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Research Article

Scutellaria barbata D. Don extracts alleviate SARS-CoV-2 induced acute lung injury by inhibiting virus replication and bi-directional immune modulation

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ABSTRACT

The emergence of SARS-CoV-2 variants and drug-resistant mutants emphasizes the urgent need to develop novel antiviral agents. In the present study, we examined the therapeutic effect of the Chinese medicinal herb, *Scutellaria barbata* D. Don (SBD), against SARS-CoV-2 infection both *in vitro* and *in vivo*. Using a viral replicon particle (VRP)-based mouse model of SARS-CoV-2 infection, our study revealed that SBD extracts can reduce viral load in mouse lungs and alleviate the viral induced pneumonia. *In vitro* antiviral determination further validated the direct acting antiviral efficacy of SBD extracts against SARS-CoV-2 replication. Mechanistic studies demonstrated that SBD can act against SARS-CoV-2 replication by targeting both 3-chymotrypsin-like and papain-like cysteine proteases, via a combination of multiple active constituents. Moreover, SBD can modulate the host inflammation response in a bi-directional manner, which also contribute to the mitigation of viral induced acute lung injury. In summary, our study provides SBD as a promising therapeutic agent to combat SARS-CoV-2 infections that merit further development.

INTRODUCTION

The coronavirus disease 2019 (COVID-19), which is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has resulted in more than 776 million reported cases and caused 7 million deaths globally. On May 5th, 2023, the World Health Organization (WHO) declared that COVID-19 is no longer a global health emergency; however, SARS-CoV-2 variants of concern (VOC) that escape immune response from previous infection or vaccination continue to circulate around the world and remain a substantial threat to human health (Yisimayi et al., 2024). Although numerous anti-SARS-CoV-2 therapeutics such as molnupiravir and nirmatrelvir/ritonavir are currently available, these antivirals possess intrinsic limitations. For instance, molnupiravir is a mutagen and should not be used by pregnant women, while nirmatrelvir/ritonavir has drug-drug interaction concerns due to its inhibitory effect against microsome enzymes (Saravolatz et al., 2023). Moreover, the emergence of mutant SARS-CoV-2 strains with antiviral resistance has further emphasized the need to develop novel effective interventions against SARS-CoV-2 infections (Liu et al., 2023; Sjaarda et al., 2023; Zuckerman et al., 2024).

Scutellaria barbata D. Don (SBD) is a widely used herb in traditional Chinese medicine (TCM), possessing a variety of pharmacological activities including anticancer, antioxidant, anti-inflammatory, antiviral, and antibacterial effects (Chen et al., 2020; Wang et al., 2020). Previously, we have identified that a pre-purified fraction of SBD shows inhibitory effect against the main protease (Mpro) of SARS-CoV-2 using a fluorescence resonance energy transfer (FRET)-based enzymatic assay (Li et al., 2021). The 3CLpro, as well as papain-like protease (PLpro) are viral-coded cysteine proteases of SARS-CoV-2, exerting critical roles during viral replication and have become attractive targets for antiviral drug development (Tan et al., 2024; Zhang et al., 2020). Besides, Huang et al. have reported that SBD blocks the cellular protease transmembrane protease serine 2 (TMPRSS2), which plays crucial role during SARS-CoV-2 entry into

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host cells (Huang et al., 2021). These data suggest that SBD is a potential inhibitor of SARS-CoV-2.

Considering SARS-CoV-2 is classified as a biosafety level 3 (BSL-3) pathogen and requires operation in BSL-3 containment laboratories, we have recently developed a replicon viral particle (VRP) system as an alternative approach for the antiviral evaluation of potential anti-SARS-CoV-2 inhibitors (Chen et al., 2024). The SARS-CoV-2 replicon genome was generated by deleting the viral Spike (*S*) gene and was packaged into the VRPs incorporating an envelope expressing the VSV glycoprotein (G), which can efficiently transduce the viral replicons into diverse cell lines and can be also used to infect mice, providing a convenient and feasible approach to evaluate the effects of antiviral agents both *in vitro* and *in vivo*. Moreover, since the SARS-CoV-2 VRPs can initiate single-cycle infections only without production of infectious progeny virions, this system can be safely handled in BSL-2 laboratories (Chen et al., 2024).

In the present study, the SARS-CoV-2 VRP system was employed to evaluate the antiviral activity of water and ethanol extracts of SBD *in vitro* and *in vivo*. It was demonstrated that SBD extracts can markedly alleviate the SARS-CoV-2 induced pneumonia in mice, by directly reducing virus replication as well as modulating the viral induced inflammatory response. Our data validate SBD as a potential candidate for further development to treat COVID-19.

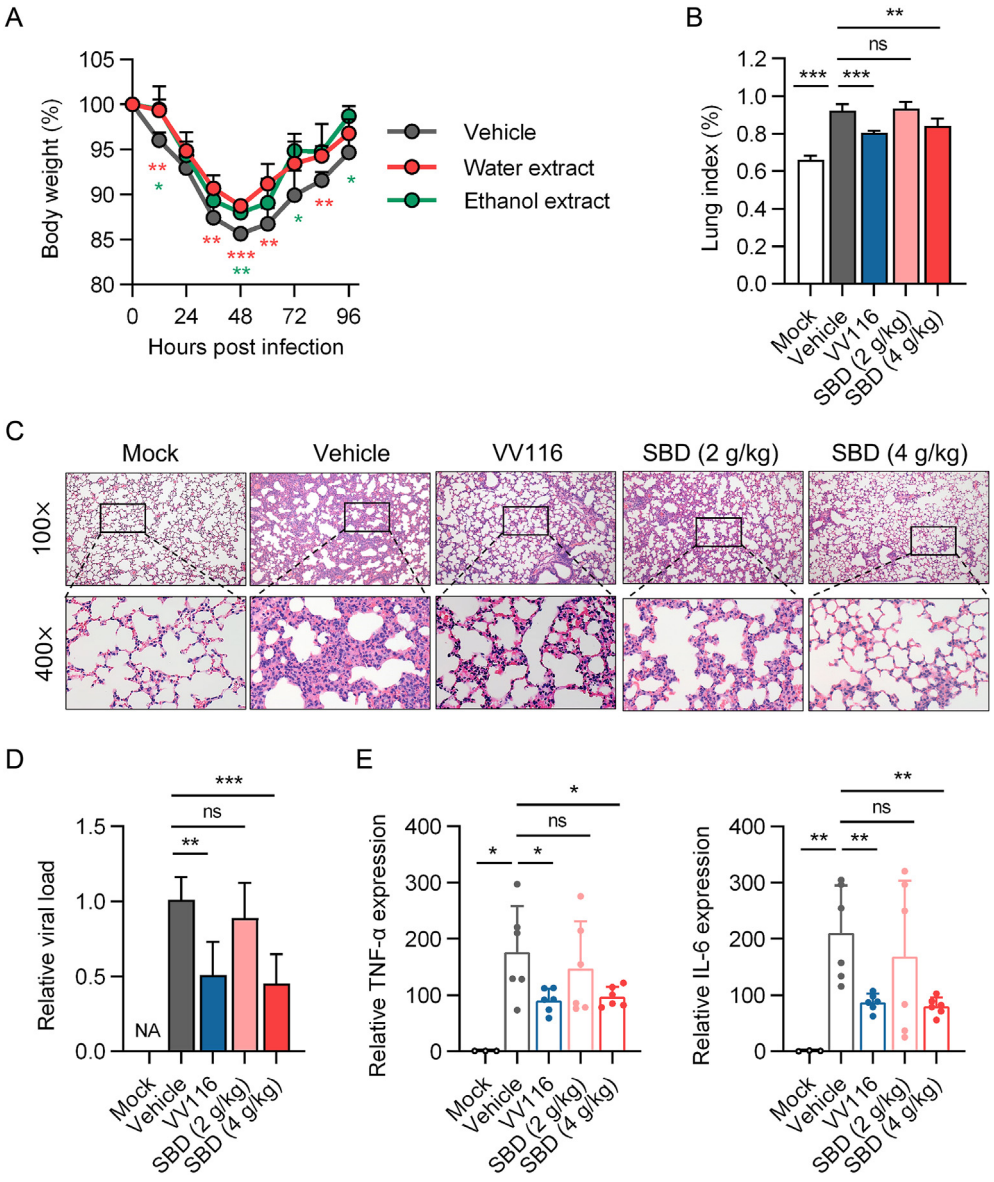

Fig. 1. *Scutellaria barbata* D. Don (SBD) extracts protect mice from SARS-CoV-2 infection. **A** Body weight changes. Female BALB/c mice (4–6 weeks old, n = 5) were intranasally inoculated with ΔS-VRP(G)-Luc at 1×10^{11} copies/mouse. Water or ethanol extract of SBD corresponding to 4 g/kg raw herbs were administered via intragastric and the treatments were given twice daily starting at 2 days prior to virus infection, for 5 days. PBS was used as vehicle control. The bodyweight changes were monitored every 12 h. **B–E** Female BALB/c mice (4–6 weeks old, n = 6) were intranasally inoculated with ΔS-VRP(G)-Luc at 2×10^{10} copies/mouse and treated with increasing doses of water extract of SBD (2 or 4 g/kg) via intragastric administration twice daily until the end of the experiment, starting at 2 days prior to virus infection. Mice receiving VV116 (100 mg/kg, starting at 2 h prior to virus infection) were used as control. At 24 hpi, the mice were euthanized and the lung tissues from each group were excised for lung index analysis (**B**), hematoxylin-and-eosin (H&E) staining (**C**), viral load determination (**D**), and expression analysis of pro-inflammatory cytokines TNF-α and IL-6 (**E**). Data are shown as mean ± SD for each group. Student's *t*-test was used to evaluate the statistical significance between indicated groups: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. ns, not significant.

RESULTS

SBD extracts reduce the viral load in SARS-CoV-2-infected mouse and alleviate viral-induced acute lung injury

To evaluate the therapeutic properties of SBD against SARS-CoV-2 infection *in vivo*, female BALB/c mice (4–6 weeks old) were infected with ΔS-VRP(G)-Luc via intranasal inoculation at a dose of 1×10^{11} copies/mouse. The water or ethanol extract of SBD corresponding to 4 g/kg of raw herbs were administered via intragastric for 5 days, starting at 2 days prior to virus challenge. The body weight change of each mouse was monitored every 12 h. As Fig. 1A shows, the body weight of mice that received vehicle treatment dropped rapidly upon infection with ΔS-VRP(G)-Luc, while both water and ethanol extracts of SBD remarkably alleviated body weight loss, implying that both water and ethanol extracts of SBD possess a protective effect against SARS-CoV-2 infection. Subsequently, we decide to focus on the water extract in the further investigations.

To better understand the mechanisms underlying the *in vivo* protective effect of SBD, another batch of BALB/c mice (4–6 weeks old) was inoculated with ΔS-VRP(G)-Luc at a dose of 2×10^{10} copies/mouse and treated with SBD extract at increasing doses corresponding to 2–4 g/kg of raw herbs. As a positive control, a group of infected mice were treated with 100 mg/kg of VV116, which is an approved oral derivative of remdesivir, a viral RNA-dependent RNA polymerase inhibitor (Xie et al., 2021). At 24 hpi, the mice were euthanized and the lung tissues were excised and weighed to calculate the lung index. As shown in Fig. 1B, the vehicle treated group showed a markedly elevated lung index compared to the mock-infected group, which is indicative of viral-induced acute lung injury. Notably, VV116 and the higher dose of SBD (4 g/kg) were able to lower the mouse lung index compared to the vehicle group, suggesting that both treatments can ameliorate the extent of SARS-CoV-2-induced pneumonia (Fig. 1B). In addition, the histopathologic changes in the lungs of the mice from each group were analyzed by H&E staining. It can be observed that the infection with ΔS-VRP(G)-Luc induced severe pathological changes in lungs of the vehicle-treated mice, characterized by interstitial pneumonia, alveolar collapse, septal thickening, and mononuclear cell infiltration; whereas SBD remarkably mitigated these tissue damages in a dose-dependent manner (Fig. 1C).

To evaluate whether the protective effect of SBD against SARS-CoV-2 infection in mice could be attributed to the decrease of viral replication, the viral genomic RNA levels in mice lungs were examined using RT-qPCR. As expected, the higher dose of SBD (4 g/kg) markedly reduced the viral loads, comparably to the reduction observed in the VV116-treated group (Fig. 1D). Furthermore, as an increase in the expression levels of the pro-inflammatory cytokines TNF-α and IL-6 in the mice lungs was detected upon infection with ΔS-VRP(G)-Luc, the treatments with VV116 and SBD (4 g/kg) significantly reduced the level of these pro-inflammatory mediators (Fig. 1E). It is worth noting that SBD may reduce the viral load and alleviated lung injury in mice by blocking the VSV-G mediated infection of ΔS-VRP(G)-Luc. To exclude this possibility, we tested the inhibitory effect of SBD against the infection of a recombinant VSV carrying a reporter GFP gene. As shown in Supplementary Fig. S1, SBD extracts did not interfere with VSV-G mediated entry at non-toxic concentrations.

Altogether, these data demonstrate the therapeutic potential of SBD extracts against SARS-CoV-2 infection *in vivo*, most likely through a dual mechanism of direct inhibition of viral replication and reduction on pro-inflammatory response in the lungs.

SBD extract inhibits viral replication by reducing the enzymatic activity of SARS-CoV-2 3CLpro and PLpro

To validate the direct-acting antiviral activity of SBD against SARS-CoV-2, the ΔS-VRP(G)-Luc virus were used to infect Vero-E6 cells in presence of increasing concentrations of the water SBD extract. As a result, the SBD extract showed inhibitory effect against SARS-CoV-2 replication, with an IC_{50} value of 1.68 mg/mL (Fig. 2A). Non-infected Vero-E6 cells were also treated with increasing concentrations of SBD extracts to evaluate their cytotoxicity, producing an CC_{50} value of 16.18 mg/mL and a reasonable selectivity index (SI = 9.6) (Fig. 2A). Similar antiviral activities were also detected using A549 and Huh7.5 cell lines (Supplementary Fig. S2). More specifically, the ΔS-VRP(G)-Luc infected cells were subjected to an immunofluorescence staining using viral NP-specific antibodies, and it showed that the SBD extract reduced viral NP expression in a dose-dependent manner (Fig. 2B). These results suggest the direct inhibitory effect of SBD against SARS-CoV-2 replication *in vitro*.

Previously, we identified the ethyl acetate fraction of SBD extract showed potential inhibitory effect against the enzymatic activity of SARS-CoV-2 main cysteine protease, 3CLpro (Li et al., 2021). To evaluate if crude extracts of SBD could also exhibit anti-SARS-CoV-2 activity by targeting the 3CLpro, a FRET-based enzymatic inhibition assay was conducted and it revealed that SBD extract blocks 3CLpro activity in a dose-dependent manner, with an IC_{50} value of 0.23 mg/mL. In addition, since a second viral cysteine protease, PLpro, also exert critical role during SARS-CoV-2 life cycle, we asked if SBD extract possess potential inhibitory effect against the enzymatic activity of PLpro. As a result, a dose-dependent inhibition of PLpro activity by SBD extract was observed through a fluorescence-based enzymatic inhibition assay, with an IC_{50} value of 0.10 mg/mL. These data demonstrated that SBD extract inhibits SARS-CoV-2 replication by a dual-mechanism of action targeting both 3CLpro and PLpro.

SBD extract acts against SARS-CoV-2 replication via a combination of multiple active constituents

The chemical composition of the SBD extracts has been investigated previously, and 141 constituents were identified collectively (Li, 2024; Ru et al., 2014). To better understand the pharmacological mechanism of SBD against SARS-CoV-2 replication, the 141 constituents were evaluated through virtual docking analysis to screen for potential active ingredients against 3CLpro or PLpro. The predicted binding affinities of each constituent to 3CLpro and PLpro are listed in Supplementary Tables S2 and S3 respectively. As summarized in Fig. 3A, 20 constituents were predicted to bind to 3CLpro with a ΔG of < -8.0 kcal/mol. These compounds were selected as hit 3CLpro inhibitors. For PLpro, although only one constituent exhibited reasonable predicted binding affinity with a ΔG of < -8.0 kcal/mol, 24 compounds in total showed higher affinity to this protease when compared to GRL0617, a well characterized PLpro inhibitor (Fig. 3B) (Shen et al., 2022). Therefore, these 24 compounds were "cherry picked" as potential PLpro inhibitors for further analysis. Interestingly, 8 compounds were predicted to possess both 3CLpro and PLpro inhibitory activities (Table 1, Fig. 3C). Indeed, numerous of the hit compounds have been reported as SARS-CoV-2 inhibitors, including ursolic acid (Lee et al., 2021), apigenin (Chaves et al., 2022), Scutellarein (Zhao et al., 2021), scutellarin (Li et al., 2022), eriodictyol (Cheng et al., 2023), baicalin (Liu et al., 2022), and Oroxylin A (Li et al., 2022) (Table 1).

Next, two of the hit compounds, ombuoside and chrysin, were tested for their anti-SARS-CoV-2 activity using the ΔS-VRP(G)-Luc. Ombuoside is predicted to show relatively high affinities to both 3CLpro and PLpro, with ΔG of −9.1 and −8.1 kcal/mol, respectively; while chrysin seems to be a specific 3CLpro inhibitor (Table 1). Ursolic acid, as one of the previously validated SARS-CoV-2 inhibitors among the SBD constituents, was tested in parallel as a positive control. As shown in Fig. 3D, ursolic acid and chrysin (but not ombuoside) exhibited inhibitory effect against SARS-CoV-2 replication, with IC_{50} values of 39.82 and 66.97 μM, respectively. Although these active compounds show low potency of inhibition when tested individually, SBD should have act against SARS-CoV-2 replication via a combination of multiple active constituents.

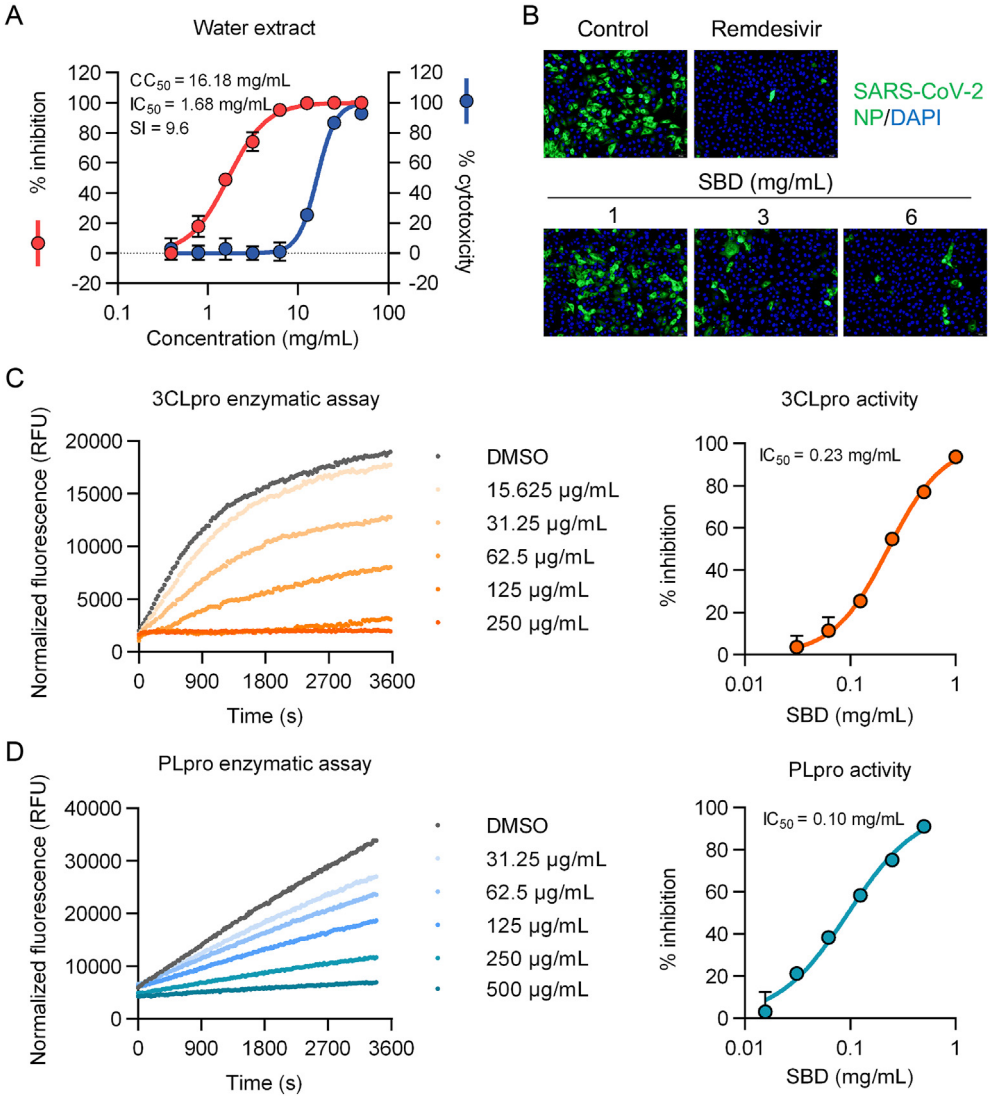

Fig. 2. *In vitro* antiviral determination of SBD against SARS-CoV-2 replication. **A** Dose-dependent inhibition of SBD against ΔS-VRP(G)-Luc replication. VeroE6 cells were infected with ΔS-VRP(G)-Luc viruses in the presence of increasing concentrations of water extract of SBD, and luciferase activity was measured at 36 hpi. The cytotoxicity assay in non-infected cells was performed using CCK-8. **B** Immunofluorescence assay. VeroE6 cells infected with ΔS-VRP(G)-Luc in the presence of negative control (DMSO), positive control (remdesivir), as well as indicated concentrations of SBD extract were stained using SARS-CoV-2 NP specific antibodies, at 36 hpi. **C** 3CLpro enzymatic inhibition assay. A FRET-based enzymatic assay of SARS-CoV-2 3CLpro was carried out in the presence of increasing concentrations of SBD extract. The fluorescence elimination curves as well as the IC_{50} curves are shown. **D** PLpro enzymatic inhibition assay. A fluorescence-based enzymatic assay of SARS-CoV-2 PLpro was carried out in the presence of increasing concentrations of SBD extract. The fluorescence elimination curves as well as the IC_{50} curves are shown. RFU, relative fluorescence units. Data are shown as mean ± standard deviation of three replicates.

SBD extract exhibits regulatory effects on host inflammatory responses

In ΔS-VRP(G)-Luc infected mouse models, SBD reduced the expression of pro-inflammatory cytokines as well as the viral load (Fig. 1). Despite it is reasonable to assume that the reduction in the level of pro-inflammatory mediators is a consequence of a decrease in viral replication, we asked whether SBD possesses direct anti-inflammatory properties additionally. To this end, Raw264.7 cells were infected with ΔS-VRP(G)-Luc or stimulated with lipopolysaccharide (LPS), followed by treatment with various concentrations of SBD extracts. As expected, the expression of a panel of pro-inflammatory mediators, including monocyte chemoattractant protein-1 (MCP-1), inducible nitric oxide synthase (iNOS), IL-6, IL-1β and TNF-α, significantly increased upon either ΔS-VRP(G)-Luc infection or LPS stimulation (Fig. 4A and B). The highest dose of SBD (10 mg/mL) strikingly reduced the ΔS-VRP(G)-Luc- or LPS-induced expression of all these pro-inflammatory factors, except for TNF-α (Fig. 4A and B). In contrast, lower doses of SBD (2.5–5 mg/mL) seem to further boost the expression of IL-6, IL-1β and TNF-α upon infection with ΔS-VRP(G)-Luc (Fig. 4A), and iNOS in response to LPS stimulation (Fig. 4B). These data suggest that SBD extracts may exhibit bi-directional modulatory effects on the host inflammatory responses independent of the stimulus.

SBD extract can regulate the host inflammation response in a bi-directional manner

To better understand the bi-directional modulatory effects of SBD on host inflammatory response, we at first examined the dose-dependent regulation of SBD on the expression of pro-inflammatory cytokines without exogenous stimuli. As a result, the expression of IL-6 and TNF-α were up-regulated by SBD in a dose-dependent manner (Fig. 5A). MCP-1

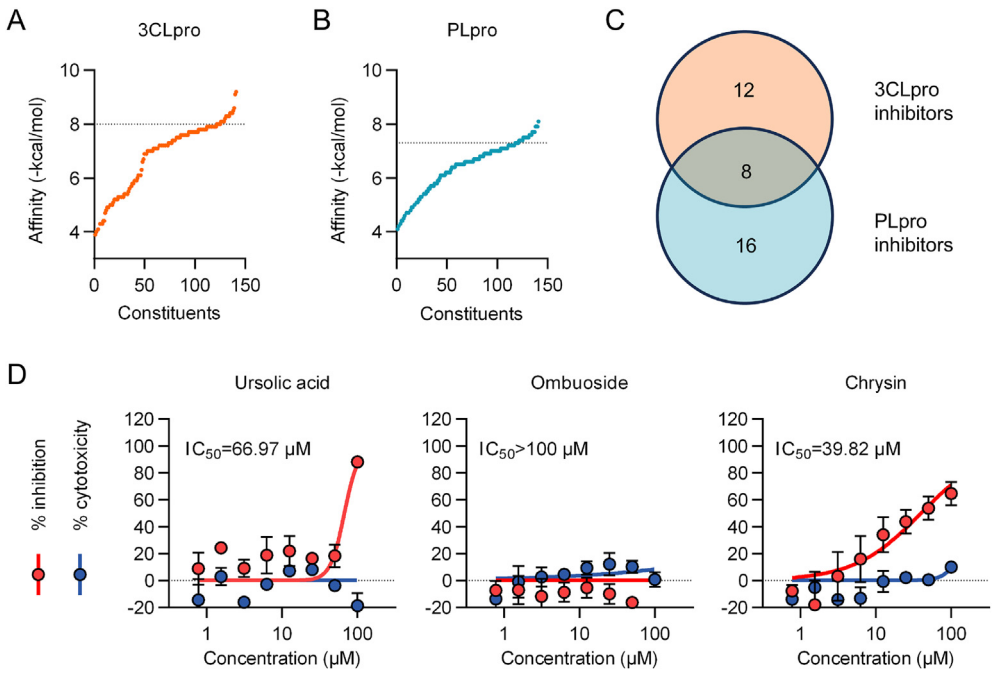

Fig. 3. Material basis underlying the anti-SARS-CoV-2 mechanism of SBD. Predicted affinity of each SBD constituent to SARS-CoV-2 3CLpro (**A**) or PLpro (**B**). The 141 constituents of SBD were docked into 3CLpro (PDB ID: 7K0E) or PLpro (PDB ID: 7CJM) and the predicted affinity is indicated by the -ΔG value. **C** Venn diagram of the numbers of predicted 3CLpro inhibitors and PLpro inhibitors from SBD constituents. **D** *In vitro* antiviral determination. Three predicted 3CLpro and or PLpro inhibitors were selected for antiviral determination using the ΔS-VRP(G)-Luc system. The cytotoxicity assay was performed using CCK-8. Data are shown as mean ± SD of three replicates.

expression reached a plateau at 2.5 mg/mL and higher concentrations of SBD (Fig. 5A). An upregulation in the expression of iNOS and IL-1β was also observed when the cells were treated with up to 5 mg/mL of SBD (Fig. 5A). However, a higher concentration (10 mg/mL) on the contrary led to a decrease in the mRNA levels of iNOS and IL-1β (Fig. 5A).

Next, we evaluated the regulatory effect of SBD in the presence of increasing doses of exogenous lipopolysaccharide (LPS). As shown in Fig. 5B, the expression levels of the inflammatory mediators MCP-1, iNOS, IL-6, IL-1β and TNF-α increased in accordance with the increasing LPS stimulation intensity (Fig. 5B). It is noteworthy that the iNOS expression saturated under the stimulations of LPS at about 0.1 ng/mL and the higher concentrations (Fig. 5B). Interestingly, the regulatory effect of SBD depends on the intensity of LPS stimulation. Generally, the expression level of the inflammatory mediators was upregulated in cells stimulated with none or low concentrations of LPS, however, as the intensity of LPS stimulation increases, the regulatory indexes of SBD become smaller, and finally converted to downregulation (Fig. 5B, Supplementary Fig. S3). As a consequence, it is visible that the expression levels of the aforementioned pro-inflammatory mediators were held almost constant in the presence of either 5 or 10 mg/mL SBD extracts, independent of the intensity of exogenous stimuli (Fig. 5B). Indeed, the higher concentration (10 mg/mL) of SBD performed better (Fig. 5B).

Together, these data firmly evidenced the bi-directional modulatory effect of SBD that contribute to the maintenance of host immune homeostasis, although the underlying specific mechanism still deserves further investigation.

DISCUSSION

Herbal medicines had played critical roles to prevent and control the COVID-19 pandemic. For instance, a traditional Chinese medicine formula, Qingfei Paidu decoction (QFPDD), was prescribed soon after the SARS-CoV-2 outbreak emerged and was recommended for the treatment of COVID-19 in China. Retrospective studies have revealed that favorable outcomes including faster recovery and lower risk of in-hospital mortality were closely associated with the clinical use of Qingfei Paidu decoction (Shi et al., 2020; Zhang et al., 2021). Development of novel anti-SARS-CoV-2 herbal medicines can greatly expand our arsenal to combat the existing and emerging SARS-CoV-2 circulating strains.

In this study, we validated the inhibitory effect of SBD extracts against SARS-CoV-2 replication both *in vitro* and *in vivo*. In addition, SBD significantly mitigated the acute lung injury induced by SARS-CoV-2 infection in mice, as a consequence of attenuated viral replication and alleviated inflammatory response.

As a direct-acting antiviral agent, SBD extracts were further demonstrated to block the hydrolytic activities of both 3CLpro and PLpro of SARS-CoV-2. Furthermore, the material basis for the anti-SARS-CoV-2 activity of SBD were analyzed, and multiple constituents of SBD have been identified as promising SARS-CoV-2 inhibitors, including ursolic acid, apigenin, scutellarein, scutellarin, eriodictyol, baicalin, oroxylin A, and chrysin (Table 1). Besides, it can be anticipated that additional active constituents, targeting to either 3CLpro, PLpro or possibly other viral/host components, should be validated in the future. Although the antiviral activity of each of the single active compound is relatively low, additive or synergistic effect can be anticipated when these active components were administered in combination. Moreover, compared to the currently approved anti-SARS-CoV-2 drugs, SBD extracts could represent a benefit, since a medication with multiple active constituents are predicted to have a lower chance of drug resistance (Gaisina et al., 2024; Zhang et al., 2023).

Apart from the direct-acting antiviral activity, SBD possess a bi-directional modulatory effect on the viral-induced inflammation. Generally, the inflammatory response of the host is a double-edged sword. The prompt and adequate inflammation plays a critical role to restrict the viral spread during early stages of a virus infection, whereas excessive inflammatory response may evocate a cytokine storm, which on the other hand leads to severe immune damages (Jamilloux et al., 2020). Therefore, the therapeutic effect of single directional immune

Table 1
The predicted binding affinity of SBD constituents against SARS-CoV-2 3CLpro and/or PLpro, and their antiviral potential.

Number	Constituent	ΔG (-kcal/mol)		Antiviral activity [a]
		3CLpro	PLpro	
1	Ursolic acid	−9.2	−7.5	Yes (Alhadrami et al., 2021)
2	Ombuoside	−9.1	−8.1	No (Fig. 3C)
3	Scutebartine C	−8.4	−7.5	NA[b]
4	Scutestrigillosin D	−8.4	−7.5	NA
5	Luteolin	−8.3	−7.4	No (Mori et al., 2023)
6	(2R)-5,7-dihydroxy-2-(4-hydroxyphenyl) chroman-4-one	−8.2	−7.9	NA
7	Chrysin-5-methylether	−8.1	−7.4	NA
8	Barbaolide E	−8	−7.6	NA
9	Barbaolide F	−8.6	–	NA
10	Scutebartine H	−8.5	–	NA
11	Scutegxbatine C	−8.4	–	NA
12	Apigenin	−8.3	–	Yes (Chaves et al., 2022)
13	Barbaolide B	−8.3	–	NA
14	Barbaolide K	−8.3	–	NA
15	6-O- Nicotinoylbarbatin A	−8.1	–	NA
16	Chrysin	−8.1	–	Yes (Fig. 3C)
17	Scutebartine I	−8.1	–	NA
18	Barbatine D	−8	–	NA
19	Scutebarbatine L	−8	–	NA
20	Scutellarein	−8	–	Yes (Zhao et al., 2021)
21	Chlorogenic	–	−7.9	NA
22	Scutellarin	–	−7.9	Yes (Li et al., 2022)
23	2-hydroxy-3-methylanthraquinone	–	−7.7	NA
24	Eriodictyol	–	−7.7	Yes (Cheng et al., 2023)
25	Isomartynoside	–	−7.7	NA
26	Baicalin	–	−7.6	Yes (Liu et al., 2022)
27	Scutebarbatine B	–	−7.6	NA
28	2',3',5,7-tetrahydroxyflavone	–	−7.5	NA
29	Cholesta-6,22,24-trien,4,4-dimethyl	–	−7.5	NA
30	Martynoside	–	−7.5	NA
31	(8S,9S,10R,13R,14S,17R)-10,13-dimethyl-17-[(E,1R,4R)-1,4,5-trimethylhex-2-enyl]-2,3,8,9,11,12,14,15,16,17-decahydro-1H-cyclopenta[a] phenanthrene	–	−7.4	NA
32	Oroxylin A	–	−7.4	Yes (Li et al., 2022)
33	24-Ethylcholest-4-en-3-one	–	−7.3	NA
34	Barbartin C	–	−7.3	NA
35	Barbatine A	–	−7.3	NA
36	Scutebarbatine A	–	−7.3	NA

Note:
[a] The antiviral activity was determined using cellular-based antiviral assays with either authentic SARS-CoV-2 or ΔS-VRP(G)-Luc viruses.
[b] NA, not applicable. The antiviral activities of these compounds have not been validated using authentic SARS-CoV-2 or ΔS-VRP(G)-Luc viruses.
-, not predicted as target of binding.

regulators, either stimulators or suppressors, depends a lot on the immune status of the host. For instance, administration of an immune stimulator in prophylaxis or early may benefit a lot, however, the delayed use especially in the status of systematic hyperinflammation may confer risk of further clinical deterioration. Vice versa, an immune suppressor may alleviate the inflammation and tissue damage in selected patients, while its misuse in early phases of illness may dampen the host antiviral immunity unanticipatedly (De Stefano et al., 2020; Jamilloux et al., 2020). So far, it is challenging to monitor the immune status in real time to decide a precise "window of therapeutic opportunity" to use the immune regulators in patients affected primarily by an infectious disease (Del Valle et al., 2020). Valuably, SBD balances both the benefits and risks of immune modulations carefully by a mode of bi-directional regulation. This herbal medicine can boost the inflammatory response in prophylaxis or early stages of infection; oppositely, it relieves the hyperinflammation by downregulating the pro-inflammatory cytokines. It is interesting to further investigate the mechanism and material basis underlying the unique bi-directional immune modulatory effect of SBD in the future.

CONCLUSIONS

The present study demonstrated the therapeutic effect of SBD against SARS-CoV-2 infection. First, SBD showed direct acting anti-SARS-CoV-2 activity both *in vitro* and *in vivo*. Second, SBD can modulate the viral induced inflammation response in a bi-directional manner. These two mechanisms of action in combination contribute to the mitigation of SARS-CoV-2 induced acute lung injury. In summary, our data validated the potential of SBD for further development as a novel COVID-19 therapeutic agent.

MATERIALS AND METHODS

Cells and viruses

African green monkey kidney epithelial cells (VeroE6) and mouse monocyte macrophage leukemia cells (Raw264.7) were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (10,000 U/ml).

The viral replicon particles of SARS-CoV-2 carrying a luciferase reporter gene, termed ΔS-VRP(G)-Luc, was generated and titrated as previously described (Chen et al., 2024).

Preparation of SBD extracts

SBD (batch no. 20231019) was purchased from Beijing Tongrentang Qilu (Jinan) Pharmacy Co., Ltd. For water extraction, 50 g SBD was boiled in 1 L deionized water for 1 h and the extract was collected by filtration. The boiling process was repeated twice. Then the two batches of extracts were combined and centrifuged at 8000 rpm for 15 min at room temperature. The supernatant was collected, concentrated and freeze dried into powder. For ethanol extraction, 50 g SBD was soaked in 1 L 95% ethanol in the round-bottomed flask and the solutions were refluxed for 1 h. The extract was collected by filtration, and the extraction process was repeated twice. Then the two batches of extracts were combined and centrifuged at 8000 rpm for 15 min at room temperature. The supernatant was collected, concentrated and freeze dried into powder. The rates of water and ethanol extractions were 25.4% and 15.6%, respectively. Both the water and ethanol extracts of SBD were dissolved in PBS for subsequent studies.

In vitro antiviral determination

Vero E6 cells seeded (1×10^5 cells/well) in white, flat-bottom 96-well plates were infected with ΔS-VRP(G)-Luc at 1 relative light unit (RLU) per cell. After 2 h of incubation, the inoculum was removed and fresh medium containing increasing concentrations of SBD extract was added. At 24 h post infection (hpi), the luciferase activity was assessed in infected cells using the Neolite Reporter Gene Assay System (PerkinElmer, MA, USA) according to the manufacturer's instructions. The luminescence signal was read using the Synergy Neo2 Multi-Mode Reader (BioTek, VT, USA). The reduction of RLU was compared to the DMSO treated cells, to evaluate the inhibition of viral replication. Non-infected cells were also treated with SBD extracts to examine the extracts' cytotoxicity using the Cell Counting Kit-8 (CCK-8; MCE, NJ, USA).

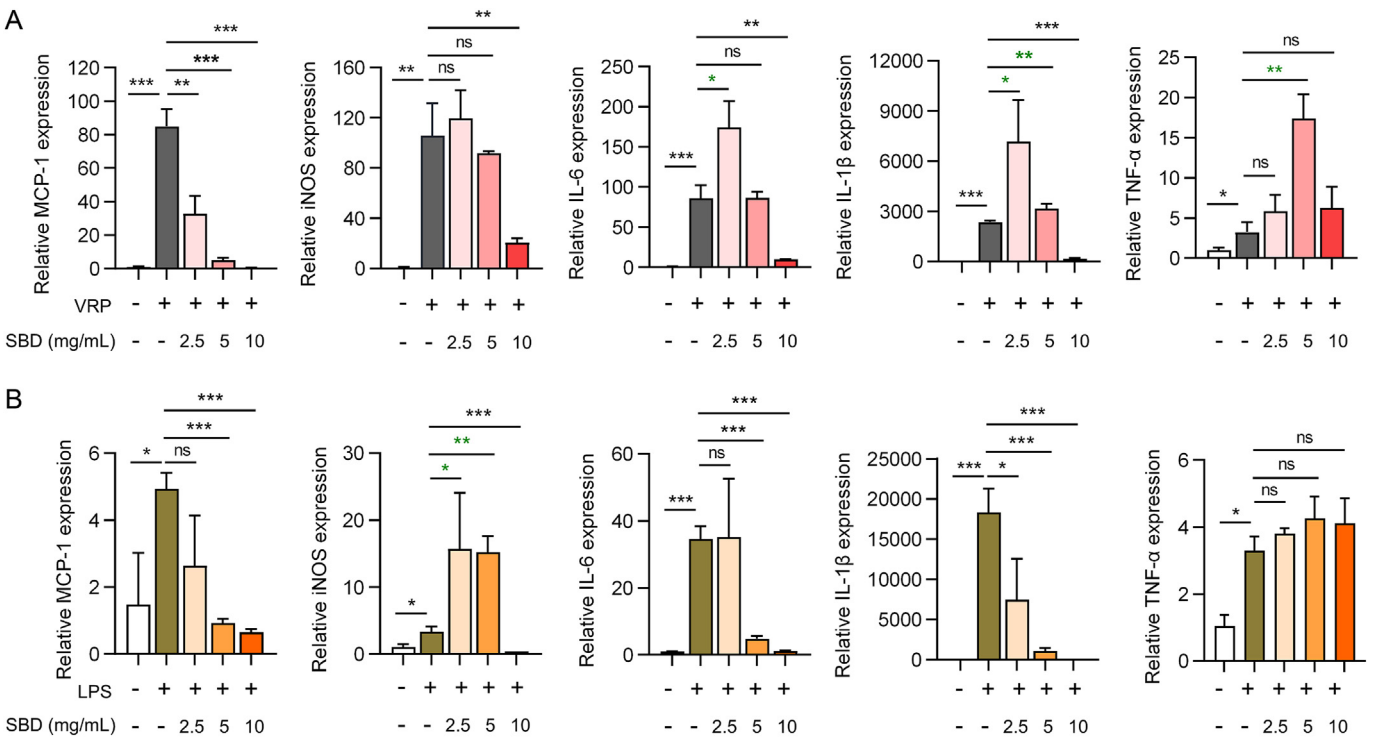

Fig. 4. Regulation of the inflammatory responses by SBD extract in mouse macrophage cells. The Raw264.7 cells were infected with ΔS-VRP(G)-Luc (**A**) or stimulated with lipopolysaccharide (LPS, 500 ng/mL, **B**) and treated with various concentrations of SBD extract for 24 h. Cells were then collected and the expression levels of pro-inflammatory factors including MCP-1, iNOS, IL-1β, IL-6 and TNF-α were detected using RT-qPCR. Mock stimulated cells were used as control. Student's *t*-test was used to evaluate the statistical significance between indicated groups: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. ns, not significant.

Immunofluorescence assay

Vero E6 cells were seeded on 24 well plates for 12 h and then infected with ΔS-VRP(G)-Luc. After 2 h of incubation, the inoculum was removed and fresh medium containing increasing concentrations of SBD extract was added. At 24 hpi, cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. Cells were then sequentially incubated with a primary antibody against the SARS-CoV-2 nucleocapsid protein (NP) (1:100, ABclonal) and the secondary fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG antibody (1:100, Thermo Fisher Scientific, Invitrogen). The cell nucleus was stained with 4,6-diamidino-2-phenylindole (DAPI, Sigma). The fluorescence images were acquired using an inverted fluorescence microscope (Olympus- IX73).

Enzymatic inhibition assay

The enzymatic inhibition assay of the viral proteases 3CLpro and PLpro were conducted as previously described (Du et al., 2021). Briefly, the recombinant SARS-CoV-2 3CLpro (250 nM at a final concentration) or PLpro (100 nM at a final concentration) and SBD extract were added in 90 μL of assay buffer (50 mM Tris–HCl, pH7.3 for 3CLpro and 20 mM Tris-buffer, pH 8.0 for PLpro) and incubated for 30 min. The reaction was initiated by adding 10 μL of 50 mM fluorogenic substrate (Dabcyl-KTSAVLQSGFRKME-Edans) for 3CLpro detected with excitation at 336/20 nm and emission at 490/20 nm using a Bio-Tek Synergy4 plate reader. For PLpro, 30 mM fluorogenic substrate Z-Arg-Leu-Arg-Gly-Gly-AMC (Z-RLRGG-AMC) was added and detected with excitation at 360/20 nm and emission at 460/20 nm.

Animal experiments

Female BALB/c mice (4–6 weeks old) were anesthetized with isoflurane and inoculated with indicated doses of ΔS-VRP(G)-Luc intranasally. The infected mice were monitored for bodyweight changes every 12 h for 4 days or euthanized at 24 h pi. For *in vivo* antiviral evaluation, 2–4 g/kg/day of SBD extracts were administered via intragastric. The treatments were given twice daily, starting at two days before virus challenge. Mice that received VV116 or vehicle (PBS) were used as positive and negative controls, respectively.

RAW264.7 cell-based inflammation model

To induce inflammatory response in the RAW264.7 cells, either ΔS-VRP(G)-Luc viruses or lipopolysaccharide (LPS) were used as stimulus. To examine the regulatory effect of SBD on inflammation, RAW264.7 cells were treated with various concentrations of SBD extract in the absence or presence of different amounts of ΔS-VRP(G)-Luc or LPS. After 24 h-incubation, the cells were harvested to determine the expression level of a panel of pro-inflammatory cytokines using RT-qPCR.

RT-qPCR

The total RNAs were extracted from the mouse lung homogenates or Raw264.7 cells, followed by reverse transcription into cDNA using PrimeScript™ RT Master Mix (Takara, Japan). qPCR was performed using the TB Green *Premix Ex Taq*II kit (Takara, Japan), using a CFX Connect real-time PCR detection system (Bio-Rad, Germany). The following cycling conditions were used: 30 s at 95 °C for denaturation, 40 cycles of 5 s at 95 °C, and 30 s at 60 °C for primer annealing and DNA polymerase extension, respectively. The $2^{-\Delta\Delta CT}$ method was used to calculate the viral genomic RNA levels or the relative expression of pro-inflammatory cytokines from each sample. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal reference gene. The primers used for qPCR analysis are listed in Supplementary Table S1.

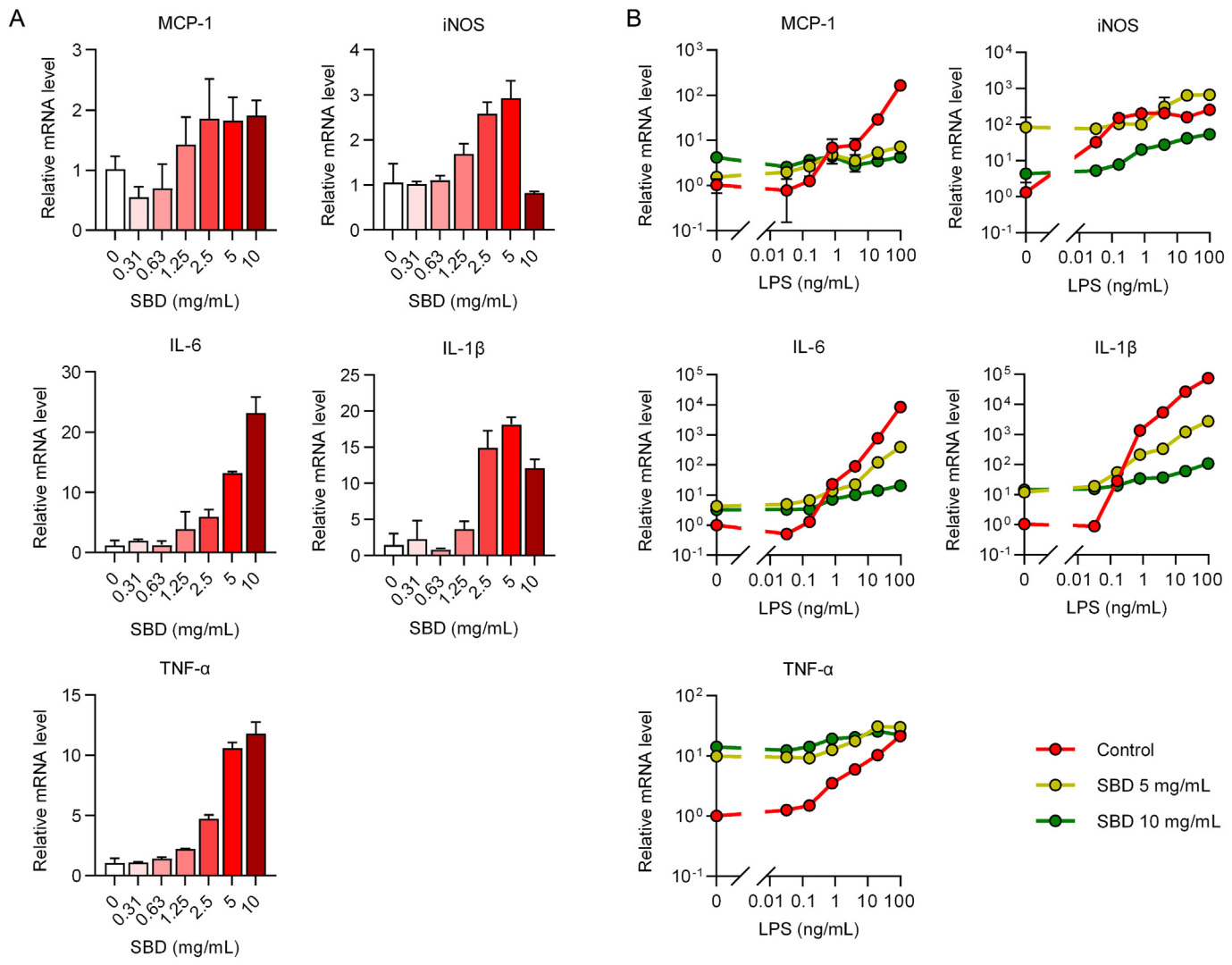

Fig. 5. Bi-directional modulation of LPS-induced inflammation by SBD extract in mouse macrophage cells. **A** The Raw264.7 cells were treated with increasing concentrations of SBD extract for 24 h, followed by RT-qPCR analysis for expressions of pro-inflammatory factors including MCP-1, iNOS, IL-1β, IL-6 and TNF-α. **B** The Raw264.7 cells were stimulated with increasing concentrations of LPS, in the absence or presence of different concentrations of SBD extract. After 24 h, the cells were collected to detect the expression levels of pro-inflammatory factors including MCP-1, iNOS, IL-1β, IL-6 and TNF-α using RT-qPCR.

Hematoxylin-and-eosin (H&E) staining

The mouse lungs were dissected and fixed in 4% formaldehyde and embedded in paraffin. The embedded tissues were cut into 5 μm thick sections and stained with H&E. The stained sections were viewed and captured using a BX53 microscope (Olympus, Japan).

Chemical composition analysis

The chemical constituents of SBD were collected through the traditional Chinese medicine systems pharmacology database and analysis platform (TCMSP; https://old.tcmsp-e.com/tcmsp.php) in combination with literature review (Li, 2024; Ru et al., 2014). Briefly, 98 compounds were recorded as SBD ingredients in TCMSP, moreover, Li et al. identified 43 additional constituents from SBD extracts. These 141 compounds were collected as the whole constituents of SBD for further analysis (Supplementary Table S2 and Table S3).

Molecular docking analysis

The 3D structures of 3CLpro (PDB ID: 7K0E) and PLpro (PDB ID: 7CJM) are retrieved from the protein database (https://www.rcsb.org/structure), while the SBD active component structure in MOL2 format is downloaded from the TCMSP database. Chemdraw software is employed to delineate the structure of the active component based on the literature, which is then saved in MOL2 format. Moreover, OpenBabel software is utilized to convert all component structures from MOL2 format to PDB format. Subsequently, the Schrodinger software is utilized to minimize the energy of the ligand compound, and AutoDock Tools 1.5.6 software is used to convert the protein and each SBD component to PDBQT format. Thereupon, AutoDock is employed for batch docking, and the resulting standard free energy changes (ΔG) were used to predict the binding affinities.

Statistical analysis

The student's *t*-test was performed to compare the significance of the difference between two groups. Results were considered significant when *P* value was lower than 0.05.

DATA AVAILABILITY

All relevant data that support the findings of this study are presented in the manuscript.

ETHICS STATEMENT

All animal procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Shandong University of Traditional Chinese Medicine (Approval No., SDUTCM20231129121).

AUTHOR CONTRIBUTIONS

Ruikun Du and Lijun Rong conceptualized the project and design of the research; Yan Ran and Zinuo Chen carried out the experiments; Lingyuan Fan participated in part of the experiment; Yan Ran, Zinuo Chen and Qinghua Cui contributed to data collection and analysis; Yan Ran, Ruikun Du, Carolina Q. Sacramento and Lijun Rong wrote the manuscript; Ruikun Du and Qinghua Cui provided grant support and supervised the study. All authors read and approved the submitted manuscript.

CONFLICT OF INTEREST

Prof. Lijun Rong is an editorial board member for *Virologica Sinica* and was not involved in the editorial review or the decision to publish this article. The authors declare no conflict of interest.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virs.2025.04.004.

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