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The potential of BEN815 as an anti-inflammatory, antiviral and antioxidant agent for the treatment of COVID-19

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A R T I C L E   I N F O

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A B S T R A C T

Background: The corona virus disease 2019 (COVID-19) pandemic has highlighted the fact that there are few effective antiviral agents for treating severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infections. Although the very recent development of vaccines is an extremely important breakthrough, it remains unclear how long-lived such vaccines will be. The development of new agents therefore remains an important goal.

Purpose: Given the multifaceted pathology of COVID-19, a combinatorial formulation may provide an effective treatment. BEN815, a natural nutraceutical composed of extracts from guava leaves (Psidium guajava), green tea leaves (Camellia sinensis), and rose petals (Rosa hybrida), had previously shown to have a therapeutic effect on allergic rhinitis. We investigated whether BEN815 possesses anti-inflammatory, antiviral and antioxidant activities, since the combination of these effects could be useful for the treatment of COVID-19.

Study design: We examined the anti-inflammatory effects of BEN815 and its principal active components quercetin and epigallocatechin gallate (EGCG) in lipopolysaccharide (LPS)-induced RAW264.7 cells and in an LPS-challenged mouse model of endotoxemia. We also assessed the antioxidant activity, and antiviral effect of BEN815, quercetin, and EGCG in SARS-CoV-2-infected Vero cells.

Methods: The principal active ingredients in BEN815 were determined and quantified using HPLC. Changes in the levels of LPS-induced pro-inflammatory cytokines interleukin (IL)-6 and tumor necrosis factor (TNF)-α were measured by ELISA. Changes in the expression levels of cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS) were analyzed using western blotting. Antioxidant assay was performed using DPPH and ABTS assay. SARS-CoV-2 replication was measured by immunofluorescence staining.

Results: BEN815 significantly suppressed the induction of IL-6 and TNF-α as well as COX-2 and iNOS in LPS-induced RAW264.7 cells. In addition, BEN815 protected against LPS-challenged endotoxic shock in mice. Two major constituents of BEN815, quercetin and EGCG, reduced the induction of IL-6 and TNF-α as well as COX-2 and iNOS synthase in LPS-induced RAW264.7 cells. BEN815, quercetin, and EGCG were also found to have antioxidant effects. Importantly, BEN815 and EGCG could inhibit SARS-CoV-2 replication in Vero cells.

Conclusion: BEN815 is an anti-inflammatory, antiviral, and antioxidant natural agent that can be used to prevent and improve inflammation-related diseases, COVID-19.

Abbreviations: ABTS, 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); ACE2, Angiotensin converting enzyme 2; CC, Cytotoxic concentration; COVID-19, Coronavirus disease 2019; COX, Cyclooxygenase; DMSO, Dimethyl sulfoxide; DPPH, 2,2-diphenyl-1-picrylhydrazyl; DRC, Dose-response curve; DXM, Dexamethasone; EGCG, Epigallocatechin gallate; ELISA, enzyme-linked immunosorbent assay; FBS, Fetal bovine serum; H&E, Hematoxylin and eosin; HPLC, High-performance liquid chromatography; IC, Inhibitory concentration; IFN, interferon; IL, Interleukin; iNOS, Inducible nitric oxide synthase; LPS, Lipopolysaccharide; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; PBS, Phosphate buffered saline; SARS-CoV-2, Severe acute respiratory syndrome coronavirus 2; SEM, Standard error of the mean; SI, Selectivity index; TNF, Tumor necrosis factor.

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Introduction

The pneumonia-like disease caused by the SARS-CoV-2 named COVID-19, has dispersed worldwide, with over 55,000,000 confirmed cases and nearly 1,500,000 deaths to December 2020. The infection pathway of SARS-CoV-2 use angiotensin converting enzyme 2 (ACE2) receptor, which is expressed in various organ of human in the same manner of SARS-CoV and Middle East respiratory syndrome coronavirus (MERS-CoV) (Tong et al., 2020). When the disease gets worse, SARS-CoV-2 stimulates the release of a lot of inflammatory mediators, for example chemokines, and cytokines, inducing cytopathic effects furthermore a “cytokine storm” that can lead to organ damage severely. COVID-19-induced cytokine storm is a phenomenon similar to bacterial endotox shock, which also increases the expression of inflammatory mediators (Altay et al., 2020; Meftahi et al., 2020; Ragab et al., 2020). These inflammatory mediators have the detrimental effects on the body’s immunity as well as on the gastrointestinal, kidney, hematologic and respiratory functions (Iwasaki and Yang, 2020). Therefore, both the inhibition of viral infection and proliferation and the dampening of the inflammatory reaction before a cytokine storm are important tools to fight against COVID-19. Even though there are upcoming promising drugs to treat COVID-19 including remdesivir, chloroquine, lopinavir, and dexamethasone, these treatment have several limitations. Remdesivir which is a direct acting antiviral drug, its efficacy and safety in SARS-CoV-2 infections need to be further explored clinically (Wang et al., 2020). Chloroquine is generally used for treating malaria and have been approved by the US FDA for COVID-19; however, they have side effects, such as worsening of nausea and vision, digestive disorders, and heart failure (Dong et al., 2020). Lopinavir/ritonavir is a combination protease inhibitor/anti-retroviral drug that has recently shown activity against SARS-CoV-2, however its clinical efficacy is controversial, requiring further evaluation (Choy et al., 2020). A rheumatoid arthritis drug, tocilizumab, another anti-retroviral HIV-1 protease inhibitor, darunavir, as well as an antiparasitic drug, ivermectin, have all been shown to be effective against the SARS-CoV-2; however, further clinical trials need to be completed to confirm the efficacy of these drugs against COVID-19 (Abd El-Aziz and Stockand, 2020). A synthetic glucocorticoid, dexamethasone, has strong anti-inflammatory and immunosuppressive properties; however, use of dexamethasone has been controversial in the application of patients with SARS-CoV-2 (Selvaraj et al., 2020). Thus, further efforts to develop new treatments are urgently needed.

Given the multifaceted pathology of COVID-19, an effective treatment would require a combinatorial formulation that achieves the effects described next (Bonafe et al., 2020; Wang et al., 2020). First, the treatment must prevent the penetration or replication of intracellular coronavirus. Second, it should have strong anti-inflammatory properties sufficient to prevent cytokine storms that occur after virus penetration. Finally, it should be beneficial if the treatment would have an antioxidant activity to prevent damage to surrounding organs from a large amount of free radicals induced by the inflammatory reaction. BEN815, a nutraceutical composed of extracts from green tea (Camellia sinensis (L.) Kuntze (Theaceae)), guava leaves (Psidium guajava L. (Myrtaceae)), and rose petals (Rosa hybrida E.H.L.Krause (Rosaceae)), which have previously shown to improve allergic inflammation (Kim et al., 2007a; 2007b). Green tea leaves and guava leaves contain plenty of polyphenols such as quercetin, catechins, kaempferol, and ellagic acid. These compounds have antioxidant, anti-inflammatory, and immunomodulation effect in addition to anti-allergic effects (Ahn et al., 2016; Tipoe et al., 2007), all of which may be relevant to the treatment of COVID-19. It can be assumed that BEN815 may similarly have diverse therapeutic activities, but further studies have not been conducted to test this possibility.

Therefore, this study aimed to characterize the anti-inflammatory, antiviral, and antioxidant activities of BEN815, identify its principal active components, and assess the ability of BEN815 and its principal active components to prevent SARS-CoV-2 replication in vitro.

Materials and methods

High-performance liquid chromatography (HPLC) analysis of BEN815

BEN815 was prepared in the same manner as described previously (Kim et al., 2007a). BEN815 contains extracts from powdered guava leaves, green tea leaves and rose petals in a 65:30:5 ratio. To determine the principal active ingredients in BEN815, the content of each, quercetin, epigallocatechin gallate (EGCG), ellagic acid, gallic acid, kaempferol, and myricetin were measured using HPLC. Six substances were selected by referring to previously reported papers (Jang et al., 2014; Kim et al., 2007a). HPLC was performed using an Agilent 1260 system (Agilent Technologies, Inc., Santa Clara, CA, USA), and 10 µl of the sample was injected in Symmetry C18 column (4.6 × 250 mm, Gemini® 5 µm, Phenomenex, Torrance, CA, USA) at a mobile phase flow rate 1.0 mL/min. The analysis condition for each component was conducted as following (Table 1). The standard compounds quercetin, EGCG, ellagic acid, gallic acid, kaempferol and myricetin were purchased from Sigma-Aldrich (>98%, Louis, MO, USA) and used to construct the calibration curve. The six components in each sample were analyzed and quantified by comparing their retention times and area of a peak in the chromatograms relative to standards. The quantification expressed as the average mg/dry g of BEN815 with three replicates.

Cell culture

RAW264.7 mouse macrophage cells obtained from Korean Cell Line Bank (Seoul, Republic of Korea), were incubated in Dulbecco’s Modified Eagle Medium (DMEM; Gibco®, Grand Island, NY, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Thermo Scientific, Rockford, IL, USA), 100 U/ml penicillin (Gibco®), and 100 µg/ml streptomycin (Gibco®) in a cell culture incubator (Sanyo Co., Osaka, Japan) under conditions of 37°C and 5% CO₂.

MTT assay

Cell viability was examined by using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich) assay. RAW264.7 cells were seeded in a 24-well plate at a density of 2 × 10⁵ cells/well and incubated for 24 h. Furthermore, cells were treated with BEN815 (6.25, 12.5, 25, 50, and 100 µg/ml) along with 100 ng/ml LPS (Sigma-Aldrich) for 20 h. After that, the MTT reagent (0.5 mg/ml) was added, and after 4 h the formazan crystals formed in the cells were dissolved with dimethyl sulfoxide (DMSO); the absorbance was measured at 570 nm using a spectrometer (BioTek, Winooski, VT, USA).

Measurement of IL-6 and TNF-α levels

RAW264.7 cells were seeded in a 24-well plate at a density of 2 × 10⁵ cells/well and incubated for 24 h, they were then exposed to concentrations of 6.25, 12.5, 25, 50, and 100 µg/ml of BEN815 or quercetin (Naturalin Bio-Resources Co., Ltd., Changsa, China) or EGCG (Naturalin Bio-Resources Co., Ltd.), and 100 ng/ml of LPS for 20 h. IL-6 and TNF-α levels in the cell culture supernatant were measured according to the manufacturer’s protocol using a mouse enzyme-linked immunosorbent assay (ELISA) kit (Thermo Fisher Scientific, Waltham, MA, USA) at 450 nm using a spectrometer (BioTek).

Western blotting

RAW264.7 cells were seeded in a 6-well plate at a density of 4 × 10⁵ cells/well and incubated for 24 h, and then, were treated with either BEN815, quercetin, or EGCG, and/or LPS, after which they were solubilized in lysis buffer (iNTiRON Biotechnology, Seongnam, Republic of Korea) as previously described (Shim et al., 2020). The specific primary antibodies, anti-COX-2 (Cell Signaling, Danvers, MA, USA; 1:1000),
anti-iNOS (Cell Signaling; 1:1000), and anti-β-actin (Cell Signaling; 1:2000) were used, and the membranes were incubated with horseradish peroxidase-linked anti-rabbit antibody (Cell Signaling; 1:5000). For quantification, the densities of COX-2 and iNOS were normalized to the density of β-actin using the Image J (1.37v; National Institutes of Health, Bethesda, MD, USA).

**Experimental animals**

Ten-week-old male, BALB/c mice (Samtako Bio Korea, Osan, Republic of Korea) were used for this test. The animals were housed in a pathogen-free chamber, and maintained under standard laboratory conditions (23 ± 2°C; relative humidity, 50 ± 5%; 12/12 h light/dark cycle). The Institutional Animal Care and Use Committee of the University of Suwon approved all animal procedures (Confirmation number: USW-IAUC-R2019-005). The research was performed in accordance with internationally accepted principles for laboratory animal use and care (Olsson et al., 2016).

**Survival in a mouse model of LPS-induced endotoxicemia**

The protective effect of BEN815 on mortality rate was assessed in mice with LPS-induced endotoxic shock, the method of survival rate modified from Jang et al. (2014). For this, five groups were established: control, LPS (10 mg/kg), LPS plus BEN815 (200 mg/kg), LPS plus BEN815 (400 mg/kg), and LPS plus dexamethasone (DXM; 2 mg/kg) groups (n = 11 per group). BEN815 was administered orally twice, at 24 h and 2 h before LPS injection, whereas DXM was administered intraperitoneally immediately after LPS injection. Mouse survival rate was observed for 72 h after LPS injection.

**IL-6 and TNF-α levels in mouse serum**

Briefly, blood was collected from the heart 3 h after LPS administration (n = 5 per group). Serum was collected by centrifugation at 1700g 4°C for 15 min. Serum IL-6 and TNF-α levels were analyzed by using mouse ELISA kits according to the manufacturer’s protocol (Thermo Fisher Scientific) at 450 nm with a spectrometer (BioTek).

**Histological evaluation of lung tissues**

The method of histopathologic evaluation of lung tissues developed by Liu et al., (2016) was used. Briefly, the lungs were excised 3 h after LPS administration, and then fixed in 10% buffered formalin, cut into 20-μm thick using a cryostat, and subsequently stained with hematoxylin and eosin (H&E) (n = 5 per group). Lung injury scores were measured according to histopathological changes, such as lung edema, inflammatory cell infiltration and accumulation, alveolar hemorrhage, and alveolar wall thickness under microscopic images. Each score was graded according to a five-point scale; ‘zero’ indicates no or minimal lung injury, ‘1’ indicates mild injury, ‘2’ indicates moderate injury, ‘3’ indicates severe injury, and ‘4’ indicates maximal injury. Lung injury score was scored as the average of the individual scores.

**Virus and Vero cell culture**

Vero cells, monkey kidney epithelial cells CCL-81 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were maintained in DMEM (Welgene Inc., Daegu, Republic of Korea) supplemented with 10% heat-inactivated FBS and 1% antibiotic-antimycotic solution (Gibco) at 37°C in a 5% CO2 atmosphere. The Korea Centers for Disease Control and Prevention (KCDC) provided SARS-CoV-2 (JCoV/KOR/KCDC03/2020). All experiments using SARS-CoV-2 were performed at Zoonotic Virus Laboratory of Institut Pasteur Korea in compliance with the guidelines of the Korea National Institute of Health, using Class III Biological Safety Cabinet (Institut Pasteur Korea, Seongnam, Republic of Korea).

**Antiviral effects of BEN815 and its principal active components and reference compounds against SARS-CoV-2 virus in Vero cells**

The experiments to determine the antiviral effect of BEN815 using Vero cells were performed as previously described (Jeon et al., 2020). Images were assessed for expression of the SARS-CoV-2 N protein and cell nuclei in virus by immunofluorescence, captured using a confocal microscope, and analyzed using Image Mining software of Institut Pasteur Korea. And then, a dose-response curve (DRC) for each sample was generated. Remdesivir (Med Chem Express, NJ, USA, Cat. No.), chloroquine diphosphate (Sigma-Aldrich), and lopinavir (SelleckChem, Houston, TX, USA) were used as references.

**Antioxidant activity assay**

**ABTS tests**

The method modified by Muanda et al. (2011) was followed. Briefly, a 7 mM 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was mixed with 2.45 mM diammonium salt in phosphate buffer saline (PBS) solution (100 mM, pH 7.4), and then left in a dark environment for 16 h. The reaction solution was diluted with ethanol so that the absorbance value is 0.7 ± 0.05 at 734 nm before use. 20 μl of samples was treated with 180 μl of ABTS solution, incubated for 10 min in a dark environment, and the absorbance was measured at 734 nm using a spectrometer (BioTek). 50% inhibitory concentration (IC50) values of the ABTS radical scavenging activity were calculated, and all experiments were conducted in triplicate.

**DPPH tests**

The method developed by Sharififar et al. (2009) was followed. Briefly, 180 μl of the 2,2-diphenyl-1-picrylhydrazyl (DPPH; 167 μM in ethanol) reaction solution was mixed with 20 μl of samples, and then incubated in a dark environment for 30 min, and the absorbance was measured at 520 nm using a spectrometer (BioTek). As a control, DPPH solution in ethanol was mixed with same volume of ethanol, and the absorbance of the solution was measured. IC50 values of the DPPH radical scavenging activity were calculated, and all experiments were conducted in triplicate.
Fig. 1. Effect of BEN815 on inflammatory responses in LPS-induced RAW264.7 cells. (A) Viability of cells treated with BEN815 and LPS assessed by MTT assay. The levels of the proinflammatory cytokines IL-6 (B), and TNF-α (C) in the supernatant of cells treated with BEN815 and LPS were determined by ELISA. Dexamethasone (DXM; 50 mg/ml) was used as a positive control. (D) The levels of COX-2 and iNOS in cells were determined by western blotting after treatment of the cells with BEN815 and LPS. For quantification, COX-2 (E) and iNOS (F) band densities were normalized to the band density of β-actin. All experiments were conducted in triplicate. *P < 0.05 compared with LPS treatment.

Fig. 2. Effect of BEN815 on LPS-induced endotoxemia in mice. (A) Survival curves for mice after oral administration of BEN815 (200 or 400 mg/kg, n = 11 per group) at 24 h and 2 h before intraperitoneal administration of LPS (10 mg/kg). Serum was collected 3 h after LPS injection (n = 5 per group) and IL-6 (B) and TNF-α (C) levels were determined by ELISA. #P, *P < 0.05 compared with LPS treatment.
Statistical analysis

Experimental results are expressed as the mean ± standard error of the mean (SEM). Comparisons between two groups were analyzed with an unpaired Student’s t-tests, and the comparisons among more than 2 groups were analyzed using the one-way analysis of variance (ANOVA) followed by Tukey’s post hoc test. Survival test was analyzed using Mantel-Cox survival analysis with a log-rank test (Prism6, GraphPad Software, Inc., La Jolla, CA, USA). P values less than 0.05 were considered to indicate statistical significance.

Results

Effect of BEN815 on proinflammatory cytokine production in LPS-induced RAW264.7 cells

To investigate the cytotoxicity of BEN815 on RAW264.7 cells, these cells were treated with BEN815 (6.25, 12.5, 25, 50, and 100 μg/ml) and LPS (100 ng/ml) for 20 h, and cell viability was measured using an MTT assay. Cell viability was not statistically different between the BEN815 with LPS treatment and the LPS treatment group, showing that BEN815 is not cytotoxic (Fig. 1A).

Cells were treated with BEN815 (6.25, 12.5, 25, 50, and 100 μg/ml), and LPS (100 ng/ml) for 20 h, and changes in the levels of LPS-induced proinflammatory cytokines IL-6 and TNF-α were measured by ELISA. Following treatment with LPS alone, IL-6 levels were 43.963 ± 256.8 pg/ml, whereas treatment with 50 and 100 μg/ml BEN815 resulted in a more than 70% inhibition of IL-6 secretion (11,346 ± 127.5 and 882.3 ± 60.81 pg/ml, respectively) (Fig. 1B). LPS treatment alone resulted in TNF-α levels of 120,746 ± 645.1 pg/ml, whereas treatment with 50 and 100 μg/ml BEN815 resulted in a greater than 40% inhibitory effect (71,572 ± 659.7 and 52,876 ± 577.7 pg/ml, respectively) (Fig. 1C). As a positive control, 50 μg/ml DXM inhibited both IL-6 and TNF-α secretion (21,460 ± 238.0 pg/ml and 83,377 ± 564.8 pg/ml, respectively). These data show that BEN815 can inhibit the release of proinflammatory cytokines as effectively as, or better than DXM.

Effect of BEN815 on COX-2 and iNOS production in LPS-induced RAW264.7 cells

RAW264.7 cells were treated with BEN815 (12.5, 25, 50, and 100 μg/ml), and LPS (100 ng/ml), and changes in the expression levels of COX-2 and iNOS were analyzed using western blotting (Fig. 1D). We found that BEN815 suppressed the increased expression of COX-2 and iNOS induced by LPS, in a concentration-dependent manner. Moreover, the inhibition of COX-2 and iNOS expression was significant starting at a concentration of 25 μg/ml of BEN815 (Fig. 1E and F).

Effect of BEN815 on the survival of mice with LPS-induced endotoxemia

We administered BEN815 (200 mg/kg or 400 mg/kg) in an LPS-induced mouse model of endotoxic shock prior to the induction of endotoxemia by LPS injection (10 mg/kg). Mouse survival was then checked for 72 h, and the results are shown in Fig. 2A. We found that the LPS-administered mice had a survival rate of only 9.1% 72 h after LPS injection, whereas mice administered BEN815 at concentrations of 400 mg/kg and 200 mg/kg had survival rates of 63.6% and 36.4% at 72 h, respectively. Treatment with DXM (2 mg/kg) resulted in a survival rate of 45.5% 72 h after LPS injection. We confirmed that IL-6 and TNF-α levels were elevated 3 h after LPS injection, and these levels were significantly reduced in mice administered BEN815 at 200 mg/kg or 400 mg/kg (Fig. 2B and C). In addition, mice in the BEN815 (400 mg/kg) group had an almost equivalent or better inhibitory efficacy compared to those in the DXM group.

Effect of BEN815 on LPS-induced histopathological changes in lung tissue

To evaluate the protective effect of BEN815 on lung lesions in LPS-administered mice, lung tissues were collected 3 h after LPS treatment and stained using H&E (Fig. 3A). As a result of measuring and averaging the scores of lung lesions in each group based on the criteria described in the Materials and methods, the LPS-treated group had a score of 3.4 ± 0.12, which represented severe damage, whereas the groups administered BEN815 at 200 and 400 mg/kg, and DXM (2 mg/kg) had scores of 2.8 ± 0.11, 2.1 ± 0.18, and 2.3 ± 0.07, respectively (Fig. 3B). The degree of lung damage induced by LPS in mice was significantly reduced by administering 400 mg/kg BEN815.

HPLC analysis of bioactive components content in BEN815

It is known that extracts of guava leaves, green tea leaves, and rose petals, which are constituents of BEN815, contain numerous polyphenolic compounds. Among them, we chose to quantify six representative substances that have anti-inflammatory or antioxidant properties, such as quercetin, EGCG, gallic acid, ellagic acid, kaempferol, and myricetin, using HPLC (Fig. 4). As a result, it was confirmed that quercetin content was 308.7 ± 0.36 mg/g of BEN815, and EGCG, 164.5 ± 0.78 mg/g, while the remaining compounds—gallic acid, ellagic acid, and kaempferol
Fig. 4. HPLC chromatogram of the BEN815 and internal standard. Chromatograms of quercetin standard and quercetin from BEN815 (A), EGCG standard and EGCG from BEN815 (B), gallic acid standard and gallic acid from BEN815 (C), ellagic acid standard and ellagic acid from BEN815 (D), and kaempferol standard and kaempferol from BEN815 (E).

Table 2
Contents of six bioactive components in BEN815.

| Name          | Concentration (mg/dry g of BEN815) |
|---------------|-----------------------------------|
| Quercetin     | 308.7 ± 0.36 mg/g                 |
| EGCG          | 164.5 ± 0.78 mg/g                 |
| Gallic acid   | 3.4 ± 0.28 mg/g                   |
| Ellagic acid  | 7.1 ± 0.11 mg/g                   |
| Kaempferol    | 2.8 ± 0.08 mg/g                   |
| Myricetin     | ND                                |

ND, not detected

content were 3.4 ± 0.28, 7.1 ± 0.11, and 2.8 ± 0.08 mg/g, respectively; however, myricetin was not detected by our analytical method (Table 2).

Effect of the principal active components of BEN815 on proinflammatory cytokine production in LPS-induced RAW264.7 cells

We found that both quercetin and EGCG could suppress LPS-induced expression of IL-6 and TNF-α in a concentration-dependent manner (Fig. 5), with quercetin having the highest efficacy. The effect of quercetin could be observed at a concentration as low as 6.25 μg/ml (Fig. 5A). Thus, among the principal active components present in BEN815, quercetin, along with EGCG, plays a major role in its anti-inflammatory activity.

Effect of the principal active components of BEN815 on COX-2 and iNOS protein levels in LPS-induced RAW264.7 cells

We found that quercetin or EGCG suppressed the expression of COX-2 and iNOS induced by LPS in a concentration-dependent manner (Fig. 6A and B). In particular, the inhibition of COX-2 and iNOS expression was significantly reduced starting at 12.5 μg/ml quercetin (Fig. 6A) and 50 μg/ml EGCG (Fig. 6D).

Effect of BEN815 and its principal active components on the replication of SARS-CoV-2 in Vero cells

Remdesivir, chloroquine, and lopinavir were used as reference drugs and had IC_{50} values of 13.1 μM (Fig. 7A), 8.98 μM (Fig. 6B), and 12.03
Fig. 5. Effect of quercetin and EGCG on proinflammatory cytokine production in LPS-induced RAW264.7 cells. The levels of proinflammatory cytokines IL-6 (A), and TNF-α (B) were measured by ELISA in the cell culture supernatant of cells treated with quercetin and LPS. The levels of the proinflammatory cytokines IL-6 (C) and TNF-α (D) were measured by ELISA in the cell culture supernatant of cells treated with EGCG and LPS. All experiments were conducted in triplicate. *P < 0.05 compared with LPS treatment.

Fig. 6. Effect of quercetin and EGCG on COX-2 and iNOS expression in LPS-induced RAW264.7 cells. (A) The levels of COX-2 and iNOS were determined by western blotting after treatment with quercetin and LPS. For quantification, COX-2 (B) and iNOS (C) densities were normalized to the density of β-actin. (D) The levels of COX-2 and iNOS were determined by western blotting after treatment with EGCG and LPS. For quantification, COX-2 (E) and iNOS (F) densities were normalized to the density of β-actin. All experiments were conducted in triplicate. *P < 0.05 compared with LPS treatment.
Fig. 7. Effects of BEN815, EGCG, and quercetin as well as reference drugs on SARS-CoV-2 infection in Vero cells. Antiviral activity of remdesivir (A), chloroquine (B), lopinavir (C), BEN815 (D), EGCG (E), and quercetin (F) was assessed by DRC. The blue circles represent the inhibition of SARS-CoV-2 infection (%) at different doses of each agent, and the red squares represent cell viabilities (%) at these doses. IC50: 50% inhibitory concentration values; CC50: 50% cytotoxic concentration values; SI: Selectivity index values (CC50/IC50). Values of mean ± SEM were calculated from duplicate experiments.

μM (Fig. 7C), respectively. BEN815 showed antiviral activity against SARS-CoV-2, with an IC50 of 34.38 μg/ml (Fig. 7D), and EGCG also showed antiviral activity against SARS-CoV-2, with an IC50 of 33.41 μM (Fig. 7E). Conversely, quercetin did not show antiviral efficacy at any concentration (Fig. 7F). Therefore, among the major components of BEN815, it can be observed that EGCG plays a major role in the antiviral effect against SARS-CoV-2.

**Antioxidant effects of BEN815 and its principal active components, quercetin and EGCG**

The antioxidant capacities of the samples are summarized in Table 2. The IC50 of BEN815 was 4.80 ± 0.03 μg/ml for DPPH radical scavenging activity and 8.08 ± 0.11 μg/ml for ABTS radical scavenging activity. The IC50 values of quercetin and EGCG were 5.90 ± 0.09 μg/ml and 3.94 ± 0.37, respectively, using DPPH assay, and 6.08 ± 0.14 μg/ml and 5.66 ± 0.11, respectively, using ABTS assay. These results indicate that BEN815, quercetin, and EGCG all showed high antioxidant activities similar to or better than vitamin C, N-acetylcysteine, and glutathione (Table 3).

**Discussion**

In response to the global health crisis caused by outbreak of SARS-CoV-2, massive efforts have been made to understand the pathogenesis of COVID-19 and to develop therapeutic interventions. Accumulating evidence suggests that damage of host tissues and organs by direct infection of SARS-CoV-2 as well as overly activated host immune
systems contribute to the pathophysiology of COVID-19 (Altabay et al., 2020; Meftahi et al., 2020). Thus, current therapeutic strategies to fight COVID-19 consist of either host-directed therapy, which is focused on alleviation of hyperactive host immune responses, or antiviral therapy that aims to prevent entry or replication of SARS-CoV-2 in host cells (Ragab et al., 2020; Iwasaki and Yang, 2020). Although drugs based on each strategy, such as the anti-inflammatory agent dexamethasone and the antiviral agent remdesivir have shown to improve clinical outcomes of COVID-19, their efficacies are far from optimal. In this scenario, it is reasonable to postulate that drugs possessing both anti-inflammatory activity against host, and antiviral activity against SARS-CoV-2 may provide an ideal option to reduce the severity and prevent progression of COVID-19.

BEN815 is a three-herb medicinal product that has been approved as a health product by the Ministry of Food and Drug Safety of Republic of Korea (No. 2009-16) based on its actions in relieving symptoms of allergic rhinitis (Kim et al., 2009). In this study, we demonstrated that BEN815 has strong anti-inflammatory activity as well as antiviral activity against SARS-CoV-2, and thereby propose BEN815 as a novel option for the treatment of COVID-19. The anti-inflammatory activity of BEN815 is supported by multiple lines of evidence. BEN815 decreased the expression of IL-6 and TNF-α in macrophages stimulated by LPS, with a high potency comparable to dexamethasone. Also, BEN815 effectively suppressed LPS-stimulated expression of COX-2 and iNOS in macrophages. Importantly, in a mouse model of LPS-induced endotoxemia, BEN815 reduced mortality rate, and significantly decreased serum levels of IL-6 and TNF-α in endotoxemic mice; this suggests that the survival benefit provided by BEN815 may be due to the suppression of cytokine storm. The antiviral activity of BEN815 against SARS-CoV-2 was demonstrated by an in vitro assay, which revealed a dose dependent inhibition of the virus proliferation by BEN815 in infected Vero cells. It is thought that antioxidants such as vitamin C may have a beneficial effect in patients with severe COVID-19 by scavenging reactive oxygen species that cause damage to surrounding organs (Horowitz et al., 2020; Ibrahim et al., 2020). Thus, the antioxidant activity of BEN815 that is as strong as vitamin C, also supports the therapeutic potential of BEN815 for the treatment of COVID-19.

Extracts of green tea leaves, guava leaves and rose petals, which are constituents of BEN815, have numerous biologically active ingredients, some of which are known to have anti-inflammatory activities and/or antiviral activities (Ahn et al., 2015; Tipoe et al., 2007). Guava leaf extract lowers expression of COX-2 and iNOS in LPS-stimulated mouse macrophage, and reduces mortality rate in a mouse model of sepsis (Jang et al., 2014). The anti-inflammatory effect of guava leaf extract is known to be mediated by quercetin, which is the most abundant among the active ingredients of BEN815. Thus, the anti-inflammatory activities of BEN815 is likely to be mediated by quercetin. Quercetin is also known to exert antiviral effects on various models of viral infection (Glinsky, 2020; Tong et al., 2020; Wan et al., 2020); however, it failed to exert antiviral effects on SARS-CoV-2 in the Vero cell cultures. Instead, we found that the antiviral activity of BEN815 is mediated by EGCG, which is richly present in green tea leaves, and is the second most abundant component among the active ingredients of BEN815 (Table 1). Previously, it was shown that EGCG inhibits protease activity of SARS-CoV-2 in silico (Ghosh et al., 2020; Mhatre et al., 2020). However, antiviral effects of EGCG on SARS-CoV-2 in infected cells have not been determined and to our knowledge, the present study is the first to demonstrate that EGCG inhibits proliferation of SARS-CoV-2 in cells.

The urgent need for COVID-19 therapeutics has accelerated the investigation of existing drugs as possible treatment options for COVID-19. Since the global outbreak of COVID-19, several currently available drugs have been tested for their efficacy against COVID-19. Some of these drugs, including dexamethasone and remdesivir, proved to be efficacious, and thereby acquired regulatory approval for use in treating COVID-19. However, these drugs lack either antiviral or anti-inflammatory activity; therefore, new drugs which combine both activities are highly needed for the efficient treatment of COVID-19.

Conclusion

BEN815 is a natural tri-herbal product that has been safely used as a health product for more than 10 years, and has shown to have anti-inflammatory and antioxidant activities as well as antiviral activity against SARS-CoV-2 in this study. However, it is necessary and urgent to further investigate the efficacy of BEN815 against COVID-19 in animal models, and ultimately in patients with COVID-19.

Declaration of Competing Interest

We confirm that there are no conflicts of interest associated with this publication and there has no significant financial support for this work that could have influenced its outcome.

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Author contributions

Jin A. Shin: Conceptualization, Data curation, Investigation, Writing-Original draft preparation. Subin Oh: Methodology, Investigation. Jong-Moon Jeong: Supervision, Conceptualization, Writing- Reviewing and Editing.

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Table 3

Table 3 Average values of DPPH and ABTS IC50.

| Compounds     | DPPH IC50 (μg/ml) | ABTS IC50 (μg/ml) |
|---------------|-------------------|-------------------|
| BEN815        | 4.80 ± 0.03       | 8.08 ± 0.11       |
| Quercetin     | 5.90 ± 0.09       | 6.08 ± 0.14       |
| EGCG          | 3.94 ± 0.37       | 5.66 ± 0.11       |
| Vitamin C     | 6.32 ± 0.03       | 7.51 ± 0.29       |
| N-acetylcysteine | 29.54 ± 0.16   | 40.36 ± 0.21      |
| Glutathione   | 38.57 ± 0.71      | 48.97 ± 1.58      |
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