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Evaluation of Physiological Activities of the Citron (*Citrus junos* Sieb. ex TANAKA) Seed Extracts

Seong Yeong Kim[1] and Kwang-Soon Shin[2]

[1]Nutrition Education, Graduate School of Education, and [2]Department of Food Science and Biotechnology, Kyonggi University, Gyeonggi 443-760, Korea

ABSTRACT: Citron seed extracts (CSEs) were made using distilled water (CSEW), ethanol (CSEE), and *n*-hexane (CSEH), to measure the total polyphenol contents, DPPH and ABTS radical scavenging activities, and anti-complementary activity. The total polyphenol content was observed the highest in CSEE (188.71 μg/mL), and occurred in the following order: CSEE>CSEW (141.11 μg/mL)>CSEH (26.19 μg/mL) at 10 mg/mL. CSEE (63.56%) and CSEW (56.61%) showed significantly higher DPPH radical scavenging activities when compared with CSEH (28.57%). ABTS radical scavenging activities of CSEE (45.53%) and CSEW (40.02%) were also observed to be higher, whereas CSEH did not show ABTS radical scavenging activity. Anti-complementary activity of CSEE (26.85%) showed a greater activity than that of CSEW (7.84%) at 1,000 μg/mL. Limonin and nomilin contents had the highest values (1.882% and 2.089%) in CSEE, and with 0.327% and 0.139% in CSEW; however, CSEH showed relatively very low values at 0.061% and 0.026%, respectively. Among the CSEs tested, CSEE as a by-product from citron may provide an important source of dietary antioxidant compounds with rich polyphenol and limonoid contents, and immunopotentiating activity, including the complement activation factor.

Keywords: citron seed extract, polyphenol, DPPH radical scavenging activity, ABTS radical scavenging activity, anti-complementary activity

INTRODUCTION

Consumers are increasingly aware of the various diet-related health problems and are demanding natural ingredients that are expected to be safe and health-promoting in foods. Health properties of the citrus fruits, recognized as one of the world's major fruit crops, is gaining widespread interest. The natural antioxidant substances in citrus fruits appear to be associated with the prevention of the degenerative processes and the reduced risks of certain chronic diseases such as cancer (1-3), osteoporosis (4) and cardiovascular diseases (5,6).

The citrus production worldwide is approximately 105 million tons per year, and the fruits of this genus are mainly used for juice-processing. The residues of the industry, such as peels, seeds and pulps, which represent about 50% of the raw processed fruit, are a potential source of valuable by-products (7). Citrus-processing produces a considerable amount of by-products, posing a potential waste problem, while at the same time providing a promising source for the food industry for their valuable technological and nutritional properties.

Citron (*Citrus junos* Sieb. ex TANAKA) is a citrus fruit that is cultivated in the northeast Asia region. In Korea, citron has been mostly consumed in thin slices combined with sugar or honey to make a thick marmalade-like syrup containing pieces of the chopped rinds. Citron is also used in various food products, such as salads, sauces, dressings, and vinegar for its flavor, as well as in bathing products. Citron is well-known for its abundant antioxidants such as vitamin C, polyphenol compounds, and limonoids (8,9). When the mature citrons are processed for tea, beverage or other food products in Korea, massive amounts of seeds are collected and discarded. Citron seeds account for 14~16% of the total citron fresh weight but are rich in limonoids as compared to the whole fruits (10,11). Despite the increasing demand for the citrus by-products as natural ingredients, a lack of well-defined environmentally friendly, and economical extraction and purification methods have precluded the commercial development of the market.

We have extracted citron seeds with water, ethanol, and *n*-hexane, and evaluated the physiological activities of the citron seed extracts (CSEs). We also performed

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Correspondence to Kwang-Soon Shin, Tel: +82-31-249-9655, E-mail: ksshin@kyonggi.ac.kr

HPLC analysis of the limonoid contents, a known bioactive substance in the citrus seeds. The results may lead to economic benefits for the citrus processing industry and citrus growers, and more importantly global health.

MATERIALS AND METHODS

Preparation of CSEs

The citrons used in the study were cultivated at Goheung-gun, Jeollanam-do, Korea and harvested in November, 2011. The citron seeds were purchased from the Ceil Food Company (Goheung, Korea). The seeds were washed twice using tap water and then dried in a 80°C incubator (Sanyo Electric Co., Ltd., Moriguchi, Japan) overnight. Dried citron seeds were ground with an electronic grinder (Hanil Electronics Corp., Weonju, Korea). One hundred grams of the ground sample (moisture content; 3.60%) was extracted with l L of distilled water, ethanol, and *n*-hexane. The extraction condition of ethanol and *n*-hexane was at room temperature for 8 h, while the distilled water was kept in the 40°C incubator (Sanyo Electric Co., Ltd.) to maintain the temperature for 8 h 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. Each lyophilized sample of CSEW (citron seeds extracted with distilled water), CSEE (citron seeds extracted with ethanol), and CSEH (citron seeds extracted with *n*-hexane) for the test was dissolved with distilled water, 75% dimethylsulfoxide (DMSO), and 100% DMSO, respectively. The positive control used for the physiological test was the commercially available grapefruit seed extract (GSE, Esfood Co., Ltd., Pocheon, Korea).

Total polyphenol contents

The total polyphenol contents were examined using the Folin-Ciocalteu method (12), 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. Exactly 1 min later, 0.15 mL of 20% sodium carbonate was added, and the mixture was then 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 by the method of Cheung et al. (13), with slight modifications. Briefly, 0.8 mL of 0.2 mM DPPH ethanolic solution was mixed with 0.2 mL of an 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.

DPPH radical scavenging activity (%) = (1 − As/Ac) × 100, where As is the absorbance in the presence of sample, and Ac is the absorbance in the absence of sample, respectively.

ABTS radical scavenging activity

The ABTS radical cation scavenging activity was carried out by the method of Re et al. (14), with slight 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 (15). Next, 1 mL diluted ABTS radical cation solution was added to a 50 μL sample (10 mg/mL). After 60 min, the absorbance was measured at 414 nm.

ABTS radical scavenging activity (%) = (1 − As/Ac) × 100, where As is the absorbance in the presence of sample, and Ac is the absorbance in the absence of sample, respectively.

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 (16). Briefly, normal human serum (NHS) was obtained from a healthy adult, after which a 50 μL aliquot of sample (1,000 μg/mL) that had been extracted from the citron seeds was mixed with equal volumes of NHS and gelatin veronal buffered saline (GVB^{2+}; pH 7.4), containing 500 mM Mg^{2+} and 150 mM Ca^{2+}. The mixtures were then incubated at 37°C for 30 min, after which the residual total hemolytic complement (TCH_{50}) was determined using IgM hemolysin-sensitized sheep erythrocytes (EA cell) at a concentration of 1×10^8 cells/mL. NHS was incubated with water and GVB^{2+} as a negative control. Polysaccharide-K (PSK), which is a known immunoactive polysaccharide from *Coliolus versicolar*, was used as a positive control in the anti-complementary activity assay.

The anti-complementary activity of the extracted samples was expressed as the percentage inhibition of the control TCH_{50}:

$$\text{Inhibition of } TCH_{50}\ (\%) = TCH_{50}\ (\text{control}) - TCH_{50}\ (\text{treated with sample})/TCH_{50}\ (\text{control})$$

HPLC analysis

Standard and sample preparation: Limonin and nomilin as standards (Enzo Life Sciences Inc., Farmingdale, NY, USA) were dissolved in acetonitrile at three different concentrations (125, 250, and 500 μg/mL) and filtrated with 0.22 μm membrane (Millipore Corp., Bedford, MA, USA). Each lyophilized sample of CSEW, CSEE, and CSEH was dissolved with water, 75% DMSO, and 100% DMSO, respectively, and also filtrated with a 0.22 μm membrane.

HPLC analysis of limonin and nomilin contents: Limonin and nomilin contents were quantified by HPLC (Varian 920, Varian Inc., Church Stretton, shropshire, UK), using a Cadenza CD-C18 column (75×4.6 mm, Imatake Corp., Kyoto, Japan). Isocratic mobile phase (acetonitrile : 10 mM acetic acid, 4 : 6) was used at a flow rate of 1 mL/min. Sample injection quantity was 5 μL; and the absorbance was measured at a wavelength of 210 nm using a UV detector. Limonin and nomilin were identified and confirmed by comparing their retention times on HPLC with their standards. The contents in CSEW, CSEE, and CSEH were calculated based on the calibration curve on each standard.

Statistical analysis

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

RESULTS AND DISCUSSION

Total polyphenol contents

A number of physical, chemical, and biological environmental agents may pose genetic risks to exposed organisms, including the induction of mutations which may be involved in the initial steps of carcinogenesis (17). In this context, one of the suggested strategies for avoiding or reducing the deleterious effects of mutagens has been the consumption of fruits and vegetables, particularly those that are proven to contain chemopreventive compounds (17,18). Polyphenols have long been considered chemopreventive agents with strong antioxidative activities (19). Polyphenols form the dietary polyphenolic antioxidant compounds which may have potential benefits in health and disease management. 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 (20). Citrus species of various origins have been assessed for their phenolic constituents and antioxidant activities (21).

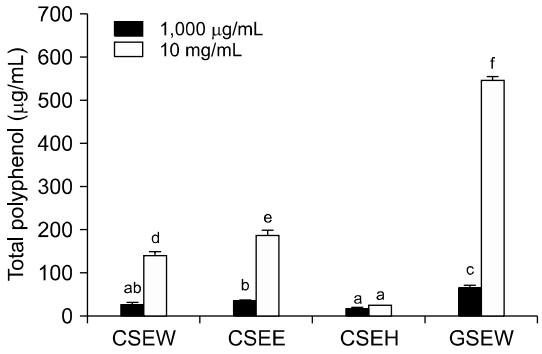

Fig. 1. Total polyphenol contents of citron seed extracts (CSEs) prepared from different solvents. CSEW, CSE using water; CSEE, CSE using ethanol; CSEH, CSE using n-hexane; GSEW, grapefruit seed extract dissolved in water on the market as positive control. Different letters mean significantly different at $P<0.05$.

The results of the total polyphenol contents of CSEs are shown in Fig. 1. The total polyphenol content was observed at the highest value in CSEE (188.71 μg/mL) ($P<0.05$), and occurred in the following order at 10 mg/mL of sample concentration: CSEE>CSEW (141.11 μg/mL)>CSEH (26.19 μg/mL). However, the total polyphenol content of CSEE showed a lower value when compared with GSEW (GSE dissolved with water, 546.53 μg/mL), used as a positive control. When compared with 1,000 μg/mL of sample concentration, the total polyphenol content of CSEE (38.53 μg/mL) and CSEW (29.71 μg/mL) showed relatively higher values ($P<0.05$) than those of CSEH (19.49 μg/mL). However, the total polyphenol contents of CSEE and CSEW also showed lower values when compared with GSEW (72.02 μg/mL) at 1,000 μg/mL.

DPPH and ABTS radical scavenging activities

As shown in Fig. 2, CSEE (63.56%) and CSEW (56.61%) showed significantly higher DPPH radical scavenging activities when compared with CSEH (28.57%) ($P<0.05$). However, the DPPH radical scavenging activities of CSEE and CSEW were observed lower when compared with GSEW (93.04%). ABTS radical scavenging activities of CSEE (45.53%) and CSEW (40.02%) were observed with similar activities, whereas CSEH did not show ABTS radical scavenging activity ($P<0.05$) (Fig. 3). ABTS radical scavenging activity of GSEW (73.80%) also showed the strongest activity. The results showed that CSEE and CSEW have higher DPPH and ABTS radical scavenging activities than CSEH. However, CSEE and CSEW showed relatively lower activities when compared with GSEW used as a positive control.

Reactive free radicals like superoxide, singlet oxygen radicals, peroxynitrite, and nitrogen dioxide radical spe-

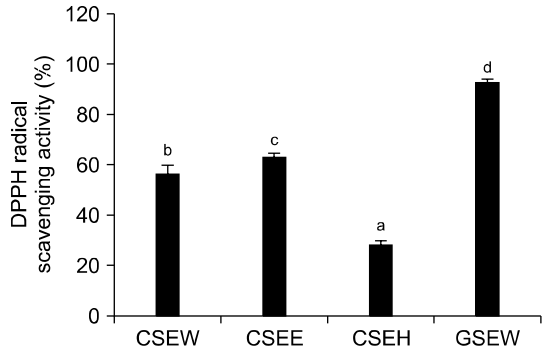

Fig. 2. DPPH radical scavenging activities of citron seed extracts (CSEs) prepared from different solvents. CSEW, CSE using water; CSEE, CSE using ethanol; CSEH, CSE using *n*-hexane; GSEW, grapefruit seed extract dissolved in water on the market as positive control. Different letters mean significantly different at $P<0.05$.

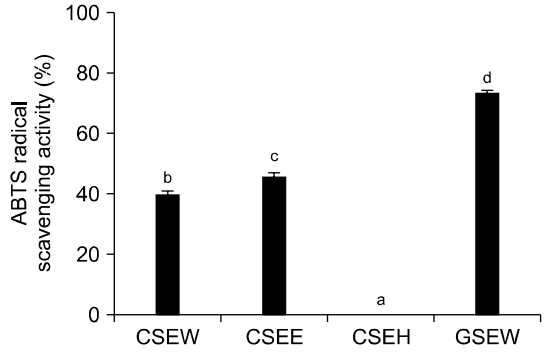

Fig. 3. ABTS radical scavenging activities of citron seed extracts (CSEs) prepared from different solvents. CSEW, CSE using water; CSEE, CSE using ethanol; CSEH, CSE using *n*-hexane; GSEW, grapefruit seed extract dissolved in water on the market as positive control. Different letters mean significantly different at $P<0.05$.

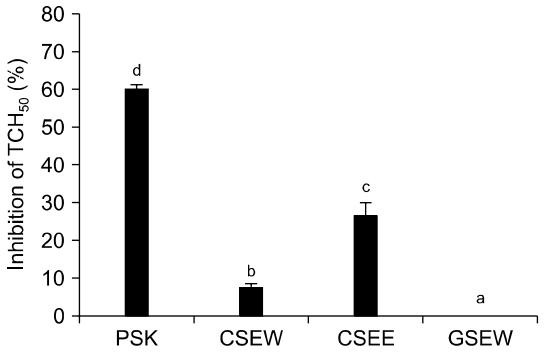

Fig. 4. Anti-complementary activities of citron seed extracts (CSEs) prepared from different solvents. CSEW, CSE using water; CSEE, CSE using ethanol; CSEH, CSE using *n*-hexane; PSK (Polysacchride-K), a known immnoactive polysaccharide from Coliolus versicolar, was used as a positive control. Different letters mean significantly different at $P<0.05$.

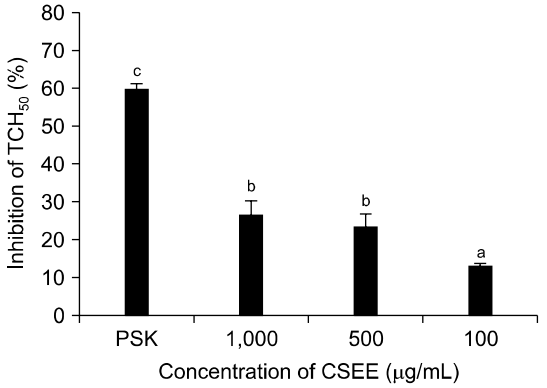

Fig. 5. Anti-complementary activities of citron seed extracts using ethanol (CSEE) as the increase of concentration. PSK (Polysacchride-K), a known immnoactive polysaccharide from Coliolus versicolar, was used as a positive control. Different letters mean significantly different at $P<0.05$.

cies generated *in vivo* are known to alter cellular structure and function; these induced alterations are thought to cause chronic degenerative diseases, including heart disease and cancer (22). The antioxidants present in the human body can reduce the incidence of such diseases (23) and provide a strong protection for the biological system. Antioxidants reduce the damages caused by reactive oxygen species (ROS), including the free oxygen centered radicals (24). A few natural antioxidants have attracted special interest for the abilities of removing free radicals (25). Fruits and vegetables are rich sources of various antioxidants that serve protective roles against various diseases, particularly cardiovascular diseases and some types of cancer (26). From our results, we saw that CSEE and CSEW as the by-products of citrons have good radical scavenging activities, including those against DPPH and ABTS radicals. Generally, phenolic compounds are significant contributors for the antioxidant activities of fruits and vegetables, which are able to scavenge radicals and chelate metals. CSEE and CSEW of the polyphenol-rich extracts showed greater antioxidant properties when compared to CSEH with lower polyphenol contents. The potential scavenging activities of the polyphenol substances may be due to the active hydrogen donor ability of hydroxyl substation.

Anti-complementary activity

The complement system plays an important role in the host defense against foreign invasive organisms such as bacteria, fungi, and viruses (27). 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 and lymphocytes and immunopotentiation (28). As shown in Fig. 4, the anti-complementary activity of CSEE (26.85%) showed to be greater than that of CSEW (7.84%) and GSEW (0%) at 1,000 μg/mL of sample concentration ($P<0.05$). However, CSEE showed a lower anti-complementary activity when compared with a positive control (60%), polysaccharide-K

(PSK), a known immuno-active polysaccharide from *Coriolus versicolor*. We also evaluated the anti-complementary acitivity of CSEE with increased sample concentrations (100, 500, and 1,000 μg/mL). The results revealed that the anti-complementary activity of CSEE increased with the increasing CSEE concentration (Fig. 5). The anti-complementary activities of CSEE at the concentrations of 1,000 and 500 μg/mL were found to be 26.85 and 23.70%, respectively. Overall, these results confirmed that CSEE produced from citron seeds had a good complement activation factor among the CSEs.

HPLC analysis

Citrus fruits contain various categories of health-promoting compounds such as flavonoids, ascorbic acid, vitamins, carotenoids, limonoids, and pectin (29). The presence of limonoids, aglycones, gludosides, and hesperidin as well as higher amount of phenolics in citrus fruits showed higher cytotoxicity in malignant cells, particularly in the limonoid aglycones-induced cell cycle arrest in breast cancer cells (30). Recently, limonoids demonstrated antiproliferative activity (31), inducing apoptosis, (2) and reducing tumor size (32,33) against colon cancer cell in an *in vivo* system. Furthermore, limonoids significantly decreased the LDL/HDL-cholesterol ratio and prolonged LDL oxidation susceptibility (34), as well as increased glutathione-S-transferase (GST) activity as the primary detoxification material in the human body (35). Limonoids also occur in significant amounts as aglycones and glycosides in the seeds (36). From the results of our study, the contents of limonin and nomilin were shown to have the highest values (1.8823% and 2.0892%) in CSEE, with 0.3268% and 0.1386% in CSEW, whereas CSEH showed relatively low values at 0.0605% and 0.0260%, respectively (Table 1). The HPLC chromatogram of standards and CSEE for analysis of limonin and nomilin are shown in Fig. 6. Limonoids including limonin and nomilin are also excellent antioxidants. Some researchers reported that the antioxidant activities shown by the citrus fruit extracts may be due to the presence of flavonoids, carotenoids, ascorbic acid, and limonoids (37). The increase of antioxidant activity is due to the increase in number of phenolic hydroxyl groups in the molecule. A study about structure-activity relationships of limonin on antioxidant activity demonstrated that the furan ring and epoxy group have the highest activity against insects. The furan moiety in limonoids is critical to enhance GST enzyme activity in various organs using animal models (35,38). Some studies also suggested that the presence of furan and an intact A-ring of limonoids are associated with increasing chemopreventive activity in cancer cells (39,40). Specifically, nomilin, which has a seven-membered A ring with acetylation at the C-1 position as well as the furan ring, showed a greater positive effect on human cardiovascular disease (41). Most of the citrus limonoids contain a furan ring at the C-17 position. The existence of the furan ring has been thought to play an important role in biological activity. Recently, *in vivo* studies demonstrated no risk of toxicity in using limonin and nomilin (36). Numerous epidemiological studies also demonstrated that the risk of cardiovascular disease and citrus fruit consumption had an inverse relationship (42).

Citrus fruits have a small edible portion and large amounts of waste materials such as the peels and seeds. The by-products of the citrus fruit-processing as sources of functional compounds and their applications in food

Table 1. Analysis of limonin and nomilin contents of citron seed extracts (CSEs) prepared from different solvents using HPLC

Samples	Limonin (%)[1)]	Nomilin (%)
CSEW	0.3268±0.0004[b]	0.1386±0.0013[b]
CSEE	1.8823±0.0007[c]	2.0892±0.0021[c]
CSEH	0.0605±0.0029[a]	0.0260±0.0026[a]

[1)]Data was expressed as means of three replications±standard deviation.
[a-c]Values with different superscripts in a column are significantly different at $P<0.05$.
CSEW, CSE using water; CSEE, CSE using ethanol; CSEH, CSE using *n*-hexane.

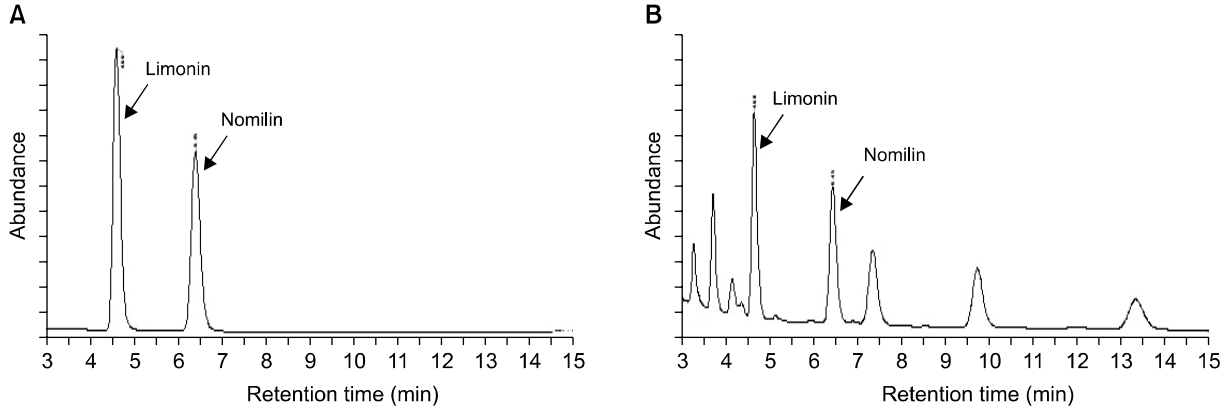

Fig. 6. HPLC chromatograms of standards (A) and citron seed extracts using ethanol (CSEE, B) for analysis of limonin and nomilin.

products are promising, considering that within the foods market, the innovative sector of the functional foods is growing fast and becoming more important. An additional advantage of using the citrus by-products is lessening the contributions to environmental waste issues.

Many artificial antioxidants, such as BHA (butylated hydroxyanisole), BHT (butylated hydroxytoluene), and TBHQ (tertiary butylhydroquinone), have been commonly used as food additives. However, these artificial antioxidants have been reported to possibly lead to the induction of cancers (43). Efforts are being made on the development of natural antioxidants from various plant resources for the purposes of health promotion. To conclude, CSEs, particularly CSEE, showed effective immunopotentiating activity of complement activation and radical scavenging activities, including those against the DPPH and ABTS radicals as rich polyphenol and limonoids. Therefore, we suggest CSEE be used as a potential antioxidant product, as well as other health-promotion and disease management products.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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