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# Anti-inflammatory effects of methanol extracts from the Antarctic lichen, *Amandinea* sp. in LPS-stimulated raw 264.7 macrophages and zebrafish

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ABSTRACT

The aim of the present study was to determine the anti-inflammatory effect of an extracts isolated from the lichen. *Amandinea* sp. was collected from the Antarctic and extracted with methanol. The basic screening of the anti-inflammatory property of the extracts was done using the NO assay. The extracts showed very little cytotoxicity, and reduced NO production in LPS-stimulated RAW 264.7 cells in a dose-dependent manner. Furthermore, the extracts inhibited LPS-induced release of pro-inflammatory cytokines such as interleukin-6 (IL-6), and tumor necrosis factor-α(TNF-α), and inflammatory mediators inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2). The extracts also reduced the cytosolic *p*-IκB-α level and the level of the nuclear factor p65. We examined the anti-inflammatory effects of the extracts using zebrafish *in vivo*. The extracts reduced the amount of reactive oxygen species (ROS) in LPS-induced zebrafish larvae and inhibited the mRNA expression of inflammatory cytokines and mediators in a tail-cutting induced model. These results are similar to those obtained *in vitro* with RAW 264.7 cells. Collectively, the data suggest that the extracts may contain one of more compounds with anti-inflammatory effects. Further studies are required to identify the candidate compound/s and to understand the mechanism of action of the extract.

## 1. Introduction

The body has a defense mechanism, called the immune system, to protect itself from infection and injury. Inflammation is the primary response of the body's immune system [1]. Several cellular and molecular events are initiated to efficiently minimize injury or infection during an inflammatory response [2]. The cytokines are generally known to be involved in the regulation of the immune response. The cytokines can be categorized as pro- and anti-inflammatory cytokines, and soluble inhibitors of pro-inflammatory cytokines [3,4]. The pro-inflammatory cytokines such as interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-12 and interferon-γ (IFN-γ) facilitate inflammation, while the anti-inflammatory cytokines such as IL-1 receptor antagonist, IL-4, IL-6, IL-10, IL-11, and IL-13 inhibit the production of pro-inflammatory cytokines or exert a number of other inhibitory effects on the inflammatory processes [5]. The NF-κB pathway is considered a prototypical pro-inflammatory signaling pathway, activated by the pro-inflammatory cytokines, and the role of NF-κB has been extensively investigated [6]. The murine macrophage cell line RAW 264.7 induced by LPS commonly used *in vitro* study on screening anti-inflammatory activity from natural compounds [7]. Upon LPS stimulation of RAW 264.7 cells, increased levels of pro-inflammatory cytokine and inflammatory mediator were observed in previous studies [8–10].

Zebrafish (Danio rerio) has many advantages as an *in vivo* animal model [11,12]. The zebrafish is widely used in various fields such as biology, embryology, molecular biology, immunology, and toxicological drug discovery studies [13–18]. The *in vivo* zebrafish anti-inflammation test model is recognized as the best tool for anti-inflammation assays [19–22].

Lichens are composite organisms that are derived from a symbiotic relationship of two different organisms, a fungus and an alga [23]. Because of their symbiotic existence, lichens can adapt well to extreme living conditions and continue to grow [24]. They are extensively

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distributed in extreme environments such as desert regions, coastal areas, forest areas and polar zones. The Antarctic is mostly shrouded in ice, and the ice-free terrestrial zone consists of <0.5% of the ground cover [25]. Even in these extreme conditions, 62 species of lichens inhabit the Barton Peninsula on the King George Island in Antarctica [26]. Lichens are known to produce several secondary metabolites that have excellent pharmacological effects [27]. The effects of such metabolites including anti-oxidative, anti-bacterial, anti-inflammatory, and anti-proliferative activities are being extensively researched [28–30]. In this paper, we examined the anti-inflammatory effect of an extracts isolated from the Antarctic lichen *Amandinea* sp. in LPS-stimulated Raw 264.7 macrophages and zebrafish.

## 2. Materials and methods

### 2.1. Collection of the lichen and preparation of the extract

Lichen samples were collected from the Antarctic on January 19, 2017. Detailed information pertaining to the sample identity and collection locality is shown in Table 1. The lichen samples were dried completely, and powdered using a pestle and mortar. Powdered lichens were extracted with methanol (1 g/10 mL) under 25 °C dark light for 2 weeks. The methanol in the extracts was evaporated using a speed vacuum concentrator. The dried extracts were then dissolved in DMSO to a final concentration of 20 mg/mL.

### 2.2. Cell culture and treatment

Murine macrophage Raw 264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin streptomycin (PS) at 37 °C in 5% $CO_2$/95% air and the medium was replaced with DMEM containing 5% FBS and 1% PS prior to treatment.

### 2.3. Cytotoxicity assay

The MTT [(3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was used to assess cell viability rate. Raw 264.7 cells ($2.5 \times 10^4$ cells/well in a 96-well plate) were incubated for 4 h and then treated with various concentrations of the lichen extracts (0, 10, 20, 40, and 80 μg/mL) for 24 h. Following the incubation, 5 μM MTT solution was added to each well, and incubated for 4 h. The medium in each well was removed, then 100 μL/well of DMSO was added, and absorbance was measured at 570 nm to determine cell viability rate. The relative cell viability rate (%) was calculated as a percentage relative to the untreated control cells.

### 2.4. NO measurement

The Griess Reagent was used to assay Nitrite production in the cell culture medium. Raw 264.7 cells ($5 \times 10^5$ cells/well in a 96-well plate) were incubated for 24 h. The medium was removed and replaced with fresh DMEM medium (5% FBS), followed by incubation at 37 °C for 4 h. The cells were treated with various concentrations of the lichen extracts (0, 10, 20, 40, and 80 μg/mL) for 1 h, and then treated with *E. coli* lipopolysaccharide (LPS, serotype O111:B4) for 24 h. The cell culture media (100 μL/well) were transferred to the wells of 96-well plates, then equal volume of Griess reagent (1% sulfanilamide and 0.1% *N*-1-naphylenediamine dihydrochloride in 2.5% phosphoric acid) was added, and the absorbance was measured at 540 nm to confirm NO concentration.

**Table 1**
The collection locality.

| Sample No. | Species | Locality Name | GPS location |
|---|---|---|---|
| 2017-Ant-064 | *Amandinea* sp. | Ardley island, Antarctica | 62°12′57.82″S, 58°56′01.87″W |

### 2.5. Measurement of IL-6 and TNF-α

The Sandwich-enzyme-linked immunosorbent assay kits (ELISA) were used to measure the concentration of IL-6 (DY406–05, R&D Systems Inc, USA) and TNF-α (DY410–05, R&D Systems Inc, USA) secreted from cells. Raw 264.7 cells ($2.5 \times 10^4$ cells/mL) were incubated for 24 h. The medium was removed and replaced with fresh DMEM medium, followed by incubation at 37 °C for 4 h. The cells were treated with various concentrations of the lichen extracts (0, 10, 20, 40, and 80 μg/mL) for 1 h, and then treated with 0.5 μg/mL E. *coli* lipopolysaccharide (LPS, serotype O111:B4) for 24 h. ELISA was conducted according to the manufacturer's instructions, and the optical density was measured immediately at 450 nm using a microplate reader.

### 2.6. Quantitative real-time PCR analysis (IL-6, TNF-α, iNOS, COX-2, IL-1β, IL-10)

Total RNA was isolated using the easy-BLUE™ Total RNA Extraction Kit (17,061, Intron biology) according to the manufacturer's instructions. RNA (1μg) was reverse-transcribed using M-MLV Reverse Transcriptase (RT001S, Sigma-Aldrich), followed by real-time PCR amplification with TOPrealTM qPCR 2x PreMIX (RT500, Enzynomics) performed using a Rotor gene 6500 (Corbett research). The target gene primer sequences are listed in Table 2. The expression data were analyzed using to the comparative cycle threshold ($C_t$) method and were normalized to the level of β-actin expression.

### 2.7. Immunoblotting

Proteins were extracted using RIPA buffer (Sigma), protease inhibitor cocktail tablets (Roche) and phosphatase inhibitor cocktail tablets (Roche) or nuclear extraction kit (ab113474, Abcam). The extracted proteins (20μg) were separated by SDS-PAGE, transferred to PVDF

**Table 2**
The list of PCR primers used in quantitative real-time PCR.

| Target | Gene | Primer orientation | Sequences | Size (bp) |
|---|---|---|---|---|
| Raw 264.7 | IL-6 | forward | 5′- CTCTGGGAAATCGTGGAAAT -3′ | 134 |
| | | reverse | 5′-CCAGTTTGGTAGCATCCATC -3′ | |
| | TNF-α | forward | 5′- CATCTTCTCAAAATTCGAGTGACAA -3′ | 175 |
| | | reverse | 5′- TGGGAGTAGACAAGGTACAACCC -3′ | |
| | iNOS | forward | 5′- GGAGCCTTTAGACCTCAACAGA -3′ | 123 |
| | | reverse | 5′- TGAACGAGGAGGGTGGTG -3′ | |
| | COX-2 | forward | 5′- GAAGTCTTTGGTCTGGTGCGTG -3′ | 133 |
| | | reverse | 5′- GTCTGCTGGTTTGGAATAGTTGC -3′ | |
| | β-actin | forward | 5′- TGTTTGAGACCTTCAACACC -3′ | 195 |
| | | reverse | 5′- AGTCTGTCAGGTCCCGGCC -3′ | |
| Zebrafish | IL-6 | forward | 5′- TGAAGGGGTCAGGATCAGCA -3′ | 108 |
| | | reverse | 5′- CACGTCAGGACGCTGTAGATT -3′ | |
| | IL-1β | forward | 5′- GCACGGCTATTCAGAGATGGT -3′ | 133 |
| | | reverse | 5′- CCAAGAATAAGCAGCACTTGGG -3′ | |
| | IL-10 | forward | 5′- TAGGATGTTGCTGGGTTGGAC -3′ | 148 |
| | | reverse | 5′- TAGTGTGATGGATGGACGGG -3′ | |
| | TNF-α | forward | 5′- TCTCAGGGCAAGAAATTCGAC -3′ | 90 |
| | | reverse | 5′- TCTCACTGCATCGGCTTTGT -3′ | |
| | iNOS | forward | 5′- CTGCGGTGGAATGAACATGG -3′ | 93 |
| | | reverse | 5′- TCTCCAGCTTCTACCTCGCTC -3′ | |
| | COX-2 | forward | 5’ -GCTGCTTTGGTGGACTTACAG -3′ | 100 |
| | | reverse | 5′- TCAGAGGAGGGCTATTGTCAG -3′ | |
| | rpp0 | forward | 5′- CTGAACATCTCGCCCTTCTC -3′ | 161 |
| | | reverse | 5′- TAGCCGATCTGCAGACACAC -3′ | |

membranes, washed with 5% skim milk in TBST buffer for 1 h, incubated with the primary antibody overnight at 4 °C. The primary antibodies were iNOS (ADI-905-431, Enzo), COX-2 (SC-166475, Santa Cruz Biotechnology), *p*-IκBα (#2859, Cell signaling), p65 (#3033, Cell signaling), PCNA (SC-25280, Santa Cruz Biotechnology) and GAPDH (SC-25778, Santa Cruz Biotechnology). Secondary antibodies were anti-mouse (HAF007, DuoSet) and anti-rabbit (SC-2537-CM, Santa Cruz Biotechnology). The blots were analyzed using an enhanced chemiluminescence (ECL) kit (Thermo Fisher).

### 2.8. Origin and maintenance of parental zebrafish

Adult zebrafish were acquired from a merchant (Ansan aquarium, Korea) and maintained at 28 °C ± 0.5 with a 14:10 h light:dark cycle [31]. Before mating, zebrafish were separated into two groups, females and males. They were put in contact in the morning and lay the eggs within 30 min in response to light.

### 2.9. Measurement of embryo toxicity

At 6 h post-fertilization (hpf), zebrafish embryos ($n = 10$) were transferred to 12-well plates containing 2 mL of embryo medium per well. The embryos were treated with various concentrations of the lichen extracts (0, 1, 10, 100, 200, and 400 μg/mL) for 120 hpf. The final DMSO concentration in the treatment solution was 1%.

The survival rate was measured every day until 120 hpf. Zebrafish were anesthetized with 0.25 mg/mL tricaine at 48 hpf, and the heart beat rate and body length were analyzed using a microscope.

### 2.10. Measurement of ROS levels in zebrafish

ROS generation in zebrafish larvae was detected using the oxidation-sensitive fluorescent probe dye, 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA, Sigma), as described previously [32]. At 3 hpf, zebrafish larvae ($n = 10$) were transferred to 12-well plates and treated with 10 μg/mL LPS (with or without lichen extract) for 24 h. After treatment, zebrafish larvae were transferred into 12-well plates and DCF-DA was added to the embryo medium at a final concentration of 20 μg/mL. After incubating for 1 h in the dark at 28.5 °C, the zebrafish larvae were washed with embryo medium and then anesthetized in 0.25 mg/mL tricaine. ROS generation was observed under fluorescence microscope and quantified using the ImageJ software.

### 2.11. 11. Tail transection model

The effect of the lichen extracts on the inflammatory responses was determined using the tail-transection model, as described previously [33]. At 96 hpf, zebrafish larvae were anesthetized in 0.25 mg/mL tricaine and the transection of the tail was performed using a scalpel. Then, the zebrafish larvae were washed with embryo medium, transferred to 6-well plates ($n = 20$/well), and treated with various concentrations of the lichen extracts (0, 25, and 50 μg/mL) and 100 μM dexamethasone for 24 h.

### 2.12. Statistical analysis

All experiments were performed in triplicate, and similar results were obtained. Results are expressed as mean ± standard error and analyzed using a student's t-test. The asterisks on the bar of each graph indicate the *P*-value ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

## 3. Results

### 3.1. Effect of Amandinea sp. extracts on cell viability and NO production

The lichen was collected from a source near the King Sejong Antarctic Station in 2017 (Fig. 1A). The species of lichen was identified as *Amandinea* sp. by PCR (data not shown).

To examine the toxicity of *Amandinea* sp. extracts on RAW 264.7 cells in the presence of LPS, we performed the MTT assay to measure cell viability rate. The extracts were found to have no cytotoxic effect on LPS-stimulated RAW264.7 cells. These showed no significant effect on viability at concentrations of 10, 20, 40 and 80 μg/mL (Fig. 1B). Next, the NO assay was used to evaluate for effects of the extracts on NO production in LPS-stimulated Raw 264.7 cells. The results showed that extracts reduced NO production in a dose-dependent manner (Fig. 1C). Based on these results, we used the extracts concentrations of 10, 20, 40 and 80 μg/mL for all subsequent experiments.

### 3.2. Effects of Amandinea sp. extracts on pro-inflammatory cytokine production

Based on the NO production results, the effects of the extracts on LPS-induced inflammatory cytokine production were further examined. The extracts suppressed the expression levels of the pro-inflammatory cytokines IL-6 and TNF-α, and reduced levels of the inflammatory mediators, iNOS and COX-2 in LPS-stimulated Raw 264.7 cells. These were measured by ELISA, qPCR, and western blotting (Figs. 2 and 3). The COX-2 protein levels were not significant but the data show that the extracts reduced LPS-stimulated cytokine production.

### 3.3. Effects of Amandinea sp. extracts on NF-κB signaling

We tested effects of the extracts on NF-κB signaling. We found that the levels of cytoplasmic *p*-IκB-α and nuclear p65 decreased as detected by western blotting (Fig. 4). These results suggest that the extracts inhibit nuclear p65 levels by reducing cytoplasmic *p*-IκB-α levels.

### 3.4. Effects of Amandinea sp. on survival rate, heart beat rate and body length in zebrafish

To determine the toxicity of the *Amandinea* sp. extracts on zebrafish, we observed the survival rate, heart beat rate, and body length. The survival rates were significantly decreased in the treatments with 200 and 400 μg/mL lichen extracts (Fig. 5A). Based on these results, we used the extracts concentrations of 25 and 50 μg/mL to study the heart beat rate and body length experiments. No significant changes in the heart beat rate and body length were detected in the treatments compared with those of the control.

### 3.5. Effect of Amandinea sp. on ROS production in LPS-treated zebrafish

We examined the inhibitory effect of the *Amandinea* sp. extracts on the overproduction of ROS in LPS-induced zebrafish larvae. The LPS-induced ROS accumulation was tested through DCF-DA staining. The DCF-DA staining on LPS-induced ROS production generated clear fluorescence images. The extracts reduced the *in vivo* ROS production in a dose-dependent manner (Fig. 6).

### 3.6. Effects of Amandinea sp. extracts on pro-inflammatory cytokine production in tail cutting-induced inflammation in zebrafish

The extracts suppressed the expression levels of the inflammatory cytokines IL-6, IL-1β, IL-10, and TNF-α, and reduced the levels of the inflammatory mediators, iNOS and COX-2 in the zebrafish tail-transection model. The expression of IL-1β and COX-2 mRNA were significantly decreased compared to that of the positive control with dexamethasone (Fig. 7).

## 4. Discussion and conclusion

We collected lichen samples on a business trip in 2017. The anti-

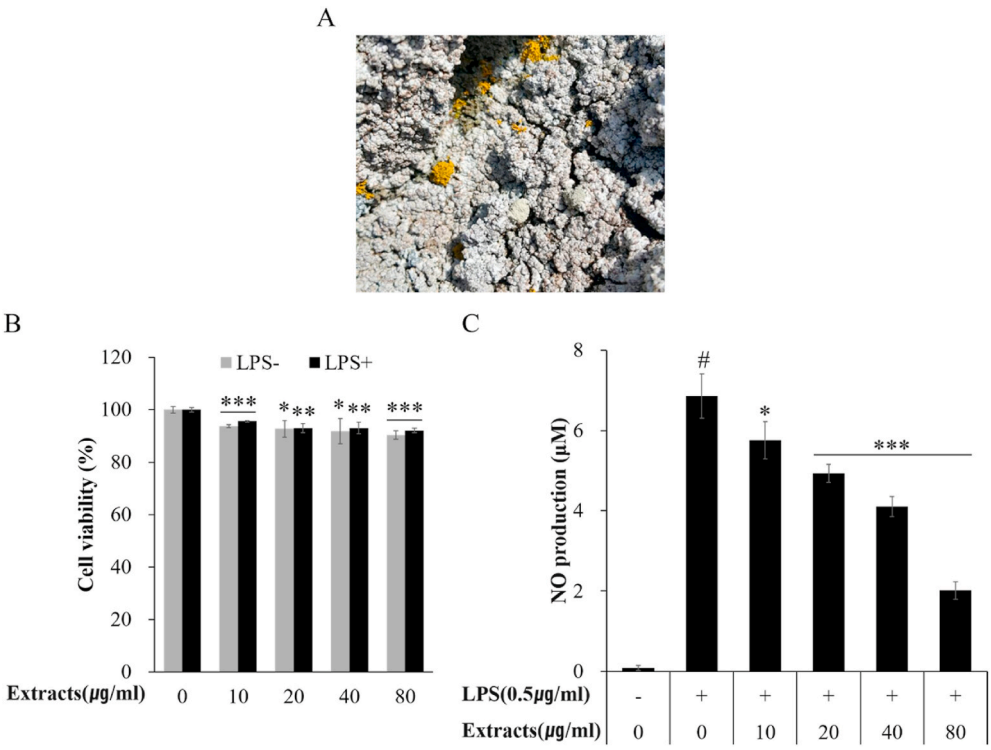


**Fig. 1.** The Antarctic lichen, *Amandinea* sp. in its natural habitat (A). Effects of the extracts of *Amandinea* sp. on cell viability rate (B), and NO production (C). These data represent the mean ± SEM of three replicates. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ vs. LPS stimulation group (#).

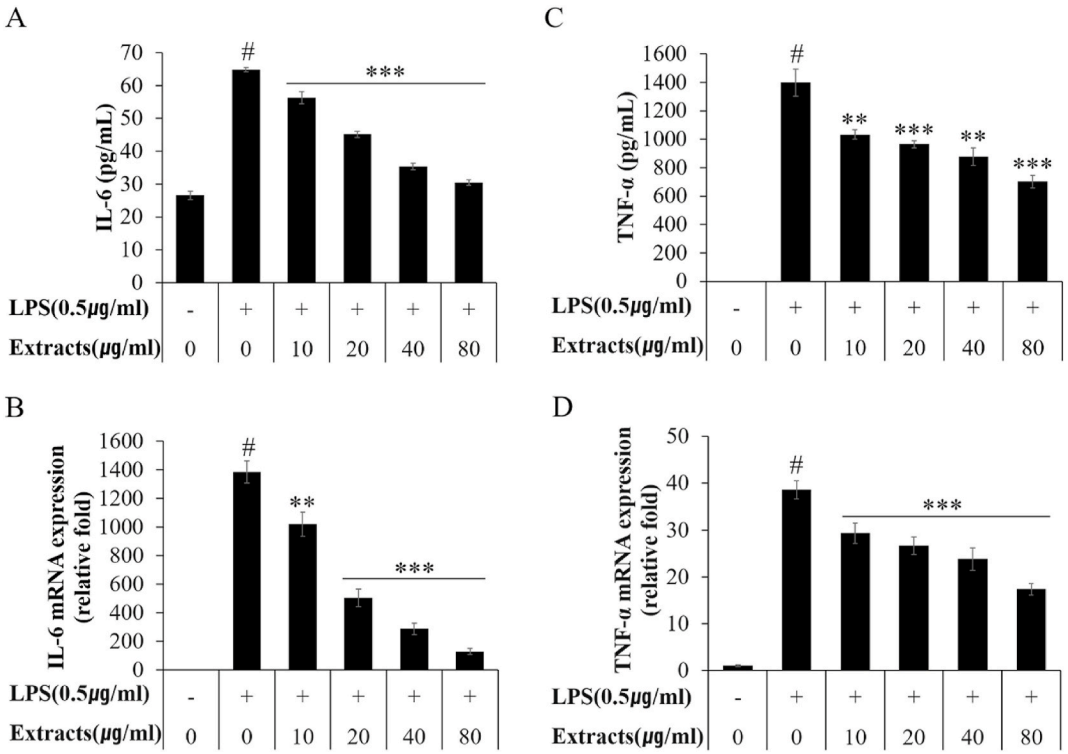


**Fig. 2.** Evaluation of Inflammatory gene expression in LPS-stimulated Raw 264.7 cells using ELISA and qPCR. Protein levels of IL-6 (A) and TNF-α (C). Relative expression of IL-6 (B) and TNF-α (D). These data represent the mean ± SEM of three replicates. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ vs. LPS stimulation group (#).

inflammatory effects of collected samples were analyzed in Macrophages Raw 264.7 cells using the MTT and NO assays (data not shown). Lichens are well known to produce diverse secondary metabolites. Secondary metabolites of lichens reported to display diverse biological activities [34–37]. The biological activities of extracts from various species of lichens have also been studied extensively, however, studies relating to *Amandinea* sp. are limited [38–40]. Therefore, to identify the anti-inflammatory effects of the extracts from *Amandinea* sp., we

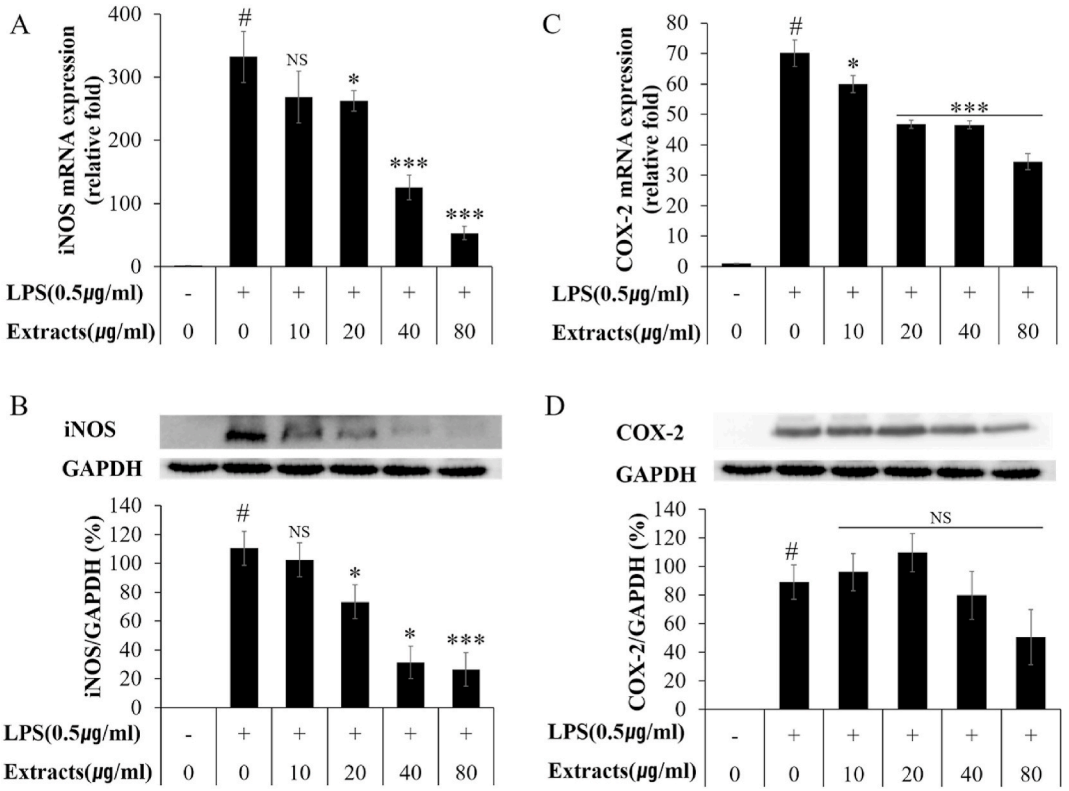


**Fig. 3.** Evaluation of Inflammatory gene expression in LPS-stimulated Raw 264.7 cells using qPCR and western blotting. Relative expression of iNOS (A) and COX-2 (C). Protein levels of iNOS (B) and COX-2 (D). These data represent the mean ± SEM of three replicates. NS, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ vs. LPS stimulation group (#).

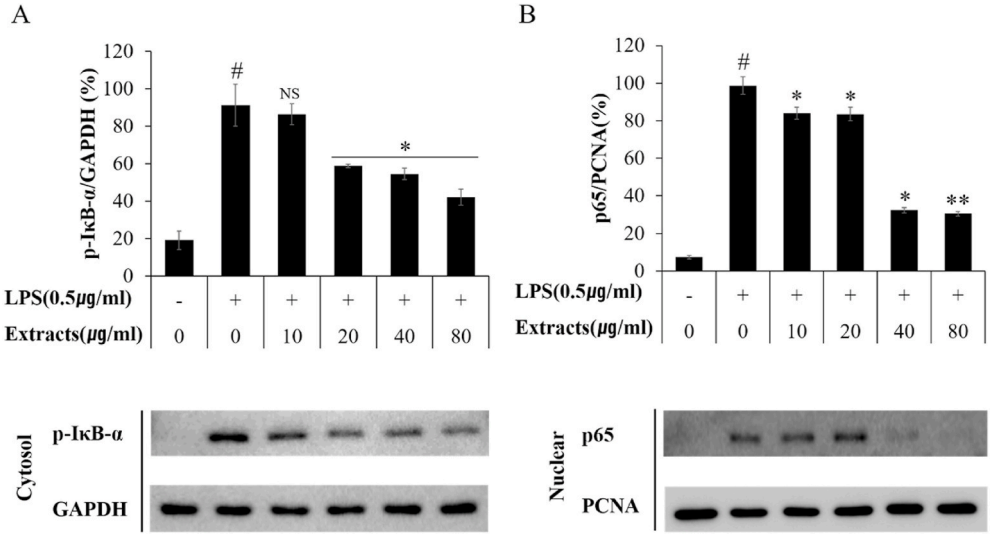


**Fig. 4.** Evaluation of protein expression on the NF-κB signaling pathway in LPS-stimulated Raw 264.7 cells using western blotting. *p*-lκB-α (A), and p65 (B). These data represent the mean ± SEM of three replicates. NS, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ vs. LPS stimulation group (#).

investigated the factors that mediate inflammatory responses.

Inflammation is known to cause various diseases, and inflammatory cells overexpress pro-inflammatory cytokines, chemokines, adhesion molecules, including COX-2 and iNOS. Macrophages produce and secrete secondary metabolites of NO, PGE2, leukotriene and pro-inflammatory cytokines [41]. We found that the extracts inhibited the production of NO, IL-6, TNF-α, iNOS and COX-2 in LPS-stimulated Raw 264.7 cells.

NF-κB is a transcription factor that plays an important role in immunity and inflammatory responses. Activated NF-κB induces the expression of various inflammatory factors [42]. The NF-κB is known to a transcription factor that regulates over 150 genes, including IL-6, TNF-α, iNOS, COX-2 and IL-1β [43]. IκB-α plays an important role in NF-κβ activation mediated by LPS, TNF, or IL-1. Activation of the IκB-α occurs by LPS-induced phosphorylation, leading to the nuclear translocation of p65 [44]. In this study, treatment with *Amandinea* sp. extracts effected in a decrease in *p*-IκBα and p-65 levels, indicating that components in the extracts inhibited NF-κB signaling, resulting in

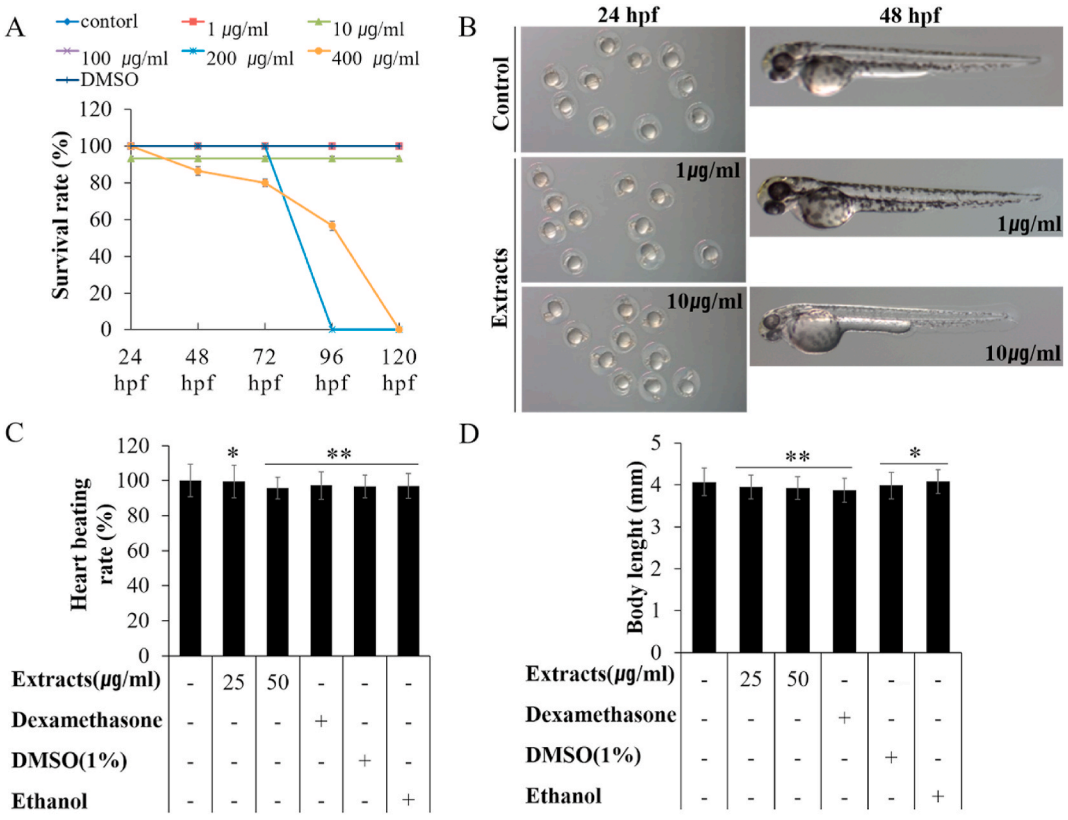


**Fig. 5.** Effects of the *Amandinea* sp. extracts on survival rate (A), morphological changes (B), heart beat rate (C), and body length of zebrafish embryo (D). $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

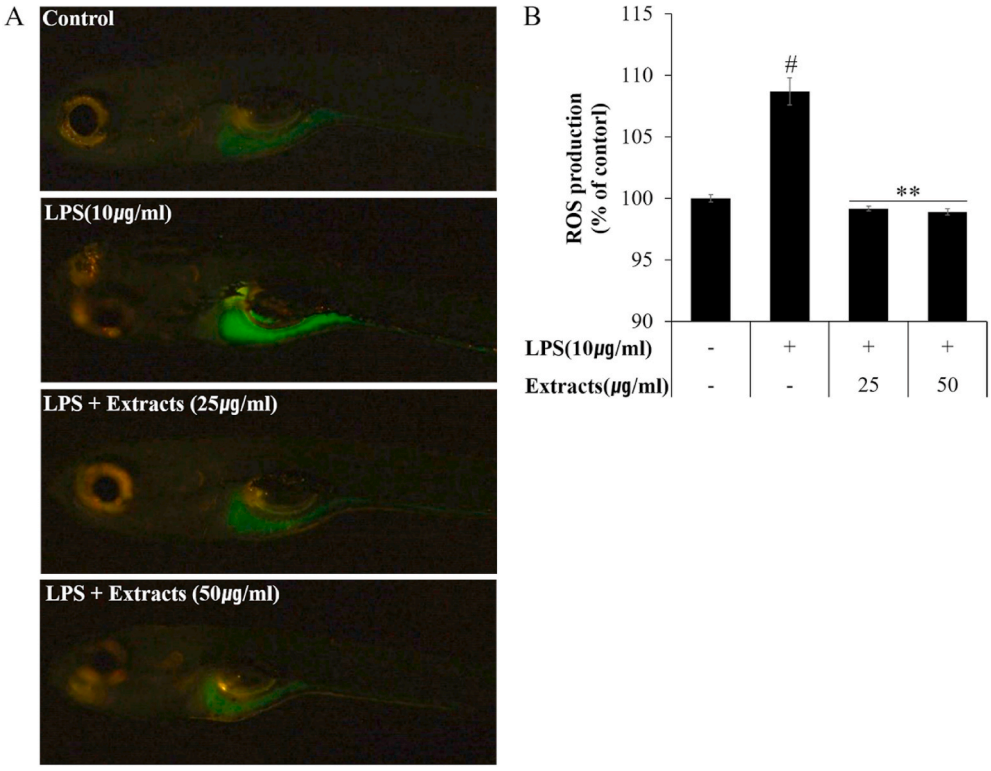


**Fig. 6.** Evaluation of ROS accumulation in LPS-stimulated zebrafish larvae using DCF-DA. The levels of ROS generation were measured by fluorescence microscope (A). The fluorescence intensities of ROS levels in individual zebrafish were quantified using an image J program (B). $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

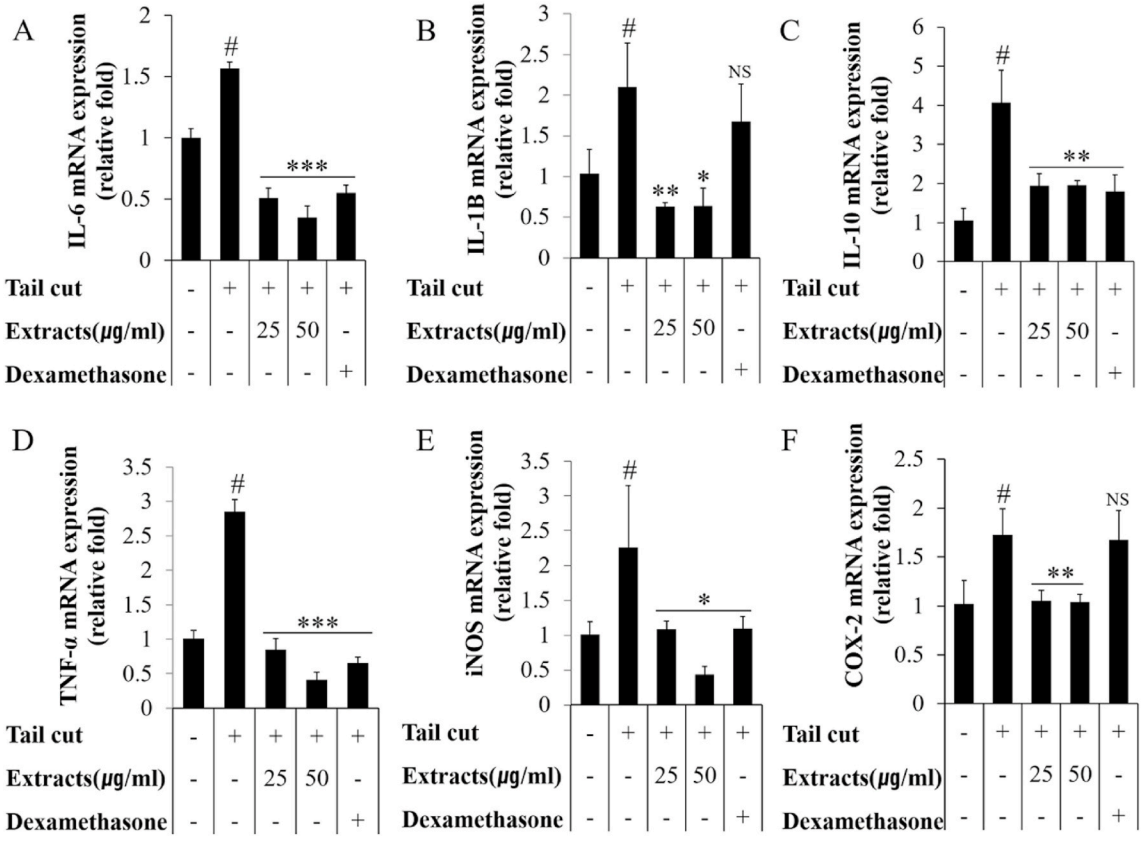


**Fig. 7.** Evaluation of Inflammatory gene expression in zebrafish larvae after tail cutting using qPCR. Relative expression of IL-6 (A), IL-1β (B), IL-10 (C), TNF-α (D), iNOS (E), and COX-2 (F). NS, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ vs. tail-cut (#).

decreased expression of cytokines and mediators such as TNF-α, IL-6, and NO. All things considered, we conclude that *Amandinea* sp. extracts contains components capable of inhibiting the inflammation reaction via NF-κB signaling.

The ROS levels are strongly associated with inflammation, and the high ROS production due to oxidative stress lead to cell or tissue injury. The ROS accumulation in activated neutrophils and macrophages induces the release of pro-inflammatory cytokines and mediators [45,46]. We observed a dose-dependent reduction in the ROS generation using *in vivo* tests with zebrafish. Moreover, the extracts reduced the mRNA expression of pro-inflammatory cytokines and mediators in the tail-transection model.

Our findings provide evidence, for the first time, that the extracts of the Antarctic lichen, *Amandinea* sp. has an anti-inflammatory effect *in vitro* and *in vivo*. Our studies suggest that the extracts of *Amandinea* sp. maybe a potential therapeutic candidate for inflammatory diseases. However, further studies are needed to test whether these effects are due to a new bioactive compound.

## Declaration of competing interest

There are no conflicts of interest to declare.

## Acknowledgements

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