KERRA, mixed medicinal plant extracts, inhibits SARS-CoV-2 targets enzymes and Feline corona virus.

Supaphorn Seetaha[1,4], Phatcharin Khamplong[1], Panatda Wanaragthai[2], Thitinan Aiebchun[1], Sucheewin Krobthongand[2], Jatuporn Rattanasrisomporn[3] and Kiattawee Choowongkomon[1,2,4]*

[1]Department of Biochemistry, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand

[2]Interdesiplinary of Genetic Engineering and Bioinformatics, Graduate School, Kasetsart University, Bangkok 10900, Thailand

[3]Department of Companion Animal Clinical Sciences, Faculty of Veterinary Medicine, Kasetsart University, Bangkok 10900, Thailand

[4]Kasetsart University Institute for Advanced Studies, Kasetsart University, Bangkok 10900, Thailand

Corresponding Author: Associate professor Kiattawee Choowongkomon, Ph.D.
Institution: Kasetsart University
Department of Biochemistry, Faculty of Science, Kasetsart University, Bangkok, 10900, Thailand
Tel: (66) 2-562-5555 ext. 647729; Fax: (+66)2-561-4627
E-mail: fsciktc@ku.ac.th
First Author: Supaphorn Seetaha
Order of Authors: Supaphorn Seetaha, Kiattawee Choowongkomon, Ph.D.

Abstract

The COVID-19 pandemic affects all parameters, especially healthcare professionals, drug and medical supply. The KERRA is a mixed medicinal plant capsule which is used for treatment of patients with high fever with food and drug administration approved by FDA Thailand. Recently, KERRA showed quicker recovery for COVID-19 patients. Therefore, it is possible that some ingredients in KERRA could inhibit SARS-CoV-2 virus. In this study, the two important replication related enzymes in SARS-CoV-2 virus, a main protease and an RNA-dependent RNA polymerase (RdRp), were used to study the effect of KERRA. The results showed that KERRA was inhibited SARS-CoV-2 main protease and SARS-CoV-2 RdRp by IC_{50} value is 49.91 ± 1.75 ng/mL and 36.23 ± 5.23 µg/mL, respectively. While KERRA displayed no cytotoxic activity on macrophage cells at concentrations lower than 1 mg/mL. Furthermore, KERRA also exhibited anti-inflammation activity and against a feline coronavirus (the Feline Infectious Peritonitis [FIP]) infection.

Keywords: COVID-19 pandemic, KERRA, SARS-CoV-2 main protease, SARS-CoV-2 RNA-dependent RNA polymerase, Anti-FIPV Activity

1. Introduction

Coronavirus disease or COVID-19, a new disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), was first identified in Wuhan, China, in December 2019 [1]. COVID-19 is a global pandemic and spread extremely quickly [2] with symptoms such as fever, cough, sore throat, asthma, or lung inflammation [3-5]. In severe cases, respiratory failure and even death may present [5]. SARS-CoV-2 is a positive-sense single-stranded RNA virus belonging to *Coronaviridae* family, *Betacoronavirus* genus which infects cells of the upper and lower respiratory tract [6]. The enzyme target for COVID-19 treatment is interesting with two enzymes. There are SARS-CoV-2 main protease is translated in the human host cells for cleavage several viral proteins into the active forms [7, 8] and the RNA-dependent RNA polymerase (RdRp) is used to replicate RNA as a genetic material of SARS-

CoV-2 virus [9, 10]. Therefore, further attempts to establish the new compounds will inhibit two important SAR-CoV-2 enzyme activities. Several inhibitors in drug discovery are derived from natural products which has been recognized as an important source of new drug discovery.

In Thailand itself, some epidermal crises also appeared in the past, such as the outbreak of Cholera during the reign of King Rama II (1820) and King Rama V (1873) which considerably damaged the lives and property of people [11]. In each epidemic, herbal medicines, as basic self-care remedies, had been alternative tools to cope with the disease. An alternative for Thais to prevent COVID-19 is herbal medicine, in particular from Encyclopaedia of Tak-Ka-Si-La, a Thai wisdom since ancient times. KERRA is one of herbal medicine from Tak-Ka-Si-La. KERRA was mixed 9-ingredients medicinal plant which includes *Dracaena loureiri* Gagnep. [12], *Tarenna hoaensis* Pit. [13], *Schumannianthus dichotomus* (Roxb.) Gagnep. [14], *Momordica cochinchinensis* (Lour.) Spreng. [15], *Citrus aurantifolia* (Christm.) Swingle. [16], *Combretum quadrangulare* Kurz. [17], *Dregea volubilis* Benth. ex Hook.f. [18], *Tiliacora triandra* Diels. [19], and *Tinospora cordifolia* [20]. All 9-ingredients medicinal plant has various pharmacological bioactivities mainly including antioxidant and anti-inflammatory.

This study explored to evaluate the inhibition two important SAR-CoV-2 enzyme activities of KERRA, exhibited anti-inflammation activity in RAW264.7 macrophage cells and against a feline coronavirus infection. The results demonstrated that KERRA have a potent anti-SARS-CoV-2 activity with no toxicity in cell culture models.

2. Materials and Methods

Inhibition of SARS-CoV-2 main protease

Preparation sample of KERRA and Fah Talai Jone (*Andrographis paniculata*) at 100 mg/mL in 100% dimethyl sulfoxide as stock solution and stored at -20 °C until used. The procedure of SARS-CoV-2 main protease inhibition was performed with fluorogenic assay [21-23] and minor modified following steps. Briefly, 200 nM SARS-CoV-2 main protease was pre-incubated with 10 µg/mL and 100 µg/mL of each inhibitor for 10 min at room temperature. After that, the relative inhibition was started by adding 40 µM fluorogenic substrate (Genscript USA, Inc.) to each well. Reaction was monitored at interval time 10 second for 10 min by fluorescence with excitation at 340 nm and emission at 430 nm (Infinite 200 PRO Microplate Reader, Tecan). The inhibitory effect of recombinant SARS-CoV-2 main protease was compared with Lopinavir and Ritonavir as commercial drugs for HIV-1 protease inhibitors. The IC_{50} measurements of KERRA and *A. paniculate* were determined with 2-fold serial dilutions. All assays were performed in triplicate. The percentage of relative inhibition and IC_{50} values were calculated from the initial velocity (V_0) as the equation and using a dose-response curve in GraphPad Prism 8 software.

$$\% \text{ Relative Inhibition} = \frac{[(V_0 \text{ Enzyme} - V_0 \text{ Blank}) - (V_0 \text{ Sample} - V_0 \text{ Blank})]}{V_0 \text{ Enzyme} - V_0 \text{Blank}} \times 100$$

Where: V_0 Enzyme = fluorogenic substrate with SARS-CoV-2 main protease
V_0 Blank = fluorogenic substrate without SARS-CoV-2 main protease
V_0 Sample = fluorogenic substrate with sample or commercial drugs

SARS-CoV-2 RNA dependent RNA polymerase inhibition of KERRA

KERRA and *A. paniculata* were screened for their ability to inhibit the SARS-CoV-2 RdRp enzyme using the RdRp (SAR-CoV-2) Homogeneous assay kit (BPS Bioscience: #78109) [24]. Firstly, 24 ng/µl RdRp enzyme was added to each well for the positive control and the test inhibitors. For the

blank added complete RdRp buffer. Then, 8-fold diluted RNAse inhibitor was added to each well. After that, 100 ug/mL inhibitors were added to each well of the test inhibitors while the positive control and blank were added the same solution without inhibitor. All reaction were pre-incubated for 30 minutes at room temperature. Then, 2 µl of RdRp reaction mixture, which consists of diluted digoxigenin-labeled RNA duplex and diluted biotinylated ATP, was mixed and incubated for one hour at 37 ºC. Afterward, 10 µl of diluted AlphaLISA anti-digoxigenin acceptor beads (PerkinElmer: #AL113C) was added and incubated on a shaker for 30 minutes at room temperature. Next, 10 µl of diluted streptavidin-conjugated donor beads (PerkinElmer: #6760002S) was added and incubated on a shaker for 30-60 minutes at room temperature. Finally, detected by measuring alpha-counts using a microplate reader (SPARK® multimode microplate reader, Tecan). All assays were performed in triplicate. The percentage of relative inhibition and IC_{50} values of each sample were analyzed using the GraphPad Prism 8 program.

Anti-Inflammatory Effect of KERRA in Lipopolysaccharide-Stimulated RAW264.7 Macrophages

RAW264.7 macrophage cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS), 1% Anti-Anti under a humidified atmosphere of 5% CO_2 at 37 °C. Cells were cultured in a 96-well plate at a density of 1×10^5 cells/well for overnight. After incubation, cells were treated with various concentrations of the sample at 1, 0.5, 0.25 and 0.1 mg/mL and co-treated with 1 µg/mL Lipopolysaccharide (LPS) incubated for 24 hours at 37 °C with 5% CO_2. After that, 50 µL media was mixed with 50 µL of Griess reagent and measured by reading absorbance at a wavelength of 540 nm. Data analysis was performed with [Anti-flammation activity (%) $=\frac{A_{control}-A_{test}}{A_{control}}$]. Cell viability was measured by PrestoBlue™ Cell Viability Reagent and reading absorbance at a wavelength of 570 nm.

Anti-FIPV Activity Assay

KERRA was prepared in 100% dimethyl sulfoxide to make a stock solution at 100 mg/mL. The stock solution was serially ten-fold diluted in DMEM, filtered using Whatman No.1 filter paper preparing a working solution before test. The final concentrations of KERRA ranged from 1 mg/mL. The KERRA stock was kept at -20 °C for further use.

The CRFK cells were prepared in 24-well plates, then 1 mg/mL KERRA was incubated at 37 °C in 5% CO_2 for 1 hour before infection by Introducing FIPV (0.01 MOI) into each well. Remove the virus-extract mixture and replaced by 500 µL maintenance medium. FIPV infected cells were used as positive control, while the cells treated with DMSO set as the negative control. The experiments were performed in triplicate. The inoculated cells were continuously incubated at 37 °C, 5% CO_2. The supernatants were collected for further viral RNA extraction (Omega Bio-tek, Inc.). The viral loads of each sample were determine using Quantitative real-time RT-PCR (qRT-PCR) (Bio-Rad, Singapore). The effective concentration that reached 50% decrease in viral replication was defined as the EC50 value.

Phytochemical profiles analysis using LC-MS/MS

Phytochemical profiling analysis was prepared using previous protocol with minor modifications [25]. Briefly, the extract powder (0.7 g) was mixed with 14 ml of ethanol. The suspension was incubated for 72 h at 8°C with a shaker at 100 rpm. Then, the solution was centrifuged at 14,000 × g for 30 min at 8°C. SPE with the extraction manifold system was used to clean up the clear upper solution. SPE was pre-conditioned using 20 ml of acetonitrile and equilibrated with 50 ml of water. The supernatants were loaded on the equilibrated SPE and eluted by 99% acetonitrile/water. The eluted fractions were evaporated under a vacuum using rotatory evaporation. To confirm the phytochemical

content, the experiments were conducted in 3-biological replications. The samples were reconstituted in 1000 µL methanol and diluted with 1000 µL of 0.2% formic acid/water before being subjected to LC-MS/MS analysis. Quality control of the samples was conducted for confirming the reproducibility data, the LC-MS/MS was approached to determine the total ion intensity of all identified compounds from three independent extraction batches and three technical injections.

The acquired raw MS files were processed with the Compound Discoverer 3.1 (Thermo Fisher Scientific) to identify phytochemicals. Peak identification, peak alignment, and peak feature extraction were all conducted in a positive mode on the data. The retention time (RT) and mass-to-charge ratio (*m*/*z*) of different injections were conducted according to the retention time deviation of 0.5 min and the mass deviation of 5 ppm. Then, the peak extraction was performed according to the set information and adduct information: mass deviation = 5 ppm, signal strength deviation = 30%, signal-to-noise ratio = 2, and fine isotopic pattern matching >90% of the precursor and the characteristic product ions. Additionally, the peak area was quantified. The target *m/z* ions were then integrated to predict the molecular formula, which was compared to mzCloud (https://www.mzcloud.org) and ChemSpider (http://www.chemspider.com) online databases for the identification and confirmation of the compounds. Furthermore, structural elucidation and transformations were suggested for each chromatographic peak by the FISh function. The FISh coverage score was calculated, and fragments on the MS/MS spectrum were auto-annotated with structure, molecular weight, and elemental composition. Among candidate metabolites obtained from mzCloud and ChemSpider with FISh, the highest MS/MS coverage scores were selected for annotation. The candidate metabolites with annotation and had mzClound best match score> 60 and FISh coverage> 20 or area >1e^9 AU were reported.

3. Results

Inhibition of SARS-CoV-2 main protease by KERRA

The SARS-CoV-2 main protease is one of the main enzymes plays a crucial role for viral replication and is highly conserved, it one of the most attractive therapeutic targets for SARS-CoV-2 inhibition. This protein has been a target for development of drug and virtual screening for several projects [21, 26-28]. Lopinavir and Ritonavir were first two drugs that suggest to use for treatment covid-19 patient [29-31]. Furthermore, a Fah Talai Jone (*A. paniculata*), was one of herbal medicine that was recommend using by FDA Thailand as an alternative drug [32-34]. Effect of these compounds were tested against SARS-CoV-2 main protease. The fluorogenic assay was used to measure the proteolytic activity of recombinant SARS-CoV-2 main protease [21, 35, 36]. The relative inhibition of KERRA, *A. paniculata*, Lopinavir and Ritonavir were determined with two concentrations that 10 µg/mL and 100 µg/mL. The results showed the dose-dependent inhibition of SARS-CoV-2 main protease of all tested compounds. At 10 µg/mL of each sample showed enzyme proteolytic inhibition lower than 50%, except KERRA showed 76.46% inhibition. The activity at 100 µg/mL of each sample represented inhibition more than 50%, except *A. paniculata* while KERRA was completely inhibited (Fig 1). The IC_{50} values of all compounds were determined for comparison. The IC_{50} values of Lopinavir and Ritonavir were 77.03 ± 8.51 µg/mL (122.50 µM) and 23.39 ± 2.52 µg/mL (32.44 µM), respectively (Fig 2A-2B). These values are similar with previously reported that IC_{50} values of Lopinavir was more than 40 µg/mL (>60 µM) and Ritonavir was more than 15 µg/mL (>20 µM) [37, 38] which indicating the reliable of our assay method. Furthermore, the IC_{50} values of *A. paniculata* extract were 29.94 ± 8.51 µg/mL (Fig 2C). This is the first report of IC_{50} of *A. paniculata* extract against main protease since it was previously purposed that andrographolide in *A. paniculata* extract targeted this main protease by molecular docking [32]. Interestingly, the KERRA showed IC_{50} value of 49.91 ± 1.75 ng/mL (Fig 2D) which showed 650 times lower than *A. paniculata* extract. These data indicated that KERRA is a candidate inhibitor for inhibit SARS-CoV-2 main protease.

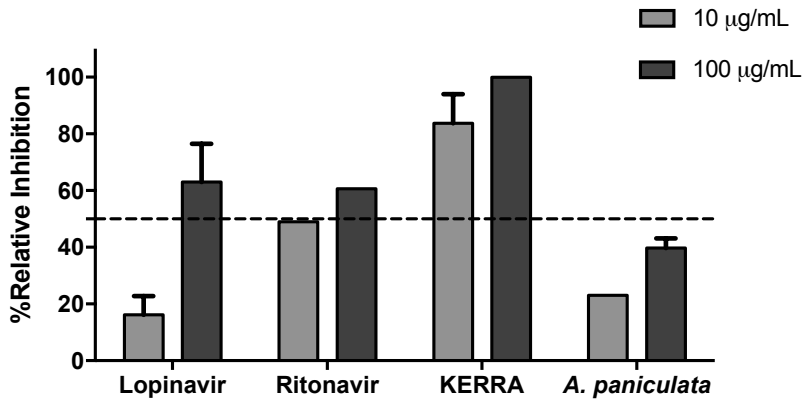

Figure 1 Relative inhibition of SARS-CoV 2 main protease with 10 and 100 μg/mL of Lopinavir, Ritonavir, KERRA and *A. paniculata*.

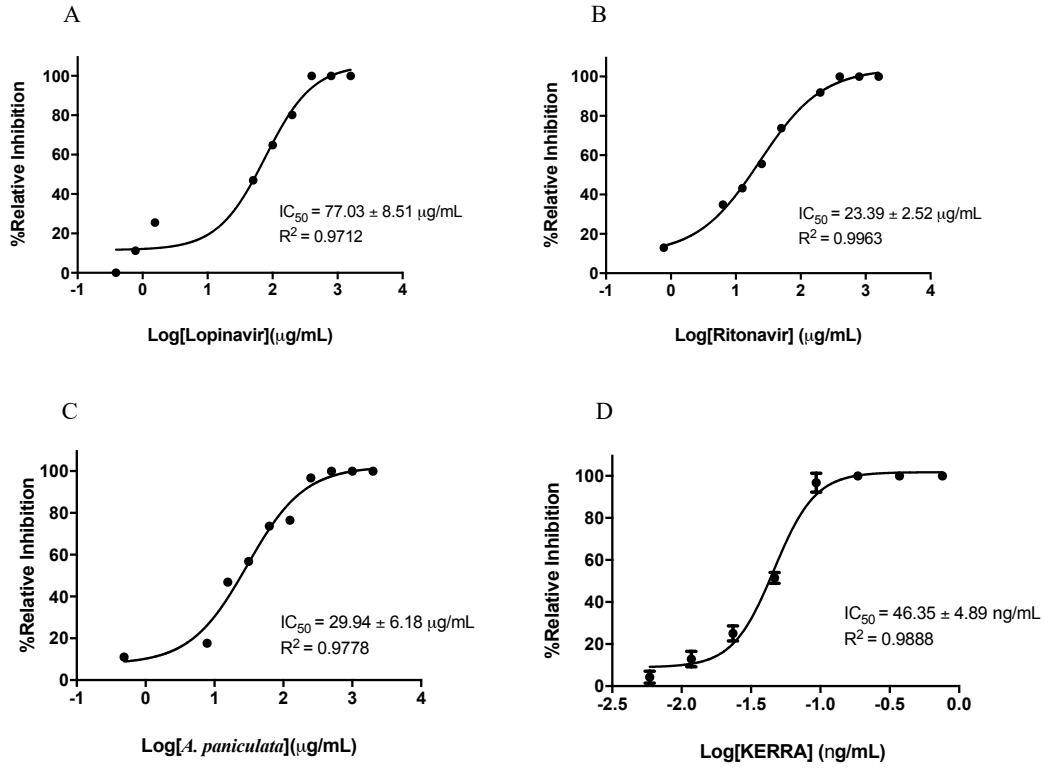

Figure 2 IC_{50} of KERRA against SARS-CoV 2 main protease with Lopinavir, Ritonavir, KERRA and *A. paniculata*.

KERRA inhibition of RdRp (SARS-CoV-2)

The other important enzyme for inhibition the virus replication is an RNA-dependent RNA polymerase (RdRp). Previously reported, Favipiravir has been effectively to inhibit the RdRp (SARS-CoV-2) activity in viral cell culture [39, 40]. However, Favipiravir is a prodrug, which are molecules with little or no pharmacological activity that are converted to the active parent drug in vivo by enzymatic or chemical reactions. Therefore, the inhibitory activity of SARS-CoV-2 RdRp assay was performed for effect of KERRA. The inhibitory activity of KERRA, *A. paniculate,* and Favipiravir were tested at 100 μg/mL using RdRp (SARS-CoV-2) Homogeneous assay kit [24]. Interestingly, the results found that KERRA showed the most effective against SARS-CoV-2 RdRp among all compounds with

57.16% (Fig 3A). This enzymatic inhibition result of Favipiravir is not surprised since it is a prodrug form. It is not fully active until metabolite by cells to active favipiravir-ribofuranosyl-5'-triphosphate (Favipiravir-RTP). The KERRA was evaluated the IC_{50} values and showed inhibitory activity against RdRp (SARS-CoV-2) with the IC_{50} value of 36.23 ± 5.23 µg/mL. While IC_{50} of RdRp (SARS-CoV-2) mostly investigated in cell base assay such as Remdesivir (2.585 ± 0.273 µM), Lycorine (1.406 ± 0.260 µM), Adefovir Dipivoxil (3.785 ± 0.866 µM), Emtricitabine (15.375 ± 3.602 µM) and Favipiravir (61.88 µM) [41-43]. Therefore, this study is the first report IC_{50} of RdRp (SARS-CoV-2) with enzyme activity assay.

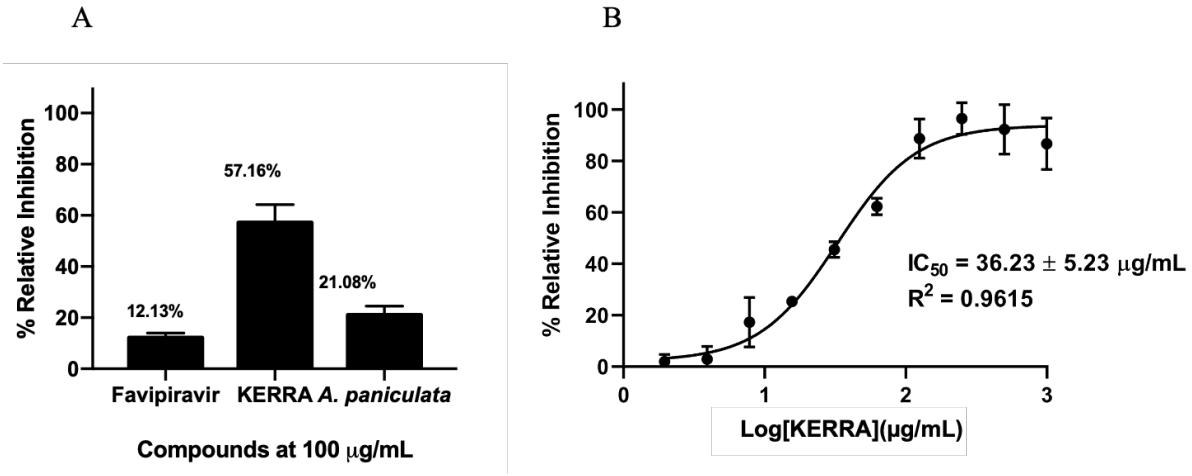

Figure 3 (A) The percentage of relative inhibition compared between Favipiravir, KERRA and *A. paniculata* at 100 µg/mL against RdRp (SARS-CoV-2) activity. (B) The IC_{50} of KERRA against RdRp (SARS-CoV-2) activity.

Effect of KERRA on Anti-inflammation activity

Firstly, observe the cytotoxicity assay, RAW 264.7 macrophage cells were treated for 24 h with various concentrations of KERRA at 1, 0.5, 0.25 and 0.1 mg/mL. The result showed that the RAW 264.7 macrophage cells treated with at 1, 0.5, 0.25 and 0.1 mg/mL KERRA found the value of cell viability at 95.51%, 96.94%, 92.65% and 91.16%, respectively. The cell viability higher than 80% indicated that KERRA not toxic to cells. (Fig 4A).

The anti-inflammation activity of macrophage cells after treated with KERRA 24 h at the concentrations 1, 0.5, 0.25 and 0.1 mg/mL were investigated the production of nitric oxide (NO) by using the Griess reagent. In this experiment, 1 µg/mL LPS and 65 µg/mL Diclofenac used for negative and positive control. The results showed that 1 mg/mL KERRA has highest anti-inflammation activity at 79.66% follow by 0.5, 0.25, 0.1 mg/mL at 59.46%, 34.99% and 7.87%, respectively (Fig 4B). This result indicated that KERRA could be anti-inflammation activity.

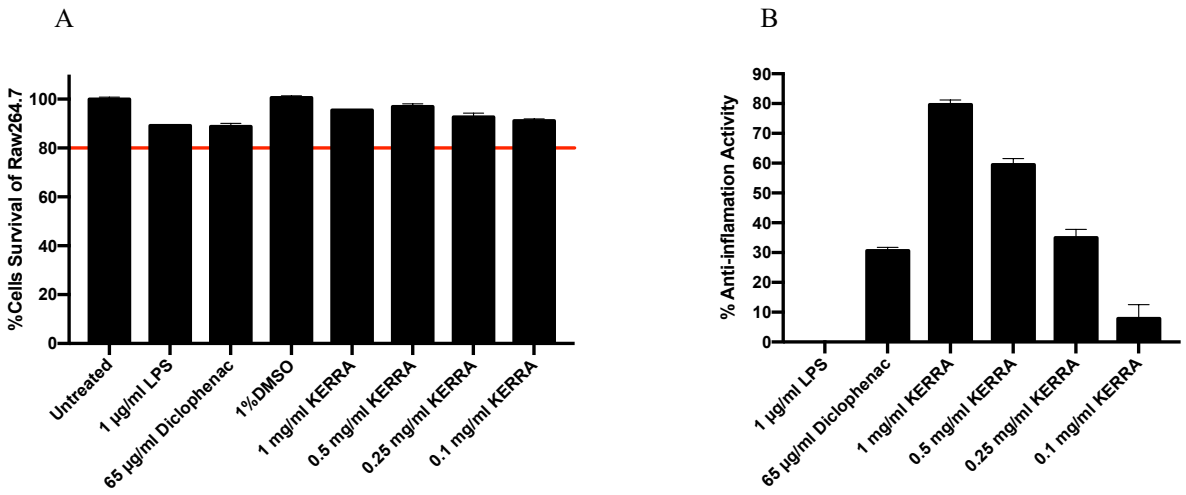

Figure 4. Cell viability and Anti-inflammation activity of RAW264.7 macrophage cells. A) Cell viability after treated with KERRA by using PrestoBlue™ Cell Viability Reagent. B) Anti-inflammation activity of RAW264.7 macrophage cells after treated 24h by various concentration of KERRA.

Anti-FIPV Activity

To test if KERRA can be effective in cellular level, the Feline coronavirus was used as a model for studying inhibition since it has similar main protease and RdRp to SAR-COV-2 and can be performed in BSL2 laboratory. The anti-FIP virus activity was performed by qRT-PCR to quantify the effect of inhibition by determining the copies number of FIP virus in the cells. The CRFK cells infected found FIP virus at 9×10^6 copies numbers after 3 days infection. The treatment with 1 mg/mL KERRA before infection was found to decrease the amount of FIP virus in the CRFK cells to 6×10^5 copies number (Fig 5). This result could be indicated that KERRA could inhibit virus propagation in the cells.

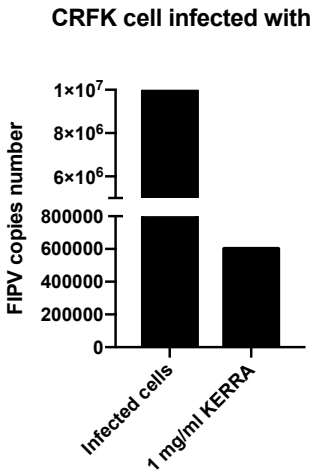

Figure 5 Copies number of CRFK cells infected with FIPV. 1 mg/mL KERRA was incubated with CRFK cells before infection.

Phytochemical profiling and metabolite qualitative analysis

The accuracy of phytochemical profiles data highly depends on the biological sampling and LC-MS/MS instrument performance. To examine whether the instrument is in good operating condition and whether the sample preparation and method applied were appropriate, the TIC of all injections is shown in Fig 6. TIC of independent batches and technical replicates revealed consistency and

reproducibility. The highest peak at approximately 6.6 minutes was shown in 9 LC-MS runs. Also, this peak exhibited good symmetry and was consistent across the three batches of the experiments (compound coefficient of variance per sample batch as 4%). Additionally, the TIC of all the detected metabolites in the 9 LC runs revealed that their profiles are extremely comparable in terms of elution time and intensity values, indicating consistency and reproducibility in batches at the overall level. The identification of metabolites by LC-MS/MS with HCD in a positive mode is well-established. A total of 415 annotated phytochemical species were identified (Supplementary 1). The Table 1 lists the top eight phytochemicals associated with the KEGG pathway.

KERERA was discovered three major components that 2-Methoxy-9H-xanthen-9-one, Isorhapontigenin, and Betaine. The presence of these annotated compounds was confirmed and validated using their respective accurate mass, experimental and calculated *m/z*, molecular formula, precursor mass error, MS2 fragmentation pattern, and well-known database matching.

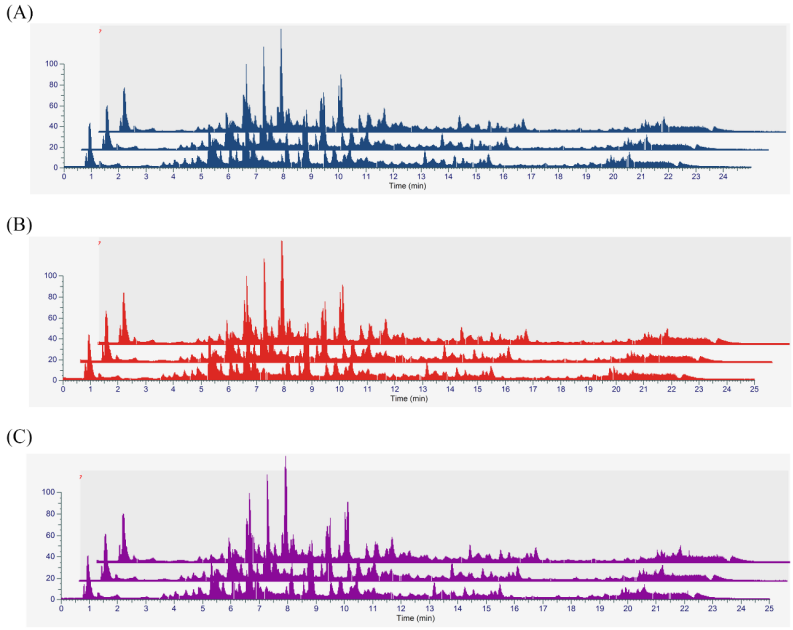

Figure 6 LC-MS/MS phytochemical profiles of KERRA. Aligned TIC profiles of the KERRA in three LC runs of 25 min. (A) KERRA batch 1, (B) KERRA batch 2 and (C) KERRA batch 3. Unit of X-axis and Y-axis are minutes and percentage intensity abundance, respectively.

Table 1. List of high abundance phytochemicals detected in KERRA.

Phytochemicals	Formular	Mass (Da)	Area (AU)×e^9	FiSh
2-Methoxy-9H-xanthen-9-one	C14 H10 O3	226.0594	7.43	13.33
Isorhapontigenin	C15 H14 O4	276.096	7.36	40.88
Betaine	C5 H11 N O2	117.0776	6.84	NA
trans-Anethole	C10 H12 O	148.0869	4.82	51.72
Eicosatetraynoic acid	C20 H24 O2	296.1734	4.05	39.51

Phytochemicals	Formular	Mass (Da)	Area (AU)×e^9	FiSh
NP-020078	C17 H28 O3	302.1847	3.28	30.15
NP-003294	C18 H16 O7	344.085	3.09	18.52
N1-(3-chlorophenyl)-2-[2-(trifluoromethyl)-4-quinolyl]hydrazine-1-carboxamide	C17 H12 Cl F3 N4 O	380.0672	2.37	43.21

4. Discussion

In vitro studies of the inhibitory activity of SARS-CoV 2 main protease of KERRA found the IC_{50} of KERRA is 49.91±1.75 ng/mL, it was lower than Ritonavir and Lopinavir which used for Covid-19. While, the inhibitory activity of RdRp (SARS-CoV-2) of KERRA compared with Favipiravir (prodrug form) at 100 µg/mL found KERRA showed the relative inhibition against RdRp (SARS-CoV-2) activity better than Favipiravir around 5-folds and represented the IC_{50} value of 36.23 ± 5.23 µg/mL. However, this study used Favipiravir in a prodrug form, it is not fully active until metabolite by cells to active favipiravir-ribofuranosyl-5'-triphosphate (Favipiravir-RTP) [44]. Therefore, the RdRp (SARS-CoV-2) inhibition of Favipiravir assay showed low inhibitory activity.

The determination of cytotoxicity to Raw264.7 cells found cell viability higher 80% at high concentration 1 mg/mL KERRA indicated that KERRA is not toxic to cells. While, 1 mg/mL KERRA is the best concentration exhibiting the highest value of anti-inflammation activity. The anti-inflammation of Diclofenac is lower than KERRA because the experiment used concentration lower than sample based on dose recommended [45]. When CRFK cells treated with KERRA showed FIP virus copies number decrease and the morphology of CRFK cells were cytopathic effected compare between untreated cells and Diclofenac. These results confirmed that KERRA against FIP virus infection.

Additional LC-MS/MS results, KERRA was discovered 2-Methoxy-9H-xanthen-9-one, which one of the major xanthones with a wide range of biological activity [46]. The second major compound is isorhapontigenin, a bioavailable dietary polyphenol, play a role for epithelial cell anti-inflammation through a corticosteroid-independent mechanism and it inhibitory effects the PI3K/Akt pathway that is insensitive to corticosteroids [47]. Moreover, isorhapontigenin have exhibited activity on SARS-COV-2 virus- infected Vero cells [48]. Next, betaine a stable and nontoxic as trimethylglycine which is widely distributed in animals, plants, and microorganisms. Betaine as an osmoprotectant and a methyl group donor display the anti-inflammatory effects in various diseases [49]. These effects were associated with protecting SAA metabolism from oxidative stress, inhibiting NF-κB and NLRP3 inflammasome activity, regulating energy metabolism, and mitigating ER stress and apoptosis [50]. Three major compounds in KERRA available in natural products and found the main function with anti-inflammatory.

5. Conclusions

KERRA is a combination of nine medicinal plants showed the inhibitory activity of SARS-CoV 2 main protease and RdRp (SARS-CoV-2) in assay with IC_{50} 49.91±1.75 ng/mL and 36.23 ± 5.23 µg/mL, respectively. KERRA displayed nontoxic to Raw264.7 cells at concentrations lower than 1 mg/mL cytotoxic activity. It was exhibited anti-inflammation activity and against FIP virus infection. Furthermore, the major compounds in KERRA were found in medicinal plants and play a bioactive that can treat many diseases the main function with anti-inflammatory. Therefore, KERRA is a candidate

for drug treatment of Covid-19 and suggesting further study with animal model and clinical trial. However, Thai medicinal plants would be most beneficial to use and could be further developed as commercial drugs.

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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Figure Captions

Figure and Legend