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Original Article

Bangpungtongsung-san reduces the expression of angiotensin-converting enzyme 2 by blocking activating protein-1 activity in stimulated human mast cells

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ABSTRACT

Bangpungtongsung-san (BTS) is a traditional prescription that has been used for inflammation and bronchial diseases. Coronavirus disease in 2019 (COVID-19), which occurs due to a severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, is a respiratory inflammatory disease, and mast cells are one of the key cells involved in the cytokine storm. In this study, we aimed to investigate the regulatory effects and mechanisms of BTS and its active compound, α-pinene, on coronavirus infections and inflammatory responses. BTS and α-pinene decreased the expression of not only angiotensin-converting enzyme 2 (ACE2) but also transmembrane protease/serine subfamily member 2 (TMPRSS2) in stimulated human mast cell line, HMC-1 cells. Additionally, BTS and α-pinene significantly suppressed the release of key inflammatory cytokines, including interleukin (IL)-1β, IL-6, IL-8, thymic stromal lymphopoietin, and tumor necrosis factor-α, without compromising cell viability. Furthermore, BTS and α-pinene decreased the expression of transcription factors c-Fos and c-Jun in activated HMC-1 cells. In conclusion, the results of the study indicated that BTS inhibited the c-Jun/c-Fos signaling pathway, leading to a decrease in the levels of ACE2, TMPRSS2, and inflammatory cytokines. This suggests that BTS may be an effective agent in mitigating the cytokine storm induced by SARS-CoV-2 infection.

Keywords Bangpungtongsung-san, SARS-CoV-2, ACE2, Mast cell

INTRODUCTION

Coronavirus disease 2019 (COVID-19) is a respiratory illness caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which has led to a global health crisis.[1] SARS-CoV-2 binds to the angiotensin-converting enzyme 2 (ACE2) receptor expressed on the surface of host cells and enters the host cell through the activation of the transmembrane protease serine 2 (TMPRSS2).[2] In this process, the virus infiltrates the host cell, disrupting its function and triggering an inflammatory response.[3] Furthermore, SARS-CoV-2 increases the levels of inflammatory cytokines such as interleukin (IL)-1β, IL-6, thymic stromal lymphopoietin (TSLP), and tumor necrosis factor (TNF)-α, leading to acute pneumonia and inflammation, which can result in cytokine storm syndrome.[4,5] Cytokine storm can provoke an excessive immune response, causing severe organ damage and respiratory failure, which are critical complications of COVID-19.[5] SARS-CoV-2 activates mast cells in the respiratory submucosa, triggering the release of inflammatory cytokines, which in turn lead to the cytokine storm that exacerbates COVID-19.[6,7] Pathological studies of COVID-19 in deceased patients revealed the accumulation of mast cells in the lungs. These mast cells express ACE2, which facilitates SARS-CoV-2 binding, and TMPRSS2, which is essential for the activation of the viral spike protein.[8] Therefore, targeting mast cells with cytokine inhibitors or strategies aimed at ACE2 could play a crucial role in developing treatments to block SARS-CoV-2 infection and inflammation.

Bangpungtongsung-san (BTS) is a traditional herbal remedy known for its diverse pharmacological effects, such as modulating the immune system, improving respiratory conditions, and providing anti-inflammatory, analgesic, and antipyretic benefits.[9-11] BTS helps regulate immune responses by reducing excessive immune activation and lowering inflammatory cytokine levels, thereby supporting immune balance.[9] It also aids in alleviating symptoms associated with respiratory diseases like bronchitis and asthma, reducing inflammation, and relieving symptoms like coughing and

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phlegm.[10] In addition, BTS provides pain relief and helps lower fever, making it effective for treating symptoms of colds and inflammatory conditions. Additionally, BTS contains decursin, imperatorin, atractylenolide III, and α-pinene, with α-pinene being a component reported to have anti-inflammatory effects.[12-14] Due to its broad therapeutic effects, BTS has been widely used in traditional medicine for strengthening immunity and addressing respiratory issues.

Building on prior studies, we proposed that BTS and α-pinene could have a positive effect on SARS-CoV-2 infection and cytokine storm. To test this hypothesis, we investigated the impact of BTS and α-pinene on the level of ACE2 and inflammatory cytokines, along with their mechanisms of action, using the human mast cell line HMC-1.

MATERIALS & METHODS

Preparation of BTS

BTS was sourced from Omniherb Co. (Seoul, Republic of Korea) and its identity was confirmed by Professor Kim HM from the Department of Science in Korean Medicine, Kyung Hee University. A voucher specimen has been officially recorded in the department's collection at Kyung Hee University. BTS is composed of *Saposhnikovia divaricate* (4g), *Angelica gigas* (4 g), *Paeonia lactiflora* (4 g), *Cnidium officinale* (4 g), *Gardenia jasminoides* (4 g), *Forsythia viridissima* (4 g), *Mentha arvensis* (4 g), *Schizonepeta tenuifolia* (4 g), *Ephedra sinica* (4 g), *Rheum undulatum* (4 g), *Atractylodes japonica* (4 g), *Platycodon grandiflorum* (6 g), *Scutellaria baicalensis* (6 g), *Glycyrrhiza uralensis* (10 g), Gypsum (6g), Talcum (4 g), and *Natrii sulfas* (6 g). BTS was prepared by boiling the dried herbs in distilled water for roughly 2 h and 30 min. The resulting extract was filtered and freeze-dried, yielding approximately 10.62%. For further use, BTS (100 mg/ml) was dissolved in distilled water and passed through a 0.22 μm syringe filter for sterilization.

Cell culture

HMC-1 cells were maintained in Iscove's Modified Dulbecco's Medium containing 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were incubated at 37°C in a humidified atmosphere with 5% CO_2. These cells were kindly provided by Eichi Mori from Osaka University, Japan. To ensure they were free of mycoplasma contamination, a Mycoplasma detection kit (iNtRON Biotech, Sungnam, Korea) was used. HMC-1 cells were treated with BTS and α-pinene (Sigma-Aldrich) for 1 h, followed by stimulation with 5 μM PMA and 100 nM A23187 (PMACI) for varying time points. The concentrations of BTS (1 mg/ml) and α-pinene (1 μg/ml) used in this experiment were based on previous studies.[9,15]

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from HMC-1 cells using the easy-BLUE RNA extraction kit (iNtRON Biotechnology, Kyunggi-do, Korea). The RNA concentration was determined with a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). For cDNA synthesis, 2.5 μg of RNA was reverse-transcribed into cDNA using the Power cDNA Synthesis Kit (iNtRON Biotechnology, Sungnam, Republic of Korea). Quantitative real-time PCR (qRT-PCR) was conducted to measure the expression levels of human ACE2, TMPRSS2, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA, using SYBR Green Master Mix and the primers listed in Table 1. The expression levels were quantified using the ABI StepOne real-time PCR system (Applied Biosystems, Foster City, CA, USA). The ΔΔCT method was applied to analyze the data, with normalization against GAPDH expression levels.

Western blotting analysis

The cells were lysed using RIPA lysis buffer supplemented with phosphatase and protease inhibitors after being washed with PBS. The total protein extract (100 μg/ml) was then separated by electrophoresis and transferred onto nitrocellulose membranes. Primary antibodies targeting ACE2, TMPRSS2, c-Jun, c-Fos, and GAPDH were purchased from Cell Signaling Technology (MA, USA) and Santa Cruz (CA, USA). The membranes were incubated overnight at room temperature with these primary antibodies, diluted 1:500 in PBST, after being blocked for 2 h in 6% bovine serum albumin. Following PBST washes, membranes were incubated with a secondary antibody conjugated to horseradish peroxidase. Protein bands were visualized using the ImageQuant LAS 500 system (Cytiva, Marlborough, MA, USA), and the intensity of the specific bands was quantified with the ImageQuant TL software (v10.1, Cytiva).

Enzyme-linked immunosorbent assay (ELISA)

Table 1. The sequence of primers used for qRT-PCR analysis

Genes		Sequences
Human ACE2	forward	GGGATCAGAGATCGGAAGAAGAAA
	reverse	AGGAGGTCTGAACATCATCAGTG
Human TMPRSS2	forward	ACTCTGGAAGTTCATGGGCAG
	reverse	TGAAGTTTGGTCCGTAGAGGC
Human GAPDH	forward	CCAAAGGGTCATCATCTCTG
	reverse	CCTGCTTCACCACCTTCTTG

Inflammatory cytokine levels in the supernatant were measured using a sandwich ELISA method. Capture antibodies (1 μg/ml) specific to the target cytokines were coated onto a 96-well microplate and incubated overnight at room temperature. After the coating process, the plate was washed with PBST and blocked with a blocking buffer for 2 h. Following this, the plate was washed again, and the samples or standard proteins were added for a 3 h incubation. Afterward, a biotin-conjugated secondary antibody (20 ng/ml or 1 μg/ml) was applied, followed by the addition of avidin-horseradish peroxidase (1:400 dilution) and substrate solutions. The results were measured using an ELISA reader at 450 nm.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium (MTT) assay

The MTT assay (Sigma-Aldrich) was employed to evaluate cell viability by measuring the conversion of a tetrazolium compound into insoluble purple formazan crystals by the mitochondria of living cells. The optical density (OD) at 562 nm was then measured to assess the level of cell vitality.

Statistical analysis

Statistical analyses and graph generation were performed using GraphPad Prism 8.0.1 software (GraphPad Software, Inc., San Diego, CA, USA). Each experiment was repeated at least three times independently. Data are presented as mean ± standard error of the mean (SEM). To compare the Blank and PMACI groups, an independent t-test was applied, while for comparisons involving three groups, such as PMACI and drug treatment groups, a one-way ANOVA was used. A P-value of less than 0.05 was considered to indicate statistical significance.

RESULTS

Effects of BTS and α-pinene on the expression of ACE2 in activated HMC-1 cells.

ACE2 has been identified as a key receptor that facilitates the entry of SARS-CoV-2 into host cells.[16] In a prior study, the expression levels of ACE2 mRNA and protein were significantly elevated in PMACI-treated HMC-1 cells compared to untreated cells.[8] To assess the effect of BTS and α-pinene on ACE2 expression in activated HMC-1 cells, we performed qRT-PCR and Western blotting analysis. The results showed that HMC-1 cells pretreated with BTS and α-pinene exhibited a significant reduction in both ACE2 mRNA and protein expression compared to cells treated with PMACI alone (Fig. 1, $P < 0.05$). Neither BTS nor α-pinene affected ACE2 expression levels when compared to unstimulated cells (data not shown).

Effects of BTS and α-pinene on the expression of TMPRSS2 in activated HMC-1 cells.

TMPRSS2 plays a crucial role in facilitating SARS-CoV-2 entry into cells, even when ACE2 expression is insufficient, making it essential for viral invasion.[17] To investigate how BTS and α-pinene affect the mRNA and protein levels of TMPRSS2 in mast cells, HMC-1 cells were pretreated with BTS and α-pinene for 1 h before being activated with PMACI. PMACI stimulation notably increased TMPRSS2 expression in HMC-1 cells, but both BTS and α-pinene effectively reduced the mRNA and protein levels of TMPRSS2 (Fig. 2, $P < 0.05$). Neither BTS nor α-pinene affected TMPRSS2 expression levels when compared to unstimulated cells (data not shown).

Effects of BTS and α-pinene on the production of inflammatory cytokines in activated HMC-1 cells.

PMACI is a mast cell activator that triggers protein kinase C and calcium signaling pathways, which play a critical role in the inflammatory response.[7] To assess whether BTS and α-pinene could suppress the production of inflammatory cytokines in PMACI-stimulated HMC-1 cells, an ELISA was performed. PMACI significantly increased the release of IL-1β, IL-6, IL-8,

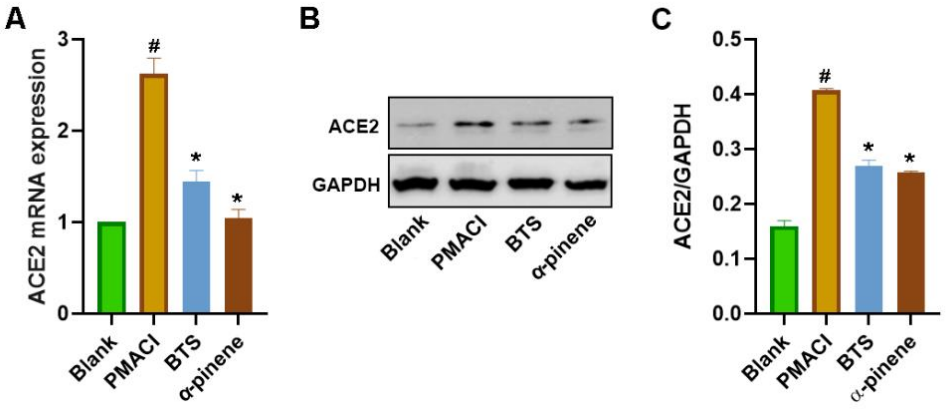

Fig. 1. Effects of BTS and α-pinene on the expression of ACE2 in activated HMC-1 cells. (A) Cells were treated with BTS (1 mg/ml) and α-pinene (1 μg/ml) for 1 h prior to the addition of PMACI, and the cells were further incubated for 5 h. The mRNA expression of ACE2 was analyzed by qRT-PCR. (B) Cells were treated with BTS (1 mg/ml) and α-pinene (1 μg/ml) for 1 h prior to the addition of PMACI, and the cells were further incubated for 24 h. The protein expression of ACE2 was analyzed by Western blotting. Results are representative of three independent experiments. (C) The relative protein level of ACE2 to GAPDH was quantified using relative intensity. Values are expressed as the means ± SEM. $^{\#}P < 0.05$. vs. untreated cell; $^{*}P < 0.05$. vs. PMACI-stimulated cells.

TNF-α, and TSLP, but this release was markedly reduced by BTS treatment (Fig. 3A-E, $P < 0.05$). Treatment with α-pinene effectively reduced the release of IL-1β, IL-6, IL-8, and TNF-α induced by PMACI treatment (Fig. 3A-D, $P < 0.05$), but had no effect on the release of TSLP. Treatment with BTS and α-pinene alone had no impact on cytokine release compared to unstimulated cells (data not shown). The MTT assay performed to evaluate cytotoxicity showed that BTS and α-pinene did not significantly affect cell viability (Fig. 3F).

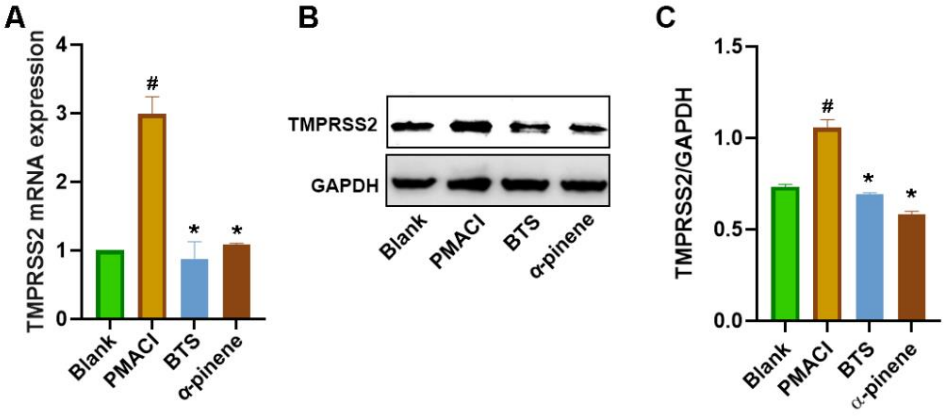

Fig. 2. Effects of BTS and α-pinene on the expression of TMPRSS2 in activated HMC-1 cells. (A) Cells were treated with BTS (1 mg/ml) and α-pinene (1 μg/ml) for 1 h prior to the addition of PMACI, and the cells were further incubated for 5 h. The mRNA expression of TMPRSS2 was analyzed by qRT-PCR. (B) Cells were treated with BTS (1 mg/ml) and α-pinene (1 μg/ml) for 1 h prior to the addition of PMACI, and the cells were further incubated for 24 h. The protein expression of TMPRESS2 was analyzed by Western blotting. Results are representative of three independent experiments. (C) The relative protein level of TMPRSS2 to GAPDH was quantified using relative intensity. Values are expressed as the means ± SEM. $^{\#}P < 0.05$. vs. untreated cell; $^{*}P < 0.05$. vs. PMACI-stimulated cells.

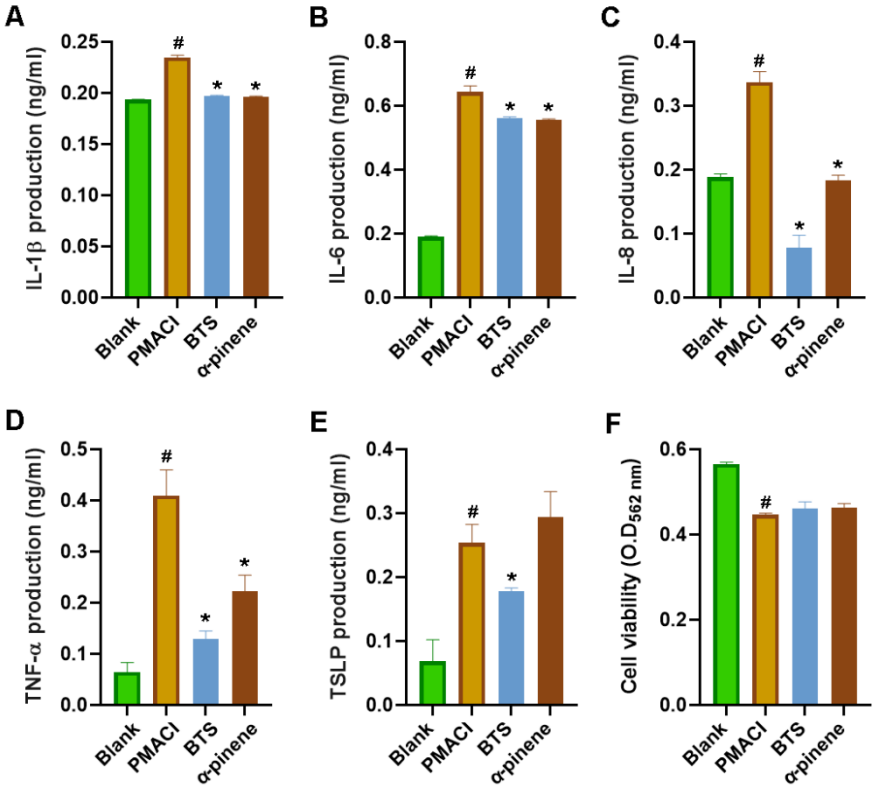

Fig. 3. Effects of BTS and α-pinene on the production of inflammatory cytokines in activated HMC-1 cells. Cells were treated with BTS (1 mg/ml) and α-pinene (1 μg/ml) for 1 h prior to the addition of PMACI, and the cells were further incubated for 8 h. (A-E) Cytokine levels were measured by ELISA. (F) Cell viability was evaluated by MTT assay. Values are expressed as the means ± SEM. $^{\#}P < 0.05$. vs. untreated cell; $^{*}P < 0.05$. vs. PMACI-stimulated cells.

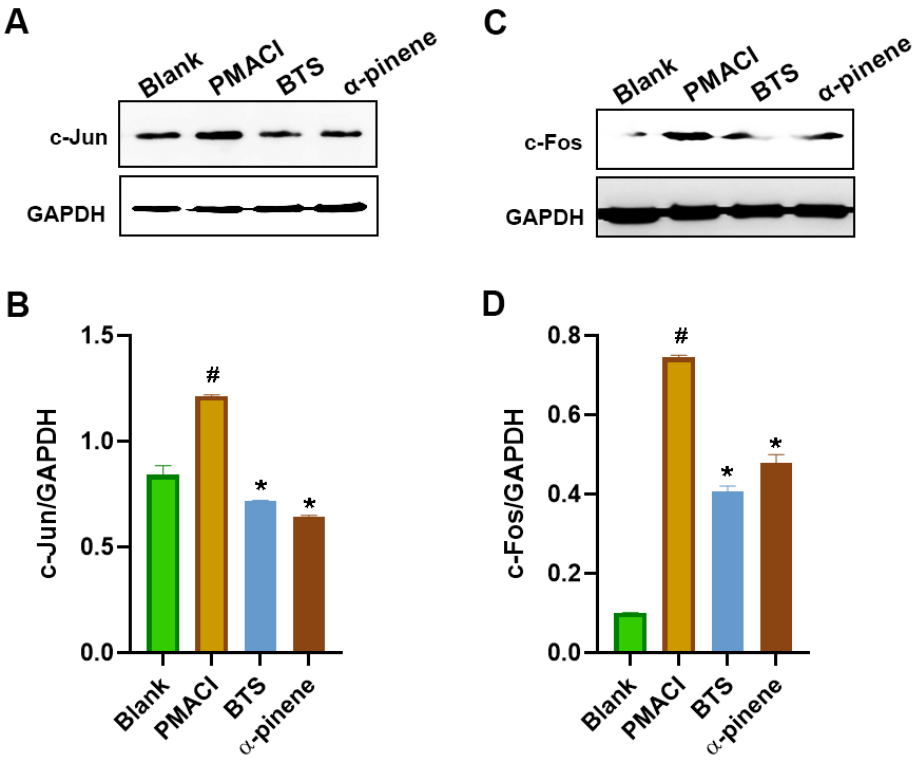

Fig. 4. Effects of BTS and α-pinene on the activation of AP-1 in activated HMC-1 cells. Cells were treated with BTS (1 mg/ml) and α-pinene (1 μg/ml) for 1 h prior to the addition of PMACI, and the cells were further incubated for 2 h. (A, C) The protein expression of c-Jun and c-Fos was analyzed by Western blotting. Results are representative of three independent experiments. (B, D) The relative protein level of c-Jun or c-Fos to GAPDH was quantified using relative intensity. Values are expressed as the means ± SEM. $^{\#}P < 0.05$. vs. untreated cell; $^{*}P < 0.05$. vs. PMACI-stimulated cells.

Effects of BTS and α-pinene on the activation of AP-1 in activated HMC-1 cells.

The transcription factor AP-1 is a dimeric complex consisting of proteins such as c-Jun and c-Fos, and is involved in regulating the inflammatory response by promoting the expression of ACE2 and various inflammatory cytokines.[7,8] In this study, we investigated whether BTS and α-pinene could modulate the expression of AP-1 in PMACI-stimulated HMC-1 cells. We found that PMACI significantly increased the protein levels of c-Jun compared to the untreated cells (Fig. 4A and B). However, BTS and α-pinene treatment effectively reduced the c-Jun expression when compared to the PMACI-treated cells (Fig. 4A and B). Additionally, the expression of c-Fos, a key component of the AP-1 complex, was also elevated by PMACI, and this increase was significantly suppressed by BTS and α-pinene treatment (Fig. 4C and D).

DISCUSSION

SARS-CoV-2 enters host cells through the use of ACE2 and TMPRSS2.[18] Tissues with high ACE2 expression are at greater risk of SARS-CoV-2 infection, and camostat mesylate, a TMPRSS2 inhibitor, can prevent infection by blocking the activation of the S protein in human lung cells.[19] Additionally, Yan et al.[20] demonstrated that blocking the interaction between ACE2 and SARS-CoV-2 can suppress viral infection. Based on this understanding, reducing the expression of ACE2 and TMPRSS2 may be an effective strategy for lowering the risk of SARS-CoV-2 infection. Mast cells are known to be an early target for SARS-CoV-2 infection and a key driver of COVID-19-related hyperinflammation.[21] Our previous studies have identified the mechanisms behind the expression of ACE2 and the cytokine storm in activated mast cells,[7,8] highlighting ACE2 as a critical mediator of mast cell inflammatory responses. We have demonstrated that dexamethasone and Eunkyo-san can reduce ACE2 and TMPRSS2 expression in activated mast cells.[7,8] BTS has been reported to regulate the expression of inflammatory cytokines, thereby mitigating the inflammatory response.[9] In this study, we showed that BTS and α-pinene effectively suppressed the expression of ACE2 and TMPRSS2 in activated mast cells, thereby contributing to a reduction in SARS-CoV-2 cell entry. These findings suggest that BTS and α-pinene have potential as therapeutic agents to modulate SARS-CoV-2 infection and could serve as promising treatment options for the prevention and management of COVID-19.

COVID-19 has the potential to excessively activate the immune system, triggering a cytokine storm that can result in severe complications such as acute respiratory distress syndrome, HLH, autoinflammatory diseases, sepsis, and even death.[22]

During viral infections, mast cells are activated and release inflammatory cytokines like IL-1β, IL-6, IL-8, TSLP, and TNF-α, which contribute to lung inflammation and fever.[23,24] Several studies have shown that the levels of inflammatory cytokines are elevated in COVID-19 patients, correlating closely with the severity of the disease.[24,25] Cytokine storms are a major cause of death in COVID-19, making the use of immune-modulating drugs essential for treatment. IL-1 antagonists, IL-6 receptor antagonists, TNF inhibitors, and corticosteroids are commonly used.[26,27] The COVID-19 treatment drug, chloroquine, inhibits mast cell signaling pathways, reducing the inflammatory activity of TSLP.[28,29] Thus, developing drugs that can simultaneously reduce ACE2 expression and retain anti-inflammatory effects is essential for the treatment of COVID-19. In the present study, BTS and α-pinene reduced the levels of inflammatory cytokines in activated mast cells. Therefore, this study suggests that BTS and α-pinene exhibit anti-inflammatory effects in activated mast cells and could mitigate the cytokine storm caused by SARS-CoV-2 infection.

In this study, BTS inhibited the secretion of all inflammatory cytokines (IL-1β, IL-6, IL-8, TSLP, and TNF-α), while α-pinene did not inhibit TSLP secretion. BTS contains various components with anti-inflammatory effects. One such component, beta-sitosterol from *Saposhnikovia divaricata*, has been reported to inhibit the expression and secretion of TSLP in activated HMC-1 cells.[30] Therefore, although α-pinene is an active component of BTS, it did not inhibit TSLP secretion, suggesting that the inhibition of TSLP production was likely due to other active components in BTS.

Mast cells activate the AP-1 signaling pathway to produce inflammatory cytokines and express ACE2 and TMPRSS2.[8] The anti-inflammatory drug dexamethasone reduces the production of mast cell-induced inflammatory cytokines and has shown beneficial effects in COVID-19 patients in randomized clinical trials.[31] According to our recent study, dexamethasone regulates the AP-1 signaling pathway in activated HMC-1 cells, inhibiting ACE2 expression.[8] Additionally, the AP-1 signaling pathway is triggered by SARS-CoV-2 particles, accessory protein 3b, nucleocapsid protein, and spike protein.[32] PMACI activates the AP-1 signaling pathway in HMC-1 cells, leading to the expression of ACE2 and inflammatory cytokines.[8] Therefore, dysfunction of AP-1 in activated mast cells can prevent inflammatory responses.[33] In this study, we confirmed that BTS and α-pinene block the AP-1 signaling pathway in activated HMC-1 cells. Thus, we propose that BTS and α-pinene could mitigate the SARS-CoV-2 infection and cytokine storm by suppressing the AP-1 signaling pathway in activated mast cells.

BTS has been reported to contain many components, including α-pinene, as determined through various analyses such as high-performance liquid chromatography and gas chromatography/mass spectrometry.[34-38] In this study, we identified that BTS and α-pinene exhibit antioxidant and anti-inflammatory effects. Therefore, we propose that α-pinene is an active ingredient in BTS involved in the regulation of ACE2 expression.

In conclusion, this study demonstrated that BTS and α-pinene inhibit the AP-1 intracellular signaling pathways, leading to a reduction in the expression of ACE2, TMPRSS2, and inflammatory cytokines. These findings suggest that BTS may effectively mitigate SARS-CoV-2 infection and cytokine storm, offering a potential pharmacological mechanism for future COVID-19 treatments.

CONFLICT OF INTEREST

The author has no conflicting financial interests.

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