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Research article

Anti-inflammatory effects of snow mushroom (*Tremella fuciformis*) drinks with different types of natural sweeteners on RAW 264.7 macrophages stimulated with lipopolysaccharide

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Abstract

Importance of work: Snow mushrooms are a food ingredient commonly consumed worldwide. However, few reports exist concerning their health benefits.

Objective: To investigate the bioactive compounds, antioxidant activities and immune response of snow mushroom drinks, including sugar and natural sweetener effects.

Materials & Methods: Snow mushrooms were soaked, boiled at 95°C for 10 min, mixed with different types of natural sweetness and sterilized by retort. Before testing, the drinks were ground, centrifuged and the supernatant was collected to test activity.

Results: The black and white snow mushroom drinks had 2.74 g/100 mL and 3.54 g/100 mL of β-glucan content, respectively. The white snow mushroom drinks with longan had the highest total phenolic content at 329.37 ± 27.23^{a} µg gallic acid/mL, followed by white snow mushroom with monk fruit, original white snow mushroom, black snow mushroom and no sugar white snow mushroom, respectively. Nonetheless, all treatments showed similar anti-inflammatory effects by decreasing nitric oxide and tumor necrosis factor-α cytokine production in lipopolysaccharide-induced RAW 264.7 macrophages.

Main finding: The snow mushroom drinks could be used as an alternative functional drink. Different types of natural sweeteners did not affect the anti-inflammatory effect.

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Introduction

Snow mushrooms (*Tremella fuciformis*) are commonly consumed in many Asian countries (Mineroff and Jagdeo, 2023). The nutrients in snow mushrooms consist of 76.6% carbohydrate, 5.7% protein, 2.9% acetyl group, 11.4% moisture, and 3.4% ash (Khondkar, 2009). This mushroom also has bioactive compounds that promote health benefits such as antioxidant, anti-inflammatory, anti-tumor, anti-aging, and immune-modulating effects (Zhang et al., 2014; Lee et al., 2016; Wen et al., 2016; Ruan et al., 2018). Studies have shown that the immunomodulating properties of snow mushrooms derive from phenolic acid and polysaccharides, especially β-glucan (Lee et al., 2012; Ruan et al., 2018).

β-glucan is the most abundant polysaccharide in mushrooms and is composed of a β-(1,3) backbone, branched via-β-(1,6) link side chains (Murphy et al., 2010). Additionally, the functions of β-glucan depend on its composition, such as the size of molecules, type of backbone structure and extraction method (Caseiro et al., 2022). Shiitake β-glucan extract decreased inflammatory gene expression compared to lipopolysaccharide (LPS) treatment on the THP-1 macrophage (Chanput et al., 2012). Similar to other studies, *Russula virescens* mushroom extract decreased an inflammation indicator, such as nitric oxide (NO) production and the tumor necrosis factor-a (TNF-α) mRNA expression on RAW 264.7 macrophages activated by LPS (Hur et al., 2012). NO and TNF-α are involved in an inflammatory response related to several immune diseases (Soufli et al., 2016). The mushroom form of β-glucan has been used as a supplement and functional food product due to its health and immunity benefits (Zhu et al., 2015). Phenolic acids, as bioactive compounds in mushrooms, also have immune-modulating effects; in particular, gentistic acid, protocatechuic acid, 4-hydroxygenzonic acid and 4-coumaric acid comprise the major phenolic acid group of snow mushrooms, which decrease NO levels and some of the pro-inflammatory cytokine production in murine macrophage cells (Li et al., 2014; Lee et al., 2016).

The bioactive compounds in edible mushrooms play a role in immune functions, which can be used for applications such as food supplements and functional food (Kumar et al., 2021). However, no report has described the immune-modulating activities of snow mushrooms in terms of functional drinks. Therefore, this study aimed to investigate the beta-glucan content, sugar profile, total phenolic content, antioxidant activity and anti-inflammatory effect of snow mushroom drinks with different types of natural sweeteners on LPS-induced RAW 264.7 macrophages.

Materials and Methods

Chemical and reagents

Gallic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2 diphenyl-l-picrylhydrazyl (DPPH) and Folin-Ciocalteu reagent were purchased from Sigma-Aldrich (St Louis, MO, USA). Dulbecco's modified eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from GIBCO (Grand Island, NY, USA). All chemicals and reagents were of analytical or biological grade.

The Murine RAW 264.7 macrophage cell line was obtained from American Type Cell Culture (ATCC number TIB-71; Rockville, MD, USA). Cells were grown in DMEM supplemented with 10% FBS in a humidified incubator at 37°C and 5% CO_2. Cells were sub-cultured every 2–3 d (Bhawamai et al., 2016).

Sample preparation

Black and white snow mushrooms in their dry form were purchased from a local supermarket in Thailand. The mushrooms were soaked and boiled at 95°C for 10 min. Mushroom drinks were prepared using different types of natural sweetness sources—original (white snow mushroom with 10% sugar in water), no sugar (white snow mushroom in water), monk fruit (white snow mushroom with 10% monk fruit in water), longan (white snow mushroom with 10% longan in water) and black snow mushroom (black snow mushroom and 10% sugar in water). Subsequently, all samples were poured into bottles and sterilized by retort. Before testing, each sample was ground and centrifuged at 3,000 rpm for 15 min. The supernatant from phase separation was collected and stored at a low temperature (4°C).

Determination of β-glucan content

The original white and black snow mushroom drinks were freeze-dried and their β-glucan contents were determined using a yeast and mushroom β-glucan assay kit (Megazyme; code K-YBGL) by the Thailand Institute of Scientific and Technological Research, Bangkok, Thailand.

Determination of sugar contents

The sugar contents in the snow mushroom drinks were detected and quantitated based on high-performance liquid chromatography (HPLC; Model Prominence LC20 series; Shimadzu; Kyoto, Japan) with a refractive index detector using a Shim-pack Gist column (5µm NH2, 4.6 mm × 150 mm; Shimadzu; Kyoto, Japan). Acetonitrile and water were used in the mobile phase at a ratio of 85:15 (volume per volume). The sugar contents of each sample were calculated from D (-) fructose, D (+) glucose, D (+) sucrose and D (+) maltose standard curves (Hunthayung and Bhawamai, 2020).

Determination of total phenolic content

The Folin-Ciocalteu method was used to analyze the total phenolic content (TPC). Samples were mixed thoroughly with 500 µL of 10% Folin-Ciocalteu reagent. Then, 2 mL of 7.5% sodium carbonate was added and incubated at room temperature. After 30 min, 1.4 mL of distilled water was added and incubated for another 2 hr. The TPC was analyzed using a Shimadzu UV-1800 spectrophotometer (Kyoto, Japan) at 755 nm. Data were expressed as micrograms of gallic acid equivalents per milliliter of sample (Bhawamai et al., 2016).

Determination of antioxidant activity

The antioxidant activity of the snow mushroom drinks was analyzed using the DPPH method (Blois, 1958), in which 50 µL of samples and 900 µL of DPPH solution were mixed and then incubated at room temperature for 30 min in the dark. Absorbance was measured at 515 nm using the Shimadzu UV-1800 spectrophotometer. Data were converted into micrograms of Trolox equivalents per milliliter of sample.

Effects of snow mushroom drinks on RAW 264.7 macrophages

Cytotoxicity

The cytotoxicity of the snow mushroom drinks was determined using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium. RAW 264.7 macrophages (1×10^4 cells per well) were seeded in 96-well plates and treated with various concentrations (10 µg/mL, 25 µg/mL or 50 µg/mL) for 24 hr. The optical density at 570 nm was measured using a microplate reader (Model FLUOstar Omega; BMG Labtech; Ortenberg, Germany), according to Bhawamai et al. (2016).

Nitric oxide levels

The production of NO levels was determined using the Griess technique by mixing 100 µL of medium supernatant with Griess reagent (1% sulphanilamide in 5% phosphoric acid with 0.1% naphthylenediamine dihydrochloride). Cells were seeded in a 24-well cell culture plate at a density of 5×10^4 cells per well and stored at 37°C and 5% CO_2 overnight. After that, the cells were stimulated with LPS at a concentration of 100 ng/mL for 24 hr. Subsequently, the cells were treated with different concentrations of snow mushroom drinks for 24 hr. Absorbance was measured at 550 nm using a microplate reader. The NO levels were calculated based on nitrite concentrations of sample-treated cells using the sodium nitrite ($NaNO_2$) standard curve (Bhawamai et al., 2016).

Anti-inflammatory cytokine

Anti-inflammatory (TNF-α and IL-6) cytokine levels were determined in medium supernatants using an enzyme-linked immunosorbent assay of mouse ELISA kit from Invitrogen (Frederick, MD, USA). The concentrations of the cytokine levels were calculated from the standard curve.

Statistical analysis

The snow mushroom drinks were assessed based on two independent replications for the measurement of the sugar content and based on three independent replications for the measurement of TPC, antioxidant activities and anti-inflammatory activities. Comparative analyses, including TPC and antioxidant activity, were performed using one-way analysis of variance and Duncan's post hoc comparison test. Cell viability, NO and anti-inflammatory cytokines were determined using a paired-sample t test to compare LPS and other groups. Significant differences between groups were evaluated at $p < 0.05$. Statistical analysis was performed using the SPSS software, version 22 (IBM Corp.; Armonk, NY, USA).

Results and Discussion

β-Glucan contents

In Thailand, both wild and cultivated mushrooms contain bioactive compounds and high levels of protein, fiber and carbohydrates as well as a low fat content (Srikram and Supapvanich, 2016). β-Glucan is a polysaccharide

in edible mushrooms that has biological activities, with researchers reporting that β-glucan from mushrooms enables health-promoting activities via anti-tumor, anti-inflammatory, anti-microbial and anti-allergic properties (Zhu et al., 2015). Thus, mushrooms have functional food ingredient applications. In the current experiment, the composition of β-glucan in the different snow mushroom drinks was determined using a sample and a yeast β-glucan assay kit. The results showed that white snow mushroom drinks had a higher β-glucan content (3.54 g/100 mL) than the black snow mushroom drink (2.74 g/100 mL). In addition, this snow mushroom extract result was higher than the β-glucan content (2.58±0.33%) reported in snow mushroom extract by Kardono et al. (2013). The difference in β-glucan content may have been due to the mushroom species, growing region and the extraction method (Zhu et al., 2015). Based on the current results, the white snow mushroom that had the higher β-glucan content was selected to develop the drinks with the different natural sweeteners.

Sugar contents

The snow mushrooms contained sugar in polysaccharide form, with mannose being the main sugar at a concentration of 31.01% total carbohydrate, followed by glucose (13.16%), glucouronic (9.86%), fructose (4.12%), galactose (3.62%) and arabinose (0.39%). However, other species of *Tremella* polysaccharides have different sugar concentration profiles (Khondkar, 2009).

Monk fruit and longan are natural sweeteners that are widely used as a sugar substitute in many products (Zhang et al., 2020; Castro-Muñoz et al., 2022). Monk fruit is the fruit of the plant called 'luo han guo', which has cucurbitane-type triterpene glycosides, known as mogrosides (EFSA Panel on Food Additives and Flavourings et al., 2019). The extract from monk fruit is extremely sweet (more than 250 times sucrose) and is low in calories. In addition, monk fruit is generally recognized as safe (GRAS) by U.S. Food and Drug Administration (2014), implying its endorsement for addition to food, drug and cosmetic products. Longan is another popular edible fruit widely cultivated in tropical countries that has a good level of sweetness and contains nutrients and bioactive constituents (Zhang et al., 2020). Due to their taste and bioactive compounds, longan and monk fruit have the potential to be used as natural sweeteners with health benefits, including antioxidant and anti-inflammatory advantages (Chen et al., 2007; Huang et al., 2012b).

In the current study, the individual sugar contents in the snow mushroom drinks were analyzed using the HPLC technique and calculated from the standard calibration curve. The results showed that sucrose was the only sugar in the original snow mushroom and black snow mushroom (4.72 ± 0.21 g/100 mL and 4.43 ± 0.70 g/100 mL, respectively), as shown in Table 1. The sugar contents in the snow mushrooms with longan included fructose (0.46 ± 0.13 g/100 mL), glucose (0.93 ± 0.11 g/100 mL) and sucrose (4.13 ± 1.05 g/100 mL). According to the results, the sucrose levels in these drinks were 5% (weight per volume) sucrose added, except for the no sugar snow mushroom and no sugar snow mushroom with monk fruit drinks. The fructose and glucose levels in the snow mushrooms with longan may have resulted from the dried longan that was added. Another study also reported glucose, fructose and sucrose as the major forms of sugar in longan (Yunchalad et al., 2008).

Total phenolic contents

The TPC levels of the snow mushroom drinks were expressed as gallic acid standard equivalents. As shown in Table 2, snow mushrooms with longan had the highest TPC (329.37 ± 27.23 µg gallic acid/mL), followed by snow mushrooms with monk fruit, original, black snow mushroom and then the no sugar drink. The variety of TPCs may have been influenced by other added ingredients such as monk fruit, longan and sucrose. Furthermore, the original snow mushrooms had a higher TPC than the no-sugar snow mushroom drink. Payet et al. (2006) reported that sucrose results in an increase in the TPC determined using the Folin-Ciocalteu method.

Table 1 Sugar content in snow mushroom drinks

Type of snow mushroom drinks	Fructose (g/100mL)	Glucose (g/100mL)	Sucrose (g/100mL)	Maltose (g/100mL)	Total sugar (g/100mL)
Black snow mushroom	nd	nd	4.43 ± 0.70	nd	4.43 ± 0.70
Original snow mushroom	nd	nd	4.72 ± 0.21	nd	4.71 ± 0.21
No sugar snow mushroom	nd	nd	nd	nd	nd
No sugar snow mushroom with monk fruit	nd	nd	nd	nd	nd
Snow mushroom with longan	0.46 ± 0.13	0.93 ± 0.11	4.13 ± 1.05	nd	5.51 ± 1.28

nd = not detected using this method;
Values are shown as mean ± SD.

Antioxidant activities

The DPPH radical scavenging method is popular and considered useful for determining antioxidant activities by inhibiting lipid oxidation (Zaman and Khalid, 2015). The snow mushrooms with longan had the highest level of antioxidant activity (111.58 ± 9.35 µg Trolox/mL), followed by snow mushrooms with monk fruit, as shown in Table 2. However, the original white, black and no sugar snow mushroom drinks did not show antioxidant activities using this method. Similarly, a study of various subfractions of methanol extract of *Tremella fuciformis* reported water subfraction resulted in the lowest and undetected antioxidant activities using Trolox equivalent antioxidant capacity and the ferric reducing/antioxidant power assay, respectively. Furthermore, hydrophobic subfractions, such as chloroform and ethyl acetate, had higher antioxidative properties than the hydrophilic subfractions (Li et al., 2014). Therefore, the different extraction methods and types of subfraction might influence antioxidant activity.

Although the tested snow mushroom drinks had antioxidant activity, especially the snow mushrooms with monk fruit and longan drinks, the immune-modulating activities, such as NO, TNF-a, and IL-6 cytokine effects, of all the snow mushroom drinks need to be assessed.

Effects of snow mushroom drinks on RAW 264.7 macrophages

Cytotoxicity

The current study investigated the effects of snow mushroom drinks on cell viability, nitric oxide level and some anti-inflammatory cytokines such as TNF-α and IL-6. To assess the cytotoxicity of the snow mushroom drinks (original snow mushroom, no sugar snow mushroom, no sugar snow mushroom with monk fruit, snow mushroom with longan and black snow mushroom), RAW 264.7 macrophages were incubated with 10 µg/mL, 25 µg/mL or 50 µg/mL of the sample for 24 hr. The cell viability of all tested conditions was greater than 85% with dose dependence (Fig. 1).

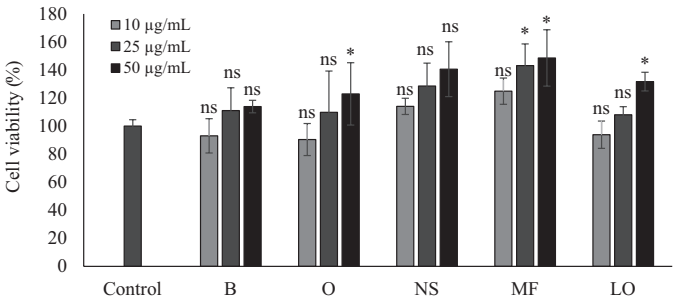

Fig. 1 Cytotoxicity of snow mushroom drink treatments on RAW 264.7 macrophage, where B = black snow mushroom, O = original snow mushroom, NS = no sugar snow mushroom, MF = snow mushroom with monk fruit, LO = snow mushroom with longan, LPS = lipopolysaccharide 100 ng/ml treated, Control = untreated; values are presented as mean ± SD (n = 3); * above bars indicate significantly ($p < 0.05$) different compared to the control; *ns* above bars indicate non-significant ($p \geq 0.05$) compared to the control.

Nitric oxide levels

Nitric oxide is produced by an inducible isoform of nitric oxide synthase, which plays an important role in immune functions; NO has been reported to be related to the cause of various inflammatory diseases and inflammatory reactions by pathogens (Sharma et al., 2007; Min et al., 2010). Furthermore, NO production has been a target of inflammation for *in vitro*, *in vivo* and clinical investigations (Yang et al., 2008; Choi et al., 2016).

The LPS extracted from the *Escherichia coli* O111:B4 bacterium has been widely used as an inflammatory stimulus to test the effects of nutrients on cellular immunity (Yang et al., 2008; Chanput et al., 2012). LPS contamination in the tested drinks in the current study was determined using an LAL chromogenic endotoxin quantitation kit,

Table 2 Total phenolic contents and antioxidant activities in snow mushroom drinks

Type of snow mushroom drink	Total phenolic contents (µg gallic acid/mL)	Antioxidant activity (µg Trolox/mL)
Black snow mushroom	62.92 ± 3.77^{c}	nd
Original snow mushroom	63.81 ± 11.34^{c}	nd
No sugar snow mushroom	47.75 ± 6.63^{c}	nd
No sugar snow mushroom with monk fruit	132.88 ± 8.95^{b}	23.37 ± 12.24^{b}
Snow mushroom with longan	329.37 ± 27.23^{a}	111.58 ± 9.35^{a}

nd = not detected using this method;
Mean ± SD in the same column superscripted with different lowercase letters are significantly ($p < 0.05$) different.

which tests the amount of endotoxin in the sample. The results showed that LPS contamination was less than 10 ng/mL (data not shown) at the testing concentrations (10, 25 and 50 ug/mL). It has been reported that LPS at concentrations in the range 10–2,000 ng/mL can induce increased secretion of inflammation-related cytokines, with secretion levels rising as the LPS concentration increased (Chanput, 2012). The current results showed that snow mushroom drinks significantly decreased NO production on LPS-stimulated RAW 264.7 macrophages (Fig. 2).

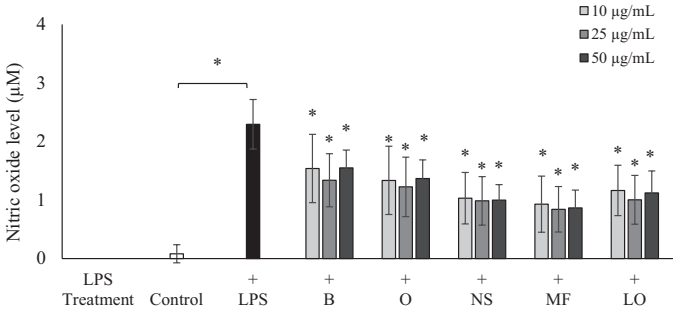

Fig. 2 Nitric oxide level of snow mushroom drinks treatments on lipopolysaccharide (LPS 100 ng/mL)-induced RAW 264.7 macrophage, where B = black snow mushroom, O = original snow mushroom, NS = no sugar snow mushroom, MF = snow mushroom with monk fruit, LO = snow mushroom with longan, LPS = LPS 100 ng/mL treatment, Control = untreated; values are presented as mean ± SD (n = 3); * above a horizontal line indicates significantly ($p < 0.05$) different compared to the control; # above each bar indicates significantly ($p < 0.05$) different compared to LPS.

Anti-inflammatory cytokine of snow mushroom drinks

The culture supernatants were measured using mouse TNF-α and IL-6 ELISA kits to investigate the effect of snow mushroom drinks on the secretion of inflammatory cytokines. In general, RAW 264.7 macrophages produced a low level of TNF-α and IL-6, while LPS stimulated RAW 264.7 macrophages as well as increased TNF-α and IL-6 cytokine production. As shown in Fig. 3, all snow mushroom drink treatments showed similar anti-inflammatory effects by decreasing TNF-α cytokine production. However, they did not significantly affect the IL-6 production of LPS-stimulated RAW 264.7 macrophages. Nonetheless, there was a downward trend in IL-6 production (Fig. 4). In addition, the various tested types of sugar and natural sweeteners (sucrose, longan and monk fruit) did not show different anti-inflammatory effects on LPS-stimulated RAW 264.7 macrophages compared to no sugar. Thus, the anti-inflammatory effects might have been influenced by the snow mushrooms.

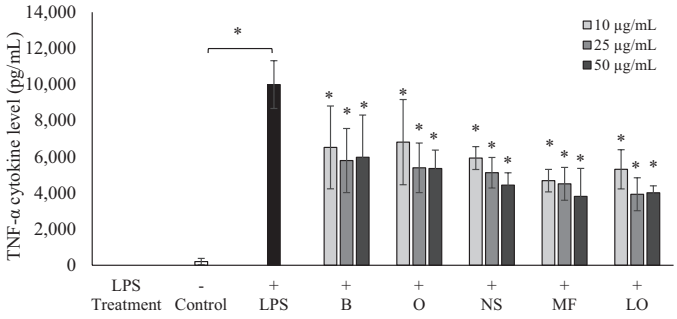

Fig. 3 Tumor necrosis factor (TNF) cytokine level of snow mushroom drink treatments on lipopolysaccharide (LPS 100 ng/mL)-induced RAW 264.7 macrophage, where B = Black snow mushroom, O = Original snow mushroom original, NS = No sugar snow mushroom, MF = Snow mushroom with monk fruit, LO = Snow mushroom with longan, LPS = lipopolysaccharide 100 ng/mL treatment, Control = untreated; value are presented as mean ± SD (n = 3); * above a horizontal line indicates significantly ($p < 0.05$) different compared to the control; # above each bar indicates significantly ($p < 0.05$) different compared to LPS.

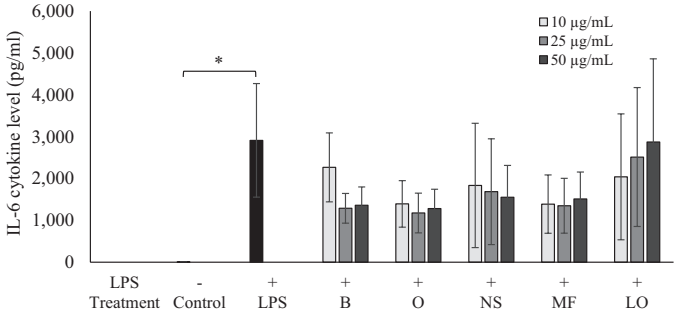

Fig. 4 IL-6 cytokine level of snow mushroom drink treatments on lipopolysaccharide (LPS 100 ng/mL)-induced RAW 264.7 macrophage, where B = black snow mushroom, O = original snow mushroom, NS = no sugar snow mushroom, MF = snow mushroom with monk fruit, LO = snow mushroom with longan, LPS = LPS 100 ng/mL treatment, Control = untreated; values are presented as mean ± SD (n = 3); * above a horizontal line indicates significantly ($p < 0.05$) different compared to the control; * above each bar indicates significantly ($p < 0.05$) different compared to LPS.

Other studies have reported that mushrooms are inhibitors of the NO level, cytokine production and gene expression in murine and human macrophage cell models (Chanput et al., 2012; Huang et al., 2012a; Hur et al., 2012; Xu et al., 2012; Gunawardena et al., 2014; Seo et al., 2018; Murphy et al., 2022). It is well known that the immune-modulating effect of polysaccharides in mushrooms is associated with β-glucan (Volman et al., 2010). Furthermore, *in vivo* study has shown that the oral intake of β-glucan extracts derived from mushrooms markedly suppressed inflammatory effects (Lavi et al., 2010; Smiderle et al., 2014). The β-glucan in mushrooms

is a heterogeneous polysaccharide of glucose consisting of a β-(1-3) backbone, branched via-β-(1->6) link side chains (Murphy et al., 2010). Numerous studies have demonstrated the immune-modulating mechanism of mushroom β-glucan, including the nuclear factor-kB (NF-kB) pathway, dectin-1 in association with toll-like receptor pathways in the cell model (Yang et al., 2008; Gantner et al., 2003; Thompson et al., 2010).

The immune-modulating mechanism of snow mushroom drinks on RAW 264.7 remains unclear, though two possible mechanisms have been proposed. First, β-glucans inhibits inflammation in macrophages through miR-155 and nuclear factor-kB (NF-kB) pathways (Ruan et al., 2018). Another mechanism involves phenolic acid, resulting in NF-kB and mitogen-activated protein kinase pathways. Both compounds suppress inducible nitric oxide synthase expression and nitric oxide production (Lee et al., 2012, 2016). Furthermore, increasing NO and TNF-α and IL-6 levels can induce inflammation, which is related to causing inflammatory diseases such as cardiovascular diseases, cancer, and joint, gut and lung inflammation diseases (Sharma et al., 2007). Thus, the current experiment demonstrated the anti-inflammatory effect of snow mushroom drinks by suppressing NO and TNF-α levels. However, further clarification is required concerning the mechanism and daily intake amount.

Conclusion

The developed snow mushroom drinks contained bioactive compounds, such as β-glucan and phenolic acid, which had anti-inflammatory effects on LPS-induced RAW 264.7 macrophages. Furthermore, the different tested types of sugar and natural sweeteners did not affect the anti-inflammatory effects. However, further investigation is necessary into the precise mechanisms of snow mushroom drinks regarding inflammatory diseases.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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