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Preparation and Functional Properties of *Dendropanax morbiferus* Kombucha

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ABSTRACT - This study aimed to prepare kombucha, a fermented tea beverage, containing *Dendropanax morbiferus* (DM) leaves and roots, and analyze its antioxidant and intracellular activities. We compared the pH change, total acidity, radical scavenging activity, and oxygen radical absorbance capacity (ORAC) of kombucha fermented with black tea alone and that with added DM leaves or roots during fermentation. Using RAW 264.7, we evaluated the effects of kombucha containing different DM parts on nitric oxide (NO) production and inflammation-related cytokine content in cells. Kombucha containing ethanol extracts of DM leaves (BTK-E-DML) and roots (BTK-E-DMR) showed higher radical scavenging activity and ORAC 3 d after fermentation than that prepared from black tea alone (BTK-Ori). In an *in vitro* experiment using RAW 264.7, samples were treated with 8 mg/mL kombucha considering cytotoxicity; the lipopolysaccharide (LPS)-induced NO content significantly reduced after BTK-E-DML and BTK-E-DMR treatments compared with that after BTK-Ori treatment. Additionally, the levels of interleukin-6 and tumor necrosis factor-alpha, which were LPS-stimulated inflammatory cytokines, significantly decreased in cells treated with BTK-E-DML and BTK-E-DMR 15 d after fermentation compared with those treated with BTK-Ori. In conclusion, these results demonstrate that kombucha fermented with the leaves and roots of DM increases antioxidant activity and can significantly regulate inflammatory responses at the cellular level.

Key words: *Dendropanax morbiferus*, Kombucha, Antioxidant, Anti-inflammation, Fermentation

The prevalence of chronic diseases such as diabetes and cardiovascular disease has gradually increased owing to unregulated dietary intake, lack of exercise, and environmental factors, and public interest on maintaining overall health has seen a corresponding growth[1]. In the case of beverages, research has focused on market expansion by reducing carbohydrate levels and addition of functional ingredients[2]. In the global market, functional drinks primarily comprise nutraceutical, energy, and sports drinks. They contain botanical extracts and other additives and are consumed to prevent dehydration, sustain continuous physical activity, and improve mental health. Fermented nonalcoholic beverages produced using various manufacturing technologies, raw materials, and microorganisms are also functional drinks with numerous health-promoting properties[3].

Kombucha is an acidic beverage produced by the fermentative action of a symbiotic culture of bacteria and yeast (SCOBY) on carbohydrate sources and contain numerous bioactive compounds derived from tea, juices, and herbal extracts[4]. Additionally, kombucha, a fermented tea with potent antioxidant and antibacterial properties, has been shown to promote gut health, enhance immune function, and prevent cardiovascular disease. The reported activity of kombucha is influenced by the type of tea used, composition of the SCOBY, and fermentation parameters. Currently, various natural products, such as ginseng berries, citrus fruits, and pineapple peels and cores, are being incorporated into the fermentation of kombucha, which is traditionally made using black, oolong, or green tea, to enhance its health-promoting properties[5-8].

Dendropanax morbiferus (DM) is a plant belonging to the *Araliaceae* family and contains numerous phytochemicals, such as rutin, chlorogenic acid, quercetin, and *p*-coumaric acid, which are used in food, medicine, and cosmetic applications[9,10]. Studies have reported that DM has various

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pharmacological effects, including antioxidant, anti-inflammatory, neuroprotective, hepatoprotective, and stress-reducing properties[10, 11]. In addition, DM has been shown to display anti-obesity and cholesterol-lowering activities in 3T3-L1 adipocytes and high-fat diet-induced obese mice[12]. Although the efficacy of DM in improving overall health and the taste and aroma components of kombucha have been studied individually using various approaches and models, the properties of kombucha prepared along with DM has not yet been reported.

This study aimed to prepare kombucha using *Gluconoacetobacter xylinus* and *Gluconobacter oxydans* by adding water or ethanol extracts of DM to black tea and to analyze the product characteristics. In addition, we examined the antioxidant activity of DM kombucha and measured its anti-inflammatory effects on LPS-induced RAW264.7 macrophages.

Materials and Methods

Materials

DM leaves (DML) and roots (DMR) were purchased from Jeju Island, and both DML and DMR extracts were prepared using 80% ethanol (v/v). The ethanol extracts were extracted at room temperature for 24 h and filtered (0.45 μm, EMD Millipore Inc., Billerica, MA, USA). After concentration using a rotary evaporator (Buchi R-100 Rotary Vap System, BUCHI Co., New Castle, DE, USA), the drying process was carried out using a freeze-dryer (FDB-5502, Operon Co., Gimpo, Korea) at -80.0°C with vacuum pressure 0.5 mm Torr for 60 h.

Fermentation

A flow diagram of the production of DM kombucha is presented in Fig. 1. After immersing two tea bags (black tea: Lipton, Hefei, China) in boiling water for 10 min, 75 g of white sugar and 12.5 g of mannitol were added to 450 mL of water, and a specific ratio of DM extract was added. After sterilization (SELA-AD100, SELABIOMEDITEK Co., Paju, Korea), it was cooled to 30°C, and then *G. xylinus* and *G. oxydans*, obtained from the Korean Culture Center of Microorganisms (Seoul, Korea), were inoculated and fermented at 30°C for 15 days. For the strain used, a single colony was cultured in the medium (5 g of yeast extract, 3 g of peptone, 25 g of mannitol in 1 L) for 48 h, and then the culture medium was removed using a centrifuge (Aventi J-E, Beckman Coulter, Brea, CA, USA).

pH and acidity measurement

A pH meter (FEP20; Mettler Toledo, Schwerzenbach, Switzerland) was used for measuring the pH. Acidity was analyzed using the neutralization titration method. After diluting the sample, 1% phenolphthalein indicator was added, and the sample was titrated against a 0.1 N NaOH solution. The acidity was quantitatively measured using acetic acid as the reference.

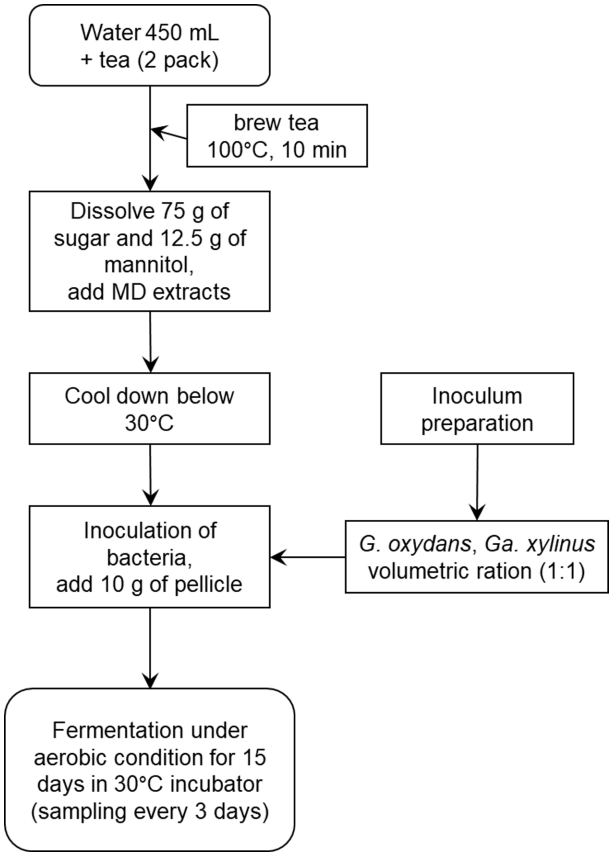

Fig. 1. Flow diagram for the preparation of kombucha with *Dendropanax morbiferus* leaf extract.

Radical scavenging and absorbance capacity assays

The antioxidant activity of DML kombucha was measured using 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, Sigma-Aldrich, St. Louis, MO, USA) and 2,2'-diphenylpicrylhydrazyl (DPPH, Sigma-Aldrich) assays. ABTS and DPPH radical-scavenging activities were measured using the methods described by Brand-Williams and Re, respectively[13,14]. The ABTS and DPPH scavenging capabilities were expressed as percentages, and ascorbic acid was used as a standard[15]. The oxygen radical absorbance capacity (ORAC) was analyzed using the method described by Ou et al., and Trolox was used as the standard[16]. The radical scavenging capacity of samples against free radical production by 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) was determined by analyzing the generation and quenching of peroxyl radicals.

Cell culture

The murine macrophage cell line, RAW264.7, was procured from the Korean Cell Line Bank (the Korean Cell Line Research Foundation, Seoul, Korea). The cells were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM) obtained from Cytiva (Seoul, Korea). The growth medium comprised 10% fetal bovine serum (FBS) sourced from Merck (Rahway, NJ, USA) and a penicillin-streptomycin solution obtained from HyClone (SV30010, Logan, UT, USA). Cell culture was performed in a controlled environment at 37°C in a 5% CO_2 incubator (Thermo Fisher Scientific, Waltham, MA, USA). Cell viability was measured using an EZ-Cytox kit per the manufacturer's instructions (DoGenBio, Seoul, Korea). For the cell viability assay, RAW264.7, cells (3×10^5) were treated with DML kombucha or original kombucha samples and cultured for 24 h. After the removal of treatments, 100 μL of EZ-Cytox diluted tenfold was added to each well. After incubation for 4 h, the plate was gently shaken for approximately 1 min, and the absorbance was measured at 450 nm.

Nitric oxide assay

Colorimetric reactions using Griess reagent were used to assess the production of nitric oxide (NO). Following the incubation period, the cells (3×10^5) were treated with kombucha samples in the presence of 1 μg/mL lipopolysaccharide (LPS) for an additional 24 h. To evaluate the transferred NO accumulation in the culture supernatants, we used equal volumes of Griess reagent, which comprised 0.2% N-(1-naphthyl)-ethylenediamine dihydrochloride and 1% sulfanilamide dissolved in 5% phosphoric acid. The resulting reaction solution was incubated for 30 min, and the absorbance was measured at 550 nm.

Measurement of inflammation-related cytokine production

RAW264.7 cells were seeded in 96-well cell culture plates at a concentration of 3×10^5 cells/mL. After removing the medium, the sample diluted with serum-free medium was added, followed by LPS at a final concentration of 1 μg/mL to stimulate for 20 h. Cell-free supernatants were collected, and cytokine levels were measured using enzyme-linked immunosorbent assay (ELISA) with mouse IL-6 and mouse TNF-α ELISA kits (BD OptEIA, San Diego, CA, USA). Absorbance was measured at 450 nm (Molecular Devices, San Jose, CA, USA).

Statistical analysis

All experimental outcomes of this investigation are expressed as the means±standard deviation (SD) using SPSS Version 24. All experimental results were analyzed using one-way ANOVA analysis, and Duncan's multiple range tests were employed to verify the significant difference between each experimental group. GraphPad Prism (Version 9.5.1; Informer Technologies, Los Angeles, CA, USA) was used to generate graphs from ANOVA and Tukey's test results and to determine significant differences between the samples. $P<0.05$, $P<0.01$, and $P<0.001$ were considered significant and marked with *, **, and ***, respectively.

Results and Discussion

Effects of DM extracts on pH and acidity in kombucha

A significant decrease was observed in the pH of original kombucha from black tea (BTK-Ori), kombucha using ethanol extract of DM leaves (BTK-E-DML), and kombucha using ethanol extract of DM roots (BTK-E-DMR) as the fermentation period increased (Table 1). Compared with BTK-Ori, the pH of BTK-E-DML and BTK-E-DMR tended to decrease rapidly after the 3rd day of fermentation but showed similar values on the 15th day of fermentation. Total acidity, similar to the change in pH, increased significantly as the fermentation period increased for all samples. On the 15th day of fermentation, a higher total acidity was observed in BTK-E-DML and BTK-E-DMR than in BTK-Ori.

Table 1. Changes in pH and total acidity of BTK-Ori and BTK-DM kombucha during fermentation

Fermentation period (days)	BTK-Ori		BTK-E-DML		BTK-E-DMR	
	pH	Total acidity (%)	pH	Total acidity (%)	pH	Total acidity (%)
0	3.96±0.00^{f}	0.03±0.00^{a}	4.02±0.01^{f}	0.03±0.00^{a}	4.02±0.01^{f}	0.03±0.00^{a}
3	3.01±0.01^{e}	0.07±0.02^{b}	2.81±0.01^{e}	0.12±0.03^{b}	2.72±0.01^{e}	0.10±0.02^{b}
6	2.45±0.01^{d}	0.22±0.02^{c}	2.67±0.00^{d}	0.24±0.00^{c}	2.33±0.01^{d}	0.16±0.02^{c}
9	2.33±0.00^{c}	0.32±0.02^{d}	2.35±0.00^{c}	0.31±0.02^{d}	2.17±0.01^{c}	0.36±0.03^{d}
12	2.28±0.00^{b}	0.32±0.02^{e}	2.24±0.01^{b}	0.34±0.02^{d}	2.12±0.01^{b}	0.38±0.02^{de}
15	2.07±0.01^{a}	0.35±0.02^{f}	2.16±0.01^{a}	0.40±0.02^{e}	2.09±0.00^{a}	0.40±0.02^{e}

Data are reported as means±standard deviation (SD) of three separate studies. Different letters indicate significant differences using Duncan's multiple range test ($P<0.05$). BTK-Ori: black tea kombucha; BTK-E-DML: black tea with DM leaf ethanolic extract kombucha; BTK-E-DMR: black tea with DM root ethanolic extract kombucha.

Table 2. Changes in radical scavenging and absorbance capacities of BTK-Ori and BTK-DM kombucha during fermentation

Fermentation period (days)	BTK-Ori			BTK-E-DML			BTK-E-DMR		
	ABTS	DPPH	ORAC	ABTS	DPPH	ORAC	ABTS	DPPH	ORAC
0	94.93±0.10^{a}	81.90±0.10^{a}	8.30±0.17^{a}	95.15±0.26^{a}	81.69±0.47^{a}	8.60±0.37^{a}	95.43±0.08^{a}	81.92±0.19^{a}	7.94±0.30^{a}
3	94.87±0.10^{a}	82.82±1.08^{b}	11.26±0.66^{c}	95.60±0.06^{d}	84.43±0.20^{b}	10.80±0.37^{bc}	95.55±0.05^{ab}	84.61±0.15^{b}	10.39±0.03^{d}
6	95.22±0.05^{b}	84.99±0.17^{c}	10.55±0.29^{b}	95.33±0.08^{abc}	85.42±0.11^{c}	11.29±0.26^{cd}	95.64±0.00^{b}	85.45±0.09^{c}	9.66±0.36^{c}
9	95.33±0.26^{bc}	85.18±0.15^{c}	10.68±0.19^{bc}	95.54±0.04^{cd}	85.55±0.18^{c}	11.48±0.21^{d}	95.59±0.13^{b}	85.46±0.09^{c}	10.00±0.12^{c}
12	95.54±0.10^{cd}	84.83±0.07^{c}	10.74±0.03^{bc}	95.26±0.06^{ab}	85.80±0.07^{c}	11.64±0.17^{d}	95.54±0.04^{ab}	85.61±0.04^{c}	10.87±0.09^{e}
15	95.63±0.02^{d}	85.57±0.29^{c}	8.58±0.14^{a}	95.38±0.02^{bcd}	85.81±0.08^{c}	10.69±0.24^{b}	95.41±0.09^{a}	86.09±0.07^{d}	9.13±0.12^{b}

Data are reported as means±standard deviation (SD) of three separate studies. Different letters indicate significant differences using Duncan's multiple range test (P<0.05). BTK-Ori: black tea kombucha; BTK-E-DML: black tea with DM leaf ethanolic extract kombucha; BTK-E-DMR: black tea with DM root ethanolic extract kombucha.

Kombucha fermentation can be detected by an increase in organic acids and a decrease in pH. The pH change in the mixture and the increased amount of specific organic acids can affect microorganism development. These changes have also been reported to affect the chemical composition of the beverage[17].

Effects of DM extracts on radical scavenging and absorbance capacities

Table 2 presents the changes in the radical scavenging and absorbance capacities of BTK-Ori and BTK-DM kombucha during fermentation. The ABTS radical scavenging rate (%) increased at the end of the fermentation process in BTK-Ori. Additionally, the DPPH radical scavenging rate of BTK-Ori increased significantly until the 6th day of fermentation (P< 0.05) and then showed no significant change until the 15th day. The oxygen radical absorbance capacity of BTK-Ori was confirmed to be significantly high on the 3rd day of fermentation (P<0.05) and then gradually decreased; ultimately on the 15th day of fermentation, the results were similar to those observed before fermentation. The BTK-E-DML sample showed a high ABTS scavenging rate on the 3rd day of fermentation (P<0.05), and a slight decrease was observed as the fermentation period increased. The DPPH scavenging rate was high on the 3rd day of fermentation and remained at the same level until the end of fermentation. The ORAC absorbance rate significantly increased on the 9th and 12th days of fermentation and tended to decrease on the 15th day. The ABTS scavenging rate of the BTK-E-DMR sample was significantly higher on the 3rd day of fermentation (P< 0.05), and the DPPH scavenging rate was significantly increased on the 15th day (P<0.05). The ORAC absorbance rate in the BTK-E-DMR sample was high on the 3rd day of fermentation and tended to decrease gradually. Polyphenols, a class of bioactive compounds found in various plant-based extracts, exhibit excellent antioxidant properties and contribute to the maintenance and promotion of health[18]. Rutin, contained in DM, is a glycoside derived from flavonolic aglycone quercetin and rutinose, and has antioxidant properties[19]. Chlorogenic acid, a water-soluble polyphenolic phenylacrylate compound, exhibits antioxidant and anti-inflammatory properties[20]. The 60% ethanol extract of DML reported exhibited activities with an IC_{50} value of 3.58 mg/mL, and fermented DML showed an IC_{50} value of 57.52 ug/mL when hydroxyl-radical scavenging activity was analyzed[21,22].

Effects of DM extracts on NO production in RAW 264.7 cells

Cell viability assays of mouse macrophage RAW264.7 cells showed cell viability above 89% in all samples (Fig. 2). Because the viability of BTK-Ori- and BTK-E-DMR-treated cells significantly decreased at a concentration of 16 mg/mL on the 15th day, a sample with a concentration of 8 mg/mL was selected for the experiment. The results of this experiment confirmed that none of the kombucha samples were cytotoxic to macrophages. In terms of cell activity, BTK-Ori exhibited the highest increase on days 6 and 9, with the highest cell viability observed on day 9. In the BTK sample, cell viability reached 89.07% at a concentration of 16 mg/mL of BTK-Ori on day 15. Similarly, cell viability in BTK-E-DMR reached 97.50% and was not found to be lower than 100% in BTK-E-DML. These results suggest that both DMR and DML have a positive effect on cell proliferation.

The nitric oxide assay was conducted to assess inflammation induced in RAW264.7 macrophages using LPS (1 μg/mL; Fig. 3). Cells were treated with kombucha samples collected at regular fermentation intervals, and the concentrations of NO in the cultured medium were compared. The results demonstrated that the LPS-positive control group had the highest NO concentration. NO production was significantly lower in cells treated with

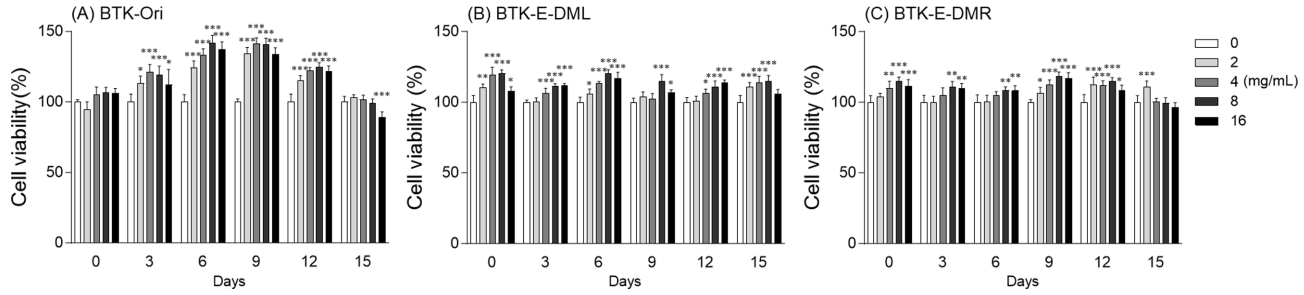

Fig. 2. Effects of *Dendropanax morbiferus* kombucha on cell viability in RAW 264.7 cells. Values are expressed as means±standard deviation (SD) for each group. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ by Tukey's multiple range test. (B) BTK-Ori: black tea kombucha; (D) BTK-E-DML: black tea with DM leave ethanolic extract kombucha; (F) BTK-E-DMR: black tea with DM root ethanolic extract kombucha.

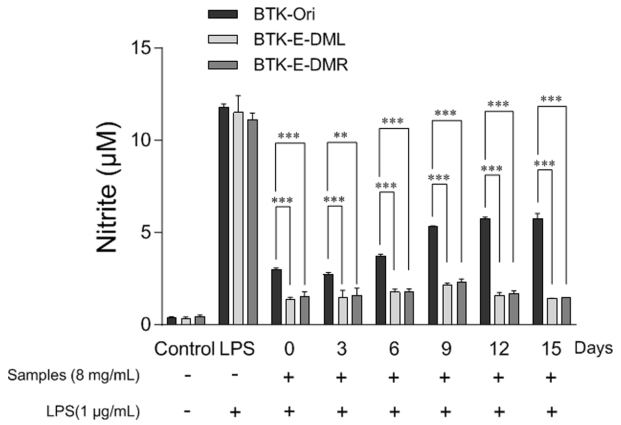

Fig. 3. Effects of *Dendropanax morbiferus* kombucha on nitric oxide production in RAW 264.7 cells. Values are expressed as means±standard deviation (SD) for each group. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ by Tukey's multiple range test. BTK-Ori: black tea kombucha; (A) BTK-E-DML: black tea with DM leave ethanolic extract kombucha; (C) BTK-E-DMR: black tea with DM root ethanolic extract kombucha.

kombucha and LPS than in the LPS-positive control group. On comparing with the NO concentrations in the BTK-Ori and BTK-E-DML groups, no difference was observed with LPS-treated groups. However, the BTK-E-DML group showed significantly lower NO production than the BTK-Ori group, particularly the cells treated with kombucha fermented between 12 and 15 days. Similar results were observed on comparing BTK-Ori and BTK-E-DMR, indicating that BTK-E-DMR had significantly lower NO production than BTK-Ori. On comparing NO release on different days of BTK-Ori treatment, the samples initially reached their lowest point on the third day and then exhibited an upward trend. However, BTK-E-DML and BTK-E-DMR-treated group showed a decrease in NO release following treatment with samples fermented for 12 to 15 days, after an initial increase for samples fermented for 6 and 9 days. NO and pro-inflammatory cytokines, such as interleukins (IL-1, IL-6, and IL-12) and TNF-α, are among the various inflammatory mediators and cytokines that are released in large quantities by activated macrophages[23]. NO regulates physiological functions such as vasodilation, neurotransmission, and immune response *in vivo*. However, NO overexpression can increase inflammatory response, damage cells and tissues, and lead to chronic inflammation[24]. Studies have confirmed that the polyphenols in DM have anti-inflammatory properties that prevent the production and activity of numerous pro-inflammatory mediators[25].

Effects of DM extracts on inflammation-related cytokines in RAW 264.7 cells

Results of ELISA on cytokine levels revealed that LPS significantly stimulated IL-6 and TNF-α production. In samples supplemented with kombucha, the production of both cytokines was significantly lower than in the LPS-positive group (Fig. 4). Compared with that in the LPS-positive control group, the IL-6 production of BTK-Ori, BTK-E-DML, and BTK-E-DMR decreased by 78% on the 15th day of fermentation (90% and 88%, respectively), indicating that kombucha has potential anti-inflammatory effects. Further, IL-6 production was significantly lower in BTK-E-DML and BTK-E-DMR than in BTK-Ori. The results regarding TNF-α production showed that cells treated with kombucha fermented until the 15th day had significantly lower levels compared to those of the LPS-positive control group. Additionally, both BTK-E-DML and BTK-E-DMR exhibited significantly lower TNF-α production compared to BTK-Ori. IL-6 is a multifunctional cytokine that plays an important role in immune system regulation and host defense against pathogens and acute stress[26]. TNF-α is an inflammatory cytokine produced by macrophages and monocytes in acute inflammation and is responsible for extensive cell signaling leading to necrosis or apoptosis[27]. Therefore, the reduction of TNF-α and IL-6 levels indicated attenuated LPS-induced inflammation in RAW264.7 macrophages. These results may be due to the higher polyphenol contents of DML and DMR.

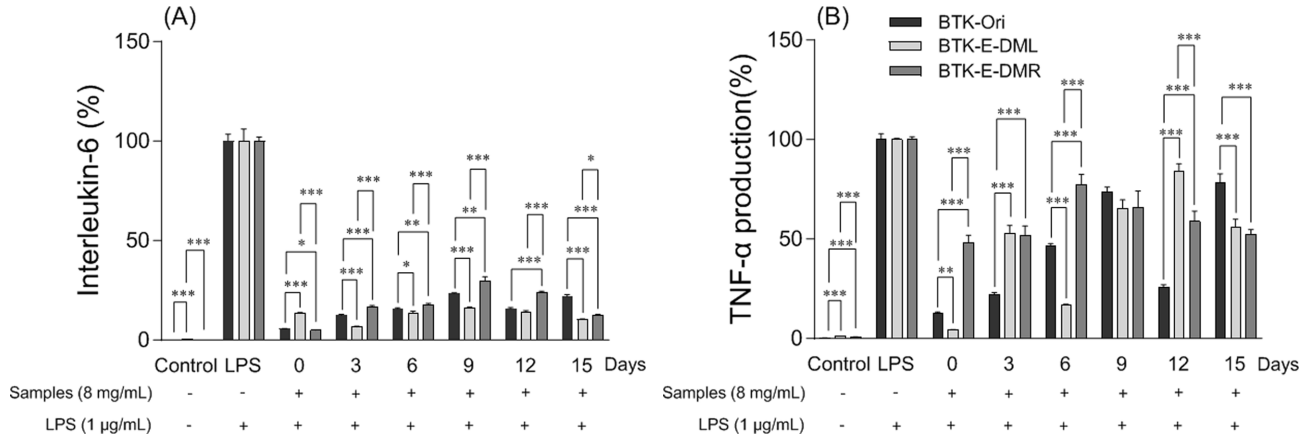

Fig. 4. Effects of *Dendropanax morbiferus* kombucha on interleukin-6 and tumor necrosis factor-alpha levels in RAW 264.7 cells. Values are expressed as means±standard deviation (SD) for each group. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ by Turkey's multiple range test. BTK-Ori: black tea kombucha; BTK-E-DML: black tea with DM leave ethanolic extract kombucha; BTK-E-DMR: black tea with DM root ethanolic extract kombucha.

Polyphenols, including catechin, rutin, quercetin, and chlorogenic acid, reduce NO production and downregulate the production of inflammatory mediators such as TNF- α, IL-1β, and IL-6[28,29].

국문요약

본 연구는 황칠나무(*Dendropanax morbiferus*, DM)의 잎과 뿌리 추출물을 함유한 발효음료인 콤부차를 제조하고, 소재의 항산화 및 세포내 활성을 분석하였다. 홍차만으로 발효한 콤부차와 발효 과정에서 DM 잎이나 뿌리 추출물을 첨가한 홍차를 사용하여 발효한 콤부차의 pH 변화, 전체 산도, 라디칼 소거능을 비교하였다. 또한 RAW 264.7 세포주를 활용하여 DM의 잎이나 뿌리 추출물을 함유한 콤부차가 세포 내 산화질소(NO) 생성 및 염증 관련 사이토카인 함량에 미치는 영향을 평가하였다. DM 잎(BTK-E-DML)과 뿌리(BTK-E-DMR)의 에탄올 추출물을 함유한 콤부차는 홍차만으로 제조한 콤부차(BTK-Ori)보다 발효 시작 3일 후 더 높은 라디칼 소거능을 나타내었다. RAW264.7 세포주를 이용한 *in vitro* 실험에서 세포독성을 고려하여 샘플을 8 mg/mL 콤부차로 처리한 결과, 지질다당류(LPS)로 유발된 NO 함량이 BTK-Ori 처리와 비교하였을 때 BTK-E-DML 및 BTK-E-DMR 처리에서 유의하게 감소하였다. 또한 LPS에 의해 자극되는 염증성 사이토카인인 인터루킨-6와 종양괴사인자-알파의 수준은 발효 15일 후 BTK-E-DML과 BTK-E-DMR을 처리한 세포에서 대조군에 비해 유의하게 감소하였다. 종합하면, 이러한 결과들은 DM의 잎 및 뿌리와 함께 발효된 콤부차는 항산화 활성이 증가되고, 세포 수준에서 염증 반응을 유의하게 조절할 수 있음을 입증하였다.

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Conflict of interests

The authors declare no potential conflict of interest.

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