Antimicrobial effects of grapefruit seed extract microfibers against mouse hepatitis virus, *Escherichia coli, Staphylococcus aureus*, and *Pseudomonas aeruginosa*

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Abstract

Grapefruit seed extract (GSE), which contains flavonoids, possesses antibacterial, antiviral, and antifungal properties. This study investigated the antimicrobial activity of cellulose-bound GSE microfibers against mouse hepatitis virus (MHV) for three weeks and against *Escherichia coli, Staphylococcus aureus,* and *Pseudomonas aeruginosa* for one week. The disinfectant was smeared on a petri dish to inactivate the MHV for three weeks or to prevent the growth of the selected bacteria for one week. As the disinfectant effect lasted at least one week, GSE microfibers could have applications in disinfecting laboratory animal facilities. The virus or bacteria mixed with the disinfectant did not cause cytopathic effects in the cells or growth on the media after one week.

Key words: Antimicrobial, disinfectant, grapefruit seed extract, microfiber

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Introduction

Facilities for laboratory animals, such as mice and rats, are well maintained to prevent pathogen contamination, which may affect the outcomes of the experiments. Each animal facility prepares facility management and user manuals that aid in consistently keeping the immediate environment clean. Animal rooms are generally swept, wiped, and disinfected with chemicals such as alcohol, aldehyde, or chloride. In 2015, we demonstrated the usefulness of a hypochlorous acid (HOCl) solution for disinfecting animal facilities by inactivating various pathogens (Goto *et al.*, 2015). However, HOCl solutions are less stable when exposed to ultraviolet radiation or sunlight, in contact with air, or when the solution temperature is higher than 25 °C. Nevertheless, these solutions are safe for animals and have a strong virucidal effect (Block and Brown, 2020; Migliarina and Fero, 2014).

Recently, the usefulness of grapefruit seed extract (GSE) as a disinfectant has been reported (Jung *et al*., 2018; Komura *et al*., 2019). The seeds and peel of grapefruits are rich sources of antioxidative components, including flavonoids, vitamin C, carotenoids, citric acid, and limonoids (Vanamala *et al*., 2006). GSE has been reported effective against several strains of bacteria (including *Bacillus cereus* spores) (Yang *et al*., 2011), viruses, fungi, and single and multicellular parasites (Heggers *et al*., 2002). GSE is also considered a food additive because of its natural origin, safety (Kim *et al*., 2016), and lack of human toxicity (Xu *et al*., 2007).

Recently, GSE bound to cellulose fibers (GSE microfibers) has been developed to enhance its disinfection potential (Alonso *et al*., 2010). The GSE solution used in this study was mixed with GSE and the microfibers. The binding of GSE to the fiber is expected to extend its antibacterial effect.

In this study, the effect of GSE microfiber on mouse hepatitis virus (MHV) and bacteria (*Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*), was investigated. The experiments aimed to determine the effective duration of disinfection after the application and drying of the GSE microfiber solution.

Materials and methods

In this study, the effects of GSE microfibers on mouse hepatitis virus (MHV) and bacteria (*Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*) were investigated. The experiments determined the effective duration of disinfection after the GSE microfiber solution was applied and dried.

The GSE microfiber solution was provided by Agenes Inc, Japan. and used directly, without dilution, according to the manufacturer's instructions. The GSP solution has a faint citrus odor but is silky and not viscous. It also contains no preservatives of any kind, as it is itself an antimicrobial agent.

MHV (strain A59), which belongs to the family *Coronaviridae* (Goto *et al*., 1995), was used in this study. MHV-A59 was propagated and assayed in mouse brain tumor-derived DBT

cells (Hirano *et al*., 1976) at 37 **°C** and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) (GIBCO BRL, Gaithersburg, MD, USA) containing 10% fetal bovine serum (GIBCO BRL, Gaithersburg, MD, USA) and 1% penicillin-streptomycin (Fujifilm Wako Pure Chemical Corporation, Osak, Japan). This experiment was conducted in a laboratory approved as P2 capable by the university committee.

E. coli (JM109) and the clinical isolates of *S. aureus* (TK-S1) and *P. aeruginosa* (TK-P-1) were used in this study. The clinical isolates were provided by Mr. Matsumura, Teikyo University. *E. coli* was cultured in Luria–Bertani agar broth (Thermo Fisher Scientific K.K. Tokyo, Japan) and incubated at 37 **°C**. *S. aureus* and *P. aeruginosa* were cultured in heart infusion (HI) broth (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) or HI and nalidixic acid cetrimide agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), respectively, and incubated at 37 **°C**.

To evaluate the efficacy of the GSE microfiber solution against MHV-A59, 900 µL of the GSE solution was dropped onto a 10 cm diameter-polystyrene sterile petri dish (Eiken Chemical Co., Ltd. Tokyo, Japan) and smeared using a spreader. The lid was placed to cover two-thirds of the dish to prevent wind from hitting the liquid directly, and the dishes were placed at 24 **°C** on a clean bench (MCV-B91F, Panasonic, Tokyo, Japan). The wind speed on the clean bench was 0.25 m/s. At specific time points (1 min, 1 week, 2 weeks, and 3 weeks) 100 µL aliquots of the virus stock (in DMEM medium) were added to the GSE-smeared dishes. The virus solution was spread over the surface of the dishes and incubated for 1 min at 24 **°C** to ensure a reaction with GSE. Afterward, the viruses were collected from the dishes, serially diluted in DMEM, and 200 μL aliquots were inoculated onto DBT monolayers in 12-well plates. The cells were incubated for 48 h in 5% CO_2 , and the cytopathic effect was observed under a microscope (Eclipse Ts2, Nikon Corp. Tokyo, Japan) at low magnification (×40). The 50% tissue culture infectivity ($TCID_{50}$/mL) was then calculated. The virus titer was measured to determine the number of viruses before reaction with GSE. Aliquots (100 μL) from the same virus stock were added to non-treated dishes, which were subjected to the same manipulation and analyses as the GSE-treated samples and used as controls at each time point.

CUF count: To 100 µl of staircase diluted (10-2~10-7) bacterial solution, add 900 µl of GES or control medium, let stand at room temperature for 1 minute, smear 100 µl on agar medium, incubate at 37°C, and count the colonies after 24 hours.

The solution (900 μL) was smeared on a 10 cm-diameter petri dish to evaluate the activity of the GSE microfiber solution against bacteria. At specific time points (1 min and 1 week), aliquots of the bacterial suspension (100 μL) were added to the dish and incubated for 1 min at 24 *°C*, and colony-forming units (CFUs) were calculated. The bacterial titer of the control culture was measured at each time point (1 min and 1 week) and compared with that of the GSE-treated bacteria. The time between sample dilution and spread plating on agar was 15 min.

We performed statistical analyses using the software IBM SPSS version 22, and a t-test and Pearson correlation analysis were performed. We assumed that the significant probability p-value, at less than 0.05, was significant.

Results

The MHV titer before addition to the GSE microfiber solution was $1.6 \times 10^{6\ TCID_{50}}$/mL. The virus was not detected in any well at 1 min and 1 week time points. $TCID_{50}$/mL of the mixed virus solution was reduced to 8.9×10^4 and 1.6×10^5 at 2 and 3 weeks, respectively (Table 1). Photographs of cells 48 hours after inoculation of DBT cells with a mixture of virus solution and GSE (Fig. 1a) and 48 hours after inoculation of cells with a mixture of virus solution and medium (control) (Fig. 1b)

The effect of the GSE microfiber against *E. coli*, *S. aureus*, and *P. aeruginosa* was evaluated after 1 min and 1 week of adding the solution to the petri dish. The CFU values for *E. coli* cultures decreased from 4.4×10^8 CFU/mL (1 min) and 2.8×10^8 CFU/mL (1 week) to undetectable levels after treatment with GSE (Table 2). The number of *S. aureus* colonies decreased from 5.0×10^8 CFU/mL to undetectable levels after treatment with GSE at both time points. After GSE treatment, *P. aeruginosa* colony numbers decreased from 6.0×10^8 CFU/mL (1 min) and 1.1×10^9 CFU/mL (1 week) to undetectable levels and 3.0×10^8 CFU/mL, respectively.

Discussion

P. aeruginosa treated with GSE smeared on a dish for one week had a lower reduction rate than other bacteria (72.7%). *P. aeruginosa* is reported to be 300-fold more resistant when present on contaminated surfaces than when in suspension (Sagripanti and Bonifacino, 2000). This increase in resistance is consistent with the findings of biofilm studies, although it precedes biofilm formation (Sagripanti and Bonifacino, 2000). The resistance of *P. aeruginosa* to GSE should be studied further to clarify these inconsistencies.

GSE is a naturally occurring antimicrobial agent and has efficacy against many pathogens such as *S. aureus*, *Candida albicans, Proteus* spp., and *Klebsiella* spp. (Al–Âni *et al*., 2011; Han *et al*., 2021). GSE exhibits antimicrobial activity by disrupting the bacterial membrane and releasing cytoplasmic contents within 15 min of contact, even at low concentrations (Heggers *et al*., 2002). Since undiluted GSE was used in this experiment, it was considered that the bacteria were killed after one minute of treatment. The spraying of chicken with GSE attached to *Salmonella typhimurium* has also been effective in reducing the number of bacteria without any discoloration of the skin or deleterious effects on the equipment used (Xiong *et al*., 1998). Besides being a disinfectant, GSE is useful in maintaining the health of laboratory animals. GSE has been shown to reduce acute pancreatitis induced by ischemia/reperfusion in rats (Dembinski *et al*., 2004). This effect following intragastric administration of GSE was suggested to arise from a reduction in the production of pro-inflammatory mediators, which may reduce the systemic inflammatory response during acute pancreatitis (Dembinski *et al*., 2004). Despite reports that GSE lacks toxicity (Kim *et al*., 2016), cell detachment was observed when the GSE fiber solution was dropped directly onto DBT cells; therefore, the direct toxicity of GSE fibers to animals may need to be evaluated.

Treatment after	Treatment with	Titer of MHV ($TCID_{50}$/mL)
1 min	Control	1.6×10^6
	GSE microfiber	ND
1 week	Control	1.6×10^6
	GSE microfiber	ND
2 weeks	Control	1.6×10^6
	GSE microfiber	8.9×10^4
3 weeks	Control	1.6×10^6
	GSE microfiber	1.6×10^5

Table 1. Effect of GSE microfiber against MHV-A59.

Table-1 a and 1 b

A 900 µL of the GSE solution was dropped onto a 10 cm diameter-polystyrene sterile petri dish and smear was made using a spreader.

At specific time points (1 min, 1 week, 2 weeks, and 3 weeks) 100 µL aliquots of the virus stock (in DMEM medium) were added to the GSE-smeared dishes.

The virus solution was spread over the surface of the dishes and incubated for 1 min at 24 °C to ensure a reaction with GSE. Controls consist of viral solution spread on non-GSE-treated dishes.

Virus titer was calculated for both samples and controls to evaluate the GSE cytopathic effect.

ND: infected cells were not detected in any wells.

	No. of bacteria (CFU/mL)					
	E. coli		*S. aureus*		*P. aeruginosa*	
	1 min	1 week	1min	1 week	1min	1 week
No treatment	4.4×10^8	2.8×10^8	5.0×10^8	5.0×10^8	6.0×10^8	1.1×10^9
Treatment with GSE microfiber	ND	ND	ND	ND	ND	3.0×10^8

Table 2. Effect of GSE microfiber against opportunistic pathogens**.**

The solution (900 µL) was smeared on a 10 cm-diameter petri dish to evaluate the activity of the GSE microfiber solution against bacteria. At specific time points (1 min and 1 week), aliquots of the bacterial suspension (100 µL) were added to the dish and incubated for 1 min at 24 °C, and colony-forming units (CFUs) were calculated. Bacteria titer was calculated before and after treatment to evaluate the GSE antibacterial effect.

ND: bacteria was not detected.

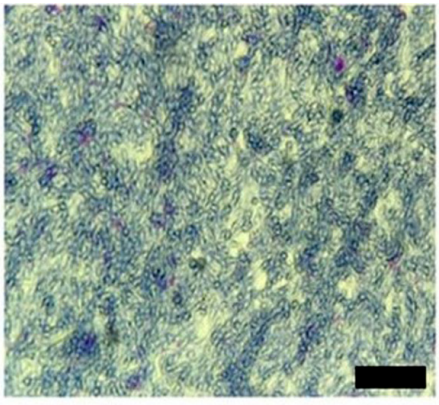

Fig. 1 (a)

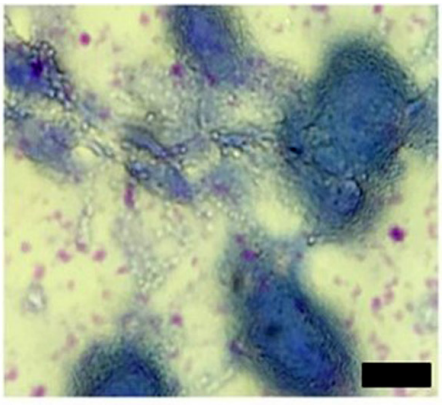

Fig. 1 (b)

Fig. 1a Photographs of cells 48 hours after inoculation of DBT cells with a mixture of virus solution and GSE
Fig. 1b 48 hours after inoculation of cells with a mixture of virus solution and medium (control)

In Japanese animal facilities, *S. aureus* and *P. aeruginosa* are among the most prevalent opportunistic pathogens, and MHV causes hepatitis in laboratory mice (Hayashimoto *et al*., 2013). Thus, we used these pathogens as targets to evaluate the disinfection ability of the GSE microfibers. These fibers are expected to have a longer bactericidal effect than GSE alone, and it has been reported that incorporating an antibacterial agent (such as orange essential oil and silver nanoparticles) into the antibacterial compound cellulose nanofibers increases the antibacterial effect and its duration (Phan *et al*., 2022). The results of this study are consistent with these previous reports.

Cleaning the animal room involves wiping away waste, food, and bedding before spraying alcohol. In this study, the usefulness of GSE as an alternative to volatile disinfectants like alcohol and the expected long-term effects after spraying were explored. Metal corrosion is a topic that has not been reported yet and needs further research,

In this study, the effect of disinfectant was observed to last for at least one week for opportunistic infections and three weeks for MHV. More than 70% of the bacteria and 90% of the MHVs were inactivated up to one and three weeks after smearing with the GSE microfiber solution. Prolonged disinfection is beneficial for the management of animal facilities. However, the toxicity of this disinfectant has not yet been reported. Therefore, we conclude that the GSE microfiber is a promising tool for disinfecting animal facilities

Competing interest:

No.

Author Contributions

The author confirms sole responsibility for the following study conception and designs, data collection, analysis and interpretation of results, and manuscript preparation.

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