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

Seong Yeong Kim* and †Kwang-Soon Shin

*Nutrition Education, Graduate School of Education, Kyonggi University, Suwon 443-760, Korea
Dept. of Food Science and Biotechnology, Kyonggi University, Suwon 443-760, Korea

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

Key words: antimicrobial activity, growth inhibition activity, trifoliate orange seed extract
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, epicatechin-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 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°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).

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 mg/ml) of TW (TSE with distilled water),

Table 1. Bacterial strains tested and their growth conditions

| Bacterial strains       | Media                  | Temperature (°C) |
|-------------------------|------------------------|-----------------|
| *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 μm) 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 ml of the bacterial suspension to a cell density of 10^6-10^7 CFU/ml was spread on appropriate solid media for each food pathogen growth. After being air-dried sterile 6 mm (diameter) paper disc was placed on the agar surface that had been inoculated with test bacteria. Each test sample 20 μl added on the paper disc, respectively and also negative control discs were 20 μl of distilled water, 75% DMSO, and 100% DMSO. Positive control disc was GSE (20 mg/ml) filtrated membrane as well. All plates were then incubated at respective optimum temperature for 2 days under microaerophilic condition. Inhibitory activity was measured (in mm) as a diameter of the observed zones. We replicated all growth inhibition tests 3 times at 400 μg/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 mm; moderate response (++), zone of inhibition diameter 8-10 mm; weak response (+), zone of inhibition diameter 6-8 mm; and no response (−), zone of inhibition diameter 6 mm.

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 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 the test pathogen at a concentration of 1% (v/v, 1-5×10^8 CFU/ml) 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 nm.

5. 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 in a shaking incubator (IS-971R, Jeio Tech., Kimpo, Korea) under 37°C 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 nm.

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 | 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 | Sample 2 | Sample 3 | Sample 4 |
|---------------------------------------------|----------|----------|----------|----------|
| Bacillus cereus KCCM 11341                  | 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. |
| Bacillus subtilis KCTC 1022                  | - 2)     | +        | ++       | +++      |
| 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 mm), +; weak inhibition (6-8 mm), ++; moderate inhibition (8-10 mm), +++; strong inhibition (> 10 mm).

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

(A) B. cereus KCCM 11341

(B) B. subtilis KCTC 1022

(C) S. aureus ATCC 19111

(D) Y. enterocolitica KCCM 41657

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. acidophilus IFO 3025 of representative efficient LAB. The results of prebiotic effects of tested samples against Lb. acidophilus 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 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. acidophilus* 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.

**REFERENCES**

Bezic N, Skocibusic M, Dinkic V, Radonic A. 2003. Composition and antimicrobial activity of *Achillea clavennae* L. essential oil. *Phytother Res* 17:1037-1040

De Buyser M-L., Dufour B, Maire M, Lafarge V. 2001. Implication of milk and milk products in food-borne diseases in France and in different industrialized countries. *Int J Food Microbiol* 67:1-17

Dorman HJ, Deans SG. 2000. Antimicrobial agents from plants: antibacterial activity of plant volatile oils. *J Appl Microbiol* 88:308-316

Farag RS, Badei AZ, Heweij FM, El-Baroty GSA. 1986. Antioxidant activity of some spices essential oils on linoleic acid oxidation in aqueous media. *J Am Oil Chem Soc* 66:792-799

Fisher K, Rowe C, Phillips CA. 2007. The survival of three strains of *Arcobacter butzleri* in the presence of lemon, orange and bergamot essential oils and their components in *vitro* and on food. *Lett Appl Microbiol* 44:495-499
Francis GA, Thomas C, O’Beirne D. 1999. Growth of *Listeria monocytogenes* on vacuum-packed cooked meats: effects of pH, aw, nitrite and ascorbate. *Int J Food Sci Nutr* 34:1-22

Gil MI, Tomás-Barberán FA, Hess-Pierce B, Holcroft DM, Kader AA. 2000. Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. *J Agric Food Chem* 48:4581-4589

Heggers JP, Cottingham J, Gusman J, Reagor L, Mccoy L, Carino E, Cox R, Zhao JG. 2002. The effectiveness of processed grapefruit-seed extract as an antibacterial agent. I. Mechanism of action and *in vitro* toxicity. *J Altern Complement Med* 8:333-340

Ionescu G, Kiehl R, Wichmann-Kunz F, Williams CH, Baum LM, Levine S. 1990. Oral citrus seed extract. *J Orthomol Med* 5:230-238

Kemp GK, Aldrich ML, Waldrup AL. 2000. Acidified sodium chlorite antimicrobial treatment of broiler carcasses. *J Food Prot* 63:1087-1092

Leistner L. 2000. Hurdle technology in the design of minimally processed foods. In Alzamora SM, Tapia MS, & López-Malo A. (Eds.). Minimally Processed Fruits and Vegetables: Fundamental Aspects and Applications. pp.13-27. Aspen Publishers, Maryland

McClements J. 2005. Food Emulsions in Principles, Practices and Techniques, (2nd ed). CRE Press, Boca Raton, FL.

Orden JA, Cid D, Ruiz-Santa-Quiteria JA, Garcia S, Martinez S, De la Fuente R. 2002. Verotoxin-producing *Escherichia coli* (VTEC) enteropathogenic *E. coli* (EPEC) and necrotogenic *E. coli* (NTEC) isolated from healthy cattle in Spain. *J Appl Microbiol* 93:29-35

Rahman A, Kang SC. 2009. *In vitro* control of food-borne and food spoilage bacteria by essential oil and ethanol extracts of *Lonicera japonica* Thunb. *Food Chem* 116:670-675

Saito M, Hosoyama H, Ariga T, Kataoka S, Yamaji N. 1998. Antiulcer activity of grape seed extract and procyanidins. *J Agric Food Chem* 46:1460-1464

Shoko T, Soichi T, Megumi MM, Eri F, Jun K, Michiko W. 1999. Isolation and identification of an antibacterial compound from grape and its application to food. *Nippon Nogeikagaku Kaishi* 73:125-128

Tauxe RV. 2002. Emerging foodborne pathogens. *Int J Food Microbiol* 78:31-41

Tian Q, Miller EG, Ahmad H, Tang L, Patil BS. 2001. Differential inhibition of human cancer cell proliferation by citrus limonoids. *Nat Cancer* 40:180-184

Tuley de Silva K. (Ed.), 1996. A manual on the essential oil industry. United Nations Industrial Development Organization, Vienna

Vercet A, Lopez P, Burgos J. 1998. Free radical production by manothermosonication. *Ultrasonics* 36:615-618