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ORIGINAL ARTICLE

Immunostimulatory Activities of a High Molecular Weight Fraction of *Cynanchum auriculatum* Royle ex Wight Root Obtained by Ultrafiltration

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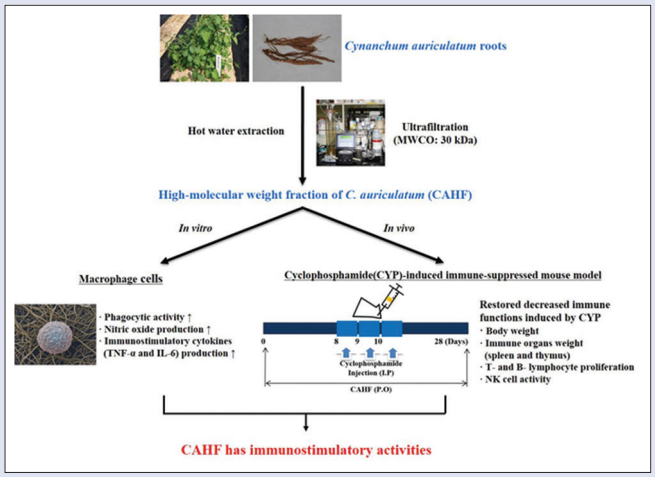
ABSTRACT

Background: The root of *Cynanchum auriculatum* Royle ex Wight has been traditionally used as a folk medicine in the eastern Asia. **Objectives:** In this study, we explored the immune-enhancing effect of the high-molecular weight fraction of *C. auriculatum* (CAHF) root extracts in RAW 264.7 macrophages and cyclophosphamide (CYP)-induced immune-suppressed mice. **Materials and Methods:** To obtain the CAHF, ultrafiltration was performed using 30-kDa molecular weight cutoff polyethersulfone membranes in a cross-flow microfiltration system. *In vitro* and *in vivo* experiments were performed to evaluate the immunostimulatory effects of CAHF. **Results:** CAHF increased the production of nitric oxide and immunostimulatory cytokines (interleukin-6 and tumor necrosis factor-α) in recombinant interferon-γ primed macrophages. In addition, the phagocytic activity of macrophage cells was enhanced by CAHF. Furthermore, CAHF treatment markedly restored the decreased body weight, immune organs (spleen and thymus) weight, natural killer cell activity, and T- and B-lymphocyte proliferation in CYP-induced immune-suppressed mice. **Conclusion:** These results suggest that CAHF is an effective immunostimulatory agent.

Key words: *Cynanchum auriculatum,* immune-suppressed model, immunostimulatory effect, macrophage, polysaccharide, ultrafiltration

SUMMARY

- Various phytochemicals including acetophenones, C_{21}-steroids, terpenoids, and alkaloids are found in the roots of *Cynanchum auriculatum* Royle ex Wight. Most studies on *C. auriculatum* have been conducted using the aforementioned low-molecular weight phytochemicals; however, the antioxidative activity of the polysaccharide fraction from *C. auriculatum* has only been recently reported. In this study, we isolated the high-molecular weight fraction of *C. auriculatum* root water extracts (CAHF), mainly comprising polysaccharides, through ultrafiltration and explored the immune-enhancing activities of CAHF in murine RAW 264.7 macrophage cells and cyclophosphamide (CYP)-induced immunosuppressed mice. We found that CAHF-enhanced phagocytic activity and the production of NO and immunostimulatory cytokines (TNF-α and IL-6) in recombinant interferon-γ-primed RAW 264.7 macrophages. Furthermore, CAHF restored the body weight, spleen and thymus weights, lymphocyte proliferation, and natural killer cell activity in a CYP-induced immune-suppressed mouse model. Our findings suggest that the immunostimulatory activities of CAHF make it an attractive novel immunopotentiator candidate.



Abbreviations used: CAHF: High-molecular weight fraction of *Cynanchum auriculatum* root extracts; Con A: Concanavalin A; CYP: Cyclophosphamide; DMEM: Dulbecco's Modified Eagle's Medium; DW: Distilled water; FBS: Fetal bovine serum; HPAEC-PAD: High-performance anion exchange chromatography coupled with pulsed amperometric detection; IL-6: Interleukin-6; LDH: Lactate dehydrogenase; LPS: Lipopolysaccharide; NO: Nitric oxide; NK cell: Natural killer cell; OD: Optical density; SD: Standard deviation; TNF-α: Tumor necrosis factor-α; rIFN-γ: Recombinant interferon-γ; RPMI: Roswell Park Memorial Institute.

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INTRODUCTION

The immune response is one of the most important biological defenses for removing and repairing a disease environment induced by the invasion of external infectious agents such as viruses, bacteria, and parasites and is divided into innate and adaptive immunity.[1] During immune responses, various immune cells such as macrophage cells, natural killer (NK) cells and lymphocytes are activated to

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remove foreign antigens.[2] Activated macrophages have critical role in maximizing the immune activity by enhancing phagocytosis and producing nitric oxide (NO) and various immunostimulatory cytokines.[3] In addition, NK cells, known as representative effector cells capable of killing cancer cells, play a pivotal role in eliminating cancer and infected cells and therefore occupy a primary line of defense element against infectious agents in initial immune responses.[4] Recently, many studies have shown that polysaccharides isolated from medicinal herbs can strengthen the immunity and cause relatively low toxicity and side effects compared to micro-organism-derived polysaccharides and synthetic drugs.[5] These medicinal plant-derived polysaccharides are known to enhance immune functions by activating immune-related cells including macrophage cells, NK cells and lymphocytes and secretion of immunostimulatory cytokines.[6] Thus, lots of researches have evaluated medicinal plant-derived polysaccharides as immunopotentiator candidates.[7-10]

Cynanchum auriculatum Royle ex Wight is distributed throughout Eastern Asia including China, Korea, and Japan and the root of *C. auriculatum* has been widely used as a traditional herbal medicine for over 1000 years in China.[11] Previous studies have shown that *C. auriculatum* possesses biological activities such as gastroprotection, antitumor, antidepressant, and neuroprotection effects.[12-15] The roots of *C. auriculatum* consist of various phytochemicals including acetophenones, alkaloids, terpenoids, and C_{21}-steroids.[16] Although most studies of *C. auriculatum* have been carried out using the low-molecular weight phytochemicals mentioned above, the antioxidative activity of the polysaccharide fraction from *C. auriculatum* was only recently reported.[17]

In this study, we made efforts to isolate the high-molecular weight fraction of *C. auriculatum* root water extracts (CAHF), which are mainly composed of polysaccharides, by ultrafiltration and also investigated the immune-enhancing activities of CAHF in RAW 264.7 murine macrophage cells and cyclophosphamide (CYP)-induced immune-impaired mice.

MATERIALS AND METHODS

Preparation of a high-molecular weight fraction of *Cynanchum auriculatum*

Two-year roots of *C. auriculatum* were cultivated in Eumseong, Chungbuk Province, Korea. Briefly, dried roots of *C. auriculatum* were extracted by hot water (95°C) for 4 h and then the residues were extracted one more time by hot water for 2 h. The water extract was filtered through a 10-μm depth cartridge filter (Pall Corporation, Port Washington, NY, USA) and concentrated at a reduced pressure. To obtain and concentrate the high-molecular weight fraction of the water extract, commercial ultrafiltration (Sartorius, Göttingen, Germany) was performed.[18] The ultrafiltration method was used to collect a >30 kDa fraction from the water extract of *C. auriculatum* root and the obtained high-molecular weight fraction (CAHF) was used in subsequent experiments.

Chemical analyses

The total sugar content of CAHF was estimated using the phenol-sulfuric acid method.[19] The content of uronic acids were determined by the m-hydroxydiphenyl sulfuric acid method.[20] The protein content was measured by the bicinchoninic acid protein assay kit (Pierce Biotechnology, Rockford, IL, USA).[21]

Monosaccharide composition

The monosaccharide composition of CAHF was analyzed by high-performance anion exchange chromatography (HPAEC) coupled with pulsed amperometric detection (PAD). The CAHF sample was dissolved in 72% H_2SO_4 for 2 h at 30°C and then distilled water (DW) was added. The reaction mixtures were hydrolyzed at 120°C for 1 h to form hydrolysates. Hydrolysates (25 μL) were injected into an ICS-5000 system (Dionex Co., Sunnyvale, CA, USA) with a CarboPac PA1 analytical column (4 × 250 mm, Dionex Co.). Isocratical elution using 18 mM NaOH was applied to separate the monosaccharides (flow rate: 1.0 mL/min). A mixture of arabinose, fucose, fructose, galactose, glucose, mannose, rhamnose, and xylose was used as a standard. Galacturonic acid and glucuronic acid were analyzed on a Dionex ICS-5000 system with CarboPac PA1 analytical column using 100 mM NaOAc and 100 mM NaOH as the mobile phase.

Cell culture and viability assay

RAW 264.7 cells, the murine monocyte/macrophage-like cells, were procured from the Korean Cell Line Bank (KCLB; Seoul, Korea) and cultured in Dulbecco's Modified Eagle's Medium (DMEM; Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Life Technologies) and 1% penicillin and streptomycin (Life Technologies) at 37°C in a humidified incubator (5% CO_2). The cells were pretreated with recombinant interferon-γ (rIFN-γ; 10 ng/mL, Sigma-Aldrich) for priming[7] and then stimulated with various concentrations of CAHF (50, 100, or 200 μg/mL) or lipopolysaccharide (LPS; 1 μg/mL, Sigma-Aldrich).

Cell viability was determined using the cell counting kit-8 (Dojindo, Kumamoto, Japan). RAW 264.7 macrophages were cultured in 96-well plates (1×10^4 cells/well) and incubated for 24 h with 50, 100, or 200 μg/mL of CAHF. The macrophages were treated with the cell counting kit-8 reagent for 120 min and optical density (OD) was determined at 450 nm (Varioskan Flash; Thermo Scientific, Waltham, MA, USA).

Measurement of nitric oxide, interleukin-6 and tumor necrosis factor-α production

A reactive radical gas, NO, was measured using Griess reagent (Sigma-Aldrich). RAW 264.7 macrophage cells were cultured in 24-well plates (1×10^5 cells/well) and treated with CAHF for 24 h. Fifty microliters of cell culture media were recovered, and equal volume of Griess reagent was added. After incubation for 15 min, NO production was monitored by OD measurements at 540 nm. The concentrations of IL-6 and tumor necrosis factor-α (TNF-α) were determined with ELISA kits (BD Bioscience, San Jose, CA, USA).

Phagocytosis assay

The phagocytic activity of macrophages was determined by a CytoSelect™ phagocytosis assay kit (Cell Biolabs, Inc., San Diego, CA, USA). The cells were preincubated with various concentrations of CAHF, complete DMEM (negative control) and LPS (positive control) for 24 h. Nonopsonized zymosan particles were introduced and incubated for 2 h. The number of cleared zymosan particles was detected at an absorbance of 405 nm.

Animals and immune-suppression

Eight weeks old female BALB/c mice (18–20 g) were obtained from Koatech Animal, Inc., (Pyeongtaek, Korea). Experimental animals were cared under following conditions (temperature: 22°C ± 1°C, dark/light cycle: 12 h, relative humidity: 50%–60%). All mice studies were performed following the guidelines of the Institutional Animal Care and Use Committee of the Korea Food Research Institute (KFRI-M-15040). Mice were randomly divided into five groups (8 mice/group). Mice

were orally administered once daily for a period of 28 days of (1) vehicle only control (p.o. physiological saline) without immunosuppression, (2) vehicle-treated negative control (p.o. physiological saline), (3) CVT-E002™ (COLD-fx®; Valeant Pharmaceuticals, Laval, QB, Canada) (p.o. 200 mg/kg) as positive control, (4) low-dose CAHF (p.o. 100 mg/kg) and (5) high-dose CAHF (p.o. 200 mg/kg). Mice in (2)–(5) groups were intraperitoneally injected with CYP (150 mg/kg) on days 8–10 after respective administration, while group (1) was administered the same volume of physiological saline. The immunostimulator CVT-E002™, a standardized extract from North American ginseng (*Panax quinquefolius*),[22] was used as a positive control. Twenty-four hours after last treatment, animals were sacrificed. The thymus and spleens were subsequently isolated and weighted. The isolated spleens were used to evaluate NK cell activity and T- and B-lymphocyte proliferation.

T- and B-lymphocyte proliferation assay

The homogenized spleen tissues were passed through a nylon mesh cell strainer (100 μm; Sigma-Aldrich) to obtain single-cell suspensions. The red blood cells were lysed by red blood cell lysis buffer (Hybri-Max™; Sigma-Aldrich). The recovered splenocytes were resuspended in Roswell Park Memorial Institute-1640 medium containing 10% FBS, 100 μg/mL streptomycin and 100 IU/mL penicillin. Five hundred microliters of splenocytes were seeded in 48-well plates (1.6×10^6 cells/well) and subsequently cultured with LPS (400 μg/mL), concanavalin A (Con A; 100 μg/mL, Sigma-Aldrich) or DW (negative control) for 72 h. Con A and LPS were used to measure the T- and B-lymphocytes proliferation, respectively.[9] One hundred microliters of the cell counting kit-8 reagent were introduced to the splenocyte cultures and then incubated for 4 h. The OD was measured at 450 nm.

Natural killer cell activity

YAC-1 lymphoma cells were obtained from the KCLB. The splenocytes were used as the effector cells, whereas YAC-1 cells were used as the target cells. Two different ratios of effector cells to target cells (50:1 and 25:1) were used. The NK cell cytotoxicity was measured with the lactate dehydrogenase (LDH) leakage assay.[23] The amount of LDH in the cell culture medium was an indicator of cytotoxicity of NK cells. The LDH activity in the culture media was determined with the LDH cell cytotoxicity assay kit (DoGen, Seoul, Korea).

Statistical analysis

Experimental values are expressed as the mean ± standard deviation. Data were analyzed by one-way analysis of variance followed by Duncan's test. A $P < 0.05$ indicates statistical significance. All statistical analyses were performed using SPSS software (SPSS, Inc., Chicago, IL, USA).

RESULTS

Chemical composition of high-molecular weight fraction of *Cynanchum auriculatum*

The chemical and monosaccharide compositions of CAHF are presented in Table 1. The yield of CAHF was 4.3% (w/w). The CAHF was mainly composed of 69.3% neutral sugars, 25.3% uronic acid and 5.4% protein. The sugar composition of CAHF was further characterized by HPAEC-PAD. The main monosaccharides in CAHF were glucose, galacturonic acid, rhamnose, galactose, and arabinose.

Table 1: Chemical and monosaccharide composition of high-molecular weight fraction of *Cynanchum auriculatum*

	CAHF
Chemical composition (%)	
Neutral sugar[a]	69.3±1.4
Uronic acid[b]	25.3±1.6
Protein[c]	5.4±0.2
Composition of sugar (mg/g)	
Arabinose	64.34±1.06
Fucose	6.33±0.13
Galactose	48.34±1.13
Glucose	312.17±5.02
Mannose	7.20±0.23
Rhamnose	14.08±0.35
Xylose	3.50±0.28
Galacturonic acid	134.09±1.25
Glucuronic acid	2.69±0.02

Values are shown as the mean±SD. [a]Phenol sulfuric acid method; [b]*m*-hydroxydiphenyl sulfuric acid method; [c]Bradford method. SD: Standard deviation; CAHF: High-molecular weight fraction of *Cynanchum auriculatum*

Effects of high-molecular weight fraction of *Cynanchum auriculatum* on murine macrophage activation

To determine whether CAHF can induce immune-enhancing activities, we examined its effects on the production of immunostimulatory cytokines (IL-6 and TNF-α) and NO in rINF-γ-primed RAW 264.7 macrophages. As shown in Figure 1a, CAHF did not exert cell cytotoxicity up to 200 μg/mL. In addition, CAHF treatment concentration-dependently increased NO, IL-6 and TNF-α production compared to the untreated control of CAHF [Figure 1b-d]. As shown in Figure 1e, phagocytosis of zymosan particles was increased by CAHF treatment. The phagocytic activity of CAHF (200 μg/mL)-treated macrophages was significantly stronger than control (zymosan-only).

Effects of high-molecular weight fraction of *Cynanchum auriculatum* on impaired immune functions in cyclophosphamide -induced immune-suppressed animals

To evaluate the effects of the CAHF on CYP-induced immune suppression, we measured the body weight gain and absolute and relative weights of the thymus and spleen. The intraperitoneal treatment with CYP resulted in reductions of body weight gain [Figure 2a] and thymus and spleen weights [Figure 2b] compared to the non-treated control group. However, oral administration of CAHF (100 and 200 mg/kg/day) significantly restored body weight gain and the spleen weight reduced by CYP. Thymus weight was also restored by CAHF administration, but the change was not significant.

The CYP treatment also inhibited T- and B-lymphocytes proliferation and NK cell activity [Figure 3]. As summarized in Figure 3a, CAHF (200 mg/kg) treatment notably recovered NK cell activity against tumor cells compared to the CYP-treated immune-suppressed group. Decreased T- and B-lymphocyte proliferation activities were also increased by CAHF treatment, but the results were insignificant [Figure 3b]. The positive control, CVT-E002™, also significantly restored body weight, thymus and spleen weights, T- and B-lymphocyte proliferation activities and NK cell activity suppressed by CYP administration.

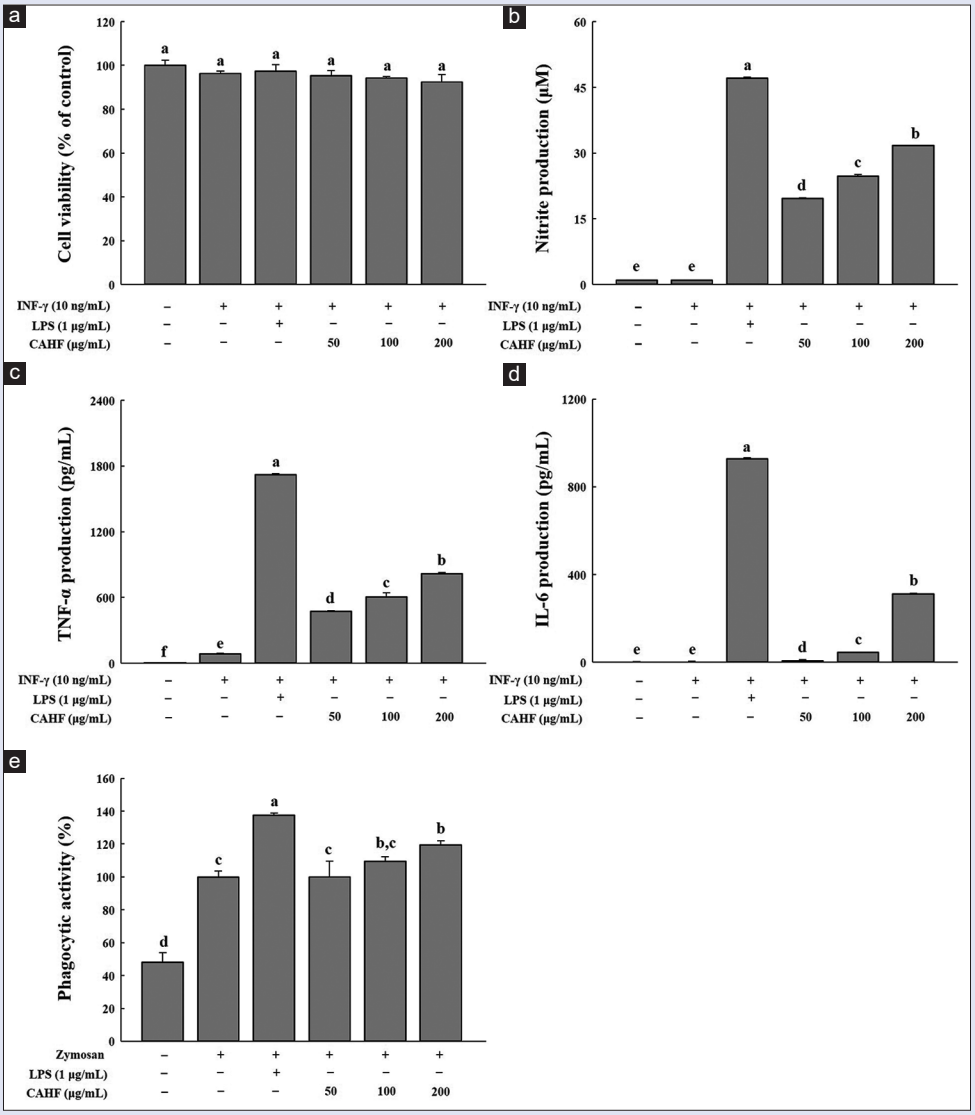

Figure 1: Effects of a high-molecular weight fraction of *Cynanchum auriculatum* on (a) cell viability, (b) nitric oxide production, (c) tumor necrosis factor-α production, (d) interleukin-6 production and (e) phagocytic activity in recombinant interferon-γ primed RAW 264.7 murine macrophages stimulated with high-molecular weight fraction of *Cynanchum auriculatum* (50, 100 and 200 μg/mL). Values with the different letters are significantly different ($P < 0.05$)

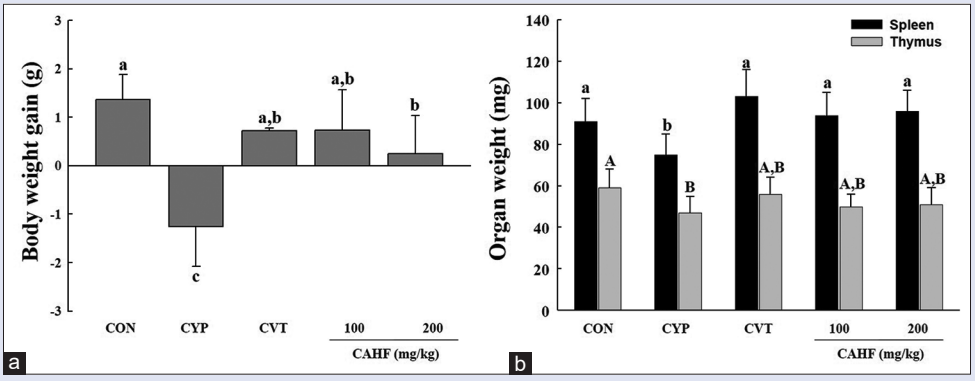

Figure 2: Effects of high-molecular weight fraction of *Cynanchum auriculatum* on (a) body weight gain and (b) immune organ weights of cyclophosphamide-induced immunosuppressed mice. High-molecular weight fraction of *Cynanchum auriculatum* (100 or 200 mg/kg) or CVT (200 mg/kg) was administered orally once daily for 28 days to cyclophosphamide-induced immunosuppressed mice. Values with different letters are significantly different ($P < 0.05$). CVT, immunostimulatory polysaccharide-rich extract of the root of *Panax quinquefolius* (North American ginseng)

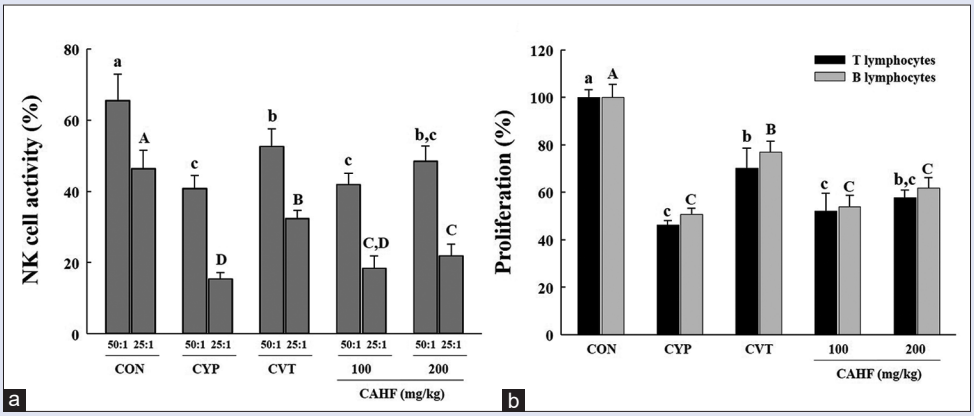

Figure 3: Effects of high-molecular weight fraction of *Cynanchum auriculatum* on natural killer cell activity and splenic lymphocyte proliferation in cyclophosphamide-induced immunosuppressed mice. (a) Natural killer cell activity was determined by lactate dehydrogenase assay. The E: T ratio indicated ratio of effector cells (splenocytes) and target cells (YAC-1 cells); (b) Concanavalin A- and lipopolysaccharide-induced T- and B-lymphocytes proliferation. Cell proliferation was measured by using CCK-8 kit. Values with different letters are significantly different ($P < 0.05$)

DISCUSSION

Recently, the use of herbal medicines as functional food materials for maintaining health has increased. Studies are actively being conducted on the development of immune-promoting materials using polysaccharides derived from herbal medicines.[24]

In this study, we explored the immune-enhancing activities of CAHF on rIFN-γ-primed RAW 264.7 murine macrophage cells and investigated the restoration effects of the CAHF on CYP-induced immune-suppression.

Macrophages play an important role in innate immunity. Macrophages are used to monitor target antigens such as bacteria, fungi, and virus-infected cells and perform a series of complex functions including detection and removal of target antigens.[25] The activated macrophages result in the induction of various immunostimulatory cytokines and NO.[26] The present study exhibited that CAHF upregulated the production of immunostimulatory cytokines (IL-6 and TNF-α) and NO in rINF-γ-primed macrophage cells compared to the negative control. NO is an important signaling molecule in the immune system that plays a defensive role against tumor cells or intracellularly infected micro-organisms.[27] Appropriate levels of NO production is considered as important factors in innate immunity.[28] Various cytokines secreted by activated macrophages are soluble proteins necessary for the regulation and signaling of other immune cells and are known to contribute to the reaction and efficiency of the entire immune system by forming a complex cytokine network between immune cells.[29] TNF-α, an immunostimulatory cytokine, promotes the expression of proteins or costimulatory factors on the surface of immature dendritic cells and converts them into mature dendritic cells. These mature dendritic cells present direct anticancer activity by interacting with T-lymphocytes, regulating T-lymphocyte activity and growth and inducing cell lysis.[30] IL-6, one of the most important immunostimulatory cytokines, acts as a stimulating factor for lymphocytes resulting in the proliferation and differentiation of lymphocytes.[31] Phagocytosis of macrophages is a very important immunological response that involves the removal of pathogens having the instance of invading the body. It is essential to maintain tissue homeostasis by removing unnecessary proteins and dead cells in the tissue.[32] Regarding that CAHF upregulated the phagocytosis of macrophages, CAHF may enhance the initial and critical step of the macrophage activation. Considering the above, these results propose that CAHF increases the immunostimulatory activities of macrophage cells.

To assess the immune-enhancing ability of CAHF on impaired immune systems, we applied a CYP-induced immune-suppressed mouse model. CYP is an alkylating agent used to treat autoimmune diseases.[33] CYP metabolites are known to inhibit the immune response of lymphocytes by alkylating DNA in splenocytes. Those that were significantly reduced include body weight, spleen and thymus indices and immunological parameters (T- and B-lymphocyte proliferation activity and NK cell activity) of CYP-treated mice. However, oral administration of CAHF restored these overall immunological functions. In general, the thymus and spleen index is known as an crucial and innate indicator of non-specific immunity and immunopotentiators that have been reported to upregulate the weight of the spleen and thymus.[34] Given that CAHF restored the reduced body weight and thymus and spleen weights induced by CYP administration, we expected that CAHF could alleviate CYP-induced immune organs weakening. NK cells are a main element of the innate immune system because they rapidly release various cytokines upon activation.[35] In addition, they induce the immune response against pathogens and have cell-killing functions in their target cells.[36] Therefore, the stimulatory activity of NK cells is closely linked to increased immunity. Lymphocyte proliferation activity is considered as an important immune function indicators and lymphocyte proliferation results in the enhancement of cellular and humoral immune responses.[37] In this study, CAHF administration recovered CYP-induced decreased lymphocyte proliferation activities and NK cell activity. Considering the above, these results suggest that CAHF administration alleviates CYP-induced immune-suppression by recovering impaired immune functions.

Pectin is an important material in the food processing field because it can form gels and films in the presence of appropriate acids and sugars.[18] Pectic polysaccharides include α-D-1,4-polygalacturonan, in which D-galacturonic acid is linked by α-1,4 bonds. However, it is reported that pectic polysaccharides, which exist in nature, have much more complicated structures. Majority of the total molecule is composed of linear homogalacturonan but rhamnogalacturonans (rhamnogalacturonan I and II), which are highly branched with various oligo-and polysaccharides, are covalently bound to homogalacturonan.[38] Many studies have reported that polysaccharides isolated from various plant types contain immunostimulatory activities.[6] Among them, pectic polysaccharides from the roots of medicinal herbs showed strong immune-enhancing

effects.[8-10,39] Considering that CAHF is mainly comprised of pectic polysaccharides components (galacturonic acid, arabinose, galactose, rhamnose, etc.), the immunostimulatory activity of CAHF likely originates from pectic polysaccharides.

CONCLUSION

To evaluate the potential of using *C. auriculatum* as a functional food material, the immunostimulatory activity of *C. auriculatum* extract was assessed. Ultrafiltration (MWCO: 30 kDa) was performed for the purpose of obtaining CAHF from water extract. CAHF enhanced phagocytic activity and the production of immunostimulatory cytokines (TNF-α and IL-6) and NO in rINF-γ-primed macrophages. Importantly, CAHF restored the body weight, thymus and spleen weights, lymphocyte proliferation and NK cell activity in a CYP-induced immune-suppressed animal model. Our findings suggest that the immunostimulatory activities of CAHF make it an attractive candidate as a novel immunopotentiator.

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Conflicts of interest

There are no conflicts of interest.

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