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Suppression of Inflammatory Mediators by Ethanol Extracts of *Pennisetum purpureum* S. (Napiergrass Taishigrass no. 2) in Activated RAW 264.7 Macrophages

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Abstract Napiergrass Taishigrass no. 2 (NT2) is a new hybrid of napiergrass (*Pennisetum purpureum* S.), and is commonly added as an ingredient in bioorganic energy soup, a plant-based drink which is thought to have health-promoting effects. Thus, NT2 is hypothesized to possess biological functions. In this study, we explored the effects of NT2 ethanol extracts on *in vitro* antioxidant activities and on modulation of immune mediators in RAW 264.7 macrophages. Results showed that ethanol extracts contained significant amount of polyphenols and chlorophylls, and possessed *in vitro* ferric-reducing antioxidant power (FRAP) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) antioxidative activities. The ethanol extracts did not show immunomodulatory activities in macrophages without lipopolysaccharide (LPS, 100 ng/ml) treatment, but significantly suppressed the LPS-stimulated production of inflammatory mediators, including nitric oxide (NO), interleukin (IL)-6, and tumor necrosis factor (TNF)-α, and such effects were associated with decreased expression of inducible NO synthase (iNOS) protein and cluster of differentiation 14 (CD14) mRNA, suggesting that NT2 has anti-inflammatory activities. On the other hand, the extracts increased matrix metalloproteinase (MMP)-9 activity, but showed no effects on Toll-like receptor (TLR) expression. In conclusion, ethanol extracts of NT2 possessed *in vitro* antioxidative activities and exhibited anti-inflammatory activities by decreasing NO, IL-6, and TNF-α production in association with decreased CD14 mRNA expression in LPS-induced RAW 264.7 macrophages, indicating its potential roles on health promotion and prentive effects against chronic diseases and such effects were and it's *in vitro* antioxidant activities, suggesting its potential roles on health promotion.

Keywords: *napiergrass, macrophage, lipopolysaccharide (LPS), anti-inflammation, antioxidation*

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1. Introduction

The immune system functions as one of the important lines of host defense against foreign pathogens in the body, and macrophages play significant roles in modulating immune functions. After being exposed to the bacterial endotoxin, lipopolysaccharide (LPS), for example, macrophages are activated to increase the production of various inflammatory mediators, including nitric oxide (NO), proinflammatory cytokines, such as interleukin (IL)-6, IL-1β, and tumor necrosis factor (TNF)-α, as well as matrix metalloproteinases (MMPs). The membrane receptors, cluster of differentiation 14 (CD14) and Toll-like receptors (TLRs), and the transcription factor, nuclear factor (NF)-κB, were indicated to be involved in this activation process [1,2,3,4]. Under physiological conditions, these mediators are essential for antipathogenic processes and are well-regulated. However, dysregulated production of these mediators is linked to various pathological or oxidative conditions, such as a

chronic inflammation status, autoimmune diseases, cardiovascular diseases, obesity, and cancers [5,6]. Therefore, compounds that possess antioxidative activities or suppress the overproduction of proinflammatory cytokines may play protective roles against inflammation and its associated diseases [7].

Napiergrass (*Pennisetum purpureum* S.) is widely used as livestock forage in Taiwan. Napiergrass Taishigrass no. 2 (NT2) is a new hybrid with shorter fibers and a better taste, so it is commonly used as one of the major ingredients in bioorganic energy soup. The bioorganic energy soup is a plant-based beverage, and is commonly consumed by healthy and illness pollutions, because it is thought to have health-promoting effects. Nowadays, scientific evidence regarding napiergrass has mainly focused on animal feeding [8,9] and renewable energy sources [10,11], but less information on its health-promoting effects was reported. Previously, the boiling-water extract of purple napiergrass was indicated to prevent *in vitro* low-density lipoprotein from oxidation, increase antioxidative enzyme activities in murine BNL hepatocytes, and decrease NO production in LPS-induced RAW 264.7 macrophages [12]. In addition, the boiling-water extract of NT2 also has *in vitro* antioxidative activities as determined by 1,1-diphenyl-2-picrylhydrazyl (DPPH) and the ferric-reducing antioxidant power (FRAP) [13]. Because only studies on boiling-water extract were reported, this study investigated the antioxidation and immunomodulation activities NT2, especially the ethanol extracts. Total polyphenol, coumaric acid, and chlorophylls contents, as well as *in vitro* antioxidant FRAP and DPPH of NT2 extracts were examined. The effects of ethanol extracts on immune-associated factors in both LPS-untreated and LPS-treated macrophages were also determined.

2. Materials and Methods

2.1. Chemicals and Biochemicals

RPMI 1640 medium, fetal bovine serum (FBS), and sodium bicarbonate were purchased from GIBCO BRL (Grand Island, NY, USA). Folin-Ciocalteau reagent, gallic acid, DPPH, LPS, absolute ethanol (≥ 99.8%), dimethyl sulfoxide (DMSO), sodium nitrite, and N-(1-naphthyl)ethylenediamine dihydrochloride were obtained from Sigma Chemical (St. Louis, MO, USA).

2.2. Preparation of NT2 Extracts

Freeze-dried NT2 powder purchased from Baoyuan Company (Taichung, Taiwan) was extracted with absolute ethanol or double-distilled water (ddH2O) (1:10 (w/v)) by a vertical rotating mixer for 24 h at room temperature. After centrifugation at 1600 xg, 4°C for 15 min, the supernatants were condensed with a rotary evaporator and adjusted to a final concentration of 1 or 2 g/ml with absolute ethanol or ddH2O, respectively. After being sterilized using 0.22-μm filters, the obtained ethanol and water extracts were used in the following experiments.

2.3. Determination of Total Phenols, Coumaric Acid, Chlorophylls, and *in vitro* Antioxidative Activities

Total polyphenol contents (TPCs) in NT2 extracts were determined according to methods described previously [14]. Briefly, the extracts were reacted with 50% Folin-Ciocalteau's reagent for 10 min, followed by addition of 2% Na_2CO_3 for another 45 min, and the OD_{755} nm was determined. Gallic acid was served as a standard, and results are expressed as milligrams of gallic acid equivalents (GAE) per gram of dry NT2.

Being one of the most abundant polyphenols in NT2 [13], coumaric acid was determined by directly measuring the diluted extracts (1000 x dilution) at 305 nm due to its mesomeric effect of absorbing UV light at low concentrations (1~10 μM) [15], and purified coumaric acid (Sigma, St. Louis, MO, USA) served as a standard. Similar to coumaric acid, chlorophylls in the extracts were directly determined spectrophotometrically at wavelengths of 665 and 649 nm. Contents of chlorophylls a and b were calculated using the formulas $13.70 \times A_{665}$-$5.76 \times A_{649}$ and $-7.60 \times A_{665} + 25.8 \times A_{649}$, respectively [16].

To explore the antioxidative activities of the extracts, antioxidant power was measured by reacting it with a FRAP reagent followed by determining the absorbance at 593 nm using trolox as a standard [17], and results are expressed as trolox equivalents (TE) in mM/g dry weight. Free radical-scavenging activity was analyzed by the addition of a DPPH solution. After determining the absorbance at 515 nm, the scavenging ability was calculated according to the following formula [18]:

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

2.4. Cell Culture and Cell Proliferation

Murine RAW 264.7 macrophages were obtained from the Bioresource Collection and Research Center (BCRC #60001, Hsinchu, Taiwan). Cells were maintained in 10% FBS supplemented with RPMI 1640 medium at 37°C in a 5% CO_2 humidified atmosphere. After being seeded for 1 day, cells were changed to 1% FBS supplemented with RPMI 1640 medium and treated with various concentrations of ethanol extracts in the presence or absence of LPS (100 ng/ml) for 24 h. Absolute ethanol was added as a negative control, and ethanol added in the medium was 0.1%. Cells and media were then collected for analyses.

To evaluate the effects of the extracts on cell proliferation, cells (10^4/well) were treated with NT2 extracts in the presence or absence of LPS for 24 h, and cell proliferation was determined using a CellTiter 96®Aqueous One Solution cell proliferation MTS assay kit (Promega, Madison, WI, USA).

2.5. Production of NO, Cytokines and MMPs

To understand the effects of NT2 on immune mediators, macrophages were treated with different concentrations of

extracts in the presence or absence of LPS (100 ng/ml) for 24 h, and secretions of NO, IL-6, and TNF-α by macrophages were analyzed. The nitrite content was determined after adding the Griess reagent (1% sulfanilamide/0.1% naphthylethylene diaminedihydrochloride in 5% H_3PO_4) with absorbance at 590 nm [19]. IL-6 and TNF-α were detected by commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D System, Minneapolis, MN, USA) following the manufacturer's instructions.

MMPs are a family of enzymes that are involved in extracellular matrix degradation, and were indicated to be associated with immune functions [20]. MMP activities were determined by a gelatin zymographic analysis. Briefly, culture medium (5 µl) was separated by 1 mg/ml gelatin containing sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) [21]. Clear bands of MMP-2 (72 kDa) and MMP-9 (92 kDa) on the gel were observed after staining with 0.5% Coomassie blue R-250, and were quantified using the Image-Pro Plus software package (Media Cybernetics, Rockville, MD, USA).

2.6. Western Blot Analysis and Real-time Polymerase Chain Reaction (PCR)

To understand the involvement of iNOS protein in modulation of NO production, cell lysate containing 15 µg total cellular protein was separated by a 10% SDS-PAGE, and was transferred onto a polyvinylidene difluoride membrane. Expression of the iNOS protein was detected by adding anti-mouse iNOS (1:200) and anti-mouse β-actin (1:1000) antibodies followed by anti-rabbit immunoglobulin G (IgG)-horseradish peroxidase (HRP) and anti-mouse IgG-HRP (1:10000), respectively (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Images were observed after reacting with enhanced chemiluminescence (ECL) Western blotting detection reagent and exposure to chemiluminescent detection film. β-Actin served as an internal control.

To explore the effects of NT2 on mRNA expressions of immune-associated factors, total cellular RNA was extracted by the TRIzol® reagent (Ambion, Carlsbad, CA, USA), followed by reverse-transcription to complementary (c)DNA by reaction with oligo(dT)$_{12-18}$ and SuperScript® III reverse transcriptase (InvitrogenTM, Carlsbad, CA, USA). The obtained cDNA was used to conduct the PCR after adding various primers (Table 1) to an ABI 7300 Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA).

Table 1. Primer sequences used in the RT-PCR

Primer	Sequence	Reference
MMP-9	F: 5'-AGTCCGGCAGACAATCCTTGCA-3' R: 5'-ATCCACGCGAATGACGCTCTGG-3'	[22]
CD14	F: 5'-CCACCGCTGTAAAGGAAA-3' R: 5'-CAGGGCTCCGAATAGAATC-3'	[23]
TLR4	F: 5'-GCAGAAAATGCCAGGATGA-3' R: 5'-TGTTTCAATTTCACACCTGGA -3'	[23]
GAPDH	F: 5'- TTCACCACCATGGAGAAGGC -3' R: 5'- GGCATGGACTGTGGTCATGA -3'	[24]

F, forward; R, reverse.

2.7. Statistical Analysis

All values are expressed as the mean ± standard deviation (SD), and the experiments were independently repeated at least 2 times. All statistical analyses were performed using SPSS vers. 19.0 (IBM, Armonk, NY, USA). A one-way analysis of variance (ANOVA) followed by post-hoc Tukey's test was performed to compare differences between groups. A p value of <0.05 was considered significantly different.

3. Results

Total polyphenols and chlorophylls in both water and ethanol extracts were determined. Table 2 shows that higher levels of polyphenols, chlorophylls, and coumaric acid, one of the major phenolic compounds in NT2 [13], were detected in ethanol extracts. As expected, chlorophylls were not detected in water extracts due to its hydrophilicity. These results paralleled the antioxidant activities, in which higher FRAP and DPPH levels were observed in the ethanol extracts (Table 2). Therefore, the ethanol extracts were used in the cell culture experiments.

Results obtained from the MTS assay showed that the ethanol extracts of NT2, at concentrations ranging 0~2.5 mg/ml showed no effects on cell proliferation, regardless of the presence or absence of LPS. In the absence of LPS, cell proliferation even slightly increased (13% compared to the control group) at a concentration of 2.5 mg/ml (Figure 1).

Table 2. Polyphenol and chlorophyll contents, as well as *in vitro* antioxidant activities of napiergrass Taishigrass no. 2 (NT2) extracts

	Extract	
	Ethanol	Water
Total polyphenols (mg GAE)	0.69 ± 0.04	0.14 ± 0.004
Coumaric acid (µg)	510.5 ± 29.1	376.9 ± 7.5
Chlorophyll a (µg)	1051.8 ± 9.1	ND
Chlorophyll b (µg)	445.1 ± 5.4	ND
Antioxidant activity		
FRAP (mM TE)	11.87 ± 0.34	0.81 ± 0.03
DPPH (%)	73.9 ± 0.7	56.3 ± 1.5

Phenol and chlorophyll contents, and antioxidant activities of the ethanol and water extracts were assessed from 1 g of dry NT2 powder. Values were presented as the mean ± SD, n = 3. GAE, gallic acid equivalents; FRAP, ferric-reducing antioxidant power; TE, trolox equivalents; DPPH, 2,2-diphenyl-1-picrylhydrazyl; ND, not detectable.

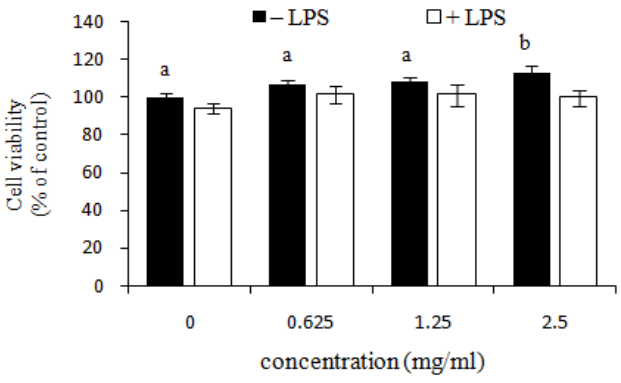

Figure 1. Effects of ethanol extracts of napiergrass Taishigrass no. 2 (NT2) on cell proliferation. RAW 264.7 macrophages were treated with various concentrations of extracts as indicated, in the absence or presence of LPS (100 ng/ml) for 24 h. Cell proliferation was measured by MTS assay. Values are means ± SDs (n = 3) from two independent experiments. ab, data that share no letters significantly differ by a one-way ANOVA (p < 0.05)

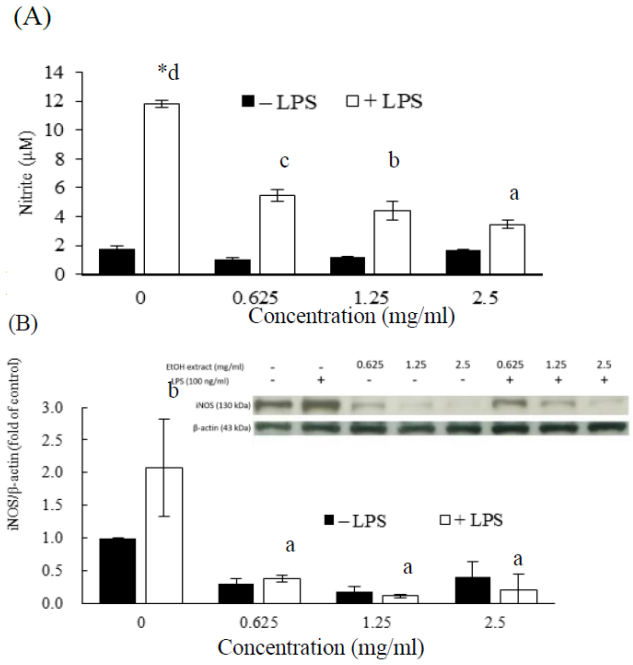

Figure 2. Effects of ethanol extracts of napiergrass Taishigrass no. 2 (NT2) on nitric oxide (NO) production (A) and inducible NO synthase (iNOS) protein expression (B) by RAW 264.7 macrophages. Cells were treated with different concentrations of extracts, in the presence or absence of lipopolysaccharide (LPS) (100 ng/ml), for 24 h. NO production was measured by the Griess reaction. iNOS protein expression was determined by Western blotting and the relative expressions were normalized to β-actin. Data are expressed as the mean ± SD from three independent experiments. * Significantly differs from the 0 (ethanol vehicle control) by Student's t-test. ab, data that share no letters significantly differ by a one-way ANOVA ($p < 0.05$)

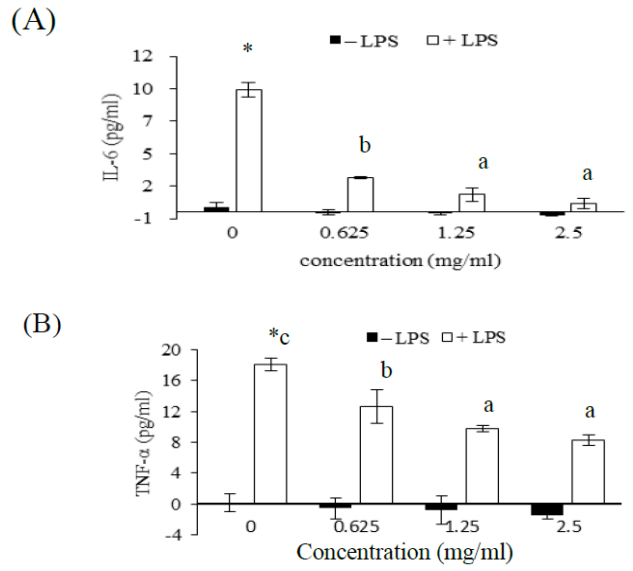

Figure 3. Effects of ethanol extracts of napiergrass Taishigrass no. 2 (NT2) on the production of interleukin (IL)-6 (A) and tumor necrosis factor (TNF)-α (B) by RAW 264.7 macrophages. Cells were treated with different concentrations of extracts, in the presence or absence of lipopolysaccharide (LPS) (100 ng/ml), for 24 h. Secretion of IL-6 and TNF-α into the cultured medium was analyzed by commercial assay kits according to the manufacturers' instructions. Data are expressed as the mean ± SD from three independent experiments. * Significantly differs from the 0 (ethanol vehicle control) by Student's t-test. abc, data that share no letters significantly differ by a one-way ANOVA ($p < 0.05$)

Figure 2 shows that the ethanol extracts did not affect NO production by macrophages, but concentration-dependently suppressed LPS-stimulated NO production by activated macrophages (Figure 2A). The decrease in NO production was mainly due to inhibition of iNOS expression, because stimulated expression of the iNOS protein was blocked by NT2 ethanol extracts (Figure 2B). Parallel to results obtained for NO, ethanol extracts did not affect the production of the proinflammatory cytokines, IL-6 or TNF-α, but significantly suppressed LPS-stimulated production of IL-6 and TNF-α in concentration-dependent manners (Figure 3). In addition to these immune mediators, the activity of MMP-9, but not MMP-2, was induced by LPS treatment, and was further enhanced by ethanol extracts, regardless of the presence of LPS (Figure 4). Being associated with LPS-stimulated inflammatory activities, the expressions of CD14 and TRL4 mRNAs were determined. As shown in Figure 5, NT2 ethanol extracts suppressed the mRNA expression of CD14, but showed no effects on TLR4 in LPS-activated macrophages.

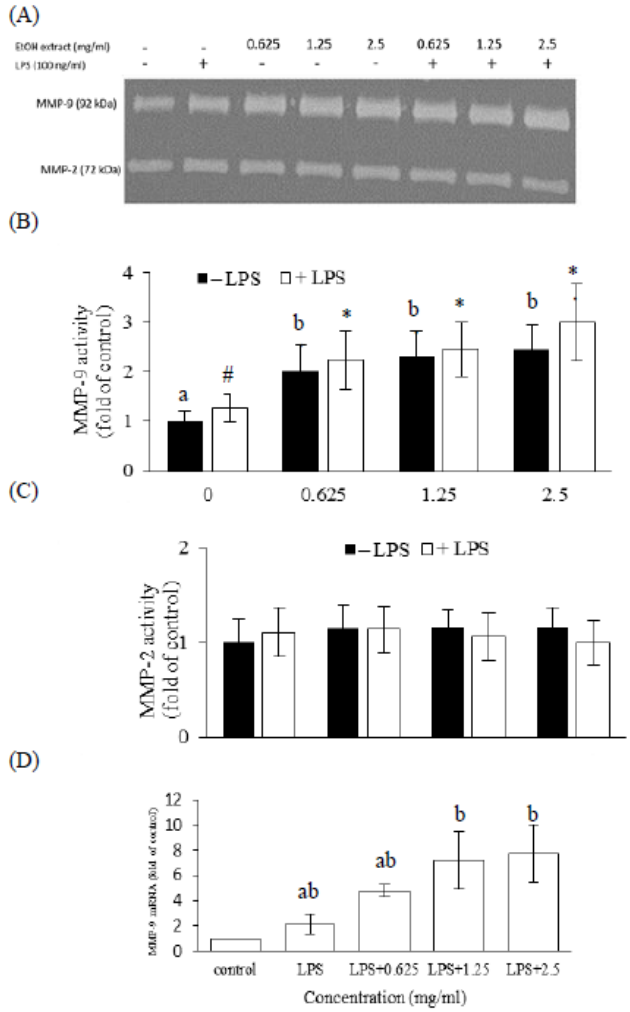

Figure 4. Effects of ethanol extracts of napiergrass Taishigrass no. 2 (NT2) on matrix metalloproteinase (MMP) activities in RAW 264.7 macrophages. Cells were treated with different concentrations of extracts in the absence or presence of lipopolysaccharide (LPS) (100 ng/ml) for 24 h. The medium was collected to analyze MMP activities by gelatin zymography (A), and clear bands were quantified by Image-Pro Plus software (B, C). mRNA expression of MMP-9 was determined by a real-time PCR (D). Data are expressed as the mean ± SD from three independent experiments. a,b,#,*, data that share no symbols significantly differ within either LPS-untreated or LPS-treated groups by a one-way ANOVA ($p < 0.05$). MMP-2, $p > 0.05$

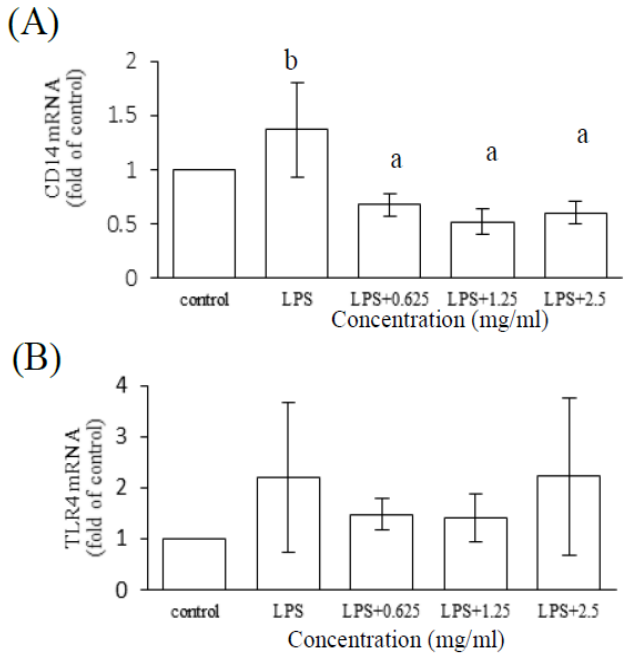

Figure 5. Effects of ethanol extracts of napiergrass Taishigrass no. 2 (NT2) on mRNA expressions of cluster of differentiation 14 (CD14) (A) and Toll-like receptor 4 (TLR4) (B) by RAW 264.7 macrophages. Cells were treated with various concentrations of the extracts in the presence of lipopolysaccharide (LPS) (100 ng/ml) for 24 h. Total cellular RNA was isolated and analyzed for mRNA expression with a real-time PCR. The relative gene expression was obtained after normalization with GAPDH. Data are expressed as the mean ± SD (n = 3). ab, data that share no letters significantly differ by a one-way ANOVA ($p < 0.05$). TLR4, $p > 0.05$

4. Discussion

In the present study, we first demonstrated that the ethanol extracts of NT2 did not affect innate immunity, but possessed anti-inflammatory activities in stimulated macrophages. Large amounts of NO produced in response to the bacterial endotoxin, LPS, or cytokines play important roles in inflammation. Prolonged inflammation is a risk factor for various inflammatory diseases and pathological conditions, including rheumatoid arthritis, cardiovascular diseases, cancers, etc. [25,26]. In addition, high concentrations of NO may react with reactive oxygen species, including superoxide, produced by macrophages leading to the generation of peroxynitrite ($ONOO^-$), a powerful oxidant [27]. The induction of inflammatory responses by LPS is a receptor-mediated process [28]. After binding to complex membrane receptors CD14/TLR4, LPS initiates signaling transduction followed by activation of nuclear NF-κB and activator protein 1 (AP1) to increase the transcription of various immune mediators, such as iNOS, IL-6, and TNF-α [29]. Therefore, the inhibitory effects of NT2 ethanol extracts on LPS-stimulated NO, IL-6, and TNF-α production are, at least partially, attributed to the decrease in CD14 expression by macrophages, and may be mediated through modulating NF-κB's effects on AP-1 activities, although confirming this possibility requires further investigation. Although different studies [30,31] have shown that LPS induced TLR4 expression in RAW 264.7 macrophages, relatively low serum (1% FBS) and low LPS (100 ng/ml) concentrations used in the present study may explain the discrepancy. Alternatively, the possibility of direct interaction of the components in the extracts with LPS leading to its decreased bioactivity of LPS cannot be ruled out.

MMPs, on the other hand, are a group of proteases involved in extracellular matrix degradation, wound healing, tissue remodeling, and chemokine activation. Dysregulated MMP-9 has been linked to inflammatory responses, and its activity can be stimulated by LPS and proinflammatory cytokines [32]. However, pieces of evidence also indicate that MMP-9 presents anti-inflammatory effects by reducing inflammatory responses in skin tissues and by regulating cytokine production in immune cells [33]. Therefore, the NT2 ethanol extracts increased MMP-9 activity indicates the potential roles of NT2 on increasing our immune responses. However, NT2 further enhances the LPS-induced MMP-9 activity, similar to the results obtained from atorvastatin and simvastatin in RAW 264.7 macrophages. The clinical significance remains to be clarified.

Different active components, including chlorophylls and polyphenols, may contribute to the antioxidation and anti-inflammatory effects of NT2 ethanol extracts. Previously, Tsai et al. [13] indicated that polyphenols in boiling water extracts of NT2 play significant roles on its antioxidant activities. Among these, coumaric acid showed higher correlation to the antioxidant capacity than most other phenolic compounds in hot water extract, and its level is 10 X higher than the ethanol or cold water extracts detected in the present study. On the other hand, the chlorophylls were not determined in Tsai's study due to its hydrophobic property. Therefore, chlorophylls are hypothesized as one of the potential bioactive components in ethanol extracts of NT2. Chlorophyll a was reported to suppress carrageenan-induced paw edema in mice and formalin-induced paw edema in rats [34], and inhibited LPS-stimulated NO production and thiobarbituric acid-reactive substances (TBARSs) in RAW 264.7 macrophages [35]. In addition, both chlorophylls a and b decreased TNF-α mRNA expression in LPS-induced HEK293 cells [34], and such an effect was associated with its *in vitro* antioxidant activity. On the other hand, coumaric acid, one of the major phenolic compounds in hot water extracts of NT2, decreased the TNF-α level in synovial tissue of rats with adjuvant-induced arthritis [36]. Rutin and quercetin, other than chlorophylls and coumaric acid, were also detected at relatively high levels in NT boiling water extract, and both compounds are able to inhibit TNF-α production and NO/iNOS protein expressions in activated RAW 264.7 macrophages [37,38]. These are all potential bioactive components in ethanol extracts of NT2 to suppress LPS-stimulated inflammatory mediators. In the present study, NT2 ethanol extracts added to culture medium contained 0.43~1.73 μg GAE/ml total polyphenols, 0.32~1.28 μg/ml coumaric acid, 0.66~2.63 μg/ml chlorophyll a, and 0.28~1.11 μg/ml chlorophyll b. Although concentrations of individual compounds in the culture medium were lower than those previously reported, these compounds may act additively or synergistically to inhibit inflammatory mediators in activated macrophages. In addition to NO and cytokines, different phytochemicals were shown to modulate MMP

activities. Among these, lutein is one of the major carotenoids found in Triticum aestivum, a plant belongs to the same Poaceae family as NT [39], and it was indicated to increase MMP-9 activity and phagocytosis in RAW 264.7 macrophages [40]. As it can be extracted by ethanol, we propose that lutein may be one of the candidates which contribute to the induction of MMP-9 activity in NT ethanol extracts.

5. Conclusions

The present study suggests that ethanol extracts of NT2 possessed anti-inflammatory activities by decreasing the production of NO, IL-6, and TNF-α in LPS-activated RAW 264.7 macrophages, and such effects were associated with increased MMP-9 activity and decreased CD14 mRNA expression. The polyphenols and chlorophylls in the extracts may play roles as anti-inflammatory agents of NT2. Therefore, the consumption of NT2 mixed with nuts or oil, which is also usually added in the bioorganic energy soup, may have potential health-promoting effects. On the other hand, the ethanol extracts of NT2 showed no significant immunoregulatory effects on RAW 264.7 macrophages.

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Statement of Competing Interests

The authors have no competing interests.

List of Abbreviations

CD14	cluster of differentiation 14
DMSO	dimethyl sulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
FBS	fetal bovine serum
FRAP	ferric-reducing antioxidant power
GAE	gallic acid equivalents
HRP	horseradish peroxidase
IL	interleukin
iNOS	inducible nitric oxide synthase
LPS	lipopolysaccharide
MMP	matrix metalloproteinase
NO	nitric oxide
NT2	napiergrass Taishigrass no. 2
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
TE	trolox equivalents
TLR	Toll-like receptor
TNF	tumor necrosis factor
TPCs	total polyphenol contents

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