Both Baicalein and Gallocatechin Gallate Efficaciously Inhibit SARS-CoV-2 Replication by Targeting M^{pro} and Sepsis in Mice

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Abstract

Severe acute syndrome coronavirus 2(SARS-CoV-2) caused the global pandemic of COVID-19 since December 2019. Although most of COVID-19's patients are mild or common, most of the severe patients have sepsis caused by the cytokine storm, which greatly increases the case fatality rate. Moreover, there is no effective drug that can resist the novel coronavirus so far, so it's urgent to develop antiviral drug for the SARS-CoV-2. In our research, we screened 29 compounds with a score lower than -6 from 35 flavonoid compounds by molecular docking. (-)-Gallocatechin gallate, (+)-Gallocatechin and Baicalein were identified to have potent inhibit activity with IC_{50} 5.774±0.805μM, 13.14±2.081μM and 5.158±0.928μM by FRET assay. Subsequently, we conducted molecular docking experiments, which showed that (-)-Gallocatechin gallate, (+)-Gallocatechin and Baicalein were non-covalently bound to M^{pro} through π-π stacking and hydrogen bonds in the Cys145 catalytic site. We further evaluated the effect of (-)-Gallocatechin gallate and Baicalein on cytokine storm use a mouse model of sepsis. (-)-Gallocatechin gallate and Baicalein significant reduced sepsis severity based on weight, murine sepsis score and survival rate and reduced the inflammatory factors level such as TNF-α, IL-1α, IL-4 and IL-10. Overall, (-)-Gallocatechin gallate and Baicalein may be potential drugs for symptomatic treatment of COVID-19.

Introduction

Corona Virus Disease 2019, is named by the World Health Organization (WHO) as "COVID-19"[1]. According to WHO's assessment, the current COVID-19 outbreak can be described as a global pandemic, with significant adverse impacts on human health and life[2]. As of May 7, 2021, there were more than 157 million confirmed cases and 3.28 million deaths worldwide. According to the Chinese Center for Disease Control and Prevention, most of COVID-19's are mild and common, with clinical manifestations of fever, cough, fatigue, myalgia and diarrhea, and most of them have a good prognosis. Severe patients often develop respiratory failure, sepsis, septic shock and multiple organ failure one week later. Sepsis caused by cytokine factor storms greatly increases the mortality of critically ill patients [3]. At present, there is no specific drug for novel coronavirus. Therefore, the most urgent task is to research and develop a specific drug for anti-SARS-CoV-2.

Coronaviruses are a large family of viruses known to cause colds and more serious illnesses such as Middle East Respiratory syndrome (MERS) and severe acute respiratory syndrome (SARS)[4]. Novel Coronavirus is a novel coronavirus strain that has never been found in human body before. It is an RNA virus with enveloped genome and linear single plus strand. The virus particles are round or elliptic, about 60-140nm in diameter, Plus strand RNA means that the virus enters the cell to direct protein synthesis, and it replicates itself by making a negative strand by RNA polymerase[5]. The viral genome encodes replicases, four structural proteins (spines, envelopes, membranes, and nucleocapsid proteins), 16 non-structural proteins (NSPs), and nine accessory proteins, Among them, non-structural proteins play a very important role in virus replication and transcription[6, 7]. NSP5 is the main protease of SARS-CoV-2, which can cut polyproteins translated by viral RNA. The main protease cleaves 12 smaller proteins from the

polyprotein, which will be involved in the replication of viral RNA. Therefore, the main protease of coronavirus is an important potential drug target, which is crucial to inhibit virus replication[8].

In recent years, more and more attention has been paid to natural products extracted from plants and animals. At present, many natural products have been proved to have therapeutic effects on diseases[9]. Since the COVID-19 outbreak, scientists have done a lot of research, screening SARS-CoV-2 inhibitors by molecular docking[10-14], but few have done further enzymatic tests. In our study, (-)-Gallocatechin gallate, (+)-Gallocatechin and Baicalein were selected from 35 natural flavonoid products as potent inhibitors of SARS-CoV-2 M^{pro} by molecular docking and enzyme activity screening. We also used a mouse model of fecal dilution-induced sepsis to evaluated the effects of (-)-Gallocatechin gallate and Baicalein on cytokine storm. They all showed a good effect in reducing the severity of sepsis. This suggests that (-)-Gallocatechin gallate and Baicalein may be promising candidates for the treatment of COVID-19.

Materials And Methods

Drugs and reagents

The 29 test compounds were mainly obtained from Topscience Co.Ltd (Shanghai, China). Cefpirome sulfate was obtained from Yuanye Bio-Technology Co., Ltd (Shanghai, China). (-)-Gallocatechin gallate and Baicalein was purchased from Topscience Co., Ltd (Shanghai, China). The enzyme activity inhibitor screening kit was purchased from Beyotime Biotechnology (Shanghai, China). ELISA kit was provided by Enzymelink Biotechnology Co., Ltd (Shanghai, China).

Molecular modeling

The protein structure (PDB ID 6Y2F), which was resolved by Zhang et al., in 2020[8], was extracted from the RCSB Protein Data Bank (PDB) and prepared us[15][15]ing the Protein Preparation Wizard module in Schrödinger 2017 to remove all crystallographic water molecules, correct side chains with missing atoms, add hydrogen atoms and assign protonation states and partial charges with the OPLS_2005 force field. Finally, the crystal structure was minimized until the root-mean-square deviation (RMSD) of the nonhydrogen atoms reached less than 0.3 Å. The 22 flavonoids were prepared using the LigPrep module of the Schrödinger 2017 molecular modeling package to add hydrogen atoms, convert 2D structures to 3D, generate stereoisomers and determine the ionization state at pH 7.0 ± 2.0 with Epik[16]. Using the prepared receptor structure, a receptor grid file was generated around original ligand of the protein and the 22 flavonoids were docked to the receptor using Glide XP protocol.

Protease activity assay

Enzyme activity inhibitor screening adopts fluorescence resonance energy transfer method. For the screening of M^{pro} inhibitor and IC_{50} measurement, 300nM M^{pro} was incubated with compounds at 37°C for 5 min in assay buffer, then 20μM substrate, Dabcyl-KTSAVLQSGFRKME-Edans, was added to

the reaction system and incubated for 10 min at 37 ℃before detection. The IC_{50} was calculated by plotting the relative fluorescence units (RFU) against various concentrations of M^{pro} inhibitors by use of a dose-response curve in GraphPad Prism 7 software. Results Inhibition rate (%) = (RFU100% enzyme activity control-RFU sample) / (RFU100% enzyme activity control-RFU blank control) × 100%.

Experimental Animals

Male C57BL/6 (6-8 weeks, 20-22g) mice were purchased from Academy of Military Medical Sciences of People's Liberation Army (Beijing, China). All animal care and experimental procedures complied with guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of Nankai University (Permit No. SYXK 2014-0003). All experimental protocols were approved by the Animal Experiment Committee of Tianjin International Joint Academy of Biomedicine (approval no. SYXK (JIN) 2017-0003).

Modeling and animal grouping

In this experiment, a mouse sepsis model was established by intraperitoneal injection of 10% fecal diluent. The mice were randomly divided into five groups (n = 7): Saline group, 10% fecal dilution only group, fecal dilution with cefpirome sulfate (CS) group (100 mg/kg) and fecal dilution with (-)-gallocatechin gallate (50 and 100 mg/kg) treatment groups and fecal dilution with baicalein (100and 200 mg/kg). Intraperitoneal injection of 10% fecal dilution was given to model group and treatment group At day 0.For the treatment group, intraperitoneal injection of cefpirome sulfate (100 mg/kg) and (-)-gallocatechin gallate (50 and 100 mg/kg) and baicalein (100 and 200 mg/kg) were given 2h before fecal dilution injection. Saline group received normal saline by the same procedure.

Weight monitoring

Measure and record the weight of each group at the 0th hour, 24th hour, and 48th hour of the model

Murine sepsis score (MSS)

The murine sepsis score (MSS) was tested according to the introduction of reference[17].

Histological

The heart, liver, spleen, lung and kidney were fixed with 10% formaldehyde at the 48th hour. After two days of fixation, embedded in paraffin and sectioned into 4μm slices. Put the prepared slices in a 60℃ over to dry, and then dewax them as usual. Hematoxylin staining for 3-5 min, Then rinse with ultra-pure water for 10-15 min，hydrochloric ethanol separation for 10-20 seconds and dye with eosin for 3 min. After dehydration and transparency, add neutral gum to seal the slice.

ELISA for the detection of inflammatory factors

The levels of IL-1α、TNF-α、IL-4、IL-10 in serum of fecal dilution-induced sepsis mice were determined by enzyme-linked immunosorbent assay (ELISA) kit. The experimental process strictly followed the

instructions of the ELISA kit.

Statistical analysis

Data was presented using the Prism version 7.0 software as the means ± SD. Differences between experimental and control group were assessed by Student's t test. Significant differences among multiple groups were detected by one-way ANOVA. $P < 0.05$ was considered as statistically significance.

Results

Molecular docking

Recently, a large of studies reported the screening result of NPs, especially flavonoids, as anti-SARS-CoV-2 inhibitors based on *in-silico* drug discovery approaches. Therefore, we selected some flavonoid drugs for molecular docking with M^{pro} main protease. We docked 35 flavonoids to SARS-CoV-2 M^{pro}. The 2D structures of the 35 flavonoids and the corresponding Glide XP docking scores are listed in Table 1. 29 of the 35 flavonoid compounds show docking scores lower that -6.0, which indicates that these compounds might have effective inhibition on SARS-CoV-2 M^{pro} activity. These 29 flavonoid compounds were processed to the following biological assays.

Drug screening of SARS-CoV-2 M^{pro} inhibitors according to molecular docking

With the established FRET assay system, we screened 29 flavonoids compounds, which molecular docking score lower than -6, to identify potential SARS-CoV-2 M^{pro} inhibitors. The inhibitors at 20μM final concentrate were pre-incubated with 0.3μM M^{pro} at 37℃ for 5 min before 20μM FRET substrate was added. Inspiringly, three compounds, (-)-Gallocatechin gallate (10), (+)-Gallocatechin (25) and Baicalein (27) had fine inhibition and inhibit rate more than 50% against M^{pro}(Figure.1A). But other compounds, which have higher docking score, such as Quercetin-3-O-β-D-glucose- 7-O-β-D-gentiobiosiden, Schaftoside, Vitexia-glucoside, Nicotiflorin and Isorhamnetin 3- O-neohesperidin, did not exhibit detectable inhibitory activity.

Inhibitory activities of SARS-CoV-2 M^{pro} by the (-)-Gallocatechin gallate, (+)-Gallocatechin and Baicalein and their structural basis

In the enzyme activity screening assay, we gained the exciting result, three potent flavonoid compounds were confirmed as SARS-CoV-2 inhibitors. To further evaluate the inhibitory activity of this three compounds, we detected their inhibition in a gradient concentrate, and calculated the 50% inhibitory concentration values (IC_{50}), respectively. The IC_{50} of (-)-Gallocatechin gallate, (+)-Gallocatechin and Baicalein are 5.774±0.805μM, 13.14±2.081μM and 5.158±0.928μM respectively (Figure.1B). Among them, (-)-Gallocatechin gallate and Baicalein showed better inhibitory effect, and the IC_{50} was lower than 10μM.

The docked binding poses and the interaction details of (-)-Gallocatechin gallate, (+)-Gallocatechin and Baicalein with SARS-CoV-2 M^{pro} are presented in Figure.2. In the structure of (-)-Gallocatechin gallate binded with SARS-CoV-2 M^{pro} (Figure.2A), the dihydrobenzopyran ring of (-)-Gallocatechin gallate interacts with the imidazole side chain of His41 through π-π stacking. The 5- and 7-hydroxyl of dihydrobenzopyran form hydrogen bonds with the backbone oxygen of Asp187 and Arg188. The 4-hydroxyl of trihydroxyphenyl forms hydrogen bonds with the backbone oxygen of Leu141, the backbone nitrogen of Gly143 and the backbone nitrogen of Cys145. The 3- and 4-hydroxyl of trihydroxybenzoate form hydrogen bonds with the backbone oxygen of Phe140 and the side chain oxygen Glu166.

In the structure of (+)-Gallocatechin binded with SARS-CoV-2 M^{pro} (Figure.2B), the 3- and 7-hydyoxyl of (+)-Gallocatechin form hydrogen bonds with the backbone oxygen of His164 and the side chain oxygen of Thr25. The 3'-, 4'-and5'-hydroxyl of (+)-Gallocatechin form hydrogen bonds with the backbone oxygen of His164, the imidazole side chain of His 163, the side chain oxygen Ser144 and the side chain oxygen Asn142.

In the structure of Baicalein binded with SARS-CoV-2 M^{pro} (Figure.2C), the 2-phenyl of Baicalein interacts with the imidazole side chain of His41 through π-π stacking. The 6- and 7-hydroxyl of Baicalein form hydrogen bonds with the backbone nitrogen of Cys145, the backbone nitrogen of Gly13, the backbone oxygen of Leu141 and the imidazole side chain of His 163.

(-)-gallocatechin gallate and baicalein attenuates fecal dilution -induced sepsis in mice

As shown in figure. 3A, we evaluated the effect of (-)-gallocatechin gallate and baicalein on sepsis use a mouse model. The change of body weight was displayed in figure. 3B. Compared with the control group, the weight of the model group was significantly reduced. The weight of the mice in the treatment group was higher than model group, and the efficacy of baicalein was better than (-)-gallocatechin gallate. The mouse sepsis score (MSS) of model group was significant higher than control group at the 24h and 48h after the model was established, but the group treated with (-)-gallocatechin gallate (50 and 100 mg/kg) and baicalein (100 and 200 mg/kg) attenuated the symptoms caused by sepsis (Figure.3C). Observing the mortality of mice every 6 hours, it was found that 3 mice died in the model group, and the mortality rate reached 42%, while the mortality rate of the treatment group was reduced to 28% except for the low-dose (-)-gallocatechin gallate group. It showed that (-)-gallocatechin gallate and baicalein can improve the survival rate of sepsis caused by fecal dilution(Figure.3D).

(-)-gallocatechin gallate and baicalein inhibits the release of pro-inflammatory cytokines in serum

COVID-19 and sepsis both have the pathological manifestation of cytokines storm, so we detected the level of pro-inflammatory factors in the serum of septic mice. As showed in figure. 3E, compared with the control group, the level of serum inflammatory factors IL-1α、TNF-α、IL-4 and IL-10 in the model group was significantly increased, while they were markedly decreased after treated with (-)-gallocatechin gallate and baicalein. These data suggested that (-)-gallocatechin gallate and baicalein could down regulate the level of serum inflammatory cytokines induced by sepsis.

We also evaluated the effect of (-)-gallocatechin gallate and baicalein on tissue damage induced by sepsis. We compared the pathological sections of the heart, liver, spleen, lung, and kidney of each group of mice and found that the damage to the spleen of the model group mice was serious, and the white pulp was significant enlarged. And a large amount of inflammatory infiltration was found in the heart, liver, lung, and kidney. The damage was obvious and the cell arrangement was disordered. But after (-)-gallocatechin gallate and baicalein intervention, the above lesions were alleviated (Figure. 4).

Discussion

The epidemic of COVID-19 urgently requires new treatment strategies[6, 7, 18, 19]. Currently, there is no specific drug for SARS-CoV-2, but a large number of compounds have been shown to have good binding affinity with M^{pro} through virtual screening on the structure[13, 20, 21]. For example, the team from the University of Alberta, Canada, found that GC376, an earlier protease inhibitor pro-drug used to treat feline coronavirus infection, and its mother GC373 were effective in inhibiting SARS-CoV and SARS-CoV-2 M^{pro} in the nanomole range[22-25]. Antiretroviral drugs such as lopinavir and nefinavir for patients with acquired immune deficiency syndrome (AIDS) and HIV-1 infection that they can bind to the main protease of novel coronavirus and inhibit virus replication[26]. However, in clinical trials, such drugs are given at a high dose, resulting in low patient compliance[27]. In China, many scientists have turned their attention to natural products, such as flavonoids[28], alkaloids[29, 30] and green tea polyphenols[31], etc., in an attempt to find effective drugs against novel coronavirus from some natural products. For these compounds, more rigorous studies may be carried out to realize the transition of drugs from laboratory to clinical, which will be further developed as potential therapeutic drugs against novel coronavirus.

In our study, the binding affinity of 35 natural flavonoids compounds with SARS-CoV-2 M^{pro} was compared through computer simulation of molecular docking. The results showed that (-)-Gallocatechin gallate, (+)-Gallocatechin and Baicalein had high affinity with M^{pro}, and (-)-Gallocatechin gallate, (+)-Gallocatechin and Baicalein exhibit significant inhibition with IC_{50} 5.774±0.805μM, 13.14±2.081μM and 5.158±0.928μM, respectively. And structurally, (-)-Gallocatechin gallate, (+)-Gallocatechin and Baicalein were non-covalently bound to SARS-CoV-2 M^{pro} through π-π stacking and hydrogen bonds in the Cys145 catalytic site. This indicates that experimental antiviral activity tests are necessary in order to develop more effective and reliable anti-SARS-CoV-2 drugs.

Novel coronavirus causes an extremely active inflammatory response, known as a cytokine storm, followed by severe inflammation of lung. Sepsis is the most common complication in severe and critical COVID-19 patients, with an incidence of 42% in the survival group and 100% in the non-survival group[32]. Many severe and critical patients have clinical manifestations of sepsis or septic shock, including fever, intractable hypotension, microcirculatory dysfunction, severe metabolic acidosis and varying degrees of multiple organ dysfunction. COVID-19 complicated with sepsis undoubtedly increases the difficulty of treatment and seriously affects the prognosis of patients. In this study, we evaluated the inhibitory effect of (-)-Gallocatechin gallate and Baicalein, the SARS-CoV-2 inhibitors we screened by FRET assay, on

sepsis using a mouse sepsis model. Both of them showed an effective effect in relieving sepsis in mice in terms of body weight, MSS score and survival rate, and they effectively inhibited the expression of inflammatory factors such as IL-1α、TNF-α, IL-4 and IL-10, which are often referred to as “cytokines storm” in sepsis.

Conclusions

In summary, the data indicated that (-)-Gallocatechin gallate and Baicalein may be potential candidates against COVID-19. At the same time, our study also shows that natural products of small molecular bioactivity may be a useful source of SARS-CoV-2 M^{pro} inhibitors and can be a powerful line of defense against COVID-19.

Declarations

Ethics approval and consent to participate

All animals care and experimental procedures conformed guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of Nankai University (Permit No. SYXK 2014-0003)

Consent for publication

Not applicable.

Availability of data and material

Data available on request from the authors.

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

Data curation, Ting Xiao and Mengqi Cui; Formal analysis, Shanfa Ren and Jiali Bao; Funding acquisition, Honggang Zhou and Cheng Yang; Investigation, Peipei Zhang and Liang Zhang; Methodology, Dandi Gao; Project administration, Honggang Zhou; Resources, Mingjiang Li; Software, Caijuan Zheng; Supervision, Jianping Lin; Validation, Ming Wang; Visualization, Ronghao Sun; Writing – original draft, Ting Xiao; Writing – review & editing, Dongmei Li.All authors read and approved the final

manuscript. All data were generated in-house, and no paper mill was used. All authors agree to be accountable for all aspects of work ensuring integrity and accuracy.

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Not Applicable

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Tables

Due to technical limitations, table 1 is only available as a download in the Supplemental Files section.

Figures

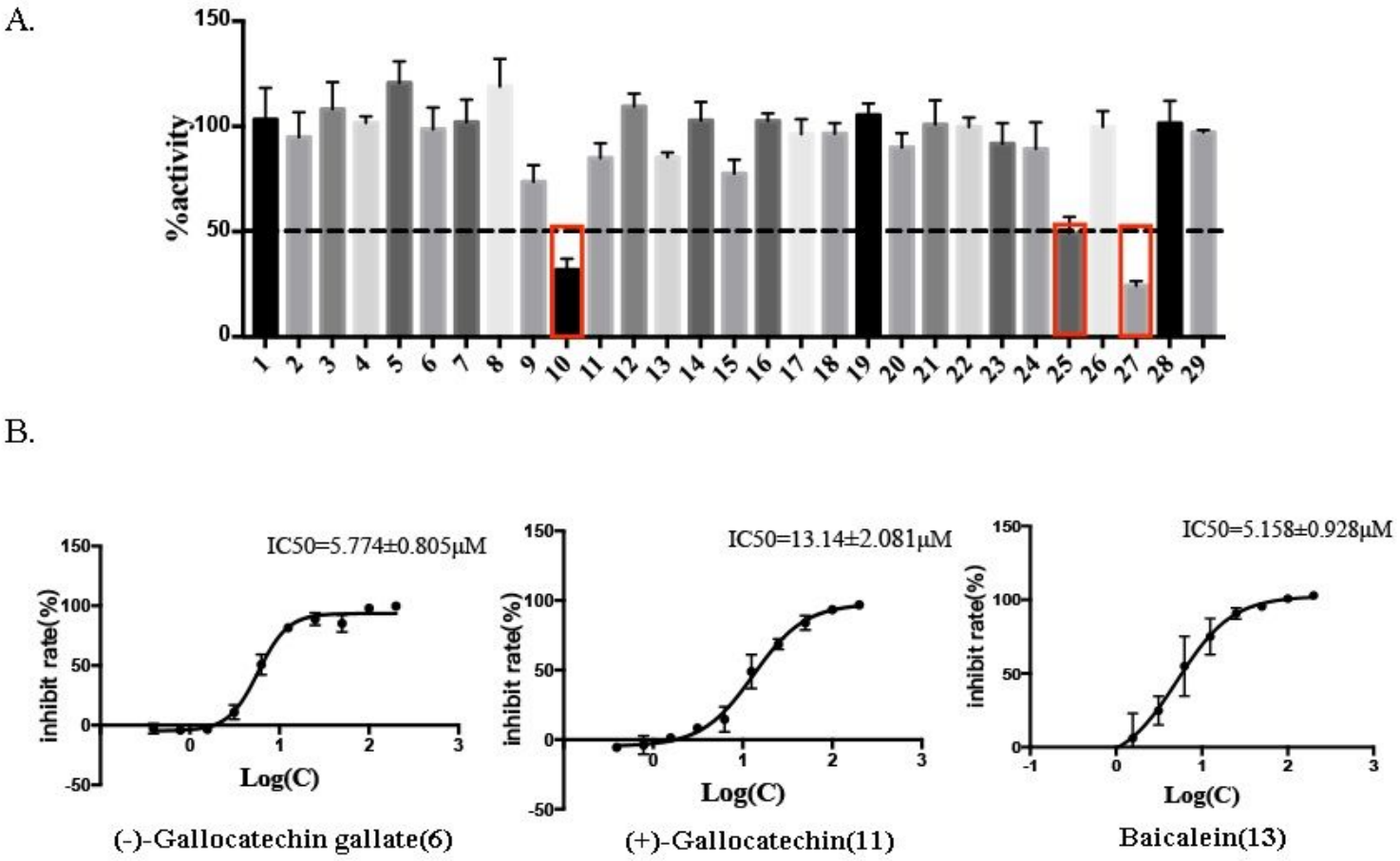

Figure 1

Screening of 29 flavonoids, docking score lower than -6, against SARS-CoV-2 Mpro using the FRET assay. A.40 μM compound (final concentration 20 μM) was pre-incubated with 0.3μM SARS-CoV-2 M pro at 30°C for 10 minutes, and then 200 μM FRET substrate was added to the reaction mixture to initiate the reaction. The excitation wavelength is 340nm and the emission wavelength is 490nm for fluorescence measurement. Results Inhibition rate (%) = (RFU100% enzyme activity control-RFU sample) / (RFU100% enzyme activity control-RFU blank control) × 100%. The results are average ±standard deviation of three repeats. B. The inhibitory assay of (-)-Gallocatechin gallate, (+)-Gallocatechin and baicalein show efficient inhibition for Mpro. Error bars: mean ± S.D. of three independent replicates.

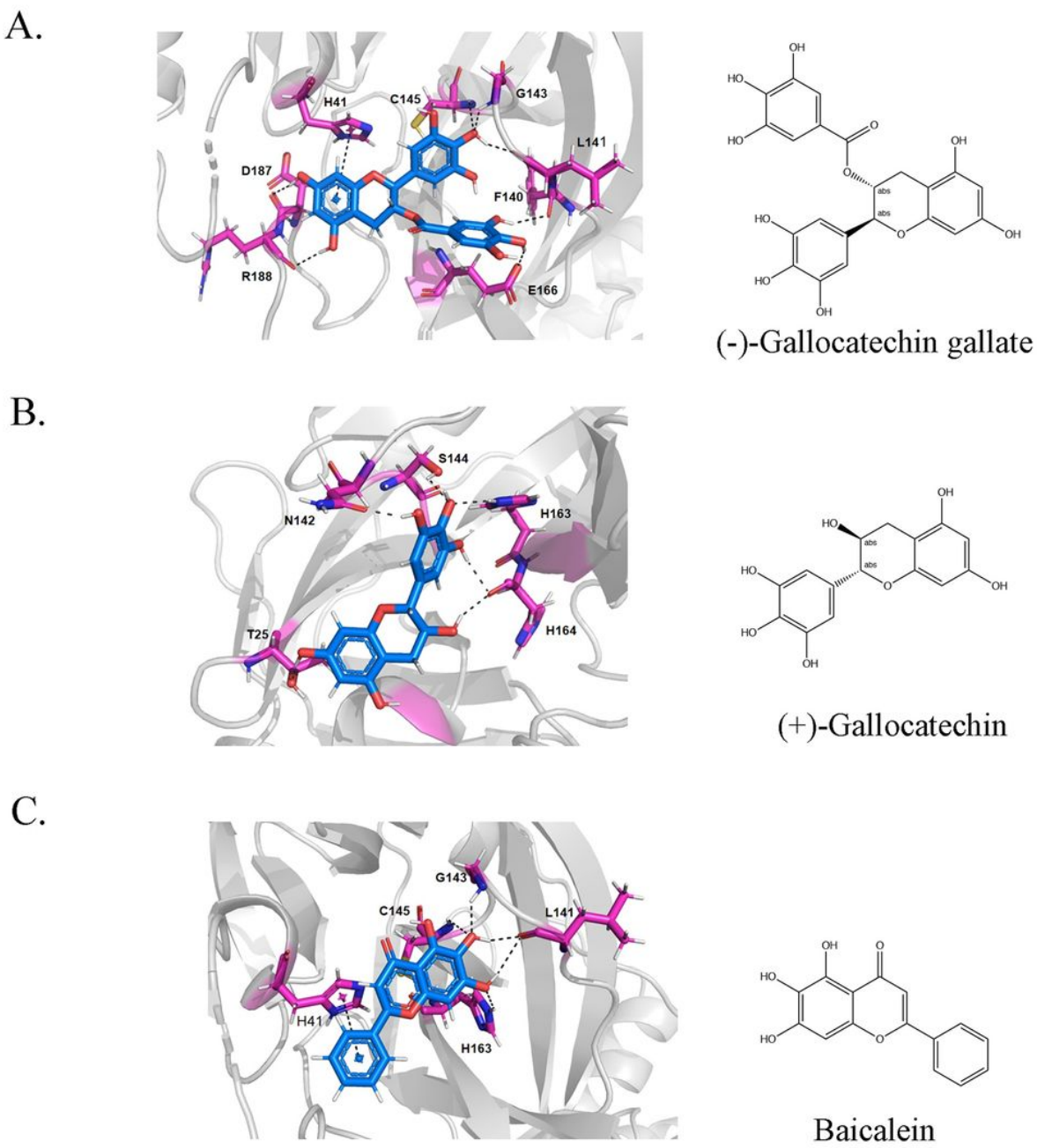

Figure 2

Docked conformations of (-)-Gallocatechin gallate, (+)-Gallocatechin and Baicalein in SARS-CoV-2 Mpro proteases. A. (-)-Gallocatechin gallate docking to the SARS-CoV-2 Mpro X-ray structure (NCBI PDB accession no. 6Y2F). B. (+)-Gallocatechin docking to the SARS-CoV-2 Mpro X-ray structure (NCBI PDB accession no. 6Y2F). C. Baicalein docking to the SARS-CoV-2 Mpro X-ray structure (NCBI PDB accession no. 6Y2F).

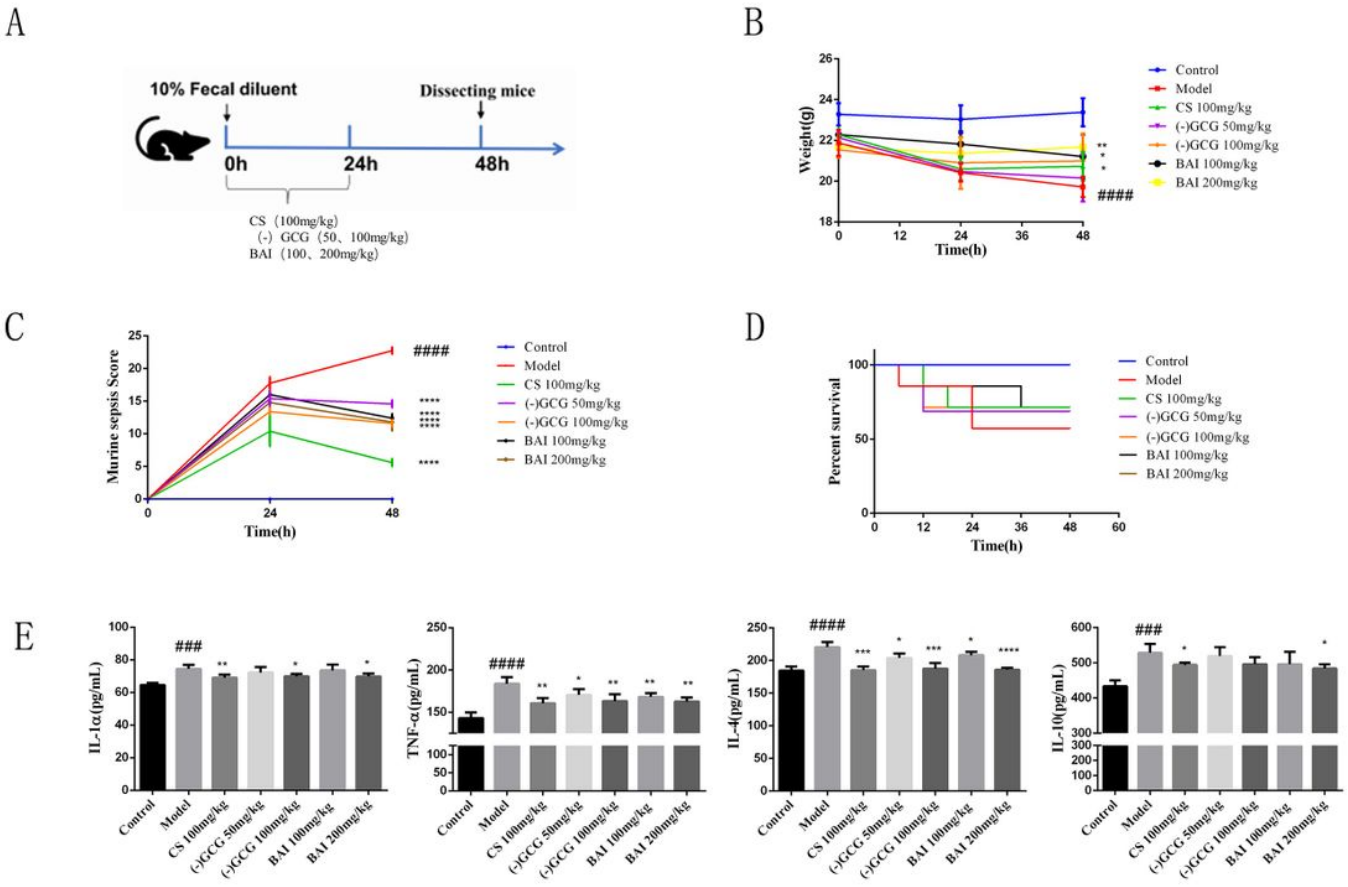

Figure 3

Effect of (-)-gallocatechin gallate and baicalein on fecal dilution -induced sepsis in mice. A. Experimental scheme of mice sepsis induced by fecal diluent. B. Changes in body weight in different groups of mice In review (Control, Model, 100mg/kg cefpirome sulfate, 50mg/kg (-)-gallocatechin gallate and 100mg/kg (-)-gallocatechin gallate, 100mg/kg baicalein and 200mg/kg baicalein). C. The effect of (-)-gallocatechin gallate and baicalein on murine sepsis score (MSS) of the different groups of mice described above. D. The effect of (-)-gallocatechin gallate and baicalein on the survival time of the different groups of mice described above. E. Serum IL-1α、TNF-α、 IL-4 and IL-10 were detected by ELISA. Values are presented as the mean ± SEM (n = 5), ##P < 0.01, ####P < 0.0001,significantly different from control group; *P < 0.05、 **P < 0.01, ***P < 0.001, ****P < 0.0001, significantly different from model groups.

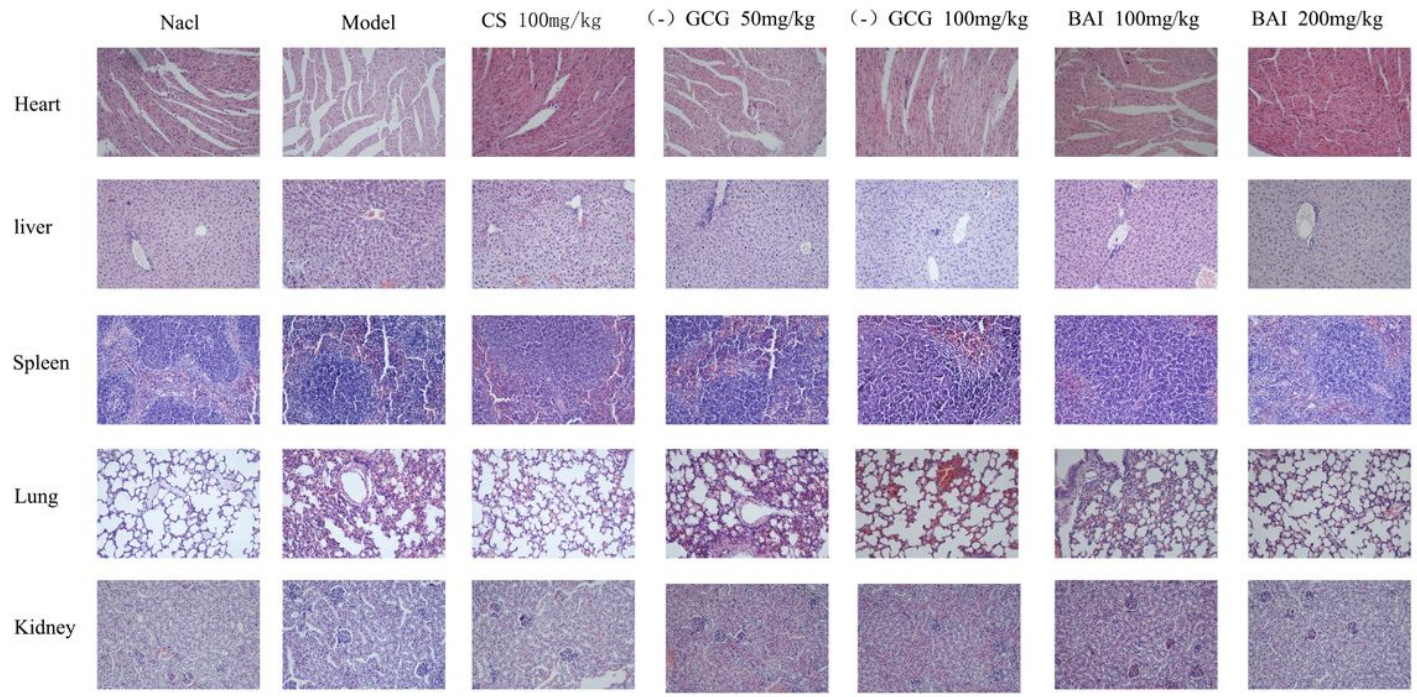

Figure 4

Effect of (-)-gallocatechin gallate and baicalein on serum proinflammatory cytokine expression. HE staining was used to evaluate the effect of (-)-gallocatechin gallate and baicalein on fecal dilution - induced pathological changes (20×magnification).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table1.docx