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Immunomodulatory effects of *Cervi Parvum Cornu* and *Acanthopanax sessiliflorus* mixtures *in vitro* and *in vivo*

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Abstract
The aim of this study was to evaluate the immunomodulatory effects of *Cervi Parvum Cornu* (C) and *Acanthopanax sessiliflorus* (A) *mixtures* (CA) *in vitro* and *in vivo*. For the *in vitro* evaluations, phagocytosis, natural killer (NK) activity, nitric oxide (NO), pro-inflammatory cytokines, as well as the mRNA expression of LP-BM5, were measured using enzyme-linked immunosorbent assay (ELISA) or real-time polymerase chain reaction (RT-PCR). CA treatment increased phagocytosis, NK cell activity, and NO production, and decreased pro-inflammatory cytokine level. LP-BM5 expression was also reduced in treated CA. In the *in vivo* study, we evaluated changes in the levels of the major histocompatibility complex (MHC) I and II, CD4 and 8(+) T-cells, Th1/Th2 cytokines, NK cell activity, and leukocytes in response to forced swimming exercise (FSE) using flow cytometry (FACS) and ELISA. Oral CA intake increased the levels of MHC I and II, CD4 and 8(+) T-cells, Th1 cytokines, T-cell proliferation, and NK cell activity. CA treatment also decreased Th2 cytokine level, increased B-cell proliferation, leucocyte counts, and the production of several immunoglobulins. Taken together, these results suggest that the CA mixture may improve the immune response by controlling the Th1/Th2 cytokine balance, making it a useful addition to immunomodulatory interventions.
Keywords: *Immunomodulation, Cervi Parvum Cornu, Acanthopanax sessiliflorus.*

1. Introduction

Over the past decade we have seen a steady increase in the interest in functional foods, especially those that modulate the immune system. As the corona virus disease 2019 (COVID-19) outbreak continues to put strain on health systems around the world, it is inevitable that people's interest in strengthening their immune system will also grow [1,2]. Immunity can be divided into innate and acquired immunity, and various mechanisms underlying the activation and regulation of each have been indentified [3,4]. When foreign antigens, such as viruses or bacteria, enter the body, the body's antigen presenting cells (APCs) identify the antigen and activate the immune defense system [5].

Viruses and bacteria generally enter the APCs via interacting with the major histocompatibility complex (MHC) I or II, respectively. MHC I typically binds to the T cell receptors (TCR) of CD8 (+) T-cytotoxic cells, while MHC II binds to receptors expressed on CD4 (+) T-helper (Th) cells [6]. Among the cytokines involved in immunity, interleukin (IL)-2 and interferon (IFN)-gamma (γ) are associated with Th1 responses and IL-4, 6, 10 and TNF-α are associated with Th2 responses. These cytokines are primarily produced by the CD4(+) Th cells. Disparities in the Th1 and Th2-type cytokines may negatively impact the immune responses, [7,8] resulting in the reduction of T- and B-cell functions and decreased CD8(+) Th cells and natural killer (NK) cell activities. These decreases may impair antigen neutralization and produce abnormalities in immunoglobulin (Ig) production and white blood cell counts [9-12].

In addition to the influence of external antigens, the immune system can also be affected by nutritional status, aging, mental stress, and exercise. Of these, exercise is thought to have the most impact on the physical stress experienced by people in modern society, with these outcomes being heavily dependent on its intensity and frequency [13]. Regularly planned exercise therapy has a positive effect on the body's immune system, but

unplanned and irregular one-time exercise has been known to lower immunity [14]. The forced swimming exercise used in this study was originally designed to evaluate improvements in immune function and evaluate changes in muscle strength and despair behavior [15]. Swimming is a high-intensity exercise requiring 70-85 VO2 (%) oxygen, [16] making it an additional method for suppressing immunity in addition to the viral injection method used before [17].

Cervi Parvum Cornu (C), also called deer velvet, has been used as a therapeutic agent for thousands of years in China and Korea. This plant has been reported to antioxidant activity, splenocyte proliferation ability, in addition to several immunomodulatory properties [18-22]. *Acanthopanax sessiliflorus* (A) belongs to the Araliaceae family and is known to be effective in the treatment of hypertension and immune stimulation [23,24]. However, little is known about the immunomodulatory effects of mixing these two compounds, i.e., CA. In this study, we investigated the immunomodulatory effects of CA *in vitro* and *in vivo*.

2. Materials and Methods

2.1 Preparation of plant materials

C, A, and CA extracts were obtained from Mom&young bio (Cheongju, Korea) with the CA product mixed at a 1:1 ratio. The C, A, and CA extracts were sealed to protect against light and air and stored at -20 °C until use.

2.2 Cell culture and treatments

The RAW264.7, Yac-1, and SC-1 (CRL-1404) cell lines were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA), and grown in RPMI-1640, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin and cultured as previously described [25].

2.3 Experimental mice and treatment

The animal experimental protocol was approved by the Institutional Animal Care and Use Review Committee of Kyung Hee University (KHGASP-20-277). Balb/c mice (N=6, female, 6-week-old) and C57BL/6N mice (N=64, male, 4-week-old) were purchased from Saeronbio, Inc. (Uiwang, Korea). The animal care facility was established using the same parameters as before [17]. Balb/c mice were used in *ex vivo* experiments, and C57BL/6N mice were randomly divided into eight groups: wild type (WT), forced swimming exercise-control (FSE-C), positive control [PC; FSE + red ginseng 300 mg/kg body weight (b.w.)], C200 (FSE + C 200 mg/kg b.w.), A200 (FSE + A 200 mg/kg b.w.), CA50 (FSE + CA 50 mg/kg b.w.), CA100 (FSE + CA 100 mg/kg b.w.), and CA200 (FSE + CA 200 mg/kg b.w.). Oral administration of C, A, and CA lasted for two weeks and swimming exercises were completed using an acrylic water bath (W 40 cm × L 25 cm × H 18 cm) at 25 °C and a water pressure of 14 L/min. Mice were forced to swim until they could no longer float on their own, at which point, the exercise was terminated [15]. After the forced swimming, spleen and blood samples were collected and used for downstream analysis.

2.4 Water soluble tetrazolium salt (WST) assay

Cell viability was confirmed using the EZ-CYTOX kit (Daeillab Service Co., Ltd, Seoul, Korea). Brietly RAW 264.7 cells were seeded in 96-well plates at a density of 1×10^4 cells/well and then allowed to grow for 1 day. These cells were then treated with varying concentrations of C, A, and CA (0-1000 μg/mL) and incubated for another day. Then each well was treat with 20 μL/200 μL EZ-CYTOX and incubated for 3 h. The plate was gently shaken for 30 sec before measuring the absorbance at 450 nm using an ELISA reader (Bio-Rad Laboratories, Hercules, CA, USA).

2.5 Cytokine ELISA

Raw 264.7 cells and isolated splenocytes were seeded at a density of 2×10^6 cells/well and 5×10^5 cells/well, respectively in 96-well plates for assay. Raw 264.7 cells were treated with C, A, or CA and lipopolysaccharide (LPS, 5 µg/mL; Gibco-BRL, Grand Island, NY, USA) to stimulate IL-1β, -6, and TNF-α (for 1 day) levels. The splenocytes were treated with concanavalin A (Con A, 5 µg/mL; Sigma-Aldrich, St. Louis, MO, USA) or LPS (5 µg/mL) to stimulate IL-2, -4, -6, -10, TNF-α (for 1 day), -12, -15 (for 2 days), and IFN-γ (for 3 days) levels. Samples were then evaluated using R&D DuoSet ELISA Development kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

2.6 NO evaluations

Raw 264.7 cells were seeded at a density of 2×10^6 cells/well in 96-well plates and treated with C, A, and CA as before and the supernatant was collected. Animal serum samples were collected from centrifuged (560 × g at 4°C for 20 min) whole blood, and the NO levels in were evaluated using the nitric oxide assay kit (Abcam, Massachusetts, UK) according to the manufacturer's protocol.

2.7 Phogocytosis activity

Raw 264.7 cells were seeded at a density of 1×10^4 cells/well in 96-well plates and then treated with CA as before. Their phagocytic activity was then evaluated using the Cytoselect 96-well phagocytosis assay (zymosan substrate) kit (Cell Biolabs Inc., San Diego, CA, USA), according to the manufacturer's instruction.

2.8 NK cell activity

Splenocytes from the experimental mice were seeded at a density of 5×10^5 cells/well in 96-well plates and co-cultured with Yac-1 cells (ATCC) (density, 1×10^4 cells/well). After a 4-h incubation at 37 °C, and 5% CO_2, cell supernatants were transferred to a new plate and NK-cell activity was measured using a CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega Corporation, Madison, WI, USA).

2.9 RT-PCR

To evaluate changes in SC-1/LP-BM5 virus replication, SC-1 (CRL-1404) and SC-1 (LP-BM5 murine leukemia virus) cell lines were co-cultured in 12-well plates at a density of 1×10^5 cells/well, stabilized for 4 h, treated with C, A, and CA, and cultured for 24 h (37°C, 5% CO_2). Changes in the penetration of the SC-1/LP-BM5 virus were also evaluated using SC-1 (CRL-1404) cells seeded at a density of 1×10^6 cells/well in a 6-well plate and stabilized for 4 h, and then the culture solution of SC-1 (LP-BM5) was additionally added. In addition, cells were treated with C, A, and CA and incubated at 37°C in an atmosphere of 5% CO_2 for 24 h. Following treatment, all cells were collected and the homogenized for RNA extraction, cDNA synthesis, and RT-PCR were performed as described previously [26]. The following primers were used for the RT-PCR: GAPDH, 5'-CATGGCCTTCCGTGTTCCTA-3' and 5'-GCGGCACGTCAGATCCA-3'; LP-BM5, 5'-CCAATGTGTCCATGTCATTT-3' and 5'-GCGATGAGCAGAGAGAGAAAG-3'.

2.10 Flow cytometry (FACS)

Splenocytes were dispensed in Eppendort tubes at a density of 3×10^6 cells/tube, washed, and then probed with each of the following primary antibodies: anti-mouse CD45-PE/CY7, anti-mouse CD8a FITC, and anti-mouse CD4 PE (SouthernBiotech, Birmingham, AL, USA), MHC class I monoclonal antibody, and MHC class II monoclonal antibody (Thermo Fisher Scientific, Waltham, MA, USA) for 30 min on ice. Then, the cells were washed twice in a flow cytometry staining buffer (Thermo Fisher Scientific) and then were resuspended in flow

cytometry staining buffer and counted using a CytoFLEX (Beckman Coulter, Brea, CA, USA). The results were analyzed using the CytExpert 2.2 program (Beckman Coulter).

2.11 T- and B-cell proliferation

Splenocytes were seeded at a density of 5×10^5cells/well in 96-well plates and treated with Con A (5 µg/mL) or LPS (5 µg/mL) to promote T- or B-cell proliferation. After two days (37°C, 5% CO_2), T- and B-cell proliferation was measured using an Ez-CyTox kit (Daeil Lab Service) according to the manufacturer's instructions.

2.12 Evaluating immunoglobulin productions

Serum from whole blood samples was used to evaluated IgE, IgA, and IgG levels in experimental animals using relevant mouse ELISA kits (Abcam) according to the manufacturer's protocols.

2.13 Leukocyte counts

Leukocytes were isolated from whole blood treated with 2% EDTA (Sigma-Aldrich) and mixed with DPBS and, Histopaque®-1077 (Sigma-Aldrich), then centrifuged for 20 min (556 × g at 4°C). Finally, the leukocyte-enriched supernatant was removed and mixed with new DPBS and leukocytes were counted.

2.14 Statistical analysis

All the results of the *in vitro* and *in vivo* experiments are presented as the mean ± standard deviation (SD). The significance of the effects of C, A, and CA was determined using a one-way analysis of variance (ANOVA) and Duncan's multiple range test, and student's *t*-test using SPSS Statistics 22.0 software (SPSS Inc., Chicago, IL, USA). A p value of < 0.05 was considered significant.

3. Results and discussion

3.1 Effects of CA treatment on NO production in RAW 264.7 cells

NO levels significantly increased, by 16.3 and 58.9%, in the PC50 and PC200 groups, respectively compared to those observed in the normal control (NC). NO levels also increased in the C (50: 40.1%, 200: 57.1%) and A (50: 48.6%, 200: 64.9%) groups compared with those in the NC group. The CA-treated groups (50: 53.9%, 100: 56.7%, and 200: 71.3%) also exhibited significantly increased NO production in a dose-dependent manner compared with the NC group ($p < 0.05$; Fig. 1A)

3.2 Effect of CA treatment on RAW 264.7 phagocytosis

The phagocytic activity of PC (50: 21.4%, 200: 33.4%), C (50: 4.6%, 200: 15.0%), and A (50: 10.2%, 200: 17.3%) groups was significantly increased compared to that of the C4 group. The CA-treated groups (50: 7.6%, 100: 14.7%, and 200: 22.0%) also exhibited significantly increased phagocytosis in a dose-dependent manner compared to the C4 group ($p < 0.05$; Fig. 1B).

3.3 Effects of CA treatment on NK activity against Yac-1 cells

NK cell activity of the PC (50: 10.4%, 200: 30.4%), C (50: 5.5%, 200: 12.1%), and A (50: 8.7%, 200: 15.4%) groups was significantly increased compared to that of the NC group. The CA-treated groups (50: 11.0%, 100:

12.1%, and 200: 23.6%) exhibited significantly increased NK cell activity in a dose-dependent manner compared to the NC group ($p < 0.05$, Fig. 1C).

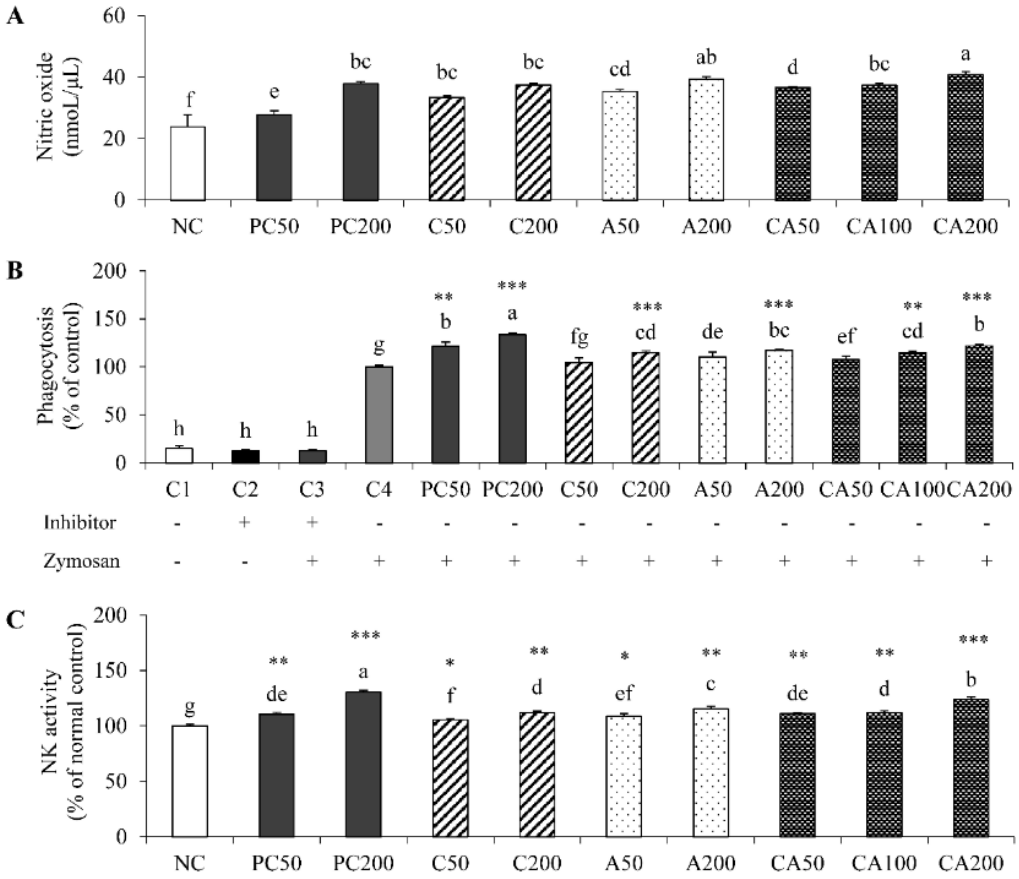

Figure 1. Effect of *cervi parvum cornu* (C), *acanthopanax* (A), and CA mixtures on NO production, phagocytosis, and NK cell activity. NC, normal control; PC, positive control (red ginseng 50 and 200 μg/mL); C, *cervi parvum cornu* 50 and 200 μg/mL; A, *acanthopanax* 50 and 200 μg/mL; CA, C and A mixture (50 and 200 μg/mL); C1-C4 (control). Values are recorded as the mean ± SD (n=3), and the different superscript letters (alphabet) indicate significance at $p < 0.05$. Significant differences from each control were evaluated using student's *t*-test: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

3.4 Effect of CA treatment on pro-inflammatory cytokine production in RAW 264.7 cells

Pro-inflammatory cytokine (IL-1β, IL-6, and TNF-α) levels are shown in Figure 2. The levels of IL-1β, IL-6, and TNF-α increased in LPS-treated RAW 264.7 cells (LPS-C) compared to those in the NC. The levels of IL-1β decreased in a dose dependent manner in the CA-treated groups (50: 15.4%, 100: 19.4%, and 200: 26.8%) compared with that in the LPS-C group (Fig. 2A). The CA-treated group also exhibited a dose dependent decrease in IL-6 levels (50: 13.5%, 100: 22.1%, and 200: 29.9%) compared to the LPS-C group (Fig. 2B). TNF-α levels were significantly and dose-dependent decreased in the CA-treated groups (50: 9.0%, 100: 18.6%, and 200: 27.2%) compared to those in the LPS-C group ($p < 0.05$; Fig. 2C).

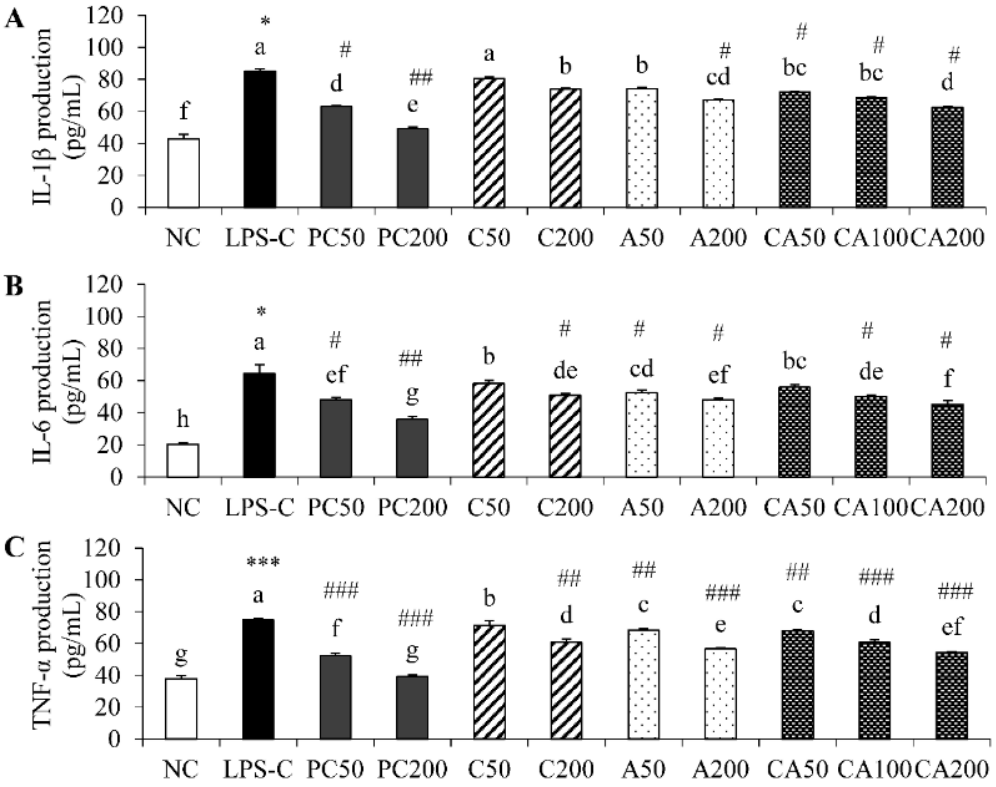

Figure 2. Effects of *cervi parvum cornu* (C), *acanthopanax* (A), and CA mixtures on the level of pro-inflammatory cytokines in RAW 264.7 cells. (A) IL-1β, (B) IL-6, (C) TNF-α. NC, normal control; LPS-C, LPS (5 μg/mL)-control; PC, positive control (red ginseng 50 and 200 μg/mL); C, *cervi parvum cornu* 50 and 200 μg/mL; A, *acanthopanax* 50 and 200 μg/mL; CA, C and A mixture (50 and 200 μg/mL). Values are presented as the mean ± SD (n=3), and the different superscript letters (alphabet) indicate significance at $p < 0.05$. Significant differences from each control were evaluated using student's *t*-test: $^{*}p < 0.05$, $^{***}p < 0.001$, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$.

3.5 Effect of CA on the replication and penetration of the SC-1/LP-BM5 virus

An increase (more that 5-fold) in LP-BM5 transcription was observed in the control (C) group upon transduction with the SC-1/LP-BM5 virus compared to that in the NC group. In addition, LP-BM5 transcription was decreased in a dose-dependent manner in cells treated with CA (50: 19.3%, 100: 29.2%, 200: 32.7%) (Fig. 3A). SC-1/LP-BM5 penetration was confirmed based on the presence of the viral genome. Viral DNA increased by 52% in the control (C) (versus the NC group). In addition, the LP-BM5 DNA decreased, in a dose-dependent manner in the CA-treated groups (50: 39.3%, 100: 47.8%, 200: 55.6%) compared with those in the C group ($p < 0.05$; Fig. 3B).

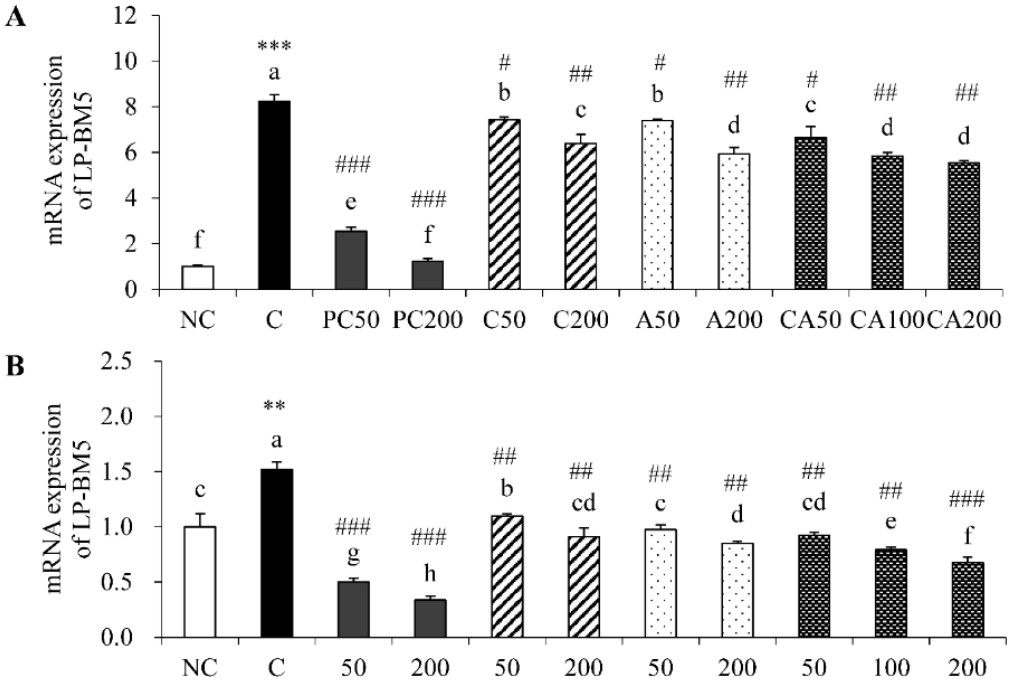

Figure 3. Effects of cervi parvum cornu (C), acanthopanax (A), and CA mixtures on replication and penetration of LP-BM5 virus in SC-1 cells. (A) Inhibition of LP-BM5 replication, (B) inhibition of viral penetration. NC, normal control; C, virus induction control; PC, positive control (red ginseng 50 and 200 μg/mL); C, cervi parvum cornu 50 and 200 μg/mL; A, acanthopanax 50 and 200 μg/mL; CA, C and A mixtures (50 and 200 μg/mL). Values are presented as the mean ± SD (n=3), and the different superscript letters (alphabet) indicate significance at $p < 0.05$. Significant differences from each control were evaluated using student's t-test: $^{**}p < 0.01$, $^{***}p < 0.001$, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$.

3.6 Effects of CA on weight gain and organ weight in mice subjected to forced swimming exercise

The effects of CA on weight gain, food intake, food efficiency rate (FER), and organ weight are summarized in Table 1. No significant differences were observed in weight gain, food intake, FER, or all organ weights between any of the groups ($p < 0.05$).

Table 1: Effects of CA on weight gain, food intake, FER and organ weight of C57BL/6N mice exposed to forced swimming exercise induced immunosuppression.

		Forced swimming exercise						
Groups	*WT*	*FSE-C*	*PC*	*C 200*	*A 200*	*CA 50*	*CA 100*	*CA 200*
Weight gain*	6.05 ± 0.69^{ab}	5.99 ± 0.63^{ab}	5.46 ± 0.85^{b}	5.88 ± 0.31^{ab}	6.20 ± 0.65^{a}	5.91 ± 0.39^{ab}	5.99 ± 0.31^{ab}	6.15 ± 0.56^{a}
Food intake**	2.75 ± 0.16^{ns}	2.74 ± 0.19	2.74 ± 0.18	2.78 ± 0.19	2.80 ± 0.15	2.78 ± 0.18	2.79 ± 0.14	2.77 ± 0.16
FER***	1.96 ± 0.22^{ab}	1.94 ± 0.21^{ab}	1.77 ± 0.27^{b}	1.90 ± 0.10^{ab}	2.01 ± 0.21^{a}	1.92 ± 0.13^{ab}	1.94 ± 0.10^{ab}	1.99 ± 0.18^{a}
Organ weight (g)								
Spleen	0.09 ± 0.01^{ns}	0.09 ± 0.01	0.09 ± 0.00	0.09 ± 0.00	0.09 ± 0.00	0.09 ± 0.01	0.09 ± 0.00	0.09 ± 0.01
Liver	1.31 ± 0.07^{ns}	1.34 ± 0.06	1.39 ± 0.07	1.32 ± 0.07	1.31 ± 0.04	1.33 ± 0.05	1.35 ± 0.04	1.32 ± 0.06
Kidney	0.31 ± 0.01^{a}	0.28 ± 0.03^{b}	0.29 ± 0.01^{ab}	0.29 ± 0.01^{ab}	0.29 ± 0.01^{b}	0.29 ± 0.01^{ab}	0.29 ± 0.01^{b}	0.29 ± 0.01^{ab}

Values are presented as the mean ± standard deviation (n = 8), and different superscript letters indicate significance at $p < 0.05$. WT, wild type; FSE-C, forced swimming exercise (FSE)-control; PC, positive control (FSE + red ginseng 300 mg/kg body weight); C 200 [FSE + *Cervi Parvum Cornu* (C) extract 200 mg/kg body weight]; A 200 [FSE + *Acanthopanax* (A) extract 200 mg/kg body weight]; CA 50 (FSE + C and A 50 mg/kg body weight); CA 100 (FSE + C and A 100 mg/kg body weight); CA 200 (FSE + C and A 200 mg/kg body weight). *Weight gain (g/2 weeks) = final body weight (g) – initial body weight (g). **Food intake, g/day/mouse. ***FER (Food efficiency rate) = weight gain (g)/total food consumption (g) x 100.

3.7 Effect of CA on the expression of MHC I, MHC II and the production of CD4(+), and CD8(+) T-cells from primary mouse splenocytes

MHC I expression was significantly decreased (28.9%) in the FSE-C group compared with that in the WT group, and significantly increased (43.7%) in the PC group compared with that in the FSE-C group. The the C200 (7.6%), A200 (25.4%), and CA (50: 14.6%, 100: 30.0%, 200: 35.7%) groups displayed significant increases in MHC I expression. MHC II expression was significantly decreased (17.6%) in the FSE-C group compared with that in the WT, and significantly increased (18.8%) in the PC group compared with that in the FSE-C group. The C200 (4.3%), A200 (11.5%), and CA (50: 4.6%, 100: 10.7%, 200: 16.2%) groups exhibited significant upregulation of MHC II expression ($p < 0.05$; Fig. 4A and C) compared to the FSE-C group.

CD4(+) T cell counts were significantly decreased (57.7%) in the FSE-C group compared with those in the WT control, and significantly increased (76.3%) following PC treatment. In addition, the C200 (14.7%), A200 (35.7%), and CA (50: 21.8%, 100: 45.4%, 200: 58.2%) groups demonstrated significantly increased CD4(+) T cell counts. CD8(+) T cell counts significantly decreased (73.2%) in the FSE-C group compared to those in the WT group and were significantly increased (more than 2-fold) in the PC group. The C200 (45.1%), A200 (148.0%), and CA (50: 69.3%, 100: 116.0%, 200: 155.1%) groups exhibited significantly increased CD8(+) T cell counts ($p < 0.05$; Fig. 4B and D).

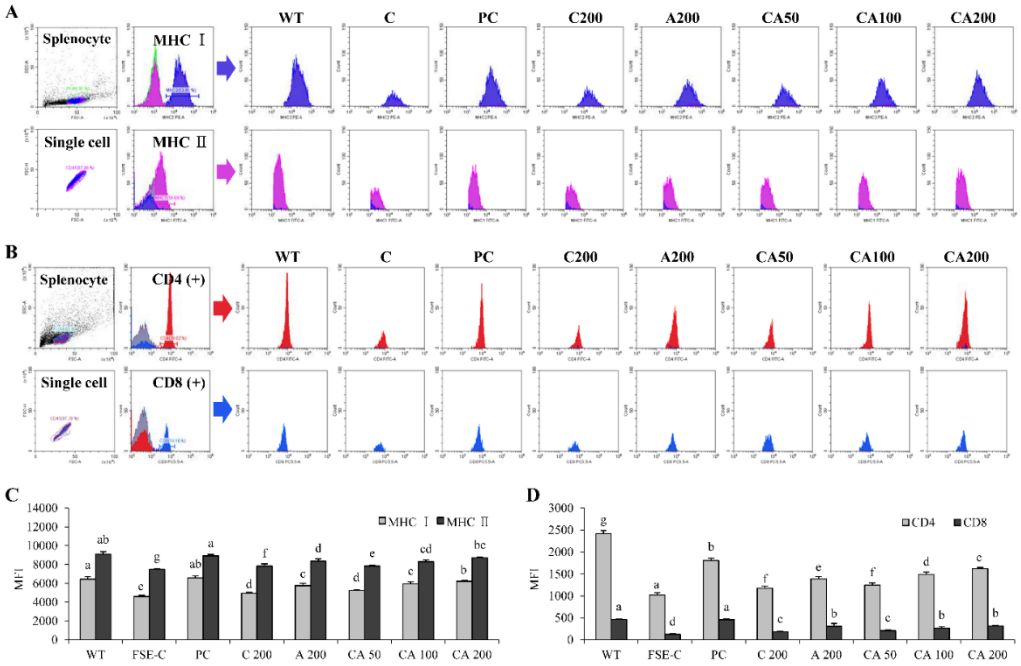

Figure 4. Effects of *cervi parvum cornu* (C), *acanthopanax* (A), and CA mixtures on MHC I and II expression, and CD4(+) and CD8(+) counts in splenocytes from C57BL/6N mice with induced immunosuppression following forced swimming exercises. (A) expression of MHC I and MHC II evaluated by FACS, (B) CD4(+) and CD8(+) counts evaluated by FACS, (C) mean fluorescence intensity (MFI) for MHC I and MHC II, (D) MFI for CD4(+) and CD8(+) cells. WT, wild type; FSE-C, forced swimming exercise-control; PC, positive control (red ginseng 300 mg/kg b.w.); C, *cervi parvum cornu* 200 mg/kg b.w.; A, *acanthopanax* 200 mg/kg b.w.; CA, C and A mixtures (50, 100, and 200 mg/kg b.w.). Values are presented as the mean ± SD (n=3), and the different superscript letters (alphabet) indicate significance at $p < 0.05$.

3.8 Effect of CA on T- and B-cell proliferation in primary mice splenocytes

T-cell and B-cell proliferation in ConA treated splenocytes from FSE-C animals significantly decreased (54.5% and 37.4%, respectively) compared to that in the WT group. The PC (85.1%), C200 (19.1%), A200 (34.4%), and CA (50: 22.0%, 100: 45.4%, 200: 60.8%) groups exhibited significantly increased T-cell proliferation compared to the FSE-C group. In addition, the PC (43.2%), C200 (11.1%), A200 (22.6%), and CA (50: 17.7%, 100: 25.6%, 200: 38.3%) groups exhibited significantly increased B-cell proliferation compared to the FSE-C samples. The CA treatment group exhibited a dose-dependent increase in T-cell and B-cell proliferation compared with the FSE-C group ($p < 0.05$; Table 2).

3.9 Effect of CA on Th1-type cytokine production in primary mouse splenocytes

The levels of Th1-type cytokines IL-2 and IFN- γ are presented in Table 2. The level of IL-2 and IFN-γ in Con A-treated splenocytes from FSE-C mice was shown to be significantly decreased (76.3% and 40.7%) when compared to that in the WT control. The PC (283.8%), C200 (133.2%), A200 (188.7%), and CA (50: 156.7%, 100: 192.1%, 200: 250.6%) groups exhibited significant upregulation of IL-2 compared to the FSE-C group. In addition, the PC (51.4%), C200 (4.0%), A200 (21.3%), and CA (50: 13.0%, 100: 26.0%, 200: 32.7%) groups exhibited significant upregulation of IFN- γ compared to the FSE-C group. CA treatment was also shown to induce a dose-dependent increase in Th1-type cytokine production ($p < 0.05$).

3.10 Effect of CA on Th2-type cytokine production in primary mouse splenocytes

The level of Th2-type cytokines IL-4, IL-6, IL-10, and TNF-α is presented in Table 2. The level of both IL-4 and IL-10 was upregulated (140.2% and 262.5%, respectively) in Con A-treated splenocytes from the FSE-C group compared to that in the WT control. The PC (49.2%), C200 (16.7%), A200 (22.9%), and CA (50: 18.5%, 100: 25.2%, 200: 37.6%) groups exhibited significantly decreased IL-4 production compared to FSE-C group. Further, the PC (67.5%), C200 (30.1%), A200 (45.3%), and CA (50: 34.8%, 100: 45.3%, 200: 58.4%) groups exhibited significantly decreased IL-10 production. The level of IL-6 and TNF-α in LPS-treated splenocytes was significantly increased (195.9% and 106.8%, respectively) in the FSE-C group compared to that in the WT control. The PC (42.7%), C200 (10.4%), A200 (20.9%), and CA (50: 13.5%, 100: 25.1%, 200: 36.2%) groups exhibited significantly decreased IL-6 production compared to the FSE-C group. Further, the PC (42.0%), C200 (11.8%), A200 (24.1%), and CA (50: 16.5%, 100: 23.2%, 200: 31.1%) groups also exhibited significantly decreased TNF-α production in the CA treatment groups demonstrated some degree of dose-dependence ($p < 0.05$).

Table 2: Effects of CA on various parameters in splenocytes and blood of C57BL/6N mice exposed to forced swimming exercise induced immunosuppression

		Forced swimming exercise						
Groups	*WT*	*FSE-C*	*PC*	*C 200*	*A 200*	*CA 50*	*CA 100*	*CA 200*
Splenocytes								
IL-2 (pg/mL)	461.5 ± 20.48^{a}	109.6 ± 4.40^{g}	420.6 ± 24.53^{b}	255.5 ± 13.57^{f}	316.3 ± 13.44^{d}	281.3 ± 12.73^{e}	320.1 ± 8.48^{d}	384.1 ± 6.35^{c}
IFN-γ (pg/mL)	678.9 ± 13.31^{a}	402.6 ± 7.39^{h}	609.5 ± 7.73^{b}	418.6 ± 7.24^{g}	488.3 ± 7.82^{e}	455.0 ± 16.78^{f}	507.3 ± 4.21^{d}	534.4 ± 13.52^{c}
IL-4 (pg/mL)	65.72 ± 5.00^{g}	157.9 ± 10.66^{a}	80.2 ± 7.42^{f}	131.5 ± 5.76^{b}	121.8 ± 7.85^{cd}	128.7 ± 1.83^{bc}	118.1 ± 1.87^{d}	98.5 ± 7.27^{e}
IL-6 (pg/mL)	170.5 ± 9.84^{h}	504.5 ± 7.97^{a}	289.1 ± 14.36^{g}	451.9 ± 13.74^{b}	399.2 ± 7.94^{d}	436.6 ± 14.67^{c}	377.9 ± 8.98^{e}	321.9 ± 8.73^{f}
IL-10 (pg/mL)	51.0 ± 3.75^{e}	185.0 ± 12.05^{a}	60.2 ± 11.27^{e}	129.3 ± 13.89^{b}	101.3 ± 7.78^{c}	120.6 ± 8.42^{b}	101.3 ± 7.78^{c}	77.0 ± 5.43^{d}
TNF-α (pg/mL)	99.1 ± 4.71^{f}	204.9 ± 22.83^{a}	118.9 ± 14.97^{e}	155.5 ± 3.77^{cd}	171.1 ± 10.92^{bc}	157.4 ± 8.48^{c}	157.4 ± 8.48^{c}	141.1 ± 5.02^{d}
T cell (%)	100.0 ± 3.03^{a}	45.5 ± 1.63^{g}	84.2 ± 2.83^{b}	54.2 ± 2.22^{f}	61.1 ± 2.72^{e}	55.5 ± 3.29^{f}	66.1 ± 0.76^{d}	73.1 ± 3.20^{c}
B cell (%)	100.0 ± 5.95^{a}	62.6 ± 4.31^{f}	89.6 ± 2.61^{b}	69.5 ± 2.75^{e}	76.7 ± 2.33^{cd}	73.7 ± 0.67^{de}	78.6 ± 2.95^{c}	86.5 ± 1.38^{b}
Blood								
Leukocyte	388.0 ± 17.22^{a}	166.8 ± 17.29^{f}	338.8 ± 26.34^{b}	228.3 ± 9.29^{e}	288.0 ± 7.26^{c}	258.0 ± 8.29^{d}	300.0 ± 14.40^{c}	329.5 ± 8.81^{b}
NO (μmol/mL)	106.9 ± 5.17^{f}	191.0 ± 9.29^{a}	121.3 ± 10.70^{e}	180.8 ± 7.42^{b}	160.2 ± 7.88^{c}	166.5 ± 2.69^{c}	161.9 ± 2.35^{c}	147.2 ± 4.69^{d}
IgE (ng/mL)	3.9 ± 0.64^{a}	2.6 ± 0.45^{b}	3.1 ± 0.45^{b}	2.6 ± 0.42^{b}	2.8 ± 0.29^{b}	2.9 ± 0.25^{b}	3.1 ± 0.41^{b}	3.2 ± 0.29^{b}
IgA (ng/mL)	78.7 ± 3.56^{a}	53.7 ± 3.60^{e}	67.3 ± 4.15^{b}	58.2 ± 1.03^{d}	61.6 ± 0.88^{cd}	60.5 ± 0.66^{d}	61.9 ± 1.39^{cd}	65.7 ± 1.08^{bc}
IgG (ng/mL)	101.2 ± 1.53^{a}	67.4 ± 2.77^{g}	92.9 ± 2.53^{b}	73.1 ± 2.32^{f}	82.3 ± 2.02^{d}	77.9 ± 1.58^{e}	81.3 ± 1.07^{de}	86.5 ± 2.09^{c}

Values are presented as the mean ± standard deviation (n = 8), and different superscript letters indicate significance at $p < 0.05$.
WT, wild type; FSE-C, forced swimming exercise (FSE)-control; PC, positive control (FSE + red ginseng 300 mg/kg body weight); C 200 [FSE + *Cervi Parvum Cornu* (C) extract 200 mg/kg body weight]; A 200 [FSE + *Acanthopanax* (A) extract 200 mg/kg body weight]; CA 50 (FSE + C and A 50 mg/kg body weight); CA 100 (FSE + C and A 100 mg/kg body weight); CA 200 (FSE + C and A 200 mg/kg body weight); IL, interleukin; IFN-γ, interferon-gamma; TNF-α, tumor necrosis factor alpha; NO, nitric oxide; Ig, immunoglobulin.

3.11 Effect of CA treatment on IL-12 and IL-15 productions and NK-cell activity in primary mouse splenocytes

The level IL-12 and IL-15 in the various treatment groups is summarized in figure 5. The level of both IL-12 and IL-15 was significantly downregulated (54.2% and 63.9%, respectively) in Con A-treated splenocytes from FSE-C animals compared to that in the WT control. The PC (63.3%), C200 (16.1%), A200 (21.2%), and CA (50: 20.9%, 100: 26.8%, 200: 33.4%) groups demonstrated significantly increased IL-12 production (Figure. 5A) compared to the FSE-C group. In addition, the PC (128.1%), C200 (44.0%), A200 (72.6%), and CA (50: 60.3%, 100: 78.6%, 200: 92.6%) groups also showed significantly increased IL-15 production (Figure. 5B) compared to the FSE-C group. CA groups also demonstrated a dose-dependent increase in the expression of these factors compared to the FSE-C group ($p < 0.05$).

NK-cell activity was significantly decreased (54.6%) in the FSE-C group compared to that in the WT control. Further, the PC (131.6%), C200 (33.8%), A200 (56.6%), and CA (50: 54.4%, 100: 72.0%, 200: 97.0%) groups were also shown to demonstrate some degree of dose-dependence with respect to these responses ($p < 0.05$; Fig. 5C).

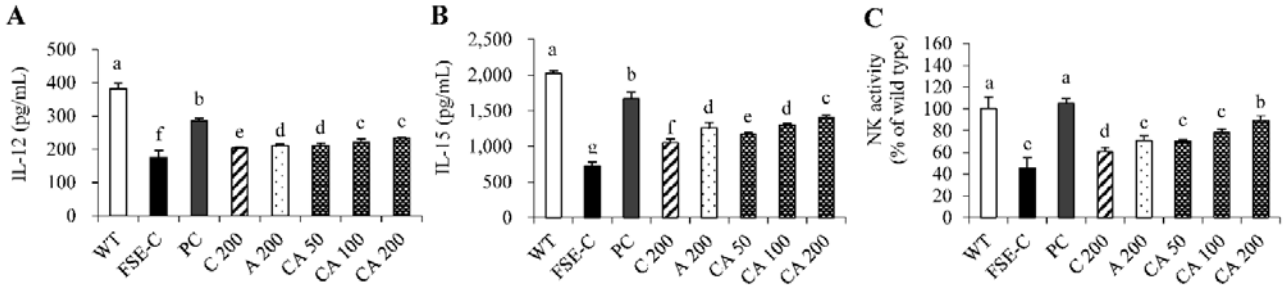

Figure 5. Effects of *cervi parvum cornu* (C), *acanthopanax* (A), and CA mixture on IL-12 (A), and IL-15 (B) levels, and natural killer (NK) cell activity in splenocytes from C57BL/6N mice with induced immunosuppression following forced swimming exercises. WT, wild type; FSE-C, forced swimming exercise-control; PC, positive control (red ginseng 300 mg/kg b.w.); C, *cervi parvum cornu* 200 mg/kg b.w.; A, *acanthopanax* 200 mg/kg b.w.; CA, C and A mixture (50, 100, and 200 mg/kg b.w.). Values represent the mean ± SD (n=3), and the different superscript letters (alphabet) indicate significance at $p < 0.05$.

3.12 Effect of CA on serum NO and immunoglobulin production

Serum NO levels significantly increased (78.7%) in FSE-C group compared to those in the WT control. Further, the PC (36.5%), C200 (5.3%), A200 (16.1%), and CA (50: 12.9%, 100: 15.2%, 200: 22.9%) groups demonstrated significantly decreased NO production compared to the FSE-C group. The CA groups also demonstrated a dose-dependent decrease in NO production compared to the FSE-C group ($p < 0.05$; Table 2).

IgE, IgA, and IgG levels were decreased by 34.7%, 31.8%, and 33.4%, respectively in the FSE-C group compared to those in the WT control. However, no significant differences were observed in IgE production between any of the groups and when compared with that in the FSE-C group. Further, the PC (25.3%), C200 (8.4%), A200 (14.7%), and CA (50: 12.7%, 100: 15.4%, 200: 22.5%) groups demonstrated significantly increased IgA levels. IgG levels were also significantly increased in the PC (37.9%), C200 (8.5%), A200 (22.1%), and CA (50: 15.6%, 100: 20.6%, 200: 28.4%) groups compared to those in the FSE-C group ($p < 0.05$; Table 2).

3.13 Effect of CA treatment on blood leukocyte counts in mice subjected to forced swimming exercise

Leukocyte numbers were significantly decreased (57.0%) in the FSE-C group compared to those in the WT control. While leukocyte numbers were shown to increase significantly in the PC (103.1%), C200 (36.9%), A200 (72.7%), and CA (50: 54.7%, 79.9%, 200: 97.6%) groups compared to those observed in the FSE-C group. CA treatment was once again demonstrated to exhibit a dose-dependent effect with respect to these responses when compared with the FSE-C group ($p < 0.05$; Table 2).

COVID-19 has caused a drastic change in the everyday habits of most of the world population. This crisis has brought human health, and specifically the idea of immunity into sharp focus increasing the demand for functional foods and natural immune boosters [27,28]. Many natural products, including tumeric, red ginseng, and β-glucan are known to enhance immunity and have been the subject of extensive evaluation [29,31]. C is known to improve hematopoietic function, anti-inflammatory responses, and immune function. Among the constituents of C, the phosphatidylcholine fatty acid chain has been linked to the proliferation of lymphocytes, and thus the regulation of the immune response [32-35]. In addition, A has been reported to stimulate the immune system by regulating the levels of several cytokines [24]. However, the synergistic effects of C and A are still unknown. Therefore, we designed this study to evaluate the immunomodulatory effects of CA mixtures *in vitro* and *in vivo*. Cytotoxicity tests performed before the start of this project demonstrated that all of the compounds used in this analysis were nontoxic (data not shown).

Macrophage phagocytosis occurs in almost all tissues, and is one of the primary intrinsic immune responses in response to pathogen challenge [36]. Various intermediaries are produced during phagocytosis, including NO, which acts as a critical mediator for antigen killing in both the innate and innate and adaptive immune responses [37]. However, excessive NO secretion can be toxic to normal cells and can lead to the onset of immune diseases, inflammation, and septic shock [38]. Activated macrophages secrete several cytokines (IL-1β, IL-6, and TNF-α) and stimulate the adaptive immune system to remove antigens. Among the various cytokines, TNF-α plays a role in localizing infection, and induces inflammation at the site of infection, thereby facilitating the host defense response. However, excessive amounts of TNF-α, and other cytokines, can cause the cytokine storm syndrome which may be extremely detrimental to the host [39-41]. Therefore, proper production of NO and TNF-α in macrophages is critical for the proper regulation of the immune system. Here, treatment with CA not only increased NO expression, but also decreased the expression of pro-inflammatory cytokines IL-1β, IL-6, and TNF-α compared to that observed in the LPS-C group *in vitro*. In addition, we confirmed that phagocytosis increases in CA-treated groups.

When pathogens, including viruses, infiltrate the body, they enter the host cells and start replicating, thereby inducing the immune response which either blocks or clears these pathogens. NK cells recognize major histocompatibility complex (MHC) I and are activated by macrophage-derived cytokines to selectively clear infected cells [42]. In our *in vitro* study we used, Yac-1 cells as invaders to evaluate changes in NK-activation in splenocytes treated with various concentrations of CA; a concentration of 200 μg/mL of the CA mixture was found to increase NK activity. Once this was confirmed we went on to evaluate CA-mediated inhibition of viral replication and penetration using LP-BM5, a murine leukemia virus (MulV), model. The LP-BM5 virus used in this study has been reported to cause hypertrophy of the spleen and lymphatic systems, and disorders in the T- and B-cells [43]. Treatment with CA inhibited the replication as well as the penetration of the LP-BM5 virus *in vitro*.

Positive body management including proper exercise improves the body's adaptive immune response via cell-mediated and positive humoral responses [44]. However, irregular exercise, such as one-time activity, can result in abnormal signaling in the immune response such as increasing oxidative stress, inducing inflammation, abnormal lymphocyte proliferation and allergic reactions [45,46]. Kwak et al. reported that lymphocyte

proliferation in splenocytes decreased in mice that were forced to swim without pre-exercise [47]. Thus, we designed a novel immune suppression model for our *in vivo* evaluations, which relied on changes in the adaptive immune response induced by forced swimming rather than viral infection. Using this model we were able to show that oral CA intake induced a significant increase in MHC I and II expression and CD4(+) and CD8(+) T-cells counts compared to that observed in the FSE control. T-cells mediate apoptosis during the adaptive immune response and can be categorized as CD4(+) T-cells and CD8(+) T-cell, and CD4(+) is called T-helper cell, and CD8(+) is called a cytotoxic T-cell. When antigen presenting cells (APCs) recognize virus-like antigens, MHC molecules select T-cells and interact with T-cell co-receptors to mediate antigen clearance [48]. MHC I is expressed by almost all nucleated cells, while MHC II is only expressed by APCs, such as macrophages, B-cells, dendritic cells, and thymic epithelial cells Although the functions of the MHC I and II appear similar, they react to specific antigens by recognizing different mediators, i.e., CD8 (+) and CD4 (+) T-cells, respectively [49].

Sudden high-intensity exercise has been reported to decrease macrophage and NK cell function and decrease the ratio of CD4(+) to CD8(+) co-factors [50]. In this study, NK cell activity increased in mice treated with CA compared to that observed in the FSE-C group. CD4(+) T cells are of two types (Th1 and Th2), where Th1 cells are primarily responsible for macrophage activation and Th1-type cytokine (IL-1β and IFN-γ) secretion, while Th2 cells stimulate B cells and are involved in antibody production via the secretion of Th2-type cytokines (IL-4, IL-6, IL-10, and TNF-α). Immune function is regulated by the appropriate balance and complementary regulation of Th1- and Th2-type cytokine production [51,52]. IL-12 and IL-15 activate NK cell activity, and NK cells bind to virus-infected cells via several pathway and eliminate APCs following the receipt of active signals [53]. In this study, oral CA intake induced significant increases in Th1-type, IL-12, and IL-15 cytokine levels compared to that observed in the FSE-C group, while Th2-type cytokines like IL-4, IL-10, IL-6, and TNF-a were shown to be secreted at abnormally high levels in an immunosuppression study [54].

IL-6 and TNF-α are known to activate the NF-κB pathway and regulate immune cell activity [7,8]. In this study, oral CA intake induced significant decreases in Th2-type cytokine and NO levels compared with that in the FSE-C group. High-intensity short-term exercise has been reported to increase NO production, [55] reduce the number of white blood cells and B cells which may affect antibody production. Here CA increased leukocyte counts IgA/IgG production compared to FSE-C (Fig. 6).

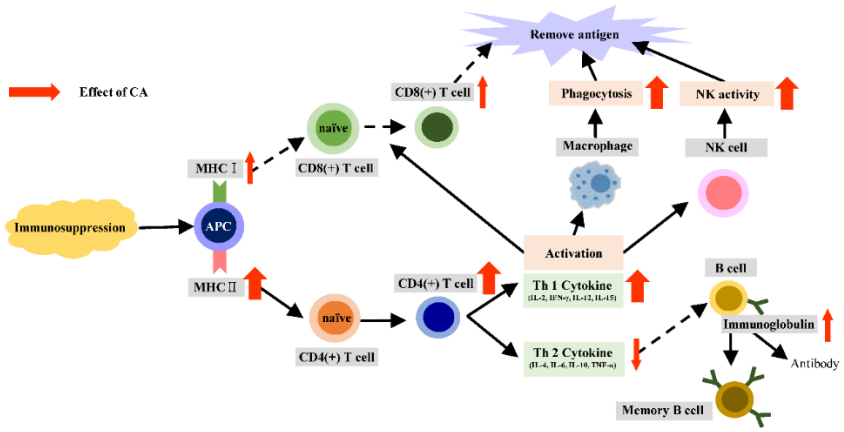

Figure 6. Effects of *cervi parvum cornu* (C), *acanthopanax* (A), and CA mixtures on immunosuppression**.** CD, cluster of differentiation; IFN-γ, interferon-gamma; IL, interleukin; MHC, major histocompatibility complex; Th, T-helper; TNF-α, tumor necrosis factor alpha

4. Conclusions

We investigated the immunomodulatory effects of CA mixtures *in vitro* and *in vivo*. We found that CA treatment increased NO production, phagocytosis and NK cell activity, and decreased pro-inflammatory cytokines production and inhibited viral replication and penetration *in vitro*. Furthermore, we found that oral CA intake increased MHC I and II expression, CD4(+), CD8(+) T-cell counts, IL-12 and IL-15 levels, NK-cell activity, B-cell proliferation, and leukocyte counts in FSE mice. Taken together our results suggest that CA has the potential to be developed into a novel, effective immunomodulatory for functional supplementation.

Disclosure statement

The authors report no conflict of interest.

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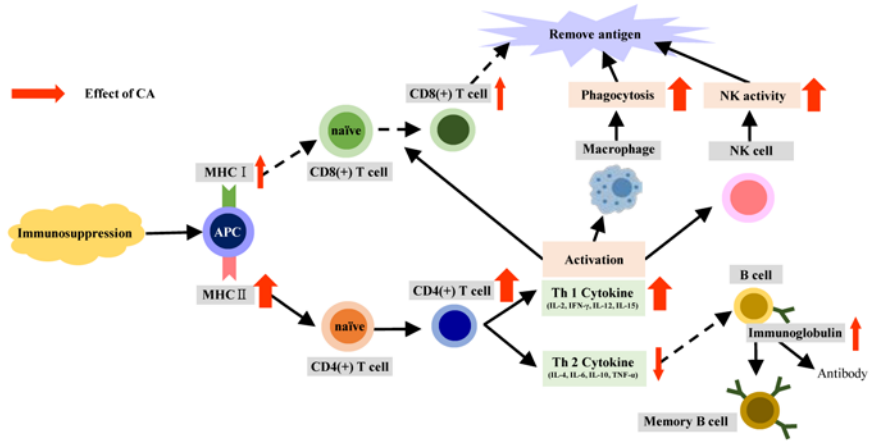
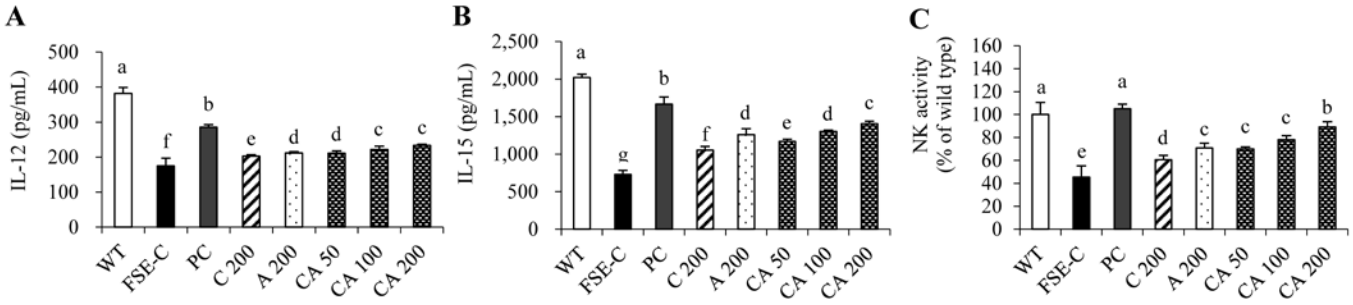
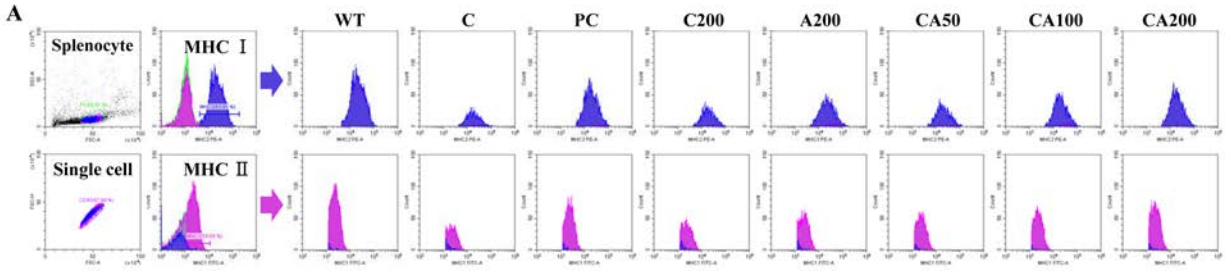
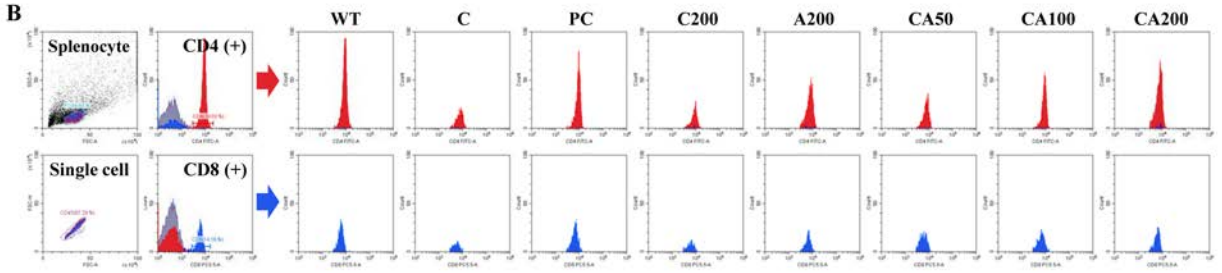
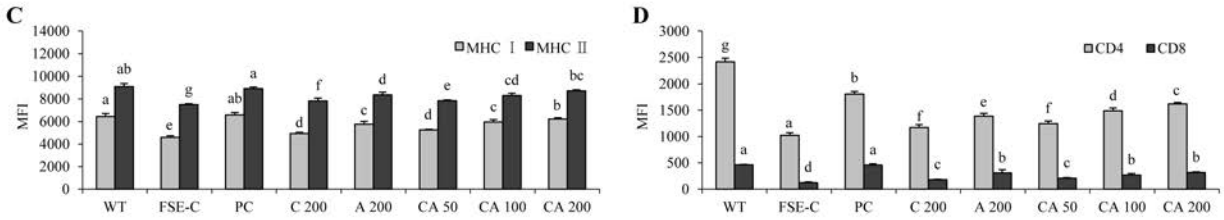
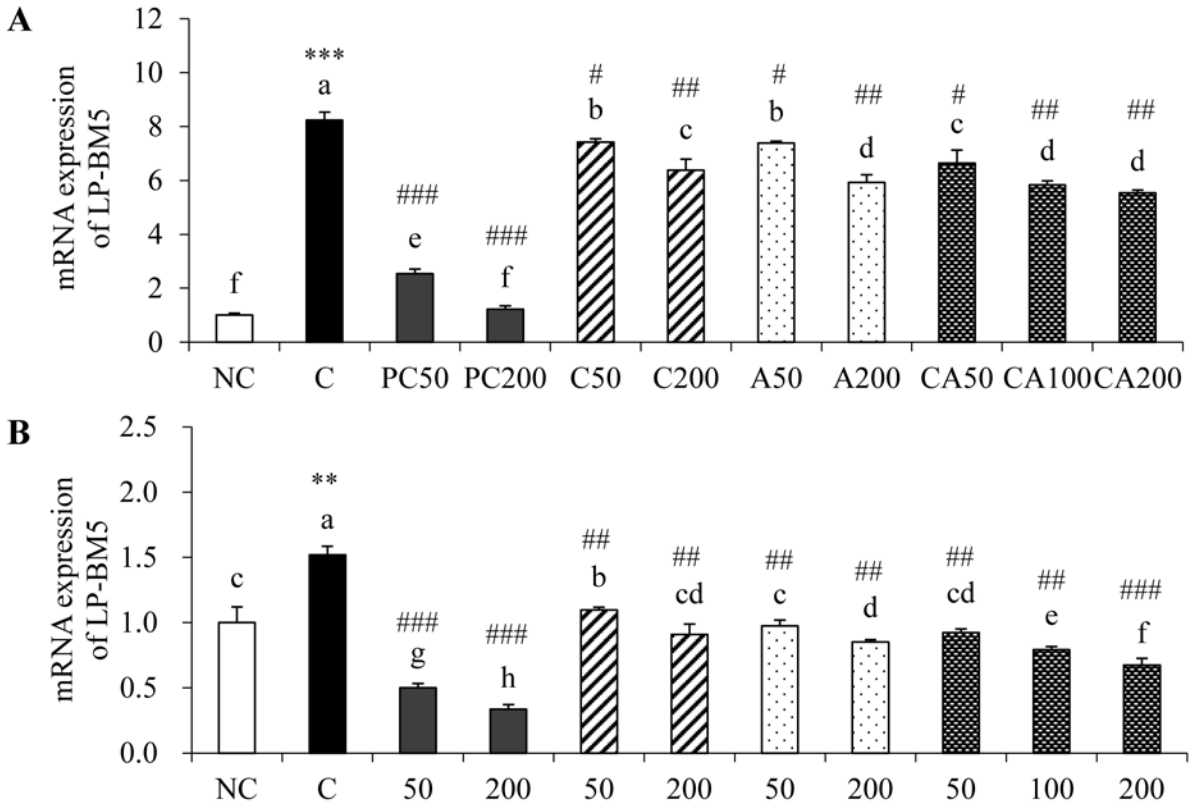

Remove antigen
Effect of CA
CD8(+) T cell
Phagocytosis
NK activity
naïve
Macrophage
NK cell
MHC I
CD8(+) T cell
Immunosuppression
APC
Activation
B cell
MHC II
CD4(+) T cell
Th 1 Cytokine
(IL-2, IFN-γ, IL-12, IL-15)
naïve
Immunoglobulin
Th 2 Cytokine
(IL-4, IL-6, IL-10, TNF-α)
Antibody
CD4(+) T cell
Memory B cell

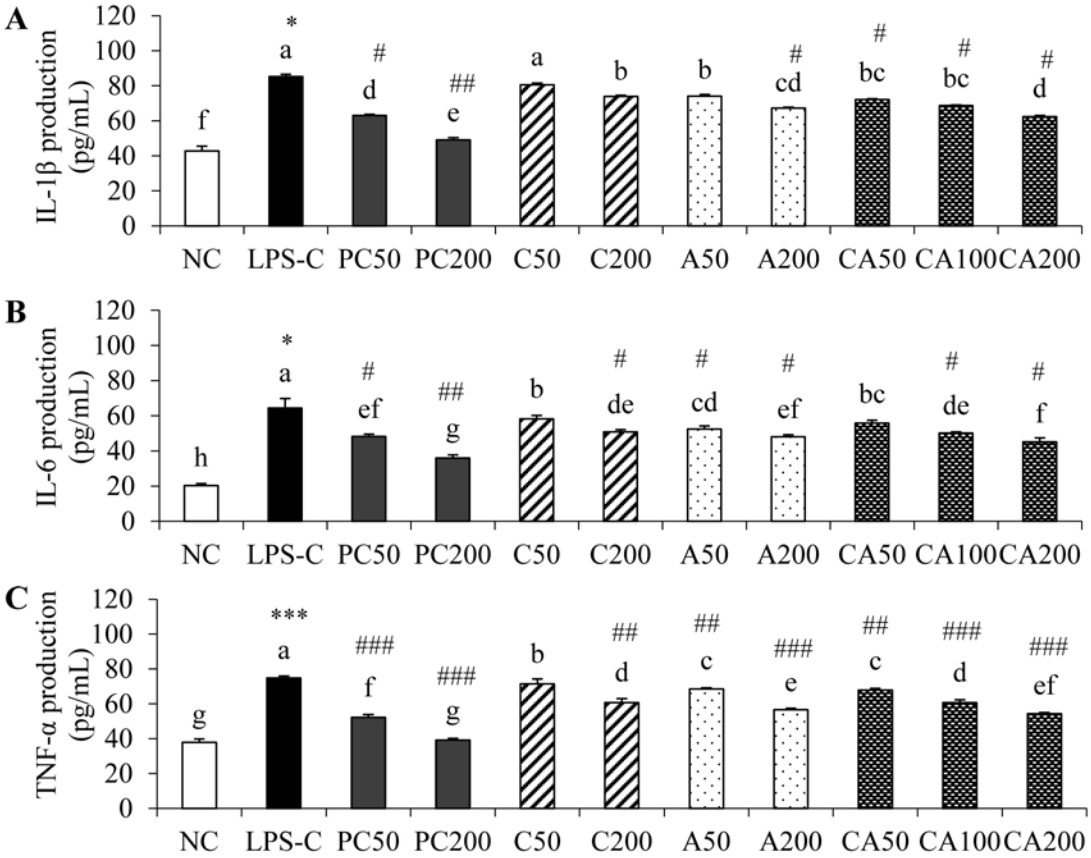

A
IL-12 (pg/mL)
B
IL-15 (pg/mL)
C
NK activity
(% of wild type)
WT
FSE-C
PC
C 200
A 200
CA 50
CA 100
CA 200

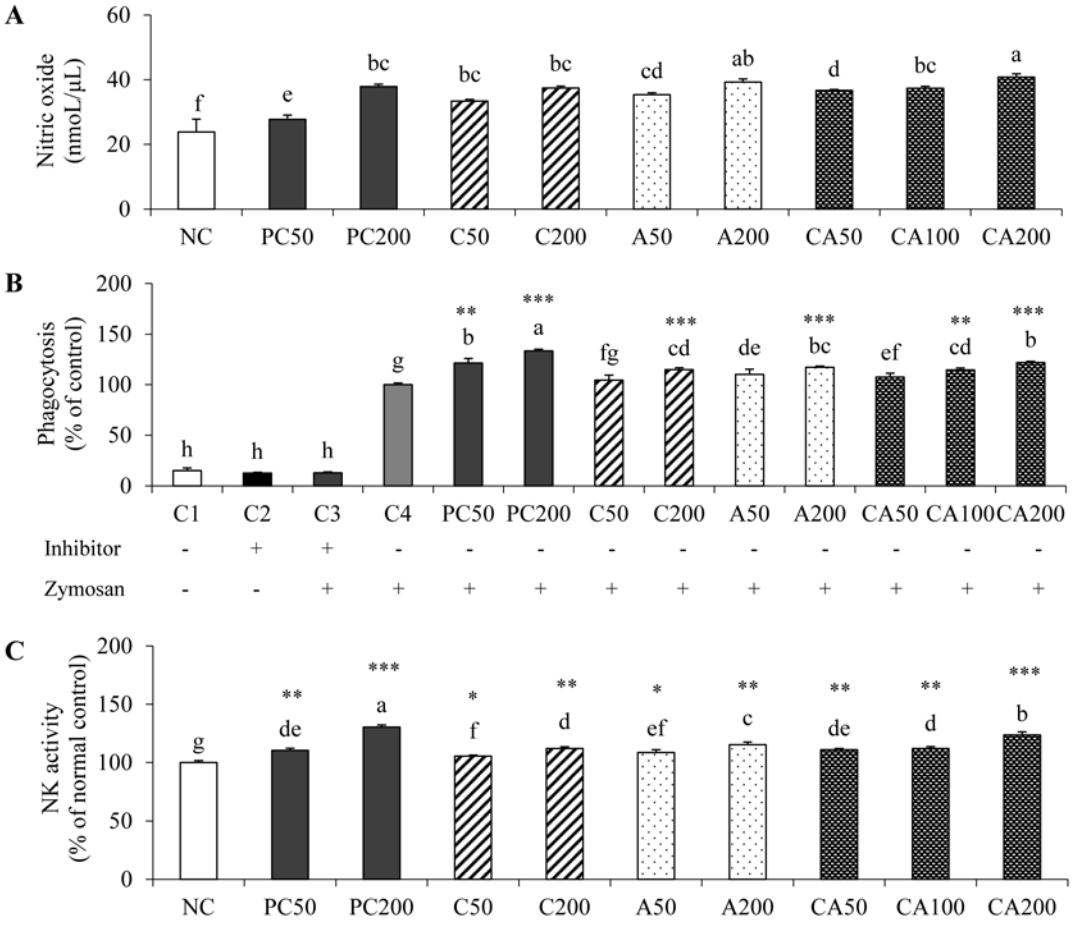

A
Splenocyte
MHC I
Single cell
MHC II
WT
C
PC
C200
A200
CA50
CA100
CA200


B
Splenocyte
CD4 (+)
Single cell
CD8 (+)
WT
C
PC
C200
A200
CA50
CA100
CA200


C
MHC I
MHC II
MFI
D
CD4
CD8
MFI
WT
FSE-C
PC
C 200
A 200
CA 50
CA 100
CA 200


A
mRNA expression of LP-BM5
12
10
8
6
4
2
0
***
a
f
###
e
###
f
#
b
##
c
#
b
##
d
#
c
##
d
##
d
NC
C
PC50
PC200
C50
C200
A50
A200
CA50
CA100
CA200
B
mRNA expression of LP-BM5
2.5
2.0
1.5
1.0
0.5
0.0
**
a
c
###
g
###
h
##
b
##
cd
##
c
##
d
##
cd
##
e
###
f
NC
C
50
200
50
200
50
200
50
100
200


A
IL-1β production (pg/mL)
120
100
80
60
40
20
0
f
*
a
#
d
##
e
a
b
b
#
cd
#
bc
#
bc
#
d
NC
LPS-C
PC50
PC200
C50
C200
A50
A200
CA50
CA100
CA200
B
IL-6 production (pg/mL)
h
*
a
#
ef
##
g
b
#
de
#
cd
#
ef
bc
#
de
#
f
C
TNF-α production (pg/mL)
g
***
a
###
f
###
g
b
##
d
##
c
###
e
##
c
###
d
###
ef


A
Nitric oxide (nmoL/µL)
60
40
20
0
f
e
bc
bc
bc
cd
ab
d
bc
a
NC
PC50
PC200
C50
C200
A50
A200
CA50
CA100
CA200
B
Phagocytosis (% of control)
200
150
100
50
0
h
h
h
g
**
b
***
a
fg
***
cd
de
***
bc
ef
**
cd
***
b
C1
C2
C3
C4
PC50
PC200
C50
C200
A50
A200
CA50
CA100
CA200
Inhibitor
Zymosan
C
NK activity (% of normal control)
200
150
100
50
0
g
**
de
***
a
*
f
**
d
*
ef
**
c
**
de
**
d
***
b
NC
PC50
PC200
C50
C200
A50
A200
CA50
CA100
CA200