Inhibition of a broad range of SARS-CoV-2 variants by antiviral phytochemicals in hACE2 mice

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ABSTRACT

Although several vaccines and antiviral drugs against SARS-CoV-2 are currently available, control and prevention of COVID-19 through these interventions is limited due to inaccessibility and economic issues in some regions and countries. Moreover, incomplete viral clearance by ineffective therapeutics may lead to rapid genetic evolution, resulting in the emergence of new SARS-CoV-2 variants that may escape the host immune system as well as currently available COVID-19 vaccines. Here, we report that phytochemicals extracted from *Chlorella* spp. and *Psidium guajava* possess broad-spectrum antiviral activity against a range of SARS-CoV-2 variants. Through chromatography-based screening, we identified four bioactive compounds and subsequently demonstrated their potential antiviral activities *in vivo*. Interestingly, in hACE2 mice, treatment with these compounds significantly attenuates SARS-CoV-2-induced proinflammatory responses, demonstrating their potential anti-inflammatory activity. Collectively, our study suggests that phytochemicals from edible plants may be readily available therapeutics and prophylactics against multiple SARS-CoV-2 strains and variants.

1. Introduction

Since the outbreak was first reported in Wuhan, China in December 2019 (Lu et al., 2020), severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has rapidly spread across the globe. To date, 262 million people were infected, and approximately 2% of the patients died from coronavirus disease 2019 (COVID-19) (WHO, 2020). COVID-19 pandemic has been posing great pressure on the current medical care system with a serious social and economic burden. Recently, several vaccines have been developed and exhibited impressive protective effects on SARS-CoV-2 infection (Krammer, 2020). However, current COVID-19 vaccination programs have not been successfully operated due to the difficulties in commercial accessibility and economic issues in some regions and countries (Grubaugh et al., 2021), causing continuous

viral spreading and outbreaks of variants of viruses. Thus, the development of the new antiviral therapy is urgently needed.

Generally, tremendous effort and time are required for developing an effective and safe drug. In order to save such efforts, 'drugs repurposing' is considered as a promising strategy for overcoming the urgent situation of the SARS-CoV-2 pandemic. For example, a recent trial with 'remdesivir', a clinically proven anti-Ebola virus disease (EVD) drug, has shown a positive antiviral effect on COVID-19 (Cohen and Kupferschmidt, 2020). Moreover, chloroquine and its derivatives, which are used for the treatment of malaria, have been examined for their potential efficacy for the treatment of multiple diseases, such as COVID-19, tissue disorders, and several types of cancers, including colon, breast, prostate, and bladder cancers (Cortegiani et al., 2020).

In general, most drugs are designed to exert their drug efficacies with

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a highly specific mechanism of action (MoA) that targets only certain molecules or signaling pathways, thus the target coverage by those drugs is specific but narrow and limited. For this reason, viruses often evolve a means to evade such drug MoA, ultimately resulting in incurring drug-resistant strains or variants. Therefore, drugs with multi-component and multi-target (MCMT) would be an ideal candidate, especially for antiviral therapy (Ramsay et al., 2018). Since coronaviruses own the largest genome among RNA viruses, and their genomes encode miscellaneous viral proteins involved in the host immune evasion and viral pathogenesis, MCMT drugs will be suitable for limiting coronavirus-induced pathogenesis.

Some diets or herbs have long been used as natural medicine or remedy from ancient times. Indeed, the medicinal efficacies of plant-derived organic compounds, such as betulinic acid derivatives and huperzine A are clinically proven for their biological activities (Ebeling et al., 2014; Stevaert et al., 2021; Yang et al., 2013). For example, SP-303, a mixture of natural proanthocyanidins isolated from the latex of *Croton lechleri*, has been being evaluated in phase II clinical trials for the treatment of genital herpes (El Sayed, 2000). Moreover, Calanolide A, a phytochemical from the *Calophyllum langigerum*, is under clinical development as a potential AIDS drug by the U.S. National Cancer Institute (Shu, 1998). Therefore, bioactive compounds from edible plants possessing antiviral properties could be a readily available low-cost natural supplement, especially for the control of diseases caused by newly emerged or re-emerged viruses.

Here, we evaluated the efficacies of 11 plant extracts for their anti-SARS-CoV-2 activity. The 11 plant extracts used for this study are previously known for their antiviral activity against the porcine epidemic diarrhea virus (PEDV), an *Alphacoronavirus* (Yu et al., 2021). Further chromatography-based screening analysis identified four bioactive compounds, pheophytin a, pheophytin b, β-caryophyllene, and Jeju-guajavone A from the bulk plant extract of *Chlorella* spp. (green algae) and *Psidium guajava* (guava). We have measured the antiviral properties of identified compounds by *in vitro* and *in vivo* systems using Vero cell lines or animal models with adapted golden Syrian hamsters and hACE2 transgenic (TG) mice. Interestingly, we found that combination treatment of animals with those compounds by oral administration exhibited significant synergistic antiviral and anti-proinflammatory activities. Taken together, our study identifies plant-derived novel bioactive compounds that possess antiviral properties against a broad range of SARS-CoV-2 strains and their variants. Furthermore, our finding suggests that phytochemicals from edible plants could be a novel candidate for developing readily available low-cost antiviral therapeutics and preventive measures.

2. Material and methods

2.1. Ethics statement

All animal experiments were approved by the Medical Research Institute, a member of the Laboratory Animal Research Center of Chungbuk National University (LARC) (approval number CBNUR-1538-21) and were conducted in strict accordance with and adherence to relevant policies regarding animal handling as mandated under the Guidelines for Animal Use and Care of the Korea Centers for Disease Control (KCDC). The handling of the virus was performed in an enhanced biosafety level 3 (BSL3) containment laboratory as approved by the Korea Centers for Disease Control and Prevention (protocol KCDC-14-3-07).

2.2. Cell and viruses

Vero cells (CCL-81, ATCC) were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/ streptomycin (PS) (Gibco). CBNU-nCoV01 (S clade), CBNU-nCoV11 (a

B.1.1.7 variant), CBNU-nCoV21 (a B.1.351 variant), and CBNU-nCoV40 (a B.1.1.529 variant) were propagated in Vero cells in Dulbecco's modified Eagle medium (DMEM; Gibco, Grand Island, NY) supplemented with 1% penicillin-streptomycin (Gibco) and TPCK (tosylsulfonyl phenylalanyl chloromethyl ketone)-treated trypsin (0.5 μg/mL; Worthington Biochemical, Lakewood, NJ) in a 37 °C incubator supplemented with 5% CO_2 for 72 h.

2.3. Isolation and characterization of active compounds from Chlorella spp. based on bioactivity-guided fractionation

Chlorella spp. powder (SNU2020-11,100 g) was extracted with 1 L of 95% of EtOH *via* stirring at 3000 rpm (90 min × 3 times), and the solvent was evaporated *in vacuo* at 35 °C. The 95% EtOH extract (2.1 g) was subjected to reversed-phase MPLC (Medium Pressure Liquid Chromatography) with 120 g C_{18} pre-packed column using EtOAc/MeOH/H_2O mixtures (0–40 min, 0/70/30 → 0/100/0; 40–110 min, 0/100/0 → 50/ 50/0; 110–130 min, 50/50/0 → 100/0/0, flow rate: 15 mL/min) to afford nine subfractions (Fr. C1–C9). Fr. C7 (37.7 mg) and C8 (13.7 mg) were subjected to semipreparative HPLC (High-Performance Liquid Chromatography) with YMC Triart C18 column (250 × 10 mm i.d., 5 μm) using A/B mixtures (A-MeOH/H_2O = 7/3, B- EtOAc). Pheophytin b (**2**) (0.3 mg) and pheophytin a (**1**) (1.1 mg) were isolated from fr. C7 (0–25 min, 50% B isocratic, flow rate: 3 mL/min) and fr. C8 (0–26 min, 54% B isocratic, flow rate: 3 mL/min), respectively.

2.4. Purification of active compounds from Psidium guajava by bioactivity-guided isolation

Dried leaves of *P. guajava* (SNU2019-03, 100 g) were extracted with 5 L of 95% EtOH *via* ultrasonication at room temperature (90 min × 3 times), and the solvent was evaporated *in vacuo* at 40 °C. The 95% EtOH extract (9.8 g) was suspended in H_2O (1 L) and partitioned with *n*-hexane (3 × 1 L), EtOAc (3 × 1 L), *n*-BuOH (3 × 1 L), and Water. The *n*-hexane part (1.1 g) was subjected to silica gel CC and eluted with *n*-hexane/EtOAc mixtures (1:0 to 0:1) to afford five fractions (Fr. 1–5). Fr. 1 (53.9 mg) was chromatographed on a silica gel column using *n*-hexane/EtOAc mixtures (1:0 to 9:1) to afford β-caryophyllene (**3**) (15.0 mg). Fr. 3 (320.2 mg) was subjected to reversed-phase MPLC using MeOH/ H_2O (0.1% formic acid) mixtures (0–60 min, 15/85 → 100/0; 60–80 min, 100/0 → 100/0, flow rate: 15 mL/min) to afford five subfractions (Fr. 3.1–3.5). Fr. 3.4 was further purified by using semipreparative HPLC with YMC Triart C18 column (10 × 250 mm, 5 μm) using CH_3CN/H_2O (0.1% formic acid) mixtures (0–65 min, 91%–93% CH_3CN, flow rate: 3.6 mL/min) to yield Jejuguajavone A (**4**) (1.2 mg).

2.5. Cytopathic effect inhibition assay against SARS-CoV-2

Vero cells were cultured overnight in 96-well plates at a density of 2 × 10^4 cells/well. The cells were infected with SARS-CoV-2 at a multiplicity of infection (MOI) of 0.01. The virus was then removed, and cells were further cultured with a fresh medium containing different concentrations of test samples. After 72 h, the cells were fixed with 10% formalin (v/v) at room temperature. The formalin was discarded, and the cells were visualized by staining the monolayer with 1% crystal violet (w/v) in 20% ethanol (v/v). Viral growth was measured in Vero cells with three independent trails as previously described.

2.6. Cytotoxicity assay

The cytotoxicity of test samples in Vero cells was evaluated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Vero cells were seeded on 96-well plates (2 × 10^4 cells per well) and grown for 24 h before use. Cells were treated with test samples (20 μM) and incubated for 72 h. To avoid the toxic effects of the solvent used, the final concentration of dimethyl sulfoxide (DMSO) in the

culture media was maintained at 0.5% (v/v). 20 μL of MTT reagent (2 mg/mL) was treated to each well, followed by incubation for 4 h further. The supernatant was carefully removed and 100 μL of DMSO was added to each well to dissolve the formazan crystals. The optical density was measured at 550 nm and the statistical significance was determined by comparing it to the vehicle group.

2.7. Animal experiments

Groups of golden Syrian hamsters were anesthetized with isoflurane and then infected intranasally with $10^{5.0}$ TCID$_{50}$/mL of SARS-CoV-2 diluted in PBS. Three hamsters per group were euthanized at 2, 4, and 6 dpi, and nasal turbinate and lungs were collected to measure tissue virus titers. Groups of 15 six-week-old female hACE mice (KRIBB, Korea) were lightly anesthetized with isoflurane and inoculated intranasally (i. n.) with $10^{4.0}$ TCID$_{50}$/mL of either CBNU-nCoV01 or CBNU-nCoV11 virus and with $10^{3.5}$ TCID$_{50}$/mL of CBNU-nCoV21, and with $10^{4.0}$ TCID$_{50}$/mL of CBNU-nCoV40 in a volume of 50 μL. Mice were monitored daily for morbidity and assessed by measuring body weight losses 7 or 14 dpi. Lung samples from three mice per group were aseptically collected at 3, 5, and 7 dpi for virus titration.

2.8. Measurements of cytokines

Bronchoalveolar lavage fluid (BALF) samples were collected from mouse lungs at 3 and 5dpi. A multiplex biometric immunoassay containing fluorescently-dyed microspheres conjugated with a monoclonal antibody specific for a target protein was used for cytokine measurement according to the manufacturer's instructions (Bio-Plex Mouse Cytokine Assay; Bio-Rad Inc., Hercules, CA). BALF samples (20 μL) were incubated with antibody-coupled beads specific for Interleukin 1 beta (IL-1β), IL-6, tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), granulocyte-macrophage colony-stimulating factor (GM-CSF), keratinocyte chemoattractant (KC-1/CXCL1), monocyte chemoattractant protein-1 (MCP-1/CCL-2), and C–C Motif Chemokine Ligand 5 (CCL-5). The complexes were washed, incubated with biotinylated detection antibody and streptavidin-phycoerythrin. Cytokine levels in BALF samples were then determined using a multiplex array reader from Luminex™ Instrumentation System (Bio-Plex Workstation, Bio-Rad Laboratories, Hercules, CA, USA).

2.9. Statistical analysis

To assess significant differences in values for viral titers statistical analyses were done. Asterisks indicate the statistical significance between vehicle-administered and treated groups determined by two-way analysis of variance (ANOVA) and a subsequent Dunnett test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; and ****, $P < 0.0001$). All statistical analyses were performed using GraphPad Prism version 9.0 for Windows (GraphPad Software, La Jolla, CA).

3. Results

3.1. Plant extracts with anti-SARS-CoV-2 activity

Previously we have shown that 11 plant extracts from the Korea Bioactive Natural Material Bank (KBNMB) exhibited the antiviral activity against porcine epidemic diarrhea virus (PEDV), an *Alphacoronavirus* (Supplemental Fig. 1) (Yu et al., 2021). Since these plant extracts have antiviral properties against coronavirus, we reason that these candidates might also exert their antiviral activities against CBNU-nCoV01, an S clade strain of SARS-CoV-2 (*Betacoronavirus*) isolated in South Korea in February 2020. To confirm our hypothesis, we evaluated the level of the cytopathic effect (CPE) of CBNU-nCoV01 with or without treatment of the plant extracts using Vero cells. Six out of eleven plant extracts tested showed various degrees of antiviral

activities against CBNU-nCoV01 (Supplemental Table 1). Prior to the analysis of the antiviral activity, we measured the cytotoxicity of the selected compounds. Although Jejuguajavone A exhibited the lowest cell viability (81%) which is not statistical differences with other compounds, most of single and combinational compounds showed above 100% cell viability until 72 h (Fig. 1b). Then, we compared the antiviral efficacy of the selected compounds against SARS-CoV-2 by measuring the EC$_{50}$ value under each or combinatorial treatment condition (Fig. 1c, Supplemental Fig. 3). While individual treatment of pheophytin a (9.20 ± 1.36 μM) and pheophytin b (5.50 ± 0.13 μM) showed moderate antiviral activity, the combinatorial treatment with pheophytin a and pheophytin b (EC$_{50}$ value of 3.82 ± 0.19 μM) together exhibited a higher activity (Fig. 1c). Similarly, compared to treatment with each β-caryophyllene (3.23 ± 0.12 μM) or Jejuguajavone A (3.22 ± 0.14 μM) alone, combinatorial treatment together with pheophytin derivatives showed greatly increased antiviral efficacy (Fig. 1c), demonstrating a synergistic antiviral effect (EC$_{50}$ of 1.27 ± 0.03 μM and 2.45 ± 0.05 μM, respectively).

Interestingly, we found that strong antiviral activity was consistently shown from certain fractions of the plant extracts which contain chlorophyll or carotenoid derivatives. To further confirm whether chlorophyll derivatives indeed possess antiviral properties, we chose green plants such as *Chlorella* spp. and *Psidium guajava* (common name; Guava), in which the chlorophyll derivatives are abundantly contained, in order to purify the chlorophyll derivatives and use them for further study.

3.2. Identification of active constituents in Chlorella spp. and P. guajava

To identify bioactive compounds that possess antiviral activity in *Chlorella* spp, and P. *guajava*, we performed a fractionation analysis with the bulk plant extracts and divided them into subfractions by reversed-phase liquid chromatography (MPLC). For *Chlorella* spp., we obtained 9 subfractions (C1 – C9) by MPLC, and we evaluated the antiviral activity of those subfractions using the CBNU-nCoV01 infection model. Among the samples tested, C1, C7, and C8 exhibited antiviral properties (Supplemental Table 2). However, other fractions (C2 – C6), which do not contain chlorophyll and carotenoid derivatives, failed to show the potential of antiviral activity, corroborating our hypothesis that chlorophylls and carotenoids may exert an anti-SARS-CoV-2 effect. High-resolution mass spectrometry (HRMS) analysis further showed that while the C1 fraction contains mixed bioactive compounds, the major components presented in C7 and C8 are pheophytins a and b (Supplemental Table 2). Thus, we determined to focus on pheophytins for further analysis, and isolated pheophytins a and b from C8 and C7, respectively (Fig. 1a and Supplemental Table 3).

For isolation of the bioactive compounds possessing antiviral property in P. *guajava*, we performed a similar analysis as we did for *Chlorella* spp. We obtained four fractions from the bulk extract of the P. *guajava* leave and identified n-hexane, EtOAc, n-BuOH, and H$_2$O as major components. To measure their antiviral property, we monitored CBNU-nCoV01-induced CPE after treatment of those compounds. Among tested, the n-hexane fraction was shown to have the strongest CPE inhibitory activity. Thus, we chose the n-hexane fraction to identify the bioactive molecules with antiviral properties. By spectroscopic analysis, we identified two compounds, β-caryophyllene and Jejuguajavone A as major active constituents in the n-hexane fraction (Fig. 1a and Supplemental Fig. 2). Finally, we confirmed that β-caryophyllene and Jejuguajavone A exert strong antiviral activity by monitoring the inhibitory effect on CBNU-nCoV01-induced CPE (Supplemental Table 3). Based on these studies, we chose four bioactive compounds, pheophytin a, pheophytin b (from *Chlorella* spp.), β-caryophyllene, and Jejuguajavone A (from P. *guajava*) as candidates for further *in vitro* and *in vivo* studies.
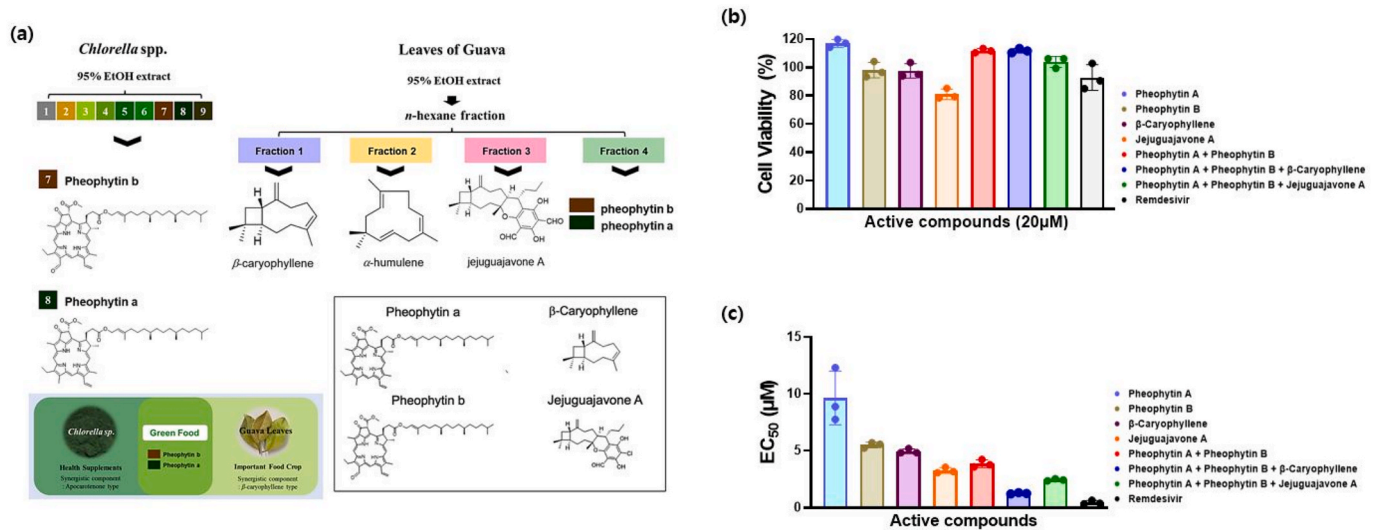
Fig. 1. Summary of discovery of active compounds from *Chlorella* spp. and P. *guajava*. (a) A schematic representation of the present studies summarizing the process of deduction of potential constituents with synergistic combination from active fractions of *Chlorella* spp. and P. *guajava*. The four compounds were isolated from the extract of *Chlorella* spp. and leaves of P. *guajava*, respectively. Pheophytin a and b are also easily obtainable from the leaves of P. *guajava*, where these compounds are predominant. (b) Cell viability and (c) reduction of SARS-CoV-2 replication in Vero cells by MTT assay. Cytotoxicity of each compound was evaluated at the concentration of 20 μM due to solubility.

3.3. Synergistic antiviral effects by combinatorial treatment of phytochemicals from the Chlorella spp. and the P. guajava in vitro and in vivo

To demonstrate the therapeutic potential of the identified compounds against SARS-CoV-2 *in vivo*, we adopted golden Syrian hamsters and human ACE2 protein-expressing transgenic mice (hACE2 TG mice) as an animal model for *in vivo* infection study. As for the initial trial, golden Syrian hamsters ($n = 9$/group) were infected with CBNU-nCoV01 ($10^{5.0}$TCID$_{50}$/mL) and followed by oral administration of a
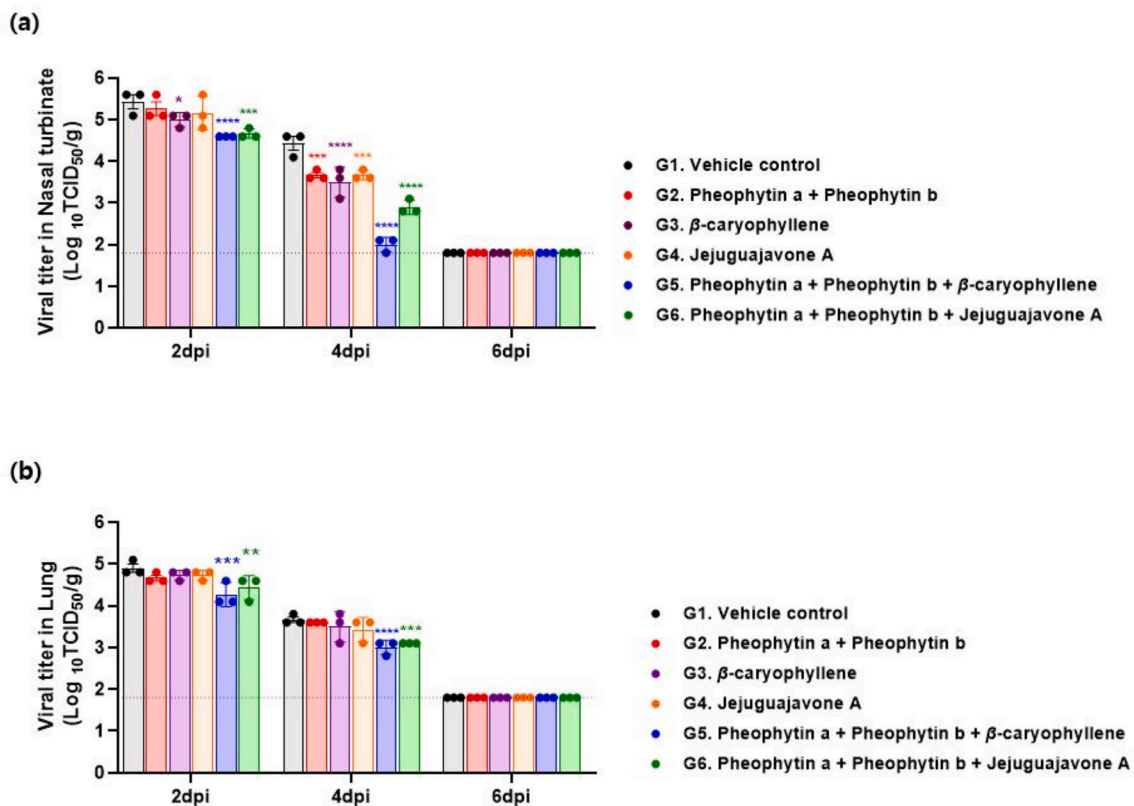

Fig. 2. The synergistic anti-SARS-CoV-2 activity of active compounds in a golden Syrian hamster model. Samples for each test group were dissolved in DMSO/EtOH/PEG400 mixed solvent and distilled water (5:5:50:40, v:v:v:v) and administered orally at 25 mg/kg/day. Groups of hamsters were sacrificed at 2, 4, and 6 dpi. (a) Viral titers in the nasal turbinates and (b) in lung tissues were measured by determining the numbers of TCID$_{50}$ per gram. The *p* values were calculated by using a two-way ANOVA, with Dunnett's post-test ($p < 0.05$, *; $p < 0.01$, **; $p < 0.001$, ***; $p < 0.0001$, ****).

single or combination of the purified phytochemicals once a day for 5 days. Then we monitored the inhibitory effect of the compounds by measuring virus titers in the upper respiratory tract (nasal turbinates). Consistent with the results obtained from the *in vitro* experiments, the treatment of the compounds showed various degrees of antiviral activity (Fig. 2). The control group (G1) exhibited the highest viral titer (5.4 $\log_{10}TCID_{50}/g$) at 2 days post-infection (dpi), persisting until 4 dpi. However, the groups with combinatorial treatments of the compounds (G5 and G6) showed significantly decreased viral titers at 2 dpi and displayed drastic antiviral effects at 4 dpi in the upper respiratory tract (Fig. 2a). Altogether, these results demonstrate that oral administration of the phytochemicals from the edible plant can be effective antiviral therapeutics against SARS-CoV-2 infection.

To test whether the combinatorial treatment of the compounds can exert an antiviral effect against a broad range of the SARS-CoV-2 strains and variants, we utilized hACE2 TG mice for the infection animal model. For evaluating the coverage of the antiviral protection, we used various SARS-CoV-2 strains including, CBNU-nCoV01 (S clade, Fig. 3a–c), CBNU-nCoV11 (an alpha variant isolate, Fig. 3d–f), and CBNU-nCoV21 (a beta variant isolate, Fig. 3g–i) strains. For measuring the antiviral efficacy, we monitored the change of body weight, mouse survival rate, and a level of viral titer in the lower respiratory tract.

Intriguingly, while the body weights of the mice from the control groups were sharply dropped, the mice of the groups treated with a combination of the phytochemicals exhibited moderated decrease until 7–8 dpi, and the bodyweight started increasing at 8–9 dpi by infection of all types of SARS-CoV-2 strains tested (Fig. 3a, d, and 3g). Surprisingly, although all mice of control groups died at 7 dpi (CBNU-nCoV01; Fig. 3b and CBNU-nCoV11; Fig 3e) and 5 dpi (CBNU-nCoV21; Fig. 3h), the mice groups with the compound treatment exhibited 20–80% survival rates (Fig. 3b, e, and 3h). Notably, among the various combination treatment conditions, the mixture of pheophytins with β-caryophyllene or

Jejuguajavone A exhibited the best efficacy. The evaluated virus titers corresponding to the experimental groups also exhibited the consistent antiviral effects of the phytochemicals (Fig. 3c, f, and 3i).

Next, we evaluated the antiviral therapeutic effects of these active compounds against the new variant, Omicron (B.1.1.529), which was first reported to the WHO on November 24, 2021 (Fig. 4). We also checked body weight losses, survival rates, and lung virus titers in the Omicron variant-infected hACE2 mice. Body weights of the control group (G1) revealed a 4–5% decrease at 3–4 dpi, while almost no body weight loss was observed among G2, G3, and G4 groups (Fig. 4a). It should be noted that Omicron variant-infected mice showed a tendency toward less severe disease compared to mice infected with other SARS-CoV-2 variants, and all mice infected with the Omicron variant survived, even without phytochemical treatment (Fig. 4b). These results suggest that the Omicron variant may be less pathogenic than other SARS-CoV-2 variants. Our results agree with the recent findings that patients infected with Omicron show decreased disease severity when compared to earlier variants (Abdullah et al., 2021). Then, we next assessed the virus titers in the lung tissues at 3, 5, and 7 dpi. Compared to the G1 group, G2, G3, and G4 groups exhibited significantly lower virus titers at 3 and 5 dpi, while G3 (mixture of pheophytins with β-caryophyllene) group showed non-detection of viral titers at 5 dpi, hence indicating viral clearance in the lower respiratory tracts (Fig. 4c). Our findings suggest that a combination of the phytochemicals induced a potent antiviral activity against a broad range of SARS-CoV-2 variants including the Omicron variant.

Altogether, these data provide evidence that oral administration of plant-derived compounds can be potential means for antiviral therapeutics targeting a broad range of SARS-CoV-2 including variants.

Fig. 3. The synergistic anti-SARS-CoV-2 activity in an hACE2 mouse. Samples for each test group were dissolved in DMSO/EtOH/PEG400 mixed solvent and distilled water (5:5:50:40, v:v:v:v) and administered orally at 25 mg/kg/day. (a–c) Groups of mice were infected with $10^{4.0}TCID_{50}/mL$ of CBNU-nCoV01 (S clade) (d–f). Each hACE2 TG mouse was infected with $10^{4.0}TCID_{50}/mL$ of CBNU-nCoV11 (a B.1.1.7 variant isolated in Korea) (g–i). Each hACE2 TG mouse was infected with $10^{3.5}TCID_{50}/mL$ of CBNU-nCoV21 (a B.1.351 variant isolated in Korea); the $TCID_{50}$ detection limit (1.8 $\log_{10}TCID_{50}/g$) is indicated by a dashed line. † indicates experiment not performed due to no surviving mice. The *p* values were calculated using a two-way ANOVA, with Dunnett's post-test ($p < 0.05$, *; $p < 0.01$, **; $p < 0.001$, ***; $p < 0.0001$, ****).
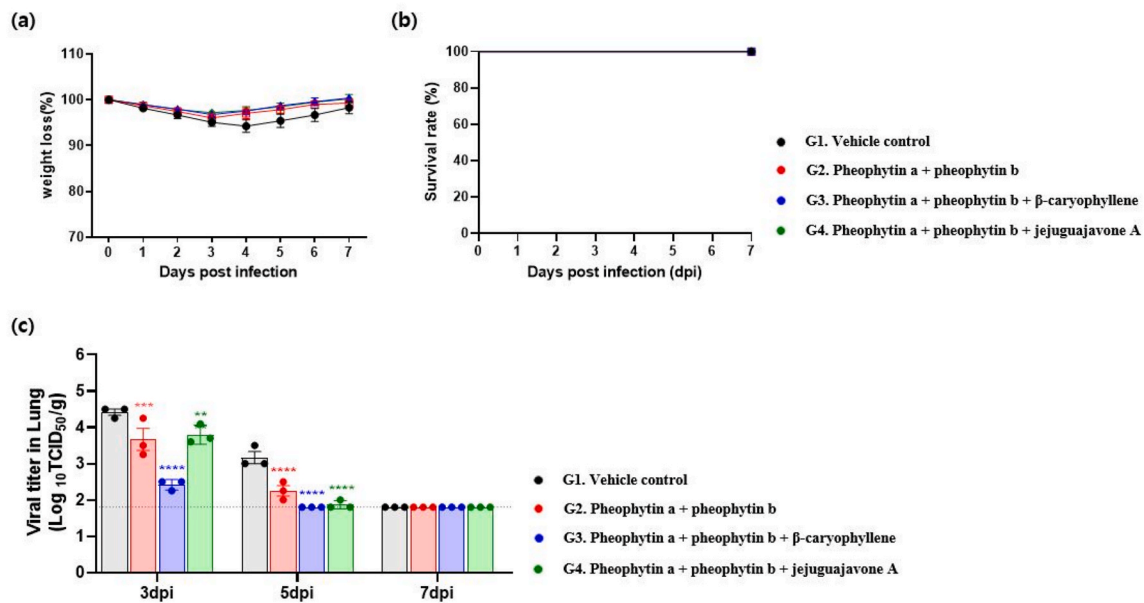
Fig. 4. Protective effects of active compounds against Omicron variant in hACE2 mouse. Samples for each test group were dissolved in DMSO/EtOH/PEG400 mixed solvent and distilled water (5:5:50:40, v:v:v:v) and administered orally at 25 mg/kg/day. (a–c) Groups of hACE2 TG mice were infected with $10^{4.0}$ TCID$_{50}$/mL of CBNU-nCoV40 (a B.1.1.529 variant isolated in Korea); the TCID$_{50}$ detection limit (1.8 log$_{10}$ TCID$_{50}$/g) is indicated by a dashed line. The *p* values were calculated using two-way ANOVA, with Dunnett's post-test ($p < 0.05$, *; $p < 0.01$, **; $p < 0.001$, ***; $p < 0.0001$, ****).

3.4. Attenuated cytokine and chemokine production by the phytochemicals

Recent studies have reported that remarkably elevated levels of proinflammatory cytokines by abnormal immune responses, termed 'cytokine storm', are closely associated with the severity and mortality of COVID-19 (Henderson et al., 2020; Mehta et al., 2020; Tufan et al., 2020). Since combinatorial treatment of the compounds could protect the animals from SARS-CoV-2-induced death, we reasoned that phytochemical treatment might attenuate the activation of proinflammatory responses. To confirm this hypothesis, we monitored the induction level of proinflammatory cytokines and chemokine using
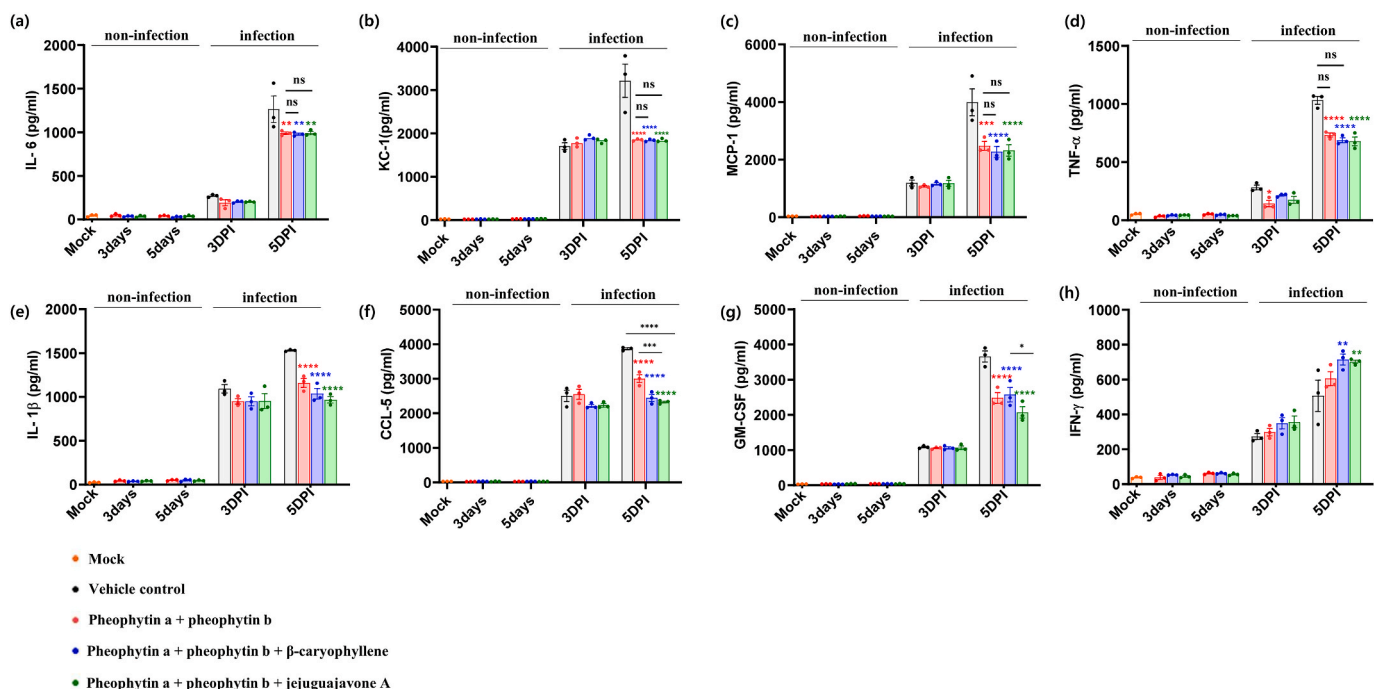

Fig. 5. Cytokine and chemokine production by SARS-CoV-2-infected mice treated with selected compounds. Samples for each test group were dissolved in DMSO/EtOH/PEG400 mixed solvent and distilled water (5:5:50:40, v:v:v:v) and administered orally at 25 mg/kg/day. Groups of mice were infected with $10^{5.0}$ TCID$_{50}$/mL of CBNU-nCoV01 (S clade). Cytokine production in lung BALF at 3 and 5dpi was analyzed including (a) IL-6, (b) KC-1, (c) MCP-1, (d) TNF-α, (e) IL-1β, (f) CCL-5, (g) GM-CSF, and (h) IFN-γ by BioPlex analysis. *Statistical significance as compared to the vehicle control group was determined by a two-way ANOVA. † indicates experiment not performed due to no surviving mice. The *p* values were calculated using a two-way ANOVA, with Dunnett's post-test ($p < 0.05$, *; $p < 0.01$, **; $p < 0.001$, ***; $p < 0.0001$, ****).

SARS-CoV-2-infected hACE2 TG mice. Mice were infected with CBNU-nCoV01 at $10^{5.0}$TCID$_{50}$/mL, and the BALF was collected twice at 3 dpi and 5 dpi. Then, the level of IL1-β, IL-6, TNF-α, IFN-γ, GM-CSF, KC-1, MCP-1, and CCL-5 was measured by Bio-Plex mouse cytokine assay (Fig. 5). We found that treatment with the compounds alone does not trigger induction of cytokines and chemokines (Fig. 5, non-infection group). However, upon SARS-CoV-2 infection, groups treated with various combinations of compounds showed significantly decreased levels of the proinflammatory cytokines and chemokines at a late time point (5 dpi) compared to the non-treated control group (Fig. 5, infection group). Notably, distinct patterns of inhibitory effect were observed by the compound combination. For example, the single treatment with pheophytin a and b showed a comparable inhibitory effect compared to the case of combinatorial treatment (Fig. 5a–d for IL-6, CXCL1/KC-1, MCP-1, and TNF-α). However, for other cytokines tested (Fig. 5e–g for IL1-β, CCL-5, and GM-CSF), compared to the case of the single treatment, a significantly increased inhibitory effect was observed by combinatorial treatment. Thus, these results suggest that distinct mechanisms are involved in attenuating the SARS-CoV-2-induced proinflammatory responses by the phytochemicals. Further study is required to address this interesting question. Intriguingly, unlike other cytokines, the induction level of IFN-γ, a critical cytokine that regulates antiviral immune responses, was significantly increased by compound treatment (Fig. 5h). Given that IFN-γ is critical for eliminating the viral replication through activating cytotoxic T cells (Yoo et al., 2021), these results suggest that treatment with these phytochemicals might boost antiviral innate immune responses. Taken together, these results demonstrate that the administration of a combination of selected plant-derived bioactive compounds could modulate SARS-CoV-2-induced host antiviral immune responses.

4. Discussion

Despite the extraordinarily rapid development of vaccines against SARS-CoV-2, the emergence of new SARS-CoV-2 variants is a continuous threat to human global public health. Unsuccessful virus control and the unbalanced distribution of vaccines and therapeutics have allowed the pandemic to rage on in large populations. The emergence of novel SARS-CoV-2 variants due to rapid viral evolution has resulted in a rapid global spread, such as was seen with the recent outbreak of the Omicron variant in Africa (Sahoo and Samal, 2021). Moreover, emerging novel variants may cause reverse zoonotic infection in animals potentially resulting in the establishment of novel viral reservoirs (Halfmann et al., 2020; Schlottau et al., 2020; Tiwari et al., 2020). To overcome these hurdles, a broader spectrum and easily available antiviral agents are urgently needed. In addition to the rapid development of potential therapeutics, especially for sudden emerging and re-emerging pathogen outbreaks, affordable and easily accessible preventive tools would be ideal.

In this study, we identified edible plant-based phytochemicals possessing antiviral properties against a broad range of SARS-CoV-2 variants. We found that four compounds, pheophytin a, pheophytin b, β-caryophyllene, and Jejuguajavone A from Chlorella spp. and P. guajava could suppress viral replication both *in vitro* and *in vivo* using hamsters and hACE2 TG mouse animal models. Furthermore, combinatorial treatment with the mixture of pheophytins with β-caryophyllene or Jejuguajavone A showed significant synergistic antiviral effects resulting in the protection of infected animals from the lethal challenge with SARS-CoV-2 variants, including Omicron (Fig. 4). The detailed modes of action for the antiviral activities of each compound were not investigated in this study. However, given that the mechanism of action for their known biological activities is distinct (Fernandes et al., 2007; Gomes et al., 2009; Pi-Yu et al., 2018; Zhang et al., 2019), presumably, the antiviral efficacies of the combination of these compounds are derived from the manifestation of MCMT. Taken together, our results provided strong evidence that combinatorial treatment with these compounds is efficacious against SARS-CoV-2 infections, including

recently emerge variant strains.

The innate immune system inhibits pathogen entry and prevents viral replication, making it a crucial component of host defense. However, studies reported that SARS-CoV-2 causes a delay in the innate immune response, hampering the immune system and resulting in uncontrolled and exacerbated inflammatory responses (Coperchini et al., 2020; Csermely et al., 2005; Kasuga et al., 2021). In this study, we show that combinatorial administration of compounds derived from Chlorella spp. and P. guajava attenuates the induction of proinflammatory responses (Fig. 5). Intriguingly, induction of IFN-γ was slightly, but significantly, upregulated in response to these compounds. Proinflammatory cytokines and chemokines tested here are critical drivers of 'cytokine storm' induced by myeloid-derived immune cells (e.g., macrophage) upon SARS-CoV-2 infection (Pedersen and Ho, 2020; Zhang et al., 2021). To test whether the antiviral phytochemicals also have immunomodulatory effects, we evaluated the immune-modulatory effects of each phytochemical compound on polyinosinic-polycytidylic acid (poly(I:C))-stimulated RAW264.7 macrophages. Results confirmed that these compounds markedly attenuate the production of pro-inflammatory cytokines and chemokines in a dose-dependent manner (Supplemental Fig. 4). These results suggest that phytochemicals may be used as mitigation strategy for viral infection through suppression of the TLR3 pathway.

In contrast, IFN-γ, which is predominantly produced by activated T cells or natural killer (NK) cells, plays a pivotal role in viral clearance (Schoenborn and Wilson, 2007). Hence, although it remains to be clarified, it is reasonable to speculate that the improved survival following treatment with phytochemicals is likely due to boosting of the antiviral function of T cells and NK cells, along with attenuation of the abnormally activated proinflammatory myeloid cells. It is noteworthy that, unlike the pheophytins, β-caryophyllene and Jejuguajavone A exert additional chemotaxis inhibitory properties which may inhibit proinflammatory cell migration to the site of infection. Therefore, the anti-inflammatory and immunomodulatory properties of these compounds along with their antiviral effects suggest that they should be considered as additional COVID-19 therapeutic interventions.

During the series of recent outbreaks, including the Delta and Omicron variants, it has become apparent that there is an urgent need, particularly in the least developed and developing countries without access to COVID vaccines and therapeutics, for economically and practically accessible alternative treatment methods. Moreover, having such interventions with readily available low-cost natural supplements will be beneficial for control of the newly emerged or re-emerged viruses before specifically targeted drugs or vaccines are developed. These results suggest that bioactive phytochemicals with antiviral properties from edible plants could be a promising therapeutic option for the treatment of COVID-19, especially for those populations without access to the antiviral drugs or SARS-CoV-2 vaccines (Gu et al., 2020). Altogether, the phytochemical compounds tested in this study showed positive attributes that might be evaluated in clinical studies as well as an adjunctive treatment. We believe that the findings provided might well cover a research problem and may establish new research options, particularly in the development of therapeutic bioproducts.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2022.105371.

References

Abdullah, F., Myers, J., Basu, D., Tintinger, G., Ueckermann, V., Mathebula, M., Ramlall, R., Spoor, S., de Villiers, T., Van der Walt, Z., 2021. Decreased severity of disease during the first global omicron variant covid-19 outbreak in a large hospital in tshwane, South Africa. Int. J. Infect. Dis. 28, 38–42.

Cohen, J., Kupferschmidt, K., 2020. 'A very, very bad look'for remdesivir. Science 370, 642–643.

Coperchini, F., Chiovato, L., Croce, L., Magri, F., Rotondi, M., 2020. The cytokine storm in COVID-19: an overview of the involvement of the chemokine/chemokine-receptor system. Cytokine Growth Factor Rev. 53, 25–32.

Cortegiani, A., Ingoglia, G., Ippolito, M., Giarratano, A., Einav, S., 2020. A systematic review on the efficacy and safety of chloroquine for the treatment of COVID-19. J. Crit. Care 57, 279–283.

Csermely, P., Agoston, V., Pongor, S., 2005. The efficiency of multi-target drugs: the network approach might help drug design. Trends Pharmacol. Sci. 26, 178–182.

Ebeling, S., Naumann, K., Pollok, S., Wardecki, T., Vidal-y-Sy, S., Nascimento, J.M., Boerries, M., Schmidt, G., Brandner, J.M., Merfort, I., 2014. From a traditional medicinal plant to a rational drug: understanding the clinically proven wound healing efficacy of birch bark extract. PLoS One 9, e86147.

El Sayed, K.A., 2000. Natural products as antiviral agents. Stud. Nat. Prod. Chem. 24, 473–572.

Fernandes, T.M., Gomes, B.B., Lanfer-Marquez, U.M., 2007. Apparent absorption of chlorophyll from spinach in an assay with dogs. Innovat. Food Sci. Emerg. Technol. 8, 426–432.

Gomes, B.B., Barros, S.B., Andrade-Wartha, E.R., Silva, A.M., Silva, V.V., Lanfer-Marquez, U.M., 2009. Bioavailability of dietary sodium copper chlorophyllin and its effect on antioxidant defence parameters of Wistar rats. J. Sci. Food Agric. 89, 2003–2010.

Grubaugh, N.D., Hodcroft, E.B., Fauver, J.R., Phelan, A.L., Cevik, M., 2021. Public health actions to control new SARS-CoV-2 variants. Cell 184, 1127–1132.

Gu, H., Chen, Q., Yang, G., He, L., Fan, H., Deng, Y.-Q., Wang, Y., Teng, Y., Zhao, Z., Cui, Y., 2020. Adaptation of SARS-CoV-2 in BALB/c mice for testing vaccine efficacy. Science 369, 1603–1607.

Halfmann, P.J., Hatta, M., Chiba, S., Maemura, T., Fan, S., Takeda, M., Kinoshita, N., Hattori, S.-i., Sakai-Tagawa, Y., Iwatsuki-Horimoto, K., 2020. Transmission of SARS-CoV-2 in domestic cats. N. Engl. J. Med. 383, 592–594.

Henderson, L.A., Canna, S.W., Schulert, G.S., Volpi, S., Lee, P.Y., Kernan, K.F., Caricchio, R., Mahmud, S., Hazen, M.M., Halyabar, O., 2020. On the alert for cytokine storm: immunopathology in COVID-19. Arthritis Rheum. 72, 1059–1063.

Kasuga, Y., Zhu, B., Jang, K.-J., Yoo, J.-S., 2021. Innate immune sensing of coronavirus and viral evasion strategies. Exp. Mol. Med. 53, 723–736.

Krammer, F., 2020. SARS-CoV-2 vaccines in development. Nature 586, 516–527.

Lu, H., Stratton, C.W., Tang, Y.W., 2020. The Wuhan SARS-CoV-2—what's next for China. J. Med. Virol. 92, 546–547.

Mehta, P., McAuley, D.F., Brown, M., Sanchez, E., Tattersall, R.S., Manson, J.J., 2020. COVID-19: consider cytokine storm syndromes and immunosuppression. The lancet 395, 1033–1034.

Pedersen, S.F., Ho, Y.-C., 2020. SARS-CoV-2: a storm is raging. J. Clin. Invest. 130, 2202–2205.

Pi-Yu, C., Huang, M.-Y., Huang, W.-D., Lin, K.-H.R., Shiau-Ying, C., Chi-Ming, Y., 2018. Study of chlorophyll-related compounds from dietary spinach in human blood. Not. Bot. Horti Agrobot. Cluj-Napoca 46, 309–316.

Ramsay, R.R., Popovic-Nikolic, M.R., Nikolic, K., Uliassi, E., Bolognesi, M.L., 2018. A perspective on multi-target drug discovery and design for complex diseases. J. Transl. Med. 7, 1–14.

Sahoo, J.P., Samal, K.C., 2021. World on alert: WHO designated South African new COVID strain (omicron/B. 1.1. 529) as a variant of concern. Biotica Res. Today 3, 1086–1088.

Schlottau, K., Rissmann, M., Graaf, A., Schön, J., Sehl, J., Wylezich, C., Höper, D., Mettenleiter, T.C., Balkema-Buschmann, A., Harder, T., 2020. SARS-CoV-2 in fruit bats, ferrets, pigs, and chickens: an experimental transmission study. The Lancet Microb. 1, e218–e225.

Schoenborn, J.R., Wilson, C.B., 2007. Regulation of interferon-γ during innate and adaptive immune responses. Adv. Immunol. 96, 41–101.

Shu, Y.-Z., 1998. Recent natural products based drug development: a pharmaceutical industry perspective. J. Nat. Prod. 61, 1053–1071.

Stevaert, A., Krasniqi, B., Van Loy, B., Nguyen, T., Thomas, J., Vandeput, J., Jochmans, D., Thiel, V., Dijkman, R., Dehaen, W., 2021. Betulonic acid derivatives interfering with human coronavirus 229E replication via the nsp15 endoribonuclease. J. Med. Chem. 64, 5632–5644.

Tiwari, R., Dhama, K., Sharun, K., Iqbal Yatoo, M., Malik, Y.S., Singh, R., Michalak, I., Sah, R., Bonilla-Aldana, D.K., Rodriguez-Morales, A.J., 2020. COVID-19: animals, veterinary and zoonotic links. Vet. Q. 40, 169–182.

Tufan, A., Güler, A.A., Matucci-Cerinic, M., 2020. COVID-19, immune system response, hyperinflammation and repurposing antirheumatic drugs. Turk. J. Med. Sci. 50, 620–632.

WHO, 2020. Coronavirus Disease (COVID-19): Weekly Epidemiological Update.

Yang, G., Wang, Y., Tian, J., Liu, J.-P., 2013. Huperzine A for Alzheimer's disease: a systematic review and meta-analysis of randomized clinical trials. PLoS One 8, e74916.

Yoo, J.-S., Sasaki, M., Cho, S.X., Kasuga, Y., Zhu, B., Ouda, R., Orba, Y., de Figueiredo, P., Sawa, H., Kobayashi, K.S., 2021. SARS-CoV-2 inhibits induction of the MHC class I pathway by targeting the STAT1-IRF1-NLRC5 axis. Nat. Commun. 12, 1–17.

Yu, J., Sreenivasan, C., Uprety, T., Gao, R., Huang, C., Lee, E.J., Lawson, S., Nelson, J., Christopher-Hennings, J., Kaushik, R.S., 2021. Piglet immunization with a spike subunit vaccine enhances disease by porcine epidemic diarrhea virus. npj Vaccin. 6, 1–5.

Zhang, J., Wu, H., Yao, X., Zhang, D., Zhou, Y., Fu, B., Wang, W., Li, H., Wang, Z., Hu, Z., 2021. Pyroptotic macrophages stimulate the SARS-CoV-2-associated cytokine storm. Cell. Mol. Immunol. 18, 1305–1307.

Zhang, R., Zhu, X., Bai, H., Ning, K., 2019. Network pharmacology databases for traditional Chinese medicine: review and assessment. Front. Pharmacol. 10, 123.