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Antimicrobial Activity of Trifoliate Orange (*Poncirus trifoliate*) Seed Extracts on Gram-Positive Foodborne Pathogens

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탱자씨 추출물의 그람양성 식중독균에 대한 항균효과

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국문요약

물(water, TW), 에탄올(ethanol, TE) 및 핵산(*n*-hexane, TH)을 이용하여 탱자씨 추출물(trifoliate orange seed extracts, TSEs)을 각각 제조한 후 그람양성 식중독균 6종(*Bacillus cereus* KCCM 11341, *Bacillus subtilis* KCTC 1022, *Listeria monocytogenes* ATCC 12692, *Staphylococcus aureus* ATCC 19111, *Streptococcus mutans* KCTC 3065 및 *Yersinia enterocolitica* KCCM 41657)에 대한 항균활성과 *Lactobacillus acidophilus* IFO 3025에 대한 프레바이오틱 효과(prebiotic effect)를 측정하였다. 그 결과, 탱자씨 핵산추출물(TH)은 *S. aureus* ATCC 19111에 대해 배양시간이 증가함에 따라 대조군에 비해 강한 증식저해활성을 보였으며, 탱자씨 에탄올 추출물(TE)은 약간의 증식저해활성을 보였다. 특히, 배양 81시간 후 대조군은 90.31%의 증식활성을 보인 것에 반해 탱자씨 핵산추출물과 에탄올추출물은 64.95%와 75.50%의 증식활성을 각각 보였다. 탱자씨 추출물의 *Lb. acidophilus* IFO 3025에 대한 프레바이오틱 효과는 대조군에 비해 증식활성을 보이지는 않았으나, 적어도 생육저해효과를 보이지는 않았다. 따라서 탱자씨의 핵산 및 에탄올 추출물은 *S. aureus* ATCC 19111에 대한 항균활성물질로서의 가능성을 제시하였다.

Key words: antimicrobial activity, growth inhibition activity, trifoliate orange seed extract

INTRODUCTION

In recent years, consumers are becoming more conscious of the nutritional value and safety of their food and ingredients. Preference for natural foods and food ingredients that are believed to be safer, healthier and less subject to hazards is increasing compared to their synthetic counterparts (Farag et al. 1986). Several natural ingredients have already been isolated from plant materials, such as oil seeds, cereal crops, vegetables, fruits, leaves, roots, spices, and herbs (Gil et al. 2000).

Citrus fruits and juices have long been recognized to contain secondary metabolites including antioxidants such as ascorbic acid, flavanones, phenolics and pectin that are important to human nutrition. Limonoids are secondary metabolites present in all citrus fruit tissues (Tian et al. 2001), but the efficient activities of their seeds have not been established.

Citrus oils are complex mixtures of natural compounds (approximately 400 compounds) whose content depends on the specific citrus cultivar, extraction and separation methods. Unlike many of the exotic plant extracts that have been proposed as new antimicrobials (Dorman & Deans 2000), citrus oils have been a part of the human diet for hundreds of years and thus have been

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generally recognized as safe (GRAS) by the Food and Drug Administration (FDA). Individual citrus oil components have demonstrated antimicrobial activity against major foodborne pathogens (Fisher et al. 2007).

Grapefruit seed extract (GSE) has been shown to possess antibacterial, antiviral, antifungal and antiparasite properties (Ionescu et al. 1990). It contains large quantities of polyphenolic compounds, such as catechins, epicatechin, epocatechin-3-*O*-gallate, dimeric, trimeric and tetrameric procyanidins (Saito et al. 1998). These beneficial actions of GSE have partly been attributed to the antioxidative activity of citrus flavonoids, such as naringenin (Shoko et al. 1999). The safety of GSE has been tested in several areas, with Heggers et al. (2002) showing that GSE was not detrimental to human fibroblast skin cells *in vitro*. On the other hands, little information is available on studies relating to the antimicrobial activity of trifoliate orange (*Poncirus trifoliate*). And also among the different parts of the trifoliate orange, seeds are one of the major byproducts which do not have significant use. Based on this information, we focused on evaluating the antimicrobial activity of the trifoliate orange seed extract (TSE) against Gram-positive foodborne pathogens.

MATERIALS AND METHODS

1. Preparation of TSEs

Trifoliate orange was cultivated at Yeongcheon, Kyungsangbuk-do, Korea and harvested in November, 2011. Dried trifoliate orange seed was purchased from Cheongmyung Medicinal Herb Company (Kwangju, Korea). To obtain only the trifoliate orange seed, foreign materials including the peel etc. were removed by naked eye identification and then ground with an electronic grinder (Hanil Electronics Corp., Weonju, Korea). One hundred of grams of ground sample (moisture content; 10.61%) was extracted with 1 ℓ of distilled water, ethanol, and *n*-hexane. The extraction condition of ethanol and *n*-hexane was at room temperature for 8 hr, while the distilled water was kept in the 40℃ incubator (Sanyo Electric Co. Ltd, Moriguchi, Japan) to maintain the temperature for 8 hr with stirring, respectively. Each extracted sample was centrifuged at 6,000×g for 20 min. The supernatant was concentrated with a rotary vacuum evaporator and then lyophilized, which was finally used as the extracted sample. The positive control used for antimicrobial activity testing was commercially available GSE (Esfood Co. Ltd., Pocheon, Korea).

2. Gram-positive Foodborne Pathogens and Culture Conditions

Bacillus cereus KCCM 11341, *Bacillus subtilis* KCTC 1022, *Listeria monocytogenes* ATCC 12692, *Staphylococcus aureus* ATCC 19111, *Streptococcus mutans* KCTC 3065, and *Yersinia enterocolitica* KCCM 41657 were used as Gram-positive foodborne pathogens in this study. *B. cereus* KCCM 11341 and *Y. enterocolitica* KCCM 41657 were purchased from Korea Culture Center of Microorganisms (Seoul, Korea). *B. subtilis* KCTC 1022 and *S. mutans* KCTC 3065 were Korea Collection for Type Culture (Daejon, Korea), and *L. monocytogenes* ATCC 12692 and *S. aureus* ATCC 19111 were American Type Culture Collection (Manassas, USA). *Lactobacillus acidophilus* IFO 3025 was used for the efficient lactic acid bacteria (LAB) for testing the prebiotic potential of the test samples. The media and culture conditions for these strains are shown in Table 1. Stock cultures of these strains were activated in their appropriate media and conditions twice and then they were used to test for antimicrobial activity.

3. Antimicrobial Activities of TSEs using Disc Diffusion Method on Gram-positive Foodborne Pathogens

Each test sample (20 ㎎/㎖) of TW (TSE with distilled water),

Table 1. Bacterial strains tested and their growth conditions

Bacterial strains	Media	Temperature (℃)
Bacillus cereus KCCM 11341	Nutrient agar	30
Bacillus subtilis KCTC 1022	Nutrient agar	30
Listeria monocytogenes ATCC 12692	Brain heart infusion agar	37
Staphylococcus aureus ATCC 19111	Nutrient agar	37
Streptococcus mutans KCTC 3065	Brain heart infusion agar	37
Yersinia enterocolitica KCCM 41657	Tryptose agar	37
Lactobacillus acidophilus IFO 3025	MRS agar	37

TE (TSE with ethanol), and TH (TSE with *n*-hexane) for antimicrobial test was dissolved with distilled water, 75% dimethylsulfoxide (DMSO), and 100% DMSO, respectively. And then they were further filtrated with membrane filter (0.22 ㎛) before use. Paper disc agar diffusion method was used to assess the antimicrobial activity of TSEs on the test microorganisms' growth. An aliquot 0.1 ㎖ of the bacterial suspension to a cell density of 10^6-10^7 CFU/㎖ was spread on appropriate solid media for each food pathogen growth. After being air-dried sterile 6 ㎜ (diameter) paper disc was placed on the agar surface that had been inoculated with test bacteria. Each test sample 20 ㎕ added on the paper disc, respectively and also negative control discs were 20 ㎕ of distilled water, 75% DMSO, and 100% DMSO. Positive control disc was GSE (20 ㎎/㎖) filtrated membrane as well. All plates were then incubated at respective optimum temperature for 2 days under microaerophilic condition. Inhibitory activity was measured (in ㎜) as a diameter of the observed zones. We replicated all growth inhibition tests 3 times at 400 ㎍/disc and then determined the antimicrobial activity by assigning one of the following values, based on the estimated diameter size of the zone of inhibition produced by each test sample, as follows: strong response (+++), zone of inhibition diameter > 10 ㎜; moderate response (++), zone of inhibition diameter 8-10 ㎜; weak response (+), zone of inhibition diameter 6-8 ㎜; and no response (−), zone of inhibition diameter 6 ㎜.

4. Growth Inhibition Curves of TSEs on Gram-positive Foodborne Pathogens

Growth inhibition curves of TSEs on Gram-positive foodborne pathogens were measured. First, we selected the test samples showing strong antimicrobial responses against food pathogens using the disc diffusion method and then determined the growth inhibition curve as a function of increasing time. Thirty milliliter of growth media appropriate for each food pathogen growth was added in a 100 ㎖ ⊿-flask and then autoclaved at 121℃ for 15 min. Three hundred of microliter (10 ㎎/㎖) of the test sample "sterilized" via the filter membrane and the test pathogen at a concentration of 1% (v/v, 1-5×10^8 CFU/㎖) were added. Each flask was incubated in a shaking incubator (IS-971R, Jeio Tech., Kimpo, Korea) under the respective optimal temperatures with gentle shaking. The absorbance of each culture sample at increasing time points was measured by the UV-Visible Spectrophotometer (UV-2401PC, Shimadzu Corp., Kyoto, Japan) at 600 ㎚.

5. Prebiotic Effects of TSEs on LAB

Prebiotic effects of TSEs on *Lb. acidophilus* IFO 3025 were measured. Briefly, 30 ㎖ of MRS broth for *Lb. acidophilus* IFO 3025 was added in a 100 ㎖ ⊿-flask and then autoclaved at 121℃ for 15 min. Three hundred of microliter (10 ㎎/㎖) of the test sample "sterilized" via the filter membrane and *Lb. acidophilus* IFO 3025 at a concentration of 1% (v/v, 1-5×10^8 CFU/㎖) were added. Each flask was incubated in a shaking incubator (IS-971R, Jeio Tech., Kimpo, Korea) under 37℃ of optimal temperature with gentle shaking. The absorbance of each culture sample at increasing time points was measured by the UV-Visible Spectrophotometer (UV-2401PC, Shimadzu Corp., Kyoto, Japan) at 600 ㎚.

RESULTS AND DISCUSSION

1. Antimicrobial Activities of TSEs using Disc Diffusion Method on Gram-positive Foodborne Pathogens

TSEs were made using distilled water, ethanol, and *n*-hexane. The yields of these 3 TSEs were as follows (Table 2): water extract (TW, 20.85%) > ethanol extract (TE, 5.39%) > *n*-hexane extract (TH, 0.44%). The water extract yield was more 3.89 times that of the ethanol extract.

And antimicrobial activities of TSEs prepared from different solvents were determined via the paper disc agar diffusion method, against Gram-positive foodborne pathogens. The results were shown in Table 3. TE showed the strong antimicrobial activity against *B. subtilis* KCTC 1022 and *Y. enterocolitica* KCCM 41657, and TH showed strong antimicrobial activity against *S. aureus* ATCC 19111 and *Y. enterocolitica* KCCM 41657 and moderate antimicrobial activity against *B. cereus* KCCM 11341, respectively. TW and negative control (distilled water, 75% DMSO, and 100% DMSO) did not show any antimicrobial activity against any of the tested strains (data not shown), whereas GW, which was the positive control showed the strongest antimicrobial activity against all strains.

Table 2. Lyophilized powder yield of trifoliate orange seed extracts (TSEs) prepared from different solvents

Sample[1)]	TW	TE	TH
Yield (%)	20.85	5.39	0.44

[1)] TW; TSE with water, TE; TSE with ethanol, TH; TSE with *n*-hexane.

Table 3. Antimicrobial activity of trifoliate orange seed extracts (TSEs) prepared from different solvents on Gram-positive foodborne pathogens using disc diffusion method

Gram positive microorganism	Sample[1)]			
	TW	TE	TH	GW
Bacillus cereus KCCM 11341	−[2)]	+	++	+++
Bacillus subtilis KCTC 1022	−	+++	+	+++
Listeria monocytogenes ATCC 12692	−	+	+	+++
Staphylococcus aureus ATCC 19111	−	++	+++	+++
Streptococcus mutans KCTC 3065	−	−	−	+++
Yersinia enterocolitica KCCM 41657	−	+++	+++	+++

1) TW; TSE with water, TE; TSE with ethanol, TH; TSE with n-hexane, GW; grapefruit seed extract (GSE) was dissolve in water which was available on the market.

2) −; no inhibition (6 ㎜), +; weak inhibition (6-8 ㎜), ++; moderate inhibition (8-10 ㎜), +++; strong inhibition (> 10 ㎜).

2. Growth Inhibition Curves of TSEs on Gram-positive Foodborne Pathogens

The disk diffusion assay is a rapid and practical approach to screen a large number of potential antimicrobials. However, the method is limited by the diffusion rates of the active compounds in the agar media and does not account for the potential effect

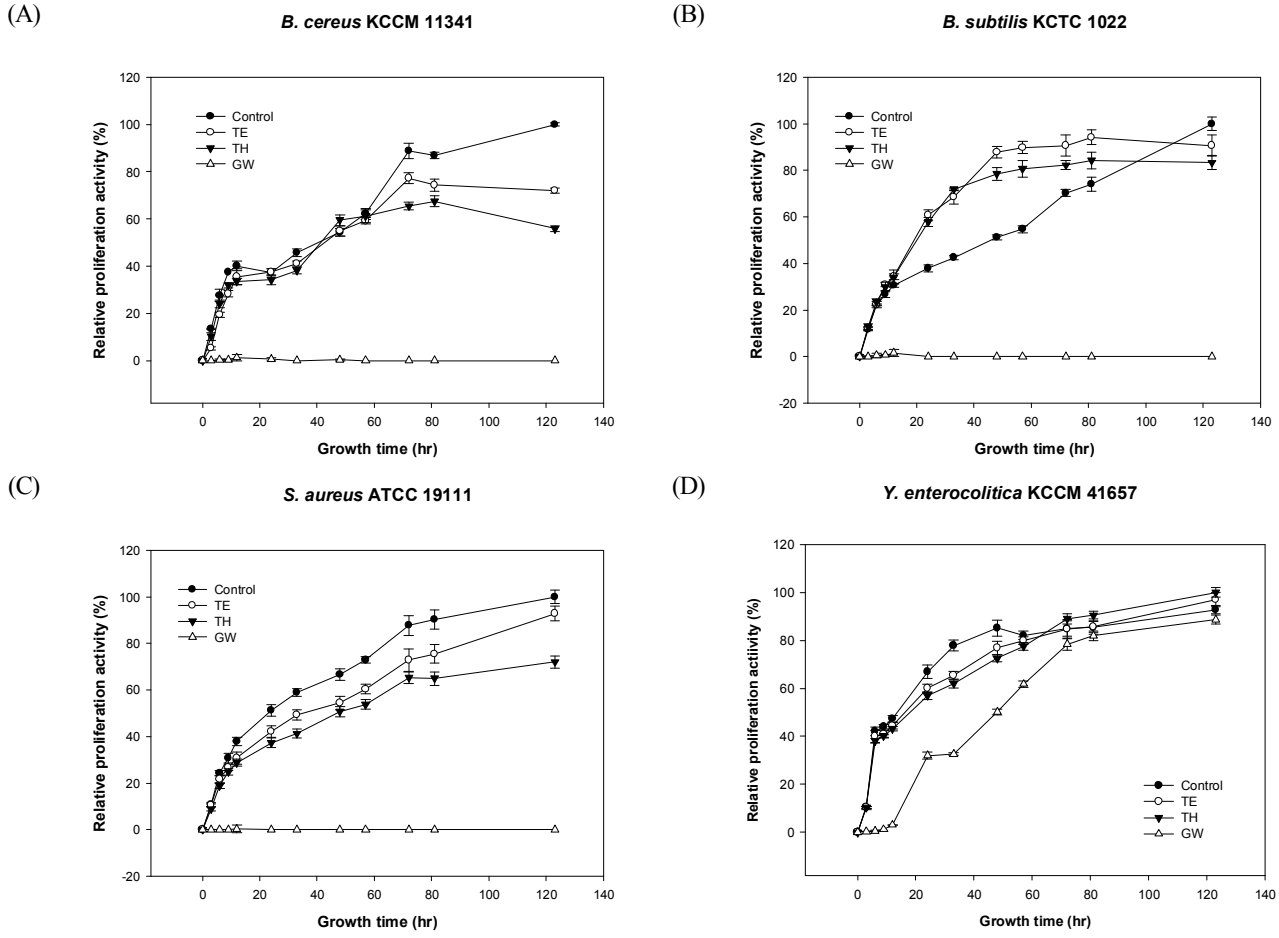

Fig. 1. Growth inhibition effects of trifoliate orange seed extracts (TSEs) prepared from different solvents on Gram-positive foodborne pathogens (A-D).

of a food matrix. Therefore, more study was needed to clarify this point. Based on the data, we selected the TSEs with antimicrobial activities and the pathogens they responded against: TE, TH, and GW, and *B. cereus* KCCM 11341, *B. subtilis* KCTC 1022, *S. aureus* ATCC 19111, and *Y. enterocolitica* KCCM 41657. Namely, the effects of growth inhibition on selected pathogenic bacteria by TE, TH, and GW were determined as the increase of growth time. The results showed that relative proliferation activity of *S. aureus* ATCC 19111 on tested samples decreased with the increase of growth time when compared with control. Especially, TE, TH, and GW showed relatively higher effects as 90.31, 75.50, 64.96, and 0% at 81 hr of growth time. That is to say, *S. aureus* ATCC 19111 showed the growth inhibition activity in all tested samples during all growth time and the order was as follows: GW > TH > TE > control (Fig. 1). And *Y. enterocolitica* KCCM 41657 also showed the growth inhibition activity in all tested samples, but which showed relatively lower growth inhibition activity when compared with the inhibition effect of *S. aureus* ATCC 19111. *B. cereus* KCCM 11341 showed the growth inhibition activity after growth time of 60 hr except for GW sample. *B. subtilis* KCTC 1022 showed the growth inhibition activity in only GW which rather showed the proliferation activity in the other tested samples until 80 hr when compared with the control.

3. Prebiotic Effects of TSEs on LAB

TE, TH, and GW were also examined prebiotic effect against *Lb. acidophius* IFO 3025 of representative efficient LAB. The results of prebiotic effects of tested samples against *Lb. acidophius* IFO 3025 did not showed the differences in TE, TH, and Control, whereas GW rather showed the growth inhibition activity during initial growth time until 24 hr (Fig. 2).

There is increasing consumer demand for fresh, healthy, convenient and additive-free ready-to-eat vegetables that are safe and nutritious (Tuley de Silva 1996; Francis et al. 1999). A broad spectrum of microbial pathogens can contaminate human food and cause illnesses after they or their toxins are consumed (Tauxe 2002). Consumption of raw food has been suspected or contaminated worldwide as the most likely source of infection in diverse outbreaks which occurred during the last decade (Orden et al. 2002). The representative pathogen has been implicated in various countries with outbreaks caused by *S. aureus* (De Buyser et al. 2001). *S. aureus* is a member of the normal skin and nasal flora in 25-30% of humans, but is also a common pathogen

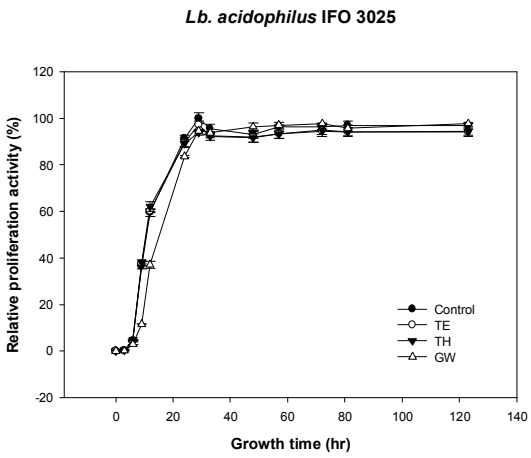

Fig. 2. Prebiotic effects of trifoliate orange seed extracts (TSEs) prepared from different solvents on the growth of *Lactobacillus acidophilus* IFO 3025.

causing a plethora of infections ranging from mild skin and wound infections to more serious infections such as septicaemia, endocarditis, osteitis, and toxic shock syndrome. The primary site of infection is often the skin or a wound from where the organism can spread to the blood stream and subsequently to other tissues and organs. Abscess formation with massive invasion of polymorphonuclear leukocytes is a hallmark of *S. aureus* infections, as well as severe tissue damage due to the production of numerous toxins and enzymes.

Nowadays, the approaches that can be adopted in food preservation include: (a) aseptic handling and packaging, (b) the mechanical removal of microorganisms by washing or filtration, (c) destruction of microorganisms by physical or chemical sanitization and finally (d) the inhibition of pathogens or saprophytes through environmental control. Inhibition of microbial growth through environmental control is achieved through the addition of synthetic chemical compounds (antimicrobial preservatives) such as trisodium phosphate, acidified calcium sulfate, organic acids (e.g., lactic, acetic) and acetyl pyridinium chloride with an inhibitory or bactericidal/fungicide activity (Kemp et al. 2000). Although approved for use in food processing, in the last years, natural antimicrobials have attracted considerable attention due to the increased consumer awareness on the aspects of food quality and safety.

Several methods have been also used to extend the storage life of green produce, such as high hydrostatic pressure, high intensity ultrasound and gamma irradiation. However, those treatments can also affect the sensory properties of food products,

alter the structures of proteins or produce free radicals that affect the flavor of fruit (Vercet et al. 1998). Therefore, much interest exists in developing sanitizers with antimicrobial activities and without toxicity in order to maintain sensory quality and extend shelf-life of minimally processed vegetables and fruit.

With the increase of bacterial resistance to antibiotics, there is considerable interest in investigating the antimicrobial effects of natural substances and different extracts against a range of bacteria, to develop other classes of natural antimicrobials useful for infection control or for the preservation of food. In this study, TH was found to be the most growth inhibition activity against *S. aureus* ATCC 19111 except for GW. Rahman and Kang (2009) reported that Gram-positive bacteria were found to be more susceptible to the essential oil and various solvent extractions than Gram-negative bacteria. Because, the hydrophilic cell wall structure of Gram-negative bacteria is constituted essentially of a lipopolysaccharide that blocks the penetration of hydrophobic oil and avoids the accumulation of essential oils in target cell membrane (Bezic et al. 2003). Based on this report, we assumed the reason that Gram-positive bacteria *S. aureus* ATCC 19111 was found to be more sensitive to the TH (*n*-hexane extraction), TE (ethanol extraction) of trifoliate orange seed than those of TW (water extraction).

Thermal processing is one of the main techniques used to destroy foodborne pathogens and ensure food safety of fruit juices. However, the severity required to traditional heating treatments in order to ensure its microbiological stability results in poor sensory and nutritional quality. An approach that is aiming to fulfill these somewhat conflicting goals is the application of the hurdle technology concept, which intelligently combines multiple preservative factors, optimizing food quality by diminishing the intensity of each single hurdle (Leistner L 2000). Under this perspective the use of mild thermal treatment in combination with other hurdles, such as the use of natural antimicrobials, to reach the desired inactivation effect represents an alternative for the development of minimally processed foods.

In conclusion, among TSEs, TH has a good growth inhibition activity against *S. aureus* ATCC 12692 and TE has a slight growth inhibition activity. And TSEs did not show any prebiotic effects against *Lb. acidophius* IFO 3025 as well as growth inhibition activity at least. From these results we confirmed the possibility of TSEs as antimicrobial material instead of GSE which will contribute commercial availability and low cost used food processing byproduct. Thus TH and TE of TSEs are promising natural antimicrobial agents with potential applications in the food or pharmaceutical industries for the control of pathogenic bacteria.

Similar to most bioactive compounds, antimicrobial agents are chemically reactive species, which can cause considerable problems when embedded into a complex food system, such as negative effects on the physical stability or integrity of the food chemistry as well as the degradation of the biological activity of bioactive compounds (McClements 2005). Therefore, further research is needed in order to obtain information regarding the practical effectiveness of TH or TE to prevent the growth of foodborne and spoilage microbes under specific application conditions.

CONCLUSION

Trifoliate orange seed extracts (TSEs) was prepared from different solvents of water (TW), ethanol (TE), and *n*-hexane (TH) which was measured antimicrobial activities against 6 Gram-positive foodborne pathogens. Among TSEs, TH has a good growth inhibition activity and TE showed a slight growth inhibition activity against *S. aureus* ATCC 19111. From these results, we confirmed that TSEs using *n*-hexane and ethanol can be used as antimicrobial materials, instead of GSE.

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