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Anti-Mutagenic and Immunomodulatory Effects of Astragali Radix Extract on a Cyclophosphamide-Induced Immunosuppressed Mouse Model

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Abstract: Although the immunomodulatory effects of Astragali Radix extract (AR) have been documented, its anti-mutagenic activity, a problem arising from chemotherapeutic agents, is rarely reported. Therefore, the anti-mutagenic and immunomodulatory effects of AR were investigated using a cyclophosphamide (CPA)-induced immunosuppressed mouse model to develop an alternative immunomodulatory agent. The fluid-bed-dried aqueous extract of AR containing 37.5% dextrin and exopolymers purified from *Aureobasidium pullulans* SM-2001 (EAP) were used in this study. The therapeutic potentials of AR at doses ranging from 100 mg/kg to 400 mg/kg was estimated by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) based cytotoxicity and splenocyte proliferation assay, body weight and lymphatic organ weight measurements, hematological measurements, serum and spleen cytokine level measurements, natural killer (NK) cell activity measurements, real-time RT-PCR expressions of splenic mRNA, a micronucleus test, histopathological observations, and immunohistochemical measurements. In CPA-treated mice, a clear immunosuppressive effect was observed for all tested parameters. However, the oral administration of AR (100, 200, and 400 mg/kg) showed dose-dependent and favorable inhibitory activities on CPA-induced immunosuppression and mutagenicity as compared to 200 mg/kg EAP. Furthermore, AR (100–400 mg/kg) up-regulated the nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB) which are related to NK-, T-, and B-cell activation, with no critical cytotoxicity. The results of this study clearly demonstrate that AR at an appropriate oral dose could act as a potential alternative agent with significant anti-mutagenicity and immunomodulatory properties.

Keywords: anti-mutagenicity effect; Astragali Radix; *Astragalus membranaceus* Bunge; cyclophosphamide; immunomodulatory effect; mouse model

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1. Introduction

The nutritional status of the human body is actively involved in various physiological activities and plays an important role in the defense against autoimmune infections and the occurrence of related diseases [1]. Natural products increase the defense against an immune infection and act as true immunomodulators to suppress excessive immunity [2–4]. Recent advances in analytical techniques have helped to identify immunomodulators with low side effects [5]. Certain nutrients play an essential role in maintaining an appropriate

immune response, where deficiency or excessive intake of these nutrients can adversely affect immune cells [6]. In particular, carotenoids, phenolic compounds, flavonoids, and vitamins present in medicinal plants exhibit pharmacological activities, including anti-inflammatory and immunomodulatory effects through antioxidant mechanisms [7,8].

Cyclophosphamide (CPA), a representative anticancer drug, is commonly used alone or in combination with other drugs [9]. CPA is known to cause severe panleukopenia by damaging the hematopoietic system and lymphatic tissue and is therefore used in bone marrow transplantation to suppress the growth of abnormal cells [10,11]. CPA metabolites release free radicals that cause chromosomal damage and exhibit mutagenicity by alkylating DNA [9,12]. In addition, CPA treatments resulting in panleukopenia, myelosuppression, and immunosuppression cause significant reduction in various immune cytokines, such as tumor necrosis factor (TNF)-α, interferon (IFN)-γ, and interleukin (IL)-1β, 6, and 12 [11,13]. CPA shows significant immunomodulatory properties at low doses; thus, in order to augment its effects to existing therapies, it can be used to modify the immunosuppressive tumor microenvironment [14]. Therefore, the CPA-induced immunosuppressed mouse model has been actively used as a basic animal model to evaluate the immunomodulatory effects and anti-mutagenic activity of candidate substances [11,13,15–19].

Polysaccharides, such as β-1,3/1,6-glucan, cause activation of cells involved in the body's defense mechanism [20,21] and exert anticancer effects through immunomodulation [22]. The purified exopolymers from *Aureobasidium pullulans* SM-2001 (EAP), used as a control drug in this study, contain 13% β-1,3/1,6-glucan as the main ingredient [16,23]. Various pharmacological properties of EAP including improvement of osteoporosis [23], promotion of fracture healing [24], kidney protection [25], anti-inflammatory effects on xylene-induced acute inflammation [26], therapeutic effects on ovalbumin-induced asthma [27], protective effects against cisplatin-induced kidney damage [25], protective effects against experimental osteoarthritis [28], and inhibitory effects on ligation-induced experimental periodontitis and alveolar bone damage [29], have been reported. Furthermore, EAP has shown immunomodulatory effects, particularly in CPA-induced immunosuppressed mice [11,13,16,29,30].

Astragalus membranaceus Bunge, a commonly used traditional medicine and an immunomodulatory agent for fatigue, diarrhea, anorexia, and viral infections in China and Republic of Korea [31–33], has significantly superior Th1 regulatory effects compared to other natural products [33]. *A. membranaceus* extract activates IFN-γ and decreases IL-4 content in pediatric asthma patients [34] and significantly inhibits the increase in Th2-responsive cytokine content in asthma experimental animal models [35]. It also decreases IL-4 content in forced swimming exercise and diet-restricted rat models [36]. Although *A. membranaceus* is used for athletes' adaptability and anti-fatigue activity through immunomodulatory effects [31] using a CPA-induced immunosuppressed mouse model, a commonly used animal-model for immunomodulation and anti-mutagenicity evaluation [11,13,16,29,30], its anti-mutagenic activity, which is a problem in chemotherapeutic agents including CPA, is rarely reported. Therefore, it is necessary to evaluate the systematic immunomodulatory effects, including anti-mutagenicity. Thus, in this study, the systematic anti-mutagenic and immunomodulatory effects of Astragali Radix (AR; Roots of *A. membranaceus* Bunge) extract were evaluated using a CPA-induced immunosuppressed mouse model.

2. Materials and Methods

2.1. Test Material

Brown color powdered extract of Astragali Radix (AR; Roots of *A. membranaceus* Bunge) was prepared and supplied by a sponsor (Aribio H&B, Jecheon, Republic of Korea; Lot No: ARHH1001). Briefly, the aqueous crude extract of AR was prepared at 100 °C for 4 h using 10-fold distilled water and evaporated at 30 brix. The evaporated extract was further fluid-bed-dried and mixed with 37.5% dextrin to maintain its powder form. Light brown powder of EAP containing 13% β-1,3/1,6-glucan (Glucan Corp., Busan, Republic

of Korea) was used as a reference drug. AR and EAP were stored in a refrigerator at 4 °C to protect from light and degeneration until further use. Some specimens of AR (Code No: AR2022Ku01) and EAP (Code No: EAP2021Ku01) were deposited in the herbarium of the Medical Research Center for Herbal Convergence on Liver Disease, Daegu Haany University (Daegu, Republic of Korea).

2.2. High-Performance Liquid Chromatography Analyses

Calycosin 7-O-β-D-glucoside content in the AR extract was quantified using an Agilent HPLC 1100 series (Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with a UV-visible absorbance detector (Agilent Technologies, Inc.) and a Capcell Pak C18 UG120 column (4.6 mm × 250 mm, 5 μm; Osaka Soda Co. Ltd., Osaka, Japan). AR extract and standard calycosin 7-O-β-D-glucoside were dissolved in acetonitrile and filtered through a 0.45 μm membrane filter. The column temperature was maintained at 35 °C during the analysis, and calycosin 7-O-β-D-glucoside was analyzed at a wavelength of 260 nm. The mobile phase consisted of a mixture of distilled water (containing 0.1% phosphoric acid) and acetonitrile (containing 0.1% phosphoric acid) (87:13). The specific conditions for HPLC analysis are provided in Table S1. Standard calycosin 7-O-β-D-glucoside, purchased from Chengdu Biopurity Phytochemicals Co., Ltd., Chengdu, China, was used as a positive control. The 10 μL of each sample were injected at a flow rate of 1.0 mL/min, and the results were quantified as the relative peak area based on the standard peak area and the concentration.

2.3. Laboratory Animals

All experimental animals, six-week-old male SPF/VAF Outbred CrlOri:CD1 (ICR) mice (OrientBio, Seungnam, Republic of Korea), were treated in accordance with the animal ethics guidelines approved by the Laboratory Animal Ethics Committee of Daegu Haany University [Approval No.: DHU2022-047; 24 May 2022]. Eighty mice were obtained and acclimatized for seven days. Mice showing constant body weights (normal weight gains during the acclimatization period) were selected for this study. Afterwards, six groups comprising ten mice each were formed and used in this study. In total 60 mice were used, having an average weight of 35.15 ± 1.68 g.

The experimental animals were divided into the following groups:

1. Intact vehicle control: distilled-water administered intact control mice;
2. CPA control: CPA-treated and distilled-water administered control mice;
3. EAP_{200}: CPA-treated and EAP (200 mg/kg; containing 26 mg/kg of β-1,3/1,6-glucan) administered mice;
4. AR_{100}: CPA-treated and AR (100 mg/kg) administered mice;
5. AR_{200}: CPA-treated and AR (200 mg/kg) administered mice;
6. AR_{400}: CPA-treated and AR (400 mg/kg) administered mice.

2.4. Induction of Immunosuppression

Immunosuppression was induced based on the methods reported in our previous studies [11,13,16,30] with slight modifications. Briefly, 150 and 110 mg/kg of CPA (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in sterile physiological saline and intraperitoneally administered using a 1 mL syringe with a 26G needle at 3 days and 1 day before euthanasia on the final day, respectively, at an oral dose of 10 mL/kg. In the normal medium control group, the same amount of sterile physiological saline was administered instead of CPA.

2.5. Dose Frequency

Once daily for 42 days, 100, 200, and 400 mg/kg of AR or 200 mg/kg of EPA (containing 26 mg/kg of β-1,3/1,6-glucan) were orally administered at a dose of 10 mL/kg to the experimental animals using a 1 mL syringe attached with a metal belt. The lowest oral dose level of 100 mg/kg of AR was selected based on the previous immunomodulatory

activity study of AR [37] and the organ protective effects test of AR using CPA-treated mice [38]. In addition, oral doses of 200 and 400 mg/kg were selected as middle and lower dosages of AR, by using a common ratio of 2, in the present study. In the normal medium and CPA control groups, only sterile distilled water (as a medium instead of a drug) was orally administered at the same dose and frequency to apply the same correction stress for oral administration.

2.6. Observation Items

Changes in body weight, thymus, spleen, and submandibular lymph node weights were directly measured using an electronic scale (Precisa Gravimetrics AG, Dietikon, Switzerland).

Hematological examinations for total white (WBC) and red blood cell (RBC) counts, white blood cell differential count, neutrophil, acidophilus, basic neutrophil and monocyte ratio, hemoglobin level, hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and platelet count were performed using an automatic complete blood count (CBC) analyzer (Abbott Laboratories, Abbott Park, IL, USA). RBC and WBC counts were measured using electrical impedance and light scattering [39], and packed cell volume (PCV) was measured using the microhematocrit method [40]. MCV was obtained using the formula PCV $\times$ 10/RBC ($10^6/mm^3$) and MCH was calculated using the formula hemoglobin—Hb (g/100 mL) $\times$ 10/RBC ($10^6/mm^3$). Whereas MCHC was automatically calculated using an automatic hemocytometer via the formula Hb (g/100 mL) $\times$ 100/PCV.

IFN-γ, TNF-α, IL-1β, IL-6, and IL-12 levels in the serum and the spleen, and NK cell activity in the peritoneal cavity and the spleen; changes in NF-κB, IFN-γ, TNF-α, IL-1β, IL-6; and IL-12 mRNA expressions in the spleen were determined using real-time RT-PCR in accordance with the method described by Bustin [41]. The oligonucleotides used for real-time RT-PCR are listed in Table S2. Splenic and peritoneal NK cell activity was measured using a standard ^{51}Cr release assay [30].

Histopathological changes in lymphoid organs; total thymus and cortical thickness, total spleen and white medulla thickness, white medulla number, number of CD3+, CD4+, CD8+, TNF-α+, IL-1β+, and inducible nitric oxide synthase (iNOS+) immunoreactive cells in spleen tissue; CD3+, CD4+, CD8+, TNF-α+, IL-1β+, and iNOS+ immune responses in spleen tissue; and the number of cells were determined using the avidin-biotin peroxidase complex (ABC) test method as proposed by Hsu et al. [42]. The primary antisera and detection kits for immunohistochemistry used in this study are listed in Table S3.

The cytotoxicity and splenocyte proliferation activity of the splenocytes harvested on the final day following euthanasia (T-cell proliferation promoter, Concanavalin A (ConA; Sigma-Aldrich, St. Louis, MO, USA) and B-cell proliferation promoter, measurement of proliferation activity of spleen cells by lipopolysaccharide (LPS; Sigma-Aldrich, St. Louis, MO, USA) treatment) were confirmed using an MTT assay [43].

Anti-mutagenicity was determined through a micronucleus test on the bone marrow smear collected on the final day following euthanasia, including the number of polychromatic erythrocytes (PCE), polychromatic erythrocyte ratio (PCE/PCE + normochromatic erythrocytes—NCE), micronucleated polychromatic erythrocytes (MN), and changes in the number of contained PCE; MNPCE (micronucleated polychromatic erythrocytes), were determined via nuclear staining [44].

2.7. Statistical Analyses

The experimental data were expressed as the means $\pm$ the standard deviation (S.D.) of ten animals. The different dose groups were compared using multiple comparison tests including the Levene's test, the least significant differences multi-comparison (LSD) test, the Kruskal-Wallis H test (a non-parametric comparison test), and the Mann–Whitney U (MW) test as demonstrated in our previous studies [11,13]. SPSS ver. 14 (IBM SPSS Inc., Armonk, NY, USA) was used for the statistical analyses [11,13], and the differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Content of Calycosin 7-O-β-D-Glucoside in AR Extract

HPLC analysis of the AR extract used in this study detected the calycosin 7-O-β-D-glucoside at a concentration of 0.34 mg/g, quantified as the relative peak area based on the standard peak area and the concentration (Figure 1).

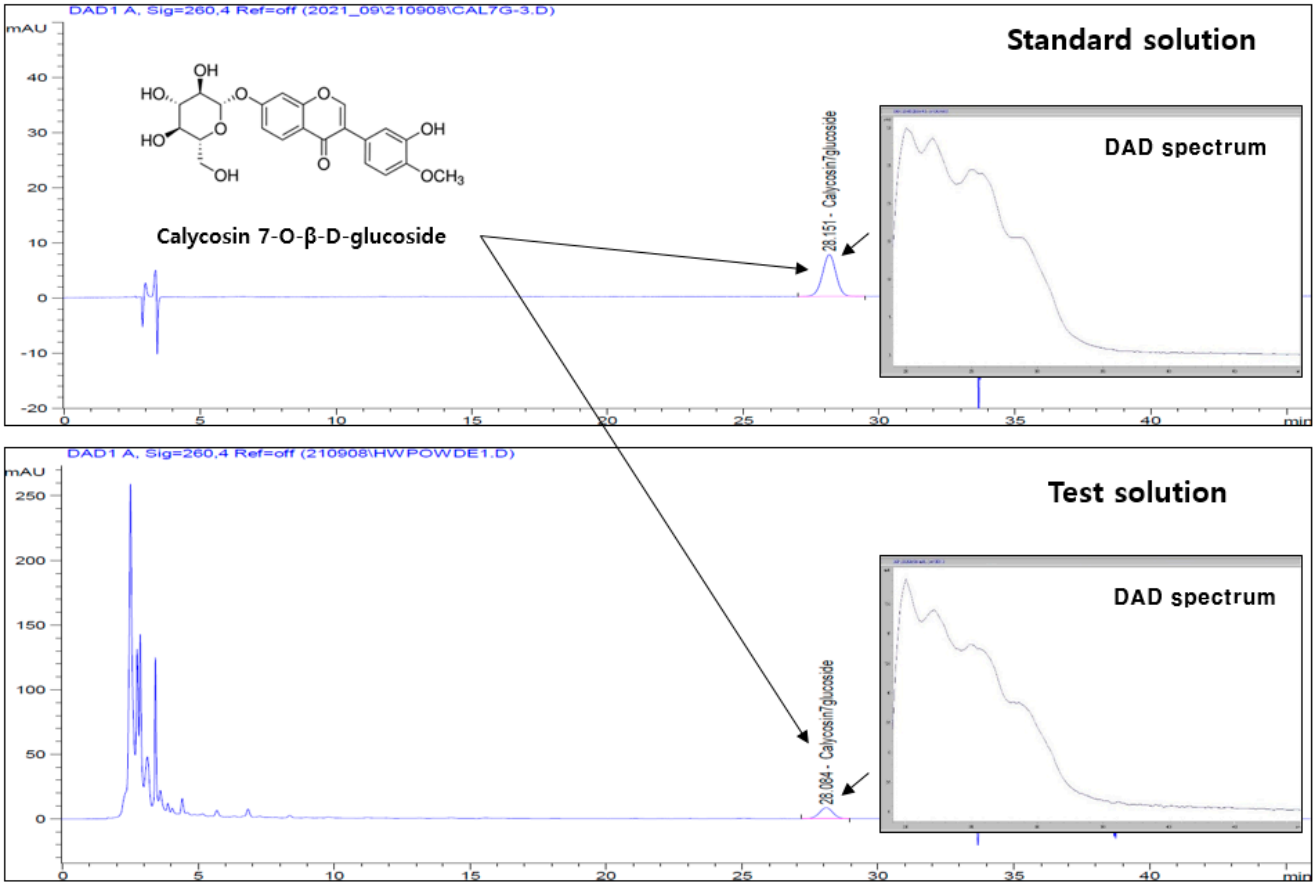

Figure 1. HPLC chromatogram of calycosin 7-O-β-D-glucoside in Astragali Radix extract.

3.2. Changes in Body Weight

Compared with the normal vehicle control group, no significant changes in body weight or weight gain were observed in the CPA control group. In addition, no significant changes in body weight or weight gain were observed in the EAP_{200}, AR_{100}, AR_{200}, and AR_{400} groups during the 42-day experimental period as compared to the CPA control group (Table 1).

3.3. Cytotoxicity to Splenocytes

When examining the results of the MTT cytotoxicity evaluation on splenocytes collected and cultured from the normal vehicle control group at the time of euthanasia, no significant changes were observed in cell viability for all AR-treated groups (0.01–10 mg/mL) compared to the untreated control group (0 mg/mL; Figure 2).

Table 1. Body weight gain in intact and CPA-induced immunosuppressed mice.

Groups	Body Weights at:				Body Weight Gains [B–A]
	First Test Substance Administration [A] *	First CPA Treatment	Second CPA Treatment	At Sacrifice [B] *	
Controls					
Intact	32.21 ± 2.24	40.56 ± 2.00	40.82 ± 2.01	37.93 ± 2.16	5.72 ± 0.84
CPA	32.30 ± 1.57	40.69 ± 1.65	40.81 ± 1.54	37.77 ± 1.54	5.47 ± 0.61
EAP_{200}	32.09 ± 1.66	40.30 ± 1.80	40.32 ± 1.78	37.37 ± 1.96	5.28 ± 0.80
Test materials					
AR_{400}	32.34 ± 1.64	40.78 ± 1.81	40.97 ± 1.75	37.99 ± 1.76	5.65 ± 0.87
AR_{200}	32.24 ± 2.12	40.51 ± 2.23	40.59 ± 2.36	37.65 ± 2.42	5.41 ± 0.85
AR_{100}	31.96 ± 1.42	40.62 ± 1.96	40.60 ± 2.07	37.61 ± 2.09	5.65 ± 1.15

CPA = cyclophosphamide administered group; EAP_{200} = *A. pullulans* purified exopolymer administered group at a dose of 200 mg/kg; AR_{400}, $_{200}$, and $_{100}$ = Astragali Radix (Roots of *A. membranaceus* Bunge) extract administered mice groups at doses of 400, 200, and 100 mg/kg, respectively. * All animals were fasted overnight (approximately 18 h; water was not restricted) before sacrifice.

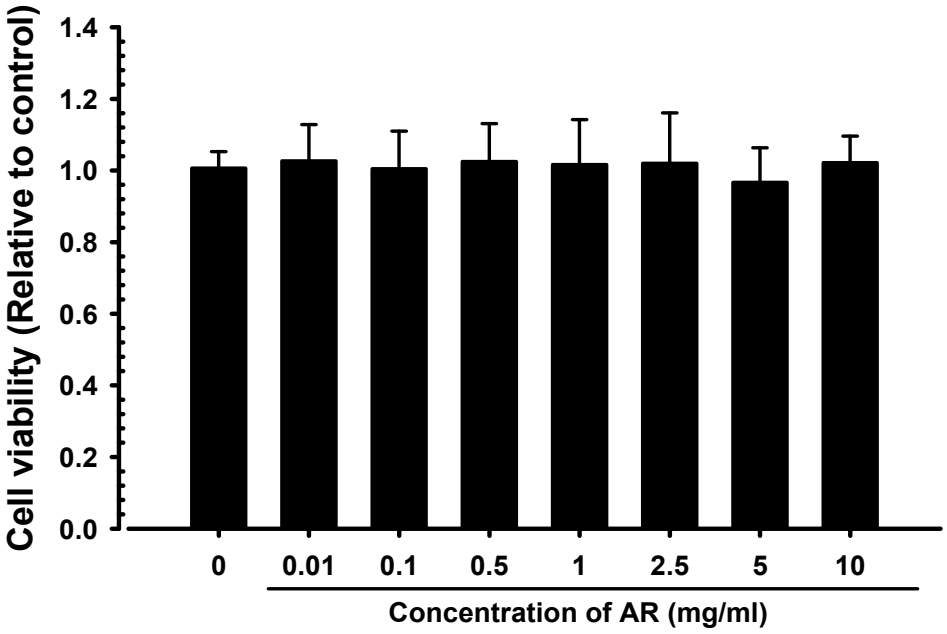

Figure 2. Effects of AR on the normal splenocytes' viability. Values are expressed as means ± S.D. of six independent experiments; AR = Astragali Radix (Roots of *A. membranaceus* Bunge) extract; cells were treated with 0 (control), 0.01, 0.1, 0.5, 1, 2.5, 5 and 10 mg/mL of AR dissolved in media and incubated for 72 h.

3.4. Changes in Splenocyte Proliferation Rate by ConA

Lymphocyte proliferation induced by ConA is a commonly used method for detection of T lymphocyte immunity in vitro [11,30]. The splenocytes collected and cultured on the final day following euthanasia in the CPA control group showed a significant ($p < 0.01$) decrease in ConA-induced cell proliferation compared to splenocytes in the normal vehicle control group. However, a significant ($p < 0.01$) increase in ConA-induced cell proliferation was observed in all test substance administered groups, including EAP, compared to spleen cells in the CPA control group. In particular, the AR groups showed a dose-dependent increase in cell proliferation rate, and the AR (100 mg/kg) administered group showed an inhibitory effect on cell proliferation reduction comparable to that of EAP (200 mg/kg; Figure 3).

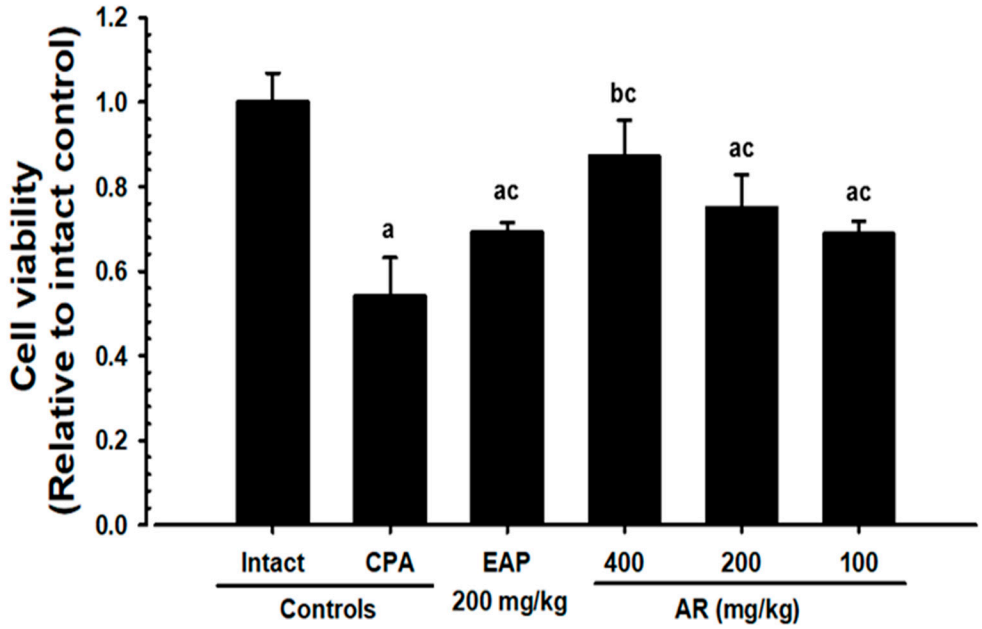

Figure 3. Effects on the ConA-induced splenocytes' proliferation. Values are expressed as means ± S.D. of ten mice; CPA = cyclophosphamide; EAP = exopolymers purified from *A. pullulans*; AR = Astragali Radix (Roots of *A. membranaceus* Bunge) extracts; ConA = Concanavalin A; DT3 = Dunnett's T3; Cells were treated with 5 μg/mL of ConA dissolved in media and incubated for 48 h; [a] $p < 0.01$ and [b] $p < 0.05$ as compared with intact control by the DT3 test; [c] $p < 0.01$ as compared with CPA control by the DT3 test.

3.5. Changes in Splenocyte Proliferation Rate by LPS

Lymphocyte proliferation induced by LPS is often used to detect B-lymphocyte immunity in vitro [11,30]. The splenocytes collected and cultured on the final day following euthanasia in the CPA control group showed a significant ($p < 0.01$) decrease in LPS-induced cell proliferation compared to splenocytes in the normal vehicle control group. However, a significant ($p < 0.01$) increase in LPS-induced cell proliferation was observed in all test substance-administered groups, including the AR_{400} group, compared to the spleen cells in the CPA control group. In particular, AR groups showed a dose-dependent increase in cell proliferation rate, and the AR_{100} group showed an inhibitory effect on the reduction in CPA-induced splenocyte proliferation comparable to that of EAP_{200} (Figure 4).

3.6. Micronucleus Test on Bone Marrow Cells

Bone marrow cells collected and smeared on the final day following euthanasia in the CPA control group showed a significant ($p < 0.01$) increase in the number of MNPCE and a decrease in the number and ratio of PCE compared to those in the normal mounted control group. However, a significant ($p < 0.01$) decrease in the number of MN and an increase in the number and ratio of PCE were confirmed in all test substance administered groups, including the AR_{200} group, compared to the CPA control group. In particular, AR groups caused a dose-dependent decrease in MNPCE and an increase in the number and ratio of PCE. In addition, AR (100 mg/kg) showed an inhibitory effect on the increase in CPA-induced MNPCE and a decrease in the number and ratio of PCE compared to EAP (200 mg/kg; Table 2, Figure 5).

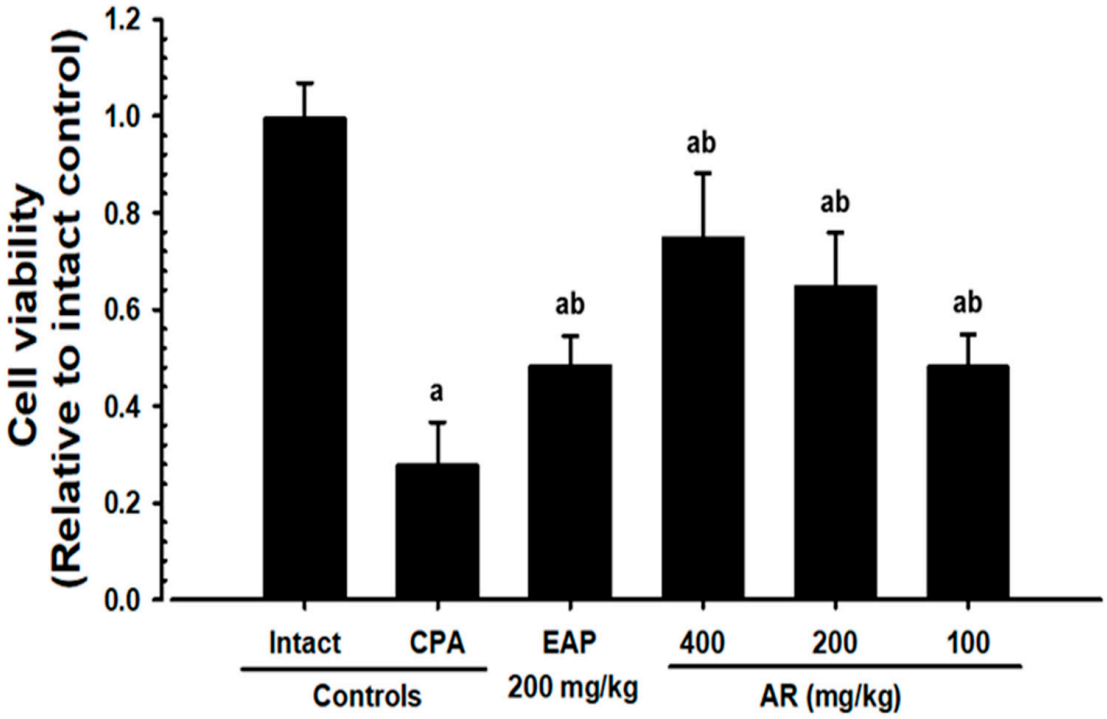

Figure 4. Effects on the LPS-treated splenocyte proliferation. CPA = cyclophosphamide; EAP = exopolymers purified from *A. pullulans*; AR = Astragali Radix (Roots of *A. membranaceus* Bunge) extracts; LPS = Lipopolysaccharide; THSD = Tukey's honest significant difference; cells were treated with 10 μg/mL of LPS dissolved in media and incubated for 48 h; [a] $p < 0.01$ as compared with intact control by THSD test; [b] $p < 0.01$ as compared with CPA control by THSD test.

Table 2. Results of the micronucleus assay of bone marrow cells collected from individual intact or CPA-induced immunosuppressed mice at sacrifice.

Groups	Bone Marrow Cell Smear Cytology		
	MNPCE Numbers (Cells/2000 PCEs)	**PCE Numbers (Cells/500 Erythrocytes)**	**PCE/(NCE + PCE)**
Controls			
Intact	0.70 ± 0.67	240.30 ± 20.94	0.48 ± 0.04
CPA	84.60 ± 10.05 [d]	129.10 ± 7.43 [a]	0.26 ± 0.01 [d]
EAP_{200}	55.40 ± 11.21 [de]	169.40 ± 11.82 [ac]	0.34 ± 0.02 [de]
Test materials			
AR_{400}	32.90 ± 16.72 [de]	219.10 ± 11.18 [bc]	0.44 ± 0.02 [e]
AR_{200}	44.90 ± 13.63 [de]	194.00 ± 21.34 [ac]	0.39 ± 0.04 [de]
AR_{100}	56.20 ± 10.60 [de]	168.40 ± 15.58 [ac]	0.34 ± 0.03 [de]

CPA = cyclophosphamide administered group; EAP_{200} = *A. pullulans* purified exopolymer administered group at a dose of 200 mg/kg; $AR_{400, 200,}$ and $_{100}$ = Astragali Radix (Roots of *A. membranaceus* Bunge) extract administered mice groups at doses of 400, 200, and 100 mg/kg, respectively; MN = Micronucleus; PCE = Polychromatic erythrocytes; NCE = Normochromatic erythrocytes; MNPCE = MN containing PCE; THSD = Tukey's honest significant difference; DT3 = Dunnett's T3; [a] $p < 0.01$ and [b] $p < 0.05$, compared with intact control by THSD test; [c] $p < 0.01$ as compared with CPA control by THSD test; [d] $p < 0.01$ as compared with intact control by the DT3 test; [e] $p < 0.01$ as compared with CPA control by the DT3 test.

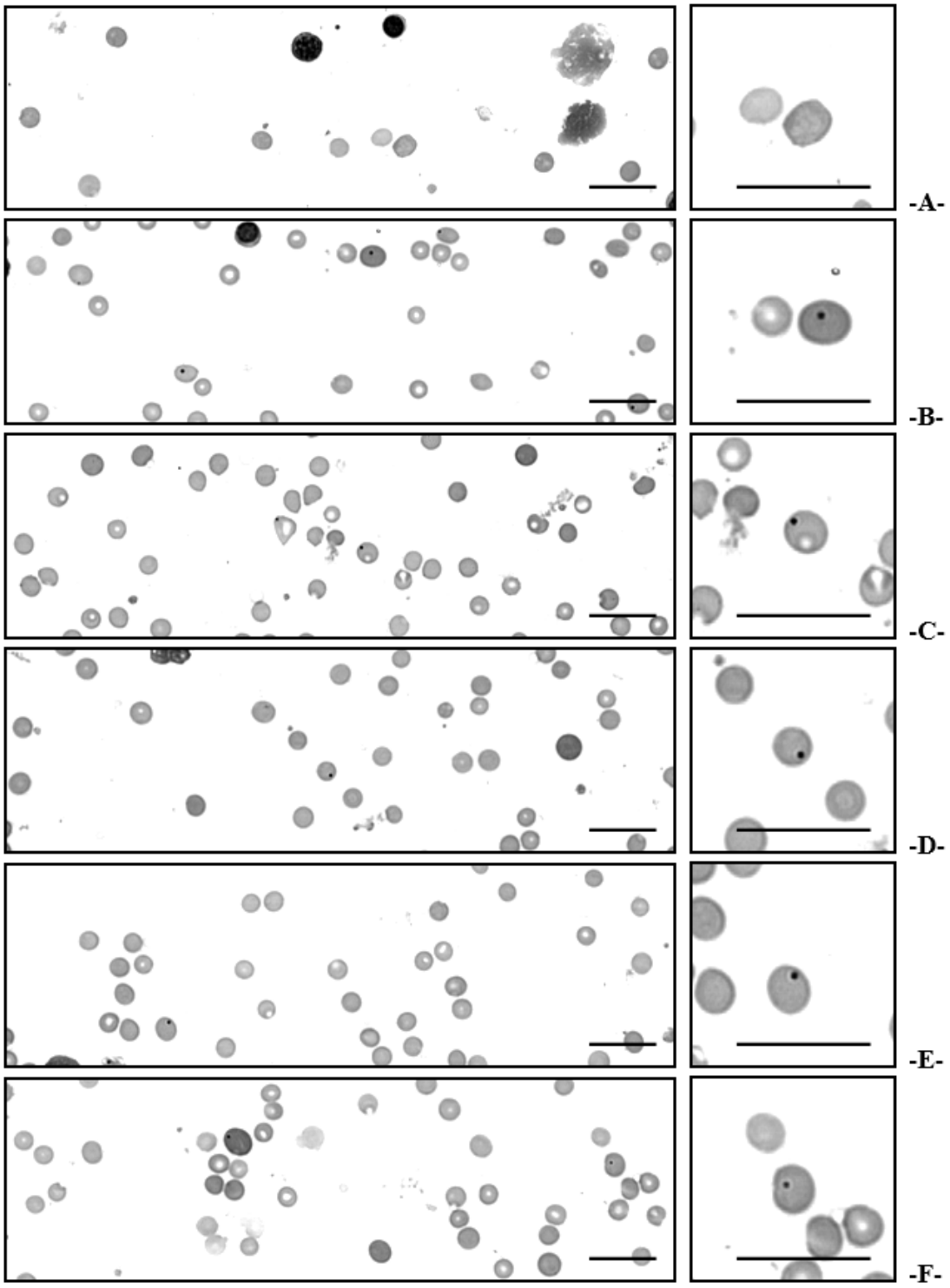

Figure 5. Representative cell smear cytological images of the bone marrow cells collected from individual mice in each group upon euthanasia. (**A**) Intact vehicle control: distilled-water administered intact mice; (**B**) CPA control: cyclophosphamide-treated and distilled-water administered control mice; (**C**) EAP_{200}: CPA treated and *A. pullulans* purified exopolymers (EAP at a dose of 200 mg/kg) administered mice; (**D**) AR_{400}: CPA treated and AR (400 mg/kg) administered mice; (**E**) AR_{200}: CPA treated and AR (200 mg/kg) administered mice; (**F**) AR_{100}: CPA treated and AR (100 mg/kg) administered mice; AR = Astragali Radix (Roots of *A. membranaceus* Bunge) extracts; PCE = Polychromatic erythrocytes; MN = Micronucleus; NCE = Normochromatic erythrocytes; MNPCE = MN contained PCE; all May-Grunwald and Giemsa stain; scale bar = 20 μm.

3.7. Changes in Lymph Organ Weight

In the CPA control group, significant ($p < 0.01$) reductions in the relative and absolute weights of the thymus gland, spleen, and submandibular lymph nodes were observed as compared to those in the control group. However, significant ($p < 0.01$) increases were observed in the thymus, spleen, and submandibular lymph node weights in all test substance administered groups, including the AR_{100} group, compared to the CPA control group. In particular, the AR administered groups showed a dose-dependent increase in the absolute and relative weights of lymph organs. In addition, the AR_{100} group exhibited an inhibitory effect on the reduction in CPA-induced lymph organ weight, comparable to that of the EAP_{200} group (Table 3).

Table 3. Lymphatic organ weights in intact and CPA-induced immunosuppressed mice.

Groups	Absolute Weights (g)			Relative Weights (% of Body Weights)		
	Thymus	**Spleen**	**LN**	**Thymus**	**Spleen**	**LN**
Controls						
Intact	0.069 ± 0.007	0.123 ± 0.009	0.015 ± 0.003	0.181 ± 0.021	0.326 ± 0.024	0.039 ± 0.007
CPA	0.014 ± 0.003 [a]	0.048 ± 0.008 [a]	0.003 ± 0.001 [c]	0.037 ± 0.006 [a]	0.127 ± 0.023 [a]	0.008 ± 0.002 [c]
EAP_{200}	0.028 ± 0.007 [ab]	0.068 ± 0.008 [ab]	0.006 ± 0.001 [cd]	0.073 ± 0.017 [ab]	0.181 ± 0.025 [ab]	0.015 ± 0.012 [cd]
Test materials						
AR_{400}	0.046 ± 0.010 [ab]	0.084 ± 0.012 [ab]	0.009 ± 0.002 [cd]	0.121 ± 0.023 [ab]	0.221 ± 0.039 [ab]	0.023 ± 0.005 [cd]
AR_{200}	0.035 ± 0.008 [ab]	0.075 ± 0.007 [ab]	0.007 ± 0.001 [cd]	0.094 ± 0.019 [ab]	0.200 ± 0.022 [ab]	0.018 ± 0.004 [cd]
AR_{100}	0.028 ± 0.010 [ab]	0.067 ± 0.007 [ab]	0.006 ± 0.001 [cd]	0.073 ± 0.024 [ab]	0.179 ± 0.021 [ab]	0.015 ± 0.002 [cd]

CPA = cyclophosphamide administered group; EAP_{200} = *A. pullulans* purified exopolymer administered group at a dose of 200 mg/kg; AR_{400}, $_{200}$, and $_{100}$ = Astragali Radix (Roots of *A. membranaceus* Bunge) extract administered mice groups at doses of 400, 200, and 100 mg/kg, respectively; LN = submandibular lymph node; THSD = Tukey's honest significant difference test; DT3 = Dunnett's T3; [a] $p < 0.01$ as compared with intact control by THSD test; [b] $p < 0.01$ as compared with CPA control by THSD test; [c] $p < 0.01$ as compared with intact control by the DT3 test; [d] $p < 0.01$ as compared with CPA control by the DT3 test.

3.8. Hematological Changes

Significant ($p < 0.01$) reductions in total leukocyte and RBC count, hemoglobin (HGB), HCT, and platelet count, that is, pan-leukocyte and thrombocytopenia due to significant hematopoietic inhibition, and aplastic anemia, were observed in the CPA control group compared to the normal vehicle control group. However, no change was observed in the leukocyte fractionation count in the CPA control group. Conversely, the decrease in total leukocyte and RBC count, HGB, HCT, and platelet count due to hematopoietic inhibition by CPA administration was significantly ($p < 0.01$) suppressed by administration of all test substances, including AR (100 mg/kg). In particular, in the AR administered groups, a dose-dependent hematopoietic promoting action was observed. In the AR_{100}, CPA-induced inhibition of the hematopoietic system was reduced, comparable to that in the EAP_{200} group (Table 4).

3.9. Changes in Blood Cytokine—IFN-γ, TNF-α, IL-1β, IL-6 and IL-12 Contents

In the CPA control group, blood IFN-γ, TNF-α, IL-1β, IL-6, and IL-12 levels were significantly ($p < 0.01$) decreased compared to those in the normal vehicle control group. However, in all test substance administered groups, including EAP_{200}, the levels of IFN-γ, TNF-α, IL-1β, IL-6, and IL-12 increased significantly ($p < 0.01$; $p < 0.05$) compared to those in the CPA control group. In particular, the AR administered groups showed a dose-dependent increase in the blood cytokine content. In the AR_{100} group, the inhibitory effects of reduction in the CPA-induced blood IFN-γ, TNF-α, IL-1β, IL-6, and IL-12 content were comparable to those of the EAP_{200} group (Table 5).

Table 4. Hematological values in intact and CPA-induced immunosuppressed mice.

Groups	Controls		Reference	Test Materials		
	Intact	CPA	EAP_{200}	AR_{400}	AR_{200}	AR_{100}
WBC (K/μL)	7.61 ± 0.48	0.55 ± 0.14 ^{c}	1.09 ± 0.10 ^{cd}	3.09 ± 1.00 ^{cd}	2.14 ± 0.79 ^{cd}	1.08 ± 0.15 ^{cd}
Differential count						
LYM%	77.93 ± 2.41	78.55 ± 2.26	78.05 ± 2.58	78.04 ± 1.40	77.98 ± 1.36	78.30 ± 2.18
NEU%	15.71 ± 2.37	15.74 ± 1.91	15.79 ± 1.94	15.88 ± 0.97	15.73 ± 1.71	15.50 ± 1.75
MONO%	3.94 ± 1.51	4.16 ± 1.12	4.21 ± 1.25	4.14 ± 1.22	4.18 ± 1.12	4.17 ± 1.35
EOS%	0.23 ± 0.13	0.24 ± 0.20	0.24 ± 0.21	0.25 ± 0.19	0.25 ± 0.16	0.25 ± 0.19
BASO%	0.31 ± 0.14	0.30 ± 0.12	0.31 ± 0.14	0.28 ± 0.15	0.30 ± 0.15	0.31 ± 0.14
RBC (M/μL)	8.42 ± 0.60	4.95 ± 0.44 ^{c}	6.25 ± 0.26 ^{cd}	7.40 ± 0.34 ^{cd}	6.87 ± 0.50 ^{cd}	6.24 ± 0.25 ^{cd}
HGB (g/dL)	19.81 ± 1.26	12.11 ± 0.45 ^{c}	15.23 ± 0.69 ^{cd}	17.99 ± 1.36 ^{d}	16.74 ± 0.45 ^{cd}	15.16 ± 0.72 ^{cd}
HCT (%)	44.71 ± 1.27	27.13 ± 1.49 ^{a}	34.24 ± 1.11 ^{ab}	40.46 ± 2.08 ^{ab}	7.49 ± 1.68 ^{ab}	34.04 ± 1.32 ^{ab}
MCV (fl)	53.28 ± 3.01	55.35 ± 7.50	54.85 ± 2.55	54.78 ± 3.81	54.74 ± 3.12	54.67 ± 3.78
MCHC (pg)	23.68 ± 2.70	24.63 ± 2.35	24.38 ± 0.88	24.34 ± 2.00	24.49 ± 2.00	24.32 ± 1.37
MCHC (g/dL)	44.38 ± 3.65	44.75 ± 2.85	44.54 ± 2.89	44.54 ± 3.72	44.73 ± 2.22	44.58 ± 2.35
PLT (×10 K/μL)	151.45 ± 15.57	66.19 ± 12.30 ^{c}	85.33 ± 4.14 ^{cd}	111.08 ± 13.66 ^{cd}	99.09 ± 9.94 ^{cd}	85.38 ± 5.45 ^{cd}

CPA = cyclophosphamide administered group; EAP_{200} = *A. pullulans* purified exopolymer administered group at a dose of 200 mg/kg; AR_{400}, $_{200}$, and $_{100}$ = Astragali Radix (Roots of *A. membranaceus* Bunge) extract administered mice groups at doses of 400, 200, and 100 mg/kg, respectively; THSD = Tukey's honest significant difference; DT3 = Dunnett's T3; ^{a} $p < 0.01$ as compared with intact control by THSD test; ^{b} $p < 0.01$ as compared with CPA control by THSD test; ^{c} $p < 0.01$ as compared with intact control by the DT3 test; ^{d} $p < 0.01$ as compared with CPA control by the DT3 test.

Table 5. Serum cytokine levels in intact and CPA-induced immunosuppressed mice.

Groups	Controls		Reference	Test Materials		
	Intact	CPA	EAP_{200}	AR_{400}	AR_{200}	AR_{100}
IFN-γ (pg/mL)	91.84 ± 11.57	24.55 ± 10.21 ^{a}	49.08 ± 12.32 ^{ab}	76.16 ± 15.26 ^{b}	61.40 ± 12.12 ^{ab}	48.49 ± 10.46 ^{ab}
TNF-α (pg/mL)	83.85 ± 13.43	15.48 ± 3.01 ^{d}	32.27 ± 12.40 ^{dg}	53.12 ± 12.85 ^{df}	40.57 ± 10.39 ^{df}	32.12 ± 6.34 ^{df}
IL-1β (ng/mL)	28.01 ± 5.26	5.90 ± 1.43 ^{a}	10.31 ± 2.31 ^{ac}	15.20 ± 2.71 ^{ab}	11.75 ± 2.07 ^{ab}	10.29 ± 1.98 ^{ac}
IL-6 (pg/mL)	37.35 ± 15.28	7.24 ± 2.01 ^{d}	13.74 ± 2.75 ^{ef}	21.57 ± 6.11 ^{f}	16.11 ± 2.22 ^{ef}	13.69 ± 2.59 ^{ef}
IL-12 (ng/mL)	23.43 ± 12.21	3.22 ± 1.18 ^{d}	6.37 ± 0.74 ^{ef}	9.65 ± 2.11 ^{f}	7.66 ± 1.02 ^{ef}	6.38 ± 0.57 ^{ef}

CPA = cyclophosphamide administered group; EAP_{200} = *A. pullulans* purified exopolymer administered group at a dose of 200 mg/kg; AR_{400}, $_{200}$, and $_{100}$ = Astragali Radix (Roots of *A. membranaceus* Bunge) extract administered mice groups at doses of 400, 200, and 100 mg/kg, respectively; IFN = Interferon; TNF = Tumor necrosis factor; IL = Interleukin; THSD = Tukey's honest significant difference; DT3 = Dunnett's T3; ^{a} $p < 0.01$ as compared with intact control by THSD test; ^{b} $p < 0.01$ and ^{c} $p < 0.05$, compared with CPA control by THSD test; ^{d} $p < 0.01$, and ^{e} $p < 0.05$, compared with the intact control by the DT3 test; ^{f} $p < 0.01$ and ^{g} $p < 0.05$, compared with CPA control by the DT3 test.

3.10. Changes in Cytokine—IFN-γ, TNF-α, IL-1β, IL-6, and IL-12 Content in Spleen Tissue

The CPA control group showed significant ($p < 0.01$) reductions in splenic IFN-γ, TNF-α, IL-1β, IL-6, and IL-12 content compared to the normal vehicle control group. However, in all test substance administered groups, including AR_{400}, the spleen IFN-γ, TNF-α, IL-1β, IL-6, and IL-12 content increased and were significantly ($p < 0.01$; $p < 0.05$) higher than those in the CPA control group. In particular, AR groups showed a dose-dependent increase in spleen cytokine levels. In addition, AR_{100} group showed an inhibitory effect on the reduction in CPA-induced splenic IFN-γ, TNF-α, IL-1β, IL-6, and IL-12 levels, comparable to that of the EAP_{200} group (Table 6).

Table 6. Splenic cytokine content in intact and CPA-induced immunosuppressed mice.

Groups	Controls		Reference	Test Materials		
	Intact	CPA	EAP_{200}	AR_{400}	AR_{200}	AR_{100}
IFN-γ (pg/mL)	275.76 ± 65.60	60.62 ± 12.93 ^{d}	100.81 ± 22.93 ^{df}	166.19 ± 31.37 ^{df}	137.27 ± 30.09 ^{df}	100.78 ± 21.37 ^{df}
TNF-α (pg/mL)	118.70 ± 18.51	38.21 ± 11.83 ^{a}	66.26 ± 11.05 ^{ab}	85.06 ± 12.08 ^{ab}	79.32 ± 13.11 ^{ab}	66.24 ± 10.38 ^{ab}
IL-1β (pg/mL)	74.39 ± 12.40	20.67 ± 3.49 ^{a}	34.65 ± 6.57 ^{ac}	51.48 ± 11.43 ^{ab}	41.84 ± 10.85 ^{ab}	34.54 ± 7.30 ^{ac}
IL-6 (pg/mL)	86.24 ± 16.60	20.74 ± 3.62 ^{d}	38.10 ± 10.59 ^{df}	51.11 ± 14.81 ^{df}	43.54 ± 10.47 ^{df}	38.54 ± 13.76 ^{dg}
IL-12 (pg/mL)	519.07 ± 168.94	164.09 ± 51.70 ^{d}	277.51 ± 55.11 ^{ef}	364.05 ± 71.64 ^{f}	323.64 ± 67.89 ^{f}	277.39 ± 37.30 ^{ef}

CPA = cyclophosphamide administered group; EAP_{200} = *A. pullulans* purified exopolymer administered group at a dose of 200 mg/kg; AR_{400}, $_{200}$, and $_{100}$ = Astragali Radix (Roots of *A. membranaceus* Bunge) extract administered mice groups at doses of 400, 200, and 100 mg/kg, respectively; IFN = Interferon; TNF = Tumor necrosis factor; IL = Interleukin; THSD = Tukey's honest significant difference; DT3 = Dunnett's T3; ^{a} $p < 0.01$ as compared with intact control by THSD test; ^{b} $p < 0.01$ and ^{c} $p < 0.05$, compared with CPA control by THSD test; ^{d} $p < 0.01$, and ^{e} $p < 0.05$, compared with the intact control by the DT3 test; ^{f} $p < 0.01$ and ^{g} $p < 0.05$, compared with CPA control by the DT3 test.

3.11. Changes in NK Cell Activity

The CPA control group showed a significant ($p < 0.01$) decrease in peritoneal and splenic NK cell activity compared to the control group. However, significant ($p < 0.01$) increases in NK cell activity in the peritoneal cavity and spleen were observed in all test substance administered groups, including the AR_{200} group, compared to the CPA control group. In particular, the AR administered groups exhibited a dose-dependent promotion of NK cell activation. In addition, the AR_{100} group showed an inhibitory effect on the reduction in CPA-induced NK cell activity, comparable to that of the EAP_{200} group (Figure 6).

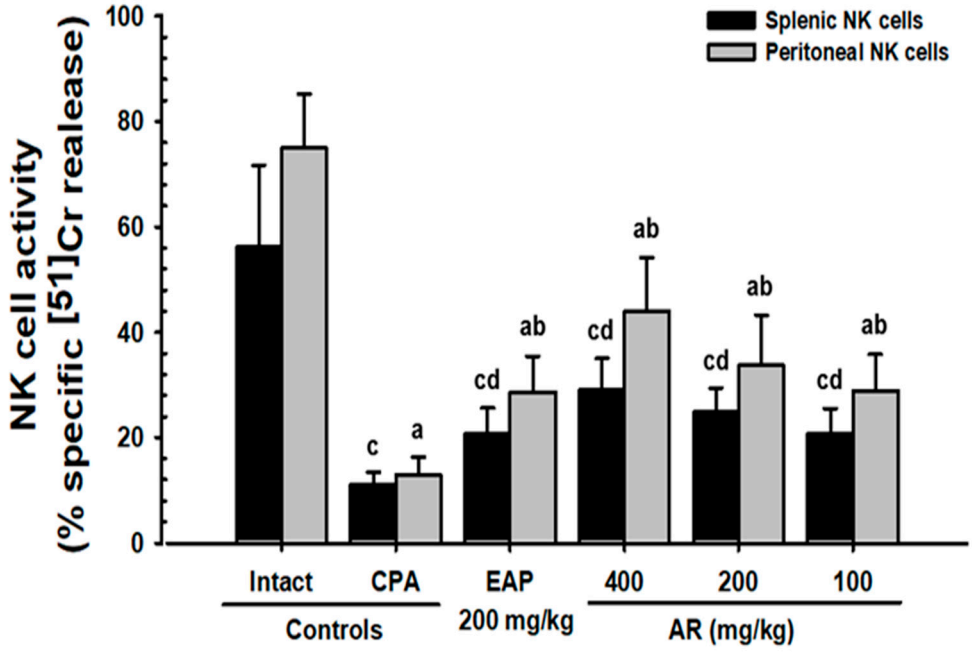

Figure 6. Effects on NK cell activity. CPA = cyclophosphamide; EAP = exopolymers purified from *A. pullulans*; AR = Astragali Radix (Roots of *A. membranaceus* Bunge) extracts; NK = Natural killer; THSD = Tukey's honest significant difference; DT3 = Dunnett's T3; ^{a} $p < 0.01$ as compared with intact control by THSD test; ^{b} $p < 0.01$ as compared with CPA control by THSD test; ^{c} $p < 0.01$ as compared with intact control by the DT3 test; ^{d} $p < 0.01$ as compared with CPA control by the DT3 test.

3.12. Changes in NF-κB, IFN-γ, TNF-α, IL-1β, IL-6, and IL-12 mRNA Expression in Spleen Tissue

In the CPA control group, a significant ($p < 0.01$) decrease in NF-κB, IFN-γ, TNF-α, IL-1β, IL-6, and IL-12 mRNA expression in the spleen tissue was observed as compared to the control group. However, NF-κB, IFN-γ, TNF-α, IL-1β, IL-6, and IL levels in spleen tissue in all test substance administered groups were significantly ($p < 0.01$) higher than those in the CPA control group, including the AR_{100} group. An increase in IL-12 mRNA expression was confirmed. In particular, the AR-administered groups showed a dose-dependent increase in NF-κB and cytokine mRNA expressions in spleen tissue. In addition, the AR_{100} group showed an inhibitory effect on the reduction in CPA-induced NF-κB and cytokine mRNA expressions, similar to that of the EAP_{200} group (Table 7).

Table 7. Splenic cytokine mRNA expression in intact and CPA-induced immunosuppressed mice.

Groups	Controls		Reference	Test materials		
	Intact	CPA	EAP_{200}	AR_{400}	AR_{200}	AR_{100}
NF-κB	1.00 ± 0.07	0.22 ± 0.06 ^{c}	0.42 ± 0.11 ^{cd}	0.69 ± 0.19 ^{cd}	0.56 ± 0.12 ^{cd}	0.42 ± 0.09 ^{cd}
IFN-γ	1.00 ± 0.08	0.22 ± 0.05 ^{a}	0.40 ± 0.07 ^{ab}	0.62 ± 0.13 ^{ab}	0.50 ± 0.12 ^{ab}	0.40 ± 0.09 ^{a}
TNF-α	1.00 ± 0.08	0.20 ± 0.04 ^{c}	0.37 ± 0.08 ^{cd}	0.54 ± 0.12 ^{cd}	0.48 ± 0.17 ^{cd}	0.36 ± 0.09 ^{cd}
IL-1β	1.00 ± 0.05	0.24 ± 0.03 ^{c}	0.43 ± 0.07 ^{cd}	0.66 ± 0.11 ^{cd}	0.57 ± 0.11 ^{cd}	0.43 ± 0.09 ^{cd}
IL-6	1.00 ± 0.06	0.28 ± 0.06 ^{a}	0.48 ± 0.10 ^{ab}	0.70 ± 0.13 ^{ab}	0.59 ± 0.13 ^{ab}	0.48 ± 0.09 ^{ab}
IL-12	1.00 ± 0.09	0.22 ± 0.03 ^{c}	0.37 ± 0.07 ^{cd}	0.59 ± 0.14 ^{cd}	0.47 ± 0.08 ^{cd}	0.38 ± 0.07 ^{cd}

CPA = cyclophosphamide administered group; EAP_{200} = *A. pullulans* purified exopolymer administered group at a dose of 200 mg/kg; $AR_{400, 200}$, and $_{100}$ = Astragali Radix (Roots of *A. membranaceus* Bunge) extract administered mice groups at doses of 400, 200, and 100 mg/kg, respectively; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; NF-κB = Nuclear factor-kappa B; IFN = Interferon; TNF = Tumor necrosis factor; IL = Interleukin; THSD = Tukey's honest significant difference; DT3 = Dunnett's T3; ^{a} $p < 0.01$ as compared with intact control by THSD test; ^{b} $p < 0.01$ as compared with CPA control by THSD test; ^{c} $p < 0.01$ as compared with intact control by the DT3 test; ^{d} $p < 0.01$ as compared with CPA control by the DT3 test.

3.13. Histopathological Changes

3.13.1. Histopathological Changes in the Thymus

In the CPA control group, atrophy characterized by a marked decrease in lymphocytes in the thymic cortex was observed as compared with the control group. The CPA control group showed a significant ($p < 0.01$) decrease in the total thymus and cortical thickness. Conversely, in all test substance administered groups, including AR_{100}, a significant ($p < 0.01$) increase in total thymus and cortical thickness was noted as compared to the CPA control group. In particular, the AR-administered groups exhibited dose-dependent inhibitory effects on thymus atrophy. In addition, the AR_{100} group showed EAP (200 mg/kg) comparable inhibitory effects on CPA-induced thymus atrophy (Table 8, Figure S1).

3.13.2. Histopathological Changes in the Spleen

In the CPA control group, atrophy was characterized by a marked decrease in the lymphocytes within the white medullary portion of the spleen compared to the control group. The significant ($p < 0.01$) decreases in spleen thickness and white medullary diameter and number, and reductions in the number of CD3+, CD4+, CD8+, TNF-α+, IL-1β+, and iNOS+ immunoreactive cells were confirmed. Meanwhile, in all test substance administered groups, including EAP_{200}, spleen thickness, white medullary diameter and number, number of CD3 +, CD4 +, CD8 +, TNF-α +, IL-1β +, and iNOS + cells were significantly ($p < 0.01$) higher than those in the CPA control group. An increase in the number of immunoreactive cells was observed. In particular, the AR-administered groups exhibited an inhibitory effect on spleen atrophy, which was characterized by a dose-dependent decrease in lymphocytes. In addition, the AR_{100} group showed EAP (200 mg/kg) comparable inhibitory effects on CPA-induced splenic lymphocyte reduction and related atrophy (Table 8, Figures S2–S5).

Table 8. Histomorphometric analysis in intact and CPA-induced immunosuppressed mice.

Groups	Controls		Reference	Test Materials		
	Intact	CPA	EAP_{200}	AR_{400}	AR_{200}	AR_{100}
Thymus-thickness						
Total (mm)	1.24 ± 0.17	0.49 ± 0.06 ^{c}	0.67 ± 0.04 ^{ce}	0.88 ± 0.10 ^{ce}	0.73 ± 0.07 ^{ce}	0.68 ± 0.08 ^{ce}
Cortex (μm)	807.34 ± 104.45	200.92 ± 25.74 ^{c}	294.55 ± 20.28 ^{ce}	377.03 ± 34.92 ^{ce}	320.39 ± 44.09 ^{ce}	291.64 ± 27.81 ^{ce}
Spleen-thickness						
Total (mm)	1.76 ± 0.20	0.85 ± 0.12 ^{c}	1.23 ± 0.24 ^{ce}	1.52 ± 0.14 ^{e}	1.34 ± 0.15 ^{ce}	1.22 ± 0.09 ^{ce}
WP (μm)	623.02 ± 75.67	238.39 ± 33.13 ^{c}	368.91 ± 43.63 ^{ce}	518.69 ± 92.95 ^{e}	426.16 ± 61.49 ^{ce}	369.84 ± 42.36 ^{ce}
WP (N/mm^2)	20.60 ± 3.06	9.40 ± 1.35 ^{d}	13.10 ± 1.10 ^{ce}	15.90 ± 1.37 ^{de}	14.30 ± 1.16 ^{ce}	13.00 ± 1.25 ^{ce}
Immunoreactive cells ($numbers/mm^2$)						
CD3+	335.40 ± 95.99	18.50 ± 4.60 ^{c}	166.80 ± 33.96 ^{ce}	269.50 ± 74.22 ^{e}	214.80 ± 65.97 ^{e}	157.20 ± 44.90 ^{ce}
CD4+	146.00 ± 16.19	12.00 ± 5.33 ^{a}	47.60 ± 15.31 ^{ab}	128.60 ± 19.93 ^{b}	75.00 ± 19.49 ^{ab}	47.00 ± 12.08 ^{ab}
CD8+	186.80 ± 36.84	12.60 ± 4.72 ^{c}	69.40 ± 18.16 ^{ce}	121.80 ± 24.97 ^{ce}	98.80 ± 16.31 ^{ce}	64.80 ± 17.18 ^{ce}
TNF-α+	288.20 ± 74.89	8.80 ± 6.12 ^{c}	82.00 ± 28.13 ^{ce}	231.80 ± 22.30 ^{e}	149.20 ± 27.15 ^{ce}	78.60 ± 23.46 ^{ce}
iNOS+	193.60 ± 55.78	12.10 ± 5.38 ^{c}	73.80 ± 21.11 ^{ce}	150.00 ± 29.38 ^{e}	110.00 ± 21.64 ^{de}	71.60 ± 18.37 ^{ce}
IL-1β+	157.80 ± 34.66	24.40 ± 12.21 ^{a}	68.40 ± 15.20 ^{ab}	146.80 ± 28.92 ^{b}	88.80 ± 15.06 ^{ab}	68.00 ± 18.31 ^{ab}
LN—thickness						
Total (mm)	1.13 ± 0.13	0.49 ± 0.09 ^{c}	0.71 ± 0.08 ^{ce}	0.91 ± 0.08 ^{ce}	0.80 ± 0.10 ^{ce}	0.71 ± 0.04 ^{ce}
Cortex (μm)	764.06 ± 107.63	233.05 ± 45.90 ^{a}	406.58 ± 78.67 ^{ab}	646.28 ± 108.56 ^{b}	461.21 ± 129.33 ^{ab}	405.70 ± 59.62 ^{ab}
FO (N/mm^2)	23.00 ± 4.55	6.50 ± 1.27 ^{c}	15.00 ± 1.94 ^{ce}	20.90 ± 3.07 ^{e}	17.20 ± 1.69 ^{de}	14.80 ± 2.15 ^{ce}

CPA = cyclophosphamide administered group; EAP_{200} = *A. pullulans* purified exopolymer administered group at a dose of 200 mg/kg; AR_{400}, $_{200}$, and $_{100}$ = Astragali Radix (Roots of *A. membranaceus* Bunge) extract administered mice groups at doses of 400, 200, and 100 mg/kg, respectively; N = Numbers; WP = White pulp; TNF = Tumor necrosis factor; IL = Interleukin; iNOS = Inducible nitric oxide synthases; LN = Submandibular lymph node, left sides; FO = Follicle; THSD = Tukey's honest significant difference; DT3 = Dunnett's T3; ^{a} $p < 0.01$ as compared with intact control by THSD test; ^{b} $p < 0.01$ as compared with CPA control by THSD test; ^{c} $p < 0.01$ and ^{d} $p < 0.05$, compared with intact control by the DT3 test; ^{e} $p < 0.01$ as compared with CPA control by the DT3 test.

3.13.3. Histopathological Changes in Submandibular Lymph Nodes

In the CPA control group, atrophy was confirmed by a significant decrease in lymphocytes in the lymph node cortex compared to the normal vehicle control group. In addition, significant ($p < 0.01$) reductions were observed in total submandibular lymph nodes, cortical thickness, and number of intracortical follicles of the CPA control group compared to the normal vehicle control group. In contrast, in all test substance administered groups, including AR_{400}, significant ($p < 0.01$) increases in the total lymph node and cortical thickness, and the numbers of follicles in the lymph node were confirmed compared to the CPA control group. In particular, AR groups exhibited an inhibitory effect on submandibular lymph node atrophy, which was characterized by a dose-dependent decrease in lymphocytes. In addition, AR (100 mg/kg) showed an inhibitory effect on reduction in CPA-induced submandibular lymphocyte and related atrophy, comparable to that of EAP (200 mg/kg; Table 8, Figure S4).

4. Discussion

During this study, the immunomodulatory effects of AR were evaluated using a CPA-induced immunosuppressed mouse model. All mice used in this study, including the CPA control group, were within the weight gain category of normal ICR mice of the same age [45,46]. No significant changes were observed in body weight or weight gain in the CPA control group compared to the normal riding control group. In addition, no significant changes were observed in body weight or weight gain in either of the three doses of AR (100, 200, and 400 mg/kg) or EAP (200 mg/kg) administered groups compared to the CPA control group during the 42-day experimental period.

The weight reduction in lymph organs due to the reduction in peripheral lymphocytes by inhibition of bone marrow hematopoiesis by CPA administration is well known [11,13,16,30]. In this experiment, significant decreases in thymus, spleen, and submandibular lymph node weights were confirmed by CPA administration. However,

the inhibitory effect of CPA on the weight reduction in lymph organs was confirmed in a dose-dependent manner by administration of all three doses of AR. In particular, in the AR_{100} group, the effect of suppressing weight loss in the thymus gland, popliteal lymph node, and spleen was comparable to that of the EAP_{200} group. The oral administration of AR (100–400 mg/kg) significantly and dose-dependently suppressed the CPA-induced hematopoietic immunosuppression-associated thymus, spleen, and submandibular lymph node weight reduction, showing considerable inhibitory effects at a dose of 100 mg/kg, comparable to that of EAP (200 mg/kg).

Pharmacologically active substances should exhibit low cytotoxicity toward normal cells and selective cytotoxicity toward denatured cells [30,47,48]. Examination of the MTT cytotoxicity results for spleen cells collected and cultured in the normal vehicle control group at the time of euthanasia showed no significant changes in cell viability in all AR-treated groups (0.01–10 mg/mL) compared to the untreated control group (0 mg/mL). Therefore, at least under the conditions of this experiment, AR does not exhibit cytotoxic or excessive proliferative effects on normal spleen cells. Furthermore, significant reductions were observed in ConA- and LPS-induced splenocyte proliferation in the CPA control group compared to the normal vehicle control group. However, a dose-dependent increase in ConA- and LPS-induced splenocyte proliferation compared to the CPA control group was confirmed in all AR-administered groups. In particular, 100 mg/kg of AR showed an inhibitory effect on the CPA-induced reduction in ConA- and LPS-induced spleen cell proliferation by CPA which was comparable to that of 200 mg/kg of EAP. Generally, ConA promotes the proliferation of T-cells, and LPS induces the division and proliferation of B-cells [11,15,30,49]. Therefore, at least when evaluating the effect of MTT-based splenocyte proliferation in this study, oral administration of AR (100–400 mg/kg) significantly suppressed cell division and proliferation of T- and B-cells by CPA in a dose-dependent manner. It is regarded as indirect evidence of improvement, showing considerable suppressing effects in the AR_{100} group, comparable to that of the EAP_{200} group.

The micronucleus test using mouse bone marrow smears is the simplest and most reliable method for genotoxicity evaluation [30]. CPA is one of the representative genotoxic substances and causes a marked increase in MNPCE, that is, genotoxicity [18,19,30]. A marked increase was confirmed in the number of MNPCE in the CPA control group. However, a dose-dependent decrease in the number of MNPCE was observed in all the AR-administered groups compared to that in the CPA control group. In particular, 100 mg/kg of AR showed anti-mutagenicity by CPA, that is, reduction in genotoxicity, comparable to that of 200 mg/kg of EAP. In contrast, the ratio of PCE (PCE/ (PCE + NCE)) is used as an indicator of the toxicity of test substances to bone marrow cells [50]. The results of this experiment confirmed that the number and ratio of PCE decreased in the CPA control group. However, a dose-dependent increase in the number and ratio of PCE was observed in all AR-administered groups compared to that of the CPA control group. In particular, reduction in bone marrow cytotoxicity by CPA in the AR_{100} group was comparable to the reduction effect of EAP_{200}. At least under the conditions of the micronucleus experiment in the present study, oral doses of 100, 200, and 400 mg/kg of AR showed a clear and convincing dose-dependent inhibition for bone marrow cytotoxicity and anti-mutagenicity by CPA, showing efficacy comparable to EAP (200 mg/kg).

CPA is an immunosuppressive agent that typically inhibits the hematopoietic system, accompanied by a decrease in the number of leukocytes and platelets in the blood, as well as a decrease in HGB and HCT, such as in aplastic anemia [11,16,30,51]. Based on the 13 hematological test results in this study, significant reductions were observed in the total leukocyte and RBC count, HGB, HCT, and platelet count of the CPA control group compared to those in the control group. Moreover, as a result of panleukopenia, no change was observed in the leukocyte differential count in this experiment. In contrast, the decrease in total leukocyte and RBC count, HGB, HCT, and platelet count due to hematopoietic inhibition by CPA administration was significantly suppressed in a dose-dependent manner by the oral administration of AR (100, 200, and 400 mg/kg). In particular, AR at a dose of

100 mg/kg showed a reduction in the inhibition of the hematopoietic system, comparable to that of 200 mg/kg of EAP. These results suggest that oral administration of AR (100, 200, and 400 mg/kg), at least under the hematological examination conditions of this study, inhibits CPA-induced panleukopenia, thrombocytopenia, and aplastic anemia in a dose-dependent manner. This is regarded as reliable evidence, showing an inhibitory effect at a 100 mg/kg oral dose of AR as compared to EAP (200 mg/kg).

TNF-α is a representative cytokine produced in various types of cells, including splenocytes, and plays an important role in the differentiation of T lymphocytes [52]. TNF-α generally activates cellular immunity and increases the function of IL-2, which promotes antibody production [52,53]. IL-1 is another type of cytokine secreted by various cells, such as macrophages, dendritic cells, lymphocytes, endothelial cells, fibroblasts, and keratinocytes. There are two types of IL-1: cell-secreted IL-1β and membrane-attached IL-1α. IL-1 plays an important role in the immune response [54]. IL-6 is secreted by Th2 cells and macrophages accompanying trauma, such as burns that cause inflammation owing to various infections and damage to surrounding tissues [55] and plays an important role against various bacteria [56]. IL-12 is produced and secreted in response to antigen stimulation in dendritic cells, macrophages, neutrophils, and human B-lymphoblastoid cells [57]. The secreted IL-12 promotes Th1 cell differentiation through stimulation of IFN-γ and TNF-α secretion. In addition, IFN-γ is secreted by CD8+ T lymphocytes, Th1 cells, and NK cells, and affects the functions of B- and T-cells and enhances the functions of NK cells and macrophages [53,54]. CPA is a typical immunosuppressant that mainly suppresses the secretion of various cytokines by significantly reducing the number of T lymphocytes [11,13,16,30]. In this study, the IFN-γ, TNF-α, IL-1β, IL-6, and IL-12 content in blood and spleen tissue were significantly reduced. Reduction in IL-6 and IL-12 mRNA expression were induced by CPA administration. However, in the AR (100, 200, and 400 mg/kg) administered groups, the levels of IFN-γ, TNF-α, IL-1β, IL-6, and IL-12 in the blood and spleen tissues were significantly higher than those in the CPA control group, and the IFN. Increases in the mRNA expression of IFN-γ, TNF-α, IL-1β, IL-6, and IL-12 were confirmed in a dose-dependent manner. In particular, AR at a dose of 100 mg/kg showed an inhibitory effect on the reduction in cytokine content and mRNA expression in CPA-induced blood and spleen tissue, comparable to that of EAP (200 mg/kg). These results, at least under the conditions of this study, provide convincing evidence that oral administration of AR (100, 200, and 400 mg/kg) significantly suppresses the CPA-induced decrease in cytokine production and expression by CPA in a dose-dependent manner, showing a suppressing effect comparable to EAP (200 mg/kg) at 100 mg/kg oral dose of AR.

As part of the immunosuppression by CPA, the function of immune-responsive cells, such as NK cells and macrophages, is significantly suppressed, and the activity of these immune-responsive cells is currently in the limelight as another concept of immune modulator development [58,59]. In the present study, significant reductions in peritoneal and splenic NK cell activity were confirmed in the CPA control group compared to the normal vehicle control group. However, in the AR (100, 200, and 400 mg/kg) groups, a significant increase in peritoneal and splenic NK cell activity was observed in a dose-dependent manner compared to the CPA control group. In particular, 100 mg/kg oral dose of AR showed an inhibitory effect on the reduction in CPA-induced NK cell activity comparable to that of 200 mg/kg of EAP. These results provide clear evidence that, at least under the conditions of this experiment, oral administration of AR (100, 200, and 400 mg/kg) significantly suppresses the decrease in NK cell activity by CPA in a dose-dependent manner, which is comparable to that of EAP (200 mg/kg) in 100 mg/kg oral dose of AR.

CPA causes a significant decrease in the number of T lymphocytes, which results in significant atrophy of lymph organs, such as the spleen, lymph nodes, and thymus [11,13,16,30]. Because T-cells always have four types of glycoproteins collectively referred to as antigen receptors, namely CD3, which bind to the surface of cell membranes in an electron noncovalent manner, they are used as surface markers for T-cells [16,30]. In addition, CD4 is a

single-chain glycoprotein with a molecular weight of 55 kDa [60] and is mainly used as a surface marker for helper T-cells and CD8 is a heterodimer where a subunit with a molecular weight of 32–34 kDa is linked with disulfide and is mainly used as a surface marker [61]. The reduction in the spleen of these CD3+, CD8+, and CD4+ cells by CPA administration is well reported [16,30]. iNOS is induced in various cells by pro-inflammatory substances, such as bacterial LPS, IL-1β, TNF-α, and IFN-γ. Nitric oxide (NO), which is formed by the oxidation of L-arginine through the action of iNOS, causes various physiological and pathological changes in the living body and induces shock and inflammatory reactions [62]. In addition, as part of immunosuppression by CPA, the reduction in iNOS expression in tissues is well known [30]. In this study, CPA administration resulted in significant atrophy of lymph organs due to a decrease in lymphocytes in the thymus cortex, white medulla of the spleen, and submandibular lymph nodes, and showed a decrease in the number of immunocompetent cells in the spleen tissue including CD3+, CD4+, CD8+, TNF-α+, IL-1β+ and iNOS+ cells. Meanwhile, oral doses of 100, 200, and 400 mg/kg of AR showed a dose-dependent inhibition in the atrophy of the thymus gland, spleen, and submandibular lymph nodes and an increase in the number of immune-competent cells in the spleen tissue, compared to that of the CPA control group. In particular, AR (100 mg/kg) inhibited CPA-induced atrophy in immune organs and reduction in immune-competent cells, comparable to EAP (200 mg/kg). These results provide reliable evidence that oral administration of AR (100, 200, and 400 mg/kg) significantly suppresses spleen and submandibular lymph node atrophy by CPA, at least under the histopathological examination conditions of this experiment, in a dose-dependent manner, showing a suppressing effect comparable to that of EAP (200 mg/kg) in 100 mg/kg oral dose of AR.

NF-κB is a protein complex that regulates DNA transcription, cytokine production, and cell survival and is found in almost all cells. NF-κB plays an important role in the body's defense response to various stimuli, such as stress, cytokines, free radicals, heavy metals, ultraviolet rays, oxidized LDL, bacteria, and viruses [63,64]. In particular, NF-κB plays an important role in immune regulation in the body's defense against infection and provides a component of the κ light chain of antibodies [65,66]. NF-κB is the most important protein that regulates the transcription of various genes involved in innate and acquired immune responses [67]. During this study, a significant decrease in NF-κB mRNA expression in spleen tissue was observed in the CPA control group compared to the normal vehicle control group. However, in the AR-treated groups, a dose-dependent, significant increase in NF-κB mRNA expression in the spleen tissue was observed compared to the CPA control group. In particular, 100 mg/kg of AR showed an inhibitory effect on the reduction in CPA-induced NF-κB and cytokine mRNA expression as compared to the EAP (200 mg/kg). Under the real-time RT-PCR test conditions of this study, these results provide conclusive evidence that oral administration of 100, 200, and 400 mg/kg of AR significantly suppress hematopoietic immunosuppression through CPA, at least in part, by increasing NF-κB expression, in a dose-dependent manner, showing a suppressing effect comparable to EAP (200 mg/kg) in 100 mg/kg oral dose of AR.

5. Conclusions

As part of the development of natural new drugs and health functional food materials for effective immune regulation, the dose-dependent immunomodulatory effects of AR aqueous crude extract were compared with that of EAP using a CPA-induced immunosuppressed ICR mouse model. The results showed that immunosuppression and mutagenicity following hematopoietic inhibition by CPA were consistent with the increase in NF-κB expression and the related activation of T-, B-, and NK-cells, thus clearly indicating suppression without cytotoxicity. The observed effects of a 100 mg/kg oral dose of AR were comparable to that of 200 mg/kg oral dose of EAP. Therefore, AR at an appropriate oral dose is considered to be of sufficient developmental value as a new anti-mutagenic and immunomodulatory agent with relatively low toxicity. However, more clearly active ingredient analysis and toxicity evaluation should be performed in the future.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app13052959/s1, Table S1: specific conditions for HPLC analysis; Table S2: oligonucleotides used for real-time RT-PCR; Table S3: primary antisera and immunohistochemistry detection kits used in this study; Figure S1: representative histopathological images of the thymus obtained from intact or CPA-induced immunosuppressed mice; Figure S2: representative histopathological images of the spleen, taken from intact or CPA-induced immunosuppressed mice; Figure S3: representative immunohistochemical images of the CD3+, CD4+ and CD8+ cells on the spleen obtained from intact or CPA-induced immunosuppressed mice; Figure S4: representative immunohistochemical images of the TNF-α+, IL-1β+ and iNOS+ cells on the spleen obtained from intact or CPA-induced immunosuppressed mice; Figure S5: representative histopathological images of the left submandibular LNn, taken from intact or CPA-induced immunosuppressed mice.

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References

1. Wu, D.; Lewis, E.D.; Pae, M.; Meydani, S.N. Nutritional modulation of immune function: Analysis of evidence, mechanisms, and clinical relevance. *Front Immunol.* **2019**, *15*, 3160. [CrossRef] [PubMed]
2. Acharya, D.; Shrivastava, A. *Indigenous Herbal Medicines: Tribal Formulations and Traditional Herbal Practices*, 1st ed.; Aavishkar Publishers: Jaipur, India, 2008; pp. 1–444.
3. Kajaria, D.; Tripathi, J.S.; Tiwari, S.K.; Pandey, B.L. Immunomodulatory effect of ethanolic extract of Shirishadi compound. *Ayu* **2013**, *34*, 322–326. [CrossRef] [PubMed]
4. Chauhan, N.S.; Singh, M.R.; Sharma, V.; Yadav, N.; Sangwan, N.S.; Singh, D. Traditional Indian knowledge of immunity from plants. In *Plants and Phytomolecules for Immunomodulation*; Sangwan, N.S., Farag, M.A., Modolo, L.V., Eds.; Springer: Singapore, 2022.
5. Heroor, S.; Beknal, A.K.; Mahurkar, N. Immunomodulatory activity of methanolic extracts of fruits and bark of *Ficus glomerata* Roxb. in mice and on human neutrophils. *Indian J. Pharmacol.* **2013**, *45*, 130–135. [CrossRef] [PubMed]
6. Ghatak, S.B.; Panchal, S.J. Investigation of the immunomodulatory potential of oryzanol isolated from crude rice bran oil in experimental animal models. *Phytother. Res.* **2012**, *26*, 1701–1708. [CrossRef] [PubMed]
7. Ullah, A.; Munir, S.; Badshah, S.L.; Khan, N.; Ghani, L.; Poulson, B.G.; Emwas, A.-H.; Jaremko, M. Important flavonoids and their role as a therapeutic agent. *Molecules* **2020**, *25*, 5243. [CrossRef]
8. Roy, A.; Das, S.; Chatterjee, I.; Roy, S.; Chakraborty, R. Anti-inflammatory effects of different dietary antioxidants. In *Plant Antioxidants and Health*; Reference Series in Phytochemistry; Ekiert, H.M., Ramawat, K.G., Arora, J., Eds.; Springer: Cham, Switzerland, 2022; pp. 573–797.
9. Kour, J.; Ali, M.N.; Ganaie, H.A.; Tabassum, N. Amelioration of the cyclophosphamide induced genotoxic damage in mice by the ethanolic extract of *Equisetum arvense*. *Toxicol. Rep.* **2017**, *4*, 226–233. [CrossRef]
10. Ayas, M.; Siddiqui, K.; Al-Jefri, A.; Al-Ahmari, A.; Ghemlas, I.; Al-Saedi, H.; Alanazi, A.; Jafri, R.; Ayas, M.F.; Al-Seraihi, A. Successful outcome in patients with Fanconi anemia undergoing T cell-replete mismatched related donor hematopoietic cell transplantation using reduced-dose cyclophosphamide post-transplantation. *Biol. Blood Marrow Transplant.* **2019**, *25*, 2217–2221. [CrossRef]
11. Kim, H.; Kim, J.W.; Kim, Y.-K.; Ku, S.K.; Lee, H.-J. Immunoenhancement effects of the herbal formula hemomine on cyclophosphamide-induced immunosuppression in mice. *Appl. Sci.* **2022**, *12*, 4935. [CrossRef]

12. Araujo-Lima, C.F.; Christoni, L.S.A.; Justo, G.; Soeiro, M.N.C.; Aiub, C.A.F.; Felzenszwalb, I. Atorvastatin downregulates in vitro methyl methanesulfonate and cyclophosphamide alkylation-mediated cellular and DNA injuries. *Oxid. Med. Cell. Longev.* **2018**, *2018*, 7820890. [CrossRef]
13. Kim, J.W.; Choi, J.-S.; Seol, D.J.; Choung, J.J.; Ku, S.K. Immunomodulatory effects of kuseonwangdogo-based mixed herbal formula extracts on a cyclophosphamide-induced immunosuppression mouse model. *eCAM* **2018**, *2018*, 6017412. [CrossRef]
14. Swan, D.; Gurney, M.; Krawczyk, J.; Ryan, A.E.; O'Dwyer, M. Beyond DNA damage: Exploring the immunomodulatory effects of cyclophosphamide in multiple myeloma. *HemaSphere* **2020**, *4*, e350. [CrossRef] [PubMed]
15. Chen, X.T.; Li, J.; Wang, H.L.; Cheng, W.M.; Zhang, L.; Ge, J.F. Immunomodulating effects of fractioned polysaccharides isolated from Yu-Ping-Feng-Powder in cyclophosphamide-treated mice. *Am. J. Chin. Med.* **2006**, *34*, 631–641. [CrossRef] [PubMed]
16. Yoon, H.S.; Kim, J.W.; Cho, H.R.; Moon, S.B.; Shin, H.D.; Yang, K.J.; Lee, H.S.; Kwon, Y.S.; Ku, S.K. Immunomodulatory effects of *Aureobasidium pullulans* SM-2001 exopolymers on the cyclophosphamide-treated mice. *J. Microbiol. Biotechnol.* **2010**, *20*, 438–445. [CrossRef]
17. Sharma, V.; Thakur, M.; Chauhan, N.S.; Dixit, V.K. Immunomodulatory activity of petroleum ether extract of *Anacyclus pyrethrum*. *Pharm. Biol.* **2010**, *48*, 1247–1254. [CrossRef] [PubMed]
18. Song, M.Y.; Ku, S.K.; Han, J.S. Genotoxicity testing of low molecular weight fucoidan from brown seaweeds. *Food Chem. Toxicol.* **2012**, *50*, 790–796. [CrossRef] [PubMed]
19. Lee, W.H.; Choi, S.H.; Kang, S.J.; Song, C.H.; Park, S.J.; Lee, Y.J.; Ku, S.K. Genotoxicity testing of Persicariae Rhizoma (*Persicaria tinctoria* H. Gross) aqueous extracts. *Exp. Ther. Med.* **2016**, *12*, 123–134. [CrossRef]
20. Raa, J. Immune modulation by non-digestible and non-absorbable beta-1,3/1,6-glucan. *Microb. Ecol. Health Dis.* **2015**, *26*, 27824. [CrossRef]
21. Thomas, S.; Rezoagli, E.; Abidin, I.Z.; Major, I.; Murray, P.; Murphy, E.J. β-Glucans from yeast—Immunomodulators from novel waste resources. *Appl. Sci.* **2022**, *12*, 5208. [CrossRef]
22. Castro, E.D.M.; Calder, P.C.; Roche, H.M. β-1,3/1,6-glucans and immunity: State of the art and future directions. *Mol. Nutr. Food Res.* **2021**, *65*, e1901071. [CrossRef]
23. Jung, M.Y.; Kim, J.W.; Kim, K.Y.; Choi, S.H.; Ku, S.K. Polycan, a β-glucan from *Aureobasidium pullulans* SM-2001, mitigates ovariectomy-induced osteoporosis in rats. *Exp. Ther. Med.* **2016**, *12*, 1251–1262. [CrossRef]
24. Lee, S.; Lee, D.K. What is the proper way to apply the multiple comparison test? *Korean J. Anesthesiol.* **2018**, *71*, 353–360. [CrossRef] [PubMed]
25. Ku, S.K.; Lee, Y.J.; Lee, S.D.; Cho, H.R.; Moon, S.B.; Kim, K.Y.; Kwon, Y.S.; Kim, J.W. Nephroprotective effect of Polycan on acute renal failure induced by cisplatin in rats. *ISRN Vet. Sci.* **2012**, *2012*, 862104. [CrossRef] [PubMed]
26. Kim, H.D.; Cho, H.R.; Moon, S.B.; Shin, H.D.; Yang, K.J.; Park, B.R.; Jang, H.J.; Kim, L.S.; Lee, H.S.; Ku, S.K. Effects of β-glucan from *Aureobasidum pullulans* on acute inflammation in mice. *Arch. Pharm. Res.* **2007**, *30*, 323–328. [CrossRef] [PubMed]
27. Ku, S.K.; Kim, J.W.; Cho, H.R.; Kim, K.Y.; Min, Y.H.; Park, J.H.; Kim, J.S.; Park, J.H.; Seo, B.I.; Roh, S.S. Effect of β-glucan originated from *Aureobasidium pullulans* on asthma induced by ovalbumin in mouse. *Arch. Pharm. Res.* **2012**, *35*, 1073–1081. [CrossRef] [PubMed]
28. Kim, J.W.; Cho, H.R.; Ku, S.K. Efficacy test of Polycan, a beta-glucan originated from *Aureobasidium pullulans* SM-2001, on anterior cruciate ligament transection and partial medial meniscectomy-induced-osteoarthritis rats. *J. Microbiol. Biotechnol.* **2012**, *22*, 274–282. [CrossRef]
29. Kim, Y.S.; Kang, S.J.; Kim, J.W.; Cho, H.R.; Moon, S.B.; Kim, K.Y.; Lee, H.S.; Han, C.H.; Ku, S.K.; Lee, Y.J. Effects of Polycan, a β-glucan, on experimental periodontitis and alveolar bone loss in Sprague-Dawley rats. *J. Periodontal. Res.* **2012**, *47*, 800–810. [CrossRef]
30. Yoo, J.H.; Lee, Y.S.; Ku, S.K.; Lee, H.J. *Phellinus baumii* enhances the immune response in cyclophosphamide-induced immunosuppressed mice. *Nutr. Res.* **2020**, *75*, 15–31. [CrossRef]
31. Yeh, T.S.; Chuang, H.L.; Huang, W.C.; Chen, Y.M.; Huang, C.C.; Hsu, M.C. *Astragalus membranaceus* improves exercise performance and ameliorates exercise-induced fatigue in trained mice. *Molecules* **2014**, *19*, 2793–2807. [CrossRef]
32. Hwang, J.; Zhang, W.; Dhananjay, Y.; An, E.K.; Kwak, M.; You, S.; Lee, P.C.; Jin, J.O. *Astragalus membranaceus* polysaccharides potentiate the growth-inhibitory activity of immune checkpoint inhibitors against pulmonary metastatic melanoma in mice. *Int. J. Biol. Macromol.* **2021**, *182*, 1292–1300. [CrossRef]
33. Latour, E.; Arlet, J.; Latour, E.E.; Juszkiewicz, A.; Łuczkowska, K.; Marcinkiewicz, A.; Basta, P.; Trzeciak, J.; Machaliński, B.; Skarpańska-Stejnborn, A. Standardized astragalus extract for attenuation of the immunosuppression induced by strenuous physical exercise: Randomized controlled trial. *J. Int. Soc. Sports Nutr.* **2021**, *18*, 57. [CrossRef]
34. Lin, Y.; Wang, B.; Luo, X. Clinical study of astragalus's preventing the recurrence of asthma in children. *Chin. J. Integ. Trad. West Med.* **2011**, *31*, 1090–1092.
35. Chen, S.M.; Tsai, Y.S.; Lee, S.W.; Liu, Y.H.; Liao, S.K.; Chang, W.W.; Tsai, P.J. *Astragalus membranaceus* modulates Th1/2 immune balance and activates PPARγ in a murine asthma model. *Biochem. Cell Biol.* **2014**, *92*, 397–405. [CrossRef] [PubMed]
36. Kuo, Y.H.; Tsai, W.J.; Loke, S.H.; Wu, T.S.; Chiou, W.F. *Astragalus membranaceus* flavonoids (AMF) ameliorate chronic fatigue syndrome induced by food intake restriction plus forced swimming. *J. Ethnopharmacol.* **2009**, *122*, 28–34. [CrossRef] [PubMed]
37. Yang, B.; Xiao, B.; Sun, T. Antitumor and immunomodulatory activity of *Astragalus membranaceus* polysaccharides in H22 tumor-bearing mice. *Int. J. Biol. Macromol.* **2013**, *62*, 287–290. [CrossRef]

38. Kim, W.; Kim, S.H.; Park, S.K.; Chang, M.S. *Astragalus membranaceus* ameliorates reproductive toxicity induced by cyclophosphamide in male mice. *Phytother. Res.* **2012**, *26*, 1418–1421. [CrossRef]
39. Simon, P.; Frankowski, M.; Bock, N.; Neukammer, J. Label-free whole blood cell differentiation based on multiple frequency AC impedance and light scattering analysis in a micro flow cytometer. *Lab. Chip.* **2016**, *16*, 2326–2338. [CrossRef]
40. Gebretsadkan, G.; Tessema, K.; Ambachew, H.; Birhaneselassie, M. The comparison between microhematocrit and automated methods for hematocrit determination. *Int. J. Blood Res. Disord.* **2015**, *2*, 1–3. [CrossRef]
41. Bustin, S.A. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J. Mol. Endocrinol.* **2000**, *25*, 169–193. [CrossRef]
42. Hsu, S.M.; Raine, L.; Fanger, H. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: A comparison between ABC and unlabeled antibody (PAP) procedures. *J. Histochem. Cytochem.* **1981**, *29*, 577–580. [CrossRef]
43. Lee, H.E.; Yang, G.; Choi, J.S.; Lee, J.Y. Suppression of primary splenocyte proliferation by *Artemisia capillaris* and its components. *Toxicol. Res.* **2017**, *33*, 283–290. [CrossRef]
44. OECD Council. OECD TG 474: Mammalian Erythrocyte Micronuclear Test. 29 July 2016. Available online: https://read.oecd-ilibrary.org/environment/test-no-474-mammalian-erythrocyte-micronucleus-test_9789264264762-en#page1 (accessed on 22 February 2023).
45. Fox, J.G.; Cohen, B.J.; Loew, F.M. *Laboratory Animal Medicine*; Academic Press Inc.: Orlando, FL, USA, 1984.
46. Tajima, Y. *Biological Reference Data Book on Experimental Animals*; Soft Science Inc.: Tokyo, Japan, 1989.
47. Chargari, C.; Toillon, R.A.; Macdermed, D.; Castadot, P.; Magné, N. Concurrent hormone and radiation therapy in patients with breast cancer: What is the rationale? *Lancet Oncol.* **2009**, *10*, 53–60. [CrossRef]
48. Park, J.C.; Lee, Y.J.; Choi, H.Y.; Shin, Y.K.; Kim, J.D.; Ku, S.K. In vivo and in vitro antitumor effects of Platycodin D, a saponin purified from Platycodi Radix on the H520 lung cancer cell. *eCAM* **2014**, *2014*, 478653. [PubMed]
49. Cerqueira, F.; Cordeiro-Da-Silva, A.; Gaspar-Marques, C.; Simoes, F.; Pinto, M.M.; Nascimento, M.S. Effect of abietane diterpenes from *Plectranthus grandidentatus* on T- and B-lymphocyte proliferation. *Bioorg. Med. Chem.* **2004**, *12*, 217–223. [CrossRef] [PubMed]
50. Chung, I.K.; Cheon, W.H.; Ku, S.K. Micronucleus test of Picrorrhiza Rhizoma aqueous extract in bone marrow cells of male ICR mice. *Toxicol. Res.* **2011**, *27*, 119–123. [CrossRef] [PubMed]
51. Hu, Q.; Xu, J.; Chen, L. Anti-mutagenicity of selenium-enriched rice on mice exposure to cyclophosphamide and mitomycin C. *Cancer Lett.* **2005**, *220*, 29–35. [CrossRef]
52. Jang, D.I.; Lee, A.H.; Shin, H.Y.; Song, H.R.; Park, J.H.; Kang, T.B.; Lee, S.R.; Yang, S.H. The role of tumor necrosis factor alpha (TNF-α) in autoimmune disease and current TNF-α inhibitors in therapeutics. *Int. J. Mol. Sci.* **2021**, *22*, 2719. [CrossRef]
53. Mehta, A.K.; Gracias, D.T.; Croft, M. TNF activity and T cells. *Cytokine* **2018**, *101*, 14–18. [CrossRef]
54. Dinarello, C.A. Overview of the IL-1 family in innate inflammation and acquired immunity. *Immunol. Rev.* **2018**, *281*, 8–27. [CrossRef]
55. Lan, T.; Chang, L.; Wu, L.; Yuan, Y.F. IL-6 plays a crucial role in HBV infection. *J. Clin. Transl. Hepatol.* **2015**, *3*, 271–276.
56. Tanaka, T.; Narazaki, M.; Kishimoto, T. IL-6 in inflammation, immunity, and disease. *Cold Spring Harb. Perspect. Biol.* **2014**, *6*, a016295. [CrossRef]
57. Hamza, T.; Barnett, J.B.; Li, B. Interleukin 12 a key immunoregulatory cytokine in infection applications. *Int. J. Mol. Sci.* **2010**, *11*, 789–806. [CrossRef]
58. Ha, E.S.; Hwang, S.H.; Shin, K.S.; Yu, K.W.; Lee, K.H.; Choi, J.S.; Park, W.M.; Yoon, T.J. Anti-metastatic activity of glycoprotein fractionated from *Acanthpanax senticosus*, involvement of NK-cell and macrophage activation. *Arch. Pharm. Res.* **2004**, *27*, 217–224. [CrossRef] [PubMed]
59. Yu, G.M.; Hwang, I.G. In vitro effect of Yuza (*Citrus junos* SIEB ex. TANAKA) extracts on proliferation of human prostate cancer cells and antioxidant activity. *Korean J. Food Sci. Technol.* **2004**, *36*, 339–344.
60. Jung, J.W.; Chun, J.H.; Lee, J.S.; Kim, S.W.; Lee, A.R.; Kim, J.; Lazarte, J.M.S.; Kim, Y.R.; Kim, H.J.; Thompson, K.D.; et al. Characterization of CD4-positive lymphocytes in the antiviral response of olive flounder (*Paralichthys oliveceus*) to nervous necrosis virus. *Int. J. Mol. Sci.* **2020**, *21*, 4180. [CrossRef] [PubMed]
61. Burnet, M. The helper T-cell responses. In *Immunology an Introduction*, 4th ed.; Tizard, I.R., Ed.; Saunders: Philadelphia, PA, USA, 1995; pp. 139–154.
62. Böger, R.H. The pharmacodynamics of l-arginine 1,2,3. *J. Nutr.* **2007**, *137*, S1650–S1655. [CrossRef]
63. Brasier, A.R. The NF-kappaB regulatory network. *Cardiovasc. Toxicol.* **2006**, *6*, 111–130. [CrossRef]
64. Gilmore, T.D. Introduction to NF-kappaB: Players, pathways, perspectives. *Oncogene* **2006**, *25*, 6680–6684. [CrossRef]
65. Liu, T.; Zhang, L.; Joo, D.; Sun, S.-C. NF-κB signaling in inflammation. *Sig. Transduct. Target. Ther.* **2017**, *2*, 17023. [CrossRef]
66. Gerondakis, S.; Siebenlist, U. Roles of the NF-kappaB pathway in lymphocyte development and function. *Cold Spring Harb. Perspect. Biol.* **2010**, *2*, a000182. [CrossRef]
67. Smith, E.M.; Gregg, M.; Hashemi, F.; Schott, L.; Hughes, T.K. Corticotropin releasing factor (CRF) activation of NF-kappaB-directed transcription in leukocytes. *Cell Mol. Neurobiol.* **2006**, *26*, 1021–1036. [CrossRef]