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RESEARCH PAPER

Anti-inflammatory Effects of *Lecania gerlachei* Extract Collected from the Antarctic King Sejong Island

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Abstract Chronic inflammation is the cause of various diseases such as rheumatoid arthritis and asthma, with a large number of people suffering from them. There have been many reports that even link cancer to inflammation, so the development of sophisticated and powerful drugs continues to be in demand. Here we demonstrate that the methanol extract of *Lecania gerlachei* (LGME), a lichen member found in the extreme Antarctic environment, exhibits anti-inflammatory activities. Treatment of lipopolysaccharide (LPS) stimulated Raw 264.7 murine macrophage cells with LGME reduced nitric oxide (NO) immune modulator production, and also down-regulated inducible nitric oxide synthase (iNOS), pro-inflammatory interleukin 6, 1β and 1α (IL-6, IL-1β and IL-1α), and tumor necrosis factor α (TNF-α) at both transcript and protein levels, in a concentration dependent manner. Furthermore, it was found that these effects were mediated by nuclear factor kappa B (NF-κB) signaling inhibition. Thus, our findings may contribute towards the development of novel inflammatory drugs.

Keywords: *Lecania gerlachei*, lichens, Antarctica, anti-inflammatory properties, nuclear factor kappa B (NF-κB) pathway

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1. Introduction

Inflammation is part of the host defense response to infection and is regulated by various mediators, including cytokines and chemokines released from immune cells such as macrophages [1]. Typical signs of inflammation are redness, heat, pain, and swelling induced by pro-inflammatory factors such as interleukin 6 (IL-6), IL-10, and tumor necrosis factor α (TNF-α) and immune mediators such as nitric oxide (NO) and Prostaglandin E2 (PGE_2) [1]. Chronic inflammation is a causal factor closely linked to various diseases including cancer, cardiovascular as well as disease arthritis and diabetes [2], and many drugs have been developed for the treatment of these inflammation-related diseases. Steroidal and non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used in pain relief management from disease-induced inflammatory reactions. However, as these drugs exhibit a number of side effects, many researchers have focused towards the development of alternative drugs [3].

In recent years, several natural products with pharmacological efficacy have been evaluated because they are less toxic and exhibit fewer side effects unlike the synthetic drugs [4]. Notably, the active ingredients of many pharmacological compounds used in clinical practice have been historically obtained from natural sources. For instance, aspirin, a widely used representative anti-inflammatory drug, derives its active pharmaceutical ingredient from natural substances extracted from salicylate-containing plants [5]. In addition, digoxin, morphine, penicillin for treatment of arrhythmia, pain, microorganism infectious disease [6], respectively, were derived from natural sources exhibiting anti-inflammatory activities, and it is believed that nature harbors many more active pharmaceutical compounds that remain to be discovered. In particular organisms that survive

in extreme environments such as the Antarctic and the Arctic regions are known to accumulate secondary metabolites that enable them to withstand these extreme conditions [7], and in many cases, such metabolites are known to exert pharmacological efficacy. For example, *Ramalina terebrata*, a lichen species collected from the Antarctic Sejong Station area, has been found to have ingredients that exhibit various bioactive properties such as antioxidant [8], antibacterial [9], anticancer [10], anti-allergic [11], and anti-melanogenesis [12]. In addition, both *Botryidiopsidaceae* sp. [13] and *Chloromonas* sp. [14] microalgae collected from seawater and freshwater near the Antarctic Sejong station, respectively, were found to carry ingredients showing anticancer activities. Moss collected from Livingstone Island in Antarctica has also been reported to contain anti-inflammatory and anti-bacterial compounds [15].

In this study, we confirmed that *Lecania gerlachei* collected from the Antarctic King Sejong Island, exhibited anti-inflammatory activities in various assays. To evaluate the anti-inflammatory activities of the *L. gerlachei* methanol extract (LGME), the lipopolysaccharide (LPS) stimulated RAW 264.7 murine macrophage cell line was used as a highly activated inflammation cell model. Our results show LGME treatment of LPS-stimulated Raw 264.7 cells reduced the increase in inducible nitric oxide synthase (iNOS) and the resultant NO production, while the increased cyclooxygenase-2 (COX-2) was only weakly decreased. Moreover, representative pro-inflammatory factors such as IL-6 and TNF-α, analyzed by both quantitative real time polymerase chain reaction (RT-qPCR) and ELISA, demonstrated a similar decrease. This reduction in inflammatory mediators and cytokines suggested that the LGME acts by regulating the nuclear factor kappa B (NF-κB) signaling. This association was further verified by examining the amount of phosphorylated nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα) released from NF-κB complex and NF-κB p65 subunit (also known as RelA) translocated to the nucleus after treatment with both LPS and the LGME. We therefore suggest that *L. gerlachei* harbors active pharmaceutical ingredients that could be potent novel drug candidates in the treatment of inflammatory diseases.

2. Materials and Methods

2.1. Biological identification and preparation of lichen extract

Lichen specimens were collected at King Sejong Antarctic Station (62°11'51.56"S, 58°48'05.41"W) in 2017 and identified by PCR analysis. PCR amplification was performed using DNA amplification kit (DSBIO, Guangzhou, China) and the mitochondrial small subunit rRNA (mtSSU rRNA) primer set (Table 1). Dried lichen thalli were incubated with methanol for a week at room temperature (22-25°C) and then filtered and dried in a rotary vacuum evaporator at 25°C. Dried extract was then dissolved in DMSO to make 20 mg/mL for all experiments.

2.2. Cell culture

The Raw 264.7 cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Before preparing cells for experiments, it was confirmed that there is no mycoplasma using kit (TaKaRa, JAPAN). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (PS), and then changed to DMEM supplemented with 5% FBS and 1% PS 6 h before

Table 1. Primers sequences used in the PCR and the RT-qPCR assay

Gene	Primer Sequences (5' → 3')		Product size (bp)
	Forward	Reverse	
mtSSU rRNA	AGCAGTGAGGAATATTGGTC	ATGTGGCACGTCTATAGCCC	815
β-actin	TGTTTGAGACCTTCAACACC	AGTCTGTCAGGTCCCGGCC	195
IL-6	CTCTGGGAAATCGTGGAAAT	CCAGTTTGGTAGCATCCATC	134
TNF-α	CATCTTCTCAAAATTCGAGTGACAA	TGGGAGTAGACAAGGTACAACCC	175
IL-1β	TGCCACCTTTTGACAGTGATG	TGTGCTGCTGCGAGATTTGA	135
IL-1α	CGCTTGAGTCGGCAAAGAAA	AGATGGTCAATGGCAGAACTGT	108
IL-10	AAGGGTTACTTGGGTTGCCA	TTCAGCTTCTCACCCAGGGA	128
COX-2	GAAGTCTTTGGTCTGGTGCGTG	GTCTGCTGGTTTGGAATAGTTGC	133
iNOS	GGAGCCTTTAGACCTCAACAGA	TGAACGAGGAGGGTGGTG	123

mtSSU rRNA: mitochondrial small subunit rRNA
IL-6: interleukin 6
TNF-α: tumor necrosis factor α
COX-2: cyclooxygenase-2
iNOS: inducible nitric oxide synthase

drug treatment. All steps were performed in 5% CO_2 in a humidified atmosphere at 37°C. These cell culture conditions were applied for all experiments.

2.3. Cytotoxicity assay

Cell viability was determined by using the 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Amresco, Solon, OH, USA) assay. Raw 264.7 cells were seeded at 2×10^4 cells/well in a 96-well plate for the assay. After 24 h at 37°C, cells were inoculated with LGME at varying concentrations (10, 20, 40, 80, and 160 μg/mL) for 24 h at 37°C. Subsequently, the medium was removed and replaced with 0.5 mg/mL MTT solution for 4 h. After discarding the solution, the formazan crystals were dissolved in 100 μL of DMSO for 10 min. The absorbance at 570 nm was measured using a microplate reader (Thermo Scientific Inc., San Diego, CA, USA).

2.4. Nitric oxide assay

Raw 264.7 cells were seeded (5×10^5 cells/well) in a 96-well plate with 10% FBS and 1% PS containing DMEM medium and incubated at 37°C in 5% CO_2 for 24 h. Medium was then removed and replaced with 5% FBS and 1% PS DMEM medium. After 4 h at 37°C, varying concentrations of LGME (10, 20, 40, 80, and 160 μg/mL) were applied and cells were cultured for 1 h before treatment with lipopolysaccharide (LPS) (0.5 μg/mL) for 24 h. NO concentrations were measured using Griess reagent in the cell supernatant. The medium was mixed with an equal volume of Griess reagent (1% sulfanilamide and 0.1% N-1-naphylenediamine dihydrochloride in 2.5% phosphoric acid). Absorbance at 540 nm was measured using a microplate reader (Thermo Scientific Inc., San Diego, CA, USA).

2.5. Cytokine assay

LPS stimulation of Raw 264.7 cells was performed using the same protocol as the NO assay up until the point before applying the Griess reagent. TNF-α and IL-6 levels were determined using ELISA kits (R&D Systems Inc., USA) according to the manufacturer's instructions. The concentration of each factor was calculated in harvested supernatants by matching with standard curves. Absorbance at 450 nm was measured using a microplate reader (Thermo Scientific Inc., San Diego, CA, USA).

2.6. Quantitative real time PCR

Quantitative reverse transcription-PCR (qRT-PCR) was performed to determine changes in the gene expression levels of IL-6, TNF-α, COX-2, and iNOS. Raw 264.7 cells were cultured in the presence or absence of varying LGME concentrations and LPS for the indicated time-periods. Collected culture cells were lysed using TRI REAGENT® (Molecular Research Center Inc., Cincinnati, OH, USA) and total RNA was extracted, according to the manufacturer's instructions. cDNA synthesis was performed using M-MLV Reverse Transcriptase (Enzynomics, Daejeon, Korea). qRT-PCR was carried out using a Rotor gene 6500 system (Corbett Research, Mortlake NSW, Australia) as follows: 10 μL TOPreal™ qPCR 2X PreMIX (Enzynomics, Daejeon, Korea), 1 μL template cDNA (synthesized with 1,000 ng of mRNA), 1 μL each primer (final 10 pmol for each primer) (Table 1) and 7 μL of deionized distilled water. The cycling conditions were as follows: 95°C for 5 min, followed by 95°C for 15 s, 58°C for 15 s, and 72°C for 15 s for 40 cycles. Data were analyzed using the comparative cycle threshold (Ct) method (2-ΔΔCq) and were normalized to α-actin.

2.7. Western blotting

Total protein was extracted using the RIPA buffer (Sigma-Aldrich, St Luis, MO, USA) containing 1× protease inhibitor cocktail tablets (Roche, Basel, Switzerland) and 1X phosphatase inhibitor cocktail tablets (Roche, Basel, Switzerland). For separated protein sample, nuclear extraction kit (Abcam, Cambridge, UK) was used according to the manufacturer's guidelines. The extracted proteins were separated by SDS-PAGE and subsequently transferred to PVDF membranes. Proteins were immunoblotted with rabbit polyclonal anti-iNOS (Enzo Life Sciences Inc., NY, USA) (1/1000), mouse monoclonal anti-COX-2 (Santa Cruz Biotechnology Inc., Dallas, TX, USA) (1/1000), rabbit monoclonal anti-p65 (Cell Signaling, Danvers, MA, USA) (1/1000), rabbit monoclonal anti-p-IκBα p65 (Cell Signaling, Danvers, MA, USA) (1/1000), mouse monoclonal anti-PCNA (Santa Cruz Biotechnology Inc., Dallas, TX, USA) (1/1000), and rabbit polyclonal anti-mouse GAPDH (Santa Cruz Biotechnology Inc., Dallas, TX, USA) (1/1000) antibodies, and after washing thrice with phosphate buffered saline with triton x-100 (PBST), they were then immunoblotted with horseradish peroxidase (HRP)-conjugated anti-rabbit and mouse secondary antibody (Santa Cruz Biotechnology Inc., Dallas, TX, USA) (1/2000). Blots were developed following incubation in enhanced chemiluminescence (ECL) kit (Thermo Fisher). Immunoblots were visualized using a Chemi-Doc instrument (Bio-Rad, Hercules, CA, USA).

2.8. Statistical analysis

The data were presented as mean ± SEM and statistical significance was determined by student t-test for the mean of each group. P-value was considered to indicate a statistically significant difference compared with non-treated (#) or only LPS-treated Raw 264.7 cells (*) (# $p < 0.001$, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$).

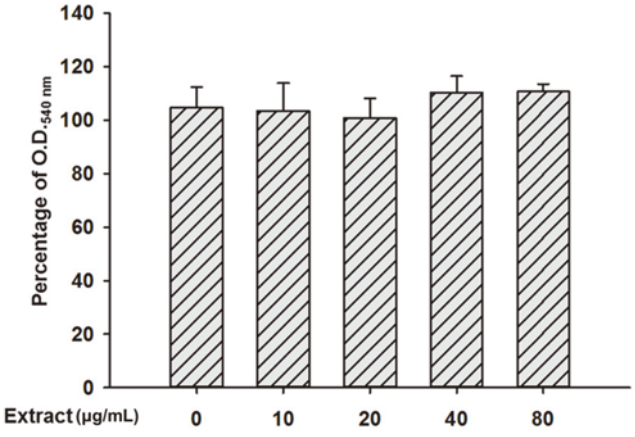

Fig. 1. Cell viability assay to confirm *Lecania gerlachei* methanol extract (LGME) cytotoxicity on LPS-stimulated Raw 264.7 cells.

3. Results and Discussion

3.1. No cytotoxicity was shown by LGME

To prevent undesired effects on cells and organs following administration of drugs, it is essential for the drugs to exert pharmacological effects without cytotoxicity. Moreover, in the case that the studied pharmacological compound exhibits cytotoxicity, analysis of *in vitro* obtained data could become unreliable due to the reduced number of viable cells in each group condition examined. In this study, cell viability was investigated by using the MTT cell proliferation assay in order to identify LGME cytotoxicity and determine non-toxic concentration for treatment. LPS-stimulated Raw 264.7 cells were treated with 0, 10, 20, 40, and 80 μg/mL of LGME. As illustrated in Fig. 1, cells did not exhibit any toxic effect from LGME treatment at concentrations below 80 μg/mL (maximum concentration) and therefore, all additional experiments were performed at the concentration range of 0 - 80 μg/mL LGME.

NO, major immune mediators and regulators causing pathological pain in the body, are mainly produced by macrophage in response to LPS [16]. High levels of NO can stimulate pro-inflammatory and destructive effects such as tissue damage, inflammation, and vasoconstriction [17]. Therefore, NO has received attention as a potential target of anti-inflammatory medicine [18]. In this context, NO production was assessed after LPS simulation and following treatment of Raw 264.7 cells with LGME. The amount of NO produced by Raw 264.7 cells markedly increased following 0.5 μg/mL LPS treatment, whereas it decreased with LGME treatment. More specifically, increasing concentration of LGME treatment (from 0 to 80 μg/mL), resulted in a graded, but intense reduction in NO production almost to the same level as in non-treatment (Fig. 2A). This result clearly argues for the presence of inflammation relieving substances in the non-toxic LGME. In addition, unlike NO, production of PGE_2, another major inflammation mediator, was also reduced, but only after treatment at the highest LGME concentration (Fig. 2B). Therefore, it can be suggested that the anti-inflammatory components contained in LGME targeted the NO rather than the PGE_2 pathway.

3.2. iNOS and COX-2 gene and protein expression levels

Since NO and PGE_2 production is controlled by iNOS and COX-2 [18], respectively, it was necessary to confirm whether the LGME-induced decrease in the production of both mediators was due to these enzymes. Changes in the mRNA and protein expression levels of these enzymes were analyzed to determine at which stage they were

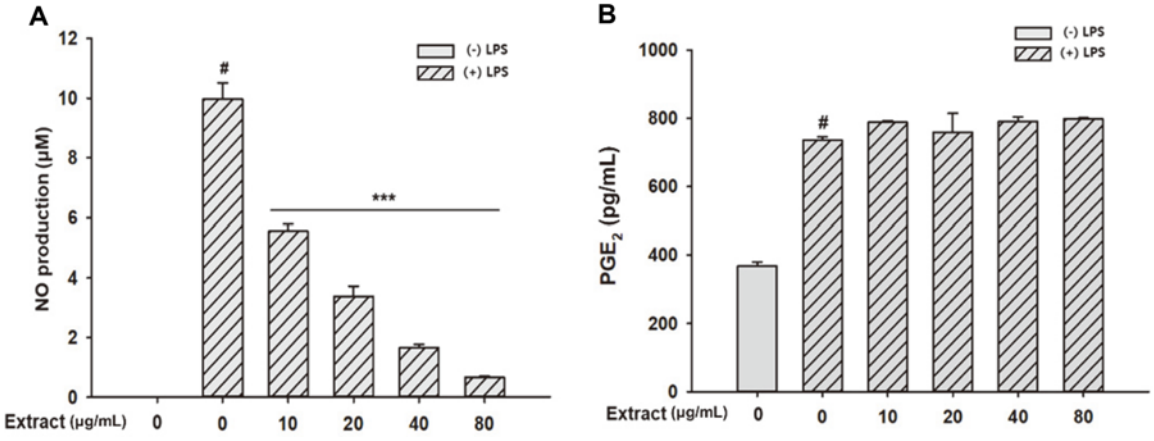

Fig. 2. Inhibitory effect of *Lecania gerlachei* methanol extract (LGME) on production of proinflammatory mediators in LPS-stimulated Raw 264.7 cells. The concentration of (A) nitric oxide (NO) and (B) prostaglandin E2 (PGE_2) were analyzed. Symbols with bars represent statistically significant difference of each condition relative to non-treated (#) and LPS-stimulated Raw 264.7 cells (*) (* $p < 0.05$, ** $p < 0.01$ and #, *** $p < 0.001$).

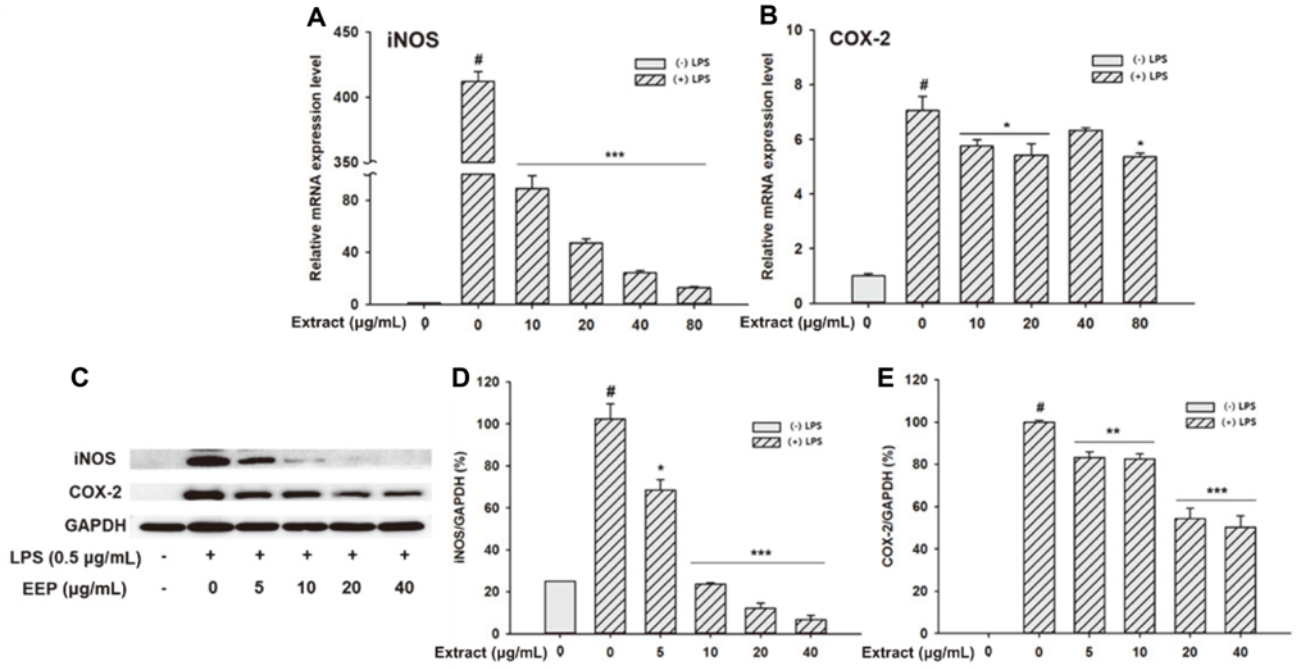

Fig. 3. Inhibitory effect of *Lecania gerlachei* methanol extract (LGME) on expression of mRNA of the enzymes involved in the production of nitric oxide (NO) and cyclooxygenase-2 (COX-2). (A) inducible nitric oxide synthase (iNOS) and (B) COX-2 mRNA expression to confirm the effect of LGME on regulation of each gene was quantified by RT-qPCR and (C) Protein level analysis was performed by western blotting. (D) iNOS and (E) COX-2 protein band intensities on membrane were quantified against GAPDH. Symbols with bars represent statistically significant difference of each condition relative to non-treated (#) and LPS-stimulated Raw 264.7 cells (*) (* $p < 0.05$, ** $p < 0.01$ and #, *** $p < 0.001$).

affected by LGME treatment. Following treatment with LPS, both iNOS (Fig. 3A, C, D) and COX-2 (Fig. 3B, C, E) mRNA and protein expression levels were highly increased, whereas those of iNOS markedly decreased with increase in LGME concentration and thus ultimately affected NO production. On the other hand, LGME had few effects on COX-2 mRNA expression and protein production, and it was further confirmed that PGE_2 production was not insensitive to LGME. All these results indicate that LGME has greater inhibitory effect on activation of the iNOS/NO pathway than the COX-2/PGE_2 pathway. The NO and PGE_2 signaling pathways, the major inflammatory-related pathways, are known to be regulated by corticosteroids [19] and NSAIDs [20], respectively. Particularly, alternatives to corticosteroid drugs are examined due to the adverse effects such as osteoporosis [19], hypertension [21], diabetes [22], and infection susceptibility [23] arising from their prolonged usage. Therefore, LGME derived chemical elements could be potential candidates for substituting corticosteroid drugs that specifically target the iNOS mechanism rather than for replacing NSAIDs that target the COX-2 mechanism.

3.3. Pro-inflammatory cytokines gene and protein expression levels

IL-6 and TNF-α are pro-inflammatory cytokines, which play various roles in intracellular signaling [24], IL-6, a pleiotropic cytokine, is rapidly produced in response to external stress, such as infection or injuries, triggering the defense system of the host [25]. It is known that many cell types including T lymphocyte, monocytes, hematopoietic cells, epithelial cells, and macrophages can produce IL-6 and abnormal expression of IL-6 might result in increased incidences of many diseases such as rheumatoid arthritis and juvenile idiopathic arthritis [25]. TNF-α is recognized as a typical pro-inflammatory factor in immune response. TNF-α is mainly secreted by activated macrophages, and its abnormal regulation has been reported as one of the causes of inflammatory diseases such as Alzheimer's disease [26], as well as many types of cancer [27]. Therefore, many drugs such as Enbrel [28], Remicade [29], and Humira [30] used in the therapeutic management of autoimmune diseases such as rheumatoid arthritis, target TNF-α. To confirm the effect of LGME treatment on the production of pro-inflammatory cytokines, RT-qPCR and ELISA analyses were performed. IL-6 mRNA and protein expression levels were sharply increased in LPS-induced relative to normal state cells and reduced about 8.4 ($p < 0.001$) and 5.3 ($p < 0.001$) times, respectively, when simultaneously treated with 10 μg/mL LGME (Fig. 4A). Further, 23.4 ($p < 0.001$) and 38.5 ($p < 0.001$) times reduction, respectively, was

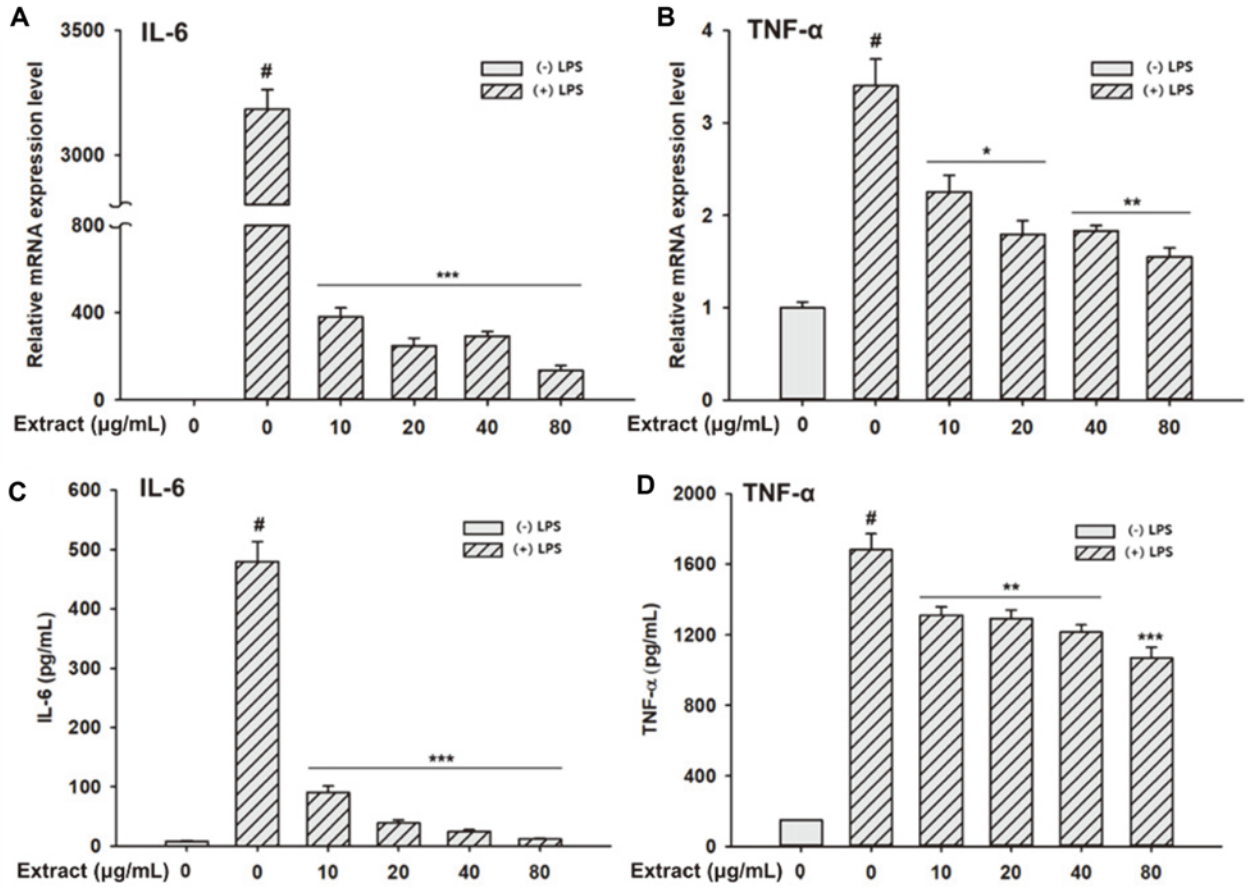

Fig. 4. Inhibitory effect of *Lecania gerlachei* methanol extract (LGME) on mRNA and protein production levels of pro-inflammatory cytokines. (A) IL-6 mRNA and (B) TNF-α mRNA expression were analyzed by RT-qPCR and (C) IL-6 and (D) TNF-α secreted protein levels were analyzed by ELISA. Symbols with bars represent statistically significant difference of each condition relative to non-treated (#) and LPS-stimulated Raw 264.7 cells (*) (* $p < 0.05$, ** $p < 0.01$ and #, *** $p < 0.001$).

observed with 80 μg/mL LGME treatment (Fig. 4C). Although TNF-α expression levels were not as substantially altered as those of IL-6, they were still significantly lowered due to LGME treatment ($p < 0.05$) (Fig. 4B, D). Additionally, mRNA expression of IL-1β and IL-1α (Fig. S1, S2), pro-inflammatory factors, decreased and IL-10, an anti-inflammatory factor, increased in a dose dependent manner (Fig. S3). All the results imply that inhibitory effect on pro- and anti-inflammatory factors expression levels following LGME treatment suggests the possibility for development of an anti-inflammatory drug in which both cytokines are targeted.

3.4. LGME-induced suppression of NF-κB pathway activation

The above inflammatory mediators and factors are produced by the regulation of transcriptional NF-κB, which plays a key role in the development of inflammatory disease [31]. Under physiological conditions, NF-κB is present in the cytoplasm in an inactivated state in conjunction with the inhibitory IκBα protein. However, external immunostimulation such as LPS, leads to the phosphorylation of IκBα and its subsequent release from the complex, thus activating the NF-κB p65 subunit, which is then transported into the nucleus where it promotes mRNA expression of various inflammatory factors [32]. Therefore, modulation of NF-κB signaling can constitute a prospective therapeutic strategy. To confirm that expression level changes were due to the effect on NF-κB signaling, which is one of the major pathways in mammalian cell inflammation processes, the p65 and phosphorylated inhibitor of kappa B (p-IκBα) protein expression levels were assessed by western blot analysis (Fig. 5A). LGME treatment enhanced phosphorylation of cytosolic p65 and inhibited phosphorylation of IκBα in LPS-stimulated Raw 264.7 cells on a concentration dependent manner (Fig. 5B, C) implying that nuclear translocation of NF-κB p65 was blocked. Concurrently, nuclear translocation of NF-κB p65 was shown to be

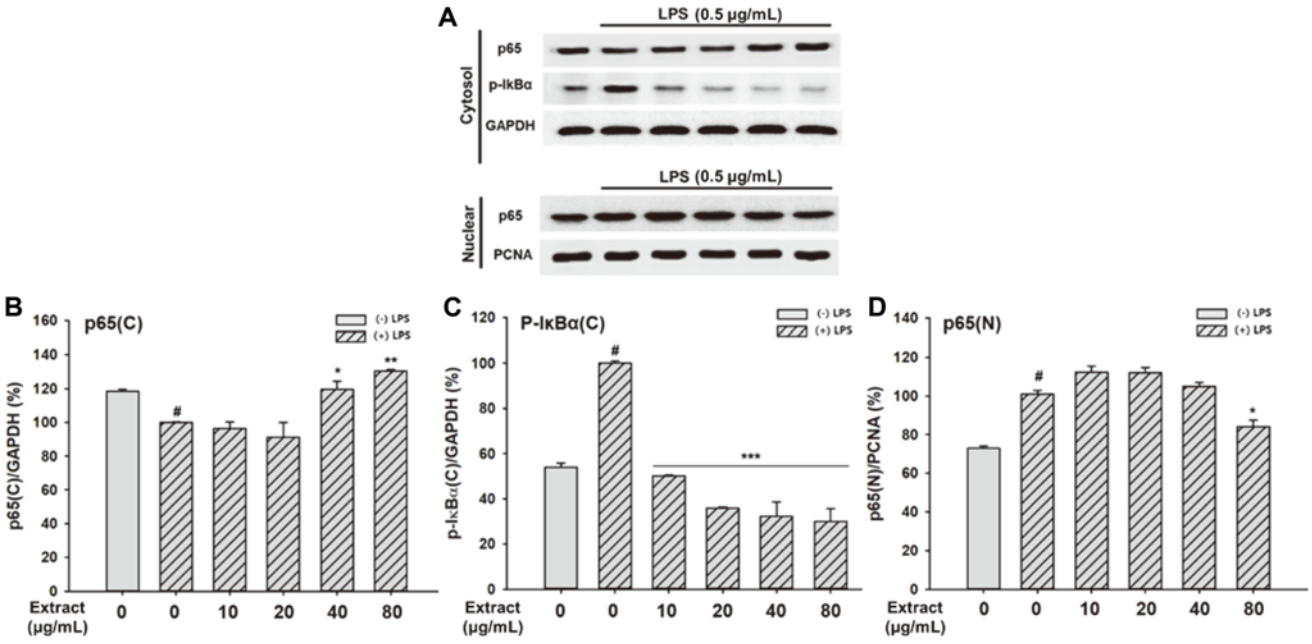

Fig. 5. Inhibitory effect of *Lecania gerlachei* methanol extract (LGME) on the nuclear translocation of NF-κB p65. (A) Quantities of each protein were confirmed on PVDF membrane with bands by western blotting. Band intensities for intracellular (B) p65 and (C) p-IκBα on membrane were quantified against GAPDH and (D) nuclear p65 was quantified against PCNA as control. Symbols with bars represent statistically significant difference of each condition relative to non-treated (#) and LPS-stimulated Raw 264.7 cells (*) (* $p < 0.05$, ** $p < 0.01$ and #, *** $p < 0.001$).

negatively correlated with LGME treatment (Fig. 5D). This result further corroborates the fact that LGME treatment was able to suppress the LPS-induced activation of NF-κB signaling in Raw 264.7 cells, thus indicating regulation of inflammation.

4. Conclusion

In this study, *L. gerlachei*, a lichen species living in the extreme Antarctic environment, was evaluated for its potential anti-inflammatory properties. We showed that LGME exhibited anti-inflammatory activities in LPS-stimulated Raw 264.7 macrophage cells, via attenuation of NO production, and through the increased expression of iNOS and various pro-inflammatory factors such as IL-6 and TNF-α at the transcript and protein levels. We additionally confirmed that the inhibitory effect of LGME on inflammation was the result of suppression of activated NF-κB signaling pathway. All these results provide evidence that LGME contains ingredients that exhibit promising anti-inflammatory activities. These bioactive elements regulate inflammation via suppression of the NF-κB signaling pathway and therefore could be considered novel therapeutic candidates in the treatment and management of inflammatory diseases.

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The authors declare no conflict of interest.

Neither ethical approval nor informed consent was required for this study.

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