

## Bioactivity of Trifoliolate Orange (*Poncirus trifoliata*) Seed Extracts

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### Abstract

Trifoliolate orange seed extracts (TSEs) were made using either distilled water (TW), ethanol (TE), or *n*-hexane (TH), to measure total polyphenol contents, DPPH and ABTS radical scavenging activities, and anti-complementary activity. The results showed that the total polyphenol content showed higher value at TE (235.24 µg/mL,  $p < 0.05$ ) than those of TW (132.65 µg/mL) and TH (165.44 µg/mL) at 10 mg/mL and TE exerted the highest DPPH radical scavenging activity (61.77%,  $p < 0.05$ ), which occurred in the following order: TE > TW (56.87%) > TH (39.78%). The results of ABTS radical scavenging activity showed that TW (34.26%) and TE (31.81%) showed similar activities, which were higher than TH (12.74%,  $p < 0.05$ ). Anti-complementary activity of TE (61% at 500 µg/mL) showed a higher activity when compared with the positive control (60% at 1,000 µg/mL) polysaccharide-K (PSK), a known immuno-active polysaccharide from *Coriolus versicolor*. Consequently, among TSEs, TE is a byproduct from trifoliolate orange and could be an important source of dietary polyphenolic antioxidant compounds and immunopotentiating activity, including complement activation.

**Key words:** trifoliolate orange seed extract, polyphenol, antioxidant activity, DPPH radical scavenging activity, anti-complementary activity

### INTRODUCTION

Synthetic antioxidants, such as butylated hydroxyanisole, butylated hydroxytoluene, and tertiary butylhydroquinone, have been widely used in foods as preservatives. However, the use of these synthetic antioxidants in foods is discouraged because of their toxicity (1) and carcinogenicity (2). For these reasons, a few natural antioxidants have attracted special interest because of their ability to remove free radicals, which are known to cause various diseases including cancer, cardiovascular disease, and aging (3). Consumers are also 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 (4).

Several natural antioxidants have been isolated from plant materials, such as oil seeds, cereal crops, vegetables, fruits, leaves, roots, spices, and herbs (5-7). Citrus fruits, a major contributor to human diet, have received attention due to their multitude of bioactive compounds. Recent *in vitro* studies have suggested these bioactive compounds have health-promoting properties and show potentials to be antioxidant, anti-proliferative, and anti-viral agents, as well as to prevent cardiovascular diseases

(8). Among them, grapefruit seed extract (GSE), a commercial product derived from the seeds and pulp of grapefruit (*Citrus paradise Macf.* Rutaceae), is a natural extract that demonstrates effective broad-spectrum bioactivities including antioxidant, bactericidal, fungicidal, anti-viral and anti-parasitic activities (9,10). GSE is also environmentally safe without toxicity to humans or animals at effective concentrations. On the other hand, only little information is available on studies relating to the bioactivity of trifoliolate orange (*Poncirus trifoliata*). Among the different parts of the trifoliolate orange, the seed is one of the major byproducts without known significant use. Based on this information, we focused on evaluating the bioactivity of the trifoliolate 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 less utilized food byproducts, seeds, which can lead to economic benefits to the citrus processing industry, citrus growers, and the global society as a whole.

### MATERIALS AND METHODS

#### Chemicals

Folin-Ciocalteu reagent, gallic acid (+)-catechin, DPPH,

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ABTS and potassium persulfate were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and IgM-hemolysin-sensitized sheep erythrocytes were purchased from Biotest Co. (Yokohama, Japan). All other chemicals used were of analytical grade.

#### Preparation of TSEs

Trifoliolate orange was cultivated at Yeongcheon, Gyeongsangbuk-do, Korea and harvested in November 2011. Dried trifoliolate orange seed was purchased from Cheongmyung Medicinal Herb Company (Gwangju, Korea). To obtain only the trifoliolate orange seed, foreign materials, including the peel, etc., were removed 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 *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. 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. For the test, each lyophilized sample of trifoliolate orange seed extracted with distilled water (TW), ethanol (TE), and *n*-hexane (TH) was dissolved with distilled water, 75% dimethylsulfoxide (DMSO), and 100% DMSO, respectively. The positive control used for bioactivity testing was commercially available GSE (Esfood Co. Ltd., Pocheon, Korea).

#### Total polyphenol contents

The total polyphenol contents were determined using the Folin-Ciocalteu method (11) adapted to a micro scale. Briefly, 0.79 mL of distilled water, 0.01 mL of appropriately diluted sample (1,000 µg/mL and 10 mg/mL) and 0.05 mL of Folin-Ciocalteu reagent were added to a 1.5-mL eppendorf tube and then mixed. After exactly 1 min, 0.15 mL of 20% sodium carbonate was added to the mixture, mixed, and allowed to stand at room temperature for 120 min. The absorbance was then read at 750 nm and the total polyphenol concentration was calculated from a calibration curve using gallic acid as a standard.

#### DPPH radical scavenging activity

The DPPH radical scavenging activity was measured according to the method described by Cheung et al. (12), with some modifications. Briefly, 0.8 mL of 0.2 mM DPPH ethanolic solution was mixed with 0.2 mL of appropriately diluted sample (10 mg/mL). The mixture was then vigorously shaken and left to stand for 10 min under

subdued light, after which the absorbance was measured at 520 nm.

#### ABTS radical scavenging activity

The ABTS radical cation scavenging activity was measured according to the method described by Re et al. (13), with some modifications. The ABTS radical cation was generated by adding 7 mM ABTS to a 2.45 mM potassium persulfate solution and then allowing the mixture to stand overnight in the dark at room temperature. The ABTS radical cation solution was then diluted with distilled water to obtain an absorbance of 1.4~1.5 at 414 nm (14). Next, 1 mL diluted ABTS radical cation solution was added to 50 µL sample (10 mg/mL). After 60 min, the absorbance was measured at 414 nm.

#### Anti-complementary activity

The anti-complementary activity was measured by a complement fixation test that was based on complement consumption and the degree of red blood cell lysis by the residual complement (15). Briefly, normal human serum (NHS) was obtained from a healthy adult, after which a 50 mL aliquot of sample (1,000 µg/mL) that had been extracted from the trifoliolate orange seed (1,000 µg/mL) was mixed with equal volumes of NHS and gelatin veronal buffered saline (GVB<sup>++</sup>; pH=7.4) containing 500 mM Mg<sup>++</sup> and 150 mM Ca<sup>++</sup>. The mixtures were then incubated at 37°C for 30 min, after which the residual total hemolytic complement (TCH<sub>50</sub>) was determined using IgM-hemolysin-sensitized sheep erythrocytes (EA cell) at a concentration of 1 × 10<sup>8</sup> cells/mL. NHS was incubated with water and GVB<sup>++</sup> as a negative control. Polysaccharide-K (PSK), which is a known immunoreactive polysaccharide from *Coliolum versicolor*, was used as a positive control in an anti-complementary activity assay.

The anti-complementary activity of the extracted samples was expressed as the percentage inhibition of the control TCH<sub>50</sub>:

$$\text{Inhibition of TCH}_{50} (\%) = \frac{\{\text{TCH}_{50} (\text{control}) - \text{TCH}_{50} (\text{treated with sample})\}}{\text{TCH}_{50} (\text{control})}$$

#### 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.

**Table 1.** Lyophilized powder yield of trifoliolate orange seed extracts prepared from different solvents

Sample <sup>1)</sup>	TW	TE	TH
Yield (%)	20.85	5.39	0.44

<sup>1)</sup>TW, trifoliolate orange seed extracted with water; TE, trifoliolate orange seed extracted with ethanol; TH, trifoliolate orange seed extracted with *n*-hexane.

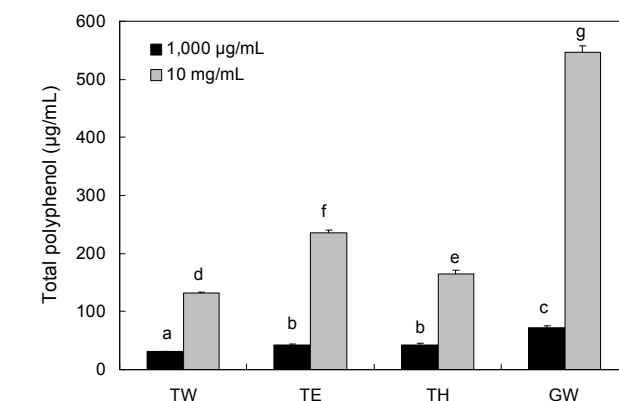
## RESULTS AND DISCUSSION

### Preparation of TSEs and total polyphenol contents

TSEs were made using distilled water, ethanol, or *n*-hexane. The yields of these three TSEs were as follows (Table 1): water extract (TW, 20.85%) > ethanol extract (TE, 5.39%) > *n*-hexane extract (TH, 0.44%). Polyphenols have long been considered chemopreventive agents because of their strong antioxidative activities (16), dietary polyphenolic antioxidant compounds that may have potential benefits in health and disease management. The relationships between antioxidant activity of plant products and their phenolic contents have been evaluated in several studies. Velioglu et al. (17) reported a high correlation between total phenolic content and antioxidant activity in selected fruits, vegetables and grain products. Citrus fruit extracts and citrus flavonoids exhibit a wide range of promising biological properties including anti-atherogenic, anti-inflammatory, anti-tumor, and strong antioxidant activities and inhibition of blood clots (18). Citrus species of various origins have been assessed for their phenolic constituents and antioxidant activities (19). The interest of finding natural antioxidants has considerably increased which can impact the management of a variety of clinical conditions and maintenance of health. The present study determined the antioxidant capacities of TSEs and the total polyphenol content that may contribute to the antioxidant activities of the TSEs. Total polyphenol contents of TSEs were evaluated using the Folin-Ciocalteu method. The total polyphenol content of TE (trifoliolate orange seed extracted with ethanol, 235.24 µg/mL) was significantly higher ( $p < 0.05$ ) than TW (trifoliolate orange seed extracted with water, 132.65 µg/mL) and TH (trifoliolate orange seed extracted with *n*-hexane, 165.44 µg/mL) at 10 mg/mL of sample concentration (Fig. 1). However, the total polyphenol content of TE was lower compared to GW (GSE dissolved with water, 546.53 µg/mL), used as a positive control.

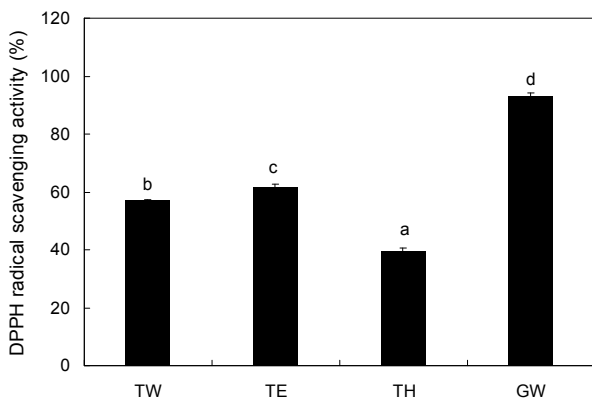
### DPPH and ABTS radical scavenging activities

Major mechanisms for the antioxidant effect of phenolics in functional foods include free radical scavenging and metal chelation activities. Reactive oxygen species (ROS), such as the superoxide radical ( $O_2^{\cdot-}$ ), hydrogen peroxide ( $H_2O_2$ ), hypochlorous acid (HOCl) and the hydroxyl radical ( $HO\cdot$ ) have been recognized to play a determining role in the pathogenesis of several human diseases (20,21). ROS-induced oxidation can result in cell membrane disintegration, membrane protein damage and DNA mutation, which can further initiate or propagate the development of diseases including cancer (22), diabetes (23), neurodegenerative diseases (24), the process of aging (25), and cardiovascular dysfunctions (26). The methods using DPPH and ABTS radical scavenging activities employ common spectrophotometric procedures for determining the antioxidant capacities of components (27). Among TSEs, TE exerted the highest DPPH radical scavenging activity (61.77%,  $p < 0.05$ ), which occurred in the following order: TE > TW (56.87%) > TH (39.78%). However, DPPH radical scavenging activity of TE had a lower observed value when compared to GW (93.04%, Fig. 2). The results of the ABTS radical scavenging activity showed that TW (34.26%) and TE (31.81%) performed similar activities, which were higher in value than those of TH (12.74%,  $p < 0.05$ ), although, ABTS radical scavenging activities of TW and TE were also lower than those of GW (73.80%, Fig. 3).

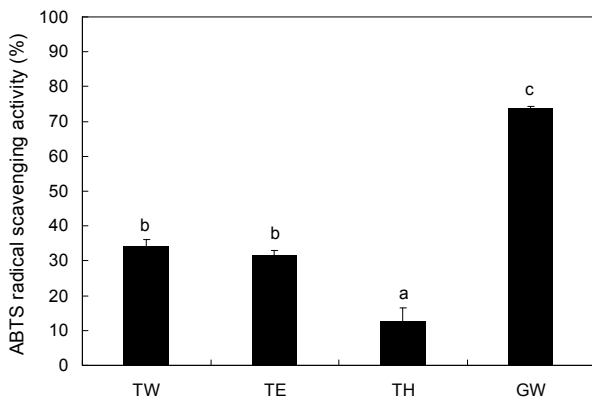


**Fig. 1.** Total polyphenol contents of trifoliolate orange seed extracts prepared from different solvents. TW, trifoliolate orange seed extracted with water; TE, trifoliolate orange seed extracted with ethanol; TH, trifoliolate orange seed extracted with *n*-hexane; GW, grape fruit seed extract dissolve with water which was commercially available.

The effect of antioxidants on DPPH and ABTS radical scavenging was thought to be due to their hydrogen-donating ability. Phytochemicals play a crucial role in health promotion and disease prevention by mechanisms related to cell differentiation, deactivation of pro-carcinogens, maintenance of DNA repair, inhibition of *N*-nitrosamine formation and change of estrogen metabolism (28). Phenolic compounds such as phenolic acids, flavonoids, stilbenes, tannins and lignans can scavenge free radicals and quench ROS and therefore provide effective means for preventing and treating free radical-mediated diseases (29). Our results also showed that TE had pre-



**Fig. 2.** DPPH radical scavenging activities of trifoliolate orange seed extracts prepared from different solvents; TW, trifoliolate orange seed extracted with water; TE, trifoliolate orange seed extracted with ethanol; TH, trifoliolate orange seed extracted with *n*-hexane; GW, grape fruit seed extract dissolve with water which was commercially available.



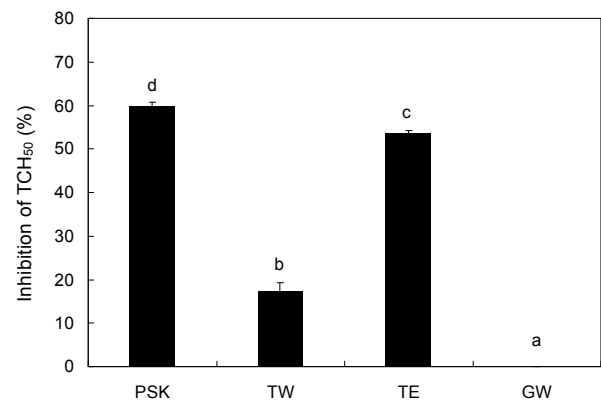
**Fig. 3.** ABTS radical scavenging activities of trifoliolate orange seed extracts prepared from different solvents. TW, trifoliolate orange seed extracted with water; TE, trifoliolate orange seed extracted with ethanol; TH, trifoliolate orange seed extracted with *n*-hexane; GW, grape fruit seed extract dissolve with water which was commercially available.

dominant DPPH radical scavenging activity (Fig. 2) and higher total polyphenol content (Fig. 1).

**Anti-complementary activity**

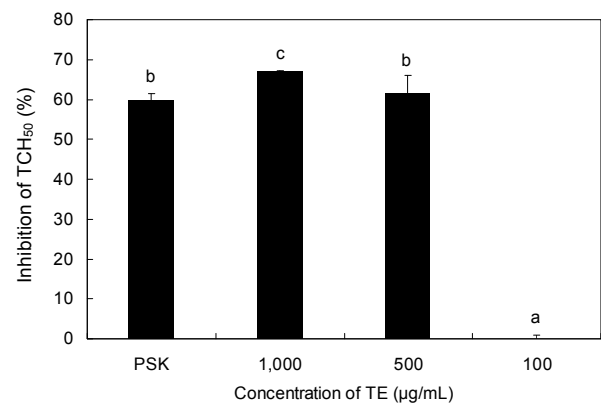
In general, the complement system plays an important role in primary defense against bacterial and viral infections. The complement system consists of over 20 proteins, which are activated by a cascade of classical, alternative and lectin pathways. Complement activation appears to be intrinsically associated with several immune reactions, including the activation of macrophages, lymphocytes and immunopotiation (30).

Anti-complementary activity of TE (53.56%) was greater than those of TW (17.38%) and GW (0%) at 1,000 µg/mL of sample concentration (Fig. 4). Interestingly, anti-complementary activity of TE showed a similar high activity when compared with positive control (60%) pol-



**Fig. 4.** Anti-complementary activities of trifoliolate orange seed extracts prepared from different solvents. TW, trifoliolate orange seed extracted with water; TE, trifoliolate orange seed extracted with ethanol; GW, grape fruit seed extract dissolve with water which was commercially available. PSK (Polysaccharide-K), which is a known immunoactive polysaccharide from *Coriolus versicolor*, was used as a positive control in an anti-complementary activity assay.

ysaccharide-K (PSK), a known immuno-active polysaccharide from *Coriolus versicolor*, at 1,000 µg/mL. Therefore, we selected TE as a complement activating material to evaluate its activity with increasing sample concentrations (100, 500, and 1,000 µg/mL). The results revealed that the anti-complementary activity increased as the TE concentration increased (Fig. 5). Furthermore, the anti-complementary activities of TE extracted from trifoliolate orange seed with concentrations of 1,000 and 500 µg/mL were found to be 67 and 61.46%, respectively, whereas the activity of PSK was 60% at 1,000 µg/mL. Taken together, these findings indicate that TE extracted from trifoliolate orange seed (61.46% at 500 µg/mL) had a higher anti-complementary activity than PSK (60% at 1,000 µg/mL). Overall, these results confirm that TSEs extracted from trifoliolate orange seed, espe-



**Fig. 5.** Anti-complementary activities of TE (trifoliolate orange seed extracted with ethanol) as the increase of concentration. PSK (Polysaccharide-K), which is a known immunoactive polysaccharide from *Coriolus versicolor*, was used as a positive control in an anti-complementary activity assay.

cially TE, had a good complement activation factor.

Trifoliolate orange is consumed mostly as fresh produce and juice and most often the peel and seed are discarded and wasted. Thus far, no report on the nutritional and health-promoting values of trifoliolate orange seed exists. Thus, trifoliolate orange seed as a byproduct could be an important source of dietary polyphenolic antioxidant compounds and immunopotentiating activity, including complement activation that may have potential benefits in health and disease management.

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