Original Article

Effects of blending wheatgrass juice on enhancing phenolic compounds and antioxidant activities of traditional kombucha beverage

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ABSTRACT

Traditional kombucha is a fermented black tea extract and sugar. Sweetened black tea (10% w/v) and wheatgrass juice (WGJ) were mixed in various ratios and used as fermentation substrate for enhancing phenolic compounds and antioxidant activity. Starter, comprising of yeast (*Dekkera bruxellensis*) and acetic acid bacteria (*Gluconacetobacter rhaeticus* and *Gluconobacter roseus*), was inoculated at 20% (v/v), and fermented statically at $29 \pm 1°C$ for 12 days. The results showed that the total phenolic and flavonoid contents and antioxidant activity of the modified kombucha were higher than those of traditional preparations. All WGJ-blended kombucha preparations were characterized as having higher concentrations of various phenolic compounds such as gallic acid, catechin, caffeic acid, ferulic acid, rutin, and chlorogenic acid as compared to traditional ones. Addition of WGJ resulted in the 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging ability of kombucha being > 90%, while the oxygen radical absorbance capacity increased from 5.0 μmol trolox equivalents/mL to 12.8 μmol trolox equivalents/mL as the ratio of WGJ increased from 0% to 67% (v/v). The highest antioxidant activity was obtained using a 1:1 (v/v) black tea decoction to WGJ ratio and 3 days of fermentation, producing various types of phenolic acids. These results suggest that intake of fermented black tea enhanced with wheatgrass juice is advantageous over traditional kombucha formulas in terms of providing various complementary phenolics and might have more potential to reduce oxidative stress.

1. Introduction

Kombucha is a well-known fermented health beverage popular in many countries [1]. Kombucha is traditionally made by fermenting sugared black tea using a symbiotic culture of acetic acid bacteria (e.g., *Acetobacter xylinum*, *Acetobacter xylinoides*, or *Bacterium gluconicum*) and yeast (e.g., *Schizosaccharomyces pombe*, *Saccharomyces ludwigii*, *Zygosaccharomyces rouxii*, *Candida* spp., or *Pichia* spp.) statically for 2 weeks. The

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fermentation product is comprised of two components: a floating cellulosic pellicle layer and a sour-tasting and slightly sparkling liquid broth [2]. It contains many compounds with antioxidant activity, such as phenolics, water-soluble vitamins, organic acids, and minerals [2,3]. Williamson et al [4] demonstrated that the antioxidant activity of kombucha, which may be attributed to the tea polyphenols, can increase the *in vivo* antioxidant ability. Oral administration of kombucha to rats exposed to pro-oxidation species also indicated the potent antioxidant properties of the fermented drink such as decrease of the degree of lipid oxidation and DNA fragmentation [5].

The antioxidant activity of kombucha corresponds highly to the fermentation substrate (tea leaves); moreover, some components of tea promote cellulose formation from acetic acid bacteria [6]. Greenwalt et al [7] showed that using green tea as the substrate could minimize the fermentation time; however, to retain its characteristic flavor, black tea is still used as the fermentation substrate in the traditional kombucha [6].

Substances with antioxidant properties offer many benefits to the human body [8]. The antioxidants found in kombucha fermentation substrates originated in tea leaves and mainly include polyphenols, especially catechins, which belong to the flavones group [5,9]. Black tea leaves in substrates used for preparing traditional kombucha account for only 1% (w/v) of the total, and in fact do not play a major role in fermentation compared with sucrose (ca. 10%, w/v). Instead, black tea contributes the sensory attributes such as flavor and taste to kombucha, and with a lesser extent on acting as a source of antioxidant substances. The beneficial effects, particularly in antioxidant activity and phenolic substance content, would be further enhanced if we supplement the traditional kombucha with other substrates such as herbs or vegetables.

Wheatgrass juice (WGJ) is an extract squeezed from the mature sprouts of wheat seeds (*Triticum aestivum*). The therapeutic qualities of WGJ have been attributed to its rich nutrient contents, including chlorophyll, vitamins (A, C, and E), bioflavonoids, minerals (iron, calcium, and magnesium), and phenolics (ferulic acid and vanillic acid) [8]. Kulkarni et al [10] reported that WGJ has high antioxidant activity partly because it contains such antioxidants as phenolic compounds and several flavonoids. Phenolics and flavonoids have been shown to remove superoxide radicals (O_2^- or HO_2^-) *in vivo* and decrease the cell damage caused by oxidative stress [9]. Wheatgrass extracts also possess superoxide scavenging and ferric reducing abilities [8,10]. Their ability to inhibit oxidative DNA damage was also demonstrated [11].

Several days of acetic acid bacteria and yeast co-culture have been shown to yield a high antioxidant activity. However, the fermentation process is long and produces a large amount of acetic acid from acetic acid bacteria, which affects the flavor of the drink. Therefore, long fermentation processes are not suitable for health beverage production. In addition, few studies have used materials besides tea leaves as the fermentation substrate for kombucha. The aim of this study is to assess the changes in kombucha's antioxidant activity and phenolic compounds during fermentation as affected by different ratios of sugared black tea decoction and WGJ.

2. Methods

2.1. Starter culture

Starter culture, or kombucha culture, was collected from a local cultivator who grows kombucha periodically in Taichung, Taiwan and maintained in sugared black tea. The culture includes both the upper pellicle layer and the lower liquid. The major bacterial components were identified as *Gluconacetobacter rhaeticus* and *Gluconobacter roseus* and the yeast component as *Dekkera bruxellensis* at Mission Biotech (Taipei, Taiwan). The starter culture was periodically maintained according to method of Chen and Liu [2], except that black tea leaves were used.

2.2. Sweetened black tea

Ten percent (w/v) sucrose was added to deionized water and heated at 100°C for 5 minutes. Next, 1% (w/v) black tea leaves (Ten-Ren Co., Taipei, Taiwan) were added, allowed to steep for 15 minutes, and then filtered through a sterile sieve.

2.3. WGJ

The wheatgrass (*Triticum aestivum* L.) was purchased from the Santa Cruz supermarket, Taichung City, Taiwan and washed with tap water and steam-blanched (100°C, 30 seconds). WGJ was extracted with a juicer (Green Power, KP-E1201; Kempo Co. Ltd., Seoul, Korea).

2.4. WGJ-blended kombucha fermentation

Sweetened black tea and WGJ were mixed in various ratios to create six groups of WGJ-blended black tea broth, with five glass jars (capacity 500 mL) in each group (Table 1). To each group, the broth was dispensed equally into five glass jars (each containing 120 mL broth) that had been previously sterilized at 121°C for 20 minutes. Each jar was then inoculated with 30 mL of previously fermented kombucha liquid starter that had been cultured in the sweetened black tea for 10 days. The jars were carefully covered with clean cheesecloth and fastened with rubber bands. The fermentation was

Table 1 – Combinations of black tea decoction and wheatgrass juice as fermentation substrates for preparing WGJ-blended kombucha.

Component	Group					
	TK	WK1	WK2	WK3	WK4	WK5
Black tea decoction (mL)[a]	120	80	60	40	0	0
Wheatgrass juice (mL)	0	40	60	80	120	120(S) [c]
Starter (mL)[b]	30	30	30	30	30	30
Total volume (mL)	150	150	150	150	150	150

TK = traditional kombucha; WK1–5 = kombucha supplemented at various ratios with wheatgrass juice.
[a] Black tea decoction contains 10% (w/v) sucrose.
[b] Starter: Freshly fermented kombucha (lower liquid portion, 30 mL) was used as inoculum or starter.
[c] Wheatgrass juice contains 10% (w/v) sucrose.

carried out in a dark incubator at 29 ± 1°C for 12 days. Sampling was performed by removing one jar from the incubator at 3-day intervals. Changes in functional components of the samples were analyzed. The jar and its content were discarded after sampling to avoid disturbing the static fermentation ascribed to the floating pellicle layer, i.e., each jar was sampled once only.

2.5. Determination of pH

The pH of the samples was measured in triplicate with an electronic pH meter (pH 720; inoLab, Weilheim, Germany) in accordance with AOAC protocols (2005).

2.6. Antioxidant determination

2.6.1. Total phenolic content
The total phenolic content (TPC) was determined using the Folin—Ciocalteu colorimetric method by Gorinstein et al [12], with modifications. The sample (0.1 mL) was mixed with 2 ml of 2% sodium carbonate. After 2 minutes, 0.1 mL of 50% Folin—Ciocalteu reagent was added and the solution was allowed to stand for 30 minutes at room temperature. The samples were measured at 750 nm versus a blank using a spectrophotometer (U-2000; Hitachi Ltd., Tokyo, Japan). The results were expressed as mg of gallic acid equivalent (GAE)/ mL of sample.

2.6.2. Total flavonoid content
The total flavonoid content (TFC) was determined using a crystalline aluminum chloride assay according to the method described by Maksimović et al [13]. The sample (1.5 mL) was added to an equal volume of 2% aluminum trichloride and allowed to stand for 10 minutes to allow the formation of a flavonoid—aluminum complex, after which the absorbance at 430 nm was recorded. The total flavonoid content was expressed as quercetin equivalents (QE) from the calibration curve.

2.6.3. Total anthocyanin content
The total anthocyanin content TAC was determined according to the modified method of Ruenroengklin et al [14]. The sample (0.1 mL) was mixed with acidified methanol (1% HCl/ methanol), stored in the dark at 4°C for 30 minutes, and then centrifuged at 9100 g for 15 minutes. The anthocyanin concentration in the supernatant was measured spectrophotometrically at 530 nm and 657 nm, and the absorbance values were indicated as A_{530} and A_{657}, respectively. The concentration was calculated using the following equation:

$$\text{Anthocyanin concentration(mM)}$$
$$= [A_{530} - 0.33 \times A_{657}/31.6] \times [\text{volume(mL)}]$$

The results are expressed as the average of triplicate measurements.

2.7. Phenolic composition

The phenolic composition was analyzed by high-performance liquid chromatography (HPLC). Filtered samples (2 mL) were passed through a membrane filter (0.45 μm) into HPLC vials. The filtrate obtained was analyzed for gallic acid, catechin, caffeic acid, ferulic acid, rutin, and chlorogenic acid by HPLC. A 10 μL of filtrate sample was separated by a reverse phase column (Mightysil RP-18 GP 250 mm × 4.6 mm, 5 μm; Kanto Corporation, Portland, OR, USA) according to the method of Nuutila et al [15]. The HPLC system (Hitachi) was equipped with an autosampler (L-2200; Hitachi) and a photodiode-array detector (L-2455; Hitachi). The injection volume was 10 μL, and the pump (L-2130; Hitachi) applied a gradient of solvent A (0.1% phosphoric acid containing 0.1% acetonitrile and 5% N, N-dimethylformamide) and solvent B (100% acetonitrile) at a flow rate of 0.8 mL/min. The gradient elution started with 100% A (0% of solvent B), linearly up to 100% B at the end of a 50-minute cycle. The analyses were monitored at a wavelength of 280 nm, and the calibration standards used for quantification of the samples were gallic acid, catechin, caffeic acid, ferulic acid, rutin, myricetin, and chlorogenic acid.

2.8. Antioxidant activity determination

2.8.1. DPPH scavenging ability
The DPPH assay was conducted according to the method of Yamaguchi et al [16]. with some modifications. First, 200 μL of sample or MeOH (control) was mixed with 100mM Tris-HCl buffer (pH 7.4, 800 μL) and then added to 1 mL of 500μM DPPH (1,1-diphenyl-2-picrylhydrazyl) in ethanol (final concentration of 250μM). The mixtures were shaken vigorously and allowed to stand in the dark at room temperature for 20 minutes, after which the absorbance was read at 517 nm using a spectrophotometer. The scavenging capacity of the sample was calculated using the following equation:

$$\text{Scavenging activity \%} = \left[1 - (A_{\text{sample}} - A_{\text{blank}})/(A_{\text{control}} - A_{\text{blank}})\right] \times 100$$

2.8.2. Trolox equivalent antioxidant capacity
The method used was as described by Miller et al [17] based on the capacity of a sample to inhibit the 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonate) radical (ABTS$^+$) relative to a reference antioxidant standard (Trolox). The ABTS radical cation was generated by the interaction of ABTS (100μM), hydrogen peroxide (50μM), and peroxidase (4.4 units/mL). Fresh ABTS$^+$ solution was prepared for each assay. To measure the antioxidant capacity, 0.25 mL of the sample was mixed with 2.25 mL of the radical solution. The absorbance was monitored at 734 nm for 10 minutes. The decrease in absorption at 734 nm after the addition of reactant was used to calculate the Trolox equivalent antioxidant capacity (TEAC) value.

2.8.3. Oxygen radical absorbance capacity
The oxygen radical absorbance capacity (ORAC) assay was performed according to the method of Ou et al [18] with some modifications. Analyses were conducted in 75mM phosphate buffer (pH 7.4) at 37°C. A freshly prepared fluorescein (150 μL of a 40nM solution) was mixed with 25 μL of the sample. The mixture was preincubated for 15 minutes at 37°C before rapidly adding 25 μL of 250mM 2,2′-azobis(2-amidino-propane) dihydrochloride using a multichannel pipette. After

incubation, fluorescence measurements (excitation, 485 nm; emission, 520 nm) were taken every 90 seconds to determine the background signal. The test was resumed, and fluoresence measurements were taken for up to 60 cycles using a microplate reader (FLUOstar Omega, BMG Labtech, Offenburg, Germany). The ORAC values were calculated using Trolox and sample concentrations and the net area under the fluorescein decay curve (AUC). The AUC was calculated as:

$$AUC = 1 + f1/f0 + \ldots + fi/f0 + \ldots + f149/f0 + (f150/f0),$$

where, $f0$ is the initial fluorescence reading and fi is the fluorescence reading at time i. The data were analyzed in Microsoft Excel to calculate the AUC. The net AUC was obtained by subtracting the AUC of the blank from that of the sample. The antioxidant activity of the test samples was expressed as μmol Trolox equivalents (TE)/mL.

2.9. Statistical analysis

Data are presented as the mean ± standard deviation, and all analyses were performed in triplicate. The results were evaluated using one-way analysis of variance and Duncan's multiple range test. The level of significance was set at $p < 0.05$. SPSS for Windows, version 10.0 (SPSS Inc., Chicago, IL, USA) was used for the analyses.

3. Results and discussion

3.1. Acidity

The pH changes for the six groups of kombucha with or without incorporating WGJ during the fermentation are shown in Fig. 1. During the fermentation, the pH of each group
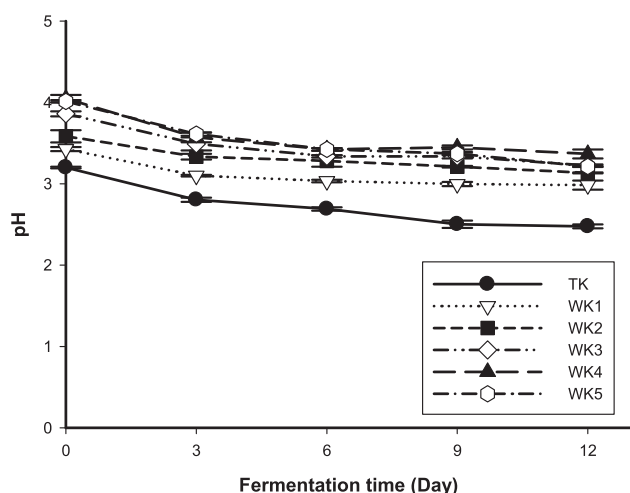
Fig. 1 — Effects of volume ratios of wheatgrass juice to black tea decoction on changes in pH of WGJ-blended kombucha during fermentation. Data are expressed as mean ± standard deviation of three samples. TK = traditional kombucha; WK1—5 = kombucha blended at various ratios with wheatgrass juice, as shown in Table 1.

decreased with increasing days of culturing, from pH 4.0 to pH 2.9. The initial pH of the medium was higher for higher ratios of WGJ to sweetened black tea. The difference in the pH is due to the culture medium substrate, which is affected by the chemical composition of black tea and WGJ [1,2]. The different substrate ratios exhibit different changes in pH but with a similar overall tendency (final pH, shape of the pH curve) during the fermentation. The result showed that changing the fermentation substrate did not affect the growth of the starter culture (yeast and acetic acid bacteria), ultimately yielding similar pH values.

3.2. TPC, TFC, and TAC

Vegetables, fruits, and tea contain high TPC, TFC, and TAC levels [19]. The TPC and TFC concentrations for all six groups increase with fermentation time (Fig. 2A and B). The increase in phenolic compounds may come from polyphenols. The microorganisms in kombucha (mainly acetic acid bacteria and yeasts) release enzymes during fermentation, which can degrade polyphenols into small molecules. Therefore, the antioxidant activity of the sample may be derived from phenolics, including flavonoids. Falcioni et al [11] indicated that wheat sprout extracts inhibit DNA oxidative damage and are effective in suppressing the superoxide radical that can lead to various diseases. Because of their ideal chemical structure for free radical scavenging activities, polyphenols have been shown to be more effective *in vitro* antioxidants than vitamins E and C [20]. The data in Fig. 2C show that there is not much difference in the TAC among the six groups, with values of approximately 0.53 ± 0.02mM to 0.65 ± 0.03mM. Anthocyanins are phenolic compounds widely present in vegetables and fruits with radical-clearing ability and antioxidant activity. The pH value is the most important factor in determining the stability of anthocyanins; under acidic conditions, anthocyanin retains its chemical structure and becomes more stable [21]. Anthocyanins are water-soluble and the fermentation environment provides high-acid conditions (Fig. 1); moreover, the anthocyanins are less strongly affected by metal ions, temperature, and structural transformations. Free anthocyanins can combine with catechin and phenolic compounds to form anthocyanin polymers, leading to a decrease in anthocyanin monomers along with a subsequent increase in anthocyanin polymers during fermentation [22]. Anthocyanins exhibit antioxidant ability due to their polyphenol-like functional structure. Extensive studies indicate that anthocyanins have strong free radical scavenging and antioxidant activities, reduce DNA mutation from oxidative stress, and reduce lipid peroxidation in colorectal, endothelial, hepatic, and breast cells [23].

3.3. Primary identification of phenolic constituents

The chromatograms of the major antioxidant component of polyphenolics, namely, phenolics, present in kombucha samples after 3 days fermentation are shown in Fig. 3. Fig. 3A presents a chromatogram of phenolic standards. Fig. 3B and 3C—G represent the chromatograms of traditional kombucha (TK) and kombucha blended at various ratios with wheatgrass juice, as shown in Table 1 (WK1—5), respectively. Some active
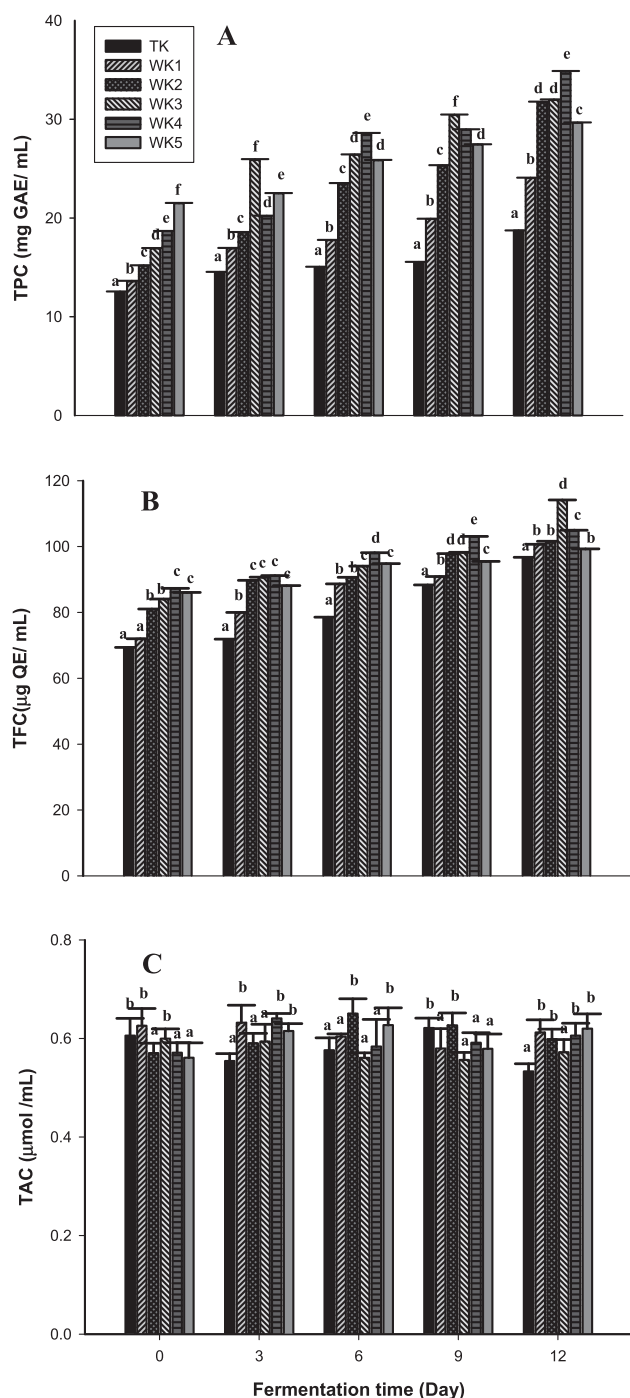
Fig. 2 − Effects of volume ratios of wheatgrass juice to black tea decoction on antioxidant components of the WGJ-blended kombucha. (A) total content of phenolic (TPC); (B) total content of flavonoids (TFC); (C) total content of anthocyanidins (TAC). Data are expressed as mean ± standard deviation of three samples. Bars topped by different letters are significantly different at $p < 0.05$. TK = traditional kombucha; WK1−5 = kombucha blended at various ratios with wheatgrass juice, as shown in Table 1.

phenolic acids, such as gallic acid, catechin, caffeic acid, ferulic acid, rutin, and chlorogenic acid, can be resolved in this system (Table 2). As the results show, caffeic acid is the most prevalent phenolic acid of the TK polyphenols, followed by gallic acid and catechin. As the proportion of WGJ increased, the caffeic acid, gallic acid, and catechin contents decreased, whereas the contents of ferulic acid, rutin, and chlorogenic acid increased. Kombucha made from black tea and WGJ substrates contains many types of phenolic acids. Tea and vegetables contain phenolics, which have been implicated in improving the health of test animals and humans [9]. Tea contains catechins, caffeine, and gallic acid, among other compounds [9]. Wheatgrass contains vitamins C and E, β-carotene, ferulic acid, and vanillic acid [10].

Phenolic acids can be derived from two nonphenolic molecules: benzoic acid and cinnamic acids. Gallic acid, vanillic acid, syringic acid, gentisic acid, and *p*-hydroxybenzoic acid are hydroxyl derivatives of benzoic acid, whereas caffeic acid, ferulic acid, sinapic acid, and *p*-coumaric acid are hydroxyl derivatives of cinnamic acid. Regarding the antioxidant structure, the −CH=CH−COOH in hydroxycinnamic acid has better antioxidant activity than the −COOH in hydroxybenzoic acid. It is likely that the −CH=CH section gains structural resonance, stabilizing the free radicals [24]. Gallic acid has higher antioxidant activity than hydroxybenzoic acid, and the antioxidant activities of hydroxycinnamic acids are ranked in descending order as follows: ferulic acid > *p*-coumaric acid > sinapic acid > *o*-coumaric acid > *m*-coumaric acid > caffeic acid > chlorogenic acid [25]. In addition, the TEAC and ORAC assays reveal that gallic acid, catechin, caffeic acid, ferulic acid, rutin, and chlorogenic acid also exhibit good antioxidant activity [18]. Some phenolic acids, such as caffeic acid, chlorogenic, ferulic, gallic acid, and ellagic acid, have been found to be pharmacologically active as antioxidant, antimutagenic, and anticarcinogenic agents [26]. The WGJ and black tea substrates provide the kombucha cultures with high contents of various phenolics and combine the high antioxidant activities of two phenolic acids: gallic acid and ferulic acid.

3.4. Antioxidant activity

3.4.1. DPPH radical scavenging ability
The use of DPPH assay is a reliable method for determining the antioxidant ability of biological substrates. The DPPH radical scavenging activity is a general assessment of the inhibition percentage of preformed free radicals by antioxidants. As shown in Fig. 4, the six beverages have good DPPH scavenging activity; the DPPH clearance percentage is as high as 85% and increases with increasing incubation time. By the 3rd day of fermentation, the beverage with the optimal proportion of WGJ exhibits 90% DPPH scavenging; WK1, WK2, and WK3 had the highest DPPH clearance until the end of the fermentation. Kombucha can be made from green tea, oolong tea, or black tea, all of which have good antioxidant capacities. Wheatgrass extracts also possess superoxide scavenging and ferric reducing power [8]. Their ability to inhibit oxidative DNA damage has also been demonstrated [11]. Brand-Williams et al [27] reported that phenolics have DPPH scavenging ability due to their phenolic content, which can provide hydrogen.
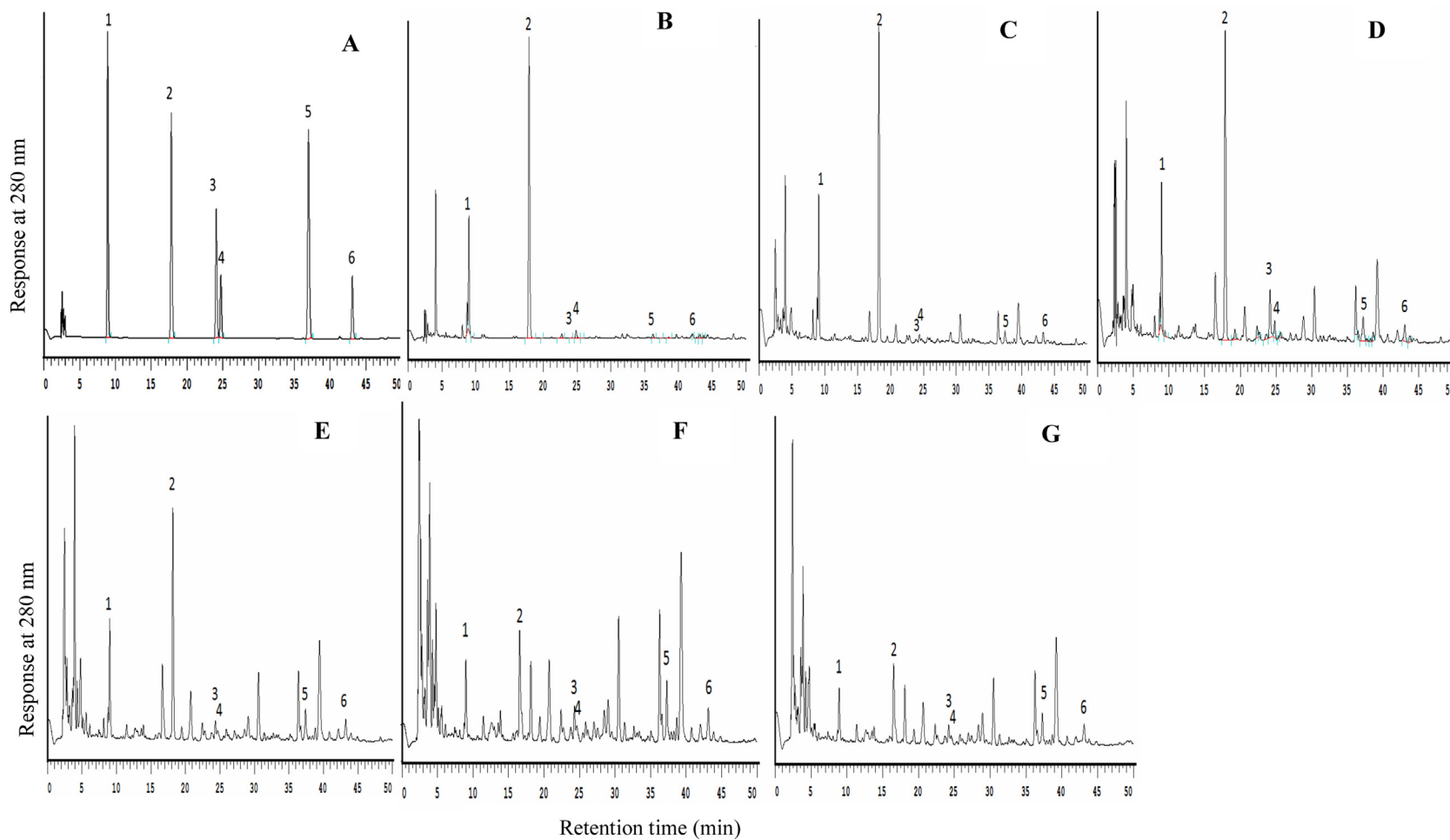
Fig. 3 — High-performance liquid chromatograms showing the standards of phenolic acids (A), TK (B), and WK1-5 (C–G). Both TK and WK1-5 were taken from samples which had fermented for 3 days. Peak identification = 1, Gallic acid; 2, Caffeic acid; 3, Chlorogenic acid; 4, Catechin; 5, Ferulic acid; 6, Rutin. TK = traditional kombucha; WK1–5 = kombucha blended at various ratios with wheatgrass juice, as shown in Table 1.

Day	Samples	Gallic acid	Caffeic acid	Catechin	Chlorogenic acid	Ferulic acid	Rutin
		mg/100 mL	mg/100 mL	mg/100 mL	mg/100 mL	μg/mL	μg/mL
0	TK	33.46 ± 0.07[a]	88.92 ± 0.03[a]	0.61 ± 0.04[b]	10.83 ± 0.03[e]	17.45 ± 0.07[e]	20.55 ± 0.11[e]
	WK1	29.41 ± 0.07[b]	82.14 ± 0.11[b]	0.63 ± 0.03[a]	13.45 ± 0.06[d]	26.01 ± 0.05[d]	38.44 ± 0.04[d]
	WK2	27.99 ± 0.06[c]	66.74 ± 0.08[c]	0.57 ± 0.04[d]	14.32 ± 0.13[c]	35.48 ± 0.05[c]	68.13 ± 0.06[c]
	WK3	23.85 ± 0.06[d]	33.12 ± 0.07[d]	0.60 ± 0.04[c]	16.43 ± 0.05[a]	45.33 ± 0.13[b]	88.00 ± 0.22[b]
	WK4	15.56 ± 0.13[e]	20.12 ± 0.05[e]	0.57 ± 0.06[d]	15.58 ± 0.12[b]	48.35 ± 0.16[a]	88.89 ± 0.11[b]
	WK5	16.68 ± 0.11[f]	19.96 ± 0.15[e]	0.56 ± 0.09[e]	15.67 ± 0.09[b]	48.01 ± 0.08[a]	91.05 ± 0.07[a]
3	TK	32.44 ± 0.05[a]	81.24 ± 0.06[a]	0.55 ± 0.03[e]	12.32 ± 0.11[d]	15.83 ± 0.07[f]	26.44 ± 0.06[f]
	WK1	27.11 ± 0.11[b]	69.45 ± 0.12[b]	0.63 ± 0.04[b]	12.98 ± 0.11[d]	23.98 ± 0.11[e]	36.36 ± 0.13[e]
	WK2	26.65 ± 0.13[c]	55.21 ± 0.11[c]	0.59 ± 0.06[d]	13.85 ± 0.10[c]	33.70 ± 0.08[c]	54.92 ± 0.12[d]
	WK3	22.98 ± 0.13[d]	32.84 ± 0.10[d]	0.59 ± 0.05[d]	14.73 ± 0.12[b]	37.95 ± 0.15[b]	63.31 ± 0.06[c]
	WK4	16.01 ± 0.07[e]	18.54 ± 0.11[e]	0.64 ± 0.04[a]	15.63 ± 0.11[a]	47.18 ± 0.11[a]	84.36 ± 0.08[b]
	WK5	16.98 ± 0.04[e]	18.93 ± 0.05[e]	0.62 ± 0.06[c]	15.46 ± 0.10[a]	24.74 ± 0.07[d]	85.93 ± 0.06[a]
6	TK	32.34 ± 0.08[a]	66.31 ± 0.06[a]	0.58 ± 0.05[d]	11.47 ± 0.08[d]	12.68 ± 0.07[f]	26.67 ± 0.05[e]
	WK1	25.47 ± 0.06[b]	53.31 ± 0.09[b]	0.60 ± 0.03[c]	11.97 ± 0.05[d]	25.38 ± 0.09[e]	24.82 ± 0.09[f]
	WK2	24.51 ± 0.08[b]	29.46 ± 0.05[c]	0.65 ± 0.05[a]	12.99 ± 0.10[c]	39.84 ± 0.12[d]	48.22 ± 0.08[d]
	WK3	21.94 ± 0.06[d]	16.35 ± 0.10[d]	0.56 ± 0.05[e]	13.89 ± 0.08[b]	50.91 ± 0.12[b]	54.75 ± 0.09[c]
	WK4	15.04 ± 0.05[e]	14.21 ± 0.11[e]	0.58 ± 0.07[d]	15.57 ± 0.10[a]	53.31 ± 0.21[a]	75.95 ± 0.10[a]
	WK5	15.32 ± 0.08[e]	10.42 ± 0.10[f]	0.63 ± 0.06[b]	13.86 ± 0.11[b]	49.12 ± 0.10[c]	74.46 ± 0.12[b]
9	TK	28.95 ± 0.11[a]	55.73 ± 0.08[a]	0.62 ± 0.04[b]	11.21 ± 0.06[d]	12.25 ± 0.11[f]	16.21 ± 0.10[f]
	WK1	23.59 ± 0.05[b]	48.94 ± 0.12[b]	0.58 ± 0.03[d]	11.56 ± 0.07[d]	24.31 ± 0.04[e]	18.64 ± 0.05e
	WK2	23.43 ± 0.05[b]	26.83 ± 0.09[c]	0.63 ± 0.05[a]	12.85 ± 0.10[c]	34.56 ± 0.04[d]	42.15 ± 0.05[d]
	WK3	21.42 ± 0.07[c]	15.63 ± 0.08[d]	0.56 ± 0.06[e]	13.98 ± 0.05[b]	44.27 ± 0.12[a]	50.54 ± 0.11[c]
	WK4	14.33 ± 0.05[d]	13.98 ± 0.05[e]	0.59 ± 0.06[c]	14.64 ± 0.04[a]	41.92 ± 0.11[b]	67.12 ± 0.08[a]
	WK5	14.95 ± 0.06[d]	12.82 ± 0.10[f]	0.58 ± 0.07[d]	12.91 ± 0.03[c]	36.81 ± 0.04[c]	64.17 ± 0.06[b]
12	TK	27.49 ± 0.10[a]	49.98 ± 0.07[a]	0.53 ± 0.04[e]	10.85 ± 0.11[d]	14.31 ± 0.04[e]	15.99 ± 0.11[f]
	WK1	22.99 ± 0.10[b]	39.44 ± 0.06[b]	0.61 ± 0.09[b]	12.93 ± 0.06[c]	18.43 ± 0.08[d]	17.34 ± 0.06[e]
	WK2	22.47 ± 0.08[b]	25.31 ± 0.08[c]	0.60 ± 0.05[c]	13.11 ± 0.06[b]	31.26 ± 0.08[c]	37.55 ± 0.05[d]
	WK3	20.35 ± 0.07[c]	14.21 ± 0.10[d]	0.57 ± 0.07[d]	14.01 ± 0.11[a]	37.46 ± 0.08[b]	40.58 ± 0.12[c]
	WK4	13.26 ± 0.04[d]	6.79 ± 0.10[e]	0.61 ± 0.09[b]	12.65 ± 0.12[c]	38.12 ± 0.10[a]	54.12 ± 0.10[a]
	WK5	12.84 ± 0.13[e]	7.83 ± 0.08[f]	0.62 ± 0.08[a]	13.53 ± 0.12[b]	31.89 ± 0.11[c]	49.95 ± 0.06[b]

Table 2 — Changes in the contents of major phenolic acids in the various types of kombucha blended with wheatgrass juice during fermentation.

Data are expressed as mean ± standard deviation of 3 samples.

[a–f] Significant differences within a column ($p < 0.05$).

TK = traditional kombucha; WK1–5 = kombucha supplemented at various ratios with wheatgrass juice, as shown in Table 1.

Nishidai et al [28] fermented vinegar using acetic acid bacteria with different substrates, revealing that the vinegar fermented using a phenolic-acid-rich substrate had the highest DPPH clearance. Phenolic compounds easily donate hydroxyl hydrogen due to resonance stabilization [17]. Lu and Foo [29] have more definitively shown that the structure in phenolic compounds can supply hydrogen, specifically in the β chain with 2,3 double bonds as well as 4-oxo and 3,5 site hydroxyl groups. This hydrogen supply confers phenolic compounds with excellent DPPH scavenging capacity. Among the DPPH radical scavenging systems, all phenolic compounds, including caffeic acid, chlorogenic acid, 3,5-dicaffeoylquinic acid, ferulic acid, rosmarinic acid, and protocatechuic acid, have free radical scavenging abilities. In fact, the free radical scavenging ability of many phenolic acids is better than that of dl-α-tocopherol or ascorbic acid [20]. WGJ contains many phenolic compounds that function as antioxidants. Therefore, increasing the proportion of WGJ to black tea in the substrate during kombucha fermentation enhances the antioxidant capacity, as the phenolic compounds supply hydrogen with excellent DPPH scavenging capacity.

3.4.2. TEAC assay

The TEAC assay is based on the inhibition of the absorbance of the ABTS radical cation by antioxidants. The TEAC results for the six beverages are shown in Fig. 5. The average value of TEAC was 0.6 μmol Trolox/mL. In TK, the TEAC capacity increased slowly with fermentation time. The WK1 sample reached its maximum capacity by the 3rd day, whereas WK3–5 samples peaked on the 6th day. This result indicates that the free radical scavenging capacity for ABTS⁺ was different from that of DPPH

Because the phenolic compounds have structural differences, each phenolic compound had a different antioxidant capacity and thus behaved differently in the antioxidant assay. In addition, the polarity, ionic conditions, and stereo structure of an antioxidant should affect its capacity evaluation [17]. The report showed that the antioxidation capacity of
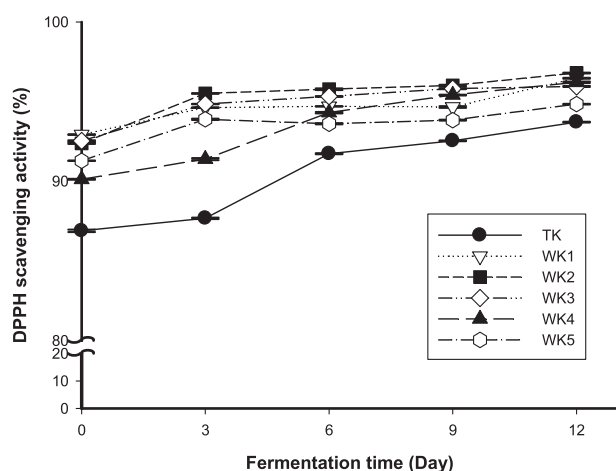
Fig. 4 — Effects of volume ratios of wheatgrass juice to black tea decoction on changes in DPPH radical scavenging ability of WGJ-blended kombucha during fermentation. Data are expressed as mean ± standard deviation of three samples. TK = traditional kombucha; WK1—5 = kombucha blended at various ratios with wheatgrass juice, as shown in Table 1.

a phenolic compound was related to the amount and position of its hydroxyl groups, and the antioxidant capacity then affects its free radical scavenging ability [24]. The antioxidant determination method is highly dependent upon the substances' reaction or the conditions of the sample during monitoring; therefore, each antioxidant determination method will yield a different result, largely due to the differences between the variables in the analysis method. Perez et al [30] compared the ORAC, TEAC, and TRAP methods in samples of red wine and white wine and did not find any
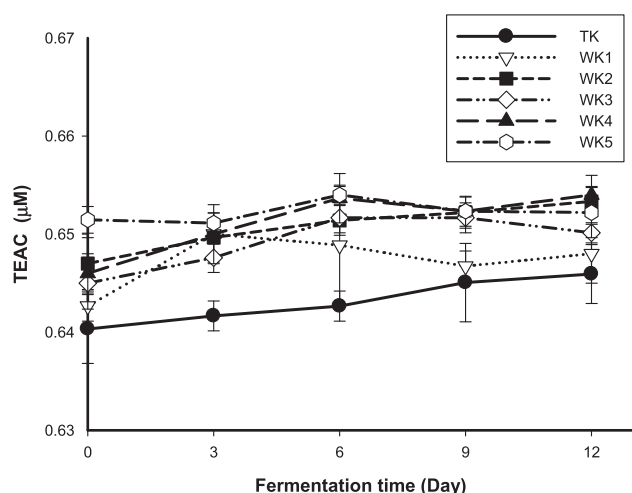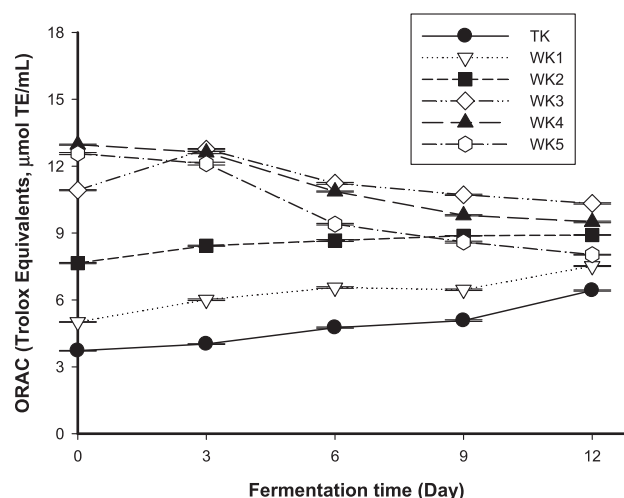
correlations between the methods. When a sample is complex or contains strongly different antioxidants, the correlation between the ORAC and TEAC methods is low because of the different kinetics and reaction mechanisms of the various antioxidants present [31].

3.4.3. ORAC

The ORAC assay is one of the methods used to evaluate the antioxidant capacity of various biological substrates, ranging from pure compounds, such as phenolic acids and flavonoids, to complex matrices, such as fruits, vegetables, and teas [9,25]. As shown in Fig. 6, the ORAC of TK increased slowly with fermentation time, reaching 6.42 ± 0.03 µmol TE/mL on Day 12, while the ORAC values of WK4—5 samples decreased with fermentation time. The ORAC values within the range of WK1—3 (WK1: 5.0—7.5 µmol TE/mL; WK2: 7.7—8.9 µmol TE/mL; WK3: 10.3—12.8 µmol TE/mL) correspond to fairly constant levels of antioxidant activity during fermentation. Dufresne and Farnworth [1] have reported that bioactive substances originate from substrates and that the derivatives from metabolites should be important sources for biological function. Falcioni et al [11] demonstrated that wheatgrass extract exhibits free radical scavenging ability, inhibits membrane injury, and minimizes oxidative DNA damage induced by free radicals. The main reason for this behavior is that wheatgrass contains phenolic compounds with flavonoids, some of which are biologically active

Peroxide free radicals, $ROO^\bullet$ (peroxyl radical), are usually used to estimate biological antioxidant activity because peroxyl radicals have long half-lives second only to those of hydroxyl radicals in toxin, pass freely through cell membranes, and are an intermediate product in cell membrane lipid peroxidation reactions [28]. The performance of WK1—3 in scavenging ORAC $_{ROO\bullet}$ indicates that cells can be

Fig. 5 — Effects of volume ratios of wheatgrass juice to black tea decoction on changes in trolox equivalent antioxidant capacity (TEAC) of WGJ-blended kombucha during fermentation. Data are expressed as mean ± standard deviation of three samples. TK = traditional kombucha; WK1—5 = kombucha blended at various ratios with wheatgrass juice, as shown in Table 1.

Fig. 6 — Effects of volume ratios of wheatgrass juice to black tea decoction on changes in oxygen radical absorbance capacity (ORAC) of WGJ-blended kombucha during fermentation. Data are expressed as mean ± standard deviation of three samples. TK = traditional kombucha; WK1-5 = kombucha blended at various ratios with wheatgrass juice, as shown in Table 1.

constantly protected from oxidative damage by peroxyl radicals. TEAC and ORAC assays have proven that phenolics have peroxyl radical scavenging ability. Phenolics can scavenge the peroxyl radical and stop the lipid oxidation chain reaction [28]. The related antioxidant activities among phenolic compounds are as follows: caffeic acid > isoeugenol > ferulic acid > p-coumaric acid [25]. Tea leaf phenolics include gallic acid, catechin, caffeic acid, and tannins [9]. Wheatgrass contains numerous antioxidant substances, such as ferulic acid, vanillic acid, vitamins C and E, and β-carotene [8]. The kombucha broth co-cultured with black tea and WGJ (WK1-3) contain phenolic compounds, which provide cations to stop the peroxyl radical chain reaction in the antioxidation reaction. Cao et al [9] noted that the ORAC method had greater specificity and was capable of responding to a greater number of antioxidant compounds than the TEAC method and that the total phenol content can affect the total antioxidant ability. Length of culture time affects the composition of the culture broth; antioxidant capacity thus increases during fermentation. Therefore, the antioxidant capacity depends on the hydrogen-bond binding ability, pH, oxidation-reduction potential, solubility, and stereo structure with oxygen of phenolic compounds [20]. These factors help to explain the differences between the antioxidant activities. In addition, the products also have higher antioxidant activity after fermentation [32].

In the present study, the analysis mechanism for the antioxidant assays depends on the reaction components. The appropriate adjustment of the fermentation substrates can shorten the culture time while maximizing the antioxidant capacity. The result shows that not only the total phenol content, total flavonoid content, and total anthocyanidin content, but also the types and contents of phenolic compounds in the culture broth, determine the comprehensive antioxidant activity of a beverage.

4. Conclusion

In our study, the culture substrate was adjusted in terms of the ratio of WGJ to black tea. The wheatgrass-blended kombucha fermented from these altered substrates reveals that each formulation presents different free radical scavenging capacity and that incubation with various substrates produces different metabolites and antioxidant activities. The highest antioxidant activity for the 1:1 WGJ:black tea substrate was obtained after 3 days of fermentation. This result clearly shows that the modified kombucha is a better free radical scavenging agent than traditional kombucha. The novel kombucha includes antioxidant activity from not only flavonoids and anthocyanins but also phenolic compounds from the culture broth. The data also showed that the antioxidation capacity of wheatgrass-blended kombucha includes contributions from the main phenolic acids, including gallic acid, catechin, and caffeic acid from the traditional kombucha and ferulic acid, rutin, and chlorogenic acid from the wheatgrass juice. Therefore, the wheatgrass-blended kombucha has higher, more stable antioxidant activity and might be recommended for consumption as a novel beverage.

Conflicts of interest

All authors declare no conflicts of interest.

REFERENCES

[1] Dufresne C, Farnworth E. Tea, kombucha, and health: a review. Food Res Int 2000;33:409–21.

[2] Chen C, Liu BY. Changes in major components of tea fungus metabolites during prolonged fermentation. J Appl Microbiol 2000;89:834–9.

[3] Bauer-Petrovska B, Petrushevska-Tozi L. Mineral and water soluble vitamins content in the kombucha drink. J Food Sci Technol 2000;35:201–5.

[4] Williamson G, Barron D, Shimoi K, Terao J. In vitro biological properties of flavonoid conjugates found in vivo. Free Radic Res 2005;39:457–69.

[5] Dipti P, Yogesh B, Kain AK, Pauline T, Anju B, Sairam M, Singh B, Mongis SS, Devendra Kumar GI, Selvamurthy W. Lead induced oxidative stress: Beneficial effects of kombucha tea. Biomed Environ Sci 2003;16:276–82.

[6] Fontana JD, Franco VC, De Souza SJ, Lyra IN, De Souza AM. Nature of plant stimulators in the production of Acetobacter xylinum ("tea fungus") biofilm used in skin therapy. Appl Biochem Biotechnol 1991;28–29:341–51.

[7] Greenwalt CJ, Ledford RA, Steinkraus KH. Determination and characterization of the antimicrobial activity of the fermented kombucha. LWT-Food Sci Technol 1998;31:291–6.

[8] Peryt B, Szymczy KT, Lesca P. Mechanism of antimutagenicity of wheat sprout extracts. Mutat Res 1992;269:201–5.

[9] Cao G, Sofic E, Prior RL. Antioxidant capacity of tea and common vegetables. J Agric Food Chem 1996;44:3426–31.

[10] Kulkarni SD, Acharya R, Nair AGC, Rajurkar NS, Reddy AVR. Determination of elemental concentration profiles in tender wheatgrass (Triticum aestivum L.) using instrumental neutron activation analysis. Food Chem 2006;95:699–707.

[11] Falcioni G, Fedeli D, Tiano L, Calzuola I, Mancinelli L, Marsili V, Gianfranceschi G. Antioxidant activity of wheat sprout extracts in vitro: inhibition of DNA oxidative damage. J Food Sci 2002;67:2918–22.

[12] Gorinstein S, Zachwieja Z, Katrich E, Pawelzik E, Haruenkit R, Trakhtenberg S, Martin-Belloso O. Comparison of the contents of the main antioxidant compounds and the antioxidant activity of white grapefruit and his new hybrid. LWT-Food Sci Technol 2004;37:337–43.

[13] Maksimović Z, Malenčić D, Kovačević N. Polyphenol contents and antioxidant activity of Maydis stigma extracts. Bioresour Technol 2005;96:873–7.

[14] Ruenroengklin N, Sun J, Shi J, Xue XJ, Jiang Y. Role of endogenous and exogenous phenolics in litchi anthocyanin degradation caused by polyphenol oxidase. Food Chem 2009;115:1253–6.

[15] Nuutila AM, Kammiovirta K, Oksman-Caldentey KM. Comparison of methods for the hydrolysis of flavonoids and phenolic acid from onion and spinach for HPLC analysis. Food Chem 2002;76:519–25.

[16] Yamaguchi T, Takamura H, Matoba T, Terao J. HPLC method for evaluation of the free radical-scavenging activity of foods by using 1,1-diphenyl-2-picrylhydrazyl. Biosci Biotechnol Biochem 1998;62:1201–4.

[17] Miller NJ, Rice-Evans CA, Davies MJ, Gopinathan V, Milner A. A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. Clin Sci 1993;84:407–12.

[18] Ou B, Hampsch-Woodill M, Prior RL. Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein the fluorescent as probe. J Agric Food Chem 2001;49:4619—26.

[19] Bar-Sela G, Tsalic M, Fried G, Goldberg H. Wheat grass juice may improve hematological toxicity related to chemotherapy in breast cancer patients: a pilot study. Nutr Cancer 2007;58:43—8.

[20] Rice-Evans C, Miller N, Paganga G. Antioxidant properties of phenolic compounds. Trends Plant Sci 1997;2:152—9.

[21] Torskangerpoll K, Andersen OM. Color stability of anthocyanins in aqueous solutions at various pH values. Food Chem 2005;89:427—40.

[22] Somers TC, Evans ME. Spectral evaluation of young red wines: anthocyanin equilibria, total phenolics, free and molecular SO_2, "chemical age". J Sci Food Agric 1977;28:279—87.

[23] Singletary KW, Jung KJ, Giusti M. Anthocyanin-rich grape extract blocks breast cell DNA damage. J Med Food 2007;10:244—51.

[24] Cuvelier ME, Richard H, Berset C. Comparison of the antioxidative activity of some acid-phenols: structure-activity relationship. Biosci Biotechnol Biochem 1992;56:324—5.

[25] Yeh CT, Yen GC. Effects of phenolic acids on human phenolsulfotransferases in relation to their antioxidant activity. J Agric Food Chem 2003;51:1474—9.

[26] Shahrzad S, Bitsch I. Determination of some pharmacologically active phenolic acids in juice by high-performance liquid chromatography. J Chromatogr 1996;741:223—31.

[27] Brand-Williams W, Cuvelier ME, Berset C. Use of a free radical method to evaluate antioxidant antivity. LWT-Food Sci Technol 1995;28:25—30.

[28] Nishidai S, Nakamura Y, Torikai K, Yamamoto M, Ishihara N, Mori H, Ohigashi H. Kurosu, a traditional vinegar produced from unpolished rice, suppresses lipid peroxidation *in vitro* and in mouse skin. Biosci Biotechnol Biochem 2000;64:1909—14.

[29] Lu Y, Foo LY. Antioxidant and radical scavenging activities of polyphenols from apple pomace. Food Chem 2000;68:81—5.

[30] Perez DD, Leighton F, Aspee A, Aliaga C, Lissi E. A comparison of methods employed to evaluate antioxidant capabilities. Biol Res 2000;33:71—7.

[31] Zulueta A, Esteve MJ, Frigola A. ORAC and TEAC assays comparison to measure the antioxidant capacity of food products. Food Chem 2009;114:310—6.

[32] Wen YL, Yan LP, Chen CS. Effects of fermentation treatment on antioxidant and antimicrobial activities of four common Chinese herbal medicinal residues by *Aspergillus oryzae*. J Food Drug Anal 2013;21:219—26.