Rhus verticillate Cortex-dependent antioxidant, anti-inflammatory, immunomodulatory effects of Korean herbal medicine, HH711, in immune cells

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ABSTRACT

Introduction: Altered oxidation/reduction, homeostasis, and inflammation appear to be hallmarks of potential cell damage and consequent functional disruption. HH711, a traditional Korean herbal medicine consisting of *Rhus vernicifluae* Cortex (RV) as the main ingredient, has been used in Korea to treat chronic fatigue, stress, indigestion, and common cold.
Methods: The aim of this study was to determine whether HH711 inhibits the generation of free radicals, such as 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals, and the production of nitrite, an index of nitric oxide (NO), nitric oxide synthase (iNOS), cyclooxygenase (COX)-2, and pro-inflammatory cytokines, in lipopolysaccharide (LPS)-treated RAW 264.7 macrophages. We also evaluated the immunoregulatory effects of HH711 by measuring Tand B cell growth and secretion of cytokines, interleukin (IL)-6, and tumor necrosis factor (TNF)-α.
Results: Our results indicated that HH711 scavenged DPPH radicals and boosted superoxide dismutase activity in an RV dose-dependent manner. HH711 significantly inhibited LPS-induced NO, iNOS, and COX-2 expression in an RV dose-dependent manner accompanied by an attenuation of TNF-α, IL-6, and IL-1β levels in macrophages. Furthermore, HH711 promoted T or B cell growth and the production of IL-6 and TNF-α.
Conclusion: These findings indicate that HH711 can be used as a natural antioxidant and immunoregulatory agent.

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Keywords:
Rhus vernicifluae Cortex
Traditional Korean Medicine
Anti-Inflammatory
Immunomodulatory
Antioxidants

Abbreviations: ROS, reactive oxygen species; RNS, reactive nitrogen species; O_2-, superoxide; NO, nitric oxide; H_2O_2, hydrogen peroxide; COX, cyclooxygenase; TNF-α, tumor necrosis factor-α; LPS, bacterial lipopolysaccharide; iNOS, inducible nitric oxide synthase; IL, interleukin; RV, *Rhus vernicifluae* Cortex; RVS, *Rhus verniciflua* Stoke; DPPH, 2,2-diphenyl-1-picrylhydrazyl; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; DMSO, dimethyl sulfoxide; SOD, superoxide dismutase; WST, water-soluble tetrazolium salt; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; ELISA, enzyme-linked immunoassay; TBST, Tris-buffered saline with Tween 20; RPMI, Roswell Park Memorial Institute; BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; C-RVS, chloroform-extracted RVS; XO, xanthine oxidase; NBT, nitro blue tetrazolium; NOS, NO synthase;

PG, prostaglandin; mPGE2, membrane-bound PGE2; NF-κB, nuclear factor-kappa B; Th, T-helper; MHC, major histocompatibility complex; RC, *Rubus coreanus*.

Introduction

Oxidative stress and inflammation are crucial events in the progression of chronic inflammatory diseases, such as hypertension, diabetes, arthritis, and atherosclerosis (Khansari *et al*., 2009, Kim *et al.*, 2009). A progressive increase in oxidative stress caused by reactive oxygen species (ROS) and reactive nitrogen species (RNS) species acts as a signaling messenger for the development and perpetuation of inflammation (Zarkovic, 2020). Representative ROS and RNS involved in oxidative damage include free radicals such as superoxide (O_2-) and nitric oxide (NO) and oxygen-derived species such as hydrogen peroxide (H_2O_2) (Del Río, 2015). Cellular mitochondria and enzymes, such as peroxisomes and cyclooxygenase (COX), continuously generate ROS, whereas RNS are produced in large amounts during an inflammatory response by macrophages, neutrophils, and other immune cells (Brüne *et al*., 2003).

Macrophages, the most plastic cells in the hematopoietic system, are found in all tissues and exhibit great functional diversity in tissue repair and immunity (Wynn *et al*, 2013). Macrophages play an essential role in the initiation, maintenance, and resolution of inflammation, being activated by various cytokines such as interferon γ and tumor necrosis factor-α (TNF-α), bacterial lipopolysaccharide (LPS), extracellular matrix protein, and other chemical mediators (Fujiwara & Kobayashi, 2005). LPS, a bacterial endotoxin, promotes the production of inflammatory factors such as inducible nitric oxide synthase (iNOS), COX-2, TNF-α, intracellular ROS, and various interleukins (ILs) in macrophages. The identification and analysis of LPS antagonists have led to the development of novel anti-LPS strategies for the treatment of inflammatory diseases (Maldonado *et al.*, 2016).

There is a growing interest in natural antioxidants found in plants owing to their safety; hence, herbs are one of the most important targets in the search for natural antioxidants (Yanishlieva *et al*., 2006). The traditional Korean herbal medicine HH711 has been prescribed for treating functional diseases with chronic symptoms such as chronic fatigue, stress, indigestion, frequent common colds, and menopausal symptoms. *Rhus vernicifluae* Cortex (RV), derived from the bark of *Rhus verniciflua* Stoke (RVS), is one of the main components of HH711. RVS is an Asian tree species of the genus Toxicodendron, which belongs to the Anacardiaceae family and is commonly known as the lacquer tree. It has been used as an herbal medicine for the treatment of abdominal symptoms, including tumors, in East Asia since the 15th century AD (Choi *et al.*, 2012). Several *in vitro* studies of RVS have shown potential antibacterial, anticancer, anti-inflammatory, and antioxidant activities (Jung *et al.*, 2007, Kitts & Lim, 2001, Suk *et al.*, 2011), while *in vivo* studies have shown its efficacy against chronic inflammatory conditions such as neurodegenerative diseases, diabetes, and arthritis (Jung *et al.*, 2006b, Lee *et al.*, 2009, Sapkota *et al.*, 2010). However,

scientific evidence supporting the health benefits of RV and HH711 is still lacking. Therefore, we aimed to evaluate the antioxidant, anti-inflammatory, and immunomodulatory effects of HH711 based on RV concentration.

Methods

Plant material and sample preparation

The HH711 sample was obtained from *Kyung Hee Hanyak* Co. (Seoul, South Korea). Briefly, 230 g of the herbal mixture was extracted by boiling in distilled water at 100 °C for 2 h. It was subsequently filtered and freeze-dried to a powdered form. The voucher specimens were stored in the Herbal Pharmacology Laboratory. The composition of HH711 is described in Table 1, and the HH711 samples were divided into four groups based on the RV content (Table 2).

No	Scientific Name	Weight (g)
1	*Rhus vernicifluae* Cortex	2.33
2	*Ziziphus jujuba* MILL.	4.13
3	*Angelica gigas* Nakai	2.06
4	*Atractylodes japonica* Koidz.	2.06
5	*Cnidium officinale* Makino	2.06
6	*Paeonia japonica* Miyabe	2.06
7	*Cornus officinalis* Siebold	2.06
8	*Citrus unshiu* Markovich	2.06
9	*Zingiber officinale* Rosco	2.06
10	*Amomum xanthoides* Wall.	1.03
11	*Amomum cadamomum* Linnaeus	1.03
Total		22.94

Table 1. Composition of HH711.

No	Group name	Concentration of RV
1	HH711-10	10%
2	HH711-30	30%
3	HH711-60	60%
4	RV	100%

Table 2. Groups of HH711 based on the concentration of *Rhus vernicifluae* Cortex. Abbreviation: RV, *Rhus vernicifluae* Cortex.

diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

The free radical scavenging ability of the extracts was tested using the DPPH radical scavenging assay, as described by Gyamfi (Gyamfi *et al*., 1999). A

solution of 0.2 mM DPPH in methanol was prepared, and 150 µl of this solution was mixed with 100 µl of the extract in methanol at different concentrations (1, 10, 100, and 1,000 µg/ml). The reaction mixture was vortexed thoroughly and incubated in the dark at room temperature for 30 min. Absorbance of the mixture was measured spectrophotometrically at 517 nm. Ascorbic acid was used as the positive control. The percentage DPPH radical scavenging activity was calculated using the following equation:

$$\%\ \text{DPPH radical scavenging activity} = \frac{(A0 - A1)}{A0} 100$$

where A0 is the absorbance of the control, and A1 is the absorbance of the extract/standard. The experiment was repeated thrice at each concentration.

Cell growth and cell number assays

Changes in RAW 264.7 cells treated with H_2O_2 were indirectly determined by measuring the absorbance of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, St. Louis, MO, USA). Briefly, 3×10^4 cells/well were seeded in 96-well plates for the MTT assay. Following exposure to the indicated amounts of H_2O_2 (Sigma-Aldrich) and HH711-10, -30, -60, or RV at different concentrations (1, 10, 100, and 1,000 µg/ml) for 24 h, the cells in 96-well plates were used for MTT assays by adding MTT solution (5 mg/ml) to each well. The plates were incubated for 4 h at 37 ℃. The medium was withdrawn from the plates by pipetting, and 200 µl dimethyl sulfoxide (DMSO) was added to each well to solubilize the formazan crystals. Optical density was measured at 570 nm using a microplate reader (Choi & Kim, 2010).

Superoxide dismutase (SOD) activity

SOD activity was measured using a dismutase colorimetric activity kit (Abcam, ab65354). After incubation at 37 ℃ for 4 h, RAW 264.7 cells were treated with HH711-10, -30, -60, or RV extracts at different concentrations (1, 10, 100, and 1,000 µg/ml) and incubated at 37 ℃ for 48 h. In brief, 20 µl of the supernatant was added to 200 µl of the water-soluble tetrazolium salt (WST) working solution with 20 µl of enzyme working solution in a new plate, and the plate was kept at room temperature for 20 min. The absorbance was measured at 450 nm using a microplate reader (Molecular Devices, USA). The SOD activity was calculated using a standard curve.

Anti-inflammatory studies

Cell culture

The murine macrophage RAW 264.7 cell line was purchased from Korea Cell Line Bank and maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 µg/l streptomycin, and

100 IU/ml penicillin at 37 ℃ in a 5% CO_2 atmosphere. Inflammation was induced by treatment with LPS at a concentration of 0.5 µg/ml (Desai *et al*., 2008).

MTT assay

MTT with formazan was used to measure cell respiration as an indicator of cell viability. Briefly, RAW 264.7 cells were seeded in 96-well plates at 1×10^4 cells/well density. After 24 h of incubation, the adherent cells were treated with various HH711-10, -30, -60, or RV concentrations. Twenty-four hours later, after changing the medium, MTT was added to a final concentration of 0.5 mg/ml, and the cells were incubated for 4 h at 37 ℃ and 5% CO_2. The medium was then removed, and the formazan precipitate was solubilized in DMSO. Absorbance was measured at 570 nm using a microplate reader (Desai *et al*., 2008).

Inhibition of NO production

The RAW 264.7 cells were seeded at a density of 1×10^4 cells/well in 24 well plates and incubated for 24 h at 37 ℃ and 5% CO_2. The medium in each well was aspirated and replaced with fresh FBS-free DMEM. Different concentrations of HH71-10, -30, -60, or RV (1, 5, 10, 50, 100, and 200 µg/ml) were prepared in FBS-free DMEM to give a total volume of 500 µl in each well of a microtiter plate. After 1 h treatment, the cells were stimulated with 0.5 µg/ml of LPS for 24 h. The presence of nitrite in cell culture media was determined using a commercial Griess reagent kit (Sigma, USA). Protocols supplied with the assay kits were followed. Briefly, 100 µl of cell culture medium with an equal volume of Griess reagent in a 96-well plate was incubated at room temperature for 10 min. The absorbance was measured at 540 nm using a microplate reader (Wang *et al*., 2007).

Measurement of cytokines

The RAW 264.7 cells were pretreated with various concentrations of HH71-10, -30, -60 or RV (1, 5, 10, 50, 100, and 200 µg/ml) and then treated with LPS (0.5 µg/ml) for 24 h. Supernatants were harvested, and the levels of proinflammatory cytokines IL-6, IL-1β, and TNF-α were measured using enzyme-linked immunoassay (ELISA) kits according to the manufacturer's protocol (Kim *et al*., 2009).

Detection of iNOS and COX 2 expression

The effects of HH711-10, -30, -60, or RV on the expression of iNOS and COX-2 in LPS-stimulated RAW 264.7 cells were examined. The membranes were blocked with 5% non-fat dried milk in Tris-buffered saline with Tween 20 (TBST) at room temperature for 1 h. After washing, the membranes were incubated in the primary antibody solution (anti-iNOS, anti-COX-2 antibodies) overnight at 4℃. The membranes were washed with TBST and incubated with a

horseradish peroxidase-conjugated secondary antibody solution for 1 h at room temperature. The blots were washed thrice in TBST, detected using enhanced chemiluminescence, and exposed to photographic films (Choi & Kim, 2010).

Immunoregulatory effects of HH711

T and B cells were seeded at a density of 1×10^4 cells/well in a 24-well culture plate and incubated for 7 days. The cells were incubated at 37 ℃ in Roswell Park Memorial Institute (RPMI) 640 containing 10% FBS and 1% penicillin-streptomycin mixture in a 37 ℃ incubator with 5% CO_2. Cell growth was observed using a microscope, and the number of cells in each sample was determined using a hemocytometer. In addition, the amounts of TNF-α and IL-6, which are cytokines secreted by immune cells into the medium, were measured using an ELISA kit (Genzyme, Cambridge, MA, USA). The culture medium was collected every day for 7 days during the culture period and centrifuge, and the amount of cytokine was measured by determining the absorbance at 450 nm of the supernatant using a microplate reader (SpectraMax® Plus 384, Molecular Devices) (Chung & Lim, 2012).

Statistical analysis

Values are expressed as the means of three replicates ± standard deviation. Statistical analyses were performed using analysis of variance followed by Duncan's multiple range test using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). A value of $p < 0.05$ was considered to indicate statistical significance (Chung & Lim, 2012).

Results

DPPH radical scavenging activity

Similar to the positive control (ascorbic acid), the RV group showed the highest scavenging ability. The scavenging efficacy of HH711 was confirmed to increase sequentially in accordance with RV concentration (Fig. 1). These results indicate that the DPPH radical scavenging ability of HH711 depends on the RV concentration.

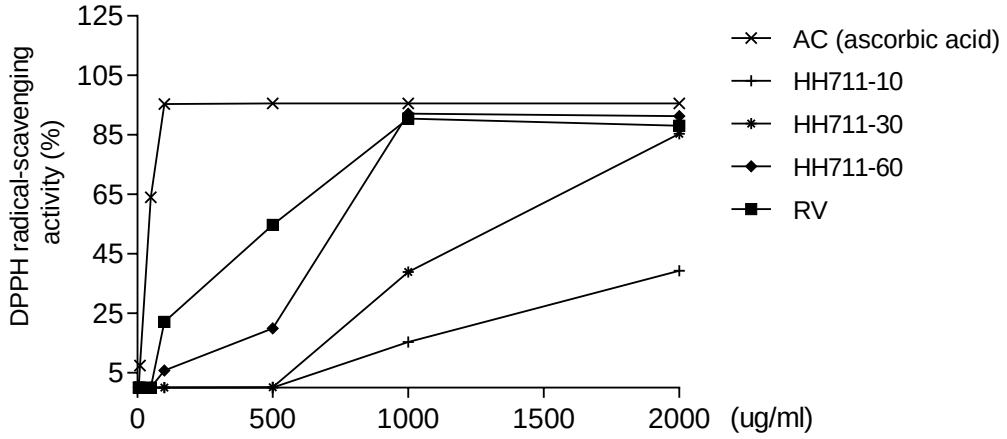


Fig. 1. DPPH radical scavenging activity of HH711-10, -30, -60, and RV extract (each group, n=6).
Abbreviation: RV, *Rhus vernicifluae* Cortex.

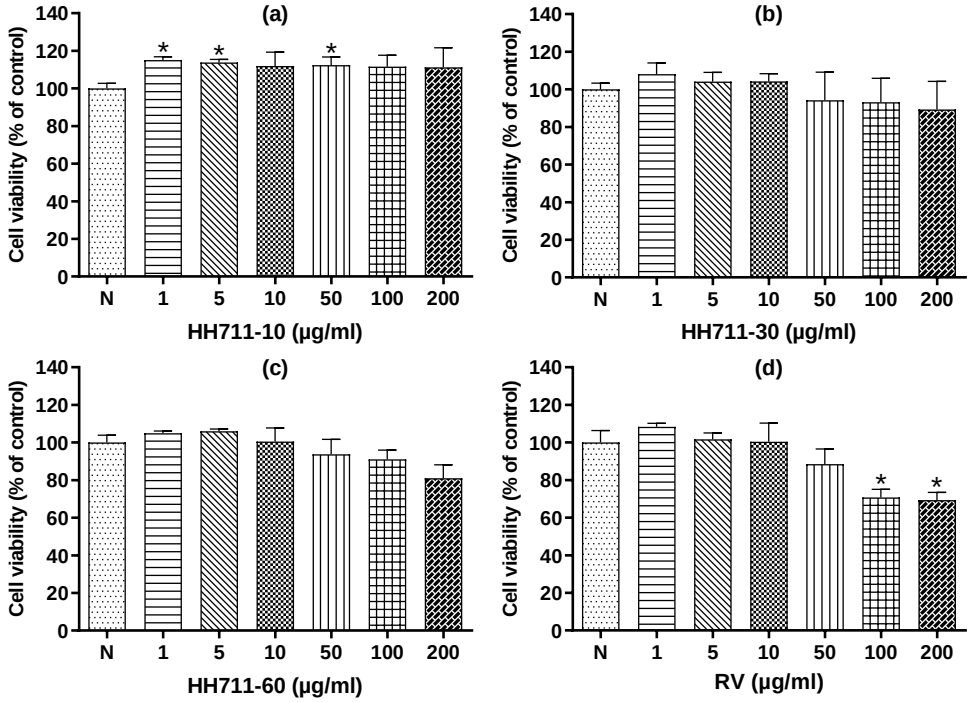


Fig. 2. Cell viability after treatment with various concentrations (1, 5, 10, 50, 100, and 200 μg/ml) of HH711-10, -30, -60, and -RV water extract. (a) HH711-10; (b) HH711-30; (c) HH711-60; (d) RV.
Abbreviation: N, Normal; RV, *Rhus vernicifluae* Cortex.
Cell viability was measured using the MTT assay.
Data are shown as mean ± SD obtained from independent experiments.

Statistical analyses were performed using analysis of variance followed by Duncan's multiple range test (each group n=3, *: $p < 0.05$).

Cell viability measurement

Cell viability at various concentrations (1, 5, 10, 50, 100, and 200 µg/ml) of HH711-10, -30, -60, and RV is shown in Fig. 2. In the HH711-10 group, cell viability increased by approximately 10% compared with that in the control group. In the HH711-30 and HH711-60 groups, cell viability decreased at concentrations above 50 µg/ml though the difference was not significant. In the RV group, cell viability decreased by over 20% at concentrations above 50 µg/ml. Based on these results, the cytotoxicity of HH711 appeared to be RV-concentration-dependent.

Protective effect of HH711 against H_2O_2 oxidative stress

Cell viability of H_2O_2-treated RAW 264.7 cells at various concentrations (1, 5, 10, 50, 100, and 200 µg/ml) of HH711-10, -30, -60, and RV are displayed in Fig. 3. At 1 and 10 µg/ml in the HH711-10 group, the cytoprotective effect significantly increased to 63% and 64%, respectively, compared to 43% in the control group. A significant increase in cell viability was also observed in the HH711-30 group (66% and 70% at concentrations of 5 and 10 µg/ml of HH711-30, respectively), the HH711-60 group (68% and 66% at concentrations of 1 and 5 µg/ml of HH711-60, respectively), and the RV group (62% at 1 µg/ml of RV). The highest cytoprotective effect in cells was observed at 10 µg/ml of HH711-30.

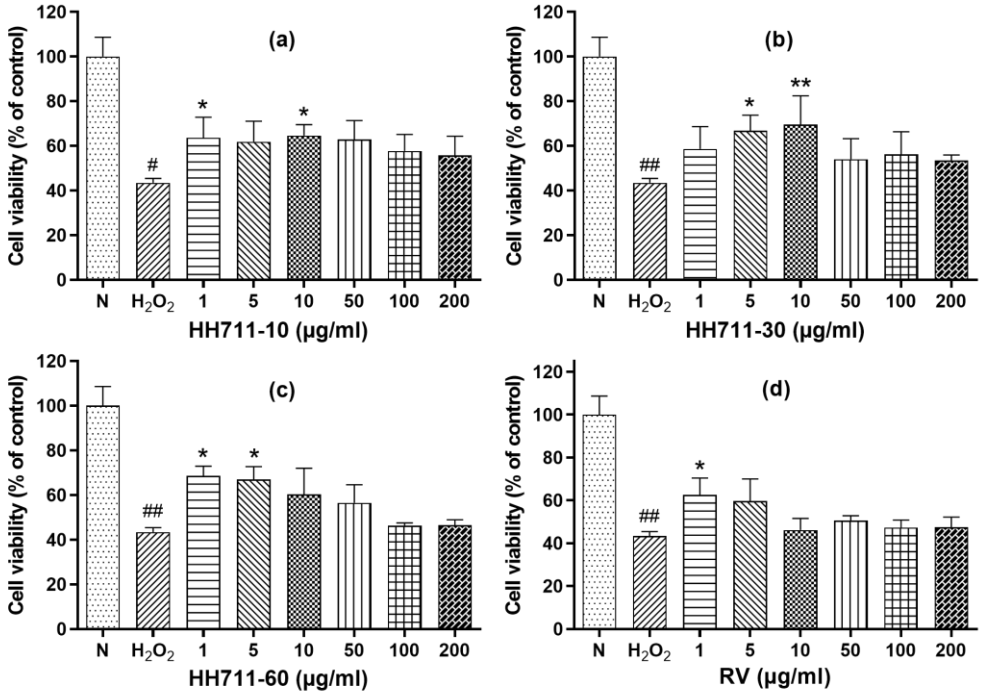


Fig. 3. Cell viability of H_2O_2-treated RAW 264.7 cells treated with various concentrations (1, 5, 10, 50, 100, and 200 μg/ml) of HH711-10, -30, -60 and RV water extract. (a) HH711-10; (b) HH711-30; (c) HH711-60; (d) RV.
Abbreviation: N, Normal; H_2O_2, hydrogen peroxide; RV, *Rhus vernicifluae* Cortex.
Data are shown as mean ± SD obtained from independent experiments. Statistical analyses were performed using analysis of variance followed by Duncan's multiple range test (each group n=3, #: compared to control, *: $p < 0.05$, **: $p < 0.01$ compared to cells cultured with H_2O_2).

Effect of HH711 on SOD activity

The effects of HH711-10, -30, -60, and RV at various concentrations (1, 10, and 100 μg/ml) on SOD activity levels in H_2O_2-treated RAW 264.7 cells are shown in Fig. 4. In the H_2O_2 treatment group, SOD activity was reduced by approximately 50% compared to that in the normal control group. At all concentrations (1, 10, and 100 μg/ml) of the HH711-10 group, SOD activity increased in a concentration-dependent manner by 12.0%, 17.7%, and 23.7%, respectively, compared to that in the H_2O_2-treated group. In the HH711-30 group, SOD activity increased significantly at all concentrations (1, 10, and 100 μg/ml) by 20.3%, 19.2%, and 30.0%, respectively. In the HH711-60 group, SOD activity was significantly increased by 9.2% and 17.4% at concentrations of 1 and 10 μg/ml, respectively, and by 15.5% at 1 μg/ml in the RV group.

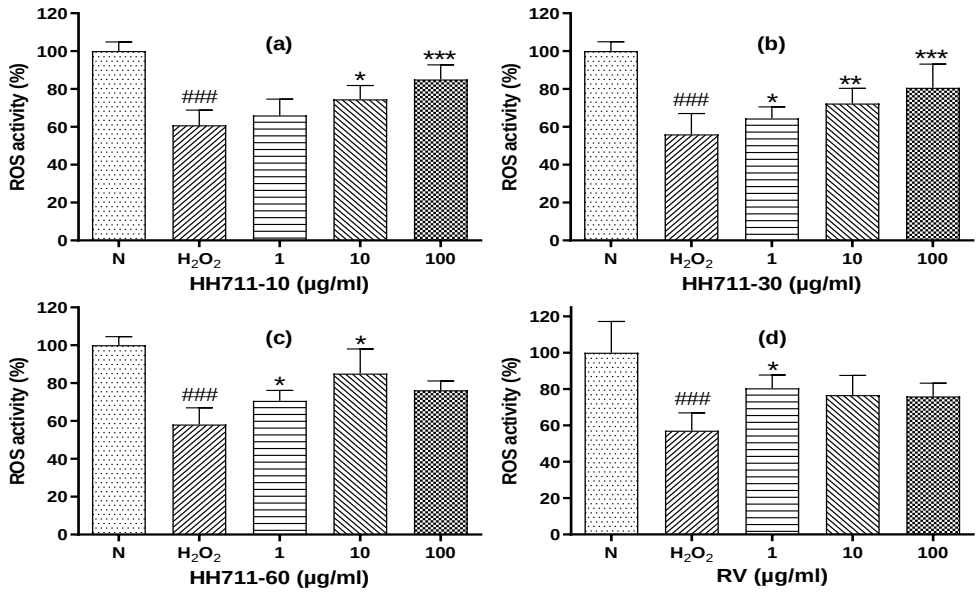


Fig. 4. Effects of HH711-10, -30, -60, and RV water extract on superoxide dismutase activity levels in H_2O_2-treated RAW 264.7 cells. (a) HH711-10; (b) HH711-30; (c) HH711-60; (d) RV.

Abbreviation: N, Normal; H_2O_2, hydrogen peroxide; RV, *Rhus vernicifluae* Cortex.
Data are shown as mean ± SD obtained from independent experiments. Statistical analyses were performed using analysis of variance followed by Duncan's multiple range test (each group n=6, #: compared to control, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$).

Anti-inflammatory effect of HH711

Cytoprotective effect of HH711 during LPS-induced inflammation

Cell viability of LPS-treated RAW 264.7 cells at various concentrations (1, 5, 10, 50, 100, and 200 µg/ml) of HH711-10, -30, -60, and RV is shown in Fig. 5. All groups showed a cytoprotective effect in the concentration range of 1 to 100 µg/ml; however, the cytoprotective effect decreased in each group at a concentration of 200 µg/ml.

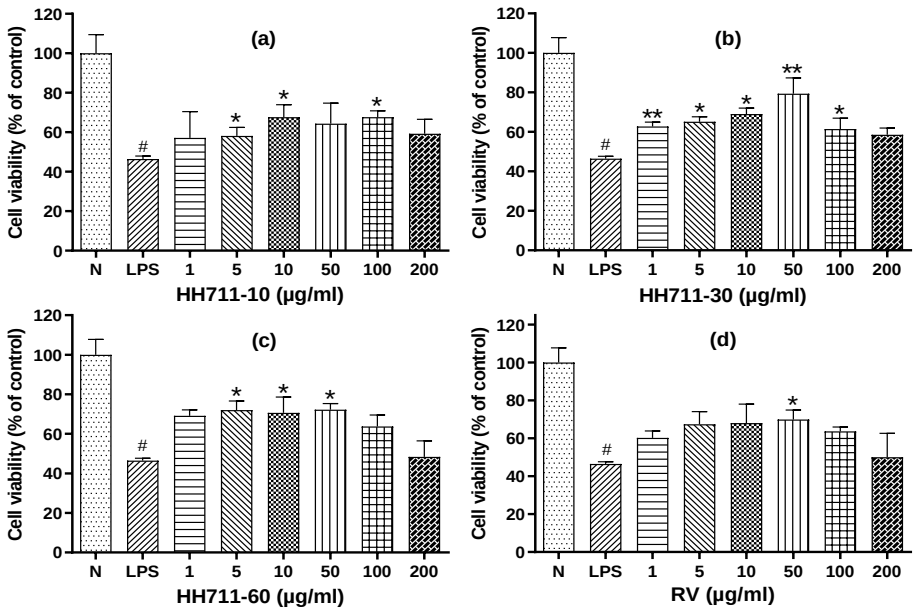


Fig. 5. Cell viability of LPS-treated RAW 264.7 cells treated with various concentrations (1, 5, 10, 50, 100, and 200 µg/ml) of HH711-10, -30, -60 and RV water extract. (a) HH711-10; (b) HH711-30; (c) HH711-60; (d) RV.
Abbreviation: N, Normal; LPS, Lipopolysaccharide; RV, *Rhus vernicifluae* Cortex.
Data are shown as mean ± SD obtained from independent experiments. Statistical analyses were performed using analysis of variance followed by Duncan's multiple range test (each group n=3, #: compared to Normal; *: $p < 0.05$, **: $p < 0.01$ compared to cells cultured with LPS.

Inhibitory effect on NO production

The effects of HH711-10, -30, -60, and RV at various concentrations (1, 5, 10, 50, 100, and 200 μg/ml) on NO production levels in LPS-treated RAW 264.7 cells are shown in Fig. 6. NO production levels in all HH711 groups and the RV group were reduced in a concentration-dependent manner compared with those in the LPS-alone-treated group.

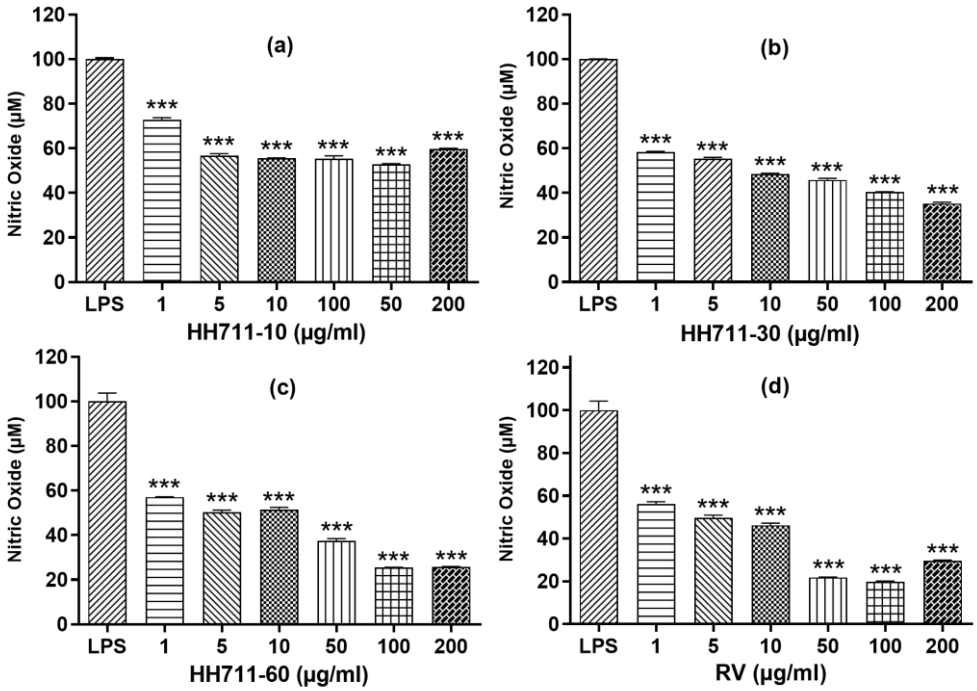


Fig. 6. Effects of HH711-10, -30, -60, and RV water extract at various concentrations (1, 5, 10, 50, 100, and 200 μg/ml) on nitric oxide production levels in LPS-treated RAW 264.7 cells. (a) HH711-10; (b) HH711-30; (c) HH711-60; (d) RV.
Abbreviation: LPS, Lipopolysaccharide; RV, *Rhus vernicifluae* Cortex.
Controls were treated with LPS.
Data shown represent means ± SD of independent experiments.
Statistical analyses were performed using analysis of variance followed by Duncan's multiple range test (each group n=3, ***: $p < 0.001$).

Inhibitory effects of HH711 on proinflammatory cytokines (TNF- α, IL-6, and IL-1 β)

The inhibitory effects of HH711-10, -30, -60, and RV at various concentrations (1, 10, and 100 μg/ml) on TNF-α, IL-6, and IL-1β production in LPS-treated RAW 264.7 cells are shown in Fig. 7. The HH711 groups exhibited

concentration-dependent inhibition on all proinflammatory cytokines, whereas the RV group showed concentration-dependent inhibition on IL-1β only.

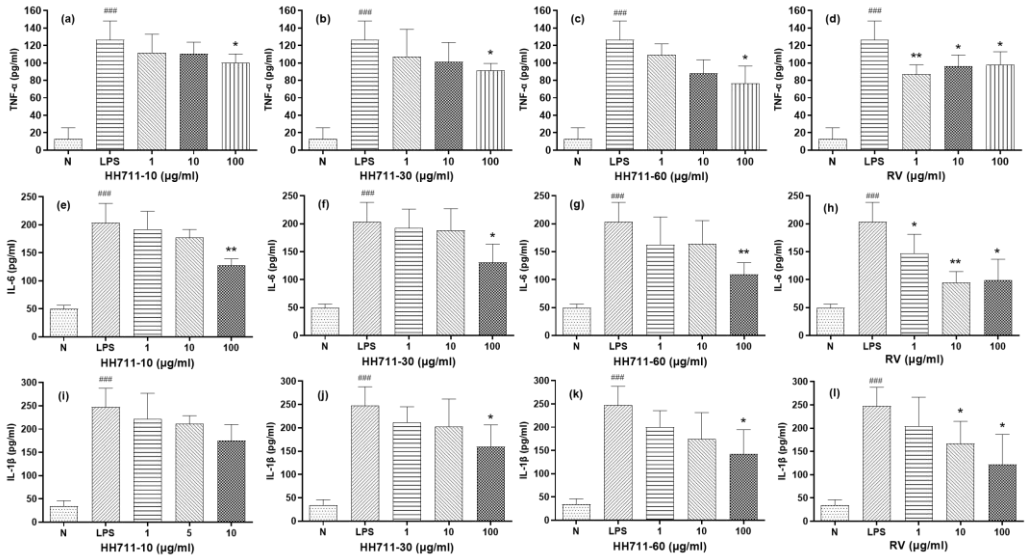


Fig. 7. Inhibitory effects of HH711-10, -30, -60, and RV water extract at various concentrations (1, 10, and 100 μg/ml) on TNF-α, IL-6, and IL-1β production in LPS-treated RAW 264.7 cells. (a) TNF-α in HH711-10; (b) TNF-α in HH711-30; (c) TNF-α in HH711-60; (d) TNF-α in RV; (e) IL-6 in HH711-10; (f) IL-6 in HH711-30; (g) IL-6 in HH711-60; (h) IL-6 in RV; (i) IL-1β in HH711-10; (j) IL-1β in HH711-30; (k) IL-1β in HH711-60; (l) IL-1β in RV.
Abbreviation: N, Normal; LPS, Lipopolysaccharide; RV, *Rhus vernicifluae* Cortex.
Data are shown as mean ± SD obtained from independent experiments. Statistical analyses were performed using analysis of variance followed by Duncan's multiple range test (each group n=5).
#: compared to Normal; *: $p < 0.05$, ***: $p < 0.01$ compared with cells cultured with LPS.

Inhibitory effect of HH711 on iNOS and COX-2 protein expression

The iNOS and COX-2 protein expression in LPS-stimulated RAW 264.7 cells treated with HH711-10, -30, -60, and RV at various concentrations (1, 10, and 100 μg/ml) is shown in Fig. 8. The iNOS and COX-2 protein expression was significantly increased by approximately 80% and 40%, respectively, in the LPS group, and all HH711 groups showed a reduction in iNOS and COX-2 protein expression. The results showed a dose-dependent inhibitory effect on iNOS and COX-2 protein expression in the low RV concentration group (HH711-10) with a reduction of the inhibitory effect in the high RV concentration groups (100 μg/ml in HH711-60 and RV groups).

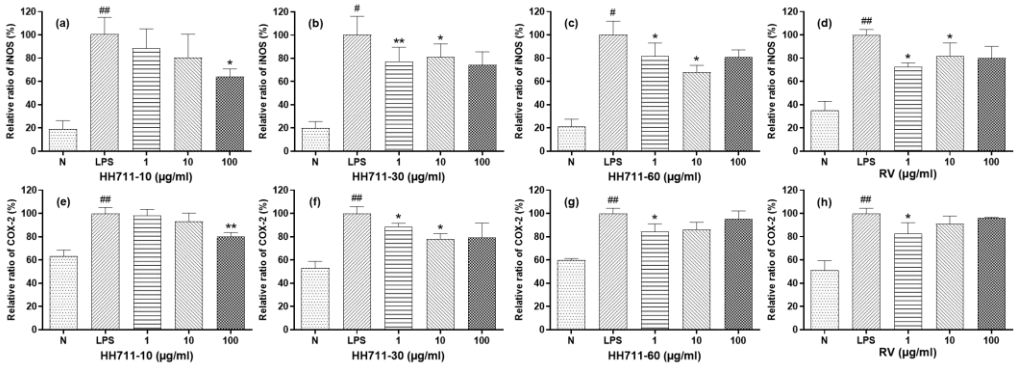


Fig. 8. iNOS and COX-2 protein expression in LPS-stimulated RAW 264.7 cells treated with HH711-10, -30, -60, and RV water extracts at various concentrations (1, 10, and 100 μg/ml). (a) iNOS in HH711-10; (b) iNOS in HH711-30; (c) iNOS in HH711-60; (d) iNOS in RV; (e) COX-2 in HH711-10; (f) COX-2 in HH711-30; (g) COX-2 in HH711-60; (h) COX-2 in RV.
Abbreviation: N, Normal; LPS, Lipopolysaccharide; RV, *Rhus vernicifluae* Cortex.
Data are shown as mean ± SD obtained from independent experiments. Statistical analyses were performed using analysis of variance followed by Duncan's multiple range test (each group n=3).
#: compared with normal; *: $p < 0.05$, **: $p < 0.01$ compared with cells cultured with LPS.

Immunoregulatory effects of HH711

Immune cell growth promotion effect of HH711

The growth of human immune T and B cells in the presence of HH711-10, -30, -60, and RV for 7 days is shown in Fig. 9. In the HH711-10, -30, and -60 groups, T cells exhibited the highest growth rates on day 6, with cell counts of 12.97×10^4, 13.53×10^4, and 10.34×10^4 cells/ml, respectively. Unlike the HH711 group, the RV group showed the highest growth rate at 9.23×10^4 cells/ml on day 5.

For B cells, the highest growth rates were 12.27×10^4, 12.72×10^4, 10.68×10^4, and 9.35×10^4 cell/ml on day 6 in HH711-10, -30, -60, and RV groups, respectively.

Cytokine secretion measurement

The IL-6 and TNF-α secretion by T and B cells after treatment with HH711-10, -30, -60, and RV extracts increased daily (Fig. 10). The highest amounts of TNF-α secretion by T cells were 3.24×10^4, 2.84×10^4, 3.01×10^4, and 3.26×10^4 pg/ml on day 6 in the HH711-10, -30, -60, and RV groups, respectively. In B cells, the highest secretion amounts of TNF-α were 3.24×10^4 (HH711-10) and 2.13×10^4 pg/ml (RV) on day 5, and 2.67×10^4 (HH711-30) and 2.13×10^4 pg/ml

(HH711-60) on day 6. The highest amounts of IL-6 secretion were 2.64×10^4, 2.84×10^4, 2.19×10^4, and 2.14×10^4 pg/ml on day 6 for both T and B cells in the HH711-10 and HH711-30 groups, respectively. The HH711-60 group showed the highest IL-6 secretion amount of 2.61×10^4 pg/ml on day 5 in T cells and 2.22×10^4 pg/ml on day 6 in B cells. In the RV group, T and B cells secreted the highest IL-6 amounts at 1.85×10^4 and 2.21×10^4 pg/ml on day 4, respectively.

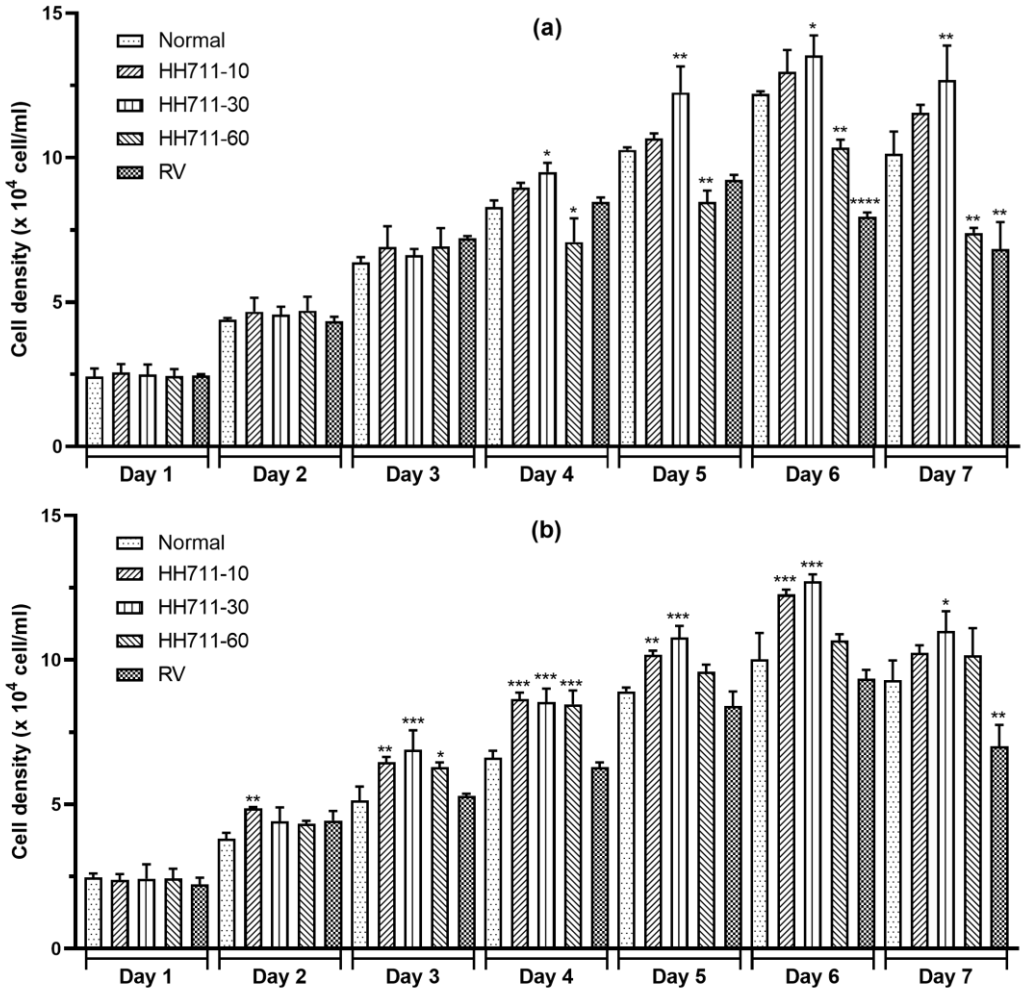
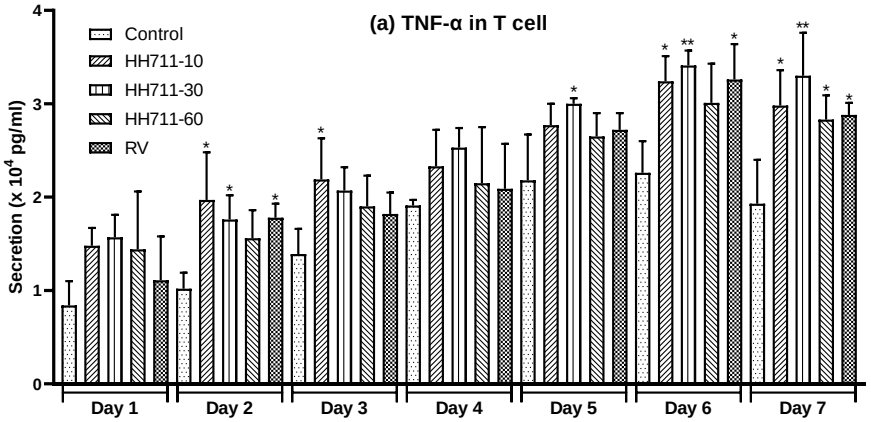
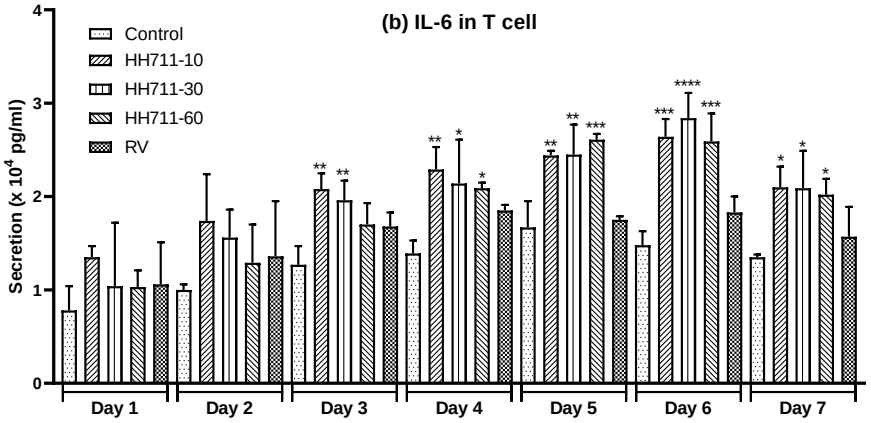
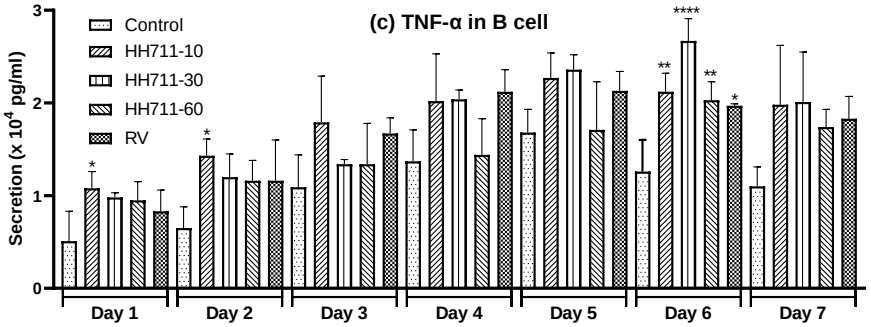


Fig. 9. Growth of human immune (a) T cells and (b) B cells after treatment with HH711-10, -30, -60, and RV extracts by several processes over a period of 7 days.
Abbreviation: RV, *Rhus vernicifluae* Cortex.
Data are shown as means ± SD of independent experiments.
Statistical analyses were performed using analysis of variance followed by Duncan's multiple range test (each group n=3, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$ compared with the normal group).


(a) TNF-α in T cell
Control
HH711-10
HH711-30
HH711-60
RV
Secretion (x 10^4 pg/ml)
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7


(b) IL-6 in T cell
Control
HH711-10
HH711-30
HH711-60
RV
Secretion (x 10^4 pg/ml)
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7


(c) TNF-α in B cell
Control
HH711-10
HH711-30
HH711-60
RV
Secretion (x 10^4 pg/ml)
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7

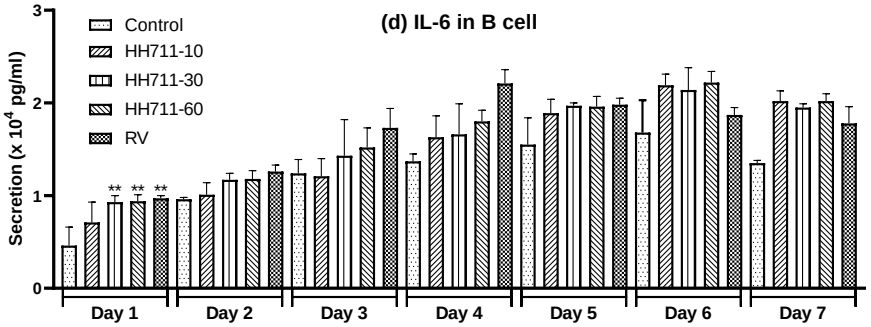

(d) IL-6 in B cell
Control
HH711-10
HH711-30
HH711-60
RV
Secretion (x 10^4 pg/ml)
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7

Fig. 10. Comparison of IL-6 and TNF- α secretion in T and B cells after treatment with HH711-10, -30, -60, and RV extracts from several processes. (a) TNF- α in T cell; (b) IL-6 in T cell; (c) TNF- α in B cell; (d) IL-6 in B cell.
Abbreviation: RV, *Rhus vernicifluae* Cortex.
Data are presented as means ± SD of independent experiments.
Statistical analyses were performed using analysis of variance followed by Duncan's multiple range test (each group n=3, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$ compared with the control group).

Discussion

This study investigated the antioxidant, anti-inflammatory, and immunomodulatory effects of HH711 at various RV concentrations. HH711 showed strong DPPH radical scavenging ability, which increased in proportion to the RV concentration. In H_2O_2-treated RAW 264.7 cells, cell viability decreased in a concentration-dependent manner, with partial cytotoxicity observed at higher RV concentrations. The highest cytoprotective effect in H_2O_2-treated RAW 264.7 cells was observed at 10 μg/ml of HH711-30. The activity of SOD, a reactive oxygen scavenger, increased the most in the HH711-10 and -30 groups, although it decreased at high concentrations of RV (HH711-60 and RV). The cytoprotective effect of HH711 in LPS-induced inflammation was confirmed in 1 to 100 μg/ml of all HH711 treatment groups. In the evaluation of the inhibitory effect on NO production, inhibition increased with higher RV concentrations. HH711 also exhibited a dose-dependent inhibitory effect on proinflammatory cytokines (TNF- α, IL-6, and IL-1β) in all HH711 RV groups. In experiments evaluating the inhibitory effect of HH711 on iNOS and COX-2 protein expression, HH711-30 inhibited the expression of iNOS most effectively, and the expression of COX-2 was effectively suppressed under high concentration of RV in the HH711-10 group. HH711 promoted immune T or B cell growth, with the maximum growth rate observed on day 6. The highest secretion levels of cytokines, such as IL-6 and TNF- α in T and B cells, were observed on day 6. These results suggested that HH711 affects the production of cytokines that regulate the overall immune response.

DPPH radical scavenging activity of HH711

The DPPH assay is considered an easy and standard colorimetric method routinely performed to evaluate the free radical scavenging potential of pure compounds, such as herbs, wheat grain, and edible seed oils (Cheng *et al*., 2006). The DPPH assay relies on the principle that the nitrogen (N) atom in the DPPH radical hydrazyl is unstable and readily accepts hydrogen (H) atoms from a scavenger molecule, such as an antioxidant (Kedare & Singh, 2011). Therefore, when the DPPH radical reacts with an antioxidant with hydrogen-donating ability, the purple DPPH radical is converted into a colourless (DPPH-H) form with a concomitant decrease in absorbance at 515 nm in methanol (Mishra *et al*, 2012). Antioxidant power can be measured in a simple and reproducible manner using

the DPPH assay. Several studies have evaluated the DPPH radical scavenging activity of RVS in a similar dose-dependent manner (Jung *et al.*, 2006a, Lim *et al*., 2001, Rha *et al*., 2014). Following the removal of urushiol, which is known to cause allergies, RVS has been consumed as a food supplement and medicine in Korea. Lee *et al*. (2018) reported that different detoxification methods may induce alterations in the major components of RVS, influencing their activities. For example, RVS extract detoxified by hot air-drying demonstrated the strongest antioxidant activity, as evaluated by the DPPH radical scavenging assay (Lee *et al.*, 2018). Jung *et al*. (2006) revealed that the RVS extract purified by ion exchange chromatography contained high amounts of phenolics and flavonoids and exhibited antioxidant activity, including DPPH radical scavenging activity (Jung *et al*., 2006a). Kim *et al*. (2015) reported the antioxidant properties of a fermented *R. verniciflua* stem bark, where the methanol extract fraction showed high radical scavenging activities, surpassing those of butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) (Kim *et al.*, 2015). Kim *et al*. (1999) showed that chloroform-extracted RVS (C-RVS) exhibited the strongest activity among various solvents, with its free phenolic acid fraction displaying stronger activity than that of BHA, BHT, and δ-tocopherol (Kim *et al*., 1999). However, the extraction time and temperature of RVS can affect DPPH radical scavenging activities. Park *et al*. (2013) demonstrated that the optimum extraction temperature and time for RVS extraction were 140 °C for 3 h, yielding the highest total flavonoid contents (Park *et al.*, 2013). In the present study, the free radical scavenging ability of HH711, as determined by the DPPH assay, was comparable to that of the positive control (ascorbic acid), and this ability increased according to the concentration of RV.

The effect of HH711 on SOD activity

SODs are metalloenzymes present in all aerobic organisms that catalyse the dismutation of superoxide radicals into oxygen and H_2O_2 (Landis & Tower, 2005). SODs form the front line of defence against toxic reduced oxygen species, which are produced as byproducts of many biological oxidations, such as the autoxidation of organic compounds and phagocytosis by macrophages (Younus, 2018). Several studies have reported the therapeutic potential of SOD. SOD plays an important role in several human diseases, including cancer, Alzheimer's disease, cystic fibrosis, rheumatoid arthritis, and diabetes (Younus, 2018). Xanthine oxidase (XO) is an enzyme that plays a role in nucleotide turnover by catalysing the oxidation of xanthine and hypoxanthine, generating superoxide radicals (Fang *et al*., 2002). The superoxide radical produced by the reaction of XO with hypoxanthine converts hypoxanthine to uric acid and reduce nitro blue tetrazolium (NBT) to NBT-diformazan (Madamanchi *et al*., 2005). Therefore, the degree of oxidation can be measured by the colour change of NBT. A previous study evaluating the SOD activity of RVS reported that purified RVS extract significantly recovered catalase activity in H_2O_2-induced RAW 264.7 cells; however it did not significantly increase the concentration of SOD (Jung *et al*., 2006a). Another study indicated that the ethyl acetate fraction of RVS and its

flavonoids protect PC-12 cells against H_2O_2-induced oxidative damage by upregulating SOD activity (Nam *et al.*, 2017). In the present study, groups with low RV concentrations (HH711-10 and HH711-30) triggered high SOD activity at high concentrations (100 µg/ml), whereas groups with high RV concentrations (HH711-60 and RV) triggered SOD activity at low concentrations (10 and 1 µg/ml, respectively).

Inhibitory effects on NO production, as well as iNOS and COX-2 protein expression

NO is a gaseous molecule derived from the amino acid L-arginine by the enzyme NO synthase (NOS) in the human body. It elicits a wide range of physiological and pathophysiological effects, including immune responses, neurotransmission, smooth muscle relaxation, and inhibition of platelet activation (Bruckdorfer, 2005). The three isoforms of NOS are encoded by distinct genes: neuronal NOS, endothelial NOS, and iNOS. The iNOS, originally found in macrophages, exists in various cell types, including epithelial cells, hepatocytes, vascular smooth muscle cells and fibroblasts (Davis *et al.*, 2001). It is not expressed in resting cells; however, compounds, such as pro-inflammatory cytokines (including TNF-α and IL-1) and endotoxins, induce iNOS expression (Garcia & Stein, 2006, Król & Kepinska, 2020). Excessive NO production results in various pathological conditions, including arthritis, colitis, and hypotension, as observed in septic shock (Davis *et al*., 2001, Sharma *et al.*, 2007). Another inflammatory factor, COX-2, is induced during inflammation, resulting in pain, swelling, and stiffness (Rajakariar *et al*., 2006). The COX-2 gene is particularly responsive to mediators of inflammation; for instance, TNF-α, IL-1α, IL-1β, and LPS induce COX-2 gene expression and subsequent prostaglandins (PGs) production (Morita, 2002). Membrane-bound PGE2 (mPGE2) synthase is also induced by these cytokines in inflammation-related cell types, including endothelial cells, osteoblasts, and monocytes/macrophages (Crofford, 1997, Martínez-Colón & Moore, 2018). The large amounts of PGE2 generated at the site of inflammation, via COX-2 and mPGE2 synthase, may be closely related to inflammation progression (Morita, 2002). Several studies have shown the anti-inflammatory effects of RVS by evaluating NO production, as well as iNOS and COX-2 mRNA expression, in LPS-stimulated RAW 264.7 cells (Choi *et al*., 2014, Jung *et al*., 2007, Ko *et al*., 2020, Lee *et al*., 2010, Oh *et al*., 2007). Ko *et al*. (2020) demonstrated that RVS suppressed iNOS and COX-2 gene expression by directly inhibiting the activation of nuclear factor-kappa B (NF-κB) and phosphorylation of the mitogen-activated protein kinase pathway (Ko *et al*., 2020). Jung *et al*. (2007) indicated that phenolic-rich fractions from RVS inhibited signalling pathways such as NF-κB and c-Jun N-terminal kinase 1/2, thereby suppressing pro-inflammatory mediators (e.g., NO, PGE2, and TNF-α) (Jung *et al*., 2007). Lee *et al*. (2010) suggested the inhibitory effects of sulfuretin, one of the major flavonoids in RVS, on LPS-induced NO, PGE2, TNF-α, and IL-1β production derived from sulfuretin-induced oxygenase-1 expression (Lee *et al*., 2010). The present study demonstrated that HH711 and RV effectively reduced

the production of LPS-induced inflammatory factors (NO, iNOS, and COX-2 protein expression) and inflammatory cytokines (TNF- α, IL-6, and IL-1β), and these effects were concentration-dependent. Compared to the findings of Choi *et al*. (2016), the present study showed an inferior inhibitory effect on NO production and similar inhibitory effects on iNOS and COX-2 expression: fermented *R. verniciflua* stem bark extract from the Choi *et al*. (2016) study showed more than 75% inhibition of NO production, 25% decrease in iNOS, and 15% decrease in COX-2 mRNA levels compared to those in LPS-treated cells (Choi *et al.*, 2016).

Immunoregulatory effects of HH711

B lymphocytes are a unique cell type in the immune system and are widely recognised for their potential to mediate human immunity by producing different antibody isotypes related to complement fixation and opsonisation (Bao & Cao, 2014). Nevertheless, increasing evidence indicates that B cell subsets may have non-classical, antibody-independent potential to amplify or suppress immune responses (Gregersen & Jayne, 2012, Mariño & Grey, 2012). These B cells can release various cytokines, including IL-6 and TNF-α, as cytokine-producing B cells (Harris *et al.*, 2000). Moreover, they modulate immune responses by promoting T-helper (Th) cell response, increasing macrophage activation, and sustaining antibody production (Bao & Cao, 2014).

When the human immune system encounters an antigen, accessory cells take up the antigen, decompose it into peptides, and release them in relation to major histocompatibility complex (MHC) molecules to T lymphocytes (Kedzierska & Koutsakos, 2020). Interactions between T-cell receptors and antigen-MHC complexes lead to the activation of CD4(+) helper T cells or CD8(+) cytotoxic T cells (Romagnani, 2000). Among murine CD4(+) T cells, there are two main subsets, Th1 and Th2, and the differential activation of the Th1 or Th2 subsets is an essential parameter implicated in the pathological outcomes of many inflammatory disorders caused by bacteria and viruses (Moss *et al*., 2004). Cytokines also play a crucial role in the selective activation of these subsets; for example, bacterial stimuli activate macrophages in the innate immune response to generate IL-12 and drive Th1 development and cell-mediated immunity (Dong, 2021).

IL-6 and TNF-α are considered major proinflammatory cytokines important in the protection from pathogens during infection. Although IL-6 was initially characterised as a growth factor for B cells and plays a significant role as a modulator of $CD4^+$ T cell effector function, thereby shaping the immune response and inflammation (Dienz & Rincon, 2009). Numerous experimental studies have shown the strong antitumour activity of the TNF superfamily by investigating the effectiveness of agonist antibodies against TNF receptors in tumour models (Croft, 2009). Members of the tumour necrosis factor receptor superfamily participate prominently in B cell maturation and function and are closely associated with increased effector activity of $CD4^+$ and/or $CD8^+$ T cells (Croft, 2009, Rickert *et al*., 2011). Taken together, B and T cells play pathological and

protective roles in inflammation, autoimmunity, and allergy through the production of a wide variety of cytokines. In this study, HH711 showed the effect of immune T or B cell growth promotion and the production of cytokines of IL-6 and TNF-α. In a previous study investigating T and B cell growth promotion in a medicinal plant, *Rubus coreanus* (RC) showed 2.2 times the growth effect of T cells and 2.4 times in B cells after 6 days (Chung & Lim, 2012). In the present study, HH711-30 had a much higher growth effect on T cells (5.4 times) and B cells (5.3 times) after 6 days. Compared with the maximum production of IL-6 and TNF-α in a previous study on RC (2.4 and 2.6 times the increase of IL-6 and TNF-α, respectively) (Chung & Lim, 2012), our study showed a similar degree of increase in IL-6 in T cells (2.7 times on the 6^{th} day of HH711-30 treatment), IL-6 in B cells (2.4 times on the 6^{th} day of HH711-60 treatment), TNF-α in T cells (2.2 times on the 6^{th} day of HH711-30 treatment), and TNF-α in B cells (2.7 times on the 6^{th} day of HH711-30 treatment).

Conclusion

Taken together, the results of our study suggest that HH711 displays RV concentration-dependent antioxidant, anti-inflammatory, and immunomodulatory activities comparable to those of a general synthetic antioxidant. Furthermore, it can be considered a good candidate for the development of anti-inflammatory and immuno-activator agents in the medical industry.

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References

Bao, Y, Cao, X. (2014). The immune potential and immunopathology of cytokine-producing B cell subsets: a comprehensive review. *Journal of Autoimmunity* **55:** 10-23.

Bruckdorfer, R. (2005). The basics about nitric oxide. *Molecular Aspects of Medicine* **26:** 3-31.

Brüne, B, Zhou, J, von Knethen, A. (2003). Nitric oxide, oxidative stress, and apoptosis. *Kidney International. Supplement***:** S22-24.

Cheng, Z, Moore, J, Yu, L. (2006). High-throughput relative DPPH radical scavenging capacity assay. *Journal of Agricultural and Food Chemistry* **54:** 7429-7436.

Choi, E, Suk, KT, Choi, H, Kim, M, Kwon, Y, Kim, M. (2016). Anti-inflammatory activities of fermented Rhus verniciflua stem bark extract and its growth inhibitory effect on Helicobacter pylori. *Korean Journal of Food Science and Technology* **48:** 502-507.

Choi, G, Kim, B. (2010). Experimental Study on the Antioxidant and Antimicrobial Properties of Dioscoreae Rhizoma. *The Journal of Korean Oriental Internal Medicine* **31:** 290-297.

Choi, HS, Seo, HS, Kim, SR, Choi, YK, Jang, BH, Shin, YC, Ko, SG. (2014). Anti-inflammatory and anti-proliferative effects of Rhus verniciflua Stokes in RAW264.7 cells. *Molecular Medicine Reports* **9:** 311-315.

Choi, W, Jung, H, Kim, K, Lee, S, Yoon, S, Park, J, Kim, S, Cheon, S, Eo, W, Lee, S. (2012). Rhus verniciflua stokes against advanced cancer: a perspective from the Korean Integrative Cancer Center. *Journal of Biomedicine and Biotechnology* **2012:** 874276.

Chung, MG, Lim, JD. (2012). Antioxidant, Anticancer and Immune Activation of Anthocyanin Fraction from Rubus coreanus Miquel fruits (Bokbunja). *Korean Journal of Medicinal Crop Science* **20:** 259-269.

Crofford, LJ. (1997). COX-1 and COX-2 tissue expression: implications and predictions. *Journal of Rheumatology. Supplement* **49:** 15-19.

Croft, M. (2009). The role of TNF superfamily members in T-cell function and diseases. *Nature Reviews: Immunology* **9:** 271-285.

Davis, KL, Martin, E, Turko, IV, Murad, F. (2001). Novel effects of nitric oxide. *Annual Review of Pharmacology and Toxicology* **41:** 203-236.

Del Río, LA. (2015). ROS and RNS in plant physiology: an overview. *Journal of Experimental Bbotany* **66:** 2827-2837.

Desai, A, Vyas, T, Amiji, M. (2008). Cytotoxicity and apoptosis enhancement in brain tumor cells upon coadministration of paclitaxel and ceramide in nanoemulsion formulations. *Journal of Pharmaceutical Sciences* **97:** 2745-2756.

Dienz, O, Rincon, M. (2009). The effects of IL-6 on CD4 T cell responses. *Clinical Immunology* **130:** 27-33.

Dong, C. (2021). Cytokine Regulation and Function in T Cells. *Annual Review of Immunology* **39:** 51-76.

Fang, YZ, Yang, S, Wu, G. (2002). Free radicals, antioxidants, and nutrition. *Nutrition* **18:** 872-879.

Fujiwara, N, Kobayashi, K. (2005). Macrophages in inflammation. *Current Drug Targets: Inflammation and Allergy* **4:** 281-286.

Garcia, X, Stein, F. (2006). Nitric oxide. *Seminars in Pediatric Infectious Diseases* **17:** 55-57.

Gregersen, JW, Jayne, DR. (2012). B-cell depletion in the treatment of lupus nephritis. *Nature reviews. Nephrology* **8:** 505-514.

Gyamfi, MA, Yonamine, M, Aniya, Y. (1999). Free-radical scavenging action of medicinal herbs from Ghana: Thonningia sanguinea on experimentally-induced liver injuries. *General Pharmacology* **32:** 661-667.

Harris, DP, Haynes, L, Sayles, PC, Duso, DK, Eaton, SM, Lepak, NM, Johnson, LL, Swain, SL, Lund, FE. (2000). Reciprocal regulation of polarized cytokine production by effector B and T cells. *Nature Immunology* **1:** 475-482.

Jung, CH, Jun, CY, Lee, S, Park, CH, Cho, K, Ko, SG. (2006a). Rhus verniciflua stokes extract: radical scavenging activities and protective effects on H2O2-induced cytotoxicity in macrophage RAW 264.7 cell lines. *Biological and Pharmaceutical Bulletin* **29:** 1603-1607.

Jung, CH, Kim, JH, Hong, MH, Seog, HM, Oh, SH, Lee, PJ, Kim, GJ, Kim, HM, Um, JY, Ko, SG. (2007)**.** Phenolic-rich fraction from Rhus verniciflua Stokes (RVS) suppress inflammatory response via NF-kappaB and JNK pathway in lipopolysaccharide-induced RAW 264.7 macrophages. *Journal of Ethnopharmacology* **110:** 490-497.

Jung, CH, Zhou, S, Ding, GX, Kim, JH, Hong, MH, Shin, YC, Kim, GJ, Ko, SG. (2006b)**.** Antihyperglycemic activity of herb extracts on streptozotocin-induced diabetic rats. *Bioscience, Biotechnology, and Biochemistry* **70:** 2556-2559.

Kedare, SB, Singh, RP. (2011)**.** Genesis and development of DPPH method of antioxidant assay. *Journal of Food Science and Technology* **48:** 412-422.

Kedzierska, K, Koutsakos, M. (2020)**.** The ABC of Major Histocompatibility Complexes and T Cell Receptors in Health and Disease. *Viral Immunology* **33:** 160-178.

Khansari, N, Shakiba, Y, Mahmoudi, M. (2009)**.** Chronic inflammation and oxidative stress as a major cause of age-related diseases and cancer. *Recent Patents on Inflammation and Allergy Drug Discovery* **3:** 73-80.

Kim, IW, Shin, DH, Choi, U. (1999)**.** Isolation of antioxidative compounds from the bark of phus verniciflua stokes screened from some Chinese medicinal plants. *Korean Journal of Food Science and Technology* **31:** 855-863.

Kim, KA, Yi, HS, Yun, HJ, Park, SD. (2009)**.** Anti-oxidative and Anti-inflammatory Effect of Fractionated Extracts of Cynomorium Songaricum. *Journal of Physiology & Pathology in Korean Medicine* **23:** 1320-1331.

Kim, M-O, Yang, J, Kwon, Y, Kim, M. (2015)**.** Antioxidant and anticancer effects of fermented Rhus verniciflua stem bark extracts in HCT-116 cells. *ScienceAsia* **41:** 322.

Kitts, DD, Lim, KT. (2001)**.** Antitumorigenic and cytotoxic properties of an ethanol extract derived from Rhus verniciflua Stokes (RVS). *Journal of Toxicology and Environmental Health. Part A* **64:** 357-371.

Ko, H, Jang, E, Kim, Y. (2020)**.** Rhus Verniciflua Stokes Extract Suppresses Expression of Metalloproteinases, iNOS and COX-2 in THP-1 Cells Via Inhibiting NF-κB and MAPK Phosphorylation. *Journal of Korean Medicine* **41:** 12-26.

Król, M, Kepinska, M. (2020)**.** Human Nitric Oxide Synthase-Its Functions, Polymorphisms, and Inhibitors in the Context of Inflammation, Diabetes and Cardiovascular Diseases. *International Journal of Molecular Sciences* **22:** 56.

Landis, GN, Tower, J. (2005)**.** Superoxide dismutase evolution and life span regulation. *Mechanisms of Ageing and Development* **126:** 365-379.

Lee, DS, Jeong, GS, Li, B, Park, H, Kim, YC. (2010)**.** Anti-inflammatory effects of sulfuretin from Rhus verniciflua Stokes via the induction of heme oxygenase-1 expression in murine macrophages. *International Immunopharmacology* **10:** 850-858.

Lee, JH, Lee, HJ, Lee, HJ, Choi, WC, Yoon, SW, Ko, SG, Ahn, KS, Choi, SH, Ahn, KS, Lieske, JC, Kim, SH. (2009)**.** Rhus verniciflua Stokes prevents

cisplatin-induced cytotoxicity and reactive oxygen species production in MDCK-I renal cells and intact mice. *Phytomedicine* **16:** 188-197.

Lee, SO, Kim, SJ, Kim, JS, Ji, H, Lee, EO, Lee, HJ. (2018)**.** Comparison of the main components and bioactivity of Rhus verniciflua Stokes extracts by different detoxification processing methods. *BMC Complementary and Alternative Medicine* **18:** 242.

Lim, KT, Hu, C, Kitts, DD. (2001)**.** Antioxidant activity of a Rhus verniciflua Stokes ethanol extract. *Food and Chemical Toxicology* **39:** 229-237.

Madamanchi, NR, Hakim, ZS, Runge, MS. (2005)**.** Oxidative stress in atherogenesis and arterial thrombosis: the disconnect between cellular studies and clinical outcomes. *Journal of Thrombosis and Haemostasis* **3:** 254-267.

Maldonado, RF, Sá-Correia, I, Valvano, MA. (2016)**.** Lipopolysaccharide modification in Gram-negative bacteria during chronic infection. *FEMS Microbiology Reviews* **40:** 480-493.

Mariño, E, Grey, ST. (2012)**.** B cells as effectors and regulators of autoimmunity. *Autoimmunity* **45:** 377-387.

Martínez-Colón, GJ, Moore, BB. (2018)**.** Prostaglandin E2 as a Regulator of Immunity to Pathogens. *Pharmacology & Therapeutics* **185:** 135-146.

Mishra, K, Ojha, H, Chaudhury, NK. (2012)**.** Estimation of antiradical properties of antioxidants using DPPH assay: A critical review and results. *Food Chemistry* **130:** 1036-1043.

Morita, I. (2002)**.** Distinct functions of COX-1 and COX-2. *Prostaglandins Other Lipid Mediat* **68-69:** 165-175.

Moss, RB, Moll, T, El-Kalay, M, Kohne, C, Soo Hoo, W, Encinas, J, Carlo, DJ. (2004)**.** Th1/Th2 cells in inflammatory disease states: therapeutic implications. *Expert Opinion on Biological Therapy* **4:** 1887-1896.

Nam, TG, Lee, BH, Choi, HK, Mansur, AR, Lee, SG, Kim, DO. (2017)**.** Rhus verniciflua Stokes Extract and Its Flavonoids Protect PC-12 Cells against H(2)O(2)-Induced Cytotoxicity. *Journal of Microbiology and Biotechnology* **27:** 1090-1097.

Oh, PS, Lee, SJ, Lim, KT. (2007)**.** Glycoprotein isolated from Rhus verniciflua Stokes inhibits inflammation-related protein and nitric oxide production in LPS-stimulated RAW 264.7 cells. *Biological and Pharmaceutical Bulletin* **30:** 111-116.

Park, H, Yoon, G, Lee, S, Jang, G, Kim, M, Meishan, L, Lee, J, Jeong, H. (2013)**.** Effects of Extraction Temperature and Time on Antioxidant Activities of Rhus verniciflua Extract. *Journal of the Korean Society of Food Science and Nutrition* **42**.

Rajakariar, R, Yaqoob, MM, Gilroy, DW. (2006)**.** COX-2 in inflammation and resolution. *Molecular Interventions* **6:** 199-207.

Rha, Y, Choi, M, Park, S. (2014)**.** Antioxidant and Anti-adipogenic Effects of Fermented Rhus verniciflua. *The Korean Journal of Culinary Research* **20:** 137-147.

Rickert, RC, Jellusova, J, Miletic, AV. (2011)**.** Signaling by the tumor necrosis factor receptor superfamily in B-cell biology and disease. *Immunological Reviews* **244:** 115-133.

Romagnani, S. (2000)**.** T-cell subsets (Th1 versus Th2). *Annals of Allergy, Asthma, and Immunology* **85:** 9-18; quiz 18, 21.

Sapkota, K, Kim, S, Kim, MK, Kim, SJ. (2010)**.** A detoxified extract of Rhus verniciflua Stokes upregulated the expression of BDNF and GDNF in the rat brain and the human dopaminergic cell line SH-SY5Y. *Bioscience, Biotechnology, and Biochemistry* **74:** 1997-2004.

Sharma, JN, Al-Omran, A, Parvathy, SS. (2007)**.** Role of nitric oxide in inflammatory diseases. *Inflammopharmacology* **15:** 252-259.

Suk, KT, Baik, SK, Kim, HS, Park, SM, Paeng, KJ, Uh, Y, Jang, IH, Cho, MY, Choi, EH, Kim, MJ, Ham, YL. (2011)**.** Antibacterial effects of the urushiol component in the sap of the lacquer tree (Rhus verniciflua Stokes) on Helicobacter pylori. *Helicobacter* **16:** 434-443.

Wang, S, Chen, Y, He, D, He, L, Yang, Y, Chen, J, Wang, X. (2007)**.** Inhibition of vascular smooth muscle cell proliferation by serum from rats treated orally with Gastrodia and Uncaria decoction, a traditional Chinese formulation. *Journal of Ethnopharmacology* **114:** 458-462.

Wynn, TA, Chawla, A, Pollard, JW. (2013)**.** Macrophage biology in development, homeostasis and disease. *Nature* **496:** 445-455.

Yanishlieva, NV, Marinova, E, Pokorný, J. (2006)**.** Natural antioxidants from herbs and spices. *European Journal of Lipid Science and Technology* **108:** 776-793.

Younus, H. (2018)**.** Therapeutic potentials of superoxide dismutase. *International Journal of Health Sciences* **12:** 88-93.

Zarkovic, N. (2020)**.** Roles and Functions of ROS and RNS in Cellular Physiology and Pathology. *Cells* **9**.