

The Immunomodulatory Activity of Mori folium, the Leaf of *Morus alba* L., in RAW 264.7 Macrophages In Vitro

ORIGINAL
ARTICLE

Da Hye Kwon¹, Ji Min Cheon², Eun-Ok Choi^{1,3}, Jin Woo Jeong^{1,3}, Ki Won Lee⁴, Ki Young Kim⁴, Sung Goo Kim⁴, Suhkmann Kim⁵, Su Hyun Hong³, Cheol Park⁶, Hye-Jin Hwang², Yung Hyun Choi^{1,3}

¹Anti-Aging Research Center and ²Department of Food and Nutrition, Dongeui University, ³Department of Biochemistry, Dongeui University College of Korean Medicine, ⁴Bio-Port Korea Inc., MarineBio-industry Development Center, ⁵Department of Chemistry, Pusan National University, ⁶Department of Molecular Biology, Dongeui University, Busan, Korea

Background: Immunoregulatory elements have emerged as useful immunotherapeutic agents against cancer. In traditional medicine, Mori folium, the leaf of *Morus alba* L. (Moraceae), has been used for various medicinal purposes; however, the immunomodulatory effects have not been fully identified. We evaluated the immunoenhancing potential of water extract of Mori folium (WEMF) in murine RAW264.7 macrophages.

Methods: RAW264.7 cells were treated with WEMF for 24 hours and cell viability was detected by an MTT method. Nitric oxide (NO) levels in the culture supernatants were assayed using Griess reagent. The productions of prostaglandin E₂ (PGE₂) and immune-related cytokines was measured using ELISA detection kits. The mRNA and protein expression levels of Inducible NO synthase, COX-2, and cytokines were assayed by reverse transcription-PCR and Western blotting, respectively. The effect of WEMF on phagocytic activity was measured using a Phagocytosis Assay Kit.

Results: WEMF significantly stimulated the production of NO and PGE₂ as immune response parameters at noncytotoxic concentrations, which was associated with the increased expression of inducible NO synthase and COX-2. The release and expression of cytokines, such as TNF- α , interleukin (IL)-1 β , IL-6, and IL-10, were also significantly increased in response to treatment with WEMF. Moreover, WEMF promoted the macrophagic differentiation of RAW264.7 cells and the resulting phagocytosis activity.

Conclusions: WEMF has the potential to modulate the immune function by regulating immunological parameters. Further studies are needed to identify the active compounds and to support the use of WEMF as an immune stimulant.

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Key Words: Mori folium, Macrophages, Immune response, Phagocytosis

INTRODUCTION

Among various immune system-related cells, macrophages are versatile cells that exist in almost all tissues and play important roles in immune responses.^{1,2} In particular, macrophages are recruited in infection sites where they are activated to perform many functions through increasing phagocytosis. This process is the first line of defense against microbial and parasitic infections and in removing senescent and dead cells, immune mediator

secretion, and antigen presentation.^{3,4} Activated macrophages also prevent the invasion of pathogens by secreting inflammatory mediators, including nitric oxide (NO), prostaglandin E₂ (PGE₂), and cytokines, such as TNF- α and interleukins (ILs).⁵⁻⁹

Recent data showed that natural compounds have been widely and safely consumed over centuries, and many studies have indicated that most natural compounds have a wide range of diverse biological activities but few side effects.^{10,11} These natural products could affect the development and progression of cancer

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Correspondence to: Yung Hyun Choi

Department of Biochemistry, Dongeui University College of Korean Medicine, 52-57 Yangjeong-ro, Busanjin-gu, Busan 47227, Korea

Tel: +82-51-850-7413, Fax: +82-51-853-4036, E-mail: choiyh@deu.ac.kr, ORCID: Yung Hyun Choi, <http://orcid.org/0000-0002-1454-3124>

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in various ways, such as inhibiting tumor cell growth, angiogenesis, and metastasis, immunomodulating, and enhancing effects of chemotherapeutic drugs. Therefore, traditional herbal medicinal resources have been investigated extensively for their immunomodulatory potential as adjuvants for therapeutic agents in immune-related functions.¹²⁻¹⁴ *Morus alba* L., white mulberry, is a deciduous tree that belongs to the Moraceae family, which is widely distributed in Asia. Mori folium, the leaf of *M. alba* L., has been used worldwide in traditional medicine from ancient times to the present for the treatment of various diseases.^{15,16} Previous studies have indicated that Mori folium possesses various pharmacological properties, including anti-microbial,¹⁷ antioxidant,^{18,19} anti-tumor,²⁰ anti-obesity,^{21,22} anti-hypotensive,²³ neuroprotective,²⁴ and anti-diabetic actions.²⁵ In addition, previous studies, as well as our recent data,²⁶ indicated that the extracts and components of Mori folium had anti-inflammatory effects in different experimental models.²⁷⁻²⁹ However, the effects and molecular mechanisms involved in such immunostimulatory actions have remained elusive. Therefore, in this study, as a part of our on-going research to find novel immunostimulatory active substances in traditional medicinal resources, we examined the immunostimulatory properties of water extract of Mori folium (WEMF) in RAW 264.7 mouse monocyte macrophages.

MATERIALS AND METHODS

1. Preparation of water extract of Mori folium, reagents, and antibodies

The dried leaves of *M. alba* were obtained from Bio-Port Korea (Busan, Korea), and WEMF was prepared as previously described.²⁶ WEMF was dissolved in a 100 mg/mL concentration with distilled water, and the stock solution was then diluted with a culture medium to the desired concentration prior to use. Dulbecco's modified Eagle's medium (DMEM) and FBS were obtained from WelGENE (Daegu, Korea). Lipopolysaccharide (LPS, *Escherichia coli* serotype 055:B5), MTT, Griess reagent, and 4,6-diamidino-2-phenylindole were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA). Inducible NO synthase (iNOS), COX-2, TNF- α , IL-1 β , IL-6, IL-10, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) oligonucleotide primers, and moloney-murine leukemia virus (M-MLV) reverse transcriptase kit were purchased from BioNEER (Daejeon, Korea). The ELISA detection kits for PGE₂, TNF- α , IL-1 β , IL-6, and IL-10 were obtained from R&D Systems (Minneapolis, MN, USA). TRIzol reagent for the isolation of RNA was purchased from Invitrogen

Life Technologies (Carlsbad, CA, USA). The primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Cell Signaling Technology (Boston, MA, USA). Peroxidase-labeled donkey, anti-rabbit, and sheep anti-mouse immunoglobulin and an enhanced chemiluminescence (ECL) detection system were obtained from Amersham (Arlington Heights, IL, USA). All other chemicals were purchased from Sigma-Aldrich Chemical.

2. Cell culture, measurement of cell viability, and morphological analysis

The RAW 264.7 macrophage cell line was obtained from the Korean Cell Line Bank (Seoul, Korea) and cultured at 37°C in 5% CO₂ containing DMEM supplemented by 10% FBS, 100 U/mL of penicillin, and 100 mg/mL of streptomycin. Cell viability studies were performed using a colorimetric MTT assay. In brief, the RAW 264.7 cells were seeded at a density of 1×10^4 cells/well in a 96 well-plate and then incubated at 37°C for 24 hours. The cells were treated with various concentrations of WEMF or 0.5 ng/mL LPS for 24 hours. After the medium was discarded, an MTT solution (5 mg/mL in PBS) was added to each well and incubated for another 3 hours at 37°C. The medium was removed and dimethyl sulfoxide was added to dissolve the formazan dye. The optical density was read at 560 nm using a microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) to determine the cell viability.³⁰ To assess the effect of WEMF on cellular morphology, the cells were photographed under an inverted microscope (Carl Zeiss, Oberkochen, Germany).

3. Measurement of nitric oxide production in RAW 264.7 macrophages

The accumulation of NO in the culture supernatants was assayed using Griess reagent. In brief, the cells were treated with various concentrations of WEMF or 0.5 ng/mL LPS for 24 hours. The supernatant was then collected and mixed with the same volume of Griess reagent for 10 minutes at room temperature in the dark. The absorbance was measured at 540 nm on a microplate reader, and NO concentrations were calculated by referencing a standard curve generated by known concentrations of sodium nitrite.³¹ A fresh culture medium was used as the blank in all experiments.

4. Measurement of prostaglandin E₂, TNF- α , and interleukin-1 β production in RAW 264.7 macrophages

To measure the production of PGE₂, TNF- α , IL-1 β , and IL-10,

the cells were cultured under the same conditions as in the NO measurement assay. The levels of PGE₂, TNF- α , IL-1 β , and IL-10 in cultured media were determined by selective ELISA kits according to the manufacturer's instructions.³²

5. RNA isolation and reverse transcription-PCR

Total RNA was isolated from the cultured cells using the TRIzol reagent according to the manufacturer's instructions and then reversely transcribed using the M-MLV reverse transcriptase kit to produce cDNAs. cDNAs were amplified by PCR using the desired primers. The PCR reaction was initiated at 94°C for 2 minutes, followed by 31 cycles at 94°C for 30 seconds, annealing temperature for 30 seconds, 72°C for 30 seconds, and the final extension step at 72°C for 5 minutes. Following amplification, the PCR products were separated by 1.5% agarose gel electrophoresis, stained with ethidium bromide, and visualized by ultraviolet illumination. In a parallel experiment, GAPDH was used as an internal control.

6. Protein extraction and Western blot analysis

The cell pellets were collected and resuspended in extraction lysis buffer (25 mM Tris-Cl [pH 7.5], 250 mM NaCl, 5 mM EDTA, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, and 5 mM dithiothreitol) for 30 minutes at 4°C. The protein concentration in the cell lysate was determined using a DC Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein from each sample were separated by SDS-PAGE at 90 V for 2 hours and transferred onto polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). The membranes were then incubated overnight at 4°C with the corresponding primary antibodies, followed by incubation with the appropriate secondary antibodies conjugated to horseradish peroxidase at room temperature for 2 hours. An ECL detection system was used to monitor the immunoreactive bands.

7. Phagocytosis assay

The phagocytic ability of RAW 264.7 cells was detected using a Phagocytosis Assay Kit (IgG-FITC) (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. Briefly, RAW 264.7 cells were plated on a 4-well chamber slide and cultured overnight at 37°C to allow adherence to the plate. After treatment with WEMF for 24 hours, Latex Beads-Rabbit IgG-FITC Complex was added directly to the culture medium at a dilution of 1:200 and incubated at 37°C for 2 hours. The cells were then gently washed twice using a supplied assay buffer. After two washes with PBS, the cells were visualized at $\times 20$ magnification

using a fluorescence microscope (Carl Zeiss). The extent of phagocytosis was determined by measuring the absorbance at 620 nm.

8. Statistical analysis

All data are presented as mean \pm SD. Significant differences among groups were determined using the unpaired Student's *t*-test. A value of $P < 0.05$ was accepted as statistically significant. All figures reflect the data obtained from at least three independent experiments.

RESULTS

1. Water extract of Mori folium did not affect RAW 264.7 cell viability

LPS, also known as lipoglycans and endotoxins, is present in the outer cell membrane of gram negative bacteria and can activate macrophages through the activation of cellular signaling

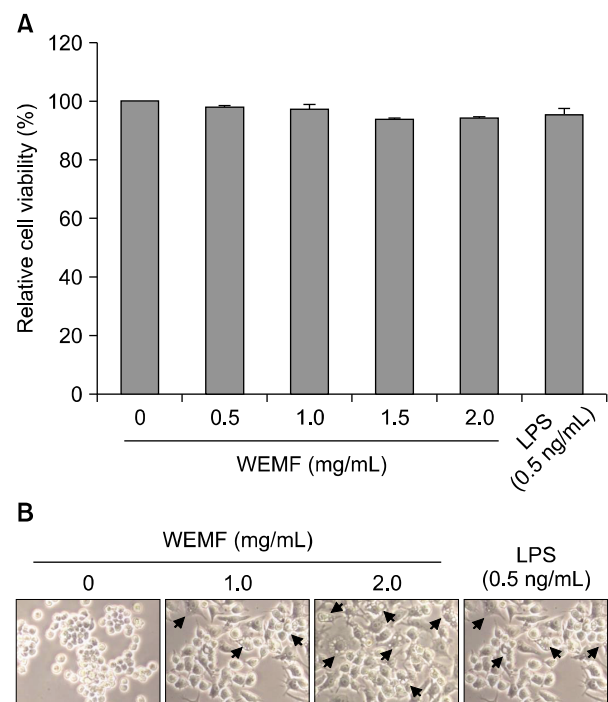


Figure 1. Effect of water extract of Mori folium on the viability and morphology of RAW 264.7 macrophages. The cells were treated with various concentrations of WEMF or 0.5 ng/mL LPS for 24 hours. (A) Cell viability was assessed with an MTT reduction assay, and the results were expressed as the mean \pm SD of three independent experiments. (B) Representative pictures of morphological changes of RAW 264.7 cells are shown. Arrows indicate activated macrophages (200 \times original magnification). WEMF, water extract of Mori folium; LPS, lipopolysaccharide.

pathways, thereby stimulating the transcription of genes, such as iNOS, COX-2, and macrophage-related cytokines.^{33,34} Thus, we used LPS as a positive substance to activate the macrophages. To determine the optimal concentration of WEMF, the RAW 264.7 cells were treated with various concentrations of WEMF for 24 hours. The MTT assay showed that up to 2.0 mg/mL WEMF and 0.5 ng/mL LPS were not cytotoxic (Fig. 1A). However, the WEMF- and LPS-treated RAW 264.7 cells increased in size, and their surfaces became crude and rough, which indicated that WEMF might activate RAW 264.7 cells (Fig. 1B). We therefore selected 2.0 mg/mL WEMF as the maximum concentration in further experiments with RAW 264.7 cells.

2. Water extract of Mori folium increased the production of nitric oxide and prostaglandin E₂ in RAW 264.7 macrophages

RAW 264.7 cells were treated with the indicated concentrations of WEMF for 24 hours in order to assess the effect of WEMF on the release of NO and PGE₂. Following stimulation with WEMF, the levels of NO and PGE₂ in the culture supernatants were determined by Griess reaction assay and ELISA, respectively. As

demonstrated in Figure 2A and 2B, the WEMF treatment significantly increased NO and PGE₂ production compared to the unstimulated control cells. LPS, as a positive control, also induced the secretion of NO and of PGE₂.

3. Water extract of Mori folium induced inducible nitric oxide synthase and COX-2 expressions at the mRNA and protein levels in RAW 264.7 macrophages

We subsequently investigated whether the inducible effects of WEMF on NO and PGE₂ production were associated with mRNA and protein expression of their synthetic enzymes, iNOS and COX-2, by applying RT-PCR and Western blot analysis, respectively. As shown in Fig. 2C and 2D, WEMF effectively increased the mRNA and protein expression of iNOS and COX-2, as did LPS, suggesting that WEMF increased NO and PGE₂ production by inducing the expression of their encoding genes.

4. Water extract of Mori folium increased the secretion and expression of cytokines in RAW 264.7 macrophages

To determine the effect of WEMF on the production of

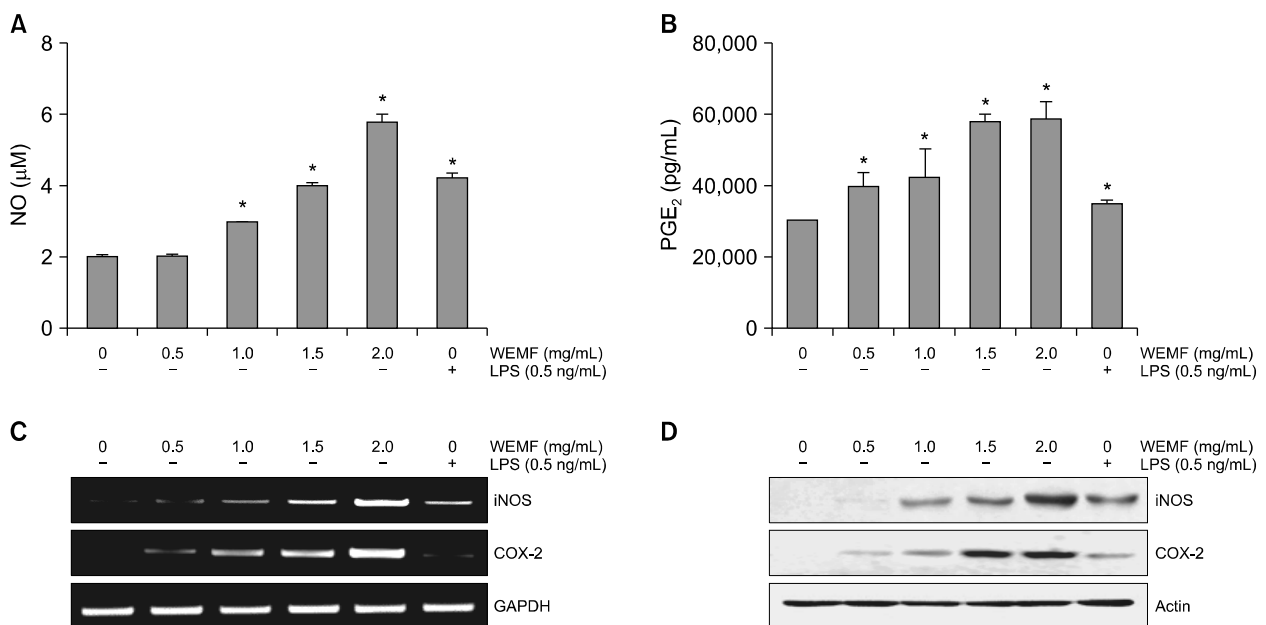


Figure 2. Induction of nitric oxide and prostaglandin E₂ production by water extract of Mori folium in RAW 264.7 macrophages. The cells were treated with the indicated concentrations of water extract of Mori folium (WEMF) or 0.5 ng/mL LPS for 24 hours. The levels of (A) NO and (B) PGE₂ in culture media were measured by Griess assay and a commercial ELISA kit, respectively. Each value indicates the mean \pm SD from three independent experiments (* $P < 0.05$ compared to the control). (C) The total RNAs were prepared for RT-PCR analysis of the iNOS and COX-2 mRNA expression using the indicated primers in Materials and Methods. (D) Cell lysates were prepared for Western blot analysis with antibodies specific for murine iNOS and COX-2. The experiment was repeated three times and similar results were obtained. GAPDH and actin were used as the internal controls for the RT-PCR and Western blot analysis, respectively. NO, nitric oxide; PGE₂, prostaglandin E₂; WEMF, water extract of Mori folium; LPS, lipopolysaccharide; RT-PCR, reverse transcription-PCR; iNOS, inducible NO synthase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

cytokines of RAW 264.7 cells, the levels of cytokines in the culture media were measured by ELISA. As presented in Figure 3, the secretion of TNF- α and ILs (IL-1 β , IL-6, and IL-10) was significantly increased following WEMF and LPS treatment. The concentrations of TNF- α in the media of 2.0 mg/mL WEMF- and LPS-stimulated cells were approximately 9.5 and 7.5 times higher, respectively, than that of untreated control cells. Consistent with the results obtained from cytokines production, the mRNA and protein levels of TNF- α and ILs were also increased by WEMF treatment in a concentration-dependent manner (Fig. 4). These data indicate WEMF positively regulated the production of cytokines at the transcriptional and translational levels in RAW 264.7 cells.

5. Water extract of Mori folium enhanced the phagocytic ability in RAW 264.7 macrophages

Because phagocytosis is the primary function of macrophages, which leads to a diverse range of anti-microbial and cytotoxic responses,^{3,4} we examined the effects of WEMF on macrophage phagocytosis. As shown in Figure 5, the phagocytic properties of RAW 264.7 macrophages were dramatically increased by WEMF

treatment.

DISCUSSION

Recently, the modulation of the immune response using natural products from herbal medicines has attracted interest as a possible therapeutic measure.^{3,35} Despite the diverse pharmacological potential of Mori folium, few studies have focused on its capacity to target immune effector cells. Therefore, we investigated whether WEMF possessed immune stimulatory potential in RAW 264.7 macrophages by examining its effects on the production of NO, PGE₂, and cytokines.

The excessive production of NO and PGE₂, which are synthesized by their inducible enzymes, iNOS and COX-2, can kill normal cells and promote inflammation. However, at certain levels, they can be used as potential therapeutic tools and quantitative indexes of macrophage activation.^{7,36} In our study, WEMF treatment was found to stimulate the production of NO and PGE₂ by promoting the expression of iNOS and COX-2 associated with morphological changes (Fig. 2), which demonstrated that WEMF is an effective activator of macrophages.

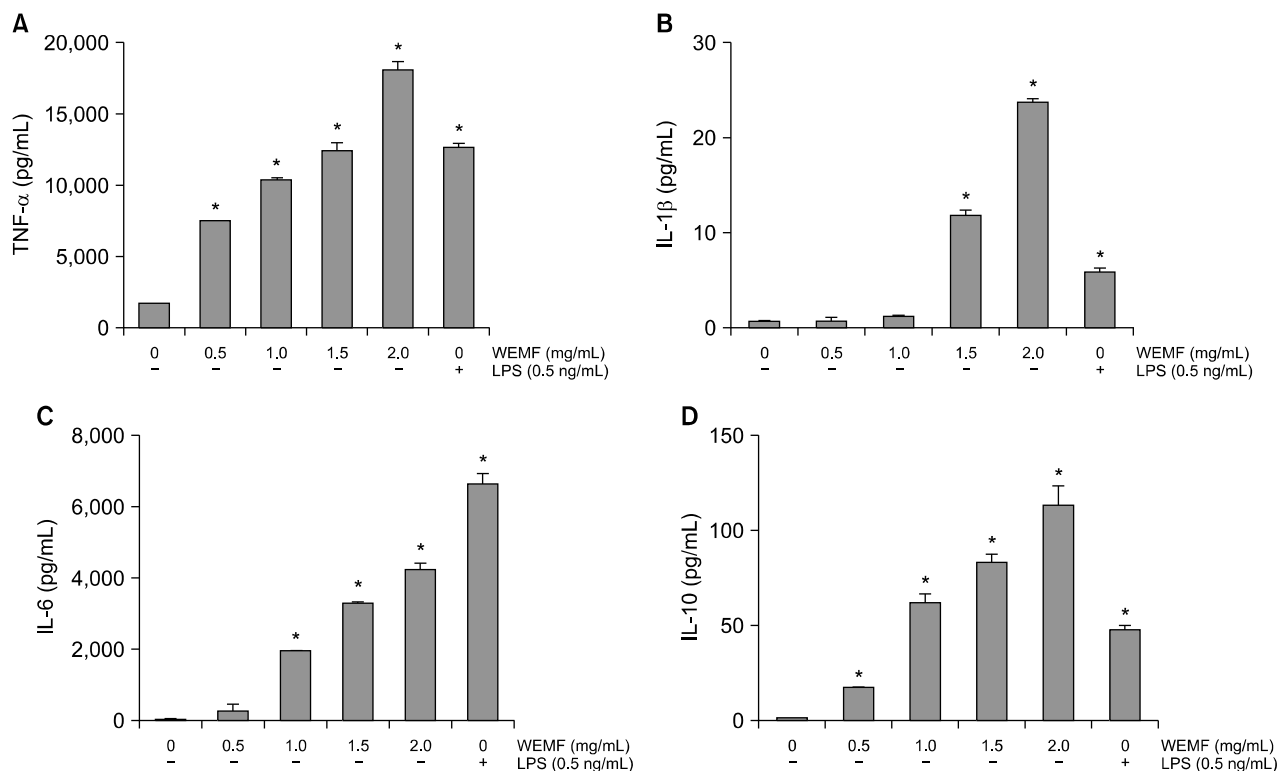


Figure 3. Increased secretion of cytokines by water extract of Mori folium in RAW 264.7 macrophages. The cells were treated with different concentrations of WEMF or 0.5 ng/mL LPS for 24 hours. The amounts of cytokines, such as (A) TNF- α , (B) IL-1 β , (C) IL-6, and (D) IL-10, in the culture supernatants were measured by ELISA kits. WEMF, water extract of Mori folium; LPS, lipopolysaccharide; IL, interleukin. Data are presented as the means \pm SD of three independent experiments (* $P < 0.05$ compared to the control).

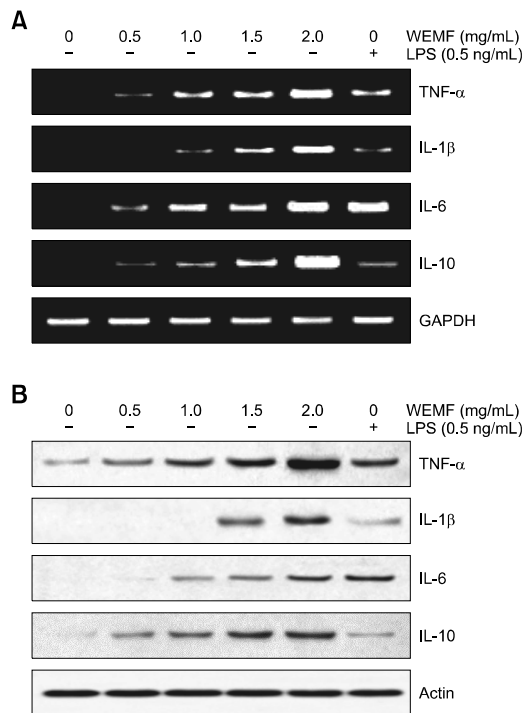


Figure 4. Effect of water extract of Mori folium on expression of cytokines in RAW 264.7 macrophages. (A) The cells were treated with different concentrations of WEMF or 0.5 ng/mL LPS for 24 hours. The total RNAs were isolated and RT-PCR was performed using the indicated primers in Materials and Methods. (B) Cell lysates were prepared for Western blot analysis with the specific antibodies against indicated cytokines. The experiment was repeated three times and similar results were obtained. GAPDH and actin were used as the internal controls for the RT-PCR and Western blot analysis, respectively. WEMF, water extract of Mori folium; LPS, lipopolysaccharide; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL, interleukin.

Various immune-related cytokines, such as TNF- α , IL-1 β , IL-6, and IL-10, are also mainly produced by activated macrophages for the modulation and orchestration of innate immunity.^{3,37} These cytokines can be produced from macrophages in response to bacterial LPS, infection, and inflammatory stimuli. They also play an important role in the immune system by aiding cytotoxic and cytostatic effects on infected or malignant cells.^{34,38} Among them, TNF- α is one of the earliest factors to be induced or activated in macrophages for eliciting tumor immunity. TNF- α plays as a key mediator of T lymphocyte and macrophage activation and exerts either beneficial or detrimental effects on mammalian cells by inducing the secretion of NO and PGE₂.^{6,39} Similarly, IL-1 β and IL-6, and IL-10 are produced by various immune cells, including macrophages. These cytokines are essential for host survival of infection, and they are required for the repair of injured tissue.^{4,34} In our investigation, WEMF significantly stimulated the release

and expression of these cytokines (Fig. 3 and 4), which indicates that it has an immunostimulatory effect on RAW 264.7 macrophages.

One of the most important roles of macrophages is phagocytosis, which comprises a variety of events that are mediated by the membrane receptors present on immune cells by binding to ligands on particle surfaces.^{40,41} Although phagocytosis is significantly involved in the acquisition of nutrients by some cells, it is also a crucial defense mechanism that provides protection against pathogen invasion and necrotic cellular debris scavenging in the immune system.^{3,4} Therefore, we further sought to determine whether WEMF increased phagocytic activity and found that WEMF significantly enhanced the capacity for phagocytosis in RAW 264.7 macrophages. The data suggested that WEMF, as a candidate immunostimulator in the innate immunity of macrophages (Fig. 5), might be used to defend against foreign particles, including bacteria, fungi, and unnecessary cellular debris.

According to our results, WEMF increased the production of immune modulators, such as NO and PGE₂, by inducing their corresponding gene and protein expression in RAW 264.7 cells. WEMF also moderated the immune response by the increased release and expression of immune-related cytokines. Furthermore, WEMF enhanced the phagocytic activity of RAW 264.7 macrophages. Although further research is needed to identify the active pharmacological constituents of WEMF and to understand the mechanisms of their actions, these findings suggest that WEMF may be a useful immune stimulant. Further in vivo studies are needed to investigate in detail the overall immunostimulatory effects of WEMF.

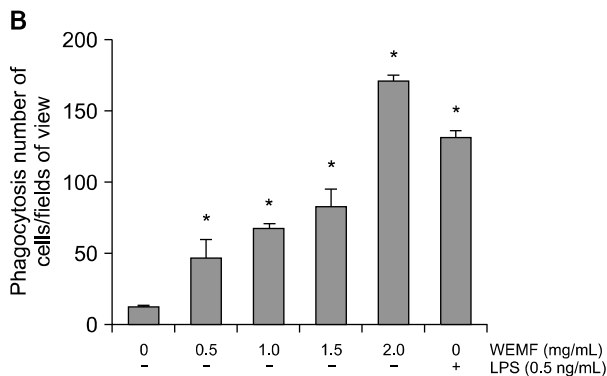
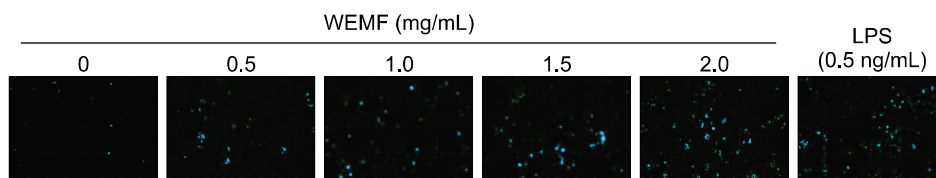
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CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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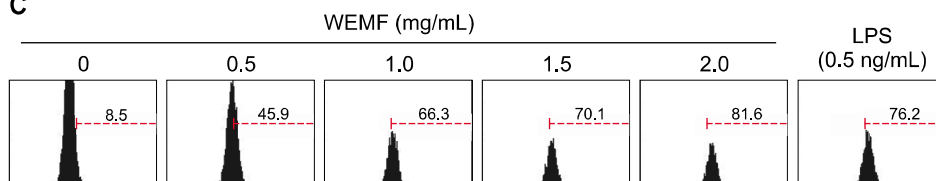


Figure 5. Effect of water extract of Mori folium on phagocytic activity in RAW 264.7 macrophages. Cells were treated with the indicated concentrations of WEMF or 0.5 ng/mL LPS for 24 hours. (A) The phagocytic cells were stained with Latex Beads-Rabbit IgG-FITC and visualized under a fluorescence microscope (200 × original magnification). (B) The number of phagocytic cells per field of view was counted and (C) the extent of phagocytosis was determined by measuring the absorbance at 620 nm. WEMF, water extract of Mori folium; LPS, lipopolysaccharide. The data were presented as means ± SD of three independent experiments (* $P < 0.05$ compared to the control).

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