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Antimicrobial Activity of Trifoliate Orange (*Poncirus trifoliate*) Seed Extracts on Gram-Negative Food-borne Pathogens

− Research Note −

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Abstract

Trifoliate orange seed extracts (TSEs) were prepared from different solvents, water (TW), ethanol (TE), and *n*-hexane (TH), and assessed for their antimicrobial activities against six gram-negative food-borne pathogens (*Escherichia coli* KCTC 1039, *Escherichia coli* O157:H7 ATCC 43895, *Salmonella* Enteritidis ATCC 3311, *Salmonella* Typhimurium KCCM 11862, *Shigella sonnei* KCTC 2518, and *Vibrio parahaemolyticus* ATCC 17802). Among the tested TSEs, TE and TH showed a slight inhibition activity on *V. parahaemolyticus* ATCC 17802, but a good growth inhibition activity on *Sal.* Typhimurium KCCM 11862. TH and TE showed steady growth inhibition activity with increasing growth time after 6 hr when compared to the control ($p<0.05$). From these results, we confirmed the possibility of TH and TE as antimicrobial materials.

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 foods and ingredients. There is an increasing preference for natural foods and food ingredients because they are believed to be safer, healthier, and pose less hazardous risk than to their synthetic counterparts (1). Several natural ingredients have already been isolated from plant materials, such as oil seeds, cereal crops, vegetables, fruits, leaves, roots, spices, and herbs (2).

Citrus fruits, a major contributor to human diet, have received attention by researchers due to their multitude of bioactive compounds. Recent *in vitro* studies suggest these bioactive compounds have health-promoting properties and show potentials to be antioxidant, anti-proliferative, and antiviral agents, as well as preventing cardiovascular diseases (3). Citrus fruits are grown commercially in more than 140 countries around the world, and oranges, grapefruits, and lemons are considered to be the top three highly consumed fruits throughout the world (4). Among them, grapefruit seed extract (GSE) is a commercial product derived from the seeds and pulp of grapefruit (*Citrus paradise Macf.* Rutaceae). GSE is a natural extract that demonstrates effective broad-spectrum bactericidal, fungicidal, antiviral and anti-parasitic activities (5,6). GSE is also environmentally safe without toxicity to humans or animals at effective concentrations. Ionescu et al. demonstrated that GSE performed as well as other antimicrobial agents tested on 770 strains of bacteria and 93 strains of fungi. On the other hand, only little information is available on studies relating to the antimicrobial activity of trifoliate orange (*Poncirus trifoliate*) and, more importantly, its seeds, which are a major byproduct with unknown significance. Based on this information, we focused on evaluating the bioactivity of the trifoliate orange seed extract (TSE). Health-promoting properties and the mechanism underlying the bioactive characteristics of TSE remain largely unknown when compared to GSE. This study is aimed at determining the health benefits of the well-utilized food by-products, seeds, which can lead to economic benefits for the citrus processing industry, citrus growers, and the global society as a whole.

MATERIALS AND METHODS

Preparation of TSEs

Trifoliate orange was cultivated at Yeongcheon, Gyeongbuk, Korea and harvested in November, 2011. Dried trifoliate orange seeds were purchased from Cheongmyung Medicinal Herb Company (Gwangju, Korea). The trifoliate orange seeds were removed from foreign materials, including the peel, etc., by naked eye identification and then ground with an electronic grinder (Hanil Electronics Corp., Wonju, Korea). One hundred grams of the ground sample (moisture content; 10.61%) was extracted with 1 L of distilled water, ethanol, or

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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°C 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).

Bacterial strains tested and growth conditions

Escherichia coli KCTC 1039, *Escherichia coli* O157: H7 ATCC 43895, *Salmonella* Enteritidis ATCC 3311, *Salmonella* Typhimurium KCCM 11862, *Shigella sonnei* KCTC 2518, and *Vibrio parahaemolyticus* ATCC 17802 were used as the gram-negative food-borne pathogens. *Lactobacillus acidophilus* IFO 3025 was the efficient bacteria used 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 used to test for antimicrobial activity.

Antimicrobial activities of TSEs using disc diffusion method on gram-negative food-borne pathogens

Each test sample (20 mg/mL) of TW (trifoliate orange seed extracted by distilled water), TE (trifoliate orange seed extracted by ethanol), and TH (trifoliate orange seed extracted by *n*-hexane) for antimicrobial test was dissolved in distilled water, 75% dimethylsulfoxide (DMSO), and 100% DMSO, respectively. The samples were further filtrated with a membrane filter (0.22 μm) before use. To assess the antimicrobial activity of TSEs on the test microorganisms' growth, paper disc agar diffusion method was performed. A 0.1 mL aliquot of the bacterial suspension at a cell density of 10^6～10^7 CFU/mL was spread on appropriate solid growth media for each food pathogen. After air-drying, a sterile 6 mm (diameter) paper disc was placed on the agar surface that had been inoculated with the test bacteria. Twenty microliter of each test sample and 20 μL of distilled water, 75% DMSO, and 100% DMSO as negative control were added on the paper disc. The positive control disc was a GSE (20 mg/mL) filtrated membrane. All plates were then incubated at their respective optimal growth temperatures for 2 days under microaerophilic conditions. Inhibitory activity was measured (in mm) as a diameter of the observed zones. All growth inhibition tests were replicated 3 times at 400 μg/disc and the antimicrobial activity was determined by assigning one of the following values based on the estimated diameter size of the zone of inhibition produced by each test sample: strong response (+++), zone of inhibition diameter>10 mm; moderate response (++), zone of inhibition diameter 8～10 mm; slight response (+), zone of inhibition diameter 6～8 mm; and no response (-), zone of inhibition diameter 6 mm.

Table 1. Bacterial strains tested and their growth conditions

Bacterial strains	Media	Temperature (°C)
Escherichia coli KCTC 1039	Nutrient agar	37
Escherichia coli O157:H7 ATCC 43895	Tryptic soy agar	37
Salmonella Enteritidis ATCC 3311	Nutrient agar	37
Salmonella Typhimurium KCCM 11862	Nutrient agar	37
Shigella sonnei KCTC 2518	Nutrient agar	37
Vibrio parahaemolyticus ATCC 17802	Marine agar	28
Lactobacillus acidophilus IFO 3025	MRS agar	37

Growth inhibition curve of TSEs on gram-negative food-borne pathogens

Growth inhibition curves of TSEs on gram-negative food-borne 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 into a 100 mL Δ-flask and then autoclaved at 121°C for 15 min. Three hundred microliter (10 mg/mL) of the test sample was filter sterilized via the membrane and the test pathogen at a concentration of 1% (v/v, 1～5×10^8 CFU/mL) was added. Each flask was gently shaken and incubated in an incubator (IS-971R, Jeio Tech., Gimpo, Korea) under the respective optimal temperatures. The absorbance of each culture sample at increasing time points was measured by a UV-Visible Spectrophotometer (UV-2401PC, Shimadzu Corp., Kyoto, Japan) at 600 nm.

Prebiotic effects of TSEs on LAB

Prebiotic effects of TSEs on *Lb. acidophilus* IFO 3025 were measured. Briefly, 30 mL of MRS broth for *Lb. acidophilus* IFO 3025 was added in a 100 mL Δ-flask and then autoclaved at 121°C for 15 min. Three hundred of microliter (10 mg/mL) 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/mL) were added. Each flask was incubated at 37°C with gentle shaking (IS-971R, Jeio Tech.) and the absorbance was

measured at 600 nm as previously described.

Statistical analysis

All values shown are the means of triplicate determinations. All statistical analyses were conducted using the Statistical Package for Social Sciences, version 12.0 (SPSS Inc., Chicago, IL, USA). The differences among samples were evaluated statistically by one-way analysis of variance (ANOVA) and Duncan's multiple tests. All data were evaluated at the 5% significance level using two-sided tests and are reported as the means±standard deviations.

RESULTS

Antimicrobial activities of TSEs using disc diffusion method on gram-negative food-borne 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%) ($p<0.05$). The TW yield was 3.89 times higher than that of the ethanol extract. The antimicrobial activity of TSEs prepared from different solvents was determined *via* the paper disc agar diffusion method against gram-negative food-borne pathogens (Table 3). TE showed a strong antimicrobial activity against *Sh. sonnei* KCTC 2518 and *V. parahaemolyticus* ATCC 17802, as did TH, which also showed strong antimicrobial activity against *Sal.* Typhimurium KCCM 11862. TW and negative control (distilled water, 75% DMSO, and 100% DMSO) did not show any antimicrobial activity against the tested strains, whereas GW, the positive control, showed the strongest antimicrobial activity against all strains.

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

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

[1)]TW: trifoliate orange seed extracted by water, TE: trifoliate orange seed extracted by ethanol, TH: trifoliate orange seed extracted by *n*-hexane. [a-c]Significantly different at $p<0.05$.

Growth inhibition curve of TSEs on gram-negative food-borne 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 of a food matrix, which awaits further study for clarification. Based on the data, we selected the TSEs with antimicrobial activities and the pathogens they responded against: TE, TH, and GW, and *Sh. sonnei* KCTC 2518, *Sal.* Typhimurium KCCM 11862, and *V. parahaemolyticus* ATCC 17802. Namely, the inhibitory effects on the growth of the selected pathogens by TE, TH, and GW were determined as a function of time. For *V. parahaemolyticus* ATCC 17802, TE and TH treated samples showed minor steady inhibition activity compared with the control, especially at 60 and 123 hr, whereas GW treated samples showed similar inhibitory activity until 48 hr. However, proliferation was observed after 57 hr in the GW treated sample when compared to the control (Fig. 1). For *Sal.* Typhimurium KCCM 11862, all tested samples demonstrated growth inhibition activity after 24 hr, from highest to lowest as follows: GW>TH>TE>control ($p<0.05$). Interestingly, TH and TE showed steady growth inhibition activity with increasing growth time after 6 hr compared to the contrsol, whereas GW the growth inhibition activity was observed from the beginning of growth time ($p<0.05$). Only GW demonstrated growth inhibition activity against *Sh. sonnei* KCTC 2518. On the contrary, bacterial proliferation activity in TH and TE treated samples occurred after 12 hr compared to the control, particularly at 24 hr ($p<0.05$).

Prebiotic effects of TSEs on LAB

TE, TH, and GW were examined for their probiotic effect, measured as the ability to increase the growth

Table 3. Antimicrobial activity of TSEs (trifoliate orange seed extracts) prepared from different solvents on gram-negative food-borne pathogens using disc diffusion method

Gram-negative foodborne pathogens	Sample[1)]			
	TW	TE	TH	GW
Escherichia coli KCTC 1039	−[2)]	+	+	+++
Escherichia coli O157: H7 ATCC 43895	−	+	+	+++
Shigella sonnei KCTC 2518	−	+++	+++	+++
Salmonella Enteritidis ATCC 3311	−	−	−	+++
Salmonella Typhimurium KCCM 11862	−	++	+++	+++
Vibrio parahaemolyticus ATCC 17802	−	+++	+++	+++

[1)]TW: trifoliate orange seed extracted by water, TE: trifoliate orange seed extracted by ethanol, TH: trifoliate orange seed extracted by *n*-hexane, GW: GSE (grape fruit seed extract) dissolve in water.

[2)]−: no inhibition (6 mm), +: slight inhibition (6~8 mm), ++: moderate inhibition (8~10 mm), +++: strong inhibition (>10 mm).

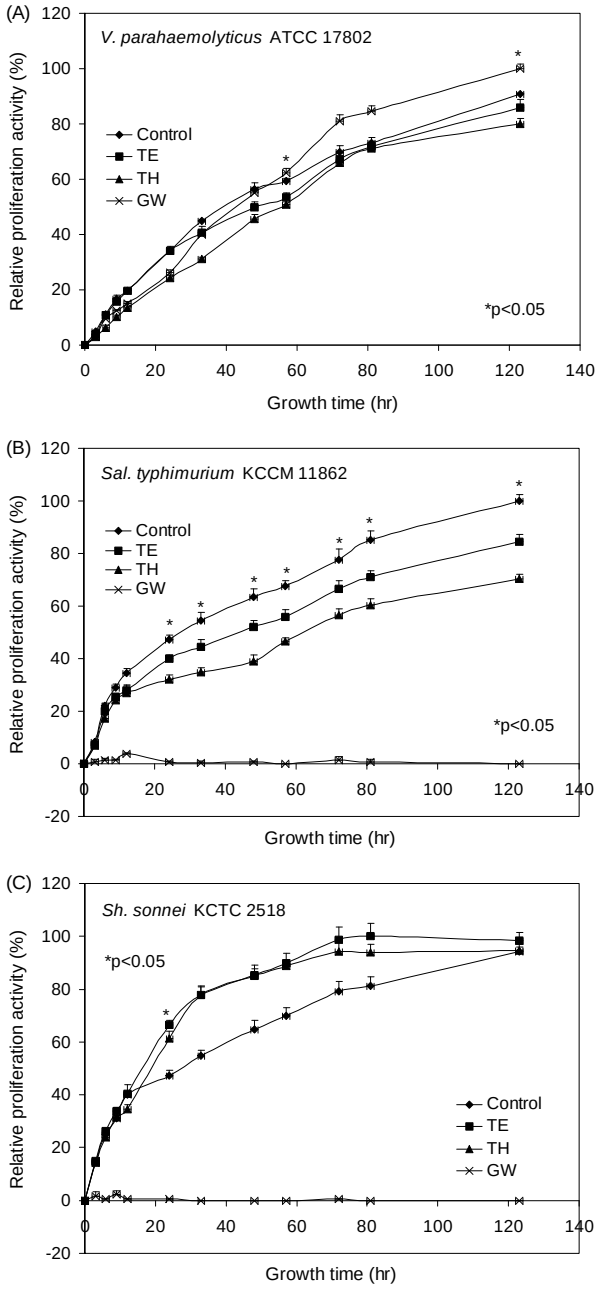

Fig. 1. Growth inhibition effects of TSEs (trifoliate orange seed extracts) prepared from different solvents on gram-negative food-borne pathogens (A～C). Control: No addition, TE: trifoliate orange seed extracted by ethanol, TH: trifoliate orange seed extracted by *n*-hexane, GW: GSE dissolved in water. *Significantly different at $p<0.05$ among the samples.

of the "good" bacteria. *Lb. acidophius* IFO 3025 was used as the representative beneficial probiotic bacteria. None of the tested samples demonstrated a prebiotic effect on *Lb. acidophius* IFO 3025; interestingly, GW appeared to be slightly inhibitory during the initial growth time to 24 hr (Fig. 2).

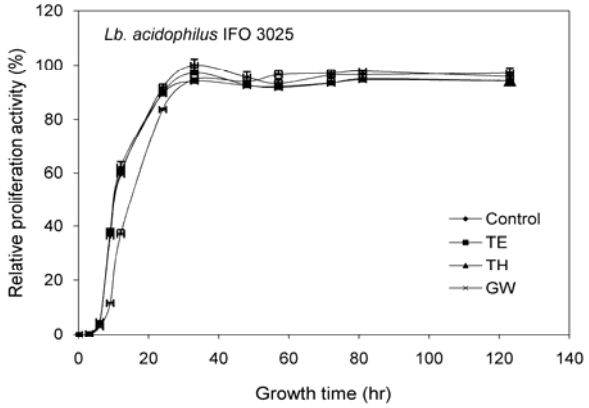

Fig. 2. Prebiotic effects of TSEs (trifoliate orange seed extracts) prepared from different solvents on the growth of *Lactobacillus acidophilus* IFO 3025. Control: No addition, TE: trifoliate orange seed extracted by ethanol, TH: trifoliate orange seed extracted by *n*-hexane, GW: GSE (grape fruit seed extract) dissolve in water.

DISCUSSION

A broad spectrum of microbial pathogens can contaminate human food and cause illnesses after they or their toxins are ingested (7). Consumption of raw foods has been suspected or contaminated worldwide as the most likely source of infection in diverse outbreaks which occurred during the last decade (8). The approaches that can be adopted in food preservation include: (a) aseptic handling and packaging, (b) mechanical removal of microorganisms by washing or filtration, (c) destruction of microorganisms by physical or chemical sanitization, and finally (d) inhibition of pathogens or saprophytes through environmental control achieved by adding synthetic chemical compounds (antimicrobial preservatives) such as trisodium phosphate, acidified calcium sulfate, organic acids (e.g., lactic, acetic) and acetyl pyridinium chloride with inhibitory or bactericidal/ fungicidal activity (9). Although approved for use in food processing in the last years, natural antimicrobials have attracted considerable attention due to the increasing consumer awareness on the aspects of food quality and safety.

Several methods have also been used to extend the storage life of green produce, such as high hydrostatic pressure, high intensity ultrasound and gamma irradiation. However, these 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 (10). Therefore, there is a growing interest in the development of sanitizers with antimicrobial activities without toxicity in order to maintain the sensory quality and extend shelf-life of minimally processed vegetables and fruit.

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 in human nutrition. Limonoids are secondary metabolites present in all citrus fruit tissues (11), but the efficient activities of their seeds have not been established. Citrus oils are complex mixtures of natural compounds (approximately 400 compounds) whose contents depend on specific citrus cultivar, extraction and separation methods. Unlike many of the exotic plant extracts that have been proposed as new antimicrobials (12), citrus oils have been a part of the human diet for hundreds of years and thus have been generally recognized as safe (GRAS) by the Food and Drug Administration (FDA). Individual citrus oil components have demonstrated antimicrobial activity against major food-borne pathogens (13).

In recent years, GSE has been shown to possess antibacterial, antiviral, antifungal and anti-parasitic properties (6). GSE contains large quantities of polyphenolic compounds, such as catechins, epicatechin, epocatechin-3-*O*-gallate, and dimeric, trimeric and tetrameric procyanidins (14). These beneficial actions of GSE have partly been attributed to the antioxidative activity of citrus flavonoids, such as naringenin (15). The safety of GSE has been tested in several areas, with Heggers et al. showing that GSE was not detrimental to human fibroblast skin cells *in vitro* (16).

There is an increasing consumer demand for fresh, healthy, convenient and additive-free ready-to-eat vegetables that are safe and nutritious. These products may be whole or fresh-cut and are usually sealed in semi-permeable packages and stored at low temperatures, they can harbor large and diverse pathogenic populations of microorganisms, such as *L. monocytogenes* and *Sal.* spp. (17). Several cases of food-borne illnesses have been attributed to the consumption of fresh-cut vegetables or fruit (18) and *Sal.* spp. is amongst the most notable causative bacterial pathogens (19).

In conclusion, among the tested TSEs, TH has a robust growth inhibition activity against *Sal.* Typhimurium KCCM 11862, while TE has a slight growth inhibition activity against *Sal.* Typhimurium KCCM 11862. TE and TH demonstrated neither a prebiotic effect on *Lb. acidophius* IFO 3025 nor a growth inhibition activity. Our data demonstrated that TSEs can be used as antimicrobial materials, instead of GSE. Thus, TSEs, as a food processing byproduct, can be a cost-friendly and safe antimicrobial product option.

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 altering physical stability or integrity of the food chemistry as well as degrading the biological activity of the bioactive compounds (20). This poses a concentration window requirement, high enough to inhibit microbial growth but low enough to minimally alter the qualitative properties of the product as imposed by food regulation. Taking into account the complexity of the TSEs mixtures and their variability in the chemical composition, further studies are highly warranted to test varying concentration ranges and the relevant food matrices in order to better characterize their potential as antimicrobials.

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