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Scutellaria baicalensis extract and baicalein inhibit replication of SARS-CoV-2 and its 3C-like protease *in vitro*

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ABSTRACT

COVID-19 has become a global pandemic and there is an urgent call for developing drugs against the virus (SARS-CoV-2). The 3C-like protease (3CL^{pro}) of SARS-CoV-2 is a preferred target for broad spectrum anti-coronavirus drug discovery. We studied the anti-SARS-CoV-2 activity of *S. baicalensis* and its ingredients. We found that the ethanol extract of *S. baicalensis* and its major component, baicalein, inhibit SARS-CoV-2 3CL^{pro} activity *in vitro* with IC$_{50}$'s of 8.52 µg/ml and 0.39 µM, respectively. Both of them inhibit the replication of SARS-CoV-2 in Vero cells with EC$_{50}$'s of 0.74 µg/ml and 2.9 µM, respectively. While baicalein is mainly active at the viral post-entry stage, the ethanol extract also inhibits viral entry. We further identified four baicalein analogues from other herbs that inhibit SARS-CoV-2 3CL^{pro} activity at µM concentration. All the active compounds and the *S. baicalensis* extract also inhibit the SARS-CoV 3CL^{pro}, demonstrating their potential as broad-spectrum anti-coronavirus drugs.

1. Introduction

Coronaviruses (CoVs) are single stranded positive-sense RNA viruses that cause severe infections in respiratory, hepatic, and various organs in humans and many other animals[1,2]. Within the 20 years of the twenty-first century, there are already three outbreaks of CoV-causing global epidemics, including SARS, MERS, and COVID-19. The newly emerged CoV infectious disease (COVID-19) has become a worldwide pandemic that needs to be controlled. There is an urgent call for drug and vaccine research and development against COVID-19.

COVID-19 was confirmed to be caused by a new coronavirus (SARS-CoV-2), whose genome was sequenced in early January 2020[3,4]. The genomic sequence of SARS-CoV-2 is highly similar to that of SARS-CoV with about 79.6% sequence identity[5] and remains stable up to now[6]. However, the sequence identities vary significantly for different viral proteins[5]. For instance, the spike proteins (S-protein) in CoVs are diverse in sequences and even in the host receptors that bind due to the rapid mutations and recombination[7]. Although it has been confirmed that both SARS-CoV and SARS-CoV-2 use ACE2 as receptor and occupy the same binding site, their binding affinities to ACE2 vary due to subtle interface sequence variations[8]. On the contrary, the 3C-like proteases (3CL^{pro}) in CoVs are highly conserved. The 3CL^{pro} in SARS-CoV and SARS-CoV-2 share a sequence identity of 96.1%, making it an ideal target for broad spectrum anti-CoV therapy. Although many inhibitors have been reported for SARS-CoV and MERS-CoV 3CL^{pro} [9–12], unfortunately none of them has entered clinical trial.

Inspired by the previous studies, several covalent inhibitors were rationally designed and experimentally shown to inhibit the 3CL^{pro} activity and viral replication of SARS-CoV-2, with some of the complex crystal structures solved[13–15]. In addition, a number of clinically used HIV and HCV protease inhibitors have been proposed as possible cure for COVID-19[16] and some of them are now processed to clinically trials[17]. Most of the reported SARS-CoV-2 3CL^{pro} inhibitors covalently target the active site cysteine. Highly potent SARS-CoV-2 3CL^{pro} inhibitors with diverse chemical structures and mode of action need to explored.

Traditional Chinese medicine (TCM) herbs and formulae have long been used in treating viral diseases. Some of them have been clinically tested to treat COVID-19[18]. Scutellariae radix (Huangqin in Chinese), the root of *Scutellaria baicalensis* Georgi, has been reported for widely used in TCM for heat clearing, fire purging, detoxification, and haemostasis. Huangqin is officially recorded in Chinese Pharmacopoeia (2015 Edition)[19] and European Pharmacopoeia (10th Edition)[20]. Its anti-tumour, antiviral, anti-microbial, and anti-inflammatory activities have been reported[21]. Remarkably, the extracts of *S. baicalensis* have exhibited broad spectrum anti-viral activities, including ZIKA[22], H1N1[23], HIV[24], and DENV[25]. In addition, a multicentre, retrospective analysis demonstrated that *S. baicalensis* exhibits more potent antiviral effects and higher clinical efficacy than ribavirin for the treatment of hand, foot, and mouth disease[26]. Several *S. baicalensis* derived mixtures or pure compounds have been approved as antiviral drugs, such as Baicalein capsule (to treat hepatitis) and

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Huangqin tablet (to treat upper respiratory infection) in China. Most of the *S. baicalensis* ingredients are flavonoids[27]. Flavonoids from other plants were also reported to mildly inhibit SARS and MERS-CoV 3CL[pro] [28,29]. Here, we studied the anti-SARS-CoV-2 activity of *S. baicalensis* and its ingredients. We found that the ethanol extract of *S. baicalensis* inhibits SARS-CoV-2 3CL[pro] activity and the most active ingredient baicalein exhibits an IC$_{50}$ of 0.39 μM. Both the ethanol extract of *S. baicalensis* and baicalein effectively inhibit the replication of SARS-CoV-2 in cell assay. The anti-SARS-CoV-2 3CL[pro] activity and antiviral activity were also reported by a recent publication[30]. We further studied the structure and activity relationship of baicalein analogues and identified four new active compounds from other herbs that inhibit SARS-CoV-2 3CL[pro] activity at μM concentration.

2. Materials and methods

S. baicalensis were purchased from Tong Ren Tang Technologies Co. Ltd. (Beijing, China). Baicalein and compounds not listed below were from J&K Scientific (Beijing, China). 5,6-Dihydroxyflavone was purchased from Alfa Aesar (Haverhill, MA). 6,7-Dihydroxyflavone was synthesised by Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China). Myricetin, quercetagetin, and herbacetin were purchased from MCE (Shanghai, China). Dihydromyricetin and myricetin were purchased from Targetmol (Boston, MA).

2.1. Construction of plasmid SARS-CoV-2 pET 3CL-21x, protein expression, and purification

The DNA of SARS-CoV-2 3CL[pro] (referred to GenBank, accession number MN908947) was synthesised (Hienzyme Biotech, Changsha, China) and amplified by PCR using primers n3CLP-Nhe (5′-CATGGCTAGCG GTTTTAGAAAAATGGCATTCCC-3′) and n3CLP-Xho (5′-CACTCTCGA GTTGGAAAGTAACACCTGAGC-3′). The PCR product was digested with *Nhe* I/*Xho* I and cloned into the pET 21a DNA as reported previously[31]. The resulting SARS-CoV-2 pET 3CL-21x plasmid encodes a 35,064 Da SARS-CoV-2 3CL[pro] with a C-terminal 6xHis-tag. The SARS-CoV-2 pET 3CL-21x plasmid was further transformed to *E. coli* BL21<DE3> for protein expression as reported[31]. The recombinant protein was purified through a nickel-nitrilotriacetic acid column (GE Healthcare, Chicago, IL) and subsequently loaded on a gel filtration column Sephacryl S-200 HR (GE Healthcare, Chicago, IL) for further purification as previously described[32].

2.2. Preparation of the ethanol extract of S. baicalensis

After minced, 5 g *S. baicalensis* was extracted with 100 ml of 70% ethanol in a 300 ml flask after sonication for 1 h at 25 °C. The solvent was filtrated and completely removed under reduced pressure at room temperature. The residue was freeze-dried overnight. The dried herbal residue was then dissolved completely to obtain a 100 mg/ml stock solution in DMSO which was stored at −20 °C before use.

2.3. Enzyme inhibition assay

A colorimetric substrate Thr-Ser-Ala-Val-Leu-Gln-pNA (GL Biochemistry Ltd., Shanghai, China) and assay buffer (40 mM PBS, 100 mM NaCl, 1 mM EDTA, 0.1% Triton 100, pH 7.3) was used for the inhibition assay. Stock solutions of the inhibitors were prepared with 100% DMSO. The 100 μl reaction systems in assay buffer contain 0.5 μM protease and 5% DMSO or inhibitor to the final concentration. First, SARS-CoV-2 3CL[pro] was diluted with assay buffer to the desired concentration. Five microlitres DMSO or inhibitor at various concentrations was pre-incubated with 85 μl diluted SARS-CoV-2 3CL[pro] for 30 min at room temperature. Then 10 μl 2 mM substrate Thr-Ser-Ala-Val-Leu-Gln-pNA (dissolved in water) was added into the above system to the final concentration of 200 μM to initiate the reaction. Increase in absorbance at 390 nm was recorded for 20 min at interval of 30 s with a kinetics mode program using a plate reader (Synergy, Biotek, Winooski, VT). The percent of inhibition was calculated by V_i/V_0, where V_0 and V_i represent the mean reaction rate of the enzyme incubated with DMSO or compounds. IC$_{50}$ was fitted with Hill1 function.

2.4. Molecular docking

The structure of SARS-CoV-2 3CL[pro] (PDB ID 6LU7)[13] and *S. baicalensis* components were prepared using Protein Preparation Wizard and LigPrep module, respectively. Then, the binding site was defined as a $20 \times 20 \times 20$ Å^3 cubic box centred to the centroid of C145. After that, molecular docking was performed using Glide. Extra precision (XP) and flexible ligand sampling were adopted. Post-docking minimisation was performed to further refine the docking results. All the above-mentioned modules were implemented in Schrödinger version 2015-4 (Schrödinger Software Suite, L.L.C., New York, NY, 2015).

2.5. Cell culture and virus

Vero cell line (ATCC, CCL-81) was cultured at 37 °C in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY) supplemented with 10% foetal bovine serum (FBS, Gibco, Grand Island, NY) in the atmosphere with 5% CO$_2$. Cells were digested with 0.25% trypsin and uniformly seeded in 96-well plates with a density of 2×10^4 cells/well prior infection or drug feeding. The virus (C-Tan-nCoV Wuhan strain 01) used is a SARS-CoV-2 clinically isolated virus strain. These viruses were propagated in Vero cells.

2.6. Antiviral activity assay

The cytotoxicity of *S. baicalensis* extract and baicalein on Vero cells were determined by CCK8 assays (DOJINDO, Kumamoto, Japan). We then evaluated the antiviral efficiency of *S. baicalensis* extract and baicalein against SARS-CoV-2 (C-Tan-nCoV Wuhan strain 01) virus *in vitro*. Cells were seeded into 96-well plates at a density of 2×10^4 cells/well and then grown for 24 h. For the full-time inhibition test, cells were pre-treated with indicated concentrations of *S. baicalensis* extract or baicalein for 1 h, and the virus (MOI of 0.01, 200 PFU/well) was subsequently added to allow infection for 2 h at 37 °C. Virus input was washed with DMEM and then the cells were treated with medium containing the drugs at various concentrations for 48 h. For the entry inhibition test, after viral infection for 2 h, both the virus input and the drugs were washed out with DMEM and the cells were treated with fresh medium without drugs for 48 h. For the post-entry inhibition test, the drugs were added only after 2 h of viral infection and maintained until the end of the experiment. The supernatant was collected and the RNA was extracted and analysed by relative quantification using RT-PCR as in the previous study[33].

2.7. RNA extraction and RT-qPCR

Viral RNA was extracted from 100 μl supernatant of infected cells using the automated nucleic acid extraction system (TIANLONG, Hangzhou, China), following the manufacturer's recommendations. SARS-CoV-2 virus detection was performed using the One Step PrimeScript RT-PCR kit (TaKaRa, Shiga, Japan) on the LightCycler 480 Real-Time PCR system (Roche, Rotkreuz, Switzerland). ORF 1ab was amplified from cDNA and cloned into MS2-nCoV-ORF1ab and used as the plasmid standard after its identity was confirmed by sequencing. A standard curve was generated by determination of copy numbers from serially dilutions (10^3–10^9 copies) of plasmid. The following primers used for quantitative PCR were 1ab-F: 5′-AGAAGATTGGTTAGATGATGATAGT-3′; 1ab-R: 5′-TTCCATCTCTAAT TGAGGTTGAACC-3′; and probe 5′-FAM-TCCTCACTGCCGTCTTGTTG ACCA-BHQ1-3′. The individual EC_{50} values were calculated by the Origin 2018 software.

3. Results and discussion

3.1. The ethanol extract of S. baicalensis strongly inhibits SARS-CoV-2 3CL^{pro}

We first prepared the 70% ethanol extract of *S. baicalensis* and tested its inhibitory activity against SARS-CoV-2 3CL^{pro}. We expressed SARS-CoV-2 3CL^{pro} and performed activity assay using a peptide substrate (Thr-Ser-Ala-Val-Leu-Gln-pNA) according to the published procedure of SARS-CoV 3CL^{pro} assay[10,34]. As shown in Figure 1(A), the crude extract exhibits significant inhibitory effect with an IC_{50} of 8.5 μg/ml, suggesting that *S. baicalensis* contains candidate inhibitory ingredients against SARS-CoV-2 3CL^{pro}.

3.2. Baicalein is the major active ingredient in S. baicalensis that inhibits SARS-CoV-2 3CL^{pro}

We then tested the inhibitory activity of four major ingredients from *S. baicalensis*: baicalein, baicalin, wogonin, and wogonoside *in vitro*. Baicalein showed the most potent anti-SARS-CoV-2 3CL^{pro} activity with an IC_{50} of 0.39 μM (Figure 1(B) and Table 1). Baicalin inhibited SARS-CoV-2 3CL^{pro} activity for about 41% at 50 μM with an IC_{50} of 83.4 μM (Figure S1), while wogonin and wogonoside were not active at this concentration. The ratio of baicalein in the crude extract determined by HPLC was 2.07% (Table S2) which is in consistent with previous reports[35]. To eliminate possible non-specific effect, the inhibitory activity of ethanol extract and all the compounds were measured in the buffer with 0.1% Triton X-100.

3.3. S. baicalensis extract and baicalein inhibit the replication of SARS-CoV-2 in Vero cells

We further tested the antiviral activity of *S. baicalensis* ethanol extract and baicalein against SARS-CoV-2 using RT-qPCR. Vero cells were pre-treated with the extract or baicalein for 1 h, followed by virus infection for 2 h. Virus input was then washed out and the cells were treated with medium containing the extract or baicalein. Viral RNA was extracted from the supernatant of the infected cells and quantified by RT-PCR. The *S. baicalensis* ethanol extract significantly reduced the growth of the virus with an EC_{50} of about 0.74 μg/ml with low cytotoxicity (SI > 675, Figure 2(A,C)). Baicalein inhibits the replication of SARS-CoV2 with an EC_{50} of 2.9 μM and SI > 172 (Figure 2(B,D)).

As the synthesis and function of SARS-CoV-2 3CL^{pro} take place inside the host cell, its inhibitors should be effective mainly at the post-entry stage. Since the above data were obtained by full-time incubation, we further tested the inhibition activity of baicalein and *S. baicalensis* extract at the entry or post-entry stage (see *Materials and Methods* section in Supporting Information for details). Both baicalein and the extract are active at post-entry stage, while the extract also shows noticeable inhibition activity at the viral entry

Table 1. The SARS-CoV-2 3CL^{pro} inhibition activity of four major flavones derived from *S. baicalensis*.
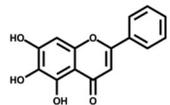
Compound	Chemical structure	IC_{50} (μM)	% Inhibition at 50 μM
Baicalein		0.39 ± 0.12	–
Baicalin		83.4 ± 0.9	41.5 ± 0.6
Wogonin		–	6.1 ± 0.8
Wogonoside		–	8.5 ± 3.3
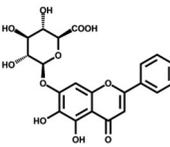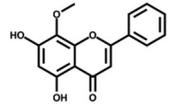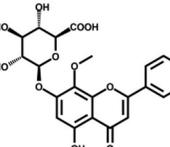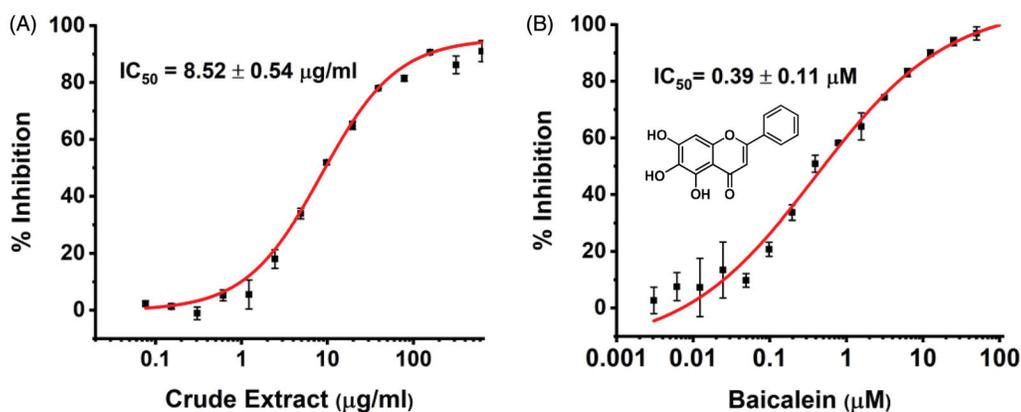
Figure 1. The *in vitro* anti-SARS-CoV-2 3CL^{pro} activity of *S. baicalensis* ethanol extract (A) and baicalein (B).

(A) IC_{50} = 8.52 ± 0.54 μg/ml

(B) IC_{50} = 0.39 ± 0.11 μM
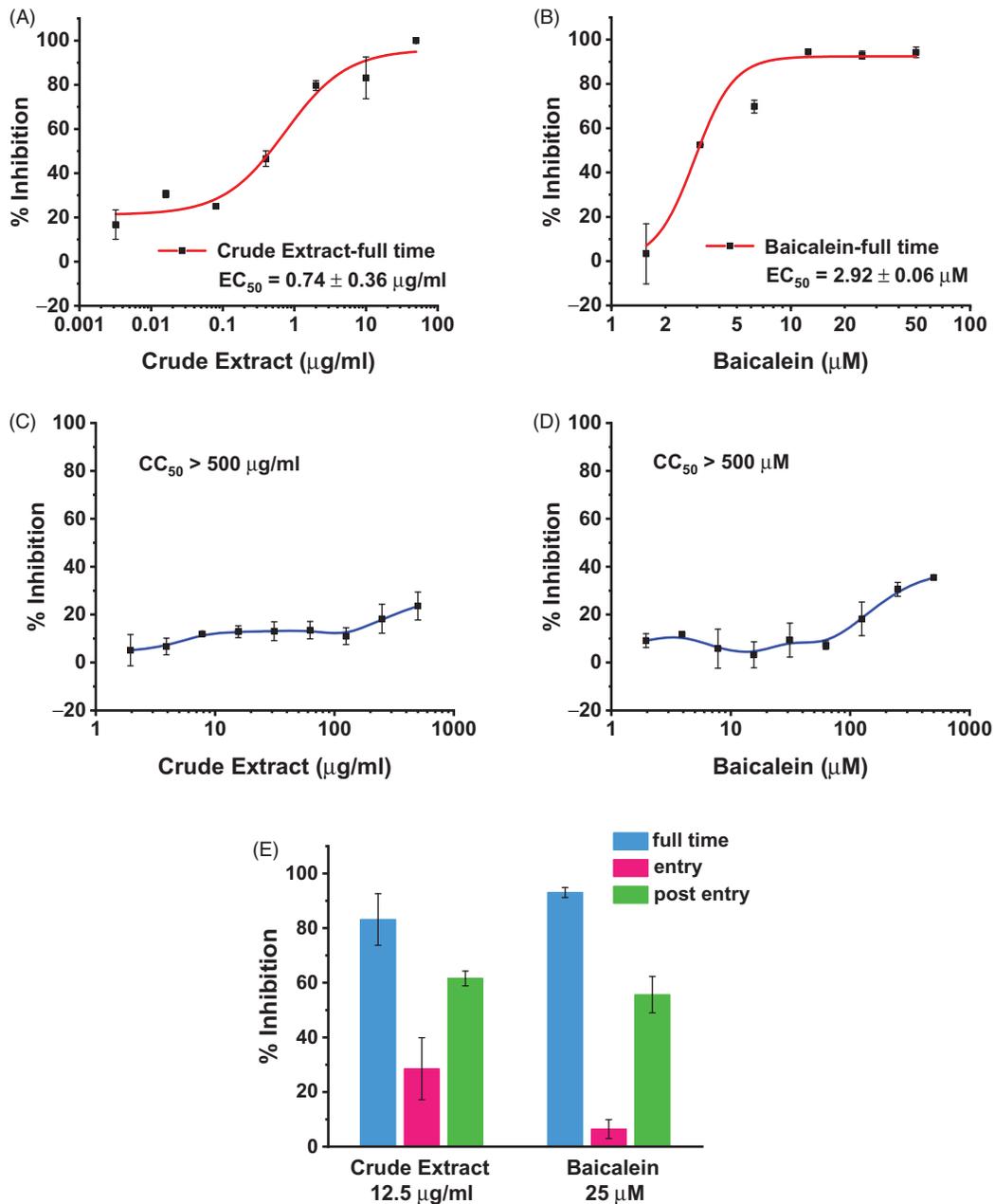
Figure 2. The antiviral activity and cytotoxicity of *S. baicalensis* extract (A, C) and baicalein (B, D) against SARS-CoV-2 in Vero cells. The effects of *S. baicalensis* extract and baicalein in different viral infection periods were also detected (E).
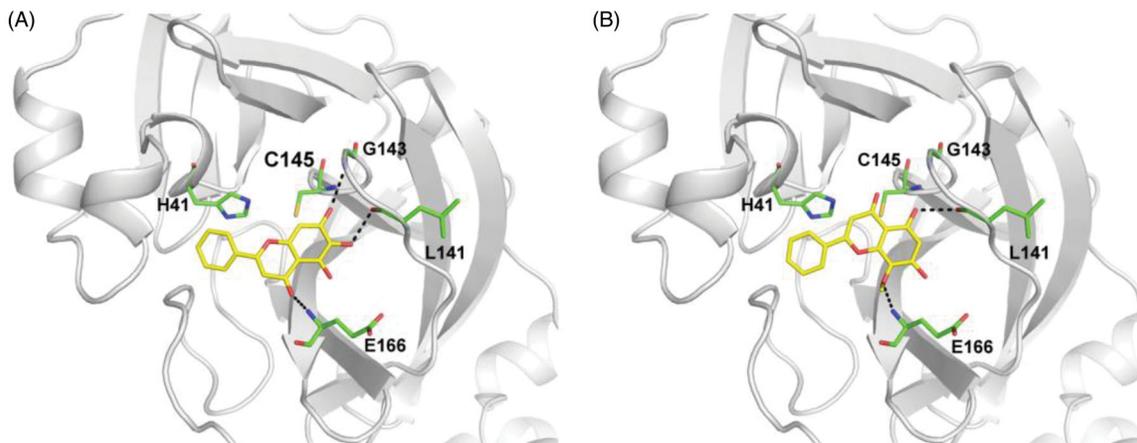
Figure 3. The interactions between SARS-CoV-2 3CL^pro and *S. baicalensis* ingredients baicalein (A) and wogonin (B) in the docking models. The overall structure and key residues of SARS-CoV-2 3CL^pro are shown as grey cartoon and green sticks, respectively. *S. baicalensis* ingredients are displayed as yellow sticks.

stage. The high activity and multiple action stages of *S. baicalensis* crude extract in the antiviral assay implies that it may also interact with other viral or host targets in addition to SARS-CoV-2 3CL^{pro} inhibition, which can be further explored in the future.

3.4. Baicalein binds SARS-CoV-2 3CL^{pro} to inhibit its activity

To confirm that baicalein and the crude extract inhibit viral replication by directly targeting SARS-CoV-2 3CL^{pro} in cells, we tested their anti-SARS-CoV-2 3CL^{pro} activity under complex cell environment. As the expression of SARS-CoV-2 3CL^{pro} is toxic to mammalian cells[36], we used the mixture of HEK293T cell lysate and purified SARS-CoV-2 3CL^{pro} for the test. Both baicalein and the crude extract showed inhibition activity under this condition (Figure S2).

We performed molecular docking to understand the inhibitory activity of *S. baicalensis* ingredients. The docking scores are listed in Table S1. In the docking model, baicalein binds well in the

Table 2. The anti-SARS-CoV-2 3CL^{pro} activity of baicalein analogue flavonoids.

Compound	Chemical structure	IC$_{50}$ (μM)	% Inhibition at 50 μM
Scutellarein		5.80 ± 0.22	–
Dihydromyricetin		1.20 ± 0.09	–
Quercetagetin		1.24 ± 0.14	–
Myricetin		2.86 ± 0.23	–
Scutellarin		–	28.9 ± 1.6
5,6-Dihydroxyflavone		–	26.6 ± 0.4
6,7-Dihydroxyflavone		–	56.7 ± 2.0
Chrysin		–	2.6 ± 1.1
Myricetin		–	30.8 ± 4.6
Herbacetin		–	59.1 ± 1.9
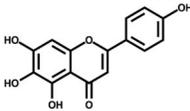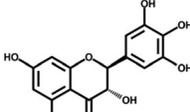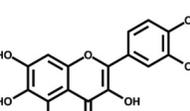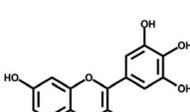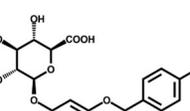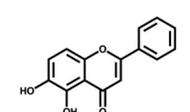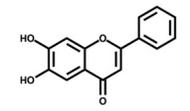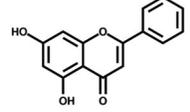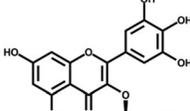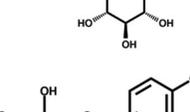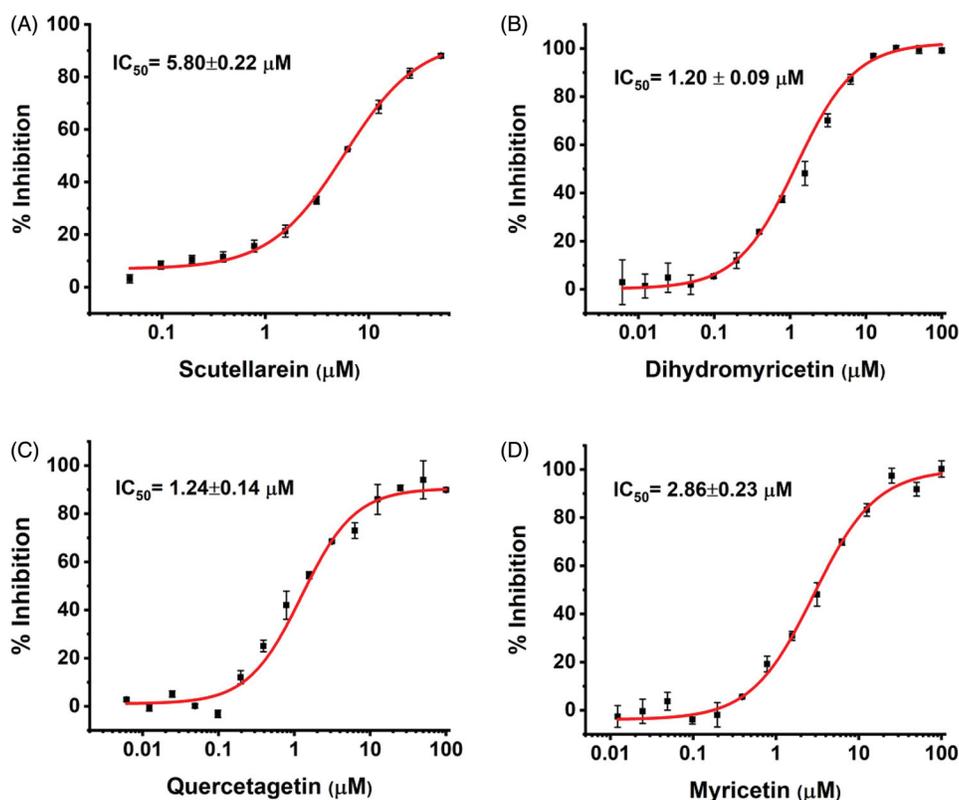
Figure 4. The SARS-CoV-2 3CL^{pro} inhibition activity of (A) scutellarein, (B) dihydromyricetin, (C) quercetagetin, and (D) myricetin.

substrate binding site of SARS-CoV-2 3CL^{pro} with its 6-OH and 7-OH forming hydrogen bond interactions with the carbonyl group of L141 and the backbone amide group of G143, respectively (Figure 3(A)). In addition, the carbonyl group of baicalein is hydrogen bonded with the backbone amide group of E166. The catalytic residues H41 and C145 are well covered by baicalein, accounting for its inhibitory effect. This computational model is in consistent with the recently reported crystal structure of SARS-CoV-2 3CL^{pro} complexed with bacailein[30]. As the 7-OH in baicalin is in close contact with the protein, there may not be enough space for glycosyl modification, explaining the low activity of baicalin. As for wogonin, the absence of 6-OH together with its additional 8-methoxyl group alters the binding orientation and weakens the binding strength (Figure 3(B)). Hydrogen bond is observed between its 5-OH and the backbone carbonyl group of L141, while the interaction with E166 by its 8-methoxy group is weaker than that formed by the carbonyl group in baicalein.

3.5. Searching for baicalein analogues that inhibit SARS-CoV-2 3CL^{pro}

Based on the docking analysis, we searched for baicalein analogues from available flavonoid suppliers and selected eight flavonoids and two glycosides for experimental testing (Table 2). The docking scores are given in Table S1. Four flavonoid compounds were found to be potent SARS-CoV-2 3CL^{pro} inhibitors. Among them, scutellarein is mainly distributed in genus *Scutellaria* and Erigerontis herba (Dengzhanxixin or Dengzhanhua in Chinese) in its glucuronide form, scutellarin. Scutellarin has long been used in cardiovascular disease treatment for its ability to improve cerebral blood supply[37]. As shown in Figure 4, scutellarein inhibits SARS-CoV-2 3CL^{pro} with an IC$_{50}$ value of 5.8 μM, while scutellarin

showed mild inhibitory activity at 50 μM concentration. The other three flavonoid compounds, dihydromyricetin, quercetagetin, and myricetin derived from *Ampelopsis japonica* (Bailian in Chinese), *Eriocaulon buergerianum* (Gujingcao in Chinese), and *Polygoni avicularis* (Bianxu in Chinese), respectively, inhibit SARS-CoV-2 3CL^{pro} with IC$_{50}$ values of 1.20, 1.24, and 2.86 μM. Interestingly, scutellarein and myricetin were reported to inhibit the SARS-CoV, indicating their potential as multi-target anti-SARS-CoV-2 agents[38].

We further showed that *S. baicalensis* extract, baicalein, and the four active baicalein analogue compounds also inhibit the activity of SARS-CoV 3CL^{pro} *in vitro* (Figure S3), demonstrating their potential as broad spectrum anti-CoV agents. For future development and *in vivo* studies, the bioavailability of these compounds needs to be considered. Although the bioavailability of baicalein is low in oral administration, its concentration can be increased at lung tissue through intratracheal administration[30]. We suggest that these compounds can be further optimised or used to search for other TCM herbs containing these compounds or substructures to develop effective treatments for COVID-19 and other coronavirus infectious diseases.

Disclosure statement

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

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