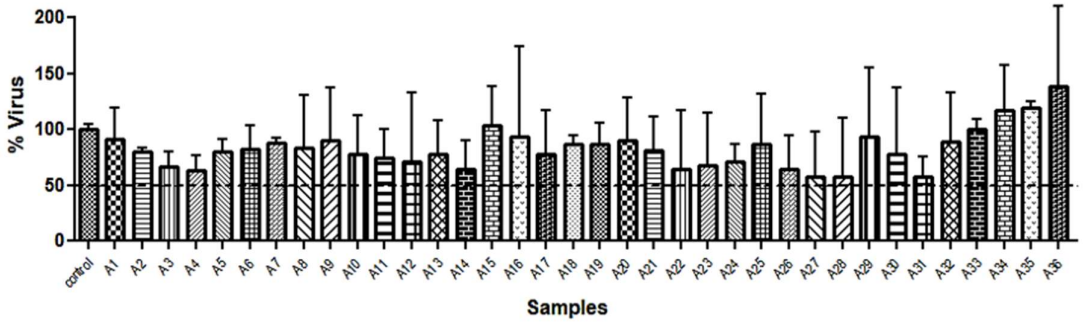

Figure 7 Partial purification of CLE; all 36 fractions screened for anti-H3N2/Vic propagation to A549 cells at an MOI = 1.
The data show the mean ± SEM of %virus from triplicated tests, 3 times repeated.

4. Conclusion

The study of CLE compounds for anti-IAV (H3N2/Vic) properties and innate immune activity in lung epithelium (A549) was studied. CLE exhibited antiviral potential *in vitro* against IAV (H3N2/Vic). The inhibitory effect acted within the infected cells. Our study concluded that CLE modulates the local host cell immune response by promoting auto- and paracrine innate antiviral signaling networks, with a greater reduction at 24 h p.i. The cytokine and chemokine proteins need to be confirmed by western blot in a further study. The purification and identification of effective bioactive compounds will be determined in further studies.

5. Ethical approval

The animal experimental design was investigated in accordance with OECD No. 478 guidelines and was approved by the Khon Kaen University ethical committee *(*NELAC 21/2557*)*. The certification of the *C. siamensis* provider was given by Sriracha Moda Co., Ltd., the crocodile farm and a commercial captive and breeding institution with CITES Secretariat number A-TH-517 and was approved on February 2, 2020.

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All authors read and approved the final manuscript. T.M. (first author): a major contributor in writing the manuscript and to the conception and design of the work; the acquisition, analysis, and interpretation of the data; and drafting and revising the work critically for important intellectual content. S.D.: critically reviewed the study proposal. J.D. (co-corresponding author): final approval of the version to be published; accountable for all aspects of the work and to ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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