Journal of *Marine Science and Engineering*

MDPI

Article

Anti-Inflammatory Activity of Mycobiont Extract of *Parmotrema austrosinense* (Zahlbr.) Hale in a Zebrafish Model

Kalidoss Rajendran [1], Ponmurugan Karuppiah [2,*], Ponmurugan Ponnusamy [3], Mohammed Rafi Shaik [4], Mujeeb Khan [4], Tae Hwan Oh [5] and Baji Shaik [5,*]

[1] Department of Education, St. Joseph University in Tanzania, Dar es Salaam P.O. Box 11007, Tanzania; doss_rk@yahoo.com
[2] Department of Botany and Microbiology, College of Science, King Saud University, P.O. Box 2455, Riyadh 11451, Saudi Arabia
[3] Biomedical Research Laboratory, Department of Botany, Bharathiar University, Coimbatore 641046, Tamil Nadu, India; drponmurugan@gmail.com
[4] Department of Chemistry, College of Science, King Saud University, P.O. Box 2455, Riyadh 11451, Saudi Arabia; mrshaik@ksu.edu.sa (M.R.S.); kmujeeb@ksu.edu.sa (M.K.)
[5] School of Chemical Engineering, Yeungnam University, Gyeongsan 38541, Republic of Korea; taehwanoh@ynu.ac.kr
* Correspondence: pkaruppiah@ksu.edu.sa (P.K.); shaikbaji@yu.ac.kr (B.S.)

Abstract: Thousands of different kinds of lichen metabolites are being examined for their biological activities, including anticancer properties. In this context, the present study aims to assess the anti-inflammatory activity of the acetone extract of *Parmotrema austrosinense* mycobiont. A lipid peroxidation assay was performed with the acetone extracts of *P. austrosinense* mycobiont, which was further used to evaluate its anti-inflammatory efficacy using a zebrafish model. Furthermore, the histopathological study was also carried out with muscle tissues and amplification of its inflammation marker. The results revealed that the lichen compound (i.e., lecanoric acid) in the acetone extract of *P. austrosinense* possesses anti-inflammatory activity. Histopathology studies confirmed the decreased numbers of neutrophil cells in the 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced zebrafishes, as confirmed by changes in the fishes' weight before and after the sample treatment, prompted by TNBS inflammation. The present results also demonstrated a dose-dependent decrease in the lipid peroxidation (LPO) levels in the muscle tissues of zebrafishes. Gene amplification studies suggested that the lichen compound might perform dose-dependent downregulation of the inflammatory gene marker of the tumor necrosis factor (TNF)-α gene; this further confirms that the extract should possess anti-inflammatory activity. As per the literature, this study is one of the most complete, comprehensive in vivo anti-inflammatory analyses in which inflammation was induced in zebrafish by using 2,4,6-trinitrobenzene sulfonic acid (TNBS). Particularly, this study successfully identified a bioactive compound isolated from the lichen *P. austrosinense*, and which exhibited decent anti-inflammatory activity.

Keywords: lichen; anti-inflammatory activity; lipid peroxidation assay; zebrafish

Citation: Rajendran, K.; Karuppiah, P.; Ponnusamy, P.; Shaik, M.R.; Khan, M.; Oh, T.H.; Shaik, B. Anti-Inflammatory Activity of Mycobiont Extract of *Parmotrema austrosinense* (Zahlbr.) Hale in a Zebrafish Model. *J. Mar. Sci. Eng.* **2023**, *11*, 1081. https://doi.org/10.3390/jmse11051081

Academic Editors: Ana Catarina Matias and Carlos Andrade

Received: 14 April 2023
Revised: 10 May 2023
Accepted: 18 May 2023
Published: 20 May 2023

1. Introduction

Phenolic compounds of lichen extracts used for biological activities are all derivatives of the orcinol type [1,2]. They are aromatic compounds containing phenolic carboxylic acids. All of these can be broadly classified into two types: depsides and depsidones [3]. Some of the depsides used in the research laboratory include lecanoric acid and erythrin. Lecanoric acid is distributed in *Parmotrema austrosinense* and *P. tinctorum* [4], while erythrin is present in *Roccella montagnei* [5]. There are wide varieties of depsidones that are used for their biological activities, such as salazinic acid, protocetraric acid, etc. [6,7]. Salazinic acid is widely distributed in *Parmotrema reticulatum* and *Parmelinella wallichiana*, whereas

protocetraric acid has been found in *Flavoparmelia caperata* [8]. The most commonly used lichen compound in the pharmacological research laboratory setting is usnic acid, which is a novel compound derived from dibenzofuran derivatives [9].

Several methods can be used to determine the anticancer abilities of lichen substances—mainly computational, in vivo, and in vitro methods [10]. Some species contain more than one metabolite. The yield of the metabolites from various lichen species must be ascertained before their clinical use. This accounts for 22% of the total dry weight of *Parmotrema tinctorum*. A large number of lichen compounds—such as anziaic acid, bonimic acid, divaricatic acid, lobaric acid, olivetoric acid, protolichesterinic acid, ramalinolic acid, spherophorin, and sekikaic acid—are associated with antibacterial action against *Bacillus subtilis*, *Mycobacterium tuberculosis*, *Mycobacterium smegmatis*, *Pneumococcus* sp., *Salmonella typhimurium*, *Staphylococcus aureus*, and *Streptococcus pyogenes* [11,12]. On the other hand, lichen substances such as caperatic acid, rangiformic acid, zeorin, atranorin, thamnolic acid, salazinic acid, psoromic acid, fumarprotocetraric acid, pannarin, and endocrocin are not effective against several test strains [13].

In India, the bioprospecting of lichen started very recently. The ethnobotanical uses of lichens by various ethnic groups in India have been documented. Over 80% of the world's population—especially in underdeveloped nations—still relies on herbal remedies as their primary form of healthcare today [14]. This has drawn substantial attention from the scientific community to investigate the mycobionts and photobionts (and their related biological activities), as a result of the influence of lichens in bioprospecting studies and their application [15]. The *Parmotrema* spp. thallus contains 23.5% lecanoric acid and 37.5% usnic acid. The medullary layer also contains protocetraric acid, physodalic acid, and physodic acid. These lichens have been identified and used throughout human history [6].

Considering their importance, the lichen compounds of specific species play a significant role in pharmacopeias due to their antimicrobial, antiviral, anti-inflammatory, antioxidant, anti-analgesic, antipyretic, antiproliferative, and anticancer activities. Moreover, they are receiving wide attention in the field of pharmacy and have already contributed to several advances in medicinal applications, including anti-snake-venom properties [16,17]. A considerable number of lichen species can cure jaundice or coughs and control rabies. The most important species are *Cladonia*, *Peltigera*, *Roccella*, and *Usnea*. Some species, such as *Parmelia sulcata* and *Lobaria pulmonaria*, can cure cranial and pulmonary diseases. *Xanthoria paritinais* has been used in the treatment of jaundice. *Cetraria islandica* displays medicinal properties for bladder, kidney, and lung diseases and heals oral inflammation. It can also control the proliferation of cancer cell lines [18]. More than a thousand different secondary metabolites of lichens have had their structures determined so far, although many of them still need to be described [19]. Cytotoxic ability, which is imparted by the presence of polyphenolic compounds in the fungal cells, is the term used to describe the most diverse biological activities of this group of organisms. Moreover, various secondary metabolites of lichens are known to exhibit anti-inflammatory properties, which are less frequently reported. Inflammation is a defense mechanism that controls and repairs the damage caused in the body, accompanying a number of diseases. In the event of inflammation, biochemical changes leading to redness, swelling, itching, pain, and increased temperature occur [20]. Particularly, depsides and depsidones have been reported to exhibit decent anti-inflammatory properties. For instance, the depside atranorin, which is present in the thalli of different species of lichen, has anti-inflammatory properties [21]. Bugni et al. studied the effects of a cyclooxygenase inhibitor [22]. The obtained results indicated that the tested depside inhibited cyclooxygenase-1 (COX-1) in a dose-dependent manner and, at the concentration of 17 μg/mL (45 μM), caused a decrease in enzyme activity by 50%. In addition, lecanoric acid is another depside produced by various lichen species, and it is also known to possess anti-inflammatory properties [23]. In this context, the present study is aimed at the isolation and characterization of bioactive compounds from the lichen mycobiont *P. austrosinense*, with a major objective of evaluating the anti-inflammatory effect of lecanoric acid in a zebrafish model.

2. Materials and Methods

2.1. Chemicals

All of the chemicals used in this study were procured from SD Fine Chem Limited, Chennai, Tamil Nadu, India. All of the chemicals were of analytical grade.

2.2. Collection and Identification of P. austrosinense

The Tamil Nadu Western Ghats (10°10′11″ N 77°03′40″ E), India, at an elevation of 2695 m (8842 ft) above sea level, is an ideal location for the present study, owing to the presence of wildlife reserves and national parks. The major reason behind the selection of this area was the availability of ethnobotanical samples obtained through prior research conducted in the region. Additionally, the region is readily accessible [24,25]. To authenticate a particular lichen sample, the representative samples were compared with the preserved samples at the National Botanical Research Institute (NBRI), Lucknow, India. The identification manual of Awasthi [13,26] was closely monitored. Thus, in addition to microscopic examination, representative samples were subjected to spot tests, chemical profiling, and thin-layer chromatography (TLC) testing to determine the lichen species. To study the morphological, structural, vegetative, and reproductive features of the lichen thalli, stereomicroscopy was used, except for anatomical sections, for which a compound light microscope was used. The lichen compounds were extracted from the thallus by the Soxhlet method, using acetone as the solvent [27].

2.3. Extraction of Bioactive Compound

Lecanoric acid was produced and extracted by the method described by Verma and Behera [28]. Briefly, Modified Bold's Basal Salt (MBBS) medium was prepared with 1% potassium nitrate and 4% sucrose in a flask and inoculated with the mycobiont isolate, and it was incubated at 25 °C for 4–8 weeks in a shaking incubator at 150 rpm agitation. During the subsequent incubation, part of the flask's contents was used for extraction of the bioactive compound with acetone through the Soxhlet apparatus. Later, the crude culture extract was concentrated with a rotary vacuum evaporator, and it was stored at −20 °C until further use. The crude extract was investigated in the presence of bioactive compounds using TLC and microcrystallization methods.

2.4. Zebrafish Collection and Maintenance

Healthy adult zebrafishes that were free from illness or deformities were selected for the present study. The average weight and length of the zebrafishes ranged from 0.18 to 0.22 g and from 2.55 to 2.95 cm, respectively. All of the fishes were habituated in a laboratory setting before the beginning of the experiments, in accordance with the Organization for Economic Co-operation and Development (OECD) recommendations [29]. For the treatment with the test sample, the zebrafishes were divided into eight different groups, each containing 12 zebrafishes (control, TNBS-induced group, standard, 200, 400, 600, 800, and 1000 µg/mL), and all maintained in different tanks [30].

2.5. Determination of LC_{50}

The zebrafishes of interest were weighed, and their weight was used to determine how much of the sample was to be injected in a 20 µL volume of phosphate-buffered saline (PBS) at pH 7.4 [31]. In this experiment, 30 G caliber injections were applied. The doses tested were 20, 40, 60, 80, and 100 µg/mL. A foundation plane was prepared beforehand to hold the fish: a soft sponge with a 1.5 cm section cut out from the center, in a Petri dish with ice water (12 °C). Prior to anesthesia, test solutions were placed into the injection. By putting ice chips into the fish water until the temperature hit 12 °C, each fish was gradually put to sleep. Holding the fish in the sponge with the abdomen facing up, it was transferred to the base plane when the fish remained still in response to any external stimuli. Without waiting, the solutions were injected intraperitoneally, and the fish were then moved to normal water (35 °C) to recover and resume their normal behaviors [32]. The fish were

monitored for 7 days following the injection [33]. From day 0 to day 7, the number of animals that died in each concentration was recorded, and the LC_{50} value was calculated.

2.6. Induction of Inflammation and Efficacy Analysis

The 2,4,6-trinitrobenzene sulfonic (TNBS) acid injection was administered based on the weight of fish, at 1 µL per 1 mg of body weight. The 2,4,6-trinitrobenzene sulfonic acid (TNBS) is a kind of hapten. It binds to a protein (tissue), turns into an antigen, and provokes immunological reactions. TNBS-induced colitis is a delayed hypersensitivity reaction to haptenized proteins, and it disrupts the epithelial barrier. For testing the efficacy, the zebrafish were divided into eight different groups: control, TNBS-induced, standard, and five different concentrations. The acetone extracts were administered at final doses of 20, 40, 60, 80, and 100 g/mL after the TNBS injections, which were timed to elicit the best inflammation in adult zebrafishes within 4 h. The zebrafish weight and the volume of TNBS to be injected were determined, and the injection was adjusted to a final volume of 20 µL in PBS. Following the same procedure as mentioned above, TNBS was injected and weight was measured before and after treatment. The zebrafishes were killed immediately to finish the determination of the biological effects upon completion of the experiment after 4 h of treatment. The experimental zebrafish model was performed in triplicate.

2.7. Histopathology Study of Muscle Tissue

After 4 h, the zebrafishes were decapitated, and their abdominal region was dissected and fixed in Dietrich's fixative before being transferred for histopathology study. The remaining tissues were immediately stored at 4 °C for extraction of DNA. The tissues were taken out of the fixative, washed with saline water, and dehydrated with 90% alcohol. Then, the tissues were cleaned up with xylene. The sample tissues were embedded and impregnated using paraffin wax. Furthermore, the embedded block containing the tissue was trimmed for easy cutting into fine sections. Later, the block containing the tissue was cut to a 5–8 µm thickness with the help of a microtone, and the cut sections adhered to the slides firmly and in the right position. Before attaching the portions to the slide, it was coated with egg albumin. The slides were stained using azan, hematoxylin, and eosin. For pathological observations, the stained sections were examined under a light microscope, and microphotographs were obtained [33].

2.8. Genomic DNA Isolation

At this stage, 100 mg of muscle tissue was placed in a mortar and pestle and thoroughly crushed after freezing with liquid nitrogen. The pulverized tissues were dissolved in 1.2 mL of digestion buffer and incubated for 12 to 18 h at 55 °C in a water bath. Equal amounts of phenol, chloroform, and isoamyl alcohol were added, gently mixed, and centrifuged at 3000 rpm for 5 min at 37 °C. Without disturbing the middle or bottom layers, the aqueous phase was carefully removed and transferred to a new tube. Half of the volume of 7.5 M ammonium acetate and twice the volume of 100% ethanol were then added, thoroughly mixed, and centrifuged at 3000 rpm for 5 min at ambient temperature. Then, 70% ethanol was used to rinse the particles, and the ethanol was then thrown away. The pellet was kept to dry in the air. For further processing, the pellet was dissolved in 30 L of nuclease-free water after it had dried. The standard procedure recommended by Lee et al. [34] for agarose gel electrophoresis was followed. A UV illuminator was used to expose the gel to UV light. Bands of orange luminous color represented DNA, and the gel was discarded after taking a picture of the DNA bands.

2.9. Quantification of Lipid Peroxidation

Lipid peroxidation was determined using the thiobarbituric-acid-reactive substances (TBARS) assay. Accordingly, the tissues treated with various concentrations of the test sample were acquired for assay. The tissue was homogenized with ice-cold KCl buffer; then, the homogenized tissue sample was collected in an Eppendorf tube and centrifuged

at 5000× *g* for 5 min, after which the pellet was rinsed and redispersed in 0.2 mL of 8.1% sodium dodecyl sulfate (SDS). Next, 1.5 mL of 20% acetic acid was added to this suspension, and the mixture was incubated for 10 min. The solution was then combined with 1.5 mL of 0.8% Tris-borate EDTA (TBA) buffer and 0.7 mL of distilled water. Later, after one hour at 95 °C, the reaction mixture was cooled to 25 °C. After adding around 5 mL of a 15:1 combination of butanol and pyridine, the reaction liquid was once more centrifuged at 5000× *g* for 15 min to remove debris. The absorbance was recorded at 532 nm. The untreated fish were taken as a control. The standard was performed with malondialdehyde (MDA). The sample values were compared with the standard, and the concentration was expressed as μM.

2.10. Amplification of Inflammatory Markers

In the present study, nitric oxide synthase (iNOS), tumor necrosis factor (TNF)-α, and β-actin primers were employed. All of the inflammatory markers (i.e., TNF-α and iNOS) were designed using the Basic Local Alignment Search Tool (BLAST) (Table 1). The primers were as follows:

Table 1. The primers used for inflammatory marker genes amplification.

Name of Primers	Sequences
iNOS	Forward—5′ACACTTCGAAAAGCAAGATGG 3′ Reverse—5′ ACGGGCATCGAAAAGCTGTA 3′
TNF-α	Forward—5′ TCATTTTGGCTGTGGGCCTT 3′ Reverse—5′ GGCGGTTCAAAATCTCACTCAC 3′
β-Actin	Forward—5′ CCATCGGCAATGAGCGTTTC3′ Reverse—5′ CATCCTGAGTCAATGCGCCA 3′

In addition to the inflammatory indicators used here, β-actin was chosen as the housekeeping control gene.

PCR kits were procured from Takara Bio Inc., Japan © (EMERALD PCR master mix), and the procedure was carried out as follows: EMERALD master mix—12.5 μL, DNA template—2.5 μL, forward and reverse primer—1 μL, distilled H_2O—8 μL. The above cocktail for each sample was amplified in the following order and conditions: initial denaturation at 95 °C for 2 min, denaturation at 95 °C for 1 min, annealing respective to the primer chosen for 30 secs, extension at 72 °C for 1 min, and a final extension at 72 °C for 5 min. Amplification was carried out in 34 cycles, the resulting PCR product was resolved in 1% agarose gel electrophoresis, and the results were interpreted.

2.11. Statistical Analysis

All of the experiments were performed in triplicate. The final results were expressed as the mean and standard deviation (mean ± SD). All of the data were checked for normal distribution and homogeneity by one-way ANNOVA. Then, the data were subjected to Tukey's test, which enabled us to identify the specific group means that were different and compare them with the values of honest significant difference.

3. Results

3.1. Determination of LC_{50}

In order to investigate the effect of dosage on the behavioral changes in the fish, several experiments were performed using varied dosages. The LC_{50} results demonstrated that, up to 10 μg/mL dosage, no mortality was recorded, nor were any behavioral changes were observed in the fish. Later, the dosages were gradually increased up to 50 μg/mL, i.e., different dosage ranges (including 10, 20, 30, 40, and 50 μg/mL) were used, and the fishes were closely observed at these dosages for up to 7 days. Notably, at these dosages—between 10 and 50 μg/mL—the fish survived, and no abnormal behavior was detected in the fish.

Encouraged by these results, the dosage was further increased, and higher dosages of 200, 400, 600, 800, and 1000 µg/mL were applied for total of 7 days. There was no evident effect in the entire dosage range, and the fish survived normally even up to 800 µg/mL. Furthermore, no prominent toxic effects or abnormal behavior were observed throughout the treatment period—even in the acute phase.

3.2. Anti-Inflammatory Activity

The inflammation induced by TNBS was dependent on the fishes' weight. The samples in the range of 200, 400, 600, 800, and 1000 µg/mL were given by gavage. The body weight of the fishes was noted. Figure 1 and Table 2 shows the initial weight of the fishes, their weight before treatment, and their weight after treatment (4 h).

Figure 1. The image represents the fishes without TNBS induction, with TNBS induction, and having recovered from inflammation after treatment with the sample. (**a**) Fish without TNBS induction. (**b**) Fish with TNBS induction (1 h later). (**c**) Fish after TNBS induction and treatment with the compound (4 h later).

Table 2. Anti-inflammatory activity of mycobiont extract towards zebrafish at different stages.

Dosage (µg/mL)	$ Weight before TNBS Injection (g)	Weight before Sample Treatment (g)	Weight after Sample Treatment (g)
Control	0.62 ± 0.01 ^{c}	0.64 ± 0.00 ^{d}	0.64 ± 0.00 ^{g}
TNBS-induced group	0.65 ± 0.01 ^{b}	0.74 ± 0.01 ^{bc}	0.77 ± 0.01 ^{b}
Standard	0.61 ± 0.01 ^{c}	0.72 ± 0.01 ^{c}	0.67 ± 0.01 ^{ef}
200	0.61 ± 0.00 ^{c}	0.63 ± 0.01 ^{d}	0.68 ± 0.00 ^{e}
400	0.70 ± 0.01 ^{a}	0.72 ± 0.01 ^{c}	0.79 ± 0.01 ^{a}
600	0.65 ± 0.01 ^{b}	0.75 ± 0.01 ^{b}	0.71 ± 0.00 ^{d}
800	0.69 ± 0.01 ^{a}	0.83 ± 0.01 ^{a}	0.74 ± 0.01 ^{c}
1000	0.62 ± 0.01 ^{c}	0.72 ± 0.01 ^{c}	0.66 ± 0.01 ^{fg}
F7,16	21.240 ***	68.146 ***	80.742 ***

$ Each value is a mean of five replicates ± SD. This means that a column followed by the same superscript ($p > 0.05$) differs significantly according to Tukey's range test. *** Significant at $p < 0.001$.

Transverse cuts were made in the abdominal muscular tissues, and slices were then stained with hematoxylin and eosin. Figure 2 denotes the control, TNBS-induced group, and treated group with standard and sample (200 µg/mL, 600 µg/mL, and 1000 µg/mL). The reference image depicts muscle tissue as it would typically appear, with intact nuclei and cytoplasm (cf. Figure 2a). The control muscle section revealed normal histological elements, such as fiber bundles, connective tissue, and the arrangement of muscle bundles. However, the fish in the TNBS-induced group displayed aberrant muscle tissue arrangements (cf. Figure 2b). The standard treatment showed recovery of tissues from the inflammation (cf. Figure 2c), where the sample-treated images showed the recovery process of muscle tissues in a dose-dependent manner (cf. Figure 2d–f). The progression of recovery of the muscle tissue arrangements was observed in the increased dose ranges. All of the morphological changes are indicated by black arrows, first indicating the neutrophil cells (Figure 2a). Later, an increased number of neutrophil cells was observed in the TNBS-induced group (Figure 2b), which recovered while treated with the samples and also showed recovered muscle tissues (Figure 2d–f). Overall, from the results, it was established that the mycobiont extracts possessed anti-inflammatory properties.

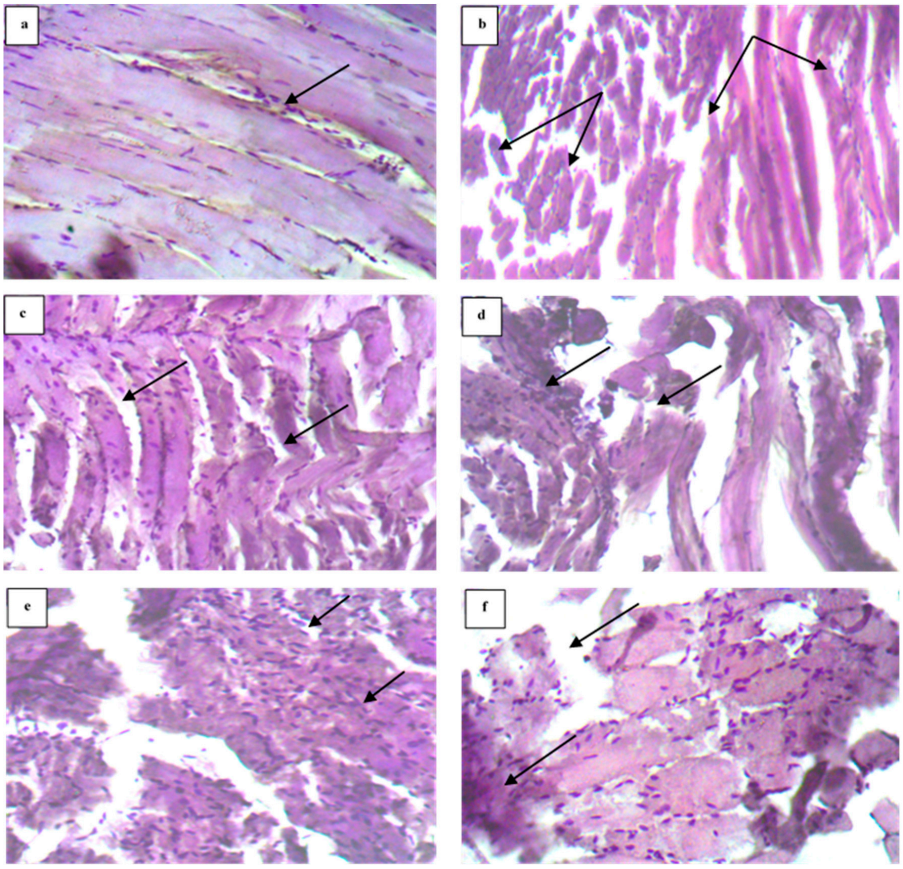

Figure 2. Histopathology of zebrafish muscle tissues: (**a**) control group; (**b**) induced group; (**c**) standard drug; (**d**) treated group (200 µg/mL); (**e**) treated group (600 µg/mL); (**f**) treated group (1000 µg/mL).

3.3. Lipid Peroxidation Assay

Figure 3 depicts the differences in lipid peroxidation (LPO) levels between the different groups. The control fish without TNBS induction showed 0.175 µM LPO release. The concentration of LPO increased in the induced group, whereas in the standard treatment the LPO level decreased. The samples showed decreasing LPO release in the range from 20 to 100 µg/mL. The figure shows the prominent levels of difference between the different groups. The graph depicts the evident change in the LPO when the inflammation was treated with the varied sample range, proving that there were significant differences in the LPO levels. The results showed that the lipid peroxidation effect ranged between 0.55 µMol (induced group) and 0.18 µMol (100 µg/mL group), whereas the variation between treatments ($F_{7,39}$ = 449.289, $p < 0.001$) was highly significant. The lipid peroxidation assay was significantly related to the extent of the mycobiont extracts of the lichen. This confirms that the samples acted on the recovery at the site of inflammation induced by TNBS.

3.4. Amplification Studies of the Inflammatory Markers

The PCR amplification of DNA extracted from the control group, induced group, and treated group (standard and sample)—gene expression of (a) β actin, (b) iNOS, and (c) TNF-α—is shown in Figure 4, which reveals that the samples evoked significant downregulation of the inflammatory gene marker TNF-α in a dose-dependent manner compared to TNBS-induced group. Among the treated groups, there was a marked decrease in the expression of TNF-α at 100 µg/mL. The standard treatment showed a low expression level compared to the sample-treated groups. There were no remarkable changes in iNOS expression levels between the groups. β-Actin was used as an internal loading control. The gene expression

studies confirmed the downregulation of the inflammatory marker TNF-α, which is an indicator of anti-inflammatory activity.

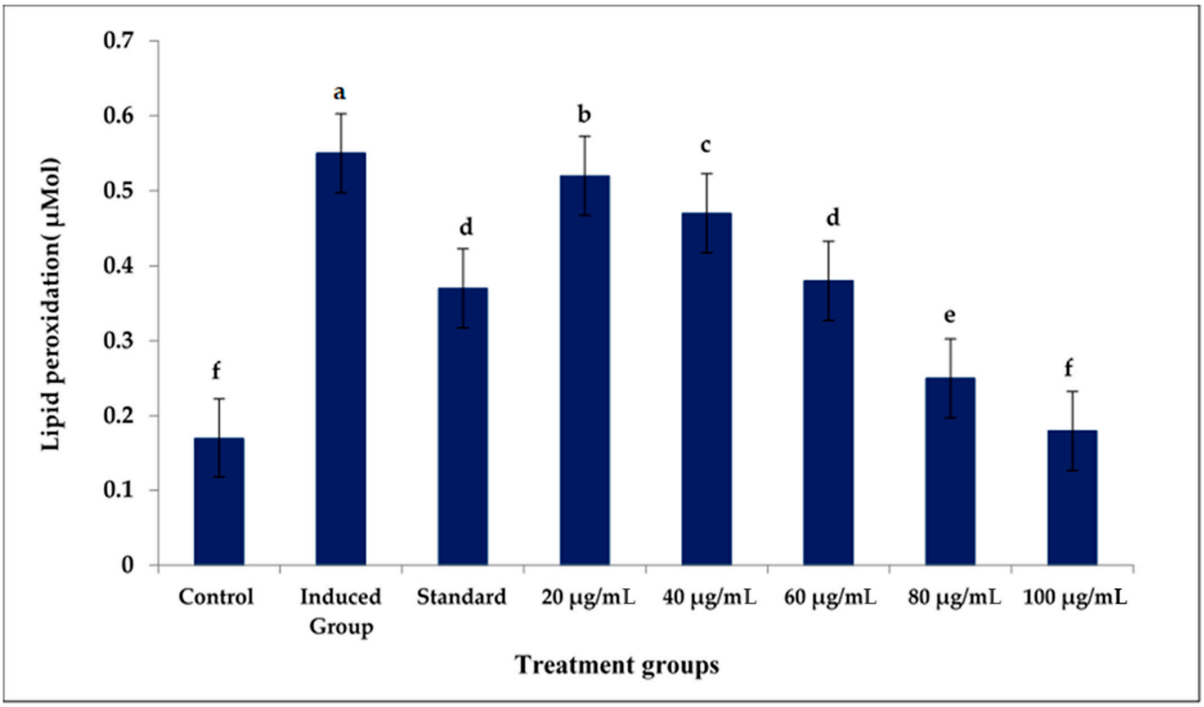

Figure 3. Lipid peroxidation activity of mycobiont extract of *Parmotrema austrosinense*. Bars bearing the same letter(s) do not significantly differ according to Tukey's range test ($p > 0.05$).

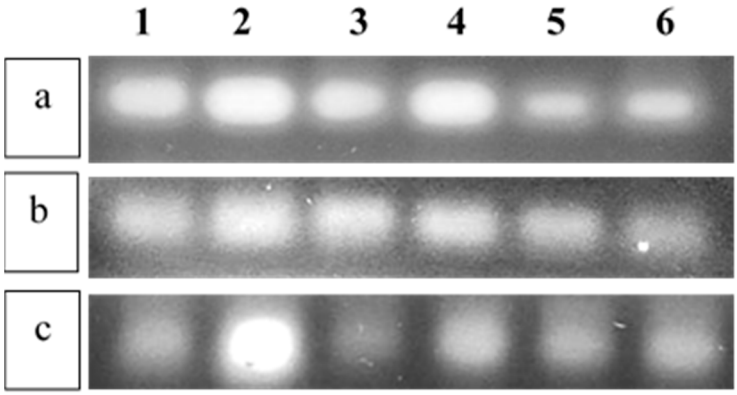

Figure 4. Gene expression analysis of treated fishes: (**a**) Beta-actin gene expression; (**b**) iNOS gene expression; (**c**) TNF-α gene expression. Lane 1—control, Lane 2—induced group, Lane 3—standard group, Lane 4—20 µg/mL, Lane 5—60 µg/mL, Lane 6—100 µg/mL.

4. Discussion

Many earlier reports have described the biological activities of lichen extracts with various organic solvents—particularly from *Parmotrema* species [35,36]. According to previously published studies, most lichen species contain a variety of potentially bioactive secondary metabolites that demonstrate excellent anti-inflammatory and antimicrobial properties [22,37]. These secondary metabolites include phenols, usnic acid, terpenes, steroids, and other depside derivatives, which exhibit several important biological properties, such as antitumor, apoptotic, and cytotoxic activities [36]. Particularly, usnic acid is a highly popular secondary metabolite of lichen, which is known to exert high antimicrobial activity and excellent antiproliferative effects on human leukemia (K562) and endometrial carcinoma [38,39]. Therefore, due to the presence of highly efficient bioactive secondary

metabolites, lichen extracts have been employed indigenously for the treatment of several different types of ailments [40]. Particularly, in herbal remedies, several genera of lichens have been used to treat a variety of diseases. A wide variety of species of lichen extracts have been used to control fever, diarrhea, infections, skin diseases, epilepsy, convulsions, and as purgatives [41]. Despite this, the lack of bulk quantity of lichen thalli and their compounds is the reason that various lichen species are still unresearched. Among these unexplored lichens, the species *P. austrosinense* is also one of the less-studied lichens. However, in recent years, mycobiont extracts have gradually been gaining popularity for research purposes, due to their diverse biological prospects [42].

In the present study, we investigated the anti-inflammatory activity of locally available *P. austrosinense*, which is a cosmopolitan lichen distributed in the humid climate of the Tamil Nadu Western Ghats. More specifically, acetone was used to extract the bioactive secondary metabolites from the mycobiont. To study the biopotential of *P. austrosinense* mycobiont extracts, a lipid peroxidation assay was performed using the acetone extract, which was further used to evaluate its anti-inflammatory efficacy using the zebrafish model. The complete study was carried out with the zebrafish model, because of the accumulated knowledge and tools available for the selected fish model, which could be utilized for further exploitation [43].

According to the results, the toxicity assay showed that the mycobiont extract had no toxic effect against zebrafishes up to 800 µg/mL. This result on toxicity is consistent with the results reported by Studzińska-Sroka and Dubino [20]. Another study reported by Poornima et al. [44] also demonstrated that toxicity against the zebrafish model was recorded at the concentration of 800 µg/mL. Thus, the mycobiont extracts were used for anti-inflammatory studies with a zebrafish model of induced inflammation. The study on the anti-inflammatory activity suggested that the mycobiont extract might contain an anti-inflammatory compound, which was found to be highly active against the inflammatory agent TNBS. A significant positive association between lichen compounds and in vivo anti-inflammatory action was demonstrated by Poornima et al. [44] and Nguyen et al. [45]. Furthermore, anti-inflammatory studies with other lichen species also evidently support the present findings [36,46]. The mycobiont acetone extract showed dose-dependent anti-inflammatory action against TNBS-induced inflammation. The lower weight in the zebrafishes with TNBS-induced inflammation was the main indicator of the presence of the anti-inflammatory compound in the extract.

Furthermore, lipid peroxidation test assays were carried out using the acetone extracts of the mycobiont. The results indicated a dose-dependent decrease in LPO levels in the sample-treated TNBS-induced zebrafishes. The TNBS-induced zebrafishes exhibited a decreased level of LPO at the concentration of 100 µg/mL. It was also noted that with increasing extract dose, a gradual decrease in the LPO level was observed. However, the results of the LPO assay in the present study are contradictory when compared to the results of an earlier published report on the acetone extract of *Parmotrema* species [47–49].

The anti-inflammatory activity of the acetone extract of the *P. austrosinense* mycobiont was confirmed by the recovery of neutrophils in the histopathological assays of TNBS-induced zebrafishes. It is possible that the reported anti-inflammatory activity of the extract could be attributed to the presence of lecanoric acid, as reported in an earlier study by Studzińska et al. [21]. According to observations, the present findings are consistent with the results obtained by Poornima et al. [44]. In contrast with our present study, the latter used oxazolone to induce inflammation in the zebrafishes.

Additionally, encouraged by these results, gene expression studies were also carried out after the TNBS-induced inflammation and lipid peroxidation tests. In comparison with the standard, a dose-dependent decrease in TNF-α gene expression was detected. These results also suggest that the aforementioned compound possibly produces a dose-dependent anti-inflammatory response. This was analyzed using TNF gene downregulation, which also resulted in a dose-dependent response, as with the increase in the dose, a greater downregulation of the TNF-α gene was detected with the PCR-amplified products. No-

tably, the results of our gene expression study are also supported by another previously published report by Lee et al. [50] with LPS-induced macrophages, where they found that after treatment with the test sample, a successful reduction in TNF-α was recorded.

5. Conclusions

In this study, a mycobiont extract of a lichen species was tested for its biopotential properties. Among the various lichen species, *P. austrosinense* is cosmopolitan in its distribution and is known to be widely found in hill stations. However, the anti-inflammatory activity of *P. austrosinense* has been little explored; thus, it was decided to investigate it's in vitro and in vivo anti-inflammatory activities using a zebrafish model in the present research. The in vivo anti-inflammatory studies showed a dose-dependent response. Accordingly, the in vitro LPO assay concluded that the extract showed a decreased LPO level at high doses. The results of PCR gene expression indicated the presence of downregulation of the inflammatory gene marker TNF-α. Therefore, the supportive in vitro and in vivo test results confirmed the anti-inflammatory activity of the mycobiont of *P. austrosinense.* These results are encouraging; however, further extensive investigations are needed to exploit the biopotential of the studied lichen extract for future medical applications.

Author Contributions: K.R.: data curation, formal analysis, and writing—original draft; P.K.: validation, review, and editing; P.P.: conceptualization, supervision, validation, visualization, review, and editing; M.R.S.: formal analysis writing—original draft; M.K.: formal analysis and writing—original draft; T.H.O.: formal analysis and resources; B.S.: formal analysis and writing—review, and editing. All authors have read and agreed to the published version of the manuscript.

Funding: The authors extend their appreciation to the Deputyship for Research and Innovation, "Ministry of Education" in Saudi Arabia for funding this research (IFKSUOR3–103–1).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data is contained within the article.

Acknowledgments: The authors extend their appreciation to the Deputyship for Research and Innovation, "Ministry of Education" in Saudi Arabia for funding this research (IFKSUOR3–103–1).

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Phi, K.-H.; So, J.E.; Kim, J.H.; Koo, M.H.; Kim, J.-H.; Kim, D.; Lee, J.H.; Lee, S.; Youn, U.J. Chemical constituents from the Antarctic lichen Usnea aurantiaco-atra and their chemotaxonomic significance. *Biochem. Syst. Ecol.* **2023**, *106*, 104581. [CrossRef]
2. Do, T.-H.; Duong, T.-H.; An, T.N.M.; Vo, T.-P.-G.; Do, V.-M.; Nguyen, N.-H.; Sichaem, J. Two new α-glucosidase inhibitory depsidones from the lichen Parmotrema cristiferum (Taylor) Hale. *Chem. Biodivers.* **2023**, *20*, e202201213. [CrossRef]
3. Susithra, E.; Meena, S.; Chamundeeswari, D.; Chekkara, R.; Varalakshmi, E. Molecular docking studies of lichen metabolites as malarial protein inhibitors: Plasmepsin II protease and dihydrofolate reductase. *J. Chem. Pharm. Res.* **2014**, *6*, 501–512.
4. Ureña-Vacas, I.; González-Burgos, E.; Divakar, P.K.; Gómez-Serranillos, M.P. Lichen Depsides and Tridepsides: Progress in Pharmacological Approaches. *J. Fungi* **2023**, *9*, 116. [CrossRef]
5. Bhat, N.B.; Das, S.; Sridevi, B.V.; Nayaka, S.; Birangal, S.R.; Shenoy, G.G.; Joseph, A. Molecular docking and dynamics supported investigation of antiviral activity of Lichen metabolites of Roccella montagnei: An in silico and in vitro study. *J. Biomol. Struct. Dyn.* **2023**, 1–14. [CrossRef]
6. Chauhan, R.; Abraham, J. In vitro antimicrobial potential of the lichen *Parmotrema* sp. extracts against various pathogens. *Iran. J. Basic Med. Sci.* **2013**, *16*, 882.
7. Yañez, O.; Osorio, M.I.; Osorio, E.; Tiznado, W.; Ruíz, L.; García, C.; Nagles, O.; Simirgiotis, M.J.; Castañeta, G.; Areche, C. Antioxidant activity and enzymatic of lichen substances: A study based on cyclic voltammetry and theoretical. *Chem. Biol. Interact.* **2023**, *372*, 110357. [CrossRef]
8. Shukla, I.; Azmi, L.; Rao, C.V.; Jawaid, T.; Kamal, M.; Alkhamees, O.A.; Alaseem, A.M.; Alsanad, S.M. Inclusive roles of protocetraric acid, a secondary metabolite from the common green shield lichen Flavoparmelia caperata in alcohol-induced hepatic injury. *Lat. Am. J. Pharm* **2022**, *41*, 613–623.
9. Morris Kupchan, S.; Kopperman, H.L. L-Usnic acid: Tumor inhibitor isolated from lichens. *Experientia* **1975**, *31*, 625. [CrossRef]

10. Anand Mariadoss, A.V.; Krishnan Dhanabalan, A.; Munusamy, H.; Gunasekaran, K.; David, E. In Silico studies towards enhancing the anticancer activity of phytochemical phloretin against cancer drug targets. *Curr. Drug Ther.* **2018**, *13*, 174–188. [CrossRef]
11. Gupta, A.; Sahu, N.; Singh, A.P.; Singh, V.K.; Singh, S.C.; Upadhye, V.J.; Mathew, A.T.; Kumar, R.; Sinha, R.P. Exploration of Novel Lichen Compounds as Inhibitors of SARS-CoV-2 Mpro: Ligand-Based Design, Molecular Dynamics, and ADMET Analyses. *Appl. Biochem. Biotechnol.* **2022**, *194*, 6386–6406. [CrossRef]
12. Tripathi, A.H.; Negi, N.; Gahtori, R.; Kumari, A.; Joshi, P.; Tewari, L.M.; Joshi, Y.; Bajpai, R.; Upreti, D.K.; Upadhyay, S.K. A review of anti-cancer and related properties of lichen-extracts and metabolites. *Anti-Cancer Agents Med. Chem.* **2022**, *22*, 115–142.
13. Awasthi, D.D. *Key to the Microlichens of India, Nepal and Sri Lanka*; J. Cramer: Stuttgart, Germany, 1991.
14. Gurib-Fakim, A. Medicinal plants: Traditions of yesterday and drugs of tomorrow. *Mol. Asp. Med.* **2006**, *27*, 1–93. [CrossRef]
15. Armin, H.; Georg, B.; Elfie, S.-w. Investigations on secondary metabolites of lichens and liehen mycobionts in culture: Production and biological activity. *Osterreichische* **2008**, *37*, 323–324.
16. Tanas, S.; Odabasoglu, F.; Halici, Z.; Cakir, A.; Aygun, H.; Aslan, A.; Suleyman, H. Evaluation of anti-inflammatory and antioxidant activities of Peltigera rufescens lichen species in acute and chronic inflammation models. *J. Nat. Med.* **2010**, *64*, 42–49. [CrossRef]
17. Shrestha, G.; El-Naggar, A.M.; St. Clair, L.L.; O'Neill, K.L. Anticancer activities of selected species of North American lichen extracts. *Phytother. Res.* **2015**, *29*, 100–107. [CrossRef]
18. Müller, K. Pharmaceutically relevant metabolites from lichens. *Appl. Microbiol. Biotechnol.* **2001**, *56*, 9–16. [CrossRef]
19. Mitrović, T.; Stamenković, S.; Cvetković, V.; Nikolić, M.; Tošić, S.; Stojičić, D. Lichens as source of versatile bioactive compounds. *Biol. Nyssana* **2011**, *2*, 1–6.
20. Studzińska-Sroka, E.; Dubino, A. Lichens as a source of chemical compounds with anti-inflammatory activity. *Herba Pol.* **2018**, *64*, 56–64. [CrossRef]
21. Studzińska, E.; Witkowska-Banaszczak, E.; Bylka, W. Związki biologicznie aktywne porostów. *Herba Pol.* **2008**, *54*, 80–88.
22. Bugni, T.S.; Andjelic, C.D.; Pole, A.R.; Rai, P.; Ireland, C.M.; Barrows, L.R. Biologically active components of a Papua New Guinea analgesic and anti-inflammatory lichen preparation. *Fitoterapia* **2009**, *80*, 270–273. [CrossRef] [PubMed]
23. Umezawa, H.; Shibamoto, N.; Naganawa, H.; Ayukawa, S.; Matsuzaki, M.; Takeuchi, T.; Kono, K.; Sakamoto, T. Isolation of lecanoric acid, an inhibitor of histidine decarboxylase from a fungus. *J. Antibiot.* **1974**, *27*, 587–596. [CrossRef] [PubMed]
24. Rajendran, K.; Poornima, S.; Ponnusamy, P. Antimicrobial and Antiproliferative Activities of Depside Compound Isolated from the Mycobiont Culture of Parmotrema austrosinense (Zahlbr.) Hale. *J. Pure Appl. Microbiol.* **2020**, *14*, 2525–2542. [CrossRef]
25. Kalidoss, R.; Mariraj, M.; Shenbagam, M.; Seles, J.M.; Prasath, K.A.; Rajaprabu, N.; Ponmurugan, P. Anti-microbial and Anti-oxidant Properties of Solvent Extract of Lichen Species Collected from Kodaikanal Hills, Western Ghats of Tamil Nadu. In *Phytomedicine*; CRC Press: Boca Raton, FL, USA, 2020; pp. 53–61.
26. Wolseley, P. A Compendium of the Macrolichens from India, Nepal and Sri Lanka. *Lichenologist* **2008**, *40*, 267–268. [CrossRef]
27. Cordeiro, L.M.; Iacomini, M.; Stocker-Wörgötter, E. Culture studies and secondary compounds of six Ramalina species. *Mycol. Res.* **2004**, *108*, 489–497. [CrossRef]
28. Verma, N.; Behera, B.C. In Vitro culture of lichen partners: Need and Implications. In *Recent Advances in Lichenology*; Springer: Berlin/Heidelberg, Germany, 2015; pp. 147–159.
29. OECD Guideline for Testing Acute Toxicity in Fishes. Available online: http://www.oecd.org/chemicalsafety/risk-assessment/1948241.pdf (accessed on 1 January 2023).
30. Xie, Y.; Meijer, A.H.; Schaaf, M.J. Modeling inflammation in zebrafish for the development of anti-inflammatory drugs. *Front. Cell Dev. Biol.* **2021**, *8*, 620984. [CrossRef]
31. Ducharme, N.A.; Reif, D.M.; Gustafsson, J.-A.; Bondesson, M. Comparison of toxicity values across zebrafish early life stages and mammalian studies: Implications for chemical testing. *Reprod. Toxicol.* **2015**, *55*, 3–10. [CrossRef]
32. Kinkel, M.D.; Eames, S.C.; Philipson, L.H.; Prince, V.E. Intraperitoneal injection into adult zebrafish. *JoVE (J. Vis. Exp.)* **2010**, *42*, e2126.
33. Slaoui, M.; Fiette, L. Histopathology procedures: From tissue sampling to histopathological evaluation. In *Drug Safety Evaluation*; Springer: Berlin/Heidelberg, Germany, 2011; pp. 69–82.
34. Lee, P.Y.; Costumbrado, J.; Hsu, C.-Y.; Kim, Y.H. Agarose gel electrophoresis for the separation of DNA fragments. *JoVE (J. Vis. Exp.)* **2012**, *62*, e3923.
35. Jain, A.; Bhandarkar, S.; Rai, G.; Yadav, A.; Lodhi, S. Evaluation of Parmotrema reticulatum taylor for antibacterial and antiinflammatory activities. *Indian J. Pharm. Sci.* **2016**, *78*, 94–102.
36. Molnár, K.; Farkas, E. Current results on biological activities of lichen secondary metabolites: A review. *Z. Für Nat. C* **2010**, *65*, 157–173. [CrossRef] [PubMed]
37. Goga, M.; Elečko, J.; Marcinčinová, M.; Ručová, D.; Bačkorová, M.; Bačkor, M. Lichen metabolites: An overview of some secondary metabolites and their biological potential. In *Co-Evolution of Secondary Metabolites*; Springer: Berlin/Heidelberg, Germany, 2020; pp. 175–209.
38. Elečko, J.; Vilková, M.; Frenák, R.; Routray, D.; Ručová, D.; Bačkor, M.; Goga, M. A Comparative Study of Isolated Secondary Metabolites from Lichens and Their Antioxidative Properties. *Plants* **2022**, *11*, 1077. [CrossRef] [PubMed]
39. Seçme, M.; Dodurga, Y. Usnic Acid Inhibits Cell Proliferation and Downregulates lncRNA UCA1 Expression in Ishikawa Endometrial Cancer Cells. *Nat. Prod. Biotechnol.* **2021**, *1*, 28–37.

40. Ingelfinger, R.; Henke, M.; Roser, L.; Ulshöfer, T.; Calchera, A.; Singh, G.; Parnham, M.J.; Geisslinger, G.; Fürst, R.; Schmitt, I. Unraveling the pharmacological potential of lichen extracts in the context of cancer and inflammation with a broad screening approach. *Front. Pharmacol.* **2020**, *11*, 1322. [CrossRef]
41. Shukla, V.; Joshi, G.P.; Rawat, M. Lichens as a potential natural source of bioactive compounds: A review. *Phytochem. Rev.* **2010**, *9*, 303–314. [CrossRef]
42. Nguyen, H.-H.; Aree, T.; Nguyen, H.T.; Tran, T.-M.-D.; Nguyen, T.-P.; Vo, T.-P.G.; Nguyen, N.-H.; Duong, T.-H. Diorygmones AB, two new guaiane-sesquiterpenes from the cultured lichen mycobiont of *Diorygma* sp. *Nat. Prod. Res.* **2023**, 1–6. [CrossRef]
43. Patton, E.E.; Tobin, D.M. *Spotlight on Zebrafish: The Next Wave of Translational Research*; The Company of Biologists Ltd.: Cambridge, UK, 2019; Volume 12, p. dmm039370.
44. Poornima, S.; Nagarjun, N.; Ponmurugan, P.; Gnanamangai, B.M.; Narasimman, S. Toxicity and anti-inflammatory study of Parmotrema austrosinense extract against oxozalone induced intestinal inflammation in zebrafish (Danio rerio) model. *Biocatal. Agric. Biotechnol.* **2019**, *21*, 101278. [CrossRef]
45. Nguyen, H.T.; Ketha, A.; Kukavica, B.; Tatipamula, V. Antiinflammatory potential of lichens and its substances. In *Inflammatory Bowel Disease*; MedDocs Publishers: Reno, NY, USA, 2021; pp. 1–9.
46. Güvenç, A.; Akkol, E.K.; Süntar, İ.; Keleş, H.; Yıldız, S.; Çalış, İ. Biological activities of Pseudevernia furfuracea (L.) Zopf extracts and isolation of the active compounds. *J. Ethnopharmacol.* **2012**, *144*, 726–734. [CrossRef]
47. Kosanić, M.M.; Ranković, B.R.; Stanojković, T.P. Antioxidant, antimicrobial and anticancer activities of three Parmelia species. *J. Sci. Food Agric.* **2012**, *92*, 1909–1916. [CrossRef]
48. Jayaprakasha, G.K.; Rao, L.J. Phenolic constituents from the lichen Parmotrema stuppeum (Nyl.) Hale and their antioxidant activity. *Z. Für Nat. C* **2000**, *55*, 1018–1022. [CrossRef]
49. Selvaraj, G.; Tinabaye, A.; Ananthi, R. In vitro antioxidant activities of Salazinic acid and its derivative hexaacetylsalazinic acid. *Int. J. Res. Eng. Technol.* **2015**, *4*, 345–355.
50. Lee, A.S.; Jung, Y.J.; Kim, D.; Nguyen-Thanh, T.; Kang, K.P.; Lee, S.; Park, S.K.; Kim, W. SIRT2 ameliorates lipopolysaccharide-induced inflammation in macrophages. *Biochem. Biophys. Res. Commun.* **2014**, *450*, 1363–1369. [CrossRef] [PubMed]