

Antioxidant Activities of Functional Beverage Concentrates Containing Herbal Medicine Extracts

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ABSTRACT: This study investigated the antioxidant activity of functional beverage concentrates containing herbal medicine extracts (FBCH) using various antioxidant assays, such as 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging activity, and reducing power assay. The total polyphenolic content of FBCH (81.45 mg/100 g) was higher than Ssanghwa tea (SHT, 37.56 mg/100 g). The antioxidant activities of FBCH showed 52.92% DPPH and 55.18% ABTS radical scavenging activities at 100 mg/mL, respectively. FBCH showed significantly higher antioxidant activities compared to the SHT (DPPH, 23.43%; ABTS, 22.21%; reducing power optical density; 0.23, $P < 0.05$). In addition, intracellular reactive oxygen species generation significantly decreased in a concentration-dependent manner following FBCH treatment. These results suggest that the addition of herbal medicine extract contributes to the improved functionality of beverage concentrates.

Keywords: antioxidant activity, herbal medicine extract, polyphenol, functional beverage, free-radical scavenging

INTRODUCTION

A stress response leads to the generation of free radicals and other reactive oxygen species (ROS), which results in lipid peroxidation, especially in cell membranes, and can alter membrane integrity, leading to tissue injury (1). Previous studies have suggested that exposure to chronic psychological stress is related to increased free radical levels and, long-term exposure to high levels of psychological stressors may cause diverse neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's diseases (2-4).

Numerous fruits and vegetables with antioxidative and radical scavenging properties have been investigated for preventing oxidative stress-related diseases (5). The advantages of using natural products to prevent oxidative stress are the following: I) low toxicity, II) unusual mixture of multiple antioxidants in the product, III) ability to react to most or all types of ROS, and IV) easy accessibility. Herbal tea is one of the most popular and widely

consumed nonalcoholic beverages among the various types of natural products available (6,7). The protective effect of these herbal teas against oxidative stress may be due to the presence of polyphenols (7-9). The neuroprotective effects of polyphenols, particularly flavonoids, against pathological conditions have been explored (10-12).

It has been reported that various medicinal herbs exert electron donating ability and hydroxyl radical scavenging activity (13,14). In the aspect of health promotion, the mixture of medicinal herbs might have more antioxidant effects than a single medicinal herb. In fact, Ssanghwa-tang is a mixture of *Paeonia lactiflora*, *Rehmanniae Radix*, *Astragalus propinquus*, *Cinnamomum cassia*, *Angelica gigas*, *Cnidii Rhizoma*, and *Glycyrrhiza uralensis*. Due to these various medicinal herbs, Ssanghwa-tang may help with anti-inflammatory and fatigue recovery functions. Moreover, it is one of the healthy drinks commonly consumed in Korea (15). Still the development of various functional beverages using medicinal herbal extracts is necessary.

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Therefore, in the present study, we developed functional beverage concentrates containing herbal medicine extracts (FBCH) and investigated the antioxidant activity of functional beverage concentrates containing FBCH using various antioxidant assays such as 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging, and reducing power assay.

MATERIALS AND METHODS

Sample preparation

A sample containing five herbal medicines [*G. uralensis* Fisch, *Lycium chinense* Miller, *A. gigas* Nakai, *Cnidium officinale* Makino, and *Scutellariae Radix* (the root of *Scutellaria baicalensis* Georgi)] was extracted with 10 volumes of 70% ethanol in a shaking incubator (SI-18, Jeio Tech Co., Ltd., Daejeon, Korea) at 200 rpm and 30°C for 7 h, and then filtered through filter paper (Whatman No. 1). Solid-phase extractions (>60%) were performed for 8 h, followed by concentration at 600~700 mmHg and 65°C, and then mixed using a stirrer 30 min to obtain homogeneous mixtures. The mixture was diluted with distilled water and adjusted to 1°Bx, and FBCH were prepared as shown in Table 1. A commercially available Ssanghwa tea (SHT, Korean traditional beverage) was purchased from a local hypermarket in Korea.

Reagents

DPPH, gallic acid, ABTS, Folin-Ciocalteu's phenol reagent, tannic acid, and butylated hydroxyanisole (BHA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sodium phosphate monobasic monohydrate, sodium phosphate dibasic heptahydrate, trichloroacetic acid, potassium sulfate, and sodium carbonate were purchased from Samchun Chemical Co. (Pyeongtaek, Korea). Potassium ferricyanide and ferric chloride were purchased from Daejung Chemicals (Siheung, Korea).

pH value, Brix (%), and color measurement

The pH values of the beverages were measured at 25°C using a pH meter (pH 510, EUTECH Instruments, Singapore), and the Brix was measured using a hand-held refractometer (PAL- α , Atago Co., Ltd., Tokyo, Japan).

The color of each sample was measured in triplicate using a color meter (CR-200, Minolta, Osaka, Japan) that recorded the L* (lightness), a* (redness), and b* (yellowness) values. Prior to color measurement, the instrument was calibrated using white and black standard tiles.

DPPH radical scavenging activity

DPPH radical scavenging activity was measured according to the method of Blois (16) with slight modifications. Samples (0.1 mL) were mixed with 0.9 mL of 0.4 mM DPPH in ethanol and incubated at room temperature for 30 min. Absorbance measurements were recorded at 517 nm. The DPPH radical scavenging activity was calculated as follows: $1 - (\text{absorbance of sample} / \text{absorbance of control}) \times 100$.

ABTS radical scavenging activity

ABTS radical was generated by reacting 7 mM ABTS with 2.6 mM potassium persulfate for 24 h (final optical density 0.7). Samples (10 μ L) were mixed with the ABTS solution (90 μ L) and the absorbance at 734 nm was determined after 30 min of incubation at room temperature (17). The ABTS radical scavenging activity was calculated as follows: $1 - (\text{absorbance of sample} / \text{absorbance of control}) \times 100$.

Ferric reducing power

The reducing power was determined using the method described by Oyaizu (18). A 2.5 mL aliquot of sample was mixed with 0.2 M sodium phosphate buffer (2.5 mL, pH 6.6) and 1.0% potassium ferricyanide (2.5 mL). The mixture was incubated at 50°C for 20 min. Aliquots of trichloroacetic acid (2.5 mL) were added to the mixture, which was then centrifuged at 3,000 rpm for 10 min. The upper layer of the solution (5 mL) was mixed with distilled water (5 mL) and 0.1% ferric chloride solution (1 mL). The absorbance was measured at 700 nm.

Total polyphenol content

Total polyphenol content of the extracts was measured using the Folin-Denis method (19). Folin-Ciocalteu reagent (1 mL) was added to 1 mL of the sample, and the reaction mixture was incubated for 5 min. After the incubation period, 1 mL of 10% Na₂CO₃ was added, and the reaction mixture was incubated at room temperature for 1 h. The absorbance at 700 nm of the reaction mixture was subsequently measured. Tannic acid (Sigma Chemical Co.) was used to prepare the standard curve.

Cell culture

The HaCaT human keratinocyte cell line was purchased from CLS Cell Lines Service GmbH (Eppelheim, Germany).

Table 1. Formulation of functional beverage concentrates containing herbal medicine extracts

Ingredients	%
Herbal medicine extracts adjusted as 1°Bx	48.6
Fructooligosaccharides	30.0
Soy protein isolate	7.0
Whey protein	3.0
Cereal enzyme powder	5.0
Dried green tea extracts	5.0
Tricalcium phosphate	1.4

ny), and cultured in Dulbecco's modified Eagle's medium (DMEM)-supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 µg/mL streptomycin sulfate. The culture medium was DMEM-supplemented with 10% bovine calf serum, 50 units/mL penicillin, and 50 µg/mL streptomycin sulfate. Cells were incubated in a humidified atmosphere of 5% CO₂ and 95% air at 37°C.

Intracellular ROS measurement

Cells pre-loaded with 10 µM 2',7'-dichlorodihydrofluorescein diacetate for 30 min were exposed to FBCH, and then lysed in 0.1% Triton X-100. The fluorescence intensity of each lysate was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm using a fluorometer (INFINITE M200, Tecan Schweiz AG, Männedorf, Switzerland).

Cell viability assay

The cytotoxicity of FBCH was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction to the corresponding blue formazan by viable cells. Cells were grown to ~80% confluence and maintained in 5% serum medium for 12 h in the presence of FBCH. The level of blue formazan was measured spectrophotometrically and used as an indirect index of cell density. Briefly, cells were exposed to MTT (1 mg/mL) for 3 h at 37°C. The medium was removed, and the cells were solubilized with dimethyl sulfoxide. After complete solubilization, the presence of blue formazan was evaluated spectrophotometrically by measuring the absorbance at 540 nm (reference, 620 nm) using an enzyme-linked immunosorbent assay plate reader. Cell viability was expressed as a percentage of the control.

Statistical analysis

Results are expressed as the mean±standard deviation. Statistical analyses were conducted using SPSS for Windows (Release 18.0K, SPSS Inc., Chicago, IL, USA). Results were analyzed by one-way analysis of variance (ANOVA/Duncan) and Student's *t*-test. Differences were considered statistically significant at *P*<0.05.

RESULTS AND DISCUSSION

Sugar content, pH, and color measurement

As shown in Table 2, the sugar content of FBCH was significantly lower than that of SHT (5.53°Bx for FBCH and 6.80°Bx for SHT). The values of pH were not significantly different between FBCH and SHT. As for color, the L*, a*, and b* values of FBCH were significantly higher than those of SHT.

Total polyphenol content

Phenolic compounds are potential antioxidants and free radical scavengers; hence, there should be a close correlation between the content of phenolic compounds and antioxidant activity (20). In this study, we determined the total polyphenol content of FBCH and SHT (Table 3). FBCH had higher total polyphenol content (81.45 mg/100 g) than SHT (37.56 mg/100 g), suggesting that FBCH would have greater antioxidant activity. This hypothesis was consistent with the results; the trend in antioxidant activity of the samples was similar to that of their total polyphenol content, indicating a correlation between the total polyphenol content and antioxidant activity. As for the Ssanghwa-tang made by the original method of the oriental medical book, the total polyphenol content was reported as 71.33 µg/mg (15), which was also lower than that of FBCH.

Table 2. pH value, sugar content, and color value of functional beverage concentrates containing herbal medicine extracts (FBCH) and Ssangwha tea (SHT)

	FBCH	SHT
pH	6.72±0.17	6.84±0.22
Sugar content (°Bx)	5.53±0.06	6.80±0.10*
L*	25.65±0.17*	21.11±0.58
a*	0.61±0.03*	0.25±0.03
b*	4.34±0.14*	0.68±0.09

Values are presented as the mean±SD.

*Significantly different at *P*<0.05 by Student's *t*-test.

L*, lightness; a*, redness; b*, yellowness.

Table 3. Antioxidant activities of functional beverage concentrates containing herbal medicine extracts (FBCH) and Ssangwha tea (SHT)

Sample	DPPH (%)	ABTS (%)	Reducing power (700 nm)	Polyphenol (mg/100 g)
FBCH	52.92±1.47 ^a	55.18±5.76 ^b	0.77±0.022 ^a	81.45±5.76*
SHT	23.43±4.52 ^c	22.21±3.67 ^d	0.23±0.003 ^d	37.56±0.67
BHA 50 ppm	37.69±3.74 ^b	42.75±1.11 ^c	0.36±0.004 ^c	—
BHA 100 ppm	57.97±0.75 ^a	79.78±2.10 ^a	0.65±0.025 ^b	—

BHA, butylated hydroxyanisole; DPPH, 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid radical scavenging activity).

Values are presented as the mean±SD.

Values in the same column with different letters (a-d) are significantly different based on Duncan's multiple range test (*P*<0.05).

*Significantly different between FBCH and SHT at *P*<0.05 (Student's *t*-test for independent groups).

Effect of herbal medicine extracts in functional beverages on antioxidant activity

A substantial body of evidence has indicated a key role for free radicals in aging and in degenerative diseases of aging, including cancer, cardiovascular disease, cataracts, and immune system and brain dysfunction (21,22). Therefore, we investigated the effect of the antioxidant activity of a FBCH using DPPH and ABTS radical scavenging activity assays.

The DPPH radical scavenging activity is widely used to evaluate the free radical scavenging properties of constituents. The method is based on the spectrophotometric measurement of the DPPH concentration change resulting from the reaction with an antioxidant (23). In this study, while SHT showed the lowest DPPH radical scavenging activity, that of FBCH was similar to BHA (100 ppm), which was used as the positive control. The DPPH radical scavenging activities of FBCH and BHA (100 ppm) were 52.92% and 57.97%, respectively (Table 3). The ABTS radical scavenging capacities of the samples were determined (24). As in the case of DPPH radical scavenging, BHA (100 ppm) exhibited the highest ABTS radical scavenging properties (79.78%), followed by FBCH (55.18%), BHA (50 ppm) (42.75%), and SHT (22.21%) exhibited lower ABTS radical scavenging activities. As for the Ssanghwa-tang made by the original method of the old oriental medical book, the DPPH scavenging effect was reported as 20.54% (15), which was lower than that of SHT.

The reducing capacities of FBCH, SHT, BHA (50 ppm), and BHA (100 ppm) were 0.77, 0.23, 0.36, and 0.65, respectively. The trend in reducing power of the samples was similar to that observed for DPPH and ABTS. Collectively, these results indicate that FBCH enhanced the antioxidant activity of the functional beverage.

Both FBCH and Ssangwha-tang had the same herbs: *G. uralensis* Fisch, *A. gigas* Nakai, and *C. officinale* Makino. However, *L. chinense* Miller and *Scutellariae Radix* were included in FBCH only. The *L. chinense* extract has antioxidant function to scavenge the DPPH radicals, intracellular ROS, hydroxyl radicals, and superoxide (25). *Scutellariae Radix* was shown to reduce the ABTS and DPPH radical formation in a dose dependent manner (26). Therefore, the addition of these two medicinal herbs may have synergic effects and could help to increase antioxidant effects similar or higher than the 50 ppm and 100 ppm of BHA.

Effect of herbal medicine extracts in functional beverages on H₂O₂-induced generation of intracellular ROS in HaCaT cells

As stated above, FBCH enhanced the antioxidant activity of the functional beverage. Next, we investigated the cellular activities of FBCH. To this end, we monitored intracellular ROS generation to investigate the effects of FBCH in HaCaT cells. FBCH was added to the cells, and the antioxidant activity of FBCH was determined using the oxidant-sensitive fluorescent dye 2',7'-dichlorofluorescein diacetate in HaCaT cells. Intracellular ROS generation induced by H₂O₂ decreased significantly in a concentration-dependent manner following FBCH treatment (Fig. 1), demonstrating its antioxidant activity.

During cellular redox, the human body constantly generates free radicals (superoxide and hydroxyl radicals) and other ROS (hydrogen peroxide, nitric oxide, peroxynitrite, and hypochlorous acid) as a result of aerobic metabolism (25). Recent studies have suggested that long-term exposure to physiological or psychological stress is associated with the production of oxidative species, which cause accumulation of oxidative damage to bio-

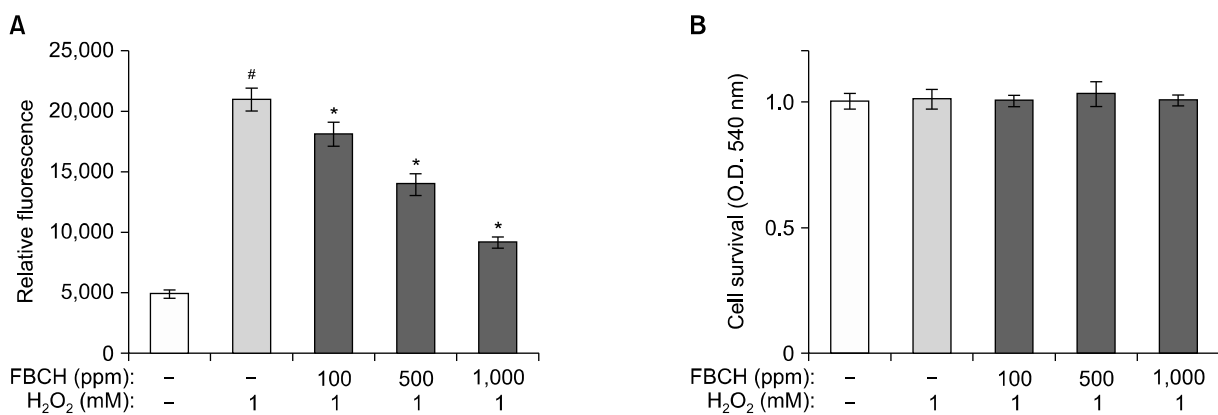


Fig. 1. Effect of functional beverage concentrates containing herbal medicine extracts (FBCH) on intracellular reactive oxygen species (ROS) production in HaCaT cells. Chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (5 μ M) was added, and the cells were incubated for 20 min in the dark. The cells were then washed and treated with 1 mM H₂O₂ for 30 min. The cells were treated with FBCH for 1 h. Subsequently, ROS-induced dichlorofluorescein formation was measured using a spectrophotometer (A). Cell viability was determined (B). Data are means \pm SD (n=3). [#]Significantly different from vehicle-treated group and H₂O₂-treated group by Student's *t*-test at *P*<0.01. ^{*}Significantly different from H₂O₂-treated group and FBCH treated groups by Duncan's multiple-range test at *P*<0.01.

molecules (lipids, proteins, and DNA) in the brain, eventually leading to diverse neurodegenerative diseases (26). Many neurodegenerative diseases, such as Alzheimer's and Parkinson's diseases, are associated with excessive production of ROS and free radicals (27). Dietary antioxidants have been shown to protect neurons against a variety of experimental neurodegenerative conditions (28). Several natural beverages, in particular herbal teas, have potential activity against a variety of oxidative stress-induced neurodegenerative diseases (29). Therefore, although further study of efficacy and safety will be needed, our data, taken together with previous reports, suggest that functional beverages containing FBCH might protect against oxidative stress caused by psychological stress and adaptation.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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