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Characteristics and antioxidant activity of royal lotus pollen, butterfly pea flower, and oolong tea kombucha beverages

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Abstract

Kombucha is a fermented functional tea beverage that is prepared by inoculating a microbial consortium comprising acetic acid bacteria and yeast into sweetened black tea and incubating it under aerobic conditions. In this study, kombucha beverages of royal lotus pollen (*Nelumbo nucifera*), butterfly pea flowers (*Clitoria ternatea*), and oolong tea (*Camellia sinensis*) leaves were prepared via fermentation with a kombucha consortium containing acetic acid bacteria and yeast for a period of 20 days. The amounts of acetic acid and ethanol in royal lotus pollen kombucha (LK), butterfly pea flower kombucha (BK), and oolong tea kombucha (OK) samples gradually increased after fermentation. In addition, the total phenolic contents (TPCs), total flavonoid contents (TFCs), 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) free radical scavenging capacities, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging capacities of all the kombucha beverages gradually increased after fermentation; the beverages exhibited the highest antioxidant capacity on day 20 of fermentation. The kombucha beverages from butterfly pea flowers and oolong tea leaves significantly inhibited hydrogen peroxide-induced reactive oxygen species (ROS) production by increasing the synthesis of antioxidant enzymes such as catalase, manganese superoxide dismutase (Mn-SOD), glutathione reductase (GRe), glutathione peroxidase-1 (GPx-1), and heme oxygenase-1 (HO-1) in human embryonic kidney-293 (HEK-293) cells. Therefore, the consumption of these antioxidant-rich kombucha beverages can reduce the risk of oxidative stress, which is the cause of many diseases.

Keywords: Antioxidant activity, Antioxidant enzyme, Kombucha, Oolong tea leaves, Butterfly pea flower, Royal lotus pollen

1. Introduction

Kombucha is a widely consumed fermented probiotic drink that has gained popularity because of its purported health benefits. Typical kombucha is prepared by fermenting a sweetened black tea via the symbiosis of a group of acetic bacteria and yeasts called "SCOBY or Tea fungus" [1]. Kombucha contains multiple species of yeast and bacteria along with organic acids, active enzymes, amino acids, polyphenols, and fructose. These make kombucha both sour and sweet, similar to cider beverages. The flavors can differ depending on the type of tea used and the herbal infusions added [2, 3]. Kombucha has recently received considerable attention as a functional beverage because of the high amounts of functional compounds it contains. Several studies have demonstrated the antioxidant activity of kombucha derived from various fruits and vegetables [3].

In this study, we prepared kombucha beverages from royal lotus pollen (*Nelumbo nucifera*), butterfly pea flowers (*Clitoria ternatea*), and oolong tea leaves (*Camellia sinensis*), and investigated their physicochemical characteristics. We also determined the contents of phenolic compounds and flavonoids, and investigated their antioxidant capacities using 2,2-diphenyl-l-picrylhydrazyl (DPPH) and 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assays.

Tea is a famous beverage in Asia. There are three types of tea preparations: unfermented green tea, fully fermented tea (black tea), and oolong tea (partially fermented) [4]. Most of the beneficial effects of tea on health have been associated with the antioxidant and free radical scavenging activity of active compounds such as polyphenols and flavonoids [5].

The butterfly pea flower is a climbing legume belonging to the Leguminosae family. It has attracted a lot of interest because all its parts are sources of nutraceuticals. This flower has potential applications in traditional medicine and exhibits a wide range of actions, including neuroprotective effects, anti-inflammatory effects, and antioxidant activity [6]. The flowers of *C. ternatea* contain an anthocyanin named "ternatin" and other phenolics and flavonoids that may be sources of natural antioxidants [7]. The aqueous and ethanol extracts from butterfly pea flowers exhibit antioxidant activity, as determined by DPPH scavenging assays [7].

The royal lotus flower belongs to the Nelumbonaceae family, which is widely distributed throughout Asia, including Thailand. Royal lotus flowers have been used in traditional medicine for the treatment of many diseases and disorders because their active compounds exert anti-inflammatory, antimicrobial, and antioxidant activity [8]. Pollen from both pink and white lotus flowers have been reported to exhibit free radical-scavenging activity [8]. Hence, royal lotus pollen are a source of natural antioxidants.

The antioxidant activity of oolong tea leaves, butterfly pea flowers, and royal lotus pollen has been studied. However, the antioxidant activity of kombucha made from these plant parts has never been studied. Our study was aimed at evaluating the cellular antioxidant activity of kombucha samples prepared using these plant materials and investigating their molecular mechanisms in the prevention of cellular oxidative stress.

2. Materials and methods

2.1 General materials

Sodium hydroxide (NaOH), sodium carbonate (Na_2CO_3), Folin-Ciocalteu's phenol reagent, ammonium dihydrogen phosphate, ABTS, and DPPH were purchased from Merck. Ascorbic acid, 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT), 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA), quercetin, and gallic acid were purchased from Sigma. Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), 0.25% trypsin-EDTA, and penicillin/streptomycin (P/S) solution were obtained from Gibco. A kombucha starter was purchased from Junior Scoby Store at Chiang Mai, Thailand.

2.2 Plant materials and preparation of kombucha beverages

Dried oolong tea leaves were obtained from the Three Horses Tea Company, Thailand. Fresh royal lotus pollen and butterfly pea flowers were purchased from Nakhon Sawan Province, Thailand. All the herbs were cleaned with distilled water and dried at 45 °C in a hot air oven for 1-2 days or until they were completely dry. The dried herbs were collected and ground for use in further processes.

The processes of kombucha starter and kombucha sample preparation were similar to those of Battikh et al. [9]. Briefly, oolong tea leaves and herbal plants (1% w/v) were steeped in 1 L of boiling water for 15 min to prepare the kombucha starter and sample, respectively. Subsequently, cane sugar (15% w/v) was added to each liquid infusion, which was then poured into a glass jar. Next, the infusion was inoculated with a liquid kombucha starter (10% v/v) and cellulose starter (3% w/v). The mixture was incubated at room temperature for 10 (kombucha starter) or 20 days (kombucha sample). Most of the kombucha starter samples contained yeast and acetic bacteria that play a role in kombucha fermentation.

2.3 Determination of pH

An electronic pH meter (Clean pH200 and pH500, China) was used to measure the pH of kombucha samples.

2.4 Determination of acetic acid by high-performance liquid chromatography (HPLC)

Kombucha samples were passed through 0.2 µm sterile syringes with PTFE filters and added into HPLC vials. Filtrate samples (20 µL) were injected into an HPLC system with a UV detector. The C-18 (5 µm, 150 mm × 4.6 mm ID) by ChemStation for LC 3D system was used for analysis. Detection was carried out at 210 nm. Acetic acid was used as the standard for the quantification of total acetic acid (g/L).

2.5 Gas chromatography (GC) analysis of ethanol

Gas chromatography (GC-2014, Shimadzu) was used to determine the ethanol contents of kombucha samples. The amount of ethanol was expressed as g/L. The chromatography device comprised a DB-1 column (30 mm ×

0.25 mm) and a flame ionization detector (FID). Propanol was used as the internal standard for ethanol determination. The device parameters were set based on those reported by Vázquez-Cabral et al. [10], with slight modifications, including an oven temperature of 60 °C, detector temperature of 60 °C, and inlet temperature of 150 °C. Helium was used as the carrier gas. Propanol was used as the internal standard for the quantification of ethanol.

2.6 Analysis of sugar content

Sugar contents were measured using the phenol-sulfuric acid method proposed by Dubois et al. [11]. The absorbance of the diluted solution was measured at 490 nm with glucose as the standard.

2.7 Measurement of total phenolic content (TPC) and total flavonoid content (TFC)

The Folin-Ciocalteu method was used to analyze TPC, as previously described [12]. The absorbance of the solution was measured at 765 nm and the amount of phenolic compounds was expressed as micrograms of gallic acid equivalent/milliliter of sample (µg GAE/mL).

TFC was analyzed using a crystalline aluminum chloride assay [12]. Finally, the absorbance of the sample at 510 nm was measured, and the mean value of the absorbance was converted into micrograms of quercetin equivalent/milliliter of sample (µg QE/mL).

2.8 ABTS and DPPH radical scavenging method

The ABTS and DPPH radical scavenging activity of kombucha samples was measured as previously described [13, 14]. The ABTS and DPPH scavenging capacities of the samples were determined according to the following formula:

$$\textit{Inhibition of ABTS and DPPH activity (\%)} = (1-((A_{sample})/(A_{control})))\times 100 \quad (1)$$

where $A_{control}$ is the absorption of ABTS and DPPH solutions in ethanol, and A_{sample} is the absorption of the sample with ABTS and DPPH. The inhibition of ABTS and DPPH activity by each sample was graphed for comparison with kombucha concentrations to determine the half maximal inhibitory concentration (IC_{50}) values, following the method of Patitungkho [15].

2.9 Cytotoxicity by MTT analysis

Human embryonic kidney-293 (HEK-293) cells were grown in DMEM containing 10% FBS and 1% P/S solution [16]. The cells were maintained at 37 °C in a CO_2 incubator. Then, they were sub-cultured routinely with 0.25% trypsin/EDTA. Cell viability by MTT assay was used to identify the appropriate concentration of kombucha samples. The absorbance value (at a wavelength of 570 nm) directly represented the relative number of cells. Cell viability (%) was calculated using the following equation:

$$\textit{Cell viability (\%)} = ((\textit{Absorbance of treated cells})/(\textit{Absorbance of control cells}))\times 100 \quad (2)$$

2.10 Determination of reactive oxygen species (ROS) levels in HEK-293 cells

The intracellular antioxidant activity of kombucha samples and unfermented tea was assayed using DCFH-DA (a fluorescent probe) to determine ROS production in cells [16]. The DCF fluorescence intensity was measured using a fluorescence microplate reader (λex/λem: 485/530 nm).

2.11 Measurement of mRNA expression by real time RT-qPCR

HEK-293 cells were seeded at 2×10^5 cells/well in a 6-well culture plate and incubated with kombucha samples for 6 h. RT-qPCR was performed using a One-step RT-qPCR kit (KAPA Biosystems) and the Mx3005p RT-qPCR system. The mRNA expression levels of several antioxidant genes were determined using the comparative cycle threshold (CT) method and normalized to the glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*, a housekeeping gene), as previously described [17]. We designed and ordered the primers of human antioxidant genes as follows: catalase (forward primer: GCAGATACCTGTGAACTGTC; reverse primer: GTAGAATGTC CGCACCTGAG), glutathione peroxidase-1 (GPx-1; forward primer: CTCTTCGAGAAGTGCGAGGT; reverse primer: TCGATGTCAATGGTCTGGAA), glutathione reductase (GRe; forward primer: CAGTGGGACT CACGGAAGAT; reverse primer: TTCACTGCAACAGCAAAACC), heme oxygenase-1 (HO-1; forward

primer: CAGGCAGAGAATGCTGAG; reverse primer: GCTTCACATAGCGCTGCA), manganese superoxide dismutase (Mn-SOD; forward primer: GCACATTAACGCGCAGATCA; reverse primer: AGCCTCCAGCAA CTCTCCTT), and GAPDH (forward primer: CGAGATCCCTCCAAAATCAA; reverse primer: GTCTTCTGGG TGGCAGTGAT).

2.12 Statistical analysis

Data were analyzed using the SPSS program, and variance was analyzed by ANOVA, using Duncan's New Multiple Range Test method (DMRT) and Tukey's Honestly Significant Difference (HSD) test at a 95% confidence interval.

3. Results

3.1 Physicochemical properties of fermented kombucha beverages

The changes in pH, total acetic acid content, ethanol content, and total sugar content during fermentation of kombucha are presented in Figure 1. Physicochemical changes during the fermentation process of three types of kombucha (oolong tea kombucha (OK), royal lotus pollen kombucha (LK), and butterfly pea flower kombucha (BK)) were investigated on days 0, 5, 7, 9, 12, 15, and 20 of fermentation at room temperature. The pH values of these kombucha samples gradually decreased with an increase in fermentation time. After 20 days of fermentation, the kombucha samples had a pH of approximately 2.59 ± 0.15, 2.58 ± 0.04, and 2.58 ± 0.03, respectively (Figure 1A). All the kombucha samples showed a similar rate of reduction in pH values. The total acetic acid content of all the kombucha samples slowly increased initially and then rapidly increased after fermentation for 12 days (Figure 1A); this phenomenon was related to the pH values. After fermentation for 20 days, OK contained the highest concentration of total acetic acid (4.18 ± 0.22 g/L), followed by LK (2.73 ± 0.25 g/L), and BK (1.20 ± 0.11 g/L) (Figure 1A). The changes in ethanol content during the kombucha fermentation process are shown in Figure 1B. At day 0 of fermentation, the ethanol contents of OK, LK, and BK were 0.17 ± 0.02, 0.20 ± 0.01, and 0.14 ± 0.01 g/L, respectively; they increased with fermentation time (Figure 1B). The highest ethanol contents of the kombucha samples were observed on day 20 after fermentation (0.48 ± 0.03, 1.34 ± 0.02, and 1.12 ± 0.01 g/L for OK, LK, and BK, respectively). In addition, the total sugar contents of OK, LK, and BK rapidly decreased after fermentation for 7 days (Figure 1C).

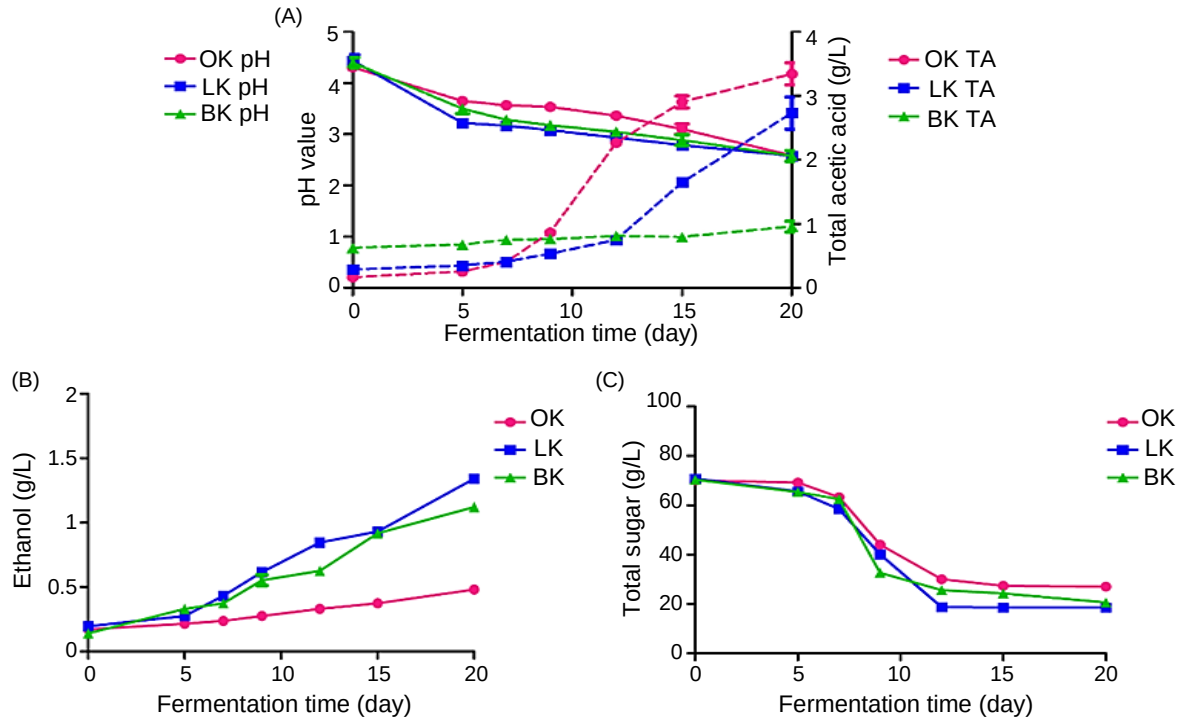

Figure 1 Physicochemical characteristics of kombucha beverages. Changes in pH values and total acetic acid (A), ethanol (B), and total sugar content (C) after fermentation with kombucha. Results were expressed as the mean ± SEM (n = 3). OK = Oolong tea kombucha, LK = Royal lotus pollen kombucha, and BK = Butterfly pea flower kombucha.

3.2 TPC and TFC of fermented kombucha beverages

The TPC of kombucha samples significantly increased with the fermentation time, and the maximum TPC was attained on day 20 of fermentation (Table 1). On day 20, OK had the highest TPC (555.00 ± 1.00 μg GAE/mL), followed by LK (403.00 ± 2.00 μg GAE/mL), and BK (269.08 ± 3.33 μg GAE/mL) (Table 1). The flavonoid contents of all the kombucha beverage samples increased after fermentation, and the highest TFC was observed after fermentation for 20 days (Table 1). OK had the highest TFC (863.89 ± 1.47 μg QE/mL), followed by LK (481.35 ± 5.32 μg QE/mL) and BK (227.65 ± 3.36 μg QE/mL).

Table 1 The TPC, TFC, and antioxidant capacities of OK, LK, and BK.

	TPC (μg GAE/mL)	TFC (μg QE/mL)	DPPH scavenging activity (%)	IC_{50} of DPPH (μL/mL)	ABTS scavenging activity (%)	IC_{50} of ABTS (μL/mL)
Day 0 of fermentation						
OK	353.60±2.36^{a}	613.38±2.43^{a}	83.61±0.34^{b}	39.20±0.98^{b}	97.76±0.00^{a}	19.76±0.32^{c}
LK	254.33±1.53^{b}	323.87±3.18^{b}	85.54±0.10^{b}	47.21±0.26^{a}	91.96±0.83^{c}	65.33±0.20^{a}
BK	163.62±5.54^{c}	165.93±2.96^{c}	88.81±0.39^{a}	35.01±0.99^{c}	93.65±0.86^{b}	56.64±0.33^{b}
Day 5 of fermentation						
OK	404.12±0.87^{a}	641.67±1.17^{a}	88.54±0.02^{a}	29.50±0.50^{b}	98.08±0.16^{a}	18.72±0.26^{c}
LK	298.33±7.64^{b}	351.23±1.00^{b}	87.58±0.03^{b}	36.27±0.21^{a}	93.12±0.13^{b}	60.25±0.10^{a}
BK	159.10±0.68^{c}	173.75±0.23^{c}	92.48±0.15^{a}	23.33±1.50^{c}	95.66±0.32^{b}	52.28±0.14^{b}
Day 7 of fermentation						
OK	454.33±5.13^{a}	682.86±2.59^{a}	90.73±0.01^{b}	25.00±1.00^{b}	98.23±0.06^{a}	17.44±0.20^{c}
LK	326.33±1.53^{b}	359.91±1.86^{b}	88.45±0.13^{c}	31.35±0.22^{a}	95.10±0.69^{b}	58.33±0.26^{a}
BK	172.33±2.52^{c}	192.58±2.48^{c}	93.15±0.01^{a}	19.27±0.22^{c}	95.98±0.87^{b}	48.45±0.33^{b}
Day 9 of fermentation						
OK	504.67±1.53^{a}	751.95±1.46^{a}	93.76±0.70^{b}	20.44±0.08^{b}	98.58±0.02^{a}	16.37±0.21^{c}
LK	351.67±2.08^{b}	391.28±11.50^{b}	92.84±0.77^{b}	26.73±0.34^{a}	96.17±1.09^{b}	53.35±0.26^{a}
BK	194.33±2.08^{c}	205.88±1.51^{c}	95.56±0.06^{a}	18.34±0.19^{b}	97.62±0.38^{a}	45.26±0.11^{b}
Day 12 of fermentation						
OK	509.00±1.00^{a}	782.33±3.45^{a}	95.17±0.18^{b}	18.33±0.20^{b}	99.56±0.02^{a}	16.28±0.04^{c}
LK	369.67±8.74^{b}	411.45±1.91^{b}	93.60±0.10^{c}	23.55±0.43^{a}	98.45±0.15^{b}	50.28±0.04^{a}
BK	207.67±3.79^{c}	210.95±0.91^{c}	96.18±0.06^{a}	17.78±0.17^{b}	99.87±0.01^{a}	39.54±0.09^{b}
Day 15 of fermentation						
OK	531.00±1.00^{a}	821.17±1.59^{a}	96.18±0.06^{b}	17.48±0.14^{b}	99.73±0.01^{b}	14.28±0.04^{c}
LK	397.00±2.65^{b}	451.60±1.49^{b}	94.39±0.35^{c}	19.59±0.06^{a}	99.72±0.11^{b}	46.32±0.06^{a}
BK	228.64±0.34^{c}	217.29±2.66^{c}	97.35±0.40^{a}	17.17±1.04^{b}	99.90±0.02^{a}	37.58±0.40^{b}
Day 20 of fermentation						
OK	555.00±1.00^{a}	863.89±1.47^{a}	97.44±0.19^{b}	17.46±0.07^{ab}	99.84±0.03^{b}	12.53±0.48^{c}
LK	403.00±2.00^{b}	481.35±5.32^{b}	95.20±0.14^{c}	18.33±1.52^{a}	99.86±0.01^{b}	46.17±0.17^{a}
BK	269.08±3.33^{c}	227.65±3.36^{c}	98.41±0.19^{a}	15.65±0.70^{b}	99.95±0.01^{a}	37.18±0.16^{b}

- Mean ± Standard Deviation (3 replications)
- ^{a,b,c} vertically different letters of each fermentation day showed a statistically significant difference at the 95 percent confidence level ($p \leq 0.05$).

3.3 Antioxidant capacities of kombucha beverages

The antioxidant activity of kombucha samples was determined using the DPPH and ABTS methods. The free radical scavenging capacities of OK, LK, and BK significantly increased after fermentation (Table 1). After fermentation for 20 days, OK, LK, and BK exhibited DPPH free radical scavenging activity with 97.44% ± 0.19%, 95.20% ± 0.14%, and 98.41% ± 019% of inhibition, respectively, and corresponding IC_{50} values of 17.46 ± 0.07, 18.33 ± 1.52, and 15.65 ± 0.70 μL/mL. In addition, the percentage of ABTS scavenging activity positively correlated with the DPPH scavenging activity. Furthermore, OK, LK, and BK exhibited potent ABTS free radical scavenging activity, with more than 99% inhibition from day 0 to day 20 of fermentation (Table 1).

3.4 Effects of kombucha beverages on cell cytotoxicity in HEK-293 cells

We selected OK (as the standard kombucha) and BK (which exhibited the highest antioxidant capacity values) to test the intracellular antioxidant activity. These kombucha samples were compared with unfermented tea (oolong tea (OT)) and butterfly pea flower tea (BT). The cytotoxicity of the kombucha samples was evaluated at various concentrations to determine the optimal concentrations for the investigation of intracellular antioxidant activity. As shown in Figure 2, in order to exclude the cytotoxic effects and allow at least 80% cell survival, we selected 2.5 and 12.5 μL/mL of kombucha samples to test the cellular antioxidant activity and mRNA expression.

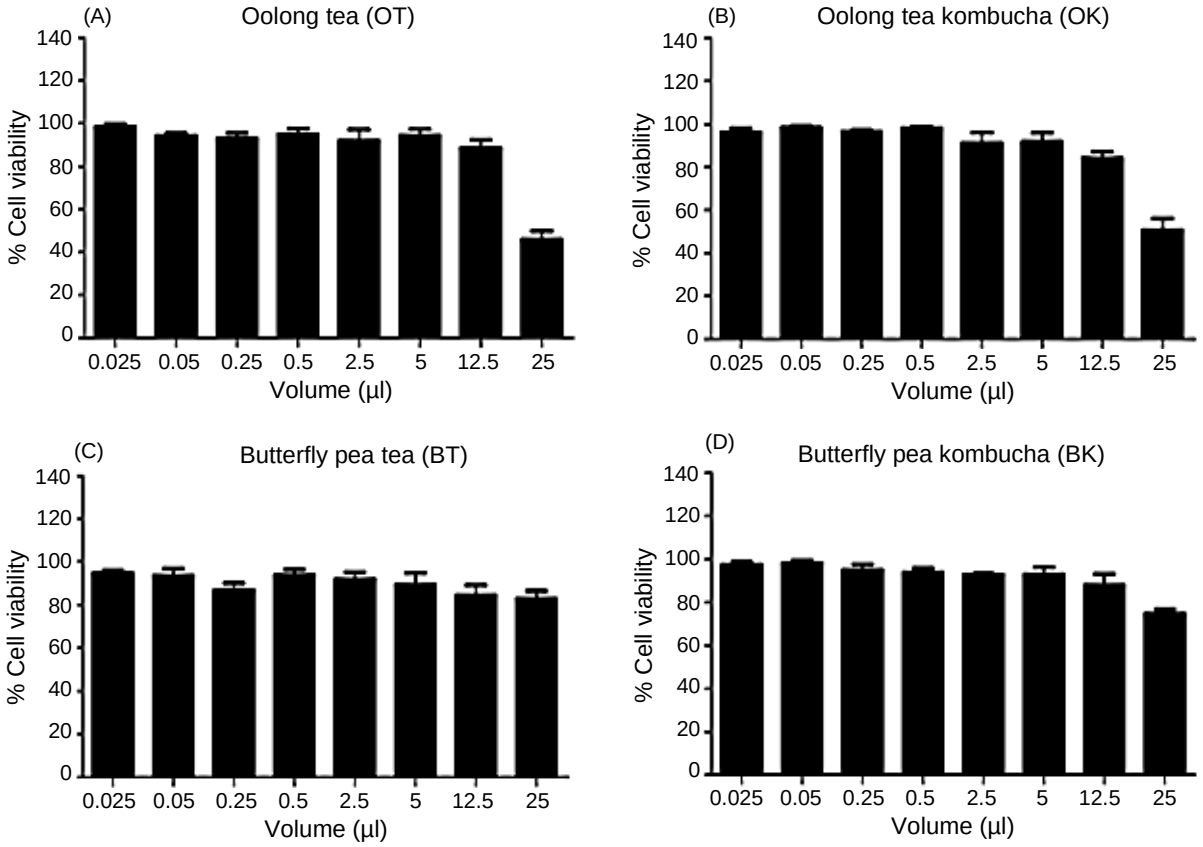

Figure 2 Cytotoxicity profiles of OT (A), OK (B), BT (C), and BK (D). Cells were treated with various concentrations of samples (0.025 - 25 μL/mL; v/v) for 36 h. Percentage of cell viability was shown as the mean ± SEM (n = 5).

3.5 Kombucha beverages from butterfly pea flower and oolong tea inhibit H_2O_2-induced intracellular ROS levels

Based on cell cytotoxicity, we determined the optimal concentrations for the investigation of intracellular antioxidant activity, and found that the range of concentrations of 2.5-12.5 μL/mL was not toxic to HEK-293 cells. Therefore, 2.5 and 12.5 μL/mL of samples were selected as the minimum and maximum concentrations, respectively, to test cellular antioxidant activity. HEK-293 cells were treated with kombucha or unfermented tea samples. Distilled water was used as the vehicle. Ascorbic acid was used as the positive control. Ascorbic acid was dissolved in distilled water so that the vehicle (control) was water. Incubating the cells with 200 μM H_2O_2 significantly increased intracellular ROS levels compared with those of the vehicle sample (control), as shown in Figure 3. Treatment with both OT and OK significantly suppressed ROS production induced by H_2O_2 in a dose-dependent manner. In addition, treatment with BT and BK at a concentration of 12.5 μL/mL also suppressed ROS production induced by H_2O_2 (Figure 3). Interestingly, OK and BK (kombucha fermented for 15 days) at a concentration of 12.5 μL/mL exhibited higher antioxidant activity in the cells compared with unfermented tea (OT and BT) (Figure 3). These results demonstrate that BK and OK exert antioxidant effects by inhibiting cellular ROS production.

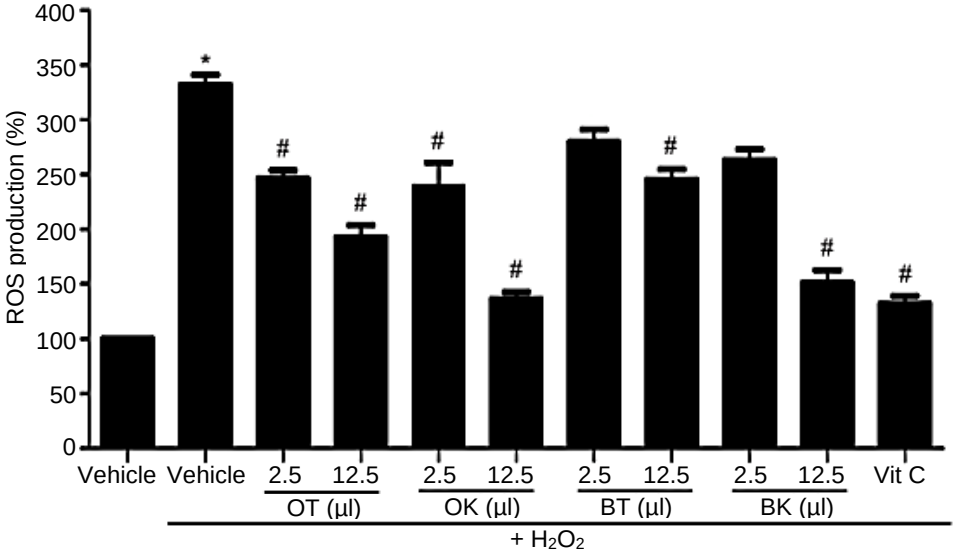

Figure 3 Antioxidant effects of OT, BT, OK, and BK in HEK-293 cells. Cells were treated with OT, OK, BT, and BK at concentration of 12.5 μL/mL (v/v) for 6 hours by using distilled water (Vehicle) and vitamin C (100 μM) (positive control). After that, cells were incubated with H_2O_2 (200 μM) for 30 minutes. The ROS levels were shown as a percentage of ROS production and represented as the mean±SEM (n=4). $^{*}P < 0.05$ vs. vehicle; $^{\#}P < 0.05$ vs. H_2O_2.

3.6 Upregulation of mRNA expression of antioxidant enzymes by kombucha samples

Next, we investigated the antioxidant effects of kombucha samples on the induction of the mRNA expression of antioxidant enzymes in HEK-293 cells. After treatment with OK for 6 h, the mRNA expression of catalase, GPx-1, GRe, and HO-1 significantly increased (Figure 4). In addition, the mRNA levels of catalase, GPx-1, GRe, Mn-SOD, and HO-1 significantly increased following treatment with BK. After fermentation for 15 days with the kombucha consortium, BK was more potent in inducing the synthesis of antioxidant enzymes than BT (Figure 4). These data demonstrated that the kombucha beverages prevented oxidative damage in the cells by upregulating the mRNA expression of several antioxidant enzymes.

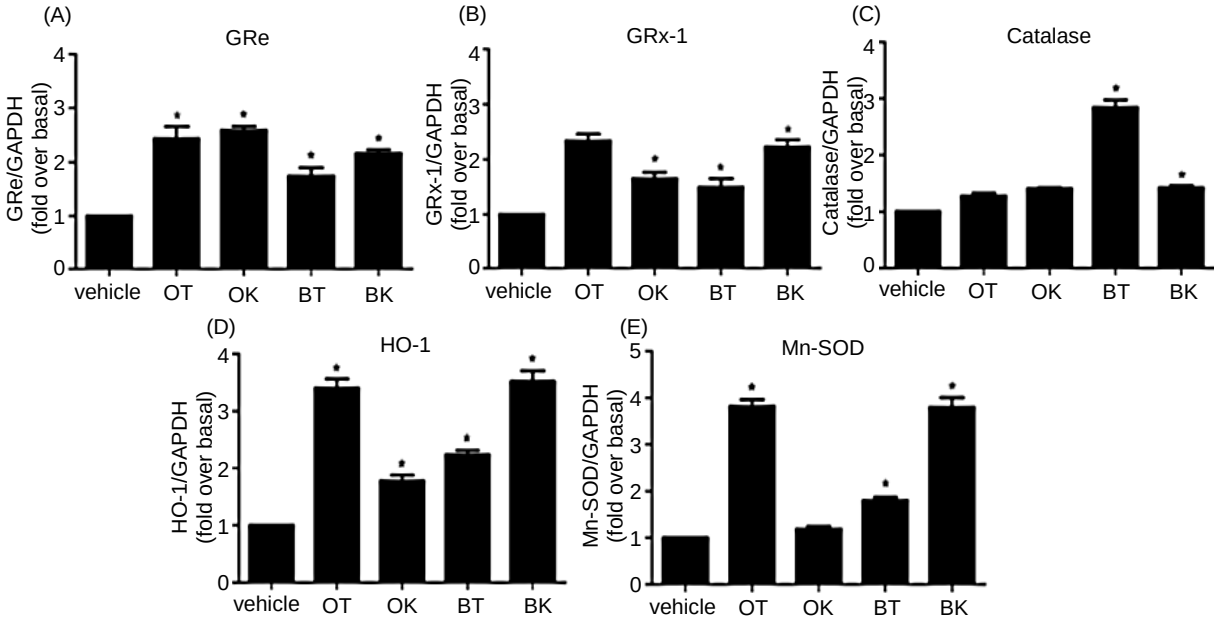

Figure 4 Kombucha beverages upregulated mRNA expression of antioxidant enzymes in HEK-293 cells. GRe (A), GRx-1 (B), Catalase (C), HO-1 (D) and Mn-SOD (E). Cells were treated with OT, OK, BT and BK at concentration of 12.5 μL/mL (v/v) and vehicle (control) for 6 hours. The mRNA levels of antioxidant enzymes were calculated and represented as mean±SEM (n=4) *P < 0.05 vs. vehicle.

4. Discussion

Kombucha beverages exhibit beneficial health effects and high antioxidant activity. Currently, herbs are used in kombucha fermentation to increase the medicinal value of various flavor compounds [2, 3]. Kombucha beverages are normally fermented for 1-2 weeks for the production of several constituents, including acetic acid and ethanol [18]. Traditional kombucha is produced using sweetened black tea fermented with the kombucha consortium under aerobic conditions. Microbiological analysis of kombucha has been carried out by researchers from various countries. The microorganisms thus identified were mainly acetic acid bacteria (*Acetobacter xylinum, Acetobacter xylinoides, Acetobacter aceti, Gluconobacter oxydans,* and *Gluconacetobacter* spp.) and yeasts (*Saccharomyces cerevisiae, Zygosaccharomyces kombuchaensis, Brettanomyces bruxellensis, Zygosaccharomyces rouxii, Schizosaccharomyces pombe,* and *Pichia* spp.) [19]. However, there have been no microbial isolations from kombucha produced in Thailand. Arikan et al. [20] determined the microbial composition of kombucha using a combination of WMS and amplicon (16S rRNA gene and ITS1) sequencing. *Komagataeibacter* and *Zygosaccharomyces* spp. were found in the cellulose and liquid parts of kombucha [20].

There are many types of kombucha that are fermented with fruit, vegetable, and dairy products. In this study, we used oolong tea, royal lotus pollen, and butterfly pea flowers to produce kombucha beverages with new flavors having different biophysical and chemical characteristics.

The pH values of kombucha beverages are different and are affected by the ingredients [21]. The amount of acetic acid is positively influenced by the fermentation time [22]. Consistent with our previous studies, the pH values decreased in the first 5 days of fermentation (Figure 1A), whereas the total acetic acid contents of all the kombucha samples gradually increased from day 7 to day 20 after fermentation (Figure 1A). The total acetic acid content correlated with the pH value. This was caused by the accumulation of microbes in the kombucha [2, 22]. Chemical analysis of the kombucha samples revealed the presence of various organic acids, such as acetic acid, gluconic acid, and glucuronic acid. Acetic acid is the major compound produced by acetic acid bacteria in the kombucha consortium. Many authors have determined the content of acetic acid in this beverage. Chen and Liu [23] determined the highest acetic acid content of 11 g/L after 30 days of kombucha fermentation. The same pattern was established by Jayabalan et al. [21], who monitored kombucha fermentation of green tea sweetened with 10% sucrose until the 18th day.

In kombucha, acetic acid bacteria convert sucrose, glucose, and fructose to acetic acid, and yeasts convert sucrose to ethanol [23]. In our study, the ethanol contents of the kombucha samples increased after 5 days of fermentation (Figure 1B). The sugar contents rapidly decreased from day 5 to day 10 (Figure 1B). Jayabalan et al. [21] used approximately 100 g/L sucrose in black tea kombucha to prepare a beverage. The ethanol content gradually increased in the kombucha sample owing to the use of sucrose as an initial ingredient of the kombucha consortium [21]. The amount of sucrose gradually decreased after fermentation as a result of conversion to glucose and fructose by yeast cells [22].

The results of DPPH and ABTS scavenging activities demonstrated that all the kombucha beverages exhibited high antioxidant activity. These kombucha samples may be used as healthy beverages to suppress oxidative stress. We found that the DPPH and ABTS scavenging activity of the kombucha samples positively correlated with the fermentation time. BK was more effective at suppressing these free radicals than LK. Moreover, the suppression correlated with the IC_{50} values of the kombucha samples. These results are similar to those of a previous study [24], which found that the antioxidant activity increased with fermentation time. The IC_{50} value of the ABTS scavenging activity of OK was the most positive. It can be said that OK is more effective in inhibiting ABTS radical activity than BK and LK. Tea polyphenols (e.g., catechins, epicatechin, and epicatechin gallate) present in kombucha might play a role in the inhibition of oxidative stress [25]. Widowati et al. [26] showed that oolong tea exhibits antioxidant activity by scavenging DPPH free radicals with an IC_{50} value of 5.005 μg/mL. BK was the most effective at inhibiting the DPPH radical. This may have been because of kaempferol, which is an antioxidant compound.

The antioxidant capacity of kombucha depends on the raw materials and fermentation time. However, if the fermentation time is too long, it may cause harm to consumers due to the high content of organic acids produced [21]. The antioxidant activity of kombucha samples, as determined by DPPH and ABTS radical scavenging assays, positively correlated with the fermentation time. Such activity might be derived from the components in kombucha, which can transfer more electrons and hydrogen atoms to free radicals than tea infusion [24]. Several previous studies have reported that black tea kombucha exhibits higher antioxidant activity, as determined by the DPPH free radical scavenging method, than non-fermented black tea [27]. Our study showed that the TPC, TFC, DPPH, and ABTS radical scavenging capacities increased with the fermentation time (Table 1). Cardoso et al. [28] indicated that the increased antioxidant capacity of fermented plant-based food was derived from the accumulation of phenolic compounds and flavonoids. The major phenolic compounds identified in kombucha were gallocatechin 3-O-gallate, epigallocatechin, catechin, and quercetin [28]. Interestingly, the TPC and TFC of kombucha were 50% higher than those in unfermented tea [29]. It is well known that plant phenolics are highly effective free radical scavengers and antioxidants. Maisuthisakul et al. [30] reported that phenolic compounds and

their derivatives, such as phenolic acids and tannins, were strongly associated with antioxidants. Oolong tea leaves also contain various phenolic compounds, such as, caffeine, gallic acids, and catechins [29]. The butterfly pea flower is an extensively used medicinal herb. It contains various bioactive molecules, such as flavonols, glycosides, kaempferol, quercetin, and anthocyanins. Both kaempferol and quercetin are major flavonoids with high reducing power, and the ability to scavenge free radicals and donate H atoms to peroxy radicals [31].

Vegetables, fruits, and herbs contain various phytochemicals that can exert antioxidant effects in several ways, such as the non-enzymatic pathway, the inhibition of free radical-derived enzymes, and the regeneration of membrane-bound antioxidants in the human body [24-26]. Oxidative stress in human cells and tissues generally occurs when the balance between antioxidant defense mechanisms and ROS production is disrupted, e.g., due to the depletion of antioxidant activity and synthesis, accumulation of ROS, or both. Over-production of ROS, which can cause DNA damage at high levels, modification of proteins and lipids, activation of apoptosis, and production of pro-inflammatory and anti-inflammatory cytokines result in cell damage and death [32]. When oxidative stress occurs in the cells, the cells attempt to counteract and restore the redox balance by upregulating transcription factors, mRNA, and protein synthesis of many proteins and enzymes related to antioxidant effects [32]. Typically, ROS are scavenged by various antioxidative defense systems, but environmental stress induces excessive amounts of ROS, which cause an imbalance in the oxidation-reduction reaction.

In the present study, we used DPPH and ABTS scavenging assays to determine the antioxidant capacity of kombucha samples. We also determined the intracellular antioxidant activity by measuring ROS levels and the mRNA levels of antioxidant genes in HEK-293 cells. The MTT assay was used to determine the optimal concentration of kombucha samples and unfermented tea (OT, OK, BT, and BK). A concentration of 12.5 μL/mL was not toxic to HEK-293 cells. However, further studies are required to determine the toxicity in the human body following prolonged fermentation. The cellular antioxidant effects of the kombucha samples in HEK-293 cells inhibited the ROS production induced by H_2O_2 (Figure 3). BK can induce the synthesis of antioxidant enzymes such as catalase, GPx-1, GRe, Mn-SOD, and HO-1. Furthermore, the mRNA expression efficiency of BK was better than that of BT (unfermented tea).

Thus, the upregulation of antioxidant enzymes and non-enzymatic antioxidant compounds in these kombucha samples played an important role in the antioxidant defense mechanisms that removed free radicals, repaired oxidative damage, and protected the cells and tissues [33]. It should be noted that the effects of the kombucha samples on the induction of mRNA expression of antioxidant enzymes (Figure 4) did not reflect their potent DPPH and ABTS free radical scavenging activity (Table 1) because there are many phytochemicals in kombucha samples that have different effects. Therefore, further studies to determine the active compounds corresponding to the antioxidant activity of kombucha are worth conducting.

5. Conclusion

We identified the cellular oxidative defense mechanisms of BK and OK. These kombucha samples elicited antioxidant effects by upregulating the synthesis of antioxidant enzymes, including catalase, HO-1, GRe, GPx-1, and Mn-SOD, in HEK-293 cells. The kombucha beverages contained potent antioxidants, the levels of which correlated with the contents of phenolic compounds and flavonoids in the kombucha samples.

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