

## Research Article

# Immune-Enhancing Activity of *Vitis coignetiae* Extract via Increasing Cytokine and Natural Killer Cell Activity in Splenocytes and Cyclophosphamide-Induced Immunosuppressed Rats

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Plant and fruit extracts exhibit fewer side effects than pharmaceuticals and can display therapeutic qualities. Consequently, they have attracted increased attention among health-conscious individuals, and many studies related to their health-promoting effects are being actively conducted. *Vitis coignetiae* is well-known for its antioxidant, anti-inflammatory, and anticancer properties. However, the immune-enhancing effects of *Vitis coignetiae* have not yet been studied. In this study, *Vitis coignetiae* extract (VCE) increased immune-related activity in Wistar rat splenocytes and cyclophosphamide (Cy)-induced immunosuppressed rats. In splenocytes, VCE was nontoxic up to 300 µg/mL and increased cell viability in the presence of Cy. VCE also recovered Cy-induced decreases in cytokine (tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), interleukin-2 (IL-2), and IL-12) levels and natural killer (NK) cell activity in splenocytes. In Cy-induced immunosuppressed rats, VCE protected against Cy-induced spleen tissue damage and decreases in body and spleen weight, cytokine (TNF-α, IFN-γ, IL-2, and IL-12) levels, and NK cell activity. We also observed recovery of immunoglobulin G (IgG) and IgM levels following VCE treatment. In conclusion, the ability of VCE to restore immune activity from an immunosuppressive state to normal levels suggests its immune-enhancing efficacy. Therefore, VCE may have the potential for application in the development of immune-enhancing functional foods and medicines.

## 1. Introduction

The immune system plays an important role in the pathophysiology of diseases, including immune disorders (psoriasis and arthritis) and cancer, in animals and humans [1, 2]. Disruption of the immune system can therefore be a starting point for disease development. Recently, several factors (excess alcohol and tobacco consumption, exposure to particulate matter 10 and 2.5, stress, and drug therapy) have been shown to reduce human and animal immunity [3–6]. Therefore, individuals are increasingly turning towards immune-boosting nutritional supplements or foods. Functional foods and botanical drug products, including natural extracts, are

known to display few side effects and confer various beneficial effects (such as immunomodulatory, anti-inflammatory, and anticancer effects) [7, 8]. Natural extracts from roots (ginseng, red ginseng, and *Heracleum moellendorffii* root), flowers (*Paulownia tomentosa*), and fruits (blueberry and black chokeberry) have shown evidence of antioxidant (reactive oxygen species chelation) and anti-inflammatory effects (decreased cytokine release and inflammatory pathway inhibition) [9–15]. Accordingly, research is being actively conducted on natural extracts, and products (foods and drugs) using natural extracts are being increasingly produced.

Cyclophosphamide (Cy) is an alkylating agent with a broad spectrum of activity in various diseases; it is widely

used as an anticancer agent, especially in chemotherapy [16]. Cy is converted to 4-hydroxycyclophosphamide/aldophosphamide in the liver, diffuses into cells in the body, and is finally converted to the active compound phosphoramidate mustard. Finally, it passes through the nuclear membrane and induces apoptosis [17]. Therefore, Cy kills both the cancer and normal cells, including immune cells. In addition, it displays side effects such as hepatotoxicity, nausea, vomiting, hemorrhagic bladder inflammation, and alopecia, and long-term therapy results in the loss of body weight (BW), thymus weight, white blood cells (WBCs), bone marrow cells, and natural T cells [18, 19]. Previous studies have utilized Cy-induced immunosuppression as a tool with which to study the immune-enhancing effects of drugs or natural products; one example is that regarding HemoHIM (Atomy Co., Korea), of which the herbal preparation showed evidence of anticancer effects, memory improvement, and immune enhancement [20, 21].

*Vitis coignetiae* Pulliat, called Meoru in Korea, contains many anthocyanins (delphinidin-3,5-diglucoside, cyanidin-3,5-diglucoside, petunidin-3,5-diglucoside, malvidin-3,5-diglucoside, and peonidin-3,5-diglucoside) in its peel and pulp, and is well-known to display excellent antioxidant and anti-inflammatory effects [22, 23]. In addition, it has been shown to decrease the expression of tumor necrosis factor alpha (TNF- $\alpha$ ) and nuclear factor kappa B (NF $\kappa$ B)-regulated genes in human breast cancer cells and inhibit migration, proliferation, and invasion in A549 human lung cancer cells [24, 25]. Though active research on its antioxidant, anti-inflammatory, and anticancer effects is being undertaken, there is a lack of such work regarding immune enhancement.

In this study, we hypothesized that *Vitis coignetiae* extract (VCE) would confer an immune-enhancing effect and aimed to verify whether the administration would increase immune-related activity in Wistar rat splenocytes and in Cy-induced immunosuppressed rats.

## 2. Materials and Methods

**2.1. Preparation of VCE.** *Vitis coignetiae* fruit (washed with distilled water) was ground using a blender for 20 s. VCE was extracted using 20% EtOH (ground fruit, 100 g with 20% EtOH, 900 g). Then, VCE was filtered using a qualitative filter paper (11  $\mu$ m, GE Healthcare, UK). The filtered solution was evaporated in a vacuum rotary evaporator. Then, the concentrated extract was lyophilized in a freeze dryer at  $-50^{\circ}\text{C}$  for 6 d.

**2.2. Component Analysis of VCE.** The separation of VCE was accomplished on a high-performance liquid chromatography (HPLC) system (Dionex UltiMate 3000 Series), equipped with a Synchronis<sup>TM</sup> C18 column (Thermo Fisher Scientific, USA). In analyses, mobile phases were A, 0.1% water solution of TFA, and B, HPLC-grade acetonitrile. The gradient condition was 0–10 min, 8% B; 10–15 min, 20% B; 15–25 min, 30% B; 25–45 min, 50% B; 45–55 min, 60% B; flow rate, 0.6 mL/min; column temperature  $27^{\circ}\text{C}$ ; wavelength 275 nm; and sample injection was 10  $\mu$ L. Standard

curves were made using external standards such as 4-hydrate benzoic acid, gallic acid, syringic acid, and quercetin for quantification. All standard agents were obtained from Sigma-Aldrich (MO, USA).

**2.3. Animals and Oral Administration of VCE.** All experiments were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Animals. This study was approved by the Institutional Animal Care and Use Committee of INVIVO Co., Ltd. (IV-RB-17-2206-20). Specific pathogen-free Wistar rats (five weeks, male) were purchased from Orient Bio Co. (Seongnam, Korea). After one week, the rats were divided into six groups (normal; control; VCE 50 mg/kg; VCE 250 mg/kg; VCE 500 mg/kg; and positive, HemoHIM 1,000 mg/kg). All rats, except those from the normal group, were orally administered with Cy (5 mg/kg) for immunosuppression. All animals' BWs were monitored weekly during the experimental period. Wistar rats were kept in a temperature-controlled room with a 12 h light and 12 h dark cycle.

**2.4. Primary Cell Culture.** The spleen tissues were collected from Wistar rats (eight weeks, male). Collected spleen tissue was carved into small pieces by a syringe needle and then forced through a 70  $\mu$ m cell strainer (SPL Life Sciences, Gyeonggi-do, Korea). Red blood cells (RBCs) were removed by RBC lysis buffer (Sigma-Aldrich). The splenocytes were maintained in RPMI-1640 with 10% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin-streptomycin (Pen-Strep, 10,000 U/mL, Invitrogen, CA, USA) in 5% CO<sub>2</sub> incubator.

**2.5. Cell Viability of Splenocytes.** In brief, primary splenocytes ( $5 \times 10^5$  cells/well) were seeded in 96-well plates and incubated at  $37^{\circ}\text{C}$  for 24 h. Then, the stabilized splenocytes were treated with VCE (indicated doses in Figure 1) and VCE with Cy 0.8 mg/mL, and incubated for 24 h in a 5% CO<sub>2</sub> incubator. Cell viability rate was measured using the WST-1 assay kit (ITSBio, Seoul, Korea) and the Sunrise<sup>TM</sup> enzyme-linked immunosorbent assay (ELISA) plate reader (Tecan, Männedorf, Switzerland).

**2.6. Nitric Oxide (NO) Release in Splenocytes.** NO production by Cy-treated splenocytes was evaluated using a Griess assay [10]. Splenocytes ( $5 \times 10^5$  cells/well) were seeded in a 96-well plate. The cells were treated with Cy (0.8 mg/mL) and VCE (0–300  $\mu$ g/mL) for 24 h, where the control group contained only RPMI-1640. Then, the supernatant (80  $\mu$ L/well) was collected and added to a new 96-well plate and mixed with the same amount of Griess reagent. Before detection of the absorbance, the mixture was shaken in the dark for 10 min. The absorbance was measured at 540 nm.

**2.7. Natural Killer (NK) Cell Activity.** NK cell activity was investigated as previously described [26]. In brief, AR42J (Cat. CRL-1492, ATCC, VA, USA) cells were used as target

cells for the NK cell activity, and primary splenocytes (from all experimental groups) were used as effector cells. Splenocytes were cocultured with AR42J cells in 96-well plates at a ratio of 25:1 in a 5% CO<sub>2</sub> incubator at 37°C for 24 h. AR42J viability rate was measured by the WST-1 assay kit.

**2.8. Western Blot Analysis.** Splenocytes were lysed in PRO-PREP™ Protein Extraction Solution (iNtRON, Cat no. 17081). The total protein concentration was determined using the Bradford assay with bovine serum albumin as the standard. The total protein (10 µg/well) was separated with 8–10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by transfer to polyvinylidene fluoride membranes (Bio-Rad, CA, USA). Specific antibodies such as iNOS (ab178945, Abcam), Phospho-Erk (9101S, CST), Erk (9102S, CST), Phospho-p38 (9216S, CST), p38 (9212S, CST), Phospho-JNK (4671S, CST), JNK (9252S, CST), Phospho-NFκB (3033, CST), NFκB (8242S, CST), and β-actin (4857, CST) were used to bind target proteins. Target proteins were detected using a chemiluminescence reagent (Surmodics IVD, Inc., USA) and then visualized and analyzed using the ChemiDoc XRS+ imaging system (Bio-Rad, CA, USA) equipped with Image Lab.

**2.9. Procytokine Levels.** Procytokine levels were performed as previously described [26]. In brief, splenocytes ( $2 \times 10^5$  cells/well) were seeded into 96-well plates with RPMI-1640 (with 10% FBS and 1% Pen-Strep), after which VCE (0–300 µg/mL) with Cy (0.8 mg/mL) was added to the wells, and then cells were incubated for 24 h in a 5% CO<sub>2</sub> incubator. Blood from all experimental Wistar rats was collected in a conical tube and separated in a centrifuge at 3,000 rpm for 15 min at 4°C to collect serum. Levels of cytokines in the culture medium from each well and serum were then measured using cytokine ELISA kits ((TNF-α and IFN-γ; R&D Systems, Minneapolis, MN, U.S.A.), Cusabio (IL-12; Biotech Co., TX, USA), Abcam (IFN-γ, immunoglobulin G (IgG), and IgM from serum; MA, USA), and MyBioSource (IL-2; CA, USA)). The results were detected using an ELISA reader.

**2.10. Complete Blood Cell (CBC) Count Analysis.** At the time of autopsy, whole blood was collected from the abdominal vena cava of experimental groups of Wistar rats. Then, it was kept in a ethylene-diamine-tetraacetic acid (EDTA)-coated tubes (BD Caribe, Ltd., USA). The total numbers of white blood cells (WBCs), lymphocytes, granulocytes, and mid-sized cells in EDTA-coated tubes were analyzed using a Mindray BC-2800 hematological analyzer (Mindray, Bath, UK).

**2.11. Histological Analysis.** Histochemical analyses were performed as previously described [26]. In brief, after the animals were euthanized, their spleens were removed, weighed, and fixed in 10% neutral-buffered formalin. The organs were then embedded in paraffin and sectioned into 4-µm-thick slices using a microtome (Thermo Scientific,

Waltham, MA, U.S.A.). Sectioned tissues were stained with hematoxylin and eosin (H&E). Spleen images were scanned using a MoticEasyScan One (Hong Kong, China) and detected using Motic DSAssistant (X86) software.

**2.12. Statistical Analysis.** All data are expressed as the mean ± standard error of the mean (SEM), and differences between groups were analyzed using one-way ANOVA (Duncan's multiple-range test). All analyses were performed using SPSS (version 23.0; SPSS, Inc. USA). Each value represents the mean of at least three independent experiments for each group. Statistical significance was set at  $p < 0.05$ .

### 3. Results

**3.1. HPLC Analysis of VCE.** Four VCE compounds were identified: three types of phenols (4-hydratebenzoic acid, gallic acid, and syringic acid) and the flavonoid quercetin (Figure 2). These phenolic compounds and flavonoids have various antioxidant, anti-inflammatory, gut microbiota-modulating, and skin health-related effects [9, 10, 27]. The immune-enhancing activity of the VCE containing these components was verified in splenocytes from Wistar and cyclophosphamide-induced immunosuppressed rats.

**3.2. Cell Viability of Splenocytes.** We investigated the viability of the VCE splenocytes obtained from Wistar rats (Figures 1(a) and 1(b)). Cell viability was measured using the WST-1 assay kit. VCE up to a concentration of 300 µg/mL was nontoxic (Figure 1(a)). Cell viability with 0.8 mg/mL of Cy was significantly decreased at 0 µg/mL of VCE ( $70.1 \pm 0.5\%$ , Figure 1(b)). In the presence of Cy with VCE, this increased at 100 µg/mL ( $76.9 \pm 1.4\%$ ) and 300 µg/mL ( $74.9 \pm 1.0\%$ ).

**3.3. NO Production in Splenocytes.** The production of NO was investigated for anti-inflammatory as well as immune-enhancing effects. Appropriate immune stimulation boosts immunity [9]. Therefore, we investigated NO production in the splenocytes of Wistar rats (Figures 1(c) and 1(d)). The production of NO was increased by VCE ( $3.43 \pm 0.17$  µM at 300 µg/mL) and VCE with Cy (at  $4.87 \pm 0.21$  µM at 300 µg/mL).

**3.4. Cytokine Levels in Splenocytes.** Immunosuppression decreases the secretion of various cytokines (IL-2, IL-12, and IFN-γ) in the body [19]. We induced immunosuppression using Cy (0.8 mg/mL) and investigated the effect of VCE on cytokine levels in Wistar rat splenocytes (Figure 3). In the presence of Cy, levels of IL-2, IL-12, IFN-γ, and TNF-α significantly decreased. At 300 µg/mL of VCE, levels of IL-2 ( $71.1 \pm 1.6$  pg/mL), IL-12 ( $12.1 \pm 0.8$  pg/mL), IFN-γ ( $53.9 \pm 1.4$  pg/mL), and TNF-α ( $137.1 \pm 2.2$  pg/mL) were increased and recovered to the levels in control cells (IL-2,  $75.1 \pm 2.0$  pg/mL; IL-12,  $13.2 \pm 0.4$  pg/mL; IFN-γ,  $71.4 \pm 1.9$  pg/mL; and TNF-α,  $133.9 \pm 3.2$  pg/mL). These results indicate that VCE increases cytokine levels in the immune system.

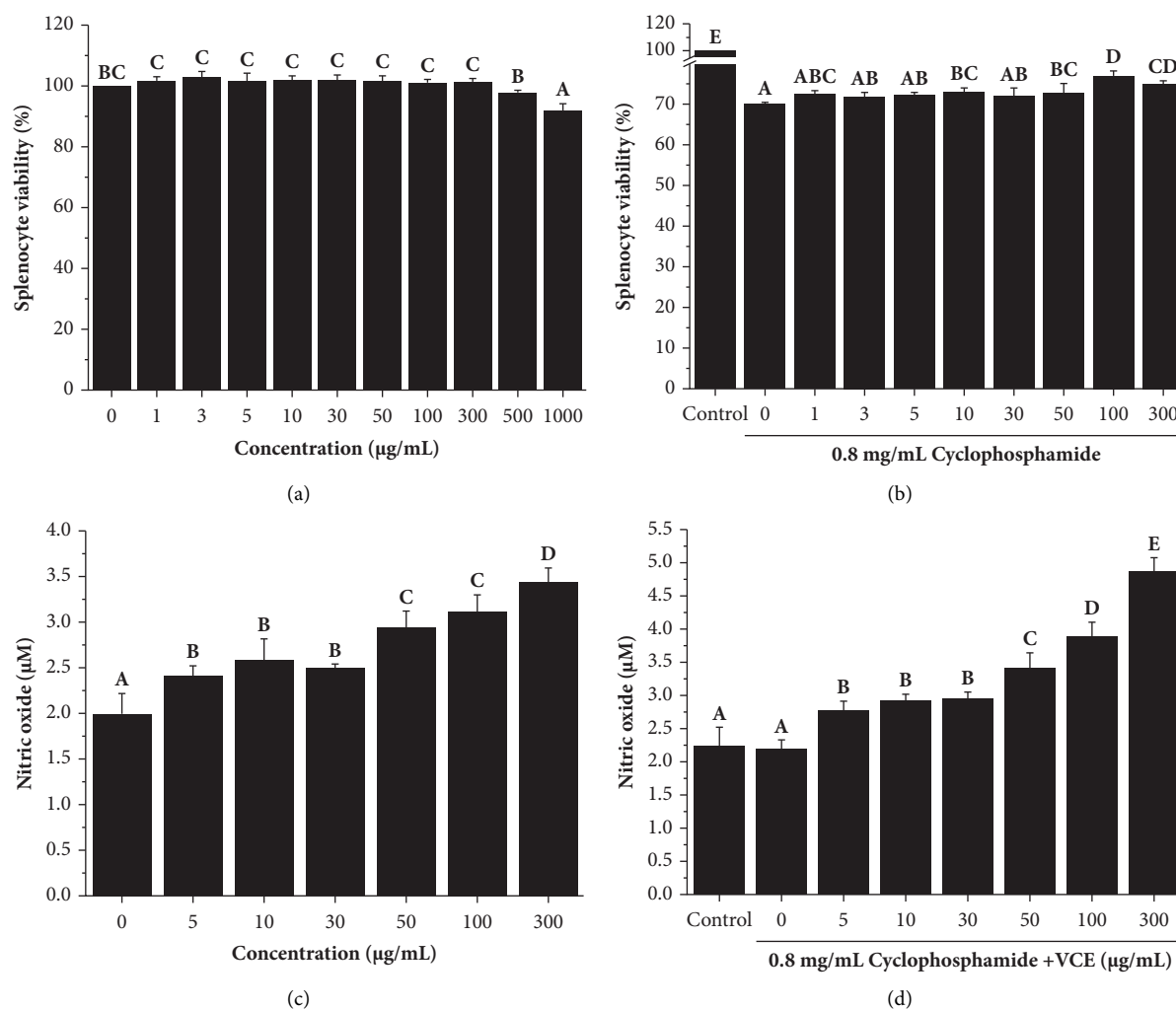


FIGURE 1: Cell viability and anti-inflammatory effects of *Vitis coignetiae* extract (VCE) on Wistar rat splenocytes. Splenocytes were treated for 24 h with (a) VCE (0–1,000  $\mu\text{g/mL}$ ) and with (b) VCE (0–300  $\mu\text{g/mL}$ ) and cyclophosphamide (Cy) (0.8 mg/mL). Nitric oxide (NO) concentration was determined using the Griess reagent. Splenocytes were pretreated with (c) VCE (0–300  $\mu\text{g/mL}$ ) and (d) Cy (0.8 mg/mL) for 24 h. Levels were detected using an enzyme-linked immunosorbent assay (ELISA). Bars labeled with different superscripts have significantly different values ( $p < 0.05$ ).

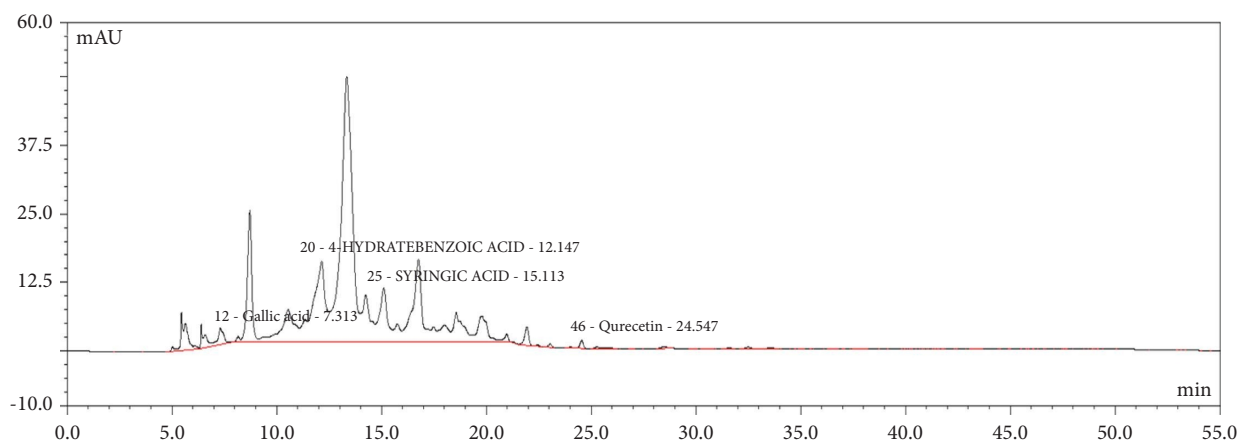


FIGURE 2: Quantitative HPLC analysis of compounds from *Vitis coignetiae* extract. HPLC chromatograms of VCE; peaks: gallic acid, hydratebenzoic acid, syringic acid, and quercetin.

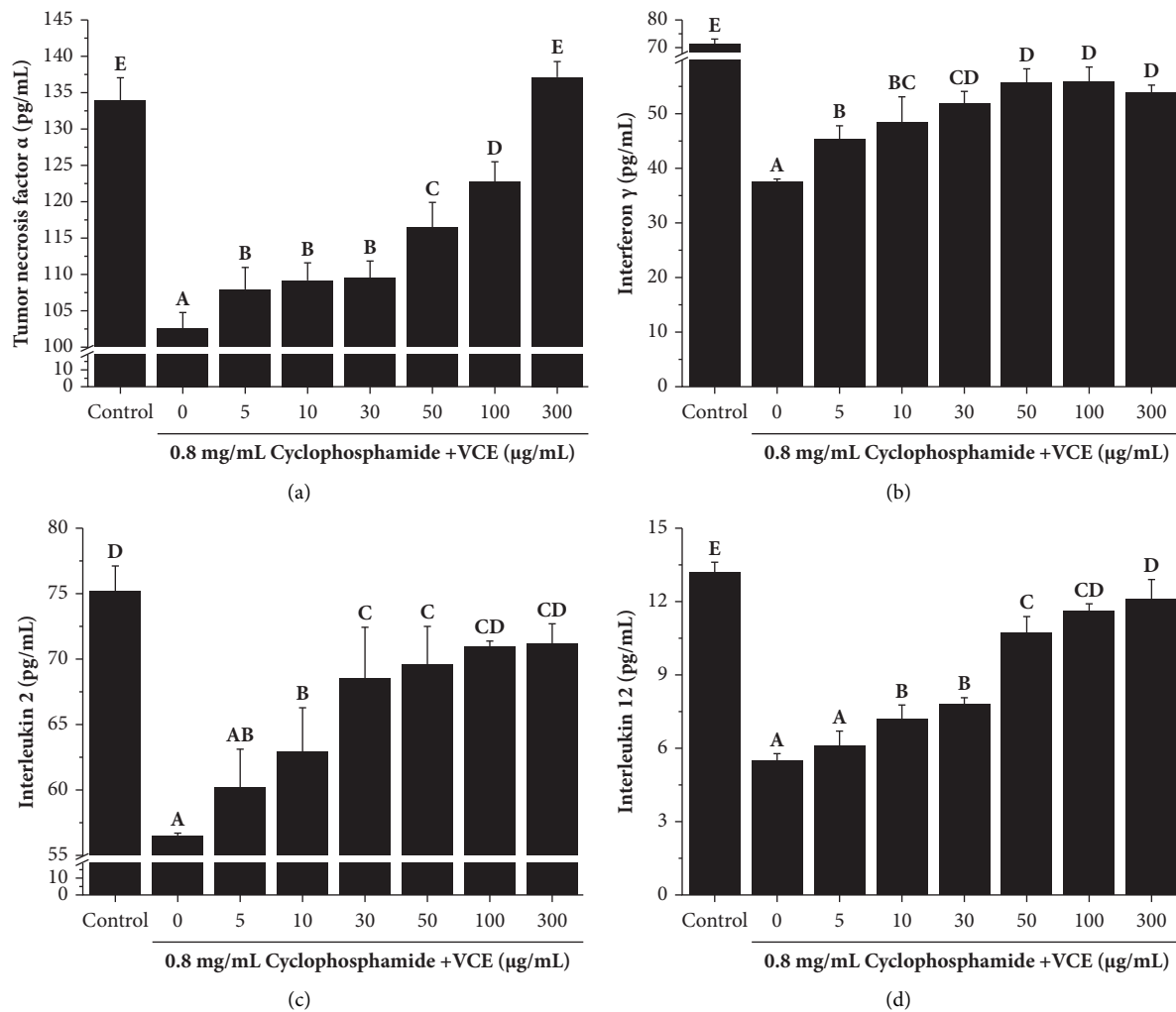


FIGURE 3: Effect of *Vitis coignetiae* extract (VCE) on the serum levels of immune-related cytokines in cyclophosphamide (Cy)-treated Wistar rat splenocytes. Splenocytes were treated with VCE (0–300 µg/mL) and Cy (0.8 mg/mL). Serum levels of (a) tumor necrosis factor alpha (TNF-α), (b) interferon gamma (IFN-γ), (c) interleukin-2 (IL-2), and (d) IL-12 were quantified using enzyme-linked immunosorbent assay (ELISA) kits. Bars labeled with different superscripts have significantly different values ( $p < 0.05$ ).

**3.5. Inflammation Pathways in Splenocytes.** A previous study showed that nonexcessive stimulation of immune pathways (such as the mitogen-activated protein kinase (MAPK) and NFκB pathways) helps to increase immunity [9]. To determine immune-related protein expression, western blot analysis was performed for the expression of inducible nitric oxide synthase (iNOS) and phosphorylation of MAPKs (Erk, JNK, and p38) and NFκB. As shown in Figure 4, immunosuppression by Cy (0.8 mg/mL) did not affect the expression of iNOS and the phosphorylation of MAPKs but suppressed the phosphorylation of NFκB. In the presence of VCE (30, 100, and 300 µg/mL), the expression of iNOS significantly increased with VCE 100 µg/mL, and concentration-dependent increases of MAPKs were observed, with the exception of p38 (Figures 4(b)–4(e)). NFκB phosphorylation increased in a concentration-dependent manner (Figure 4(f)). These results show that VCE stimulates immune-related pathways in a concentration-dependent manner.

**3.6. The Effect of VCE in Cy-Induced Immunosuppressed Rats.** Then, we investigated the immune-enhancing effects of the oral administration of VCE in Cy-induced immunosuppressed rats (5 mg/kg/d). We measured BW changes (once per week) during immunosuppression and oral administration of VCE (Figure 5(a)). In control rats (Cy-treated only group,  $346.37 \pm 5.37$  g), BW was significantly reduced compared to normal rats ( $365.23 \pm 5.73$  g). Rats orally administered with VCE (50, 250, and 500 mg/kg) and HemoHIM (positive group, 1,000 mg/kg/d) with Cy showed increased BWs at four weeks (VCE 50,  $372.12 \pm 4.83$  g; VCE 250,  $366.78 \pm 6.01$  g; VCE 500,  $383.38 \pm 3.47$  g; and positive group,  $360.80 \pm 1.93$  g). In addition, we measured the weights of the liver, spleen, and thymus in Cy-induced immunosuppressed rats (Figure 5(b)–5(d)). Cy significantly reduced spleen weight; however, liver and thymus weights did not significantly decrease. In rats administered with VCE (50, 250, and 500 mg/kg) and positive rats (HemoHIM) treated with Cy, liver and thymus weights were

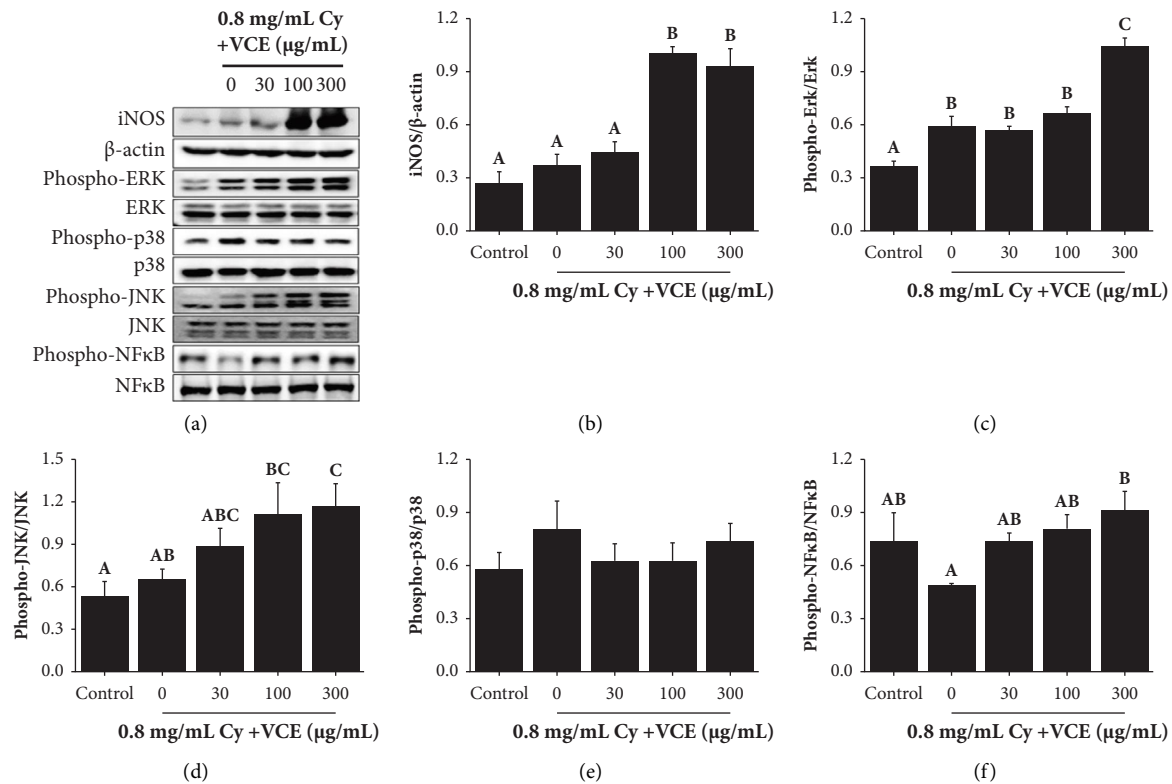


FIGURE 4: Anti-inflammatory-related protein expression and signal pathways in Wistar rat splenocytes. Splenocytes were treated with *Vitis coignetiae* extract (VCE) (30, 100, and 300 μg/mL) and cyclophosphamide (Cy) (0.8 mg/mL) for 24 h. (a) Representative image of anti-inflammatory-related protein expression and signal pathways (inducible nitric oxide synthase (iNOS), mitogen-activated protein kinase (MAPK), and NFκB). (b) Expression levels of iNOS, (c-e) phosphorylation of MAPKs, and (f) NFκB in total cell lysate, determined using western blot analysis. Bars labeled with different superscripts have significantly different values ( $p < 0.05$ ).

also unchanged, while the spleen weight increased compared to control rats. These results suggest that VCE enhances immunity by activating the immune system in the spleen.

**3.7. CBC and Cytokines in Cy-Induced Immunosuppressed Rats.** A previous study showed reductions in WBC, granulocyte, lymphocyte, midsize cell, and inflammatory cytokine (such as IL-2, IL-12, IFN-γ, and TNF-α) levels in Cy-treated splenocytes and Cy-induced immunosuppressed rats [19, 28]. Therefore, we investigated whole blood and serum cytokine levels (Figures 6 and 7). After respiratory anesthesia, whole blood was collected immediately from the vena cava, followed by an analysis of the CBC (WBC, granulocytes, lymphocytes, and midsize cells), and serum was separated. As shown in Figure 6, WBCs, granulocytes, lymphocytes, and midsize cells significantly decreased following Cy intake (5 mg/kg) and increased following VCE administration (250 mg/kg). CBC levels in VCE 50 rats were similar to those in control rats. As shown in Figure 3, inflammatory cytokine levels decreased in Cy-treated splenocytes. Therefore, we investigated the levels of inflammatory cytokines in the serum of Cy-induced immunosuppressed rats (Figure 7). Following oral administration of Cy for four weeks, IL-2, IL-12, IFN-γ, and TNF-α levels decreased. VCE administration (500 mg/kg) increased cytokine levels to levels similar to those of normal rats. In

addition, IgG and IgM levels were investigated to determine immunity (Figures 7(e) and 7(f)). Levels of IgG and IgM were decreased in control rats, while IgG increased after VCE 250 treatment and IgM increased after VCE 500 treatment. In the control group, IgG and IgM levels decreased, while these were significantly increased in the VCE 250 and positive control groups (HemoHIM). These results show that VCE can recover CBC, cytokine, IgM, and IgG levels to normal levels in immunosuppressed rats. Therefore, VCE is effective in increasing immunity.

**3.8. NK Cell Activity.** Enhancing NK cell activity protects against viral infection [29]. To determine the effect of VCE on NK cell activity, we analyzed lactate dehydrogenase (LDH) production in splenocytes (Figure 8(a)), Cy-induced immunosuppressed rats (Figure 8(b)), and AR42J cells. In Figure 8(a), NK cell activity (%) in splenocytes increased in a concentration-dependent manner with VCE. In addition, oral administration of VCE increased NK cell activity in Cy-induced immunosuppressed rats. These results indicate that VCE enhances immune function by increasing NK cell activity in splenocytes.

**3.9. Histological Assay.** We observed morphological changes in the spleen and evaluated the effects of VCE (Figure 9). In the control group, white pulp (WP) and lymph node (LN) depletion were observed using H&E staining in the spleen,



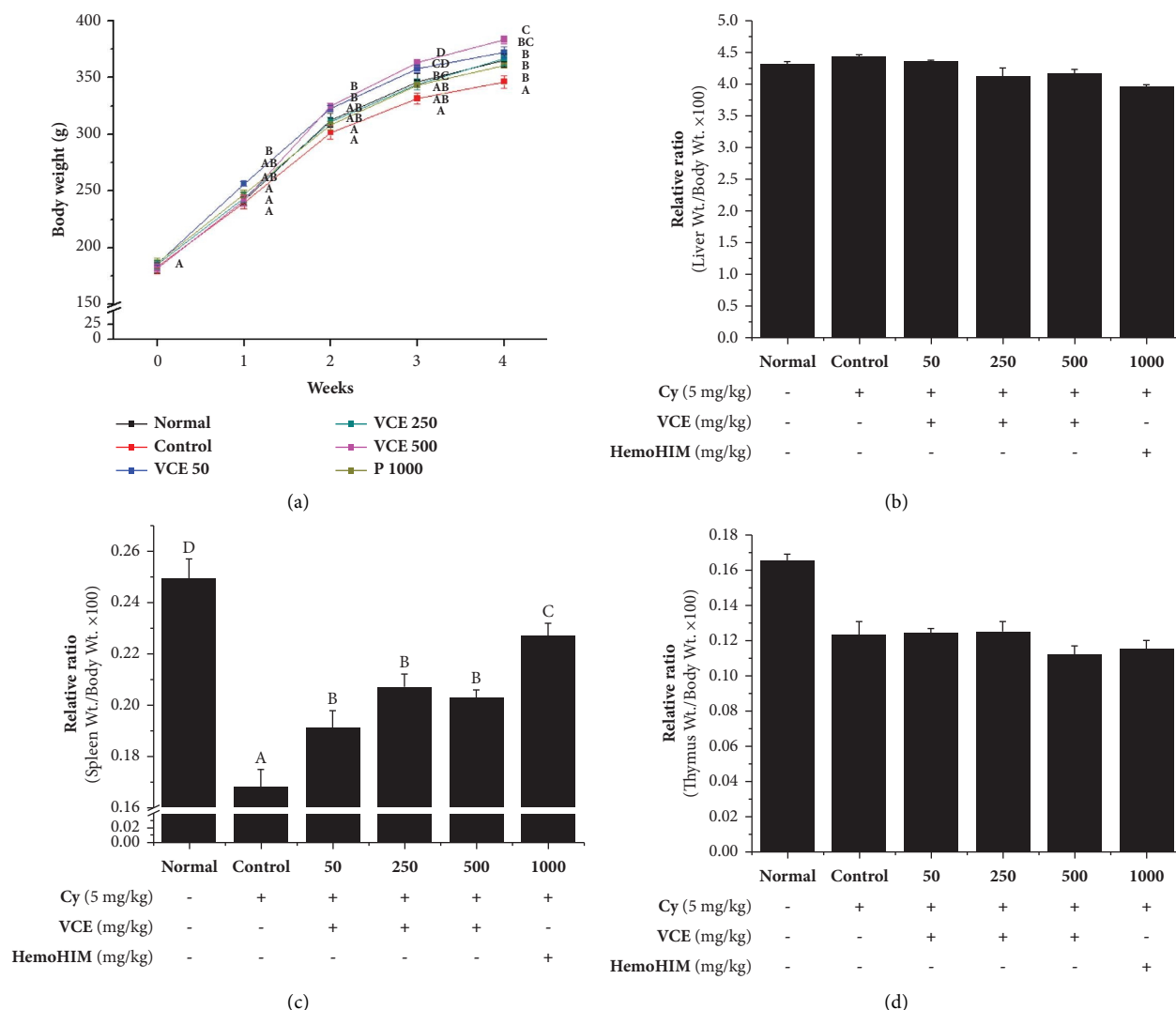


FIGURE 5: Effects of *Vitis coignetiae* extract (VCE) on body weight and liver, spleen, and thymus weights in cyclophosphamide (Cy)-induced immunosuppressed rats. Wistar rats were treated with saline, Cy (5 mg/kg/d), oral VCE (0, 50, 250, and 500 mg/kg/d), and HemoHIM (positive control; 1,000 mg/kg/d) once daily for four weeks. (a) Changes in body weight over four weeks and changes in (b) liver, (c) spleen, and (d) thymus weights. Bars labeled with different superscripts have significantly different values ( $p < 0.05$ ).

indicating immunosuppression by Cy (Figure 9(b)). VCE 50 treatment led to moderate WP quality and a marginal zone (MZ) (Figure 9(c)). In VCE 250 spleens (Figure 9(d)), WP damage was reduced compared with that in control spleens. In the VCE 500 (Figure 9(e)) and positive (Figure 9(f)) groups, the improvement was similar to that in the normal group. These results indicate the innate and adaptive immune-enhancing effects of VCE through histopathological recovery of spleen structures.

Various natural products affect the immune system [30]. Recently, natural products (flavonoids and phenols) have been shown to have various effects (such as antioxidant, anti-inflammatory, anticancer, and immune-regulating effects) and increase immunity through various actions such as cellular and nonspecific immunity as a stimulus to immune organs. Various effects of natural products defend against external harm [9]. A previous study reported that the intake of a natural extract (*Platycodon grandiflorum*) helps to

improve immunity by recovering IL levels and the collapse of spleen tissue [19]. VCE has been reported to have strong anti-inflammatory effects on macrophages and anticancer effects (proliferation, migration, and invasion) in cancer cells [22, 27]. The spleen is essential for immune system function; it is important for filtering blood, removing bacteria and infecting organisms in the bloodstream, and destroying and producing Immunoglobulins. In a previous study, Cy-induced immunosuppression induced decreases in CBC and IL levels and led to the collapse of the spleen tissue. To date, there have been no reports on the results of studying the efficacy of VCE in Cy-induced immunosuppression.

#### 4. Discussion

Our results show that VCE is a natural product containing phenols (4-hydratebenzoic acid, gallic acid, and syringic acid) and the flavonoid quercetin (Figure 2). It has been

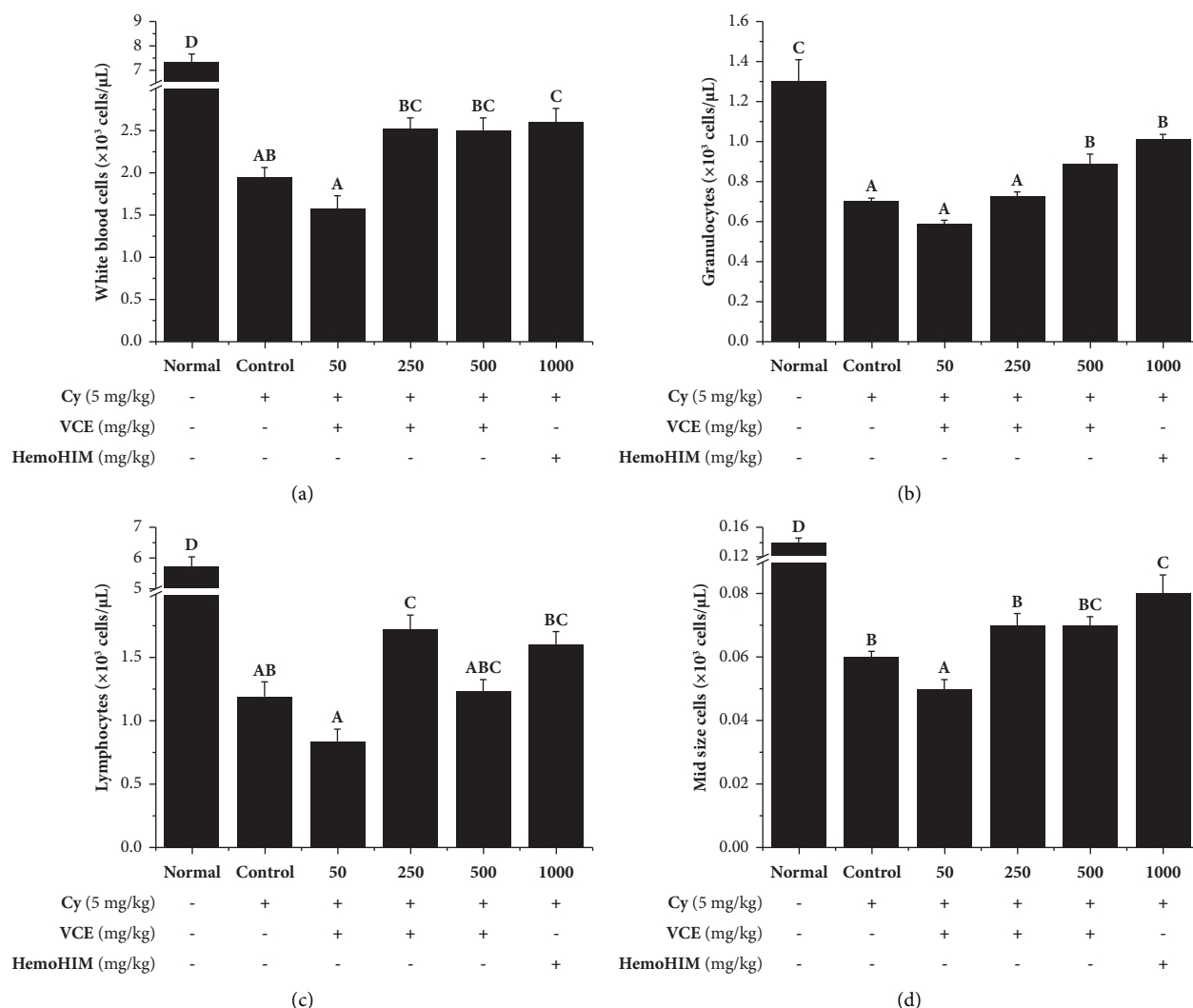


FIGURE 6: Effects of *Vitis coignetiae* extract (VCE) on (a) white blood cell, (b) granulocyte, (c) lymphocyte, and (d) midsize cell absolute counts in the whole blood of cyclophosphamide (Cy)-induced immunosuppressed rats. Wistar rats were treated with saline, Cy (5 mg/kg/d), oral VCE (0, 50, 250, and 500 mg/kg/d), and HemoHIM (positive control; 1,000 mg/kg/d) once daily for four weeks, after which whole blood samples were collected for analysis. The levels of white blood cells, granulocytes, lymphocytes, and midsize cells in the blood. Bars labeled with different superscripts have significantly different values ( $p < 0.05$ ).

suggested that VCE affects the immune regulation and could be effective in enhancing immunity. Therefore, the immune-enhancing effects of VCE were evaluated. We performed a cytotoxicity evaluation in Wistar rat splenocytes to ensure the safety of VCE. The results show that there is no cytotoxicity up to a concentration of 300  $\mu$ g/mL. Moreover, cytokine (TNF- $\alpha$ , INF- $\gamma$ , IL-2, and IL-12) levels were decreased in Cy-treated splenocytes and were recovered following VCE administration. Previous studies have shown the stimulation of inflammatory pathways (MAPK and NF $\kappa$ B pathways) and related protein expression (iNOS and cyclooxygenase-2 (COX-2)) in immune cells by various natural extracts [31, 32]. Furthermore, inflammatory pathway activation and related protein expression increased by inflammatory mediators (such as lipopolysaccharide, IL-1 $\beta$ , and TNF- $\alpha$ ) have been shown to be reduced by natural extracts [31, 33]. However, this is increased by natural

extracts due to their immune-enhancing effect [9]. Our results showed that VCE increased the expression of iNOS and phosphorylation of MAPKs (Erk and JNK) and NF $\kappa$ B. These results suggest that VCE shows immune-enhancing effects at nontoxic concentrations *in vitro*.

A decrease in immunity indicates a condition in which immune system disruption may fail to prevent harmful external intrusion. This decreases the weight of immune-related tissues (spleen and thymus) and WBC count *in vivo* [34, 35]. Our results showed that CBC and cytokine levels, and thymus and spleen weights, were reduced in Cy-induced immunosuppressed rats. Elevated cytokine regulation indicates that inflammation has occurred, while down-regulation indicates that immunity has decreased [36]. In our results, Cy-induced cytokine reduction was recovered by VCE to normal levels. In addition, WBC, lymphocyte, granulocyte, and midsize cell levels were recovered in the



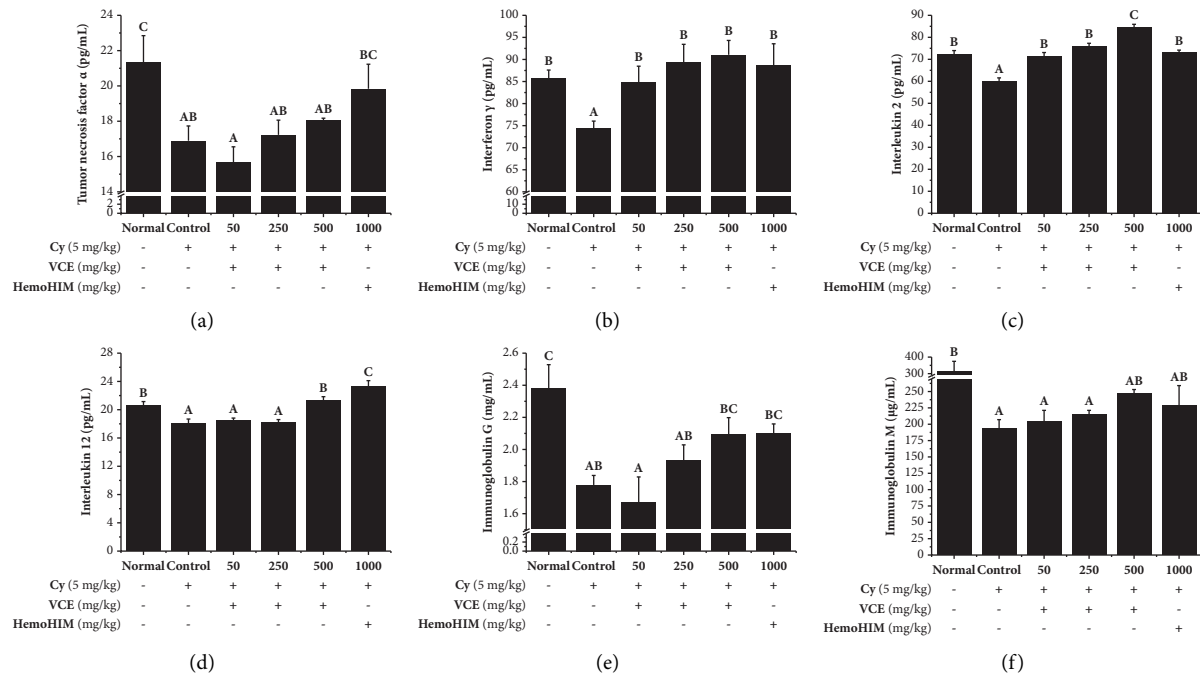


FIGURE 7: Effect of *Vitis coignetiae* extract (VCE) on the serum levels of immune-related cytokines in the serum of cyclophosphamide (Cy)-induced immunosuppressed rats. Wistar rats were treated with saline, Cy (5 mg/kg/d), oral VCE (0, 50, 250, and 500 mg/kg/d), and HemoHIM (positive control; 1,000 mg/kg/d) once daily for four weeks, after which serum levels of (a) tumor necrosis factor (TNF- $\alpha$ ), (b) interferon gamma (IFN- $\gamma$ ), (c) interleukin-2 (IL-2), (d) IL-12, (e) immunoglobulin G (IgG), and (f) IgM were quantified using enzyme-linked immunosorbent (ELISA) kits. Bars labeled with different superscripts have significantly different values ( $p < 0.05$ ).

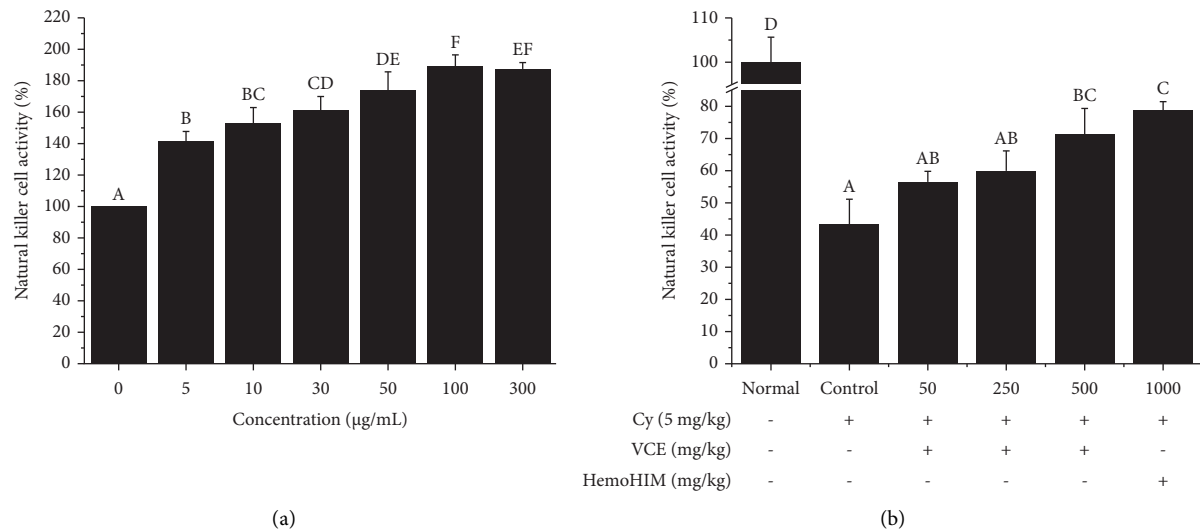


FIGURE 8: Effect of *Vitis coignetiae* extract (VCE) on natural killer (NK) cell activity in cyclophosphamide (Cy)-treated Wistar rat splenocytes and Cy-induced immunosuppressed rats. Wistar rat splenocytes were treated with VCE (0–300  $\mu\text{g/mL}$ ) for 24 h and Cy-induced immunosuppressed rat splenocytes were treated with Cy (5 mg/kg/d), oral VCE (0, 50, 250, and 500 mg/kg/d), and HemoHIM (positive control; 1,000 mg/kg/d) once daily for four weeks, after which spleens were collected for analysis. Splenocytes were cocultured with target cells (AR42J) in 96-well plates. Then, the cells were incubated for 24 h in a 5%  $\text{CO}_2$  incubator with a ratio of effector to target cells of 20 : 1. NK cell activity was calculated as the survival rate of AR42J cells compared to those of the control group. Bars labeled with different superscripts have significantly different values ( $p < 0.05$ ).

whole blood of Cy-induced immunosuppressed rats. Finally, we observed morphological changes in the spleen. In our results, the WP and LN collapsed in the Cy group and

recovered in the VCE (500 mg/kg) and positive control groups (HemoHIM). Our findings suggest that VCE-induced cytokine (TNF- $\alpha$ , IFN- $\gamma$ , IL-2, IL-12, IgG, and

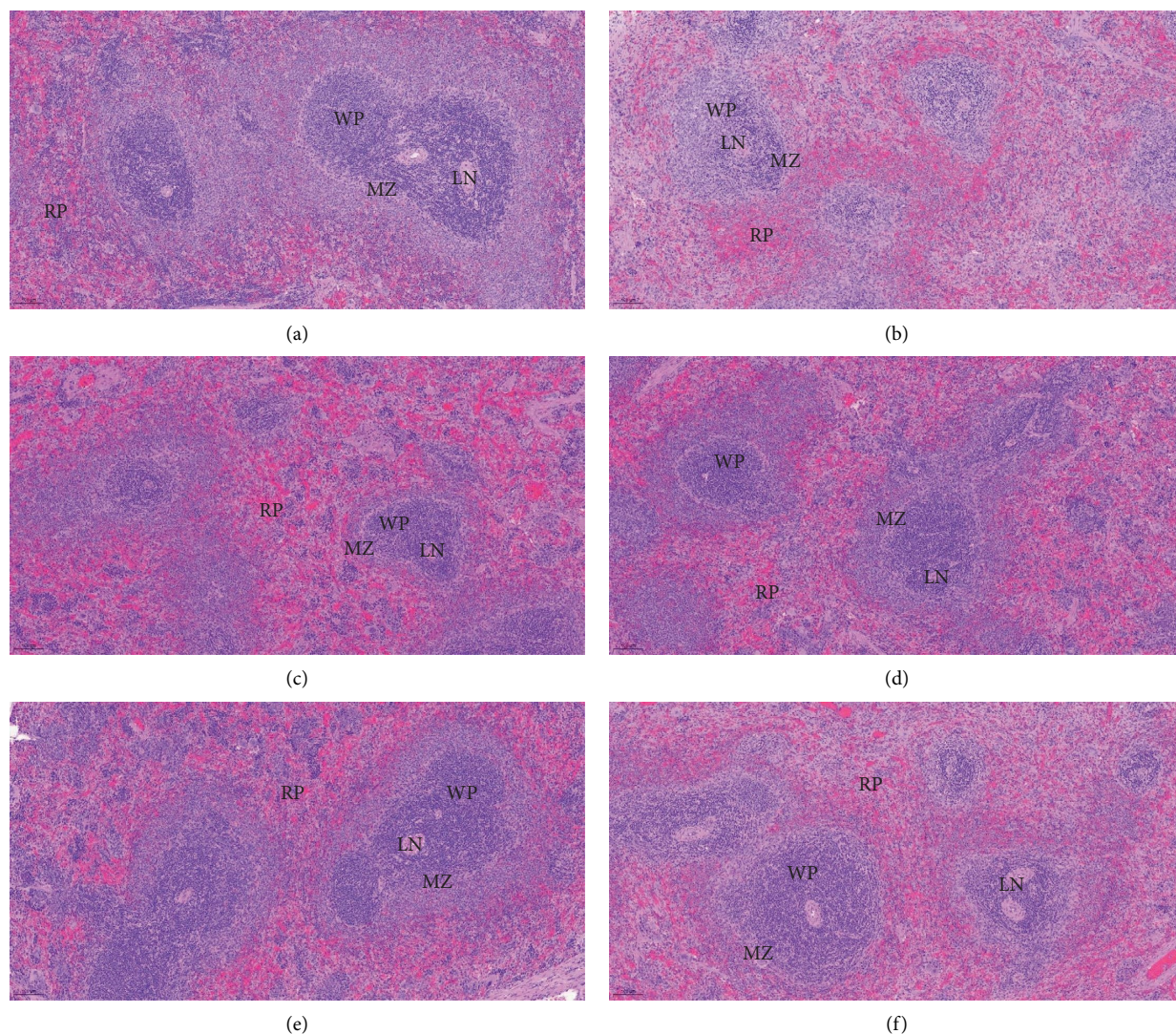


FIGURE 9: Effect of *Vitis coignetiae* extract (VCE) on immunity-associated spleen damage in cyclophosphamide (Cy)-induced immunosuppressed rats. Wistar rats were treated with saline, Cy (5 mg/kg/d), and oral VCE (0, 50, 250, and 500 mg/kg/d) once daily for four weeks, after which spleen damage was analyzed histologically. Representative images of the sectioned spleens of (a) normal rats (saline), (b) control rats (Cy only), (c-e) VCE-treated rats (treated with Cy and (c) 50 mg/kg, (d) 250 mg/kg, or (e) 500 mg/kg of VCE), and (f) positive control rats (treated with Cy and 1,000 mg/kg of HemoHIM). Scale bar = 100  $\mu$ m. CV: central vein; LN: lymph node; MZ: marginal zone; and RP: red pulp.

IgM), CBC (WBC, granulocyte, lymphocyte, and midsize cell), and NK cell activity increases in Cy-treated splenocytes and Cy-induced immunosuppressed rats, as well as the stimulation of inflammatory pathways, constitutes immune enhancement.

## 5. Conclusions

In conclusion, our findings demonstrate that VCE mitigated the decrease in cytokine levels in Cy-treated splenocytes, CBC levels in whole blood, and cytokine levels in serum, and diminished the increased thymus and spleen weight increases in Cyc-induced immunosuppressed rats. These results suggest that VCE is a healthy functional food that can boost immunity during chemical immunosuppression.

Therefore, VCE may have applications in the development of immune-enhancing functional foods and medicines. In this study, VCE was nontoxic *in vivo*; however, further toxicity assessments are needed before its use in humans [37–40].

## Data Availability

The data that support the concept of this review will be available from the corresponding author upon a reasonable request.

## Ethical Approval

Animal experiments were approved by the University of Florida Animal Care and Use Committee (IV-RB-17-2206-20).

## Conflicts of Interest

The authors declare no conflicts of interest.

## Authors' Contributions

Conceptualization, Y. M. P., M. S. K., H.J.Y., and J.G.K.; investigation, Y.M.P., H.Y.L., D.Y.S., H.Y.P., and J.G.K.; data curation, H.M.H. and H.N.J.; formal analysis, S.H.K., M.J.K., H.J.K., and J.H.K. Writing—original draft preparation: Y. M. P., M. J. K., and J. G. K.; Writing—review and editing: J.G.K. All authors have read and agreed to the published version of the manuscript.

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