In Vitro Anti-SARS-CoV-2 Activities of Curcumin and Selected Phenolic Compounds

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Abstract

Since the COVID-19 pandemic in 2020, many reports have highlighted several potential anti-SARS-CoV-2 drug candidates, including phenolic compounds. Therefore, this study aimed to evaluate the anti-SARS-CoV-2 activity of nine common phenolic compounds found in plants using the in vitro cellular infection model. The anti-SARS-CoV-2 activity of curcumin, quercetin, gallic acid, catechin, rutin, kaempferol, naringenin, coumaric acid and caffeic acid were evaluated on SARS-CoV-2-infected Vero E6 cells by using a cytopathic effect (CPE)-based assay. The anti-SARS-CoV-2 activity in human lung cells, A549 expressing human ACE2 and TMPRSS2, was evaluated by the RT-qPCR technique. S1-ACE2 interaction and 3CL protease activity assays were also performed for the potent compound. Of the nine phenolic compounds, only curcumin inhibited the SARS-CoV-2 induced CPE activity (EC_{50} of 13.63 μM) in Vero E6 cells, but with a low selective index (SI) value. Interestingly, curcumin exhibited potent anti-SARS-CoV-2 activity in A549 cells with an EC_{50} of 4.57 μM and an SI value of 7.96. S1-ACE2 interaction and 3CL protease inhibitory activities of curcumin were also observed. In conclusion, curcumin showed a moderate in vitro anti-SARS-CoV-2 activity. The true potential of curcumin as an anti-SARS-CoV-2 candidate could be further evaluated in a COVID-19 animal model.

Introduction

Since the start of the COVID-19 pandemic in 2020, many studies have been initiated to find a cure for the disease including the development of vaccines and antiviral drugs targeting the essential components for virus entry and replication. To date, mRNA-based, inactivated, and viral vector-based COVID-19 vaccines have been approved by regulatory authorities and used widely to reduce the disease severity.[1–3] However, as the COVID-19 virus, SARS-CoV-2, frequently mutates its spike protein (S protein), the effectiveness of current vaccines was affected.[4,5] Due to the waning of spike-antibodies in vaccine recipients over time,[6] vaccine booster shots are needed to maintain the optimum antibody levels against different SARS-CoV-2 variants.[7]

COVID-19 drug treatments, especially the antivirals, have showed promising benefits in preventing severe disease[8–10] and were reported to remain effective against different SARS-CoV-2 variants.[11] After remdesivir, two oral anti-SARS-CoV-2 drugs have been authorized for emergency use by the US Food and Drug Administration (FDA).[8,10] These drugs are Paxlovid, a combination of nirmatrelvir tablet (3CL^Pro main protease or MPro inhibitor) with ritonavir tablet (to slow down nirmatrelvir breakdown), and molnupiravir, a small-molecule ribonucleoside prodrug of N-hydroxycytidine that induced RNA mutagenesis by viral RNA dependent RNA polymerase.[10]

Apart from the FDA approved antiviral drugs, several other potential antiviral candidates have been highlighted by many

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studies.[12–15] These candidates include the phenolic compounds commonly found in plants and dietary sources, which have been previously studied for their potential in inhibiting the essential viral infection[16–19] and replication components, such as 3CL[pro] main protease,[12,20–23] human type-2 transmembrane serine (TMPRSS2), angiotensin-converting enzyme-2 (ACE2)[24] and viral spike S1-protein.[25] In detail, several in silico studies have uncovered the potential of rutin in binding with the SARS-CoV-2 3CL[pro] main protease and helicase and the host ACE2 receptor. At higher concentration ($EC_{50} > 100\ \mu M$), rutin was reported to inhibit several viruses such as influenza H5N1, hepatitis C, enterovirus and dengue virus.[17] Quercetin was also highlighted by many in silico studies for its potential in inhibiting the SARS-CoV-2 3CL[pro] main protease. In addition, the antiviral property of quercetin against live virus was previously shown by its antiviral activity against the SARS-CoV-1 entry into Vero E6 cells.[18] Naringenin was also considered a broad antiviral compound as many studies have reported its antiviral activities against dengue, hepatitis C, hepatitis B, zika, chikungunya, herpes simplex 1 and 2, rotavirus, yellow fever, and human immunodeficiency viruses. Furthermore, in silico studies on naringenin have reported its multi-protein target potential activities against the SARS-CoV-2 spike S1-protein, TMPRSS2, 3CL[pro] main protease and the host ACE2 protein receptor.[16] An anti-inflammatory compound, curcumin, was also a compound of interest during the COVID-19 pandemic. Curcumin was found to be effective in inhibiting the wild-type, D614G and Delta SARS-CoV-2 infections in Vero E6 cells.[19,26] Other phenolic compounds, such as gallic acid, caffeic acid, catechin, kaempferol and coumaric acid, were also reported as potential anti-SARS-CoV-2 candidates by several in silico and enzyme target-based assay studies.[20,21,27]

Based on the available evidence, some of these compounds need further validation for their potential antiviral activity against live SARS-CoV-2 virus. Although some of these compounds have been previously demonstrated for their antiviral activities against live SARS-CoV-2 virus, the antiviral screenings were limited to either certain virus variants or host cells. Therefore, the aim of our study was to screen and validate the antiviral activity of nine phenolic compounds (curcumin, quercetin, gallic acid, rutin, caffeic acid, catechin, kaempferol, naringenin, and coumaric acid), together with the well-known antivirals as positive controls (andrographolide, remdesivir, and chloroquine),[14,28] against the Malaysian clinical SARS-CoV-2 virus isolates by using cytopathic effect (CPE) based assay. The potential phenolic compound candidate was further screened for its anti-SARS-CoV-2 activity against alpha, delta and omicron SARS-CoV-2 variants. In addition, the potential phenolic compound candidate was also validated against the SARS-CoV-2 infection in human lung cells and studied for its potential targets against the essential viral infection components by performing the SARS-CoV-2 spike (S1)-human ACE2 interaction and 3CL protease or main protease (Mpro) assays.

Materials and Methods

Drugs and Compounds Preparation

All drugs and compounds (Curcumin, 95% [Alfa Aesar, Great Britain], quercetin, 95% [Sigma, USA], gallic acid, 98% [Sigma, USA], rutin hydrate, 94% [Sigma, USA], caffeic acid, 98% [Sigma, USA], andrographolide, 98% [Sigma, USA], catechin 98% [Santa Cruz Biotechnology, USA], kaempferol, 98% [ChemFaces, China], naringenin, 98% [ChemFaces, China], coumaric acid, 98% [Sigma, USA] and remdesivir [Cayman Chemical, USA]) were dissolved in 100% DMSO, except chloroquine diphosphate salt (Sigma, USA), which was dissolved in water. The stocks were prepared at 20 mM concentration and kept at $-20\ °C$ prior to use.

Cell Culture

African green monkey-derived epithelial kidney cells (Vero E6; ATCC-CRL-1586) and human lung carcinoma expressing human angiotensin I-converting enzyme-2 (hACE2) and transmembrane protease serine 2 (TMPRSS2) (A549-hACE2-TMPRSS2) were purchased from the American Type Cell Culture Collection (ATCC, USA) and InvivoGen (InvivoGen, USA), respectively. Vero cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA) and 100 units/mL penicillin–streptomycin (Gibco, USA). A549-hACE2-TMPRSS2 was cultured in DMEM (Gibco, USA) supplemented with 10% FBS (Gibco, USA), 100 μg/mL normocin (Invivogen, USA), 100 units/mL penicillin–streptomycin (Gibco, USA), 0.5 μg/mL puromycin (Invivogen, USA) and 300 μg/mL hygromycin (Invivogen, USA). All cell lines were grown at 37 °C in a 5% CO_2 incubator.

Virus Propagation

The clinical SARS-CoV-2 variants, Wuhan-Wild-Type (WT), Alpha, Delta and Omicron were isolated from COVID-19 patients' diagnostic nasal swab samples by the Virology Unit, Institute for Medical Research, National Institutes of Health, Malaysia. The viral isolates were propagated in Vero E6 cells grown in 2% FBS supplemented DMEM media at 37 °C in a 5% CO_2 incubator at a biosafety level 3 (BSL-3) lab facility, Institute for Medical Research, National Institutes of Health, Malaysia. The virus culture supernatant was collected once the viral-induced cytopathic effect (CPE) was observed and kept at $-80\ °C$ prior to use. Virus isolated from human diagnostic samples is not considered as a human biological specimen by the Medical Research and Ethics Committee (MREC), Ministry of Health, Malaysia. Therefore, informed consent was not obtained from the patients.

Virus Titer Determination

The virus titer was measured by the 50% tissue culture infective dose ($TCID_{50}$) method. Vero E6 (2×10^4 cells/well) and

A549-hACE2-TMPRSS2 (1×10^4 cells/well) cells were seeded in 96 well culture plates and grown overnight. The cells were exposed to serially diluted (10-fold) viruses and incubated for 72 hours at 37 °C in a 5% CO_2 incubator. The CPE was observed microscopically. The viral-induced CPE was measured by ATP-based assay (ViralToxGlo®, Promega, USA). The luminescent signal intensity was measured by a multimode microplate reader (GloMax® Explorer, Promega, USA). The $TCID_{50}$ values were determined by dose–response curve analysis using GraphPad Prism software (Version 7.0).

Cell Viability Assay

Vero E6 (2×10^4 cells/well) and A549-hACE2-TMPRSS2 (1×10^4 cells/well) cells were seeded in 96 well plates and incubated overnight in 2% FBS supplemented DMEM media at 37 °C in a 5% CO_2 incubator. Then, the confluent cells were exposed to a serially diluted concentration of compounds or drugs and incubated for 72 hours at 37 °C in a 5% CO_2 incubator. The healthy cell control was cells exposed to media containing 0.5% DMSO and the lysis cell control was cells exposed to media containing 0.05% Triton X 100. The cell viability was measured by either MTT assay or ATP-based assay. For the MTT assay, the MTT reagent solution was added and after 4 hours of incubation, the culture supernatant was discarded. The purple formazan was solubilized by addition of 100% DMSO. The intensity of the solubilized formazan was measured by a multimode microplate reader (FLUOstar Omega, BMG LABTECH, Germany) at 570 nm wavelength.[29] For the ATP-based cell viability assay, the ATP detection reagent (CellTiter-Glo®, Promega, USA) was added and incubated at room temperature for 10 minutes. The luminescent signal intensity was measured by a multimode microplate reader (GloMax® Explorer, Promega, USA). The half-maximal cytotoxic concentration value (CC_{50}) was determined by dose–response curve analysis using GraphPad Prism software (Version 7.0).

Antiviral Screening in Monkey Kidney Cells by Cytopathic Effect (CPE) Inhibition Assay

Vero E6 cells were seeded at 2×10^4 cells/well in a 96 well plate and incubated overnight in 2% FBS supplemented DMEM media at 37 °C in a 5% CO_2 incubator. Then, either compounds or drugs, which were serially diluted with 2% FBS supplemented DMEM media, were added to the confluent cells. In the BSL3 lab, SARS-CoV-2 virus isolate was diluted with 2% FBS supplemented DMEM media and added to the confluent cells in a 96 well plate ($25TCID_{50}$ per well) containing serially diluted concentration of either compounds or drugs. The healthy cell control was cells exposed to media containing 0.5% DMSO only, and the CPE control was cells exposed to the virus only. The test plate was incubated for 72 hours at 37 °C in a 5% CO_2 incubator. The viral cytopathic effect

(CPE) was measured by ATP-based assay (ViralToxGlo®, Promega, USA). The luminescent signal intensity was measured by a multimode microplate reader (GloMax® Explorer, Promega, USA). The half-maximal effective concentration value (EC_{50}) was determined by dose–response curve analysis using GraphPad Prism software (Version 7.0).

Antiviral Screening in Human Lung Cells by RT-qPCR Technique

A549 cells expressing human ACE2 and TMPRSS2 were seeded at 1×10^4 cells/well in a 96 well plate and incubated overnight in 2% FBS supplemented DMEM media at 37 °C in a 5% CO_2 incubator. Then, either compounds or drugs, which were serially diluted with 2% FBS supplemented DMEM media, were added to the confluent cells. In the BSL3 lab, SARS-CoV-2 virus isolate was diluted with 2% FBS supplemented DMEM media and added to the confluent cells in a 96 well plate (0.03 MOI) containing serially diluted concentration of either compounds or drugs. The healthy cell control was cells exposed to media containing 0.5% DMSO only, and the infection control was cells exposed to the virus only. The test plate was incubated for 72 hours at 37 °C in a 5% CO_2 incubator. The viral RNA from the culture supernatant was automatically extracted by KingFisher Duo Prime System (ThermoFischer Scientific, USA) using a MagMax^{TM} Viral/Pathogen Nucleic Acid Isolation Kit (Applied Biosystems^{TM}, USA). The viral RNA copy numbers were quantified by using the Applied Biosystems 7500 thermocycler (Applied Biosystems, USA). Briefly, the primer set targeting the N gene region[30] was used for the viral RNA copy number quantification. The RT-qPCR condition was as recommended by the Quantitect SYBR Green RT-PCR Kit (Qiagen, Germany). Synthetic SARS-CoV-2 RNA (VR-3276SD) (ATCC, USA) with known copy number was used as a standard for this assay.

Spike S1 (SARS-CoV-2)-ACE2 Inhibitor Assay

The effect of curcumin on the spike S1-ACE2 interaction was determined by using the Spike S1 (SARS-CoV-2): ACE2 inhibitor screening colorimetric assay kit (BPS Bioscience, Canada). Briefly, curcumin (5, 10, and 20 μM) was added to the spike S1, Fc fusion, avi-tag (2 μg/mL) pre-coated 96 well plated together with the reaction mixture containing 1X immunobuffer and ACE2-Biotin (0.4 ng/μL). The positive control was the reaction mixture without the curcumin. The reaction mixture was incubated for 10 minutes at room temperature prior to 1 hour incubation with streptavidin-horse radish peroxidase (HRP). The signal intensity was measured at 450 nm by using a microplate reader ((FLUOstar Omega, BMG LABTECH,

Germany) after 5 minutes incubation with HRP substrate and stopped by 1 M sulfuric acid.

3CL Protease (SARS-CoV-2) Assay

The effect of curcumin on 3CL protease (Mpro) activity was determined by using the 3CL protease, untagged (SARS-CoV-2) assay kit (BPS Bioscience, Canada). Briefly, the curcumin (5, 10, and 20 μM) was incubated with the reaction mixture containing assay buffer and 3CL protease (0.3 ng/μL). The positive control was the reaction mixture without the curcumin. The inhibitor control was GC376 (100 μM). The reaction mixture was preincubated for 20 minutes at room temperature with slow shaking. The reaction was started by adding 3CL protease substrate (40 μM) and incubated at room temperature for 4 hours with slow shaking. The fluorescence intensity was measured at an excitation wavelength of 350 nm and an emission wavelength of 460 nm by using a microplate reader ((FLUOstar Omega, BMG LABTECH, Germany).

Data Analysis

The dose–response curve was generated by nonlinear regression (curve fit) XY analysis using (inhibitor) versus normalized response (variable slope) algorithm (GraphPad Prism software, version 7.0). The CC_{50} and EC_{50} values were used to calculate the selectivity index value (CC_{50}/EC_{50}).

Results

Cytotoxic Activity of the Phenolic Compounds

Curcumin and gallic acid showed cytotoxic activities in Vero-E6 cells with CC_{50} values of 44.83 μM and 200 μM, respectively. Besides curcumin and gallic acid, none of the tested phenolic compounds (quercetin, rutin, caffeic acid, catechin, naringenin, kaempferol, and coumaric acid) showed any cytotoxic activity, even at the highest tested concentration of 200 μM ($CC_{50} >$ 200 μM) (Figures 1 and 2). The standard antiviral compound, andrographolide, showed cytotoxic activity with a CC_{50} value of 40.19 μM (Figure 1). The standard antiviral drug, chloroquine, showed cytotoxic activity with a CC_{50} value of 54.08 μM, while remdesivir did not show any cytotoxic activity ($CC_{50} >$ 200 μM) (Figure 2). Only the non-cytotoxic concentrations of the phenolic compounds, standard antiviral compound, and drugs were chosen for the SARS-CoV-2-induced CPE-based assay.

Effect of the Phenolic Compounds on SARS-CoV-2 Induced CPE

Antiviral screening of the selected phenolic compounds against WT Wuhan SARS-CoV-2 showed that only curcumin inhibited the viral-induced CPE with an EC_{50} value of 13.63 μM (SI =

3.29) (Figures 1 and 2). The positive control compounds and drugs, such as andrographolide, chloroquine and remdesivir, inhibited the viral-induced CPE with EC_{50} values of less than 10 μM with a high selectivity index (SI ≥ 10) (Figures 1 and 2).

Antiviral Activities of Curcumin Against SARS-CoV-2 Variants of Concern (VOCs)

Curcumin also exhibited antiviral activities against SARS-CoV-2 VOCs (Figure 3). Exposure of curcumin to alpha, delta and omicron variants of SARS-CoV-2 resulted in a reduction of CPE with EC_{50} values of ∼25 μM (extrapolated data: 25.25 μM) (SI: 1.78), 20.64 μM (SI: 2.17), and ∼25 μM (extrapolated data: 25.36 μM) (SI: 1.77), respectively (Figure 3).

Antiviral Activities of Curcumin Against SARS-CoV-2 Infection in Human Lung Cell Lines

To validate the in vitro anti-SARS-CoV-2 activity of curcumin in human lung cells, curcumin was further tested in WT Wuhan SARS-CoV-2-infected A549 lung carcinoma cells expressing the human ACE2 and TMPRSS2 genes (Figure 4). The curcumin treatment showed anti-SARS-CoV-2 activity with an EC_{50} value of 4.57 μM and a SI value of 7.96 (Figure 4).

The Effect of Curcumin on SARS-CoV-2 Spike S1-ACE2 Interaction and 3CL Protease Activity

To confirm the potential targets of curcumin in preventing SARS-CoV-2 infection of the cells, the direct inhibitory effects of curcumin on the SARS-CoV-2 spike S1-ACE2 interaction and 3CL protease activity were measured. The effect of curcumin on the S1-ACE2 interaction was observed at 10 μM and 20 μM, which reduced the interaction activity to 85% and 67%, respectively, as compared to 100% activity in the positive control (no treatment) (Figure 5). An inhibitory effect of curcumin on 3CL protease activity was also observed at 10 μM and 20 μM, which reduced the activity to 81% and 60%, respectively, as compared to the total activity in the positive control without treatment (Figure 5). There was no reduction in activity for both S1-ACE2 interaction and 3CL protease by 5 μM of curcumin (Figure 5).

Discussion

The screening of anti-SARS-CoV-2 activity in our study has showed that out of the nine phenolic compounds tested, only curcumin inhibited the viral-induced CPE in Vero E6 cells against WT, alpha, delta and omicron variants. However, the SI was considered low (SI < 5) due to the cytotoxic effect of curcumin on the uninfected Vero E6 cells. Interestingly, the anti-SARS-CoV-2 activity of curcumin was found to be more potent in the human A549-hACE2-TMPRSS2 lung cells (EC_{50}: 4.57 μM, SI: 7.96). In addition, our study has also
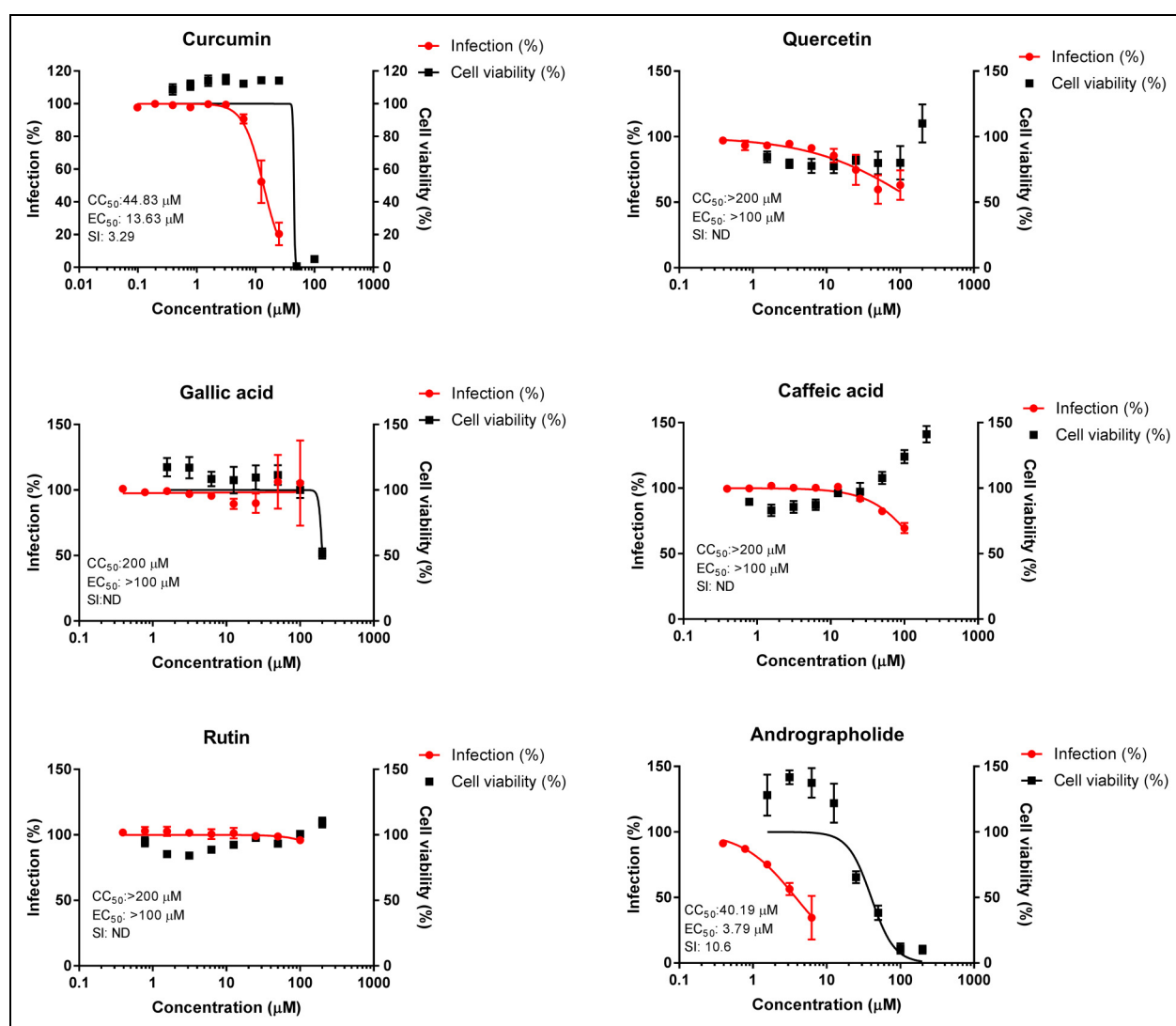
Figure 1. Anti-SARS-CoV-2 and cytotoxic activities of curcumin, quercetin, gallic acid, caffeic acid, rutin, and a standard compound, andrographolide (anti-SARS-CoV-2 plant derived compound). Antiviral and cytotoxic activities were determined by CPE inhibition assay and MTT assay, respectively after 72 hours' exposure in a 37 °C CO_2 incubator. The data were normalized to percentage of infection (left *Y*-axes: red) and percentage of cell viability (right *Y*-axes: black). Data points are mean values and bars are the standard error of mean of experiments performed in 2 to 3 independent tests.

highlighted the dual action potential of curcumin whereby the S1-ACE2 interaction and 3CL protease activities were inhibited by curcumin in a dose dependent manner.

The S protein of SARS-CoV-2 was one of the proposed target sites for curcumin by a computational study.[26] This could explain the less potent antiviral activities against alpha, delta and omicron variant infections in vero cells (EC_{50} values 20.64-25.36 μM), which could be due to viral S protein mutations that could possibly affect the binding affinity. A study conducted by Goc et al has highlighted the capability of curcumin to affect the binding and cellular entry of pseudo-typed SARS-CoV-2 virions by disrupting the interaction of spike protein with human angiotensin-converting enzyme-2 (hACE2) receptor, as well as inhibiting the essential proteases

(TMPRSS2 and cathepsin L) for entry mechanisms.[31] The multiple targets of curcumin as demonstrated by previous studies could also explain the more potent anti-SARS-CoV-2 activity of this compound in the human lung cell line used in our study, whereby the cells overexpressed human ACE2 receptor and TMPRSS2. Besides that, an in silico study conducted by Teli et al showed that curcumin was able to covalently inhibit 3CL and Mpro with good pharmacokinetic parameters and passes Lipinski's rule of five.[32]

Our in vitro findings were further supported by a study by Bormann et al,[26] which showed the anti-SARS-CoV-2 activity of curcumin in infected Vero-E6 cells, with an EC_{50} value of 7.9 μg/mL (21.44 μM). Furthermore, another study conducted by Marin-Palma et al[19] has demonstrated that curcumin
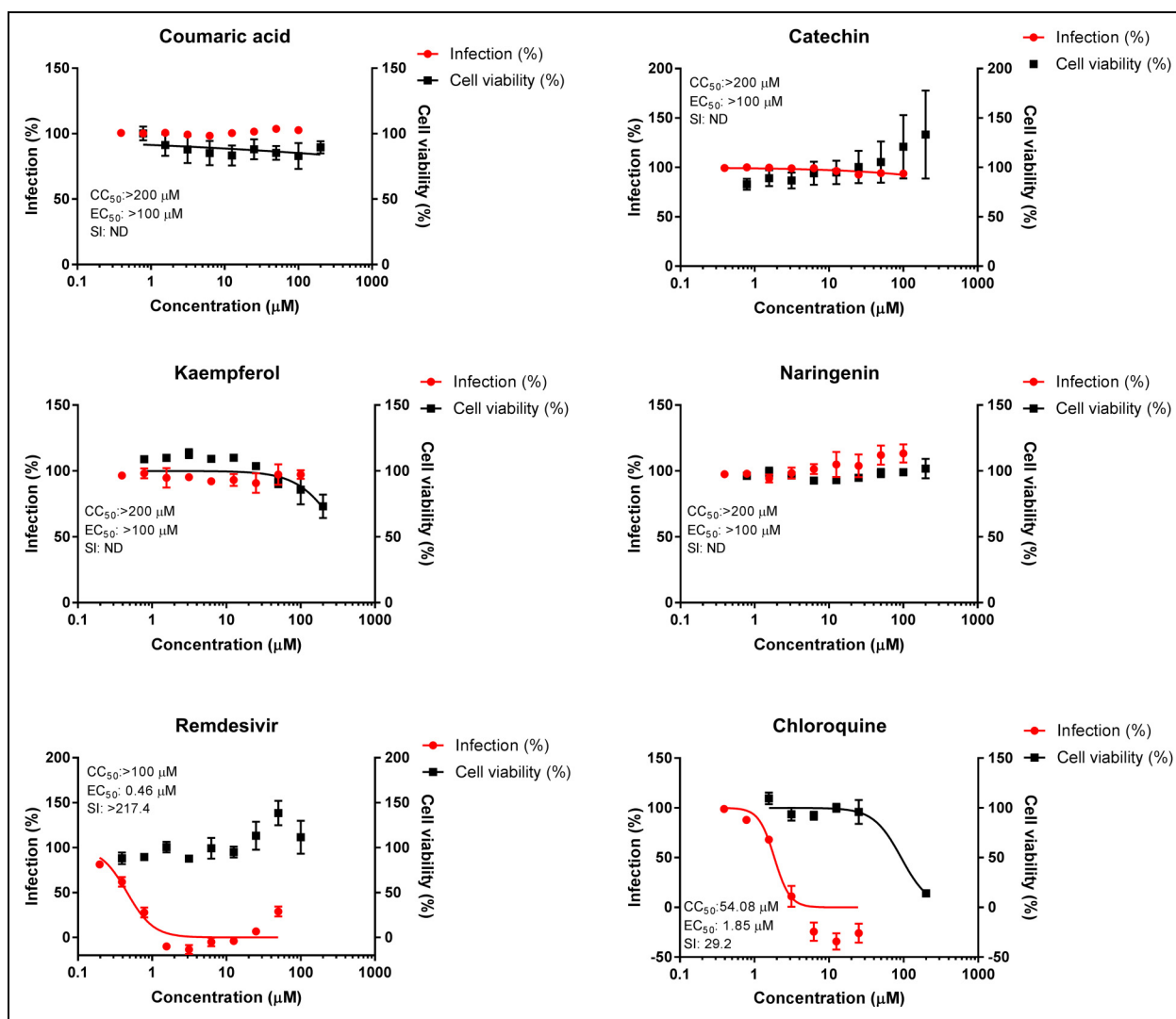
Figure 2. Anti-SARS-CoV-2 and cytotoxic activities of coumaric acid, catechin, kaempferol, naringenin, and standard antiviral drugs, remdesivir and chloroquine. Antiviral and cytotoxic activities were determined by CPE inhibition assay and MTT assay, respectively after 72 hours' exposure in a 37 °C CO_2 incubator. The data were normalized to percentage of infection (left Y-axes: red) and percentage of cell viability (right Y-axes: black). Data points are mean values and bars are the standard error of mean of experiments performed in 2 to 3 independent tests.

reduced the viral titer of the D614G mutation SARS-CoV-2 strain at different points of treatments (pre-infection, post-infection treatments and co-treatment) with EC_{50} values ranging from 3.57 to 6.03 μg/mL (equivalent to 9.69-16.37 μM) and a SI value of less than 5. In contrast to our findings, the anti-SARS-CoV-2 activity of curcumin was more potent against the Delta variant with EC_{50} values ranging from 1.14 to 1.66 μg/mL (equivalent to 3.09-4.51 μM) and SI values of 9.94 to 14.5.[19] The differences in these data could be due to the different approaches used in each study. Despite its potential as an antiviral agent, there are concerns on the poor bioavailability of curcumin in humans ($C_{max} \leq 150$ ng/mL). Therefore, the use of curcumin as a single compound treatment is not advisable and further development on curcumin formulations and drug delivery systems to

enhance its pharmacokinetic profile is warranted.[33] Indeed, several clinical trials of nano-curcumin (SinaCurcumin® Exir Nano, SinaCurcumin® Soft gel and curcumin-piperine combination) have positively affected the COVID-19 patient symptoms through its anti-inflammatory effects.[34]

Besides curcumin, other phenolic compounds, such as quercetin, rutin, kaempferol, naringenin, catechin, gallic acid, and caffeic acid, have been reported to inhibit the activity of SARS-CoV-2 3CL^{pro} main protease.[12,20–23] However, none of them inhibited the SARS-CoV-2-induced CPE in our study. This could be due to the unfavorable cellular bioavailability property, as previously showed in the study of rutin.[23] Another possible reason is the lack of viral-induced CPE inhibitory activity at the concentration of 100 μM and below as set in our study. Quercetin has been showed to significantly inhibit SARS-CoV-2 viral replication at
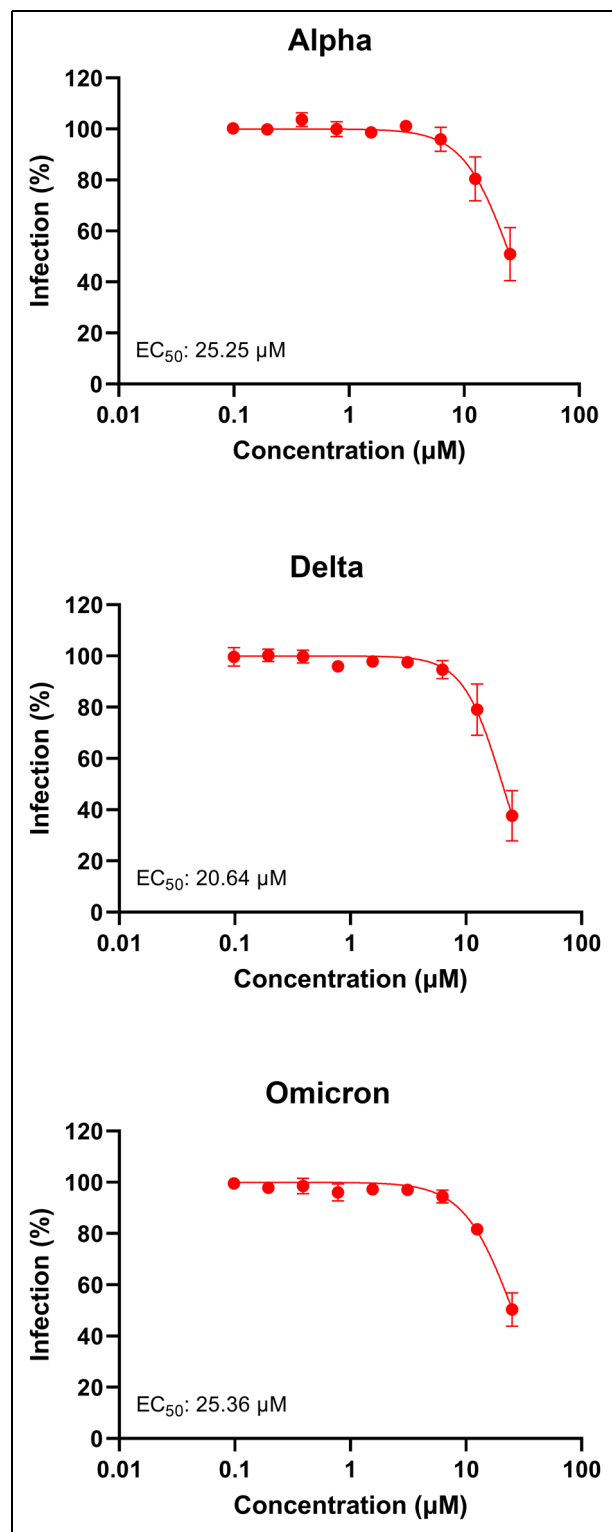
Figure 3. Anti-SARS-CoV-2 activities of curcumin against SARS-CoV-2 VOCs. Antiviral activities were determined by CPE inhibition assay after 72 hours' exposure in a 37 °C CO_2 incubator. The data were normalized to percentage of infection (*Y*-axes: red). Data points are mean values and bars are the standard error of mean of experiments performed in 3 independent tests.
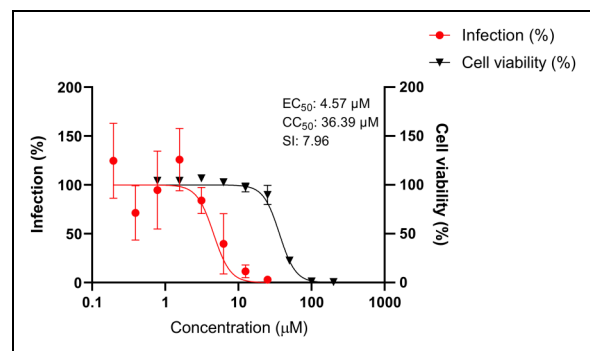

Figure 4. Anti-SARS-CoV-2 activity of curcumin against SARS-CoV-2 infection in a human lung cell line (A549-hACE2-TMPRSS2 cells). Antiviral and cytotoxic activities of curcumin were determined by RT-qPCR and ATP-based assay, respectively, after 72 hours' exposure in a 37 °C CO_2 incubator. The data were normalized to percentage of infection (*Y*-axes: red) and percentage of cell viability (right *Y*-axes: black). Data points are mean values and bars are the standard error of mean of experiments performed in 3 independent tests.
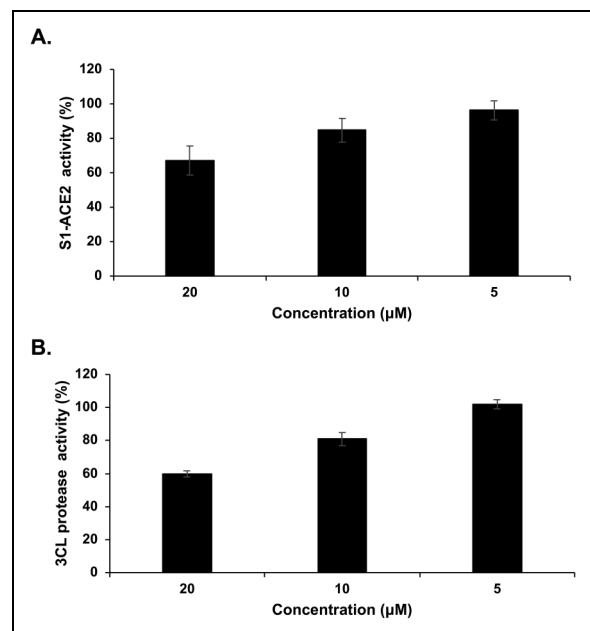

Figure 5. Inhibitory effect of curcumin on SARS-CoV-2 spike S1-ACE2 interaction and 3CL protease activity. Curcumin (5, 10, and 20 μM) was tested against the SARS-CoV-2 spike S1-ACE2 interaction (A) and 3CL protease (B) activities at room temperature for 1 and 4 hours, respectively. The data were normalized to percentage of activity. The bar charts are mean values with the standard deviation of each test in triplicates.

high concentrations of 400 to 500 μM,[22] while gallic acid has been reported to exert anti-SARS-CoV-2 activity with an IC_{50} of 108 μg/mL, which is equivalent to 635 μM.[35] In the case of naringenin, a study by Abdallah et al have showed that naringenin exerted anti-SARS-CoV-2 activity with the EC_{50} value of 28.35 μg/mL.[12] This concentration (equivalent to 104 μM) is two times higher than the reported human serum concentration

(C_{max} about 50 μM) of healthy volunteers given 600 mg of naringenin.[36]

As reported by other studies,[14,28,37] in vitro anti-SARS-CoV-2 activity was similarly showed by the positive control antivirals (andrographolide, chloroquine and remdesivir) in our study. Andrographolide is an active compound found in the medicinal plant *Andrographis paniculata* (Burm. G.) Wall. ex Nees. The compound was previously reported to exert an anti-SARS-CoV-2 activity in Vero-E6 cells and human lung cells, Calu-3, with EC_{50} values of 6.58 and 1.68 μM, respectively.[14,38] Initially, chloroquine and remdesivir were reported to inhibit SARS-CoV-2 virus replication in Vero E6 cells with EC_{50} values of 1.13 μM and 0.77 μM, respectively. However, chloroquine was later found not to inhibit the SARS-CoV-2 virus in human lung cells, Calu-3.[39] This is due to the lack of the endosomal-pH-dependent cysteine protease cathepsin L viral entry requirement in Calu-3 cells, which is a target for chloroquine.[39] Hence, this phenomenon emphasized the importance of validating the anti-SARS-CoV-2 activity of potential antiviral candidates in human lung cells, although Vero E6 cells were conveniently used for primary screening due to their measurable cytopathic effect.[13,15] Therefore, our study has also demonstrated that curcumin anti-SARS-CoV-2 activity was more potent (lower EC_{50} value) in human lung cells, A549 expressing human ACE2 and TMPRSS2 infected with the WT Wuhan SARS-CoV-2 variant. Despite the different techniques or assays used, the higher EC_{50} values for some compounds in Vero E6 cells could also be due to the increase of compound efflux activity in the cells. Inhibition of compound efflux activity by the presence of P-glycoprotein efflux inhibitor in the antiviral assay has been shown to improve the efficacy of anti-SARS-CoV-2 drugs in Vero E6 cells.[11]

Conclusions

Curcumin exhibited a moderate anti-SARS-CoV-2 activity in monkey kidney (Vero E6) and human lung (A549 expressing human ACE2 and TMPRSS2) cells. The dual targets of curcumin on S1-ACE2 interaction and 3CL protease activities were confirmed in our study. Further pre-clinical evaluation of curcumin anti-SARS-CoV-2 activity in a COVID-19 animal model is warranted. However, to make curcumin a clinically relevant candidate, the issue of bioavailability needs to be addressed.

Additional Information

The data presented in this study are available on request from the corresponding author.

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Statement of Human and Animal Rights

This article does not contain any studies with human or animal subjects.

Informed Consent

There are no human subjects in this article and informed consent is not applicable.

References

1. Zhang Y, Zeng G, Pan H, et al. Safety, tolerability, and immunogenicity of an inactivated SARS-CoV-2 vaccine in healthy adults aged 18-59 years: a randomised, double-blind, placebo-controlled, phase 1/2 clinical trial. *Lancet Infect Dis.* 2021;21(2):181-192.
2. Voysey M, Clemens SAC, Madhi SA, et al. Safety and efficacy of the ChAdOx1 nCoV-19 vaccine (AZD1222) against SARS-CoV-2: an interim analysis of four randomised controlled trials in Brazil, South Africa, and the UK. *Lancet.* 2021;397(10269): 99-111.
3. Polack FP, Thomas SJ, Kitchin N, et al. Safety and efficacy of the BNT162b2 mRNA COVID-19 vaccine. *N Engl J Med.* 2020;383(27):2603-2615.
4. Abu-Raddad LJ, Chemaitelly H, Butt AA. National Study Group for C-V. Effectiveness of the BNT162b2 COVID-19 vaccine against the B.1.1.7 and B.1.351 variants. *N Engl J Med.* 2021;385(2): 187-189.
5. Harvey WT, Carabelli AM, Jackson B, et al. SARS-CoV-2 variants, spike mutations and immune escape. *Nat Rev Microbiol.* 2021;19(7):409-424.
6. Chemaitelly H, Tang P, Hasan MR, et al. Waning of BNT162b2 vaccine protection against SARS-CoV-2 infection in Qatar. *N Engl J Med.* 2021;385(24):e83.
7. Accorsi EK, Britton A, Fleming-Dutra KE, et al. Association between 3 doses of mRNA COVID-19 vaccine and symptomatic infection caused by the SARS-CoV-2 omicron and Delta variants. *JAMA.* 2022;327(7):639-651.

8. Drozdzal S, Rosik J, Lechowicz K, et al. An update on drugs with therapeutic potential for SARS-CoV-2 (COVID-19) treatment. *Drug Resist Updat.* 2021;59:100794.

9. Fischer WA 2nd, Eron JJ Jr, Holman W, et al. A phase 2a clinical trial of molnupiravir in patients with COVID-19 shows accelerated SARS-CoV-2 RNA clearance and elimination of infectious virus. *Sci Transl Med.* 2022;14(628):eabl7430.

10. Couzin-Frankel J. Antiviral pills could change pandemic's course. *Science.* 2021;374(6569):799-800.

11. Vangeel L, Chiu W, De Jonghe S, et al. Remdesivir, Molnupiravir and Nirmatrelvir remain active against SARS-CoV-2 Omicron and other variants of concern. *Antiviral Res.* 2022;198:105252.

12. Abdallah HM, El-Halawany AM, Sirwi A, et al. Repurposing of some natural product isolates as SARS-COV-2 main protease inhibitors via in vitro cell free and cell-based antiviral assessments and molecular modeling approaches. *Pharmaceuticals (Basel).* 2021;14(3):213.

13. Chen CZ, Shinn P, Itkin Z, et al. Drug repurposing screen for compounds inhibiting the cytopathic effect of SARS-CoV-2. *Front Pharmacol.* 2020;11:592737. doi:10.3389/fphar.2020.592737

14. Sa-Ngiamsuntorn K, Suksatu A, Pewkliang Y, et al. Anti-SARS-CoV-2 activity of *Andrographis paniculata* extract and its major component andrographolide in human lung epithelial cells and cytotoxicity evaluation in major organ cell representatives. *J Nat Prod.* 2021;84(4):1261-1270.

15. Zhang ZR, Zhang YN, Li XD, et al. A cell-based large-scale screening of natural compounds for inhibitors of SARS-CoV-2. *Signal Transduct Target Ther.* 2020;5(1):218.

16. Agrawal PK, Agrawal C, Blunden G. Naringenin as a possible candidate against SARS-CoV-2 infection and in the pathogenesis of COVID-19. *Nat Prod Commun.* 2021;16(12):1934578X-211066723. doi:10.1177/1934578X211066723

17. Agrawal PK, Agrawal C, Blunden G. Rutin: a potential antiviral for repurposing as a SARS-CoV-2 main protease (mpro) inhibitor. *Nat Prod Commun.* 2021;16(4):1934578X-21991723. doi:10.1177/1934578X21991723

18. Agrawal PK, Agrawal C, Blunden G. Quercetin: antiviral significance and possible COVID-19 integrative considerations. *Nat Prod Commun.* 2020;15(12):1934578X-20976293. doi:10.1177/1934578X20976293

19. Marin-Palma D, Tabares-Guevara JH, Zapata-Cardona MI, et al. Curcumin inhibits in vitro SARS-CoV-2 infection in Vero E6 cells through multiple antiviral mechanisms. *Molecules.* 2021;26(22):6900.

20. Bahun M, Jukic M, Oblak D, et al. Inhibition of the SARS-CoV-2 3CL(pro) main protease by plant polyphenols. *Food Chem.* 2022;373(Pt B):131594.

21. Nguyen TTH, Jung JH, Kim MK, et al. The inhibitory effects of plant derivate polyphenols on the main protease of SARS coronavirus 2 and their structure-activity relationship. *Molecules.* 2021;26(7):1924.

22. Mangiavacchi F, Botwina P, Menichetti E, et al. Seleno-functionalization of quercetin improves the non-covalent inhibition of M(pro) and its antiviral activity in cells against SARS-CoV-2. *Int J Mol Sci.* 2021;22(13):7048.

23. Sancineto L, Ostacolo C, Ortega-Alarcon D, et al. L-Arginine improves solubility and Anti- SARS-CoV-2 Mpro activity of rutin but not the antiviral activity in cells. *Molecules.* 2021;26(19):6062.

24. Alzaabi MM, Hamdy R, Ashmawy NS, et al. Flavonoids are promising safe therapy against COVID-19. *Phytochem Rev.* 2022;21(1):291-312.

25. Jena AB, Kanungo N, Nayak V, Chainy GBN, Dandapat J. Catechin and curcumin interact with S protein of SARS-CoV2 and ACE2 of human cell membrane: insights from computational studies. *Sci Rep.* 2021;11(1):2043.

26. Bormann M, Alt M, Schipper L, et al. Turmeric root and its bioactive ingredient curcumin effectively neutralize SARS-CoV-2 in vitro. *Viruses.* 2021;13(10):1914.

27. Elfiky AA. Natural products may interfere with SARS-CoV-2 attachment to the host cell. *J Biomol Struct Dyn.* 2021;39(9):3194-3203.

28. Wang M, Cao R, Zhang L, et al. Remdesivir and chloroquine effectively inhibit the recently emerged novel coronavirus (2019-nCoV) in vitro. *Cell Res.* 2020;30(3):269-271.

29. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods.* 1983;65(1-2):55-63.

30. Gordon DE, Jang GM, Bouhaddou M, et al. A SARS-CoV-2 protein interaction map reveals targets for drug repurposing. *Nature.* 2020;583(7816):459-468.

31. Goc A, Sumera W, Rath M, Niedzwiecki A. Phenolic compounds disrupt spike-mediated receptor-binding and entry of SARS-CoV-2 pseudo-virions. *PLoS One.* 2021;16(6):e0253489.

32. Teli DM, Shah MB, Chhabria MT. In silico screening of natural compounds as potential inhibitors of SARS-CoV-2 main protease and spike RBD: targets for COVID-19. *Front Mol Biosci.* 2020;7:599079. doi:10.3389/fmolb.2020.599079

33. Stohs SJ, Ji J, Bucci LR, Preuss HG. A comparative pharmacokinetic assessment of a novel highly bioavailable curcumin formulation with 95% curcumin: a randomized, double-blind, crossover study. *J Am Coll Nutr.* 2018;37(1):51-59.

34. Vahedian-Azimi A, Abbasifard M, Rahimi-Bashar F, et al. Effectiveness of curcumin on outcomes of hospitalized COVID-19 patients: a systematic review of clinical trials. *Nutrients.* 2022;14(2):256.

35. El Gizawy HA, Boshra SA, Mostafa A, et al. *Pimenta dioica* (L.) Merr. bioactive constituents exert anti-SARS-CoV-2 and anti-inflammatory activities: molecular docking and dynamics, in vitro, and in vivo studies. *Molecules.* 2021;26(19):5844.

36. Rebello CJ, Beyl RA, Lertora JJL, et al. Safety and pharmacokinetics of naringenin: a randomized, controlled, single-ascending-dose clinical trial. *Diabetes Obes Metab.* 2020;22(1):91-98.

37. Choy KT, Wong AY, Kaewpreedee P, et al. Remdesivir, lopinavir, emetine, and homoharringtonine inhibit SARS-CoV-2 replication in vitro. *Antiviral Res.* 2020;178:104786.

38. Kanjanasirirat P, Suksatu A, Manopwisedjaroen S, et al. High-content screening of Thai medicinal plants reveals *Boesenbergia rotunda* extract and its component Panduratin A as anti-SARS-CoV-2 agents. *Sci Rep.* 2020;10(1):19963.

39. Hoffmann M, Mosbauer K, Hofmann-Winkler H, et al. Chloroquine does not inhibit infection of human lung cells with SARS-CoV-2. *Nature.* 2020;585(7826):588-590.