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Inhibitory effect of [6]-shogaol against 3CLpro activity and SARS-CoV-2 infection

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Abstract

Background *Zingiber officinale* Roscoe (ginger) has been traditionally used not only as a culinary ingredient, but also for its medicinal properties. In Japan, ginger-derived crude drugs are categorized into dried ginger (*Zingiberis Rhizoma* [ZR]) or steamed ginger (*Zingiberis Rhizoma processum* [ZR-P]). Heating ginger converts gingerol, the primary component of ZR, into shogaol. In this study, we aimed to evaluate the inhibitory effects of ginger and its constituents on SARS-CoV-2 infection.

Methods We assessed the inhibitory effects of ZR, ZR-P, [6]-gingerol, and [6]-shogaol on SARS-CoV-2 infection in VeroE6/TMPRSS2 cells. To understand the molecular mechanism underlying this inhibitory effect, we evaluated the activity of [6]-shogaol against the viral proteases 3 C-like protease (3CLpro) and papain-like protease (PLpro).

Results We observed that ZR-P (66.1% suppression at 50 µg/ml) and [6]-shogaol (70.0% suppression at 25 µM) exhibited significant inhibitory effects against SARS-CoV-2 infection. We found that [6]-shogaol effectively inhibited 3CLpro activities both *in vitro* (20.4% inhibition at 40 µM) and in FlipGFP reporter system (19.7% inhibition at 25 µM).

Conclusions This study highlights [6]-shogaol and ZR-P as promising natural inhibitors of SARS-CoV-2, providing new insights into the pharmacological potential of traditional ginger preparations and their active components against COVID-19.

Keywords SARS-CoV-2, Shogaol, 3CL protease, COVID-19

Introduction

Viruses often affect humans, often causing serious illness and death. In recent years, we experienced the coronavirus disease (COVID-19) pandemic, which is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Vaccines against SARS-CoV-2 have been developed; however, the emergence of new variants of SARS-CoV-2 may compromise the effectiveness of the vaccine, and various treatments against COVID-19 are needed [1]. Therapeutic agents that target key proteins involved in viral replication and transcription have been developed, including 3 C-like protease (3CLpro), papain-like protease (PLpro), and RNA-dependent RNA polymerase (RdRp) [2–4]. Remdesivir, which has received Food and Drug Administration (FDA) approval for the

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treatment of COVID-19, inhibits viral replication by targeting RdRp; however, its effectiveness is limited [5, 6]. The viral genome (ssRNA) uses the host ribosome to translate viral RNA into a long polypeptide (PP) chain. Two proteases encoded by the viral genome, 3CLpro and PLpro, cleave the newly formed PP chain to generate several nonstructural proteins (NSPs) necessary for viral replication [7]. 3CLpro generates 11 of 16 NSPs, making this protease a prime target for the development of anti-SARS-CoV-2 drugs. 3CLpro has been validated as an important antiviral target because nirmatrelvir and ensitrelvir, which are inhibitors of SARS-CoV-2 3CLpro, have been reported to reduce the risk of severe disease and death from COVID-19 [8].

Natural products have been used as medicines throughout the world since ancient times. Several studies have shown that natural products have the potential to manage and treat COVID-19 [9–11]. *Zingiber officinale* Roscoe (ginger) belongs to the family Zingiberaceae and has been used as a traditional herbal medicine as well as food and spice around the world [12–14]. It has attracted considerable attention because of its wide range

of pharmacological activities, including antioxidant, anti-inflammatory, anticancer, antiemetic, and antibacterial effects [15, 16]. *Zingiber officinale* has been reported to effectively shorten the duration of clinical recovery and improve viral clearance in patients with mild and moderate COVID-19 [17].

In Japan, crude drugs derived from *Zingiber officinale* Roscoe are defined in the 18th edition of the Japanese Pharmacopoeia and categorized into dried ginger (Zingiberis Rhizoma [ZR]) or steamed ginger (Zingiberis Rhizoma Processum [ZR-P]) (Fig. 1) [18]. Ginger contains many phytochemicals, including phenolic compounds, terpene compounds, carbohydrates, and lipids. Among these, phenolic compounds such as gingerols and shogaols have been reported as the main components of ginger [19]. When ginger is heat-treated, [6]-gingerol is converted to its dehydrated form, [6]-shogaol [19–23]. Therefore, ZR-P contains a larger amount of [6]-shogaol than ZR. Moreover, [6]-gingerol and [6]-shogaol are responsible for the medicinal effects of ZR and ZR-P, respectively [18]. However, despite the known antiviral potential of *Zingiber officinale* and its major constituents

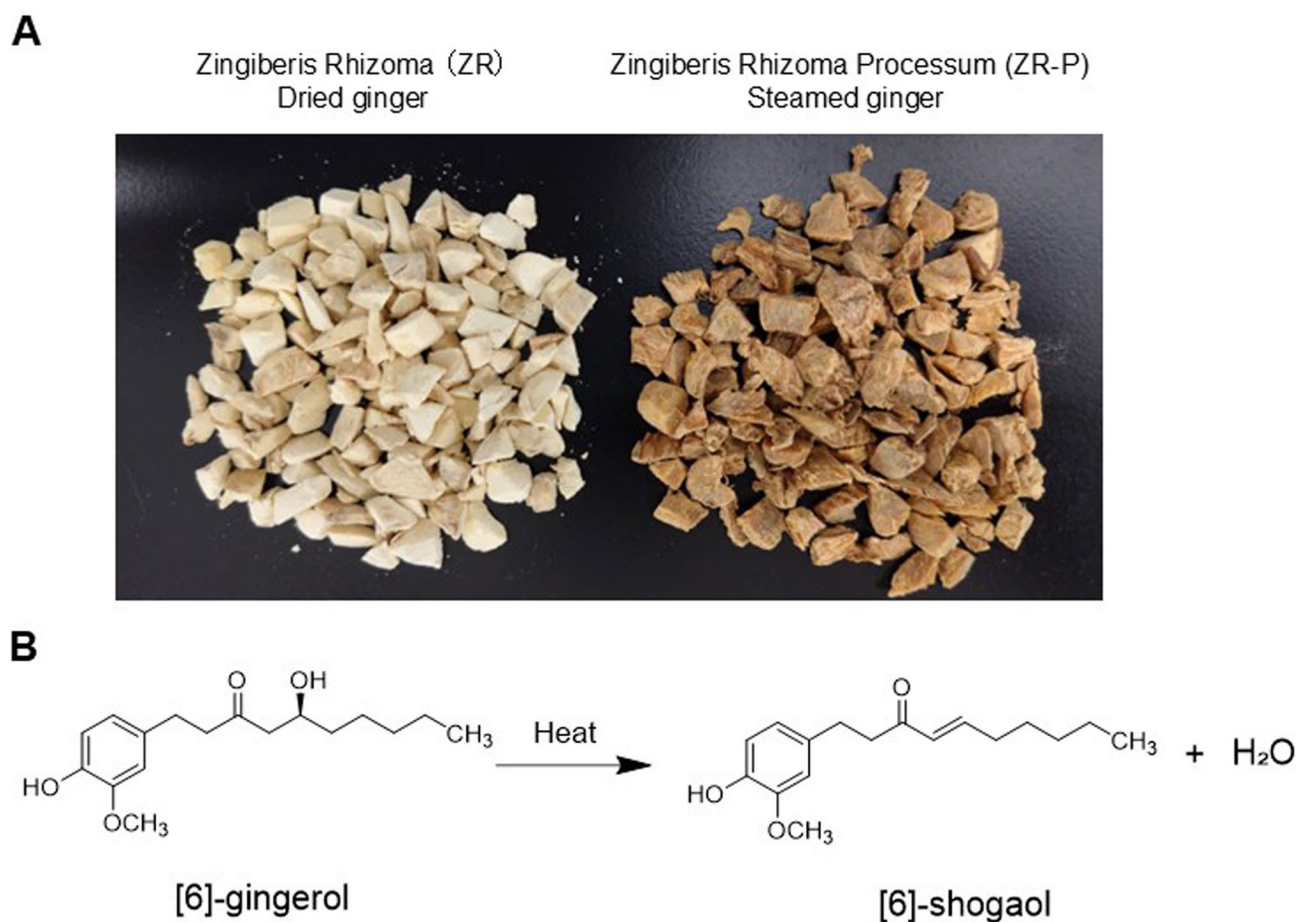


Fig. 1 **A** Zingiberis Rhizoma (Dried ginger) and Zingiberis Rhizoma Processum (Steamed ginger). **B** [6]-gingerol is converted to its dehydrated form, [6]-shogaol, through a heating process

such as [6]-gingerol and [6]-shogaol, little is known about the antiviral efficacy of ginger preparations derived from different processing methods, including ZR and ZR-P, against SARS-CoV-2. In particular, direct evidence regarding their effects on viral replication—especially through inhibition of key viral proteases such as 3CLpro—is still lacking.

In this study, we investigated the inhibitory effects of ZR and ZR-P on SARS-CoV-2 infection and found that ZR inhibited SARS-CoV-2 infection in VeroE6/TMPRSS2 cells. This could be because [6]-shogaol directly inhibited 3CLpro activity.

Materials and methods

Materials

ZR (Cat. No. 11250) and ZR-P (Cat. No. 5260) were purchased from Uchida Wakanyaku Co., Ltd. (Tokyo, Japan) and Tochimoto Tenkaido Co. Ltd. (Osaka, Japan), respectively. The extracts were prepared as previously described [24]. Briefly, the crude drugs were refluxed in 70% ethanol for 1 h, and the extracts were dried and dissolved in dimethyl sulfoxide at a concentration of 10 mg/mL. [6]-gingerol (Cat. No. 11707) and [6]-shogaol (Cat. No. 11901) were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Analytical HPLC was performed on a Jasco PU-4580 instrument equipped with a UV-4570 detector (Jasco Corp., Easton, MD, USA). The HPLC column was a CAPCELL PAK C18 ACR column (4.6×250 mm, 5 mm, Osaka Soda Co., Ltd., Osaka, Japan). A mixture of acetonitrile and water (0–10 min: 20:80, 10–50 min: from 20:80 to 100:0, 50–60 min: 100:0) was used as the solvent at a flow rate of 1.0 mL/min. Chromatograms were monitored at 254 nm. The 70% ethanol extracts of the crude drugs (ZR and ZR-P) were dissolved at a concentration of 1.0 mg/200 mL, and 20 mL was injected into the column. Following purification, each of the isolated compounds was dissolved at a concentration of 0.1 mg/mL, and 20 μ L was injected for analysis (Supplemental figure, Fig. S1).

Evaluation of anti-SARS-CoV-2 activity

The antiviral activity against SARS-CoV-2 was assessed as described in a previous study [25, 26]. Briefly, VeroE6/TMPRSS2 cells (JCRB1819, JCRB Cell Bank, Osaka, Japan), which overexpress TMPRSS2, were cultured in a 96-well plate (CellCarrier-96 Ultra, Perkin Elmer, Waltham, MA, USA), and pretreated with the reagent at 37°C for 1 h. The final concentrations of samples were 12.5, 25, and 50 μ g/mL for ZP and ZP-P, and 12.5, 25, and 50 μ M for [6]-gingerol and [6]-shogaol. Subsequently, SARS-CoV-2 (2019-nCoV/Japan/TY/WK-521/2020 strain, WK-521) was inoculated at a multiplicity of infection (MOI) of 0.5 in the presence of the reagent. The MOI was calculated based on virus titers measured by

immunofluorescence (IF) analysis in this cell line using an anti-SARS-CoV-2 S protein antibody, as previously described [26]. After 24 h of incubation at 37°C, the infected cells were fixed with 4% paraformaldehyde in D-PBS for 30 min and permeabilized with 0.2% Triton X-100 in D-PBS for 15 min. Immunostaining was performed using rabbit anti-SARS-CoV-2 Spike RBD monoclonal antibody (1:3,000, clone HL1003, GTX635792; GeneTex, Irvine, CA, USA) as the primary antibody and goat anti-rabbit IgG Alexa Fluor 488 (1:1,000, Life Technologies, Carlsbad, CA, USA) as the secondary antibody. The nuclei were stained with 1 μ g/mL of 4',6-diamidino-2-phenylindole (DAPI) solution (Dojindo Laboratories, Kumamoto, Japan). The cells were imaged using the Operetta CLS High-Content Analysis System (Perkin Elmer), and SARS-CoV-2 S- and DAPI-positive cells were counted using Harmony software (Perkin Elmer).

3CLpro and PLpro assay

The 3CLpro and PLpro assays were conducted according to previous reports, with modification [27, 28]. *E. coli* BL21(DE3) cells (DS255, BioDynamics Laboratory Inc., Tokyo, Japan) were transformed with codon-optimized N-terminal GST-tagged SARS-CoV-2 3CLpro or PLpro expression plasmids in vectors (pGEX-6X-1 for 3CLpro or pGEX-6P-1 for PLpro). The cells were grown and protein expression was induced by adding 1 mM IPTG. Cultured *E. coli* cells were harvested and washed with D-PBS. The pellet was resuspended in lysis buffer (1% Triton X-100, 20 mM Tris-HCl, pH 7.5, and 150 mM NaCl) and homogenized by sonication. The supernatants were obtained by centrifuging at $15,000 \times g$ for 10 min at 4°C. 3CLpro and PLpro were purified using GST-accept (Nacalai, Kyoto, Japan), according to the manufacturer's instructions. GST-PLpro was treated with PreScission protease (Cytiva, Tokyo, Japan) to remove GST. Because 3CLpro activity was significantly reduced during the GST removal process, purified GST-3CLpro was used for the protease assay. 3CLpro and PLpro assays were performed using the fluorogenic substrate Ac-Thz-Tle-Leu-Gln-MCA (PEPTIDE Institute Inc., Osaka, Japan) and Z-Arg-Leu-Arg-Gly-Gly-MCA (PEPTIDE Institute Inc.). The reaction mixture consisting of purified protease (3CLpro or PLpro), assay buffer (20 mM Tris-HCl pH7.5, 1 mM EDTA, and 150 mM NaCl for the 3CLpro assay, and 20 mM Tris-HCl pH7.5, 1 mM DTT, and 150 mM NaCl for the PLpro assay), and 50 μ M fluorogenic substrate was incubated for 30 min at 37°C, and the fluorescence was monitored with an excitation wavelength of 380 nm and an emission wavelength of 460 nm every 3 min using a Synergy H1 microplate reader (Agilent Technologies, Santa Clara, CA, USA). The substrate specificity and inhibitor evaluation of 3CLpro and PLpro activity are shown in Supplemental figure, Fig. S2.

A cell-based flipped green fluorescent protein (Flip-GFP) assay was performed according to a previously reported method with a minor modification [29]. 293T cells (Riken BioResource, RCB2202) were seeded in 24-well plates and grown overnight to 70–90% confluency. 293T cells were transfected with 250 ng of the pcDNA3 FlipGFP (Mpro) T2A mCherry (Addgene plasmid#163078) plasmid and 250 ng of the pcDNA3 H2B-mIFP T2A Mpro (wt) (Addgene plasmid #163079)

using TransIT-293 (Mirus Bio, Madison, WI). The pcDNA3 FlipGFP(Mpro) T2A mCherry and pcDNA3 FlipGFP(Mpro) T2A mCherry were a gift from Xiaokun Shu. After 2 h of transfection, the cells were treated with the indicated concentrations of reagents and further incubated for 46 h. Cells were washed, suspended in D-PBS, and transferred to 96-well black plates, and GFP and mCherry signals were measured using a Synergy H1 microplate reader.

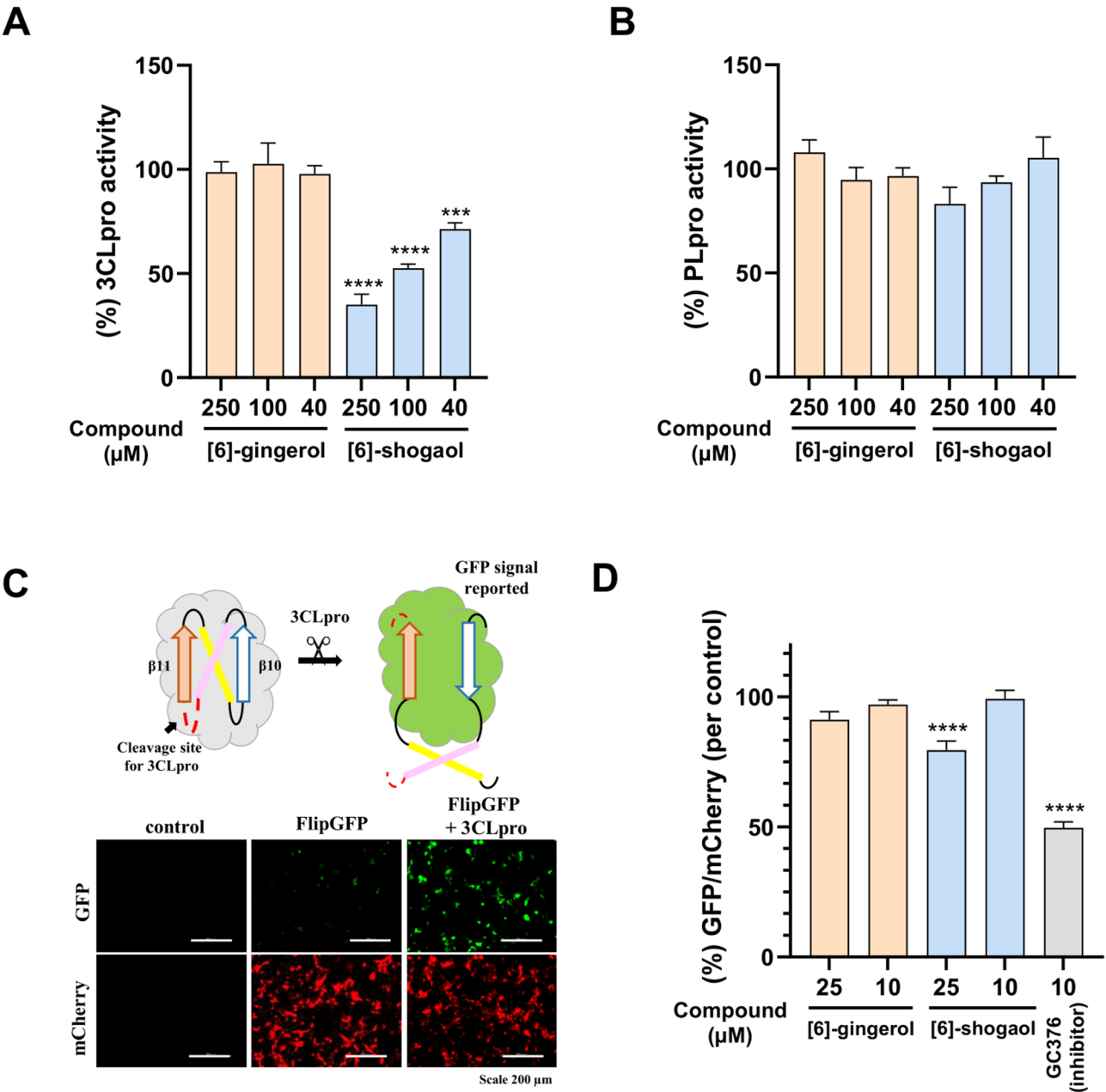


Fig. 2 The inhibitory effects of ZR-P and ZR on SARS-CoV-2 infection. **A, B** VeroE6/TMPRSS2 cells were infected with SARS-CoV-2 for 24 h in the presence of the indicated concentration of ZR-P and ZR. The percentages of cell numbers and infectivity were normalized to those of DMSO-treated cells infected with SARS-CoV-2. **C** Representative fluorescence images show SARS-CoV-2 S protein (green) and cell nucleus (blue). Scale bar, 1 mm. Values represent the mean $\pm$ SD of two independent experiments ($n=6$)

Statistical analysis

The experiments were conducted in triplicate for reproducibility. Statistical analysis was performed using a one-way analysis of variance (ANOVA) with Dunnett's test. The p -value was set at $p < 0.05$.

Results

Anti-SARS-CoV-2 activity of ZR and ZR-P

We first examined the effects of ZR (dried ginger) and ZR-P (steamed ginger) on SARS-CoV-2 infection. Both ZR and ZR-P showed virtually no cytotoxicity because more than 80% of VeroE6/TMPRSS2 cells were alive even at a high concentration of 50 $\mu\text{g/mL}$ (Fig. 2A). For an infection study, we pretreated VeroE6/TMPRSS2 cells with either serially diluted gingers (50, 25, and 12.5 $\mu\text{g/mL}$), and then cells were infected with SARS-CoV-2 in the presence of ZR and ZR-P. In the presence of ZR-P,

SARS-CoV-2 infection was significantly suppressed at 50 $\mu\text{g/mL}$ (66.1% suppression) without cytotoxicity, however ZR did not suppress infection (Fig. 2B and C).

Inhibitory effect of [6]-shogaol on SARS-CoV-2 infection

To identify the constituents of ZR-P with inhibitory effects on SARS-CoV-2 infection, we examined the effects of [6]-gingerol and [6]-shogaol at concentrations of 50, 25, and 12.5 μM (Fig. 3). [6]-gingerol showed no significant changes in either cell viability or infection rate at any of the concentrations tested. In contrast, [6]-shogaol reduced cell viability to approximately 40.6% at 50 μM , indicating considerable cytotoxicity, while exhibiting only minimal cytotoxicity at 25 μM (88.9% viability) and no detectable cytotoxicity at 12.5 μM (approximately 102.0% viability). With respect to antiviral activity, [6]-shogaol exhibited infection-suppressive effects at all tested

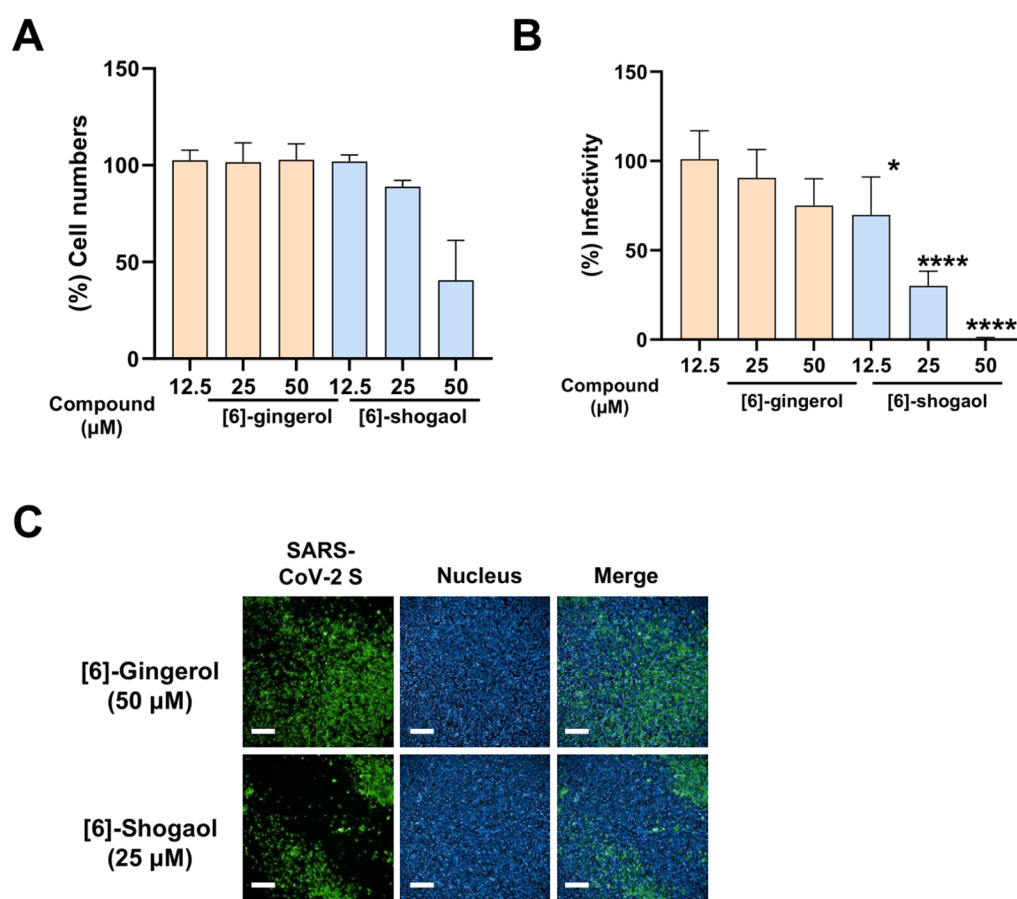


Fig. 3 The inhibitory effects of [6]-gingerol and [6]-shogaol on SARS-CoV-2 infection. **A, B** VeroE6/TMPRSS2 cells were infected with SARS-CoV-2 for 24 h in the presence of the indicated concentration of [6]-gingerol and [6]-shogaol. The percentages of cell numbers and infectivity are shown. **C** Representative fluorescence images show SARS-CoV-2 S protein (green) and cell nucleus (blue). Scale bar, 1 mm. Values represent the mean $\pm$ SD of two independent experiments ($n=6$)

concentrations. However, at 50 μM , the observed inhibition could have been influenced by cytotoxic effects. Notably, [6]-shogaol at 12.5 μM (30.2% suppression) and 25 μM (70.0% suppression) demonstrated clear antiviral effects without significant cytotoxicity, suggesting that its inhibitory activity is not attributable to cytotoxicity.

Inhibitory effect of [6]-shogaol on SARS-CoV-2 3CLpro

To understand the molecular mechanisms underlying the inhibitory effect of [6]-shogaol on viral infection, we examined whether [6]-shogaol and [6]-gingerol inhibited 3CLpro or PLpro, which are essential for viral replication. The enzymatic activity was measured using fluorescence-labeled substrates (Fig. 4). [6]-shogaol exhibited a strong concentration-dependent inhibitory effect against 3CLpro (20.4% inhibition at 40 μM), but not against

PLpro. However, [6]-gingerol had no inhibitory effect on either 3CLpro or PLpro. Thus, in *in vitro* assays evaluating the activity of recombinant protease, a comparison at middle (40 μM) and high concentrations (250 μM and 100 μM) clearly demonstrated that [6]-shogaol exhibited a more pronounced inhibitory effect on 3CLpro than [6]-gingerol.

To evaluate the effects of 3CLpro inhibition in cells, 3CLpro activity was assessed using a FlipGFP reporter system derived from a split GFP. In this system, GFP is not regenerated in the absence of proteases. Upon 3CLpro digestion, a part of the GFP undergoes a conformational change to meet another part of the GFP, resulting in the regeneration of the GFP signal (Fig. 4C). In the co-expression of 3CLpro and the FlipGFP reporter, the GFP signal was restored by the cleavage of 3CLpro.

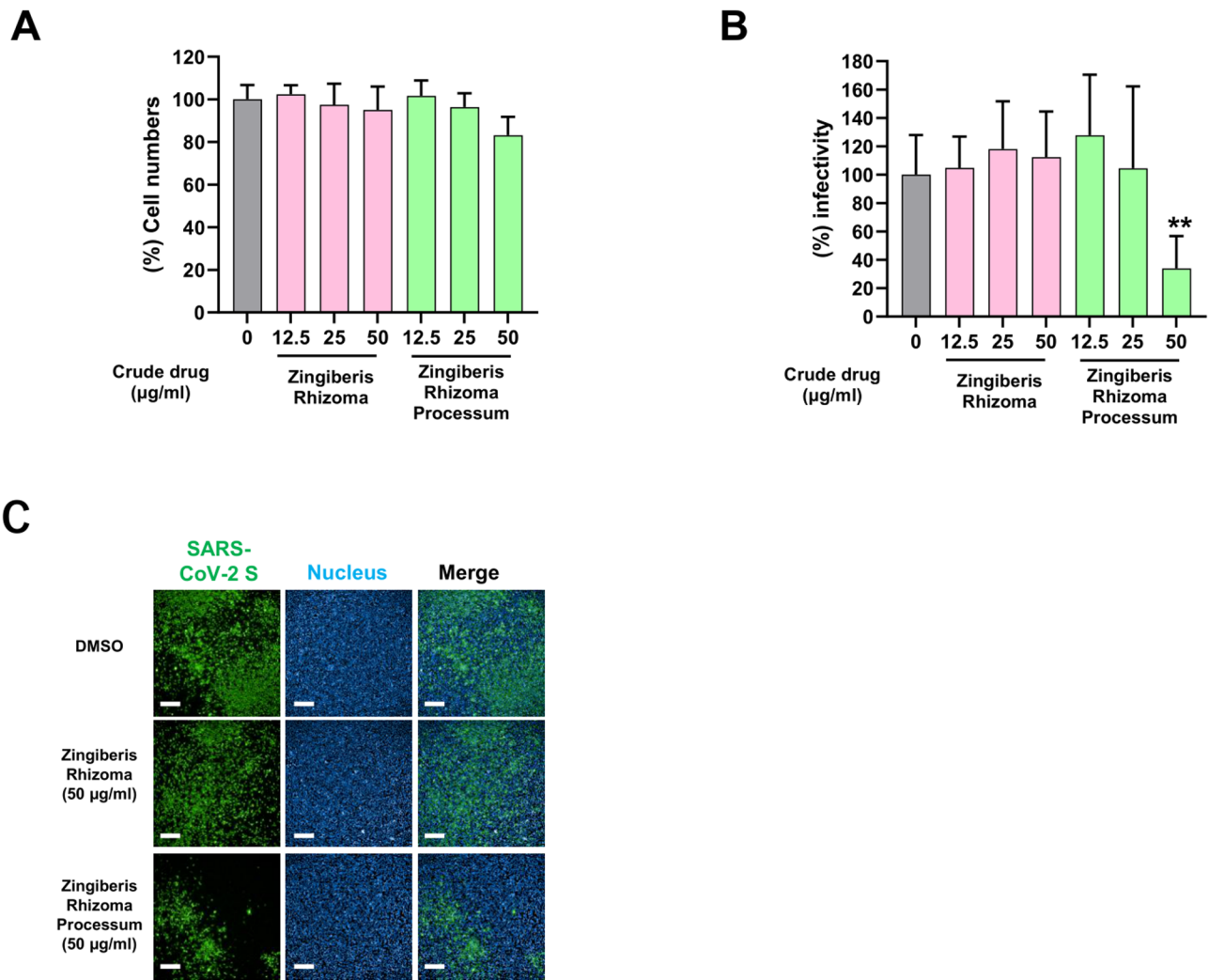


Fig. 4 The inhibitory effects of [6]-gingerol and [6]-shogaol on 3CLpro and PLpro activities. **A, B** Fluorogenic-based 3CLpro and PLpro assays were performed in the presence of [6]-gingerol and [6]-shogaol at three concentrations (40, 100, and 250 μM). **C, D** Cell-based reporter FlipGFP assay. GFP signals were restored by 3CLpro cleavage. Transfected cells were observed as mCherry signal. Values represent the mean $\pm$ SD of three independent experiments ($n = 3$)

The ratio of GFP signal per mCherry signal (the latter represents transfected cells) decreased in the presence of 25 μ M [6]-shogaol (19.7% reduction), whereas [6]-gingerol did not affect the GFP/mCherry ratio (Fig. 4D). Although the inhibitory effect of [6]-shogaol was not as strong as that of the known 3CLpro inhibitor GC376 (10 μ M), which reduced the GFP/mCherry ratio to approximately 50%. These results indicated that [6]-shogaol inhibited 3CLpro activity in *in vitro* and cell-based assays.

Discussion

Zingiber officinale Roscoe (Ginger) is used worldwide as an ingredient in cooked or steamed dishes. *Zingiber officinale* Roscoe is also an important herb used in traditional medicine owing to its beneficial anti-oxidant, anti-tumor, anti-influenza, anti-colitis, and anti-tussive effects [17]. While ZR contains gingerols as its major pungent component, gingerols are converted to shogaols upon dehydration. Therefore, the concentration of gingerols is higher in ZR, and the concentration of shogaols is increased in ZR-P during its preparation. Previous reports have described that [6]-shogaol shows significantly strong correlations between the anti-oxidant and anti-inflammatory activities of gingerols and shogaols [30]. In the present study, we demonstrated the inhibitory effects of ZR-P and [6]-shogaol against SARS-CoV-2 infection. [6]-shogaol showed a strong inhibitory effect against SARS-CoV-2 infection at a concentration of 25 μ M. Although [6]-shogaol inhibited 3CLpro in a cell-based assay, its inhibition rate was approximately 20% at the same concentration. These results suggested that the inhibitory effect of [6]-shogaol on viral infection may be due to factors other than the inhibition of 3CLpro activity. One possibility is the inhibitory effect of [6]-shogaol on phosphatidylinositol-3-kinase (PI3K)/protein kinase B (Akt) [31, 32]. Because SARS-CoV-2 enters cells via clathrin-mediated endocytosis, which activates the PI3K/Akt pathway, inhibiting this pathway might reduce viral entry and replication [33, 34]. Further studies are needed to clarify the inhibitory effects of [6]-shogaol on the viral propagation.

Conclusions

In summary, we showed that SARS-CoV-2 infection was suppressed in the presence of [6]-shogaol and ZR-P (steamed ginger). Our study suggests that [6]-shogaol has the potential to inhibit SARS-CoV-2 infection, possibly through its activity against 3CLpro. However, the effective concentration range in which [6]-shogaol suppresses SARS-CoV-2 infection and inhibits 3CLpro without inducing cytotoxicity is limited and close to the cytotoxic threshold. Therefore, further investigation is required to validate its antiviral efficacy. In addition,

although we used VeroE6/TMPRSS2 cells in this study, it will be important to assess the effects of [6]-shogaol in human-derived cell lines such as HuH-7 or A549-ACE2, as well as against different SARS-CoV-2 variants. Our results demonstrate the potential of ZR-P extract and [6]-shogaol as anti-SARS-CoV-2 agents and potential therapeutic options for COVID-19.

Abbreviations

ZR	Zingiberis Rhizoma
ZR-P	Zingiberis Rhizoma processum
3CLpro	3C-like protease
PLpro	Papain-like protease
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
COVID-19	Coronavirus disease 2019
NSP	Nonstructural proteins
HPLC	High performance liquid chromatography
TMPRSS2	Transmembrane serine protease 2
DAPI	4',6-diamidino-2-phenylindole
GST	Glutathione S-transferase
IPTG	Isopropyl β -D-thiogalactopyranoside
FlipGFP	Flipped green fluorescent protein
DTT	Dithiothreitol
PI3K	Phosphatidylinositol 3-Kinase
Akt	AKT serine/threonine kinase 1

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12906-025-05094-4>.

Supplementary Material 1.

Acknowledgements

Not applicable.

Authors' contributions

T.T. and M.K. devised and designed the study, performed formal analyses. T.H., Y.K., and H.K. contributed to the study methodology and data analysis. H.K. assisted in data curation and validation. T.T., Y.S. and M.K. supervised the study and oversaw project administration. T.T. and M.K. drafted the original manuscript. T.H. prepared Figs. 1 and 2 and Y.K. prepared Fig. 3. All authors have read, revised and approved the final version of the manuscript for publication.

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Data availability

All data generated in this study is included in the manuscript.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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