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FULL PAPER

Avian Pathology

Inhibitory effect of grapefruit seed extract (GSE) on avian pathogens

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ABSTRACT. The inhibitory activities of grapefruit seed extract (GSE) on avian influenza virus (AIV), Newcastle disease virus (NDV), infectious bursal disease virus (IBDV), *Salmonella* Infantis (SI) and *Escherichia coli* (EC) were evaluated. Original GSE contained 0.24% benzalkonium chloride (BZC), however, 0.0025% BZC solution could not inactivate bacteria. The activity of diluted GSE (×100, ×500 and ×1,000 with redistilled water) against selected viruses and bacteria was evaluated in this study. The GSE solutions were incubated with the pathogens over a period of time after which the remaining viruses were titrated and the bacterial colonies were counted. In the presence of organic material—5% fetal bovine serum (FBS), the test solutions were sprayed at 1 cm and 30 cm distances to test the efficacy of GSE in a spray form. Furthermore, the efficacy of GSE against bacteria on clothes was tested using non-woven cloth. GSE×100 reduced the viral titer of both AIV and NDV even in 5% FBS condition. IBDV showed high resistance to GSE. GSE×1,000 inactivated both SI and EC within 5 sec, even in the presence of 5% FBS. The disinfectant was able to maintain its efficacy in the spray form at 30 cm distance. GSE was also effective against SI and EC inoculated on fabric. GSE is a potential novel disinfectant against viruses and bacteria, effective even within a short contact time.

KEY WORDS: avian influenza virus, enhancement of biosecurity, grapefruit seed extract, spraying method

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Due to globalization, there are higher chances of the spread of zoonotic diseases by contagion. The enhancement of biosecurity is crucial, especially at ports such as airports, where people come and go, and at farms, where there is a higher chance of people having contact with animals. There are various disinfectants in the market, but the ones that are effective in various conditions and at the same time safe for animals are limited. For example, hypochlorous acid (HOCl) is safe for animals, and has a strong virucidal effect, but this effect is lost in its spray form at 30 cm distance [9]. Therefore, we considered the potential of grapefruit seed extract (GSE) as a novel disinfectant for animals and humans due to its desirable properties.

GSE is well known for its disinfecting property against bacteria. It has a high growth inhibition effect against gram negative bacteria such as *Pseudomonas aeruginosa* and *Escherichia coli*, as well as gram positive bacteria, such as *Staphylococcus* spp. and *Enterococcus* spp. [22]. Due to its high bactericidal effect, the application of GSE to fresh vegetables [38], food packaging [30], hypromellose gel [2], and many more commodities are being considered. GSE is considered a food additive because of its natural origin and safety [13]. However, there have been some issues with the safety and the bactericidal effect of GSE in the past. In the early 2000s, there were claims that GSE sold in the market contained benzalkonium chloride (BZC) [29], benzethonium chloride [28], and eighteen other preservatives [6]. GSE sold in Japan also contained high concentration of BZC and benzethonium chloride [25], and hence, awareness on ingredient labeling of all GSE products sold in Japan was promoted. These reports threaten the safety and efficacy of GSE.

The GSE used in the current study was checked for preservatives, and the amounts of benzethonium chloride and triclosan were at undetectable levels (tested by Mizuken Co., Ltd., Osaka, Japan). However, the solution (diluted with redistilled water (dW_2) ×100) contained 0.0024% BZC. In order to confirm that this concentration of BZC would not contribute to the bactericidal effect of GSE in this experiment, the bactericidal activity of a diluted commercially available BZC solution was also evaluated.

In the present study, the effect of GSE was evaluated on avian influenza virus (AIV), Newcastle disease virus (NDV), infectious bursal disease virus (IBDV), *Salmonella* Infantis (SI) and *Escherichia coli* (EC). Avian influenza is listed as one of the top three

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Table 1. pH of GSE

Dilution	×100	×500	×1,000
GSE	3.49 ± 0.02	4.45 ± 0.01	4.91 ± 0.00
GSE+stop solution[a)]	7.00 ± 0.00	7.06 ± 0.00	7.04 ± 0.00
Stop solution[b)]		7.07 ± 0.00	

a) 400 μl GSE + 100 μl MM + 500 μl stop solution. b) Stop solution is mixture of 1 M HEPES and FBS (7:3). Data are expressed as mean of three experiments ± standard deviation.

priorities in the tripartite alliance among World Health Organization (WHO), Food and Agricultural Organization of the United Nations (FAO), and World Organization for Animal Health (OIE) [37]; hence, it is globally important to control this disease. In Japan, Ministry of Agriculture, Forestry, and Fisheries (MAFF) have a guideline for the prevention of highly pathogenic avian influenza virus (HPAIV). This prevention manual states that the prevention of people and vehicles carrying the virus from entering the farm is crucial [18]. Furthermore, if HPAI occurs in the farm, not only vehicles and equipment, but humans in the farm should also be disinfected [19]. The recommended disinfectants against HPAIV are cationic disinfectants, chlorine-based disinfectants, and alkaline disinfectants. Among them, it is difficult to find one that can safely be applied for the disinfection of humans. Alkaline agents, especially those with pH higher than 12, are highly effective towards HPAIV [16, 23, 31, 36]. However, highly alkaline agents can easily damage human skin and mucous membrane [18], and therefore may not be suitable for humans.

GSE is a natural product, and when diluted more than 100 times, it becomes almost odorless and harmless against humans. In the present study, GSE was evaluated for its virucidal and bactericidal activity against AIV, NDV, IBDV, SI and EC. Furthermore, GSE was assessed for its virucidal and bactericidal activities in its spray form. Lastly, the bactericidal activity of GSE against SI and EC inoculated on non-woven cloth was evaluated.

MATERIALS AND METHODS

GSE and BZC solution

GSE, derived from *Citrus paradisi* (Macf.) was kindly provided by Fine Reverse Co., Ltd. (Saitama, Japan). It was diluted 100 times (GSE×100), 500 times (GSE×500) and 1,000 times (GSE×1,000) with dW_2, prior to use in the experiments. The pH values of the GSE solutions are shown in Table 1.

A commercial BZC solution (Osvan solution, Nihon Pharm Co., Ltd., Tokyo, Japan) was used in the experiment. Osvan, a 10% BZC (w/v) solution was diluted ×4,000 with dW_2 to make 0.0025% BZC solution.

Viruses

Low pathogenic AIV, A/duck/Akita/714/06 (H5N2) [12], NDV strain Sato [26] and IBDV vaccine strain D78 (Intervet Co., Ltd., Tokyo, Japan) were used to evaluate the *in vitro* virucidal effect of GSE.

Cell culture

Madin Darby Canine Kidney Cell (MDCK) cells were cultured in growth medium (GM). The GM contained Eagle's minimum essential medium (EMEM; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), supplemented with 5% fetal bovine serum (FBS), 0.3 mg/m*l* l-glutamine, 1.4 mg/m*l* $NaHCO_3$ and antibiotic–fungicide cocktail (100 IU/m*l* penicillin, 0.1 mg/m*l* streptomycin and 0.5 g/m*l* amphotericin B). Chicken embryo fibroblasts (CEF) were prepared from 10-day-old embryonated eggs [26] and cultured in the same GM as MDCK cells. MDCK cells and CEF were maintained in the maintenance medium (MM)—EMEM supplemented with 0.3 mg/m*l* l-glutamine, 1.4 mg/m*l* $NaHCO_3$ and antibiotic–fungicide cocktail.

Virus titration

For the titration of AIV, MDCK cells were prepared with MM containing 1 μg/m*l* trypsin in a 96-well cell-culture plate. At 5 days post-inoculation (dpi), cytopathic effect (CPE) was observed and hemagglutinin (HA) activity of the supernatant was checked using 0.5% chicken red blood cells. For NDV, CEF were prepared with MM without trypsin in a 96-well cell-culture plate. At 5 dpi, CPE was observed, and HA activity was checked in the same way as AIV. Virus titer of 50% tissue culture infectious dose ($TCID_{50}$)/m*l* was calculated by Behrens-Kaerbel's method [17] according to the result of HA test for AIV and NDV. For the titration of IBDV, CEF cultured in GM in a 60 mm cell-culture dish were used for plaque assay. Plaques were counted at 6 dpi. The titer was calculated as plaque forming units (PFU)/m*l*.

Bacteria culture

EC strain NBRC106373 and SI were prepared as described [8]. Bacterial number was counted on deoxycholate hydrogen sulfide-lactose (DHL) agar, and expressed as colony forming units (CFU)/m*l*.

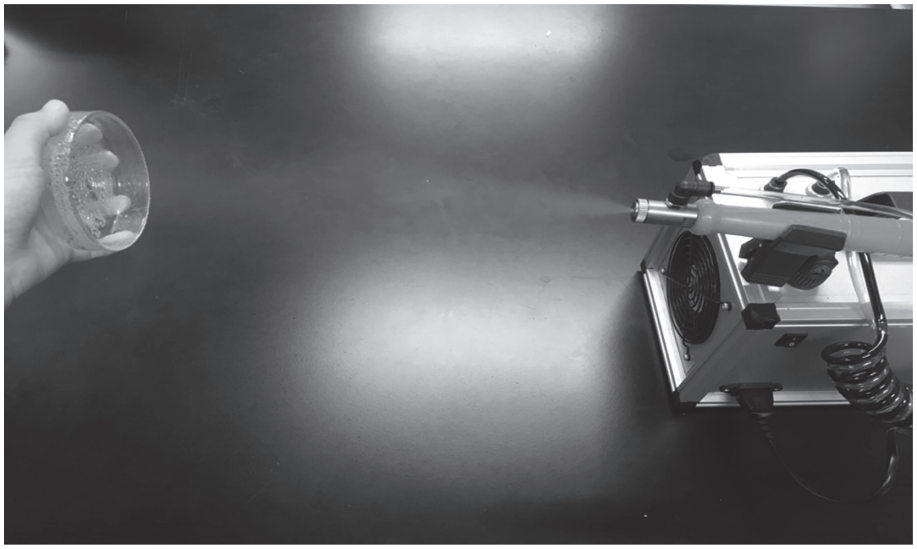

Fig. 1. Spray method. GSE collected at 30 cm away from the spray nozzle using petri dish.

Calculation of reduction factor

Reduction factor (RF) was calculated using the following equation.

RF = tpc–ta

"tpc" is virus or bacteria titer/number from untreated sample in $\log_{10}TCID_{50}$/m*l*, or $\log_{10}$PFU/m*l* or $\log_{10}$CFU/m*l* while "ta" is the titer/number of recovered virus or bacteria in $\log_{10}TCID_{50}$/m*l*, or $\log_{10}$PFU/m*l* or $\log_{10}$CFU/m*l*. The inactivation of the viruses or the bacteria was considered effective if RF>3 [1, 10, 14, 15, 20, 27, 32–34].

Evaluation of virucidal and bactericidal efficacies of GSE in aqueous phase

GSE (400 *µl*) was mixed with 100 *µl* virus/bacteria suspension in a microtube. The tube was incubated for 0, 5, 10, 30 sec (30 min for IBDV only) at room temperature (25°C ± 2°C). The activity of GSE was stopped with 500 *µl* stopping solution. Stopping solution for the virucidal activity contained FBS and 1 M HEPES (pH 7.2) in the ratio of 3:7. Stopping solution for the bactericidal activity was FBS and 1 M Tris-HCl (pH7.2) in the ratio of 3:7. The inhibitory effect of GSE on the viruses and the bacteria was also evaluated in the presence of 5% FBS (25 *µl* of FBS and 375 *µl* of GSE). As positive control, 100 *µl* viruses and bacteria were inoculated in 400 *µl* PBS, and after 30 sec incubation, the reaction was stopped with 500 *µl* stopping solution. The resulting supernatant was diluted in serial 10-fold dilutions and inoculated into the respective sensitive cells or on DHL agar. The experiment was carried out in triplicate.

Evaluation of bactericidal efficacy of BZC in aqueous phase

Four hundred micro-liter of 0.0025% BZC solution was mixed with 100 *µl* bacteria suspension in a microtube, and incubated for 0, 5, 10, 30 sec as earlier described.

Evaluation of the spray method

Sprayer with two-fluid nozzle was kindly provided by Aelph Co., Ltd. (Tokyo, Japan). Diluted GSE was sprayed in the air at 1 cm and 30 cm distances using a sprayer (Fig. 1). Sprayed GSE was collected at each distance and its efficacy against viruses and bacteria was evaluated using the same method described for the aqueous phase.

Evaluation of bactericidal efficacy of GSE (spray form) against bacteria inoculated on non-woven cloth

Bacteria suspension (100 *µl*) was inoculated on 3 × 3 cm non-woven cloth (Iwatsuki Co., Ltd., Tokyo, Japan) and dried for 30 min in a safety cabinet. The cloth was placed on a 5 × 5 cm glass inside a 90-mm glass petri dish. The petri dish was set inside a plastic box (W360 × D290 × H112 mm), and GSE was sprayed towards the cloth from a 30 cm distance (between the spray nozzle and the cloth sample). Redistilled water was sprayed in the same condition as GSE, as positive control. After 3 min of contact time, non-woven cloth was transferred into a plastic bag (stomacher bag: Organo Corp., Tokyo, Japan) containing 900 *µl* of stopping solution for bacteriological examination. The remaining bacteria cells were detached from the cloth using MINIMIX® 100CC® (Interscience Co., Ltd., Saint Nom, France). The supernatant was diluted in serial 10-fold dilutions and was inoculated on DHL agar for bacterial counting.

Evaluation of bactericidal efficacy of GSE (non-spray form) against bacteria inoculated on non-woven cloth

The inoculation of bacteria cells on the cloth was carried out using the same protocol described for the non-spray method, but

Table 2. Bactericidal effect of 0.0025% BZC

Bacteria		SI	EC
Positive control		9.11 ± 0.05[a)]	8.99 ± 0.01
Reaction time	0 sec[b)]	8.96 ± 0.23	8.59 ± 0.18
	5 sec	8.45 ± 0.02	8.34 ± 0.07
	10 sec	8.27 ± 0.04	8.44 ± 0.09
	30 sec	8.47 ± 0.01	7.65 ± 0.07

a) Colony counts are shown in $\log_{10}$, b) Reaction time. Data are expressed as mean of three experiments ± standard deviation.

Table 3. Virucidal and bactericidal efficacies of GSE against viruses and bacteria in aqueous phase

Pathogen			AIV		NDV		IBDV		SI		EC	
FBS			0%	5%	0%	5%	0%	5%	0%	5%	0%	5%
Positive control			6.50 ± 0.07[a)]	6.75 ± 0.00	7.00 ± 0.12	6.75 ± 0.00	6.84 ± 0.21	NT[b)]	8.30 ± 0.19	7.73 ± 0.19	7.98 ± 0.25	7.23 ± 0.16
Titer / number of colonies	×100	0 sec[c)]	4.91 ± 0.35	5.00 ± 0.00	7.00 ± 0.00	6.63 ± 0.06	5.62 ± 0.04	NT	5.88 ± 0.51	7.30 ± 0.00	7.15 ± 0.01	6.40 ± 0.25
		5 sec	3.08 ± 0.17	3.91 ± 0.37	<2.50 ± 0.00	3.50 ± 0.00	5.61 ± 0.07		<2.60 ± 0.00	<2.60 ± 0.00	<2.60 ± 0.00	<2.60 ± 0.00
		10 sec	2.83 ± 0.16	<2.50 ± 0.00	<2.50 ± 0.00	3.50 ± 0.00	5.60 ± 0.10		<2.60 ± 0.00	<2.60 ± 0.00	<2.60 ± 0.00	<2.60 ± 0.00
		30 sec	2.83 ± 0.16	<2.50 ± 0.00	<2.50 ± 0.00	3.50 ± 0.00	5.44 ± 0.00		<2.60 ± 0.00	<2.60 ± 0.00	<2.60 ± 0.00	<2.60 ± 0.00
	×500	0 sec	5.75 ± 0.07	5.25 ± 0.00	7.00 ± 0.00	6.75 ± 0.00	5.81 ± 0.21	NT	7.41 ± 0.24	6.84 ± 0.11	7.72 ± 0.09	6.73 ± 0.08
		5 sec	4.41 ± 0.24	5.66 ± 0.04	<2.50 ± 0.00	6.66 ± 0.04	5.70 ± 0.05		<2.60 ± 0.00	3.03 ± 0.20	<2.60 ± 0.00	3.76 ± 0.54
		10 sec	4.16 ± 0.35	5.91 ± 0.14	<2.50 ± 0.00	6.16 ± 0.14	5.67 ± 0.07		<2.60 ± 0.00	3.42 ± 0.38	<2.60 ± 0.00	3.53 ± 0.44
		30 sec	3.16 ± 0.17	5.91 ± 0.14	<2.50 ± 0.00	5.33 ± 0.20	5.73 ± 0.06		<2.60 ± 0.00	<2.60 ± 0.00	<2.60 ± 0.00	2.86 ± 0.07
	×1,000	0 sec	6.00 ± 0.11	5.63 ± 0.18	6.88 ± 0.06	6.50 ± 0.00	6.25 ± 0.13	NT	7.58 ± 0.28	7.24 ± 0.36	7.55 ± 0.03	6.75 ± 0.07
		5 sec	5.33 ± 0.32	5.91 ± 0.04	<2.50 ± 0.00	7.00 ± 0.07	5.76 ± 0.06		<2.60 ± 0.00	4.13 ± 0.36	<2.60 ± 0.00	3.99 ± 0.50
		10 sec	5.41 ± 0.20	5.83 ± 0.04	<2.50 ± 0.00	7.00 ± 0.07	5.71 ± 0.06		<2.60 ± 0.00	3.09 ± 0.23	<2.60 ± 0.00	3.58 ± 0.46
		30 sec	5.16 ± 0.39	6.16 ± 0.10	<2.50 ± 0.00	7.08 ± 0.10	5.73 ± 0.06		<2.60 ± 0.00	2.98 ± 0.18	<2.60 ± 0.00	3.05 ± 0.21

a) Virus titer or colony counts are shown in $\log_{10}$, b) Not Tested, c) Reaction time. Data are expressed as the mean of three experiments ± standard deviation.

instead of spraying GSE solution, 400 *µl* of GSE solution was directly added to the cloth for 10 sec. After the contact time, 500 *µl* stopping solution was added to stop the activity of GSE. The remaining bacteria cells were detached from the cloth as earlier described and counted on DHL agar.

RESULTS

Bactericidal effect of 0.0025% BZC

The RF was below 3 within the contact time indicating that 0.0025% BZC solution was not able to reduce the number of bacteria colonies to an ideal level (Table 2).

Virucidal and bactericidal effect of GSE in aqueous phase

Table 3 shows the effect of GSE solutions against viruses and bacteria in aqueous phase. At 0 sec (when the stopping solution was added to the GSE solution before adding viruses or bacteria), the viral titer and the bacterial count were not reduced. In the absence of organic material (0% FBS), GSE×100 inactivated AIV in 5 sec, reducing the virus titer from $10^{6.50}$ $TCID_{50}$/m*l* to $10^{3.08}$ $TCID_{50}$/m*l* (RF>3). GSE×500 was also able to reduce the viral titer to an acceptable level in 30 sec (RF>3). However, GSE×1,000 could not inactivate the viruses within 30 sec. In the presence of organic material (5% FBS), only GSE×100 could inactivate AIV in 10 sec, reducing the viral titer from $10^{6.75}$ $TCID_{50}$/m*l* to an undetectable level (<$10^{2.50}$$TCID_{50}$/m*l*; RF>3). Every dilution of GSE (×100, ×500 and ×1,000) inactivated NDV in the absence of FBS in 5 sec. However, in the presence of FBS, only GSE×100 was able to inactivate the virus in 5 sec. IBDV showed high resistance to GSE. Even 30 min of contact time was not enough to achieve RF>3 (data not shown). GSE showed high bactericidal activity against SI and EC. Without FBS, all the dilutions of GSE reduced the bacterial count to undetectable level (<$10^{2.60}$CFU/m*l*; RF>5.70). In 5% FBS condition, not all the dilutions were able to reduce the titer to undetectable limits, but they were able to achieve RF>3 in 5 sec. A similar result was observed for EC (Table 3).

Virucidal and bactericidal effect of GSE in the spray method

As shown in Table 4, it is clear that GSE sprayed at 1 cm and 30 cm distances showed similar results— the same number of viruses/bacteria was recorded at the same inactivation period at both distances. This result is consistent with that of the non-spray form of GSE shown in Table 3.

Table 4. Virucidal and bactericidal efficacies of GSE in the sprayed method

Pathogen			AIV		NDV		SI		EC	
Spray distance (cm)			1	30	1	30	1	30	1	30
Positive Control			6.00 ± 0.11[a)]	6.58 ± 0.08	6.50 ± 0.00	7.25 ± 0.00	7.11 ± 0.01	6.60 ± 0.00	8.03 ± 0.20	6.86 ± 0.01
Titer / number of colonies	×100	0 sec[b)]	6.50 ± 0.00	4.83 ± 0.31	6.50 ± 0.00	7.00 ± 0.12	7.29 ± 0.04	6.58 ± 0.01	7.45 ± 0.07	6.87 ± 0.02
		5 sec	<2.50 ± 0.00	<2.50 ± 0.00	2.58 ± 0.04	<2.50 ± 0.00	<2.60 ± 0.00	<2.60 ± 0.00	<2.60 ± 0.00	<2.60 ± 0.00
		10 sec	<2.50 ± 0.00	<2.50 ± 0.00	<2.50 ± 0.00	<2.50 ± 0.00	<2.60 ± 0.00	<2.60 ± 0.00	<2.60 ± 0.00	<2.60 ± 0.00
		30 sec	<2.50 ± 0.00	<2.50 ± 0.00	<2.50 ± 0.00	<2.50 ± 0.00	<2.60 ± 0.00	<2.60 ± 0.00	<2.60 ± 0.00	<2.60 ± 0.00
	×500	0 sec	5.33 ± 0.33	5.83 ± 0.31	6.75 + 0.00	6.75 ± 0.00	7.20 ± 0.00	6.88 ± 0.16	7.34 ± 0.02	6.88 ± 0.01
		5 sec	4.83 ± 0.10	3.83 ± 0.31	<2.50 ± 0.00	<2.50 ± 0.00	<2.60 ± 0.00	<2.60 ± 0.00	<2.60 ± 0.00	<2.60 ± 0.00
		10 sec	4.66 ± 0.20	4.08 ± 0.08	<2.50 ± 0.00	<2.50 ± 0.00	<2.60 ± 0.00	<2.60 ± 0.00	<2.60 ± 0.00	<2.60 ± 0.00
		30 sec	2.91 ± 0.14	3.16 ± 0.31	<2.50 ± 0.00	<2.50 ± 0.00	<2.60 ± 0.00	<2.60 ± 0.00	<2.60 ± 0.00	<2.60 ± 0.00
	×1,000	0 sec	5.33 ± 0.33	5.66 ± 0.27	6.75 ± 0.00	7.13 ± 0.06	7.29 ± 0.04	6.90 ± 0.15	7.49 ± 0.05	6.86 ± 0.01
		5 sec	4.83 ± 0.33	5.50 ± 0.24	2.83 ± 0.16	<2.50 ± 0.00	2.93 ± 0.16	<2.60 ± 0.00	<2.60 ± 0.00	<2.60 ± 0.00
		10 sec	5.00 ± 0.27	5.33 ± 0.31	2.58 ± 0.04	<2.50 ± 0.00	3.37 ± 0.18	<2.60 ± 0.00	<2.60 ± 0.00	<2.60 ± 0.00
		30 sec	5.00 ± 0.24	4.83 ± 0.08	<2.50 ± 0.00	<2.50 ± 0.00	<2.60 ± 0.00	<2.60 ± 0.00	<2.60 ± 0.00	<2.60 ± 0.00

a) Virus titer or colony counts are shown in $\log_{10}$, b) Reaction time. Data are expressed as the mean of three experiments ± standard deviation.

Table 5. Bactericidal effect of GSE in the sprayed method

Bacteria		SI	EC
Positive control[a)]		4.69 ± 0.03[b)]	5.06 ± 0.06
Dilution	×100	3.56 ± 0.09	4.39 ± 0.05
	×500	4.27 ± 0.05	4.82 ± 0.06
	×1,000	4.36 ± 0.08	5.06 ± 0.08

a) Redistilled water was sprayed instead of GSE, b) Colony counts are shown in $\log_{10}$.

Table 6. Bactericidal effect of GSE in aqueous phase

Bacteria		SI	EC
Positive control[a)]		6.22 ± 0.19[b)]	6.15 ± 0.18
Dilution	×100	<2.60 ± 0.00	<2.60 ± 0.00
	×500	<2.60 ± 0.00	<2.60 ± 0.00
	×1,000	<2.60 ± 0.00	<2.60 ± 0.00

a) Redistilled water was added on the cloth instead of GSE, b) Colony counts are shown in $\log_{10}$.

Bactericidal effect of GSE (spray form) against bacteria inoculated on non-woven cloth

When non-woven cloth inoculated with 100 *µl* bacteria suspension was sprayed with GSE for 10 sec with 3 min contact time, the bacteria titer was not reduced to an acceptable level (Table 5). GSE×100 reduced SI titer from $10^{4.69}$ CFU/m*l* to $10^{3.56}$ CFU/m*l*, resulting in RF<3. A similar result was observed for EC—bacteria titer was only reduced from $10^{5.06}$ CFU/m*l* to $10^{4.39}$ CFU/m*l* (RF<3).

Bactericidal effect of GSE (non-spray form) against bacteria inoculated on non-woven cloth

The non-spray method was evaluated because the spray method did not work for bacteria inoculated on cloth. After bacteria were inoculated on cloth, 400 *µl* GSE was directly added to the cloth, allowing 10 sec of contact time. Bacteria colonies were reduced to an undetectable level (<$10^{2.60}$ CFU/m*l*) even with GSE×1,000 in both bacteria strains tested (Table 6).

DISCUSSION

In the present study, GSE was tested for its virucidal and bactericidal activity against avian pathogens.

Firstly, GSE used in this experiment was assayed for its contaminants, and 0.0024% BZC was detected. BZC is an analogue of konium, which is originally present in grape fruit seed (Tokuda, Y. NMG Environmental Development Co., Ltd., Tokyo, Japan, personal communication). However, in order to confirm that the BZC content would not contribute to the bactericidal effect of GSE, bactericidal activity of 0.0025% BZC was tested using diluted Osvan. As shown in Table 2, this concentration of BZC did not show any bactericidal activity in the given contact time. Previous studies reported that GSE, void of any contaminant, can exhibit bactericidal activity [4], which is consistent with the findings of the current study.

In addition, GSE showed virucidal activity against AIV and NDV, namely enveloped viruses, but was not able to show virucidal activity against non-enveloped virus—IBDV. IBDV is resistant to many disinfectants due to its non-enveloped structure [7]; however IBDV can be inactivated by alkaline disinfectant in a short period of time (5 sec to 3 min) [20, 33]. GSE is acidic (Table 1); hence, IBDV is highly resistant to GSE.

GSE showed high efficacy against bacteria such that the bacterial count of GSE×1,000 was undetectable in 0% FBS condition after 5 sec. In 5% FBS condition, GSE×1,000 was able to reduce the bacterial count to an acceptable level (RF>3) in 5 sec. The high bactericidal activity of GSE is possibly mediated by its ability to destroy the cytoplasmic membrane of bacteria [11]. In the present study, only gram negative bacteria were used, but gram negative bacteria show more resistance to GSE, compared to gram positive bacteria [3]. Therefore, GSE is expected to have similar bactericidal activity towards gram positive bacteria, but further

study is necessary to validate this hypothesis.

The active ingredients of GSE against viruses and bacteria are still unknown; aglicons such as limonoids, flavonoid glycoside, naringen, quercetin, kaempferol, hesperigin, apigenin, and saturated or unsaturated fatty acids are possible components of GSE [5, 22, 35, 39]. The pH of the diluted GSE was 3.49–4.91 (Table 1) and this relatively low pH would not kill pathogens [14, 21, 23, 24].

GSE showed high virucidal and bactericidal effect against the pathogens within 5 sec. A previous study showed that HOCl showed virucidal and bactericidal activity within 5 sec; however HOCl lost its efficacy when sprayed at a distance of 30 cm [8, 9]. This suggested that HOCl should be sprayed at a distance less than 30 cm in order to maintain its virucidal and bactericidal activity. Thus, the efficacy of the spray form of GSE was evaluated. Generally, GSE's virucidal and bactericidal effects were not affected by the 30 cm distance (Table 4), suggesting that GSE is suitable as a spray disinfectant.

It is necessary that GSE is effective against pathogens on clothes for its suitable use in airports, seaports, farms and on humans. Hence, the efficacy of GSE against bacteria GSE was tested on fabric. The spray form of GSE was not effective against bacteria inoculated on non-woven cloth (Table 5). This was probably because only 120 μl of GSE reached the non-woven cloth within 10 sec of spraying. On the other hand, when 400 μl GSE was directly added to the bacteria inoculated on the non-woven cloth, GSE was able to inactivate the bacteria to undetectable level in 10 sec of contact time, suggesting that the bactericidal effect of GSE was dose dependent, and that GSE could kill bacteria on fabric.

GSE is an expensive disinfectant due to its manufacturing cost. The cost of GSE used in this experiment was about $132/kg (currency exchange rate on 3 December, 2018), indicating a possibly higher market price. To reduce the cost implication of using GSE as a disinfectant, higher dilution of the extract is desirable. GSE×1,000 was effective against SI and EC, however, for viruses, ×100 was the ideal dilution.

GSE has many advantages compared to other disinfectants. For example, alcohol-based disinfectants are volatile and flammable while GSE is not. Furthermore, unlike aldehyde, alkaline, and phenol based disinfectants, GSE is not harmful to skin or mucous membrane of humans and animals. Other features of GSE include minimal odor, no corrosiveness, and no staining on clothes.

Although GSE has many advantages, there are many things that need to be clarified. A previous study reported that GSE's acidity does not affect its bactericidal effect [2], but its mechanism of bactericidal and virucidal activities, or its active ingredients are still unknown. Further study is expected in this area. Lastly, GSE is a potential novel disinfectant against viruses/bacteria demonstrated by its efficacy in short contact time, in spray form and on contaminated fabric; therefore, further study on wider area of pathogens is highly anticipated in future research.

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