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Screening of antimicrobial and antiinflammatory activities of three lichenized fungal extracts collected from Northwest Anatolia (Türkiye)

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ABSTRACT: In this study, we investigated the anti-microbial and anti-inflammatory activities of the cosmopolite macrolichens *Usnea articulata* (L.) Hoffm., *Umbilicaria crustulosa* (Ach.) Lamy and *Bryoria fuscescens* (Gyeln.) Brodo & D.Hawksw hydroalcoholic extracts to contribute the potential pharmacological uses of lichens. In vitro antimicrobial activities of ethanol extracts against Gram-negative bacteria *Escherichia coli*, Gram-positive bacteria *Staphylococcus aureus*, and the yeast *Candida albicans* were presented using the Broth microdilution method. The most effective lichen extract against gram-positive bacteria *S. aureus* was *U. articulata* ethanol extract with a MIC value of 0.125 mg/ml. *U. articulata* and *B. fuscences* extracts have similar anti-fungal activities despite having MIC values of 0.5 mg/ml. The anti-inflammatory effects of the extracts on Lipopolysaccharide/Interferon-gamma (LPS/IF-γ) induced macrophage-like cellular systems (BV-2 microglia and RAW 264,7 macrophages) were evaluated by measuring P38 mitogen-activated protein kinase phosphorylation (P38MAPK), cyclooxygenase 2 (COX-2) and inducible nitric oxide synthase 2 (NOS2) mRNA and protein expression. Especially, *Usnea* and *Umbilicaria* extracts also attenuated the LPS/IF-γ induced increase in P38MAPK phosphorylation, COX-2, and NOS2 expression in both macrophage-like cells without any cytotoxicity. According to the results of our study, we suggest that the anti-inflammatory mechanism of lichen extracts might result from the inhibition of P38MAPK phosphorylation through a reduction in COX-2 and NOS2 expressions.

KEYWORDS: Inflammation; Lichens; Microglia; Lipopolysaccharide; Interferon-gamma

1. INTRODUCTION

Lichens have a wide distribution all over the world, from the Arctic to the tropics, from the plains to the mountains and some are even able to survive in difficult desert environments. Lichens are famous for their symbiotic relationship with algae, fungi, or cyanobacteria, and these unique organisms create a variety of chemical substances known as secondary metabolites or lichen acids. A broad range of unique chemical compounds can be produced with them and accepting the use of lichens as a source for colorants, cosmetics, and medicines dates back to ancient times. These compounds such as secondary metabolites and lichen acids exhibit a broad range of biological activities, including anti-oxidant, anti-biotic, anti-viral, anti-inflammatory, and anti-proliferative properties [1,2].

Numerous types of pathogenic bacteria are resistant to various medications. To overcome this phenomenon, researchers have targeted the development of new antibacterial compounds. For this purpose, a series of lichens were screened for antibacterial activity [3]. As a result, several lichen compounds (Phomol isolated from *Usnea sp.* with MIC value 21.1 µg/mL against *Staphylococcus aureus*, Floricolin C isolated from *Umbilicaria* sp. with MIC value 8 µg/mL against *Candida albicans,* etc.) were found to be active against Gram-positive and negative bacteria and even mycobacteria [4,5].

As a consequence of pathogen invasion in the body, non-sterile inflammation develops as a natural immune defense mechanism. However, in the absence of pathogens, sterile inflammation can be induced by damage-associated molecular patterns (DAMPs) released from cells or formed in the interstitial space [6]. Sentinel immune cells, mainly mast cells, dendritic cells, and macrophages, respond to inflammation by starting the processes that result in the classic signs of inflammation. On the other hand, Microglial cells are immune cells of the central nervous system and respond to inflammation using a comparable mechanism to circulating macrophages. Pathogen-associated molecular patterns (PAMPs) and DAMPs both trigger the pattern recognition receptors (PRRs) of initiator cells [7]. Mitogen-activated protein kinases (MAPKs),

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Nuclear Factor-kappa B (NF-kB), and phosphoinositide 3-kinases/protein kinase B (PI3K/AKT) signaling pathways are all activated as a result of the interaction between PAMPs (Lipopolysaccharide, LPS) and PAMPs-receptors. The interaction of PAMP/PAMP-receptor causes a powerful inflammatory response [8]. When exposed to a variety of PAMPs, macrophages quickly activate and release a wide variety of cytokines and chemokines. In response to PAMPs, macrophages not only secrete cytokines and chemokines but also activate proinflammatory inducible nitric oxide synthase (NOS2) and cyclooxygenase-2 (COX-2) enzymes [10]. In a previous study, the anti-inflammatory activity of the methanol extract of the *Umbilicaria antarctica* species was demonstrated by reducing COX-2 and iNOS mRNA levels in LPS-stimulated macrophage cells [11].

In accordance with these facts, in this study, we investigated the antimicrobial activity of three cosmopolite macrolichen species (*U. articulata*, *U. crustulosa,* and *B. fuscescens*) ethanol extract collected from Northwest Anatolia against *E. coli*, *S. aureus,* and *C. albicans.* Besides, we tested the anti-inflammatory effects of lichen extracts on macrophage-like cellular systems (BV-2 microglia and RAW 264,7 macrophages) stimulated by LPS-INF-γ.

2. RESULTS

2.1. Anti-microbial and Anti-fungal Effects of Lichen extracts

The most effective lichen extract against gram-positive bacteria *S. aureus* was *Usnea articulata* ethanol extract with a MIC value of 0.25 mg/ml. The MICs of *U. articulata*, *U. crustulosa,* and *B. fuscences* extracts were 0.5 mg/ml for gram-negative bacteria *E. coli*. *U. articulata* and *B. fuscences* extracts have similar anti-fungal activities despite having MIC values of 0.5 mg/ml (Table 1).

Table 1. MIC values of lichenized fungal extracts and against *E. coli*, *S. aureus,* and *C. albicans.*

Microorganisms	MIC Values		
	U. articulata	*U. crustulosa*	*B. fuscescens*
Escherichia coli	0.5 mg/mL	0.5 mg/mL	0.5 mg/mL
Staphylococcus aureus	0.25 mg/mL	0.5 mg/mL	>0.5 mg/mL
Candida albicans	0.5 mg/mL	>0.5 mg/mL	0.5 mg/mL

2.2. Effect of Lichen Extracts on BV-2 and RAW 264,7 Cell Viability

Firstly, the study aims to determine the non-toxic dose of extract to use in further experiments. *Usnea*, *Umbilicaria,* and *Bryoria* extracts did not show cytotoxic effects in BV-2 cells at concentrations below 80 µg/mL (Figure 1A). For RAW 264,7 cells (Figure 1B), the non-cytotoxic concentration for *Bryoria* extract is below 60 µg/mL, whereas *Usnea* and *Umbilicaria* extracts do not have cytotoxic effects at concentrations below 40 µg/mL. Based on the MTT results, non-cytotoxic concentrations of 40, 40, and 30 µg/mL were selected for the extracts of *Usnea*, *Umbilicaria,* and *Byroria*, respectively, to test the potential protective effect of the extracts against the inflammatory response induced by LPS/IFN-γ.

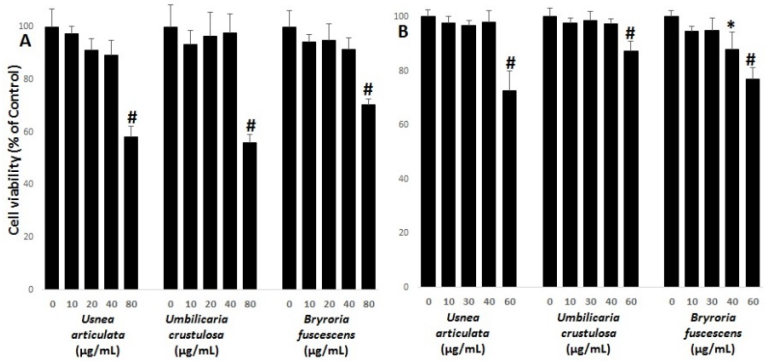

Figure 1. Effects of Lichenized Fungal Extracts on Microglia Cells BV-2 (A) and macrophage cells Raw 264,7 (B) viability. n=4, *p<0.05 vs. control, #p<0.01 vs. control.

2.3. LPS/IFN-γ Combination Induced Inflammatory Enzymes COX-2 and NOS2

The inflammatory response induced by the combination of LPS+IF-γ at varying time intervals (1, 3, and 6 hours) was tested by measuring COX-2 and NOS2 mRNA levels in both BV-2 and RAW 264,7 cells. At 3 and 6 hours, inflammatory stimuli (LPS/IFN-γ combination) increased the mRNA levels of inflammatory enzymes (COX-2 and NOS2) in BV-2 cells, but at 1 hour, there was no noticeable difference (Figure 2A). Inflammatory stimuli showed similar effects in RAW 264,7 cells to BV-2 cells. One hour of incubation with inflammatory stimuli did not significantly alter the mRNA levels of inflammatory enzymes, but 3 and 6 hours of incubation markedly elevated COX-2 and NOS2 mRNA levels (Figure 2B). 3 hours of incubation of the LPS/IFN-γ combination was chosen to induce inflammation in further experiments.

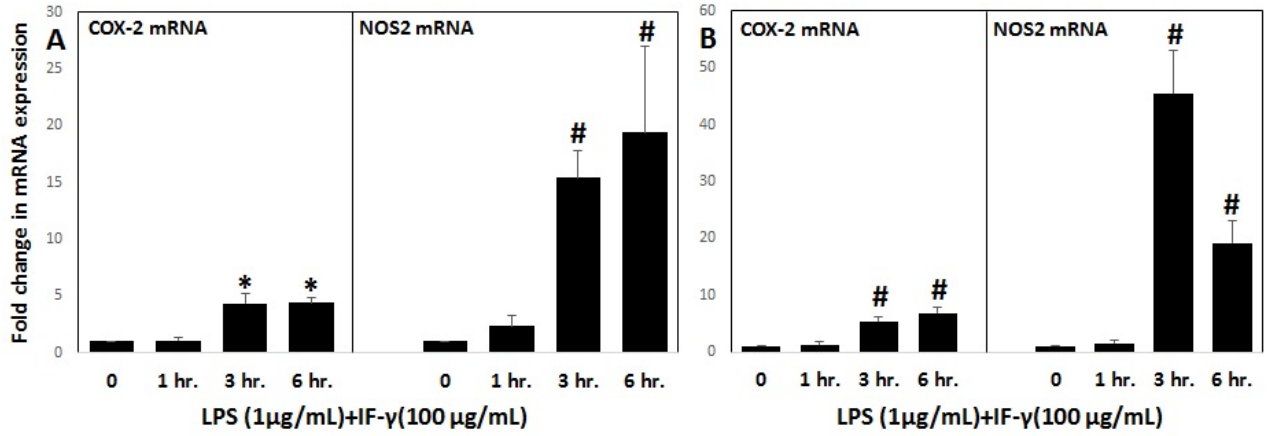

Figure 2. Fold change in mRNA expression of proinflammatory enzymes (COX-2 and NOS2) after treatment with LPS/IF-γ combination (LPS 1µg/mL+IF-γ 100µg/mL) in different time incubation periods. (A) Fold change in mRNA expression of proinflammatory enzymes in BV-2 cells, (B) Fold change in mRNA expression of proinflammatory enzymes in Raw 264.7 cells. n=3, *p<0.05 vs. control, #p<0.01 vs. control.

2.4. Lichen Extracts Reduced COX-2 and NOS-2 mRNA Levels in LPS/IFN-γ Induced Inflammation

Pretreatment with *U. articulata* and *U. crustulosa* extract caused a reduction in COX-2 and NOS2 mRNA levels in LPS/IFN-γ stimulated BV-2 (Figure 3A) and RAW 264,7 cells (Figure 3B). Interestingly, treatment with *B. fuscescens* extract alone enhanced COX-2 (3.46 fold in RAW 264,7 cell) and NOS2 (21.2 fold in RAW 264,7 cell) levels in comparison to control cells, even though it had no protective impact against the LPS/IFN-γ induced overexpression of COX-2 and NOS2.

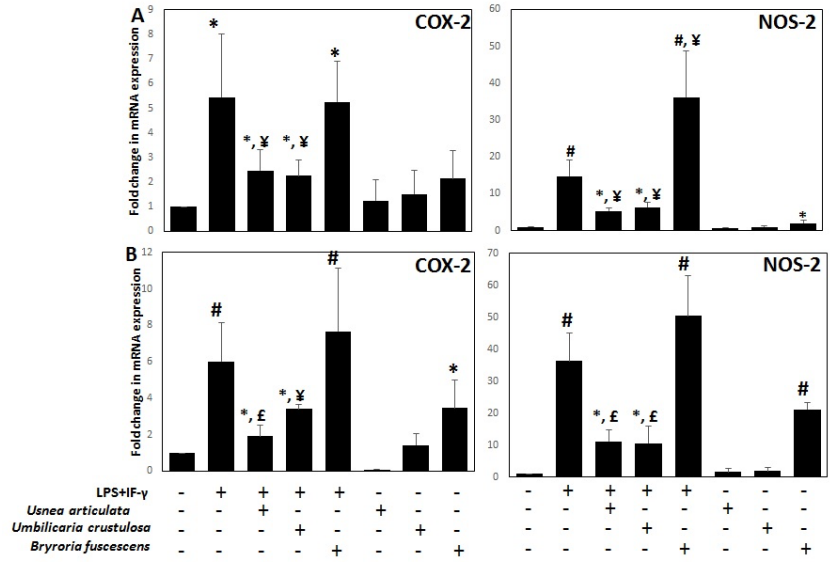

Figure 3. Effects of Lichenized Fungal Extracts (*U. articulate* 40µg/mL, *U. crustulosa* 40µg/mL, and *B. fuscescens* 30µg/mL) on LPS/IF-γ stimulated mRNA expression of proinflammatory enzymes (COX-2 and NOS2). (A) BV-2 cells were pretreated with Lichenized Fungal Extracts for 24 h. and then treated with LPS/IF-γ for 3 h. (B) Raw 264,7 cells

were pretreated with Lichenized Fungal Extracts for 24 h. and then treated with LPS/IF-γ for 3 h. n=3, #p<0.01 vs. control, *p<0.05 vs. control, £p<0.01 vs. LPS+IF-γ, ¥p<0.05 vs. LPS+IF-γ.

2.5. Lichen Extracts Reduced P38MAPK Phosphorylation, COX-2 and NOS2 Protein Levels in LPS/IFN-γ Induced Inflammation

This study has established that activation of the p38 MAPK is involved in LPS/IFN-γ-stimulated COX-2 and NOS2 expression in both BV-2 and RAW264,7 cells. In this study, LPS/IFN-γ significantly increased both COX-2 and NOS2 protein levels in BV-2 (4.2 fold COX-2 and 3.7 fold NOS2) and RAW 264,7 (4.47 fold COX-2 and 2.7 fold NOS2) cells. *Usnea* and *Umbilicaria* extracts showed anti-inflammatory effects by suppressing P38/COX-2 and p38/NOS2 signaling pathways in both BV-2 (Figure 4A) and RAW264,7 cells (Figure 4B).

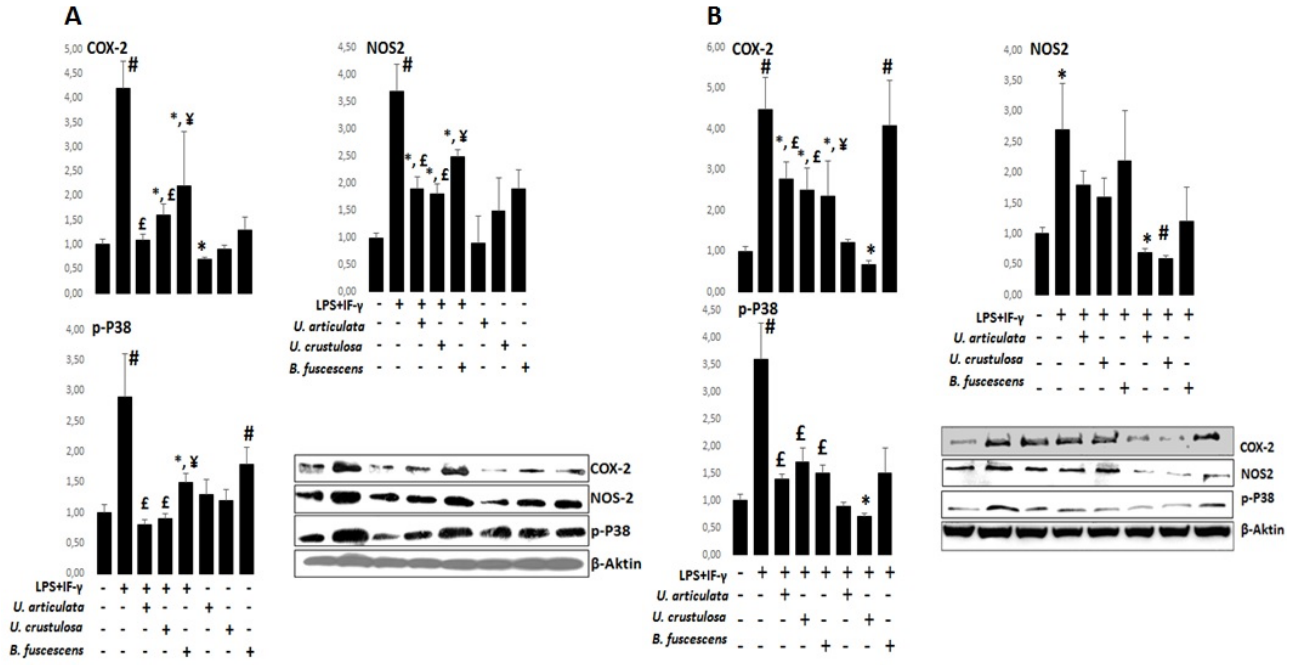

Figure 4. Effects of Lichenized Fungal Extracts (*U. articulate* 40µg/mL, *U. crustulosa* 40µg/mL and *B. fuscescens* 30µg/mL) on LPS/IF-γ stimulated protein expression of proinflammatory enzymes (COX-2 and NOS2) and P38 activation (phosphorylation). (A) BV-2 cells were pretreated with Lichenized Fungal Extracts for 24 h. and then treated with LPS/IF-γ for 3 h. (B) Raw 264,7 cells were pretreated with Lichenized Fungal Extracts for 24 h. and then treated with LPS/IF-γ for 3 h. n=3, #p<0.01 vs. control, *p<0.05 vs. control, £p<0.01 vs. LPS+IF-γ, ¥p<0.05 vs. LPS+IF-γ.

3. DISCUSSION

In our research, we examined the anti-bacterial and anti-fungal properties of three cosmopolite macrolichen species that are collected from the richest area in Anatolia in terms of oxygen level, Mount IDA (Northwest Anatolia) with regard to the inflammatory response in macrophage-like cell systems induced by the lipopolysaccharide/interferon-γ combination.

First, we tested the cytotoxic effect of lichen extracts on macrophage-like cells. The purpose of the cytotoxicity investigation was to establish the non-cytotoxic amounts of lichen extracts despite the inflammatory stimulation. We found 40 µg/mL for *U. articulata* and *U. crustulosa* and 30 µg/mL for *B. fuscescens* as the highest non-cytotoxic concentrations, and we investigated the potential protective effects of these concentrations against Lipopolysaccharide/Interferon-γ-induced inflammation.

Lipopolysaccharide-induced p38 MAPK activation plays an active role in the positive regulation of several inflammation-related genes both in vitro and in vivo [12]. It has been demonstrated that MAPKs are crucial in the up-regulation of NOS2 caused by a variety of stimuli in different cell types [13,14]. The production of prostanoid from arachidonic acid is carried out by two isoforms of cyclooxygenase (COX). Most cell types express COX-1 constitutively, which results in the production of prostanoids that are crucial to regulating cell homeostasis. The expression of COX-2 is regulated by p38 MAPK in a variety of cell types [15-17] and is activated by mitogenic and proinflammatory stimuli [18-20]. Therefore, the P38 MAPK signaling pathway has strategic importance in the treatment of inflammation. In animal models [21,22] as

well as in humans [23], it has been demonstrated that several powerful P38 MAPK inhibitors have anti-inflammatory effects against endotoxemia. In our investigation, *Usnea*, *Umbilicaria*, and *Briyoria* extracts dramatically decreased P38 phosphorylation (activation), which was significantly elevated by the combination of lipopolysaccharide/Interferon-gamma in the cell. However, only *Usnea* and *Umbilicaria* extracts have also been shown to effectively reduce NOS2 and COX-2 proinflammatory enzyme mRNA and protein levels. These findings suggest that by lowering P38 activation by inflammatory stimuli, *Usnea* and *Umbilicaria* extracts have potential as an alternative therapeutic for the treatment of inflammatory diseases. The effects of lichen secondary metabolites were the main focus of studies demonstrating the anti-inflammatory properties of lichens. Lobaric acid isolated from *Stereocaulon alpinum* showed an anti-inflammatory effect in cells by suppressing the activation of the P38/ERK/NFkB signaling pathway in mouse smooth muscle cells stimulated with Tumor Necrosis Factor-alpha (TNF-a) [24]. In another study, the secondary metabolite ramalin isolated from the Antarctic lichen *Ramalina terebrata* has been shown to have anti-inflammatory activity in cells by suppressing the activation of P38/ERK/JNK/AP-1 signaling pathway in mouse smooth muscle cells stimulated with TNF-α [25]. In a recent experiment, the Antarctic *Amandinea* sp. methanol extract blocked the LPS-stimulated NFkB signaling pathway in RAW 264,7 macrophage cells and decreased the release of cytokines and nitric oxide [26].

Studies were done on the antibacterial qualities of lichens long before their benefits for reducing inflammation were realized. In our investigation, the antibacterial and antifungal activities of lichen extracts were generally concentration-dependent. Although *U. articulata* extract had a high antibacterial impact against *E. coli*, it had a lesser antibacterial and antifungal effect on *S. aureus* and *C. albicans*. The antibacterial efficacy of *U. crustulosa* extract against *E. coli* was comparable to that of *Usnea articulata* extract, while it was less potent against *S. aureus* and *C. albicans*. *B. fuscescens* extract was discovered to be superior to other extracts in terms of its antifungal action. Previous investigations have shown the strong antibacterial and antifungal properties of the lichen secondary metabolite usnic acid and its numerous in vitro-produced derivatives [27,28]. The antibacterial screening conducted by Micheletti et al. using 12 species of lichens and 10 secondary metabolites identified in these lichens showed *C. borealis*, *C. confusa*, *S. ramulosum*, and *C. cryptochlorophaea* extracts as the most active extracts with MIC between 7.8 and 31.25 g/mL [29].

4. CONCLUSION

In conclusion, in macrophage-like cell lines, especially *U. articulata* and *U. crustulosa* extracts showed an anti-inflammatory effect by inhibiting MAPK/NOS2 and MAPK/COX-2 signaling pathways against inflammation induced by lipopolysaccharide/Interferon-gamma combination. In addition, the biological activities of lichen extracts were not limited to their anti-inflammatory effects, but also the lichen extracts especially *Usnea* extract showed antibacterial and antifungal effects. This study shows that lichen extracts exhibit anti-inflammatory effects on active macrophage-like cellular systems, suggesting that they may have therapeutic promise in the treatment of acute and/or chronic inflammatory illnesses.

5. MATERIALS AND METHODS

5.1. Lichen Specimens

Specimens belonging to *B. fuscescens* and *U. articulata* were collected from the barks of *Pinus nigra* and specimens of *U. crustulosa* were collected from the siliceous rocks in Mount Ida (Balıkesir, Türkiye) in August 2016 by Mehmet Gökhan Halıcı (Professor in Faculty of Science, Department of Biology, Erciyes University). The duplicates of the specimens studied are stored in ERCH with accession numbers of ERCH 2.246, 2.247, and 2.248 respectively.

5.2. Extraction Method

The powdered dried lichen components were then macerated in 70% ethanol for 72 hours in a 42 °C heated shaker water bath. Extraction was carried out with an extraction ratio of 1:5 (20 g of lichen materials, 100 mL of 70% ethanol). The materials were lyophilized after the ethanol solvent was removed from the extracts in a rotavapor under vacuum.

5.3. Broth Microdilution Method

According to the National Committee for Clinical Laboratory Standards, the minimum inhibitory concentration (MIC) was calculated using the broth microdilution method [30]. In this study, *E. coli* ATCC 35218, *S. aureus* ATCC 25923K, and *C. albicans* ATCC 90028 standard strains were tested. Using 96-well microtiter plates and the broth microdilution technique, the MIC value was calculated. Mueller-Tryptic soy agar for bacteria and yeast extract agar was used to prepare two-fold serial dilutions of the extracts, resulting in a concentration range of 0.125 to 0.5 mg/ml. Overnight cultures were used to prepare the bacterial inoculates, and a turbidimeter was used to adjust suspensions to 0.5 McFarland standard turbidity. 100 µl of the bacterial suspension containing 10^6 cells per milliliter was added to each well as an inoculant. For bacterial cultures, the 96-well microtiter plates with the inoculum were incubated for 24 hours at 37°C. The MIC was determined to be the lowest extract concentration that stopped observable growth.

5.4. Cell Culture

The immortalized mouse microglial cell line BV-2 was developed in the laboratory of Dr. Blasi at the University of Perugia [31] and Raw 264,7 (***ATCC*** TIB 71) macrophage cells were cultured in Dulbecco Eagle's Minimum Essential Medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Sigma-Aldrich), 100 U/mL penicillin, 100 µg/mL streptomycin (Sigma Aldrich). Standard cell culture protocol was followed [32].

5.5. MTT Cytotoxicity Assay

Bv-2 microglia and Raw 264,7 macrophages were seeded in 96-well cell culture plates at $1.5x10^4$ cells/well and 10^4 cells/well, respectively. Lichen extracts (100 µL) were added to each well in varying concentration ranges (0-80 µg/mL). The media containing the extract in the wells was removed at the end of the 24-hour incubation period and then 10 µl of MTT reagent (5 mg/ml) was added. After incubation with MTT for 4 hours, purple crystals formed in the wells were dissolved by adding 100 µl of dimethylsulfoxide. The absorbance values in the wells were recorded in the microplate reader.

5.6. Real-Time Polymerase Chain Reaction (RT-PCR) Analysis

The mRNA expression levels of COX-2 and NOS2 were estimated by qRT-PCR. Total RNA was extracted with the TRIzol® Reagent following the protocol provided by the manufacturer. Total RNA was reversely transcribed to complementary DNA (cDNA) using a Roche cDNA synthesis kit (Roche cat. no: 07 912 374 001), thermal cycler steps of conditions set by following the protocol provided by the manufacturer. Roche catalog assays (primer prob mix for COX-2, NOS2, and β-Actin) were used in this study for measuring quantitative PCR. The thermal cycling programs were carried out in a real-time PCR system (LightCycler 480) supplemented with the Roche Master Mix Kit (Roche cat. no: 14554900). The average expression level of the testing genes from qRT-PCR analysis was utilized from the result of three replicates for examination using relative quantification.

5.7. Western Blot Analysis

Cells were pretreated with lichen extract (40 µg/mL *Usnea*, 40 µg/mL *Umbilicaria*, *30 µg/mL Bryoria*) followed by treatment with LPS+IF-γ (1 µg/mL LPS+ 100 µg/mL IF-γ) combination for induction of inflammatory response. After the treatment procedure, cells were lysed in RIPA lysis buffer (25mM Tris (pH 7.4), 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40) supplemented with protease inhibitor cocktail (Complete MiniTM, Roche, Mannheim, Germany) and 1 mM Na_3VO_4. Protein concentrations were determined by using the BCA protein assay (Pierce). 30 µg protein was heated for 5 min at 94°C in Laemmli sample buffer containing 4% *β*-mercaptoethanol and loaded on 4–12% Tris-glycine SDS-PAGE gels, then transferred electrophoretically to PVDF membranes. Membranes were incubated overnight at 4°C with COX-2, NOS2, phospo-P38, and β-actin antibodies (BT LAB, Shanghai, China), Protein bands were detected with horseradish peroxidase-conjugated secondary antibodies (YL Biont, Shanghai Biotech Co. LTD., China) and visualized by West-Pico ECL reagents (Pierce).

5.8. Statistical Analysis

All data were presented as mean±SD. analyzed by SigmaPlot 15 software. Comparisons among groups were analyzed with the student-t test and $p<0.05$ was considered a significant difference.

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Conflict of interest statement: The authors declare that they have no competing interests.

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