Inhibitory Effect of Baicalin on 2019-NCOV Invasion

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Research Article

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Abstract

Objective To study the the inhibitory effect of baicalin on novel Coronavirus (2019-NCOV) entry process in vitro. **Methods** The pseudovirus system of SARS-CoV-2 S protein constructed with luciferase reporter gene was used in the study. A luciferase kit was used to detect the changes of luciferase expression in Huh-7 cells, and then the virus inhibition curve was plotted.

Results Baicalin can significantly inhibited the infection rate of pseudovirus. There was no significant difference in the virus inhibition curve between the baicalin&virus pre-incubation group and co-incubation at different concentrations, indicating that baicalin could not directly bind to virus, but inhibited the virus S protein mediated cell fusion process. We futher found that, the inhibition rate of baicalin to virus decreased significantly in the 4h group, but had no significant difference in the 0h and 2h groups at the concentration of 0.125mg/ mL, indicating that baicalin may have an inhibitory effect on virus invasion stage rather than adsorption stage, and the mediated inhibition stage occurred within 4h.

Conclusion Baicalin may mediate the fusion of SARS-CoV-2 S protein with cell surface receptor and exert anti-novel coronavirus activity by playing a role in inhibitting virus invasion in non-adsorption stage.

Introduction

In December 2019, a new infectious respiratory disease was named "COVID-19(Coronavirus Disease 2019)" by the World Health Organization (WHO) was reported[1]. The causative agent causing this disease has been successfully identified as SARS-COV-2 [2]. Until now, more than 260 million people have been diagnosed with COVID-19 of worldwide scope, resulted in more than 5.2 million deaths and posed a significant threats to to international health and the economy. SARS-CoV-2

includes two open reading frames ORF1a and ORF1b, each of which can be translated as two viral multi-protein pp1a and pp1ab by host ribosomes after entering the host cells.Then, polymeric protein precursors such as RNA-dependent RNA polymerase (RdRp) and helicase can be cutted by under main protease (Mpro) and Papain-like protease (PLpro), to produce multiple non-structural proteins, which are indispensable in viral transcription and replication[3]. Any blocking of key enzymes or proteins can inhibit the replication of SARS-CoV-2 and be one of the good targets to find antiviral agents before vaccines are available. Recently, Coronavirus drug targets have been reported include structural proteins (S protein, M protein, N protein and E protein), major proteases, papain like proteases and RNA polymerases.

Baicalin is the main component isolated from Scutellaria baicalensis Georgi (Huangqin in Chinese) roots. According to previous studies, Baicalin have the efficacy of antibacterial, antiviral, anti-inflammatory, antioxidant, anti-tumor, anti-diabetes and its complications, etc[4,5], and It have shown antiviral activities against viral pathogens by directly killing viruses, inhibiting virus replication, regulating the expression of functional proteins in host cells and other mechanisms. Baicalin was reported that not only have significant an antiviral activity on influenza virus, hepatitis virus, Coxsackie virus, respiratory syncytial

virus[6–9], but also was identified as the first noncovalent, nonpeptidomimetic inhibitor of SARS-CoV-2 3CL protease(3CLpro) in the SARS-CoV-2 infected cells. Recent report indicated that three phenolic hydroxyl groups of baicalein binds with 3CLpro at Leu141/Gly143 as well as Ser144/His163 through a series of direct and indirect hydrogen bonds, fix the free oxygen anion ring structure and prevent peptide substrates from accessing the active site so as to achieve the anti-virus action[10]. The result of molecular docking showed that baicalin and bromocryptostine can bind with N-terminal and C-terminal active sites in homology model of nonstructural protein (NSP) 14 protein of novel Coronavirus respectively. What's more, baicalin may play an antiviral role as an inhibitor of papain like protease in SARS-CoV-2. All of the studies above are focused on post-invasion replication phase, whether baicalin can plays an antiviral role in the adsorption stage has not been reported yet. In order to solve this problem, we used lentivirus-based SARS-CoV-2 spike pseudotyped virions containing a firefly luciferase gene as a reporter to mimic SARS-CoV-2 spike-driven cell entry to explore the inhibition effect of baicalin on SARS-CoV-2 and predict the possible new target protein of baicalin. These study provides data support for further study on the antiviral role of baicalin for SARS-CoV-2.

Materials And Methods

Cells,reagents,and viruses

SARS-CoV-2-Fluc (lot No.:20210121), was purchased from Beijing Tiantan Pharmaceutical Biotechnology Development Co.LTD; Luciferase Detection kit (item No.: E2520), was purchased from Promega company; fetal bovine serum (item No.:10100-147C,Gibco); DMEM High Sugar medium (item No. :11965-092,Gibco); Trypsin(item No.:25200-056, Gibco); HBSS (item No.: 14025-092/095,Gibco); MTT (itemNo.: MKBL3665V, Sigma); DMSO(item No.: 10657336,Honeywell); baicalin reference Standard(item No.:D026471), provided by China National Institute for Food and Drug Control.

Cell viability assay

The Huh-7 cell line was purchased from National Collection of Authenticated Cell Cultures and maintained in DMEM High sugar medium supplemented with 10% fetal bovine serum in a humid incubator with 5% CO_2 at 37°C. The standard solution of baicalin with different concentrations was prepared and Huh-7 SARS-COV-2 susceptible cells were incubated in the culture medium contains baicalin of for 24h. The cell viability was detected by MTT assay, and the non-toxic dose of baicalin was obtained.

SARS-CoV-2 spike pseudovirus infectivity assay

Pseudotyped entry representing viral infectivity was evaluated by measuring luciferase activity in cell lysates. The experiment was divided into blank control, negative control, positive control and test substances groups. In the 96-well plate, 50μL of pseudovirus solution diluted to 1.3×10^4 titer, 100μ L of Huh-7 cell culture solution at a density of 2×10^5 cells/ml and 50μl baicalin solution with different concentrations or equal volumes of complete medium were added into per orifice respectively for test

substances group or positive control group. What'more, the negative control group cells were incubated with equal test substance solution without pseudovirus solution, and the blank control group was supplemented with complete medium only. The virus inhibition curve was determined by luciferase kit after incubation for 24h.

Comparison test between pre-incubation group and co-incubation group

96-well plates were laid, and besides blank control, negative control and positive control group, two test article groups were set at certain concentration according to the cell survival test results. In one test article group, baicalin solution with different concentrations was incubated with 1.3×10^4 pseudovirus solution at 37℃ for 2h, and then the mixed solution were added into Huh-7 cell suspension for infection. In the other group, baicalin and pseudovirus solution at the same concentration were added into huh-7 cell suspension at the same time. After incubation for 24h, luciferase was detected both to draw the virus inhibition curve.

Pseudovirus infectivity assay at different time points

50μL of pseudovirus solution diluted to 1.3×10^4 titer and 100μL of Huh-7 cell culture solution were added into 96-well plate, then cells were incubated at 37℃with 5% CO_2. 2h, 4h and 8h post-infection, 50μL of 0.5mg/mL baicalin solution were supplemented respectively at certain concentration according to the cell survival test results. Blank group was supplemented with complete medium. Fluorescein detection was performed after post-infection 24h to draw virus inhibition curve.

Cell culture and transfection

Huh-7 cells were cultured in high-glucose Dulbecco's modified Eagle's medium

supplemented with 5% heat-inactivated FBS and 1% penicillin and streptomycin and

grown at 37℃ in a humidified atmosphere containing 5% CO_2. Cells were transfected with the indicated plasmids using Lipofectamine 3000 according to the manufacturer's specifications.

Western blotting analysis

Cells were harvested and lysed in RIPA buffer,and protein were separated on SDS polyacrylamide gels and transferred to PVDF memebranes.The membranes were blocked with 5% BSA, then was incubated with ACE2 or Actin primary antibodies at 4℃ overnight.Membranes were washed three times with TBST, then were incubated with appropriate secondary antibodies at room temperature for 1h. Membranes were subsequently developed with ProSignal Pico ECL reagent and chemiluminescent signals were acquired using Bio-Rad ChemiDoc imager

Results

1 Baicalin is an effective inhibitor of SARS-CoV-2 pseudovirus

Baicalin can significantly inhibit the infection rate of huh-7 cell pseudovirus (Fig. 1A), and according to the result of cell viability assay, it can still produce significant inhibitory effect at concentration with no cytotoxicity. (conc≤0.2mg/ml) (Fig. 1B).

2 Baicalin is not a virus neutralizer and play a role in the early stage of virus infection

Two groups were construted with (group a) or without (group b) pre-incubation of baicalin and pseudovirus solution prior to the process that huh-7 cells were incubated with baicalin and pseudovirus solution for 24h. The inhibitory effect of baicalin on pseudovirus infection was observed at different concentrations. The results showed that there was no significant difference in the infection rate curves between the two groups (Fig. 2A), indicating that baicalin could not bind to virus directly. According to the results of cell survival test, we choosed 0.125mg/mL as the maximum concentration in follow-up experiment. In the study, Huh-7 cells were incubated with 0.125mg/mL baicalin at different times after infection with SARS-CoV-2 pseudovirus. We found the virus inhibition rate was significantly reduced in the 4h group, and there was no significant difference in the 0h and 2h groups (Fig. 2B,C). These results suggest that baicalin may inhibit virus entry into cell (non-adsorption), and the mediated entry into the inhibition phase occurred within 4h.

3 The role of ACE2 in the anti-invasion effect of Baicalin

Since the first step in SARS-CoV-2 infection is binding of the virus to cell surface receptors such as angiotensin converting enzyme 2 (ACE2) on the airway epithelium, we induced ACE2 overexpression in Huh-7 cells using transduction with an ACE2-lentivirus construct. The protein level of ACE2 was successfully upregulated as shown in Fig. 3A. The effect of ACE2 overexpression on the increasing virus inhibition rates induced by Baicalin at different concentration was further investigated. The assay indicated that overexpression of ACE2 can increase the viral infection rate, which is represented by negative virus inhibition rates, and can inhibit the elevated inhibition rates induced by Baicalin especially at low concentration (Fig. 3B).

Discussion

SARS-COV-2 enters cells mainly through membrane fusion and endocytosis, which is completed by the interaction of S protein on the cell surface with receptors on the cell surface. Untill now, several cell receptors of coronaviruses have been identified, such as Amino Peptidase N (APN), Angiotensin-converting enzyme 2 (ACE2), Dipeptidyl Peptidase 4 (DPP4), etc.[11] Among them, ACE2 is the earliest confirmed cell surface receptor of SARS-COV-2 with high affinity. Studies have confirmed that the binding force of ACE2 with SARS-COV-2 virus is 10–20 times compared to SARS virus[12]. We provide an in vitro study on antiviral effect of baicalin in pseudovirus system. From the results of this study, we can

conclude that baicalin significantly inhibited the cell invasion of the pseudovirus system, which was due to the interaction between baicalin and the cell surface receptor, rather than the direct polymerization of baicalin and the virus surface S protein. In addition, the in vitro test confirmed that the virus inhibition rate had no difference with 2h and increased at 4h with the extension of the co-incubation time of virus cells, which also supported the first point of view of this study. Therefore, we boldly speculated that the inhibitory effect of baicalin on the invasion process of pseudovirus system found in this study might be caused by the effect of Baicalin on the binding process of S protein and ACE2. In order to confirm the hypothesis, we construct a ACE2 high expression cell lines and investigated the vital function of ACE2 in the anti-invasion effect of baicalin. Recently, some researchers have found that baicalin may have strong binding ability with ACE2 through molecular docking [13], which may support our findings futher. But due to laboratory security level restrictions, this study only provides a preliminary discussion and new idea for development of anti SARS-CoV-2 drug only, the specific mechanism in live virus is required for further study .

Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Meng Zhou, Rong Sun, Xia Wei and Qing-fen Zhu. The first draft of the manuscript was written by Xue Geng and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request

Ethics approval

This article does not contain any studies with human participants or animals performed by any of the authors. The Shandong Institute for Food and Drug Control Ethics Committee has confirmed that no ethical approval is required.

Consent to participate

Informed consent was obtained from all individual participants included in the study.

Consent to publish

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

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Figures

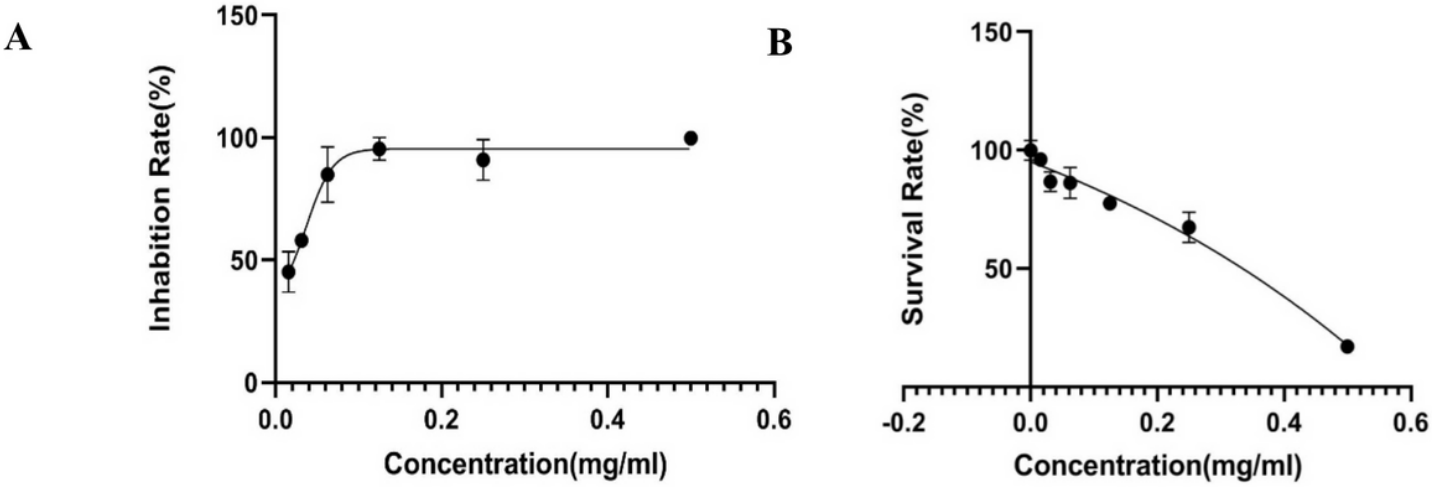

Figure 1

Baicalin is an effective inhititor of SARS-CoV-2 pseudovirus. A SARS-CoV-2 spike pseudovirus infectivity assays. Anti-virus potency of baicalin was tested at different concentrations in Huh-7 cells for 24h. **B** Cell viability assays. The MTT assay was used to examine the Huh-7 cell viability in different doses of baicalin for 24h.Data shown are mean±SD of three independent experiments.

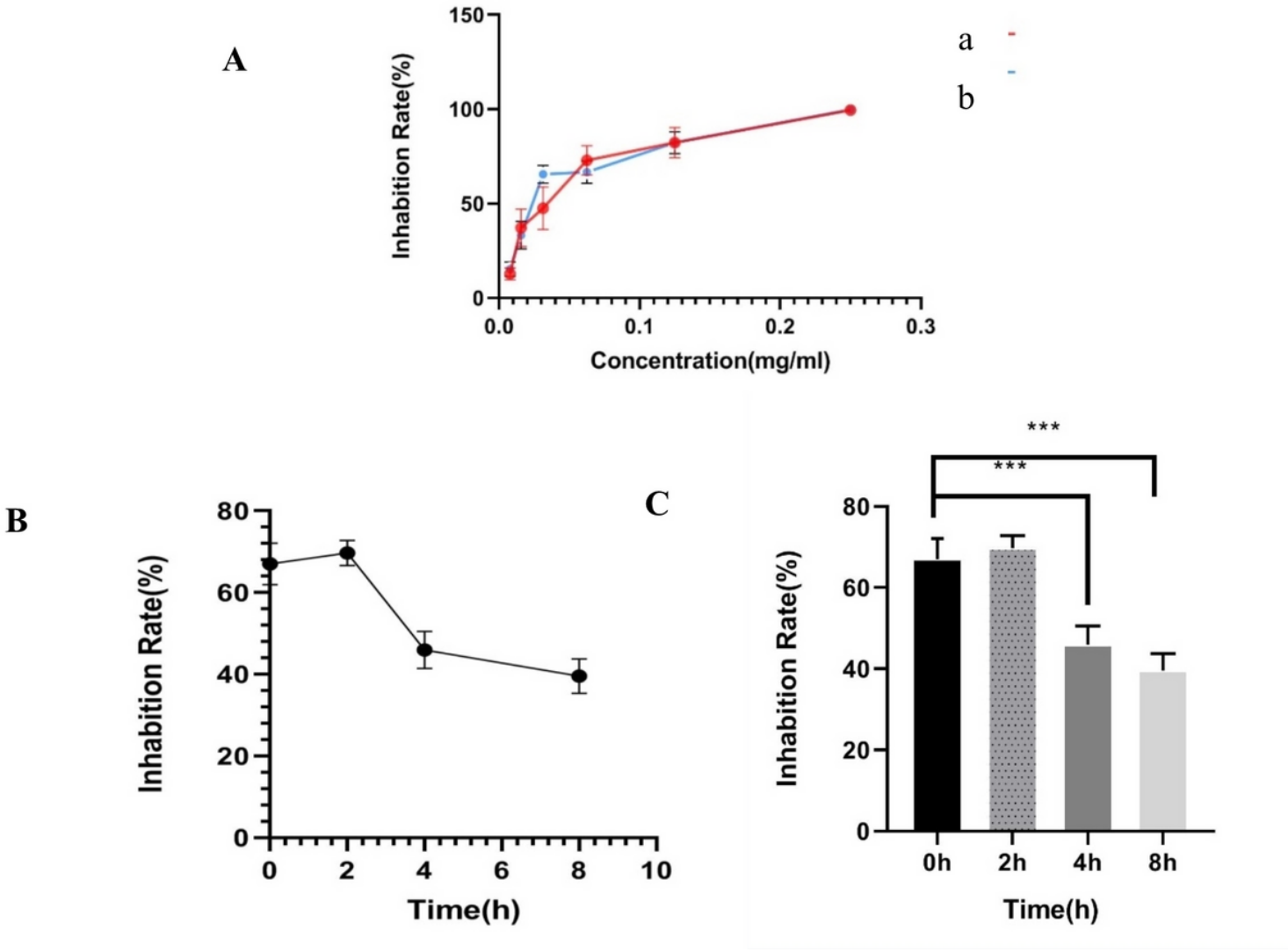

Figure 2

Baicalin is not a virus neutralizer and acts in the early stage of virus infection. A SARS-CoV-2 spike pseudovirus infectivity assays. Virus inhibition rate of Baicalin at gradient concentration for co-incubation group (group a) and pre-incubation group (group b) in Huh-7 cells. **B** SARS-CoV-2 spike pseudovirus infectivity assays. Post-infection with pseudovirus 2h,4h,8h, Baicalin was added to the culture system, and luciferase activities of all the cells were measured at 24h post-infection.Data shown are mean±SD of three independent experiments.***P 0.001.

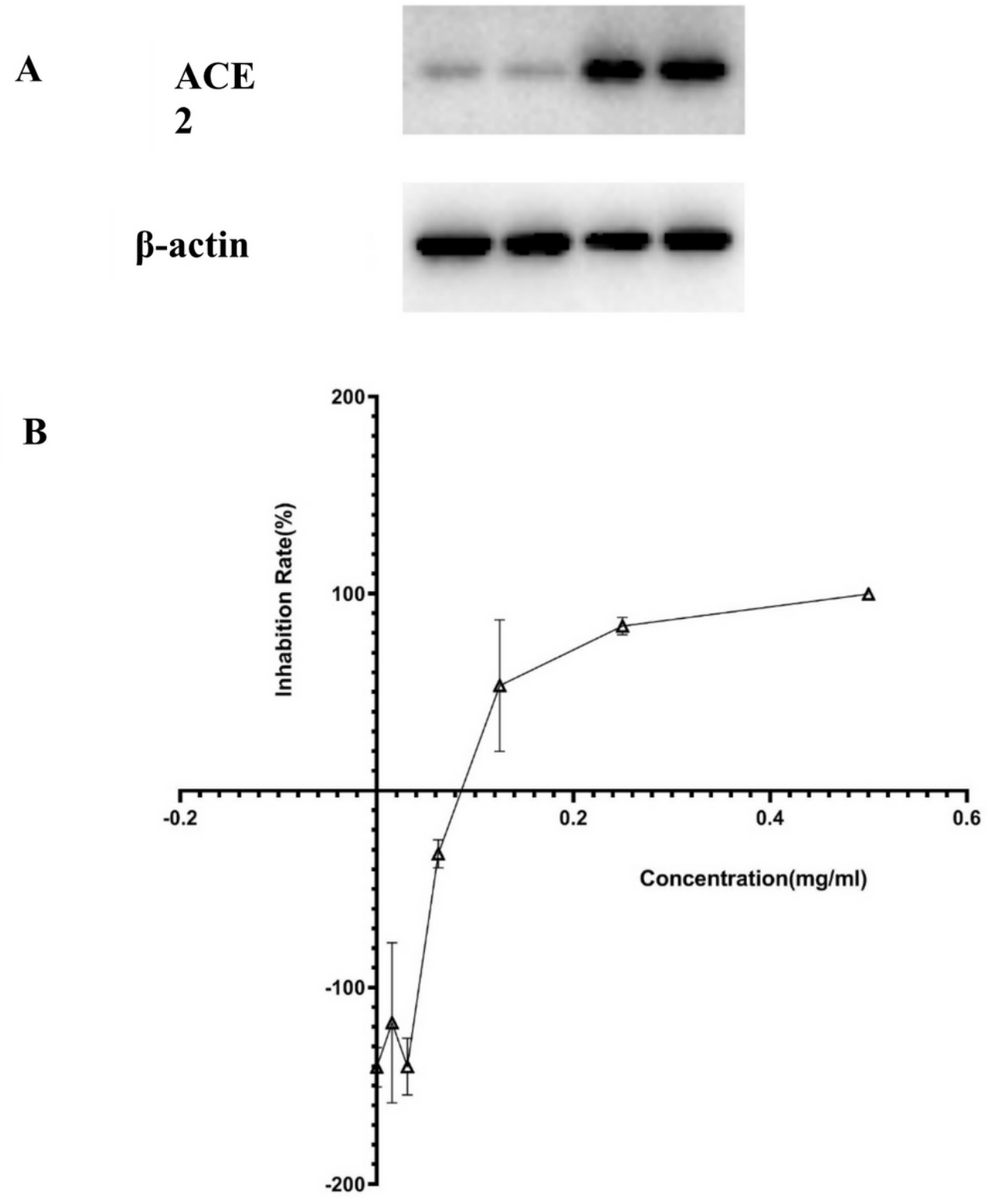

Figure 3

The role of ACE2 in the anti-invasion effect of Baicalin. A Western blot assay. The protein level of ACE2 in Huh-7 cells stably overexpressing ACE2 . **B** SARS-CoV-2 spike pseudovirus infectivity assays. Virus inhibition rate at different concentration in Huh-7 cells stably overexpressing ACE2. Data shown are mean±SD of three independent experiments.