INTRODUCTION

Coronavirus disease 2019 (COVID-19) is a respiratory syndrome caused by infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), an RNA virus belonging to coronaviridae (Zhu et al., 2020). It has been reported that SARS-CoV-2 virus infection can occur not only in respiratory epithelial cells but also in the mucous membrane of the eyes (Rothe et al., 2020). Various symptoms, ranging from fever, malaise, cough, shortness of breath, and, in severe cases, severe pneumonia, have been reported (Huang et al., 2020). In several countries, drugs like palliatives for the respiratory syndrome are being used for limited COVID-19 treatment (Gavriatopoulou et al., 2021; Varrassi & Rekatsina, 2022). Recently, Pfizer’s Paxlovid has received emergency use authorization from the FDA. (Izda et al., 2021; Parums, 2022). Even now, in vitro studies and clinical trials trying to discover effective therapies for SARS-CoV-2 infection are ongoing (Droždžal et al., 2021). In addition, as one of the therapeutic strategies targeting the SARS-CoV-2 virus directly, a method using a neutralization reaction specific to the virus surface protein has been proposed (Chatterjee et al., 2020). In the COVID-19 situation, disinfectants that are effective directly against the virus are considered one of the main strategies to inhibit the spread of infection. Still, the...
human harm of chemical disinfectants cannot be overlooked (Rai et al., 2020).

Chitin, poly(β-[1,4]-N-acetyl-d-glucosamine), is a natural high-molecular polysaccharide that can be obtained in large amounts from the shells of various crustaceans (Suneeta & Rupak, 2020). Among these chitin-derived biological materials, chitosan (poly-[D]glucosamine) and chitoooligosaccharide, which are most widely used, have been proposed as natural disinfectants with high biocompatibility, low cytotoxicity, antibacterial activity, and antiviral activity (Cheung et al., 2015; Kim et al., 2021; Kong et al., 2010; Liaqat & Eltem, 2018). In several studies, chitosan-derived substances have been reported as effective for preventing viral infections, such as HIV-1, by reducing inflammation and enhancing immunity (Fernandes et al., 2010; Jaber et al., 2021; Lodhi et al., 2014; Marmouzi et al., 2019; Sánchez et al., 2018). On the other hand, an infectious disease drug delivery system using chitosan-derived compounds as nanoparticles is also being developed (Friedman et al., 2013; Garg et al., 2019; Mohammed et al., 2017).

Here, we showed that chitosan-based substances have natural antiviral properties against SARS-CoV-2 in vitro using Vero cells. In particular, among the chitosan-based substances, we propose that low molecular weight chitoooligosaccharide can serve as a natural antiviral treatment for SARS-CoV-2.

**MATERIALS AND METHODS**

**Gel permeation chromatography of chitosan-based substances**

The molecular weight of chitosan-based substances was measured by the gel permeation chromatography analysis using the columns Ultrahydrogel™ 120 and Ultrahydrogel™ 250 (Waters™) of high-performance liquid chromatography systems (Shimadzu) (Choi et al., 2004). The mobile phase and dilution solvent were prepared in 0.1 M NaCl (with 0.1% trifluoroacetic acid). The flow rate of the mobile phase is 1 ml/min and was detected using the refractive index detector. The concentration of chitosan-based substances was diluted to 1%. The samples were analysed for 30 min, and the standard curve was prepared by analysing the standard product before exploring substances.

**Cell culture and chitosan-based substances treatment**

Vero E6 cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) (Gibco®) containing 10% fetal bovine serum (FBS) (Gibco®), 2 mM L-glutamine, 1 mM sodium pyruvate, 1500 mg/L sodium bicarbonate, 15 mM HEPES and 1% penicillin/streptomycin in a 5% CO₂ incubator at 37°C. Cells were seeded 24 h before being treated with candidate substances (AMI-1 to −4) at a different dose (25–200 μg/ml) for 24 h and observed through an inverted microscope (LionHeart FX automated Microscope, Biotek).

**Cell viability assay**

Vero E6 cells were seeded into 96-well tissue culture plates 24 h before treatment with candidate substances for 24 h and incubated with 0.04 mg/ml MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) solution (Sigma-Aldrich, USA) at 37 °C for 2 h. The cells were then incubated with 100 μl of dimethyl sulfoxide at 37°C for 30 min to solubilize the formazan precipitate’s final product of MTT metabolism. The optical density of each well was measured using a microplate reader set to 570 nm (Synergy H1, BioTek). The cytotoxicity concentration of 50% (CC₅₀) was analysed in GraphPad Prism 5.0 (GraphPad Software).

**Viral infection and cytopathic effect (CPE) observation**

Vero E6 cells were seeded into 6-well tissue culture plates 24 h before infection with SARS-CoV-2 (National Culture Collection for Pathogens [NCCP]) NCCP43326 in media containing 2% FBS for 1 h. CPE of the infected cells was observed for 96 h through an inverted microscope.

**Viral RNA extraction and detection of SARS-CoV-2 using real-time RT-PCR**

The in vitro reaction between the virus and the chitosan-based substances was performed as described (Chung et al., 2021; Kim et al., 2021). SARS-CoV-2 virus (NCCP43326) was incubated with candidate substances at room temperature for 1 h and then infected at a multiplicity of infection (MOI) 0.01 (a dose of 1 × 10⁴ PFU/well, 6-well) for 1 h to the Vero E6 cells seeded into 6-well plates. The infected cells were washed with PBS 3 times and then replaced and incubated with fresh media for 2 days. Viral RNA from the cultured media was extracted using QIAamp Viral RNA Mini Kit (QIAGEN). Real-time RT-PCR assay was performed using STANDARD M nCoV Real-Time Detection kit (SD Biosensor) or iScript™ cDNA Synthesis Kit (Bio-Rad) and iQ SYBR Green Supermix
and cultured with the first overlay 1% low-melting-point agarose and 2% FBS. For the double overlay, the infected cells were washed with PBS 3 times and cultured with 1% LMP agarose and 2% FBS containing cell culture media in a 5% CO₂ incubator for 1 h. For the fixation staining method, the infected cells were washed with PBS 3 times and cultured with the first overlay 1% low-melting-point agarose (Invitrogen) and 2% FBS containing cell culture media in a 5% CO₂ incubator at 37°C for 5 days. The second overlay was added with 1% LMP agarose and 0.01% neutral red-containing media and cultured in a 5% CO₂ incubator at 37°C for 24 h. For the fixation staining method, the infected cells were washed with PBS 3 times and cultured with 1% LMP agarose and 2% FBS containing cell culture media in a 5% CO₂ incubator at 37°C for 3 days. After solid overlay aspirated from each well, the cells were fixed with 4% paraformaldehyde for 1 h at room temperature and then stained with 0.5% crystal violet solution for 15 min. The plaque was observed using a white-light transilluminator.

SARS-CoV-2 plaque assay

Plate assay on SARS-CoV-2 was performed as described (Mendoza et al., 2020). Vero E6 cells were seeded into 6-well tissue culture plates 24 h before infection with serial diluted SARS-CoV-2 (NCCP43326) from 10⁻² to 10⁻⁶ in media containing 2% FBS for 1 h. For the double overlay method, the infected cells were washed with PBS 3 times and cultured with the first overlay 1% low-melting-point (LMP) UltraPure™ agarose (Invitrogen) and 2% FBS containing cell culture media in a 5% CO₂ incubator at 37°C for 5 days. The second overlay was added with 1% LMP agarose and 0.01% neutral red-containing media and cultured in a 5% CO₂ incubator at 37°C for 24 h. For the fixation staining method, the infected cells were washed with PBS 3 times and cultured with 1% LMP agarose and 2% FBS containing cell culture media in a 5% CO₂ incubator at 37°C for 3 days. After solid overlay aspirated from each well, the cells were fixed with 4% paraformaldehyde for 1 h at room temperature and then stained with 0.5% crystal violet solution for 15 min. The plaque was observed using a white-light transilluminator.

Detection of SARS-CoV-2 using immunofluorescence staining

Vero E6 cells were infected with SARS-CoV-2 at MOI 0.01 and incubated with the candidate compound as an indicated concentration in a 5% CO₂ incubator at 37°C for 36 h. The cells were fixed with 4% paraformaldehyde in PBS for 1 h and permeabilized with 0.1% Triton X-100 in PBS for 15 min at room temperature. The cells were incubated with anti-nucleocapsid protein (NP) rabbit antibody (200-401-A50, Rockland) (1:100 dilute) in 1% BSA in PBS for 24 h at 4°C. After washing with PBS, the cells were incubated with Alexa 488-conjugated anti-rabbit goat antibody (ab150077, Abcam) (1:250 dilute) for 1 h at room temperature, and then stained with Hoechst 33342 (H3570, Thermo Fisher) for 1 h at room temperature. Fluorescent images were obtained using LionHeart FX automated Microscope (Biotek). The fluorescent intensity of images was quantified using the Gen5 software (Biotek).

RESULTS

The average molecular weight of chitosan-based substances

The substances from Amicogen in the table were used in this study to investigate the antiviral effect of chitosan and chitosan-based substances against SARS-CoV-2 (Figure 1a). The average molecular weight of these chitosan-based substances, including chitosan, chitooligosaccharide, and water-soluble chitosan, was analysed through gel permeation chromatography, and each substance was named AMI-1 (221,620 daltons), AMI-2 (15,604 daltons) and AMI-3 (84,504 daltons), respectively (Figure 1b). Ivermectin, named AMI-4 in this study, is a substance with potential as a therapeutic agent for COVID-19 (Caly et al., 2020; Jans & Wagstaff, 2021) due to being one of the anti-parasitic agents and was used in this study to confirm the in vitro ability of antiviral effects against SARS-CoV-2.

Cytotoxicity of chitosan-based substances

Before investigating the antiviral effect of chitosan-based substances, cytotoxicity was measured in Vero cells treated with 25–200 μg/ml concentration of substances through cell viability assay. Before analysing the cell viability assay at the concentration intervals, the cytotoxic effect of the substances was observed by phase-contrast microscopy (Figure 2a). As shown in Figure 2, the cytotoxic effect of chitosan-based substances in treated cells was not observed, and AMI-3 crystallized without being absorbed into cells at two high concentrations (200 and 100 μg/ml). However, there was no cytotoxicity. As a result of quantifying cytotoxicity through the MTT assay, the chitosan-based substances have very low cytotoxicity to Vero cells in the range of 25–200 μg/ml concentration. There was no significant difference in cytotoxicity compared to the control-treated with 0.1 N HCl solvent used as a vehicle. On the other hand, AMI-4 (ivermectin) has a CC₅₀ of 13.98 μM (Figure 2b). As a result of quantifying cytotoxicity in A549 and NCI-H358 cells, both human non-small cell lung carcinomas, chitosan-based substances have low cytotoxicity, but AMI-4 has CC₅₀ of 10.76 μM and 36.98 μM, respectively (Figure S1).
Antiviral effects of chitosan-based substances on SARS-CoV-2

The SARS-CoV-2 virus was treated with 50 μg/ml of the substances for 1 h and then infected to Vero cells to validate the antiviral effects of chitosan-based substances. Viral RNA was extracted from the infected cells medium after two days post-infection and was analysed with quantitative PCR to detect SARS-CoV-2 genes (Figure 3a–c). By targeting the \( \text{RdRp} \) gene (RNA-dependent RNA polymerase) involved in RNA viral genome synthesis and the \( E \) gene (Envelope protein) involved in the component of the virus envelope, the Ct values of these genes were quantified using SARS-CoV-2 diagnosis kit (STANDARD MnCoV Real-Time Detection kit, SD Biosensor). As a result, the \( \text{RdRp} \) gene and \( E \) gene were not detected in Vero cells infected with a mixture of SARS-CoV-2 virus and AMI-2, and the \( \text{RdRp} \) gene was not detected in the case of AMI-4. Next, the antiviral effect of AMI-2 on the ability of the SARS-CoV-2 virus to generate a cytopathic effect was verified through plaque assay. As a result of observing plaque formation by treatment with two different concentrations of AMI-2 (25 and 12.5 μg/ml), it was observed that plaques were not formed by the SARS-CoV-2 virus when AMI-2 was treated at a concentration of 25 μg/ml (Figure 3e).

Considering the cytotoxicity test results, we regard that AMI-2 has an antiviral effect on SARS-CoV-2 among the substances used for viral RNA quantitation and plaque formation.

Dose-dependent antiviral effect of chitooligosaccharide

Next, dose-dependent antiviral efficacy of AMI-2 was confirmed by observation through immunofluorescence staining of the nucleocapsid protein (NP) produced in host cells with the SARS-CoV-2 virus. After infecting the SARS-CoV-2 virus with three different concentrations in the range of 25–100 μg/ml of AMI-2 for 36 h, the expression of SARS-CoV-2 NP was observed by immunofluorescence microscopy. As shown in Figure 4, the AMI-2 decreased the expression of SARS-CoV-2 NP of the infected cells in a dose-dependent manner (Figure 4a). As a result of analysing the fluorescence intensity, the expression of NP was reduced by 46.66 ± 2.70% at the 25 μg/ml of AMI-2, 92.02 ± 7.25% at the 50 μg/ml, and 95.43 ± 1.51% at the 100 μg/ml of AMI-2, respectively (Figure 4b). Taken together, the chitooligosaccharide, a chitosan-based substance, exhibited antiviral effects against the SARS-CoV-2
virus in vitro, confirming that these effects were correlated with concentration dependence.

**Antiviral effect on SARS-CoV-2 by the molecular weight of chitooligosaccharide**

Finally, the expression of the viral gene and plaque-forming unit were quantified to investigate the antiviral effect of chitooligosaccharides by molecular weight on SARS-CoV-2. The chitooligosaccharides of various molecular weights (2, 10, 30 and 50 kDa) were used in the experiment to confirm the inhibitory effect on SARS-CoV-2 replication according to molecular weight (Figure 5a). The virus was reacted with a substance at a concentration of 25 μg/ml for 1 h, then infected with Vero cells to investigate the impact of chitooligosaccharides on the inhibition of SARS-CoV-2 replication by molecular weight. The viral RNA was extracted from the infected cells 2 days post-infection and analysed with quantitative PCR to detect SARS-CoV-2 Rdrp and E genes. As a result of analysing the quantitative PCR, the expression of the Rdrp gene was reduced by 43.19% ± 28.81% at the chitooligosaccharide 2 K, 91.04% ± 5.32% at the chitooligosaccharide 30 K, and 60.76% ± 10.53% at the chitooligosaccharide 50 K, respectively (Figure 5b). The E gene expression was reduced by 50.61% ± 27.94% at the chitooligosaccharide 2 K, 83.24% ± 11.87% at the chitooligosaccharide 30 K, and 66.00% ± 3.74% at the chitooligosaccharide 50 K, respectively. In the case of chitooligosaccharide 10 K, it did not significantly reduce the gene expression of SARS-CoV-2 (Rdrp gene; 21.86% ± 15.52% reduced, E gene; 25.08% ± 23.80% reduced). Next, plaque formation was quantified to investigate whether chitooligosaccharide 30 K effectively inhibited the viral infectivity of SARS-CoV-2. The virus was reacted with 200 μg/ml of chitooligosaccharide 30 K, then infected with Vero cells, and plaque assay was performed. As a result of the plaque-forming unit measure, the plaque formation was reduced by 30.42% ± 11.88% at chitooligosaccharide 30 K (Figure 5c). Together, these results show that the chitooligosaccharide with a range of specific molecular weight has higher antiviral activity on SARS-CoV-2 in vitro than others.

**DISCUSSION**

Antimicrobial effects of chitosan, chitooligosaccharide or modified compounds derived from chitin against bacteria and viruses has already been studied (Liaqat &
Eltem, 2018; Lodhi et al., 2014; Tachaboonyakiat, 2017). Among them, it is suggested that chitosan-derived compounds were proposed as natural antiviral reagents from the results of studies showing that antiviral effects on several types of viruses, such as human immunodeficiency virus (HIV), influenza A and porcine epidemic diarrhoea virus (PEDV) (Artan et al., 2010; Kim et al., 2021; Zheng et al., 2016). It is suggested for the first time in this study that chitosan-based substances such as chitooligosaccharide can have antiviral efficacy against SARS-CoV-2 in vitro. The application of these chitosan-based substances can be expected in the natural treatment for COVID-19, which is currently an epidemic worldwide (Jaber et al., 2021; Safarzadeh et al., 2021; Sharma et al., 2021).

Commonly called low molecular weight chitosan, the molecular weight is reduced by shortening the D-glucosamine bond through an enzymatic reaction or chemical reaction with high molecular weight chitosan (El Knidri et al., 2018). Although the exact mechanism of the antibacterial and antiviral effects of low molecular weight chitosan and chitooligosaccharide is not fully understood, the antibacterial and antiviral action of chitosan on microorganisms suggests a mechanism in which the leakage of intracellular constituents of microorganisms is induced by changing the cell membrane permeability through the interaction of positively charged chitosan molecules with the negatively charged cell membrane of microorganisms (Kong et al., 2010; Kumirska et al., 2011; Nagy et al., 2011). A recent study predicts that the disinfectant feature of chitosan against other types of coronavirus will also be a neutralizing effect (Kim et al., 2021). In this study, the viruses were reacted with chitosan-based substances in vitro at room temperature for 1 h before infecting the cells. This experimental design includes a part of the host cell’s immune response and the virus-neutralizing effect by the chitosan-based substances. Recently, a molecular structure study suggested that the receptor-binding domain of spike protein, which plays a role in the primary infection mechanism of the SARS-CoV-2 virus, and chitosan...
derivatives can interact with a high binding affinity of the ligands (Modak et al., 2021). In particular, their findings are encouraging that chitosan derivatives are considered as anti-SARS-CoV-2 treatments because they have low molecular weight, and it is the advantage of being able to work against the structural features of spike proteins of B.1.7 (UK) and P.1 (Brazil) SARS-CoV-2 variants as well as wild type SARS-CoV-2. The experimental results we present in this study seem to partially corroborate the previous molecular structural predictions.

The main factors determining the average molecular weight of chitosan and chitooligosaccharides are the number of chitosanases added to the enzymatic reaction and the hydrolysis time during the manufacturing process (Jeon et al., 2001; Qin et al., 2004). Previous studies showed differences in antifungal, antibacterial, and antitumor effects according to the difference in average molecular weight of chitosan and chitooligosaccharides (Jeon et al., 2001; Kendra & Hadwiger, 1984; Omura et al., 2003). In the present study, the chitooligosaccharides with a specific molecular weight seemed suitable for antiviral activity against SARS-CoV-2 because chitooligosaccharides with 30 kDa of molecular weight were significantly superior to others. In addition, further study is needed to investigate how chitooligosaccharides of a specific molecular weight can have a higher antiviral effect than substances of other molecular weights.

In the case of ivermectin, one of the substances used in this study, as an FDA-approved drug, it was proposed as a candidate drug capable of inhibiting the replication of SARS-CoV-2 in vitro (Bello, 2021; Caly et al., 2020). However, when the cytotoxicity of ivermectin was measured in vitro using Vero, A549 and NCI-H358 cells in the present study, it appeared to have relatively high

![FIGURE 4](https://example.com/figure4.png)

**FIGURE 4** Dose-dependent antiviral effect of chitooligosaccharide on SARS-CoV-2 infected Vero cells. (a, b) Indirect immunofluorescence images of nucleocapsid protein (NP) in SARS-CoV-2 infected Vero E6 cells. Vero E6 cells were infected with SARS-CoV-2 at MOI 0.01 within indicated concentrations of the AMI-2 for 36 h. The infected cells were fixed and stained with anti-nucleocapsid protein antibody followed by Alexa 488-labelled antibody and analysed by fluorescence microscopy. The nuclei were stained with Hoechst dye. (a) Representative two different single optical sections are shown. Scale bars, 100 μm. (b) The bar graph was converted from the fluorescence intensity of the images. Data are represented as mean ± SD.
cytotoxicity compared to natural treatment candidates such as chitosan-based substances (Figure 2b and Figure S1). Previous studies have observed that ivermectin inhibits cell proliferation, arrests the cell cycle through DNA damage, and induces apoptosis via the mitochondrial pathway in vitro (Zhang et al., 2019). Considering the low cytotoxicity and antiviral ability of the chitosan-based substances identified in this study, we suggest that a natural treatment through the development of chitosan-derived substances will be suitable for infectious viral pathogens.

Our findings suggest the chitooligosaccharide may be a potent natural treatment for COVID-19. Additional studies will be needed to verify its effect by molecular weight in more detail. Furthermore, demonstration of its stability and antiviral ability in animal models is needed to develop a harmless treatment using chitooligosaccharide for infectious diseases such as COVID-19.

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### Table 1

| Compound             | Average M.W | Solvent     |
|----------------------|-------------|-------------|
| Chitooligosaccharide 2K | 2 kDa       | 0.1N HCl    |
| Chitooligosaccharide 10K | 10 kDa     | 0.1N HCl    |
| Chitooligosaccharide 30K | 30 kDa     | 0.1N HCl    |
| Chitooligosaccharide 50K | 50 kDa     | 0.1N HCl    |

### Figure 5

Antiviral effect of chitooligosaccharides of various molecular weights on SARS-CoV-2. (a) The chitooligosaccharides of various molecular weights. Separation by molecular weight to confirm the inhibitory effect of chitooligosaccharide on SARS-CoV-2 virus replication. (b) Antiviral effect of chitooligosaccharides on SARS-CoV-2 through qPCR analysis. The amount of SARS-CoV-2 virus at MOI 0.01 was incubated with 25 μg/ml of chitooligosaccharides (2, 10, 30 and 50 kDa) for 1 h at room temperature, infected with Vero E6 cells, and incubated with the chitooligosaccharides for 48h. Viral RNA was extracted from the culture medium of the cells, and the SARS-CoV-2 gene was detected through qPCR analysis. The graph of relative viral RNA was represented to RdRP gene and E gene of SARS-CoV-2. Data are represented as mean ± SD (n = 5, ns. not significant, *p < 0.05, **p < 0.001, two-way ANOVA). (c) Plaque assay of SARS-CoV-2 infected Vero E6 cells. After 1 h of the virus within 200μg/ml of chitooligosaccharide 30kDa adsorption, low-melting agar containing overlays were added. After 3 days, the cells were fixed and stained with crystal violet. The plaque forming unit is calculated by plaque number counting. Data are represented as mean ± SD (n = 3, p < 0.05, Student’s t test).
CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest. Through Amicogen (Republic of Korea), the authors have filed patent applications on the chitosan-derivates substances. The authors declare that there are no conflicts of interest.

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**SUPPORTING INFORMATION**

Additional supporting information may be found in the online version of the article at the publisher’s website.

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