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TOXICOLOGICAL AND BEHAVIOR ANALYSES OF A FOLIOSE LICHEN *PARMOTREMA TINCTORUM* (DELISE EX NYL.) HALE EXTRACT USING ZEBRAFISH (*DANIO RERIO*) MODEL

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ABSTRACT

Objective: The objective of the study was to evaluate the toxicity and behavior analysis with the extract of foliose lichen *Parmotrema tinctorum* against the adult zebrafish.

Methods: The zebrafishes were exposed to different concentrations of *P. tinctorum* extract such as 50, 100, and 200 mg/ml for 7 days of exposure, in which the mortality and behavioral responses of zebrafishes were recorded. The standard histopathological examination was conducted with the lichen extracts of *P. tinctorum*.

Results: The results revealed that *P. tinctorum* extract did not show any prominent behavior abnormalities in zebrafishes even at a high concentration of 200 mg/ml. The extract was found to have dose-dependent toxic to zebrafish and the number of neutrophil cells in the muscle bundles reduced at a high concentration. The results of the inflammatory marker gene expression using polymerase chain reaction results suggested that the dose-dependent suppression of tumor necrosis factor gene by the *P. tinctorum* lichen extract.

Conclusion: Overall, concluded that the extract might contain anti-inflammatory induction properties and further tests are required to prove apoptosis and anticancer activity using other *in vivo* or *in vitro* techniques.

Keywords: Lichen, *Parmotrema tinctorum,* Zebrafish, Toxicity, Nutraceutical effect, Histopathology, Inflammatory markers.

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INTRODUCTION

Parmotrema tinctorum lichen is a rich source of lecanoric acids, orcellinic acids, and atranorin that can be highly active against a vast number of human disorders. The proportion of secondary metabolites, the different solvent extraction procedures, and concentration of the depsides or depsidones have been optimized to be ideal for treating human disorders by our ancient forefathers several 1000 years ago. With the development of drug resistance among the pathogens, the demands for the new drug have also risen in recent years. With physicians targeting the ancient indigenous method of healing the diseases, there has been a need for the discovery of novel drugs. Tamil Nadu, the province of India in Western Ghats, is known for indigenous methodologies of curing diseases using lichen extracts. Despite many species of nutraceutical lichens and their pharmaceutical importance are documented [1-4], a very few species are in use currently. Therefore, lichen exploration is severe in recent years to exploit the biological agent from lichens with nutraceutical values. Anti-inflammatory and toxicological studies of lichen extract are carried out regularly in folk medicine. Crawford to assess the extracts of several lichens might conclude to be nutraceutical [5]. In 20th century, AIDS was the most challenging disease. However, cancer has been one of the most common diseases forever, with the huge annual deaths across the world. The rapid multiplication of many numbers of cells of the body tissues leads to the development of cancer in our body system. The affected person may have signs in the bone, inflammation in the tissues or lymph nodes. Although a number of lichen species extracts which can reveal anti-inflammatory roles have been examined, one lichen *P. tinctorum* is the least known. The extracts in such lichen species exhibit the activity of anti-inflammatory ideally. Zebrafish embryo studies can reveal the possession of anti-inflammatory activity in the *P. tinctorum* lichen extracts, but it is only after the gene analysis that such activity can be well identified.

Current safety and toxicology testing methods of different herbal products are being considered as comprising traditional preclinical studies. Potentially, zebrafish is used in *in vivo* methods to assess the cytotoxic activity of the lichen extracts. This zebrafish model is a less expensive and reliable model to study the toxicological screening [6]. Zebrafish exhibits a high resemblance to human neurobehavioral phenotypes and neurotransmitter systems [7]. The novel test is an open glass aquarium setup which is similar to the open field test for rodent's studies to assess anxiety and behavioral investigations in the zebrafish [8]. In addition, it is a fascinating research model for the toxicological studies of a novel drug to obtain results within a short period without much complications [9,10]. The proportion of balanced nutrients and dosage of dietary elements can promote the reproductive and early growth phase of zebrafish which in turn be useful to assess the toxicity and behavior analysis of lichen compounds [11].

The fungi, algae, bacteria, and actinomycetes or two or more partners are associated to form lichens. *P. tinctorum* (Nyl) Hale is an edible lichen, used in the meat and vegetable preparations by ethnic groups in India and Nepal since ancient times [12]. P. tinctorum is a prominent foliose lichen belonging to Parmeliaceae members which are mostly corticolous (found in tree barks) and saxicolous (found in rocks) in nature. The reason for the selection of lichens *P. tinctorum* and consequent explorations in their metabolite potentiality in Kodiakanal hills is due to the highly abundant of such species biodiversity set around the area. Honda *et al*. documented the antimicrobial activity of *P. tinctorum* [13]. The depsides, depsidones produced by the lichen thallus exhibited its biological

activity. Tiwari *et al*. confirmed the antifungal activity of methanol extracts of *P. tinctorum* [14]. Anjali *et al*. examined the antibacterial and antifungal activity of solvent extracts of *P. tincotrum* [15]. Vivek *et al*. and Poornima *et al*. reported that the extracts of *P. tinctorum* exhibited the DPPH radical scavenging activity [16,17]. Ganesan *et al*. reasoned the radical scavenging activity of *P. tinctorum* was due to the high phenolic content [18]. Therefore, the lichen compounds of the *P. tinctorum* can be extracted to treat microbial diseases and for application as antioxidants. Although antimicrobial and antioxidant activities of such genus can be found in the literature, anti-inflammatory properties of this genus have not been assessed extensively [19].

MATERIALS AND METHODS

Plant material

Fresh thallus of a foliose lichen, *P. tinctorum* (Delise ex Nyl.) Hale collected from Kodaikanal hills of the Western Ghats in southern India was used for the present study. The altitude of the hills was 2130 m above mean sea level, and latitude and longitude of the hills, respectively, were 10°14'17.21"N and 77°29'21.06"E. The lichen specimens were identified based on the lichen identification key of [20]. The samples were characterized by studying their external morphology, reproduction structures, anatomical features, and chemical profile referred from the lichen manual of Culberson and Krishnan (1970). The color test was done on medulla of lichen thallus using test reagents such as potassium hydroxide (K), calcium hydroxide (C), and Paraphenylene diamine (PD) as per the protocol of [21].

Extraction of secondary metabolites from *P. tinctorum*

Bioactive secondary metabolites were extracted from the powder (5 g) thallus of *P. tinctorum* by the Soxhlet methods [22]. The extraction was carried out using 250 ml of methanol and heated up to 80ºC for 8 h. The crude lichen extracts were concentrated using a rotary vacuum evaporator and stored at −5°C temperature until further use.

Identification of lichen secondary metabolites by thin layer chromatography (TLC)

Five gram of air-dried lichen samples was blended with 100 ml of acetone at room temperature in a magnetic stirrer. The filtrate was separated through Whatman No. 1 filter paper and evaporated to dryness. TLC was used to identify the bioactive secondary metabolites present in the lichen extract using Toluene: Dioxane: Acetic acid (36:9:1) solvent system containing a TLC glass cabinet. The developed TLC plates were sprayed with sulfuric acid, air-dried, and observed the spots under Ultraviolet light of 253~254 nm [23].

Animals and experimental design

Adult zebrafishes (*Danio rerio,* AB wild type strain) that were healthy and devoid of any malformations or infections were chosen for the study. Before initialization of the experiment, fishes were acclimatized in aquarium glass tanks (20 L) containing biologically filtered and dechlorinated water at 23±2°C with 10:14 h light and dark cycles. In order to mimic the natural habitat of zebrafishes, the tanks were filled with gravel to a height of 2 cm, and a popular aquarium plant (*Cabomba aquatica*) was submerged. Fishes were fed twice a daily with commercial fish flakes containing protein pellets (Hindustan level limited, Bangalore). Zebrafishes with an average weight of 0.26±0.03 g and length of 32±0.4 mm were selected. Later, the fishes were removed from the recirculating groups, placed in static groups, and fasted for 24 h before experimentation. The pH and dissolved oxygen content of the water was maintained at 6.8–7.3 and 5.4 mg/l, respectively. Fishes were grouped and separated into various aquarium tanks for the treatment of lichen extracts to check the inflammation if any in the muscle tissues. Zebrafishes were placed in the novel test tank devoid of *P. tinctorum* extract immersion as a drug free system for comparison purposes.

LC_{50} determination of *P. tinctorum* extract using zebrafish

To obtain the lethal concentration (LC_{50}) in terms of acute toxicity of *P. tinctorum* extract, we followed the guidelines of Organization for Economic Cooperation and Development for testing the chemicals [24]. The various concentrations of *P. tinctorum* extract from 2 to 10 µg/ml and then from 10 to 50 µg/ml were initially chosen. Finally, 50, 100, and 200 µg/ml dosage of *P. tinctorum* extract was standardized. Immersion administration of *P. tinctorum* lichen extracts was diluted in distilled water as required concentration and the control groups had contact with aquarium water only along with regular feed material. In the control group, seven untreated fishes were maintained in a separate tank. The fishes were not fed for 24 h before (or) during the experimentation to maintain the constant exposure concentrations. Similarly, the control group was also starved. Fishes from the control as well as treated groups were sacrificed after 7 days of exposure.

Behavior analysis of zebrafish treated with *P. tinctorum* extract

A rectangular aquarium measuring the size of 15×25×20 cm (width×length×height), divided equally into two horizontal sections and demarcated by eight rectangles. The water level was maintained at 18.0 cm in height, producing a final volume of 6.9 l [25]. A video camera was positioned on the front of the device to capture the exploration of the entire environment. Behavior analysis of treated and untreated fishes in terms of time spent at the top (s), top entries (n), freezing frequency (n), freezing duration (s), and swimming pattern was observed. The observations such as total time spent in upper 2/3 and latency to upper 2/3 entry indicative of anxiogenic state, pattern of movement anxiety like and anti-inflammatory activity of *P. tinctorum* extract were also recorded [26].

Histopathology of muscle tissue of zebrafish treated with *P. tinctorum* extract

P. tinctorum extract treated and untreated control zebrafishes were anesthetized and decapitated to get their muscle tissues and then fixed in Dietrich's fixative solution (30 ml ethanol, 1 ml formalin, 2 ml glacial acetic acid and 58 ml of distilled water for 100 ml). They were washed thrice with a cacodylate buffer at 4°C for 10 min and post-fixed in 1% osmium tetroxide at 4°C for 90 min. They were stained with *en bloc* staining solution containing 0.5% uranyl acetate. They were dehydrated with absolute ethanol and the clearing of tissues was done by subjecting to a clearing agent xylene. Finally, they were embedded in melted paraffin wax and sliced to get thin sections with the help of a microtome (Thermo scientific Microm- HM 34 OE). Then the tissue sections were mounted on microscope slides using haematoxylin solution and documented under a light microscope [27].

Extraction of genomic DNA from the muscle tissues of zebrafish

Around 100 mg of muscle tissues of zebrafish treated with *P. tinctorum* extract were smashed in a pestle and mortar using liquid nitrogen. The ground tissues were dissolved in 1.2 ml digestion buffer (pH 6.4) and incubated overnight in a water bath at 55°C. Equal volume of Phenol-Chloroform-Isoamyl alcohol mixed in the ratio 25:24:1 was added and centrifuged at 3000 rpm for 5 min at room temperature. The aqueous phase was carefully removed to which ½ the volume of 7.5M ammonium acetate and twice the volume of absolute ethanol were added. They were centrifuged once again at 3000 rpm for 5 min at room temperature. The pellet containing the genomic DNA of zebrafish was removed carefully and rinsed with 70% ethanol. The isolated DNA was run on an Agarose gel electrophoresis to separate the DNA fragments [28,29].

Inflammatory marker gene expression through polymerase chain reaction (PCR) due to *P. tinctorum* extract treatments

In order to check any inflammation present in the muscle tissues of zebrafish due to *P. tinctorum* extract treatments, the inflammatory markers such as iNOS (induced Nitric Oxide Synthesis) gene and tumor necrosis factor (TNFα) and a housekeeping gene, β-Actin were used. The inflammatory marker and housekeeping genes were designed with NCBI primer BLAST tool search and the specific primers used were given in the (Table 1). The genomic DNA extracted from the mussel tissues of zebrafish along with the specific primers as cDNA was performed using a TAKARA©'s EMERALD PCR master mix. The PCR cocktail contained 5 µl

of genomic DNA, 5 µl of 25–fold diluted cDNA template mixed with 2.5 µl of each primer and 12.5 µl of EMERALD PCR master mix to a final volume of 25 µl. Amplification of DNA was carried out in 34 cycles using a Thermal cycler (Lark L125, Chennai, India) and the resulting PCR product was resolved in 1% Agarose gel electrophoresis [30]. The conditions applied for amplification of genomic DNA for inflammatory marker gene expression was given in the (Table 2).

Statistical analysis

The data generated in the present study were subjected to analyze the mean±standard error and one-way analyze of variance.

RESULTS

LC_{50} determination of *P. tinctorum*

The lichen extract was subjected to LC_{50} analysis in the range of 2, 4, 6, 8 and 10 µg/ml concentrations using adult zebrafish observed for 7 days. The results indicated that there were no mortality and behavioral changes of *P. tinctorum* extract treated zebrafish up to 10 µg/ml concentration. The dosage of *P. tinctorum* extract range was increased to 10, 20, 30, 40 and 50 mg/ml observed for 7 days. In all the dosages tested, fishes showed normal behavior and no mortality was observed between 10 and 50 µg/ml. The dosage level further increased to 50, 100 and 200 mg/ml and kept under treatment for 7 days. The results indicated that there was no evident effect in the entire dosage range up to 200 mg/ml of *P. tinctorum* extract tested. The test samples did not show any prominent effect and abnormal behavior of zebrafishes throughout the treatment period in acute phase.

Behavioral analysis of zebrafishes

The behavioral analysis of zebrafish treated with *P. tinctorum* extract was carried out for 5 min at every 30 min intervals during the first 4 h exposure and then 5 min for every 24 h until 7th day of exposure. Behavior analysis such as anxiety, swimming patterns, aggression, freezing, and top entry were noted. Anxiety of the fishes was noted as soon as they were drawn into the test tanks. The fishes in the control tank rise from darker region to the light region within 1 min and 30 s and the fishes in the *P. tinctorum* extract treated tank took 1.20–1.40 min to reach the light region (Fig. 1a-f). These observations showed that there was no immediate prominent effect on the behavior analysis of fishes treated with *P. tinctorum* extract. Swimming patterns of the fishes were observed throughout the course of 7 days exposure and fishes in the entire drug treated tanks showed the same swimming patterns as the fishes in the control tank. There was no change in the swimming patterns. The fishes were also observed for aggression behavior for 7-day exposure similar to that of untreated control fishes. The results further showed that there were no physical changes such as contraction of the dorsal region or any behavioral changes such as swimming toward or against the other fishes in a peculiar manner. The fishes were also observed freezing throughout 7 days of exposure to *P. tinctorum* extract.

Table 1: Inflammatory marker genes and specific primer pairs used for amplification of genomic DNA extracted from the muscle tissues of zebrafish treated with *Parmotrema tinctorum* extract

Gene Name	Functions	Forward and Reverse Primer Sequences (5'& 3')
iNOS	Inflammatory	Fwd: 5' ACACTTCGAAAAGCAAGATGG 3' Rev: 5' ACGGGCATCGAAAAGCTGTA 3'
TNF- α	Inflammatory	Fwd: 5' TCATTTTGGCTGTGGGCCTT 3' Rev: 5' GGCGGTTCAAAATCTCACTCAC 3'
β-Actin	Housekeeping gene	Fwd: 5' CCATCGGCAATGAGCGTTTC3' Rev: 5' CATCCTGAGTCAATGCGCCA 3'

Fwd: Forward primer sequence, Rev: Reverse primer sequence

Table 2: Conditions applied for amplification of genomic DNA extracted from the muscle tissues of zebrafish treated with *Parmotrema tinctorum* extract

PCR cycles	Temperature (°C)	Time
Initial denaturation	95	2 min
Denaturation	95	1 min
Annealing chosen	Respective to the primer	30 s
Extension	72	1 min
Final extension	72	5 min

Histopathology of muscle tissues of zebrafish

Fig. 2 showed the histopathology of muscle tissues of zebrafishes treated with different concentrations of *P. tinctorum* extract such as 50, 100, and 200 mg/ml. The untreated control zebrafishes showed the normal appearance of nuclei and muscle tissues. In addition, they showed the normal histological structures such as fiber bundles, connective tissues, and arrangement of muscle bundles, whereas *P. tinctorum* extract treated zebrafishes exhibited the muscle bundles in normal arrangements as dose-dependent manner. There was no damage in neutrophil cells in the muscles of zebrafishes treated even in the high dosage (200 mg/ml) of *P. tinctorum* extract. The results suggested the presence of an increased number of neutrophil cells.

Inflammatory marker gene expression due to *P. tinctorum* extract treatment in zebrafishes

The gel images revealed that the test samples evoked a significant suppression of upregulation of TNFα and iNOS genes when compared with that of the untreated control zebrafishes in dose dependent manner. The different doses of *P. tinctorum* extract significantly decreased the expression of TNFα and iNOS genes, in which 60 µg/ml dose had shown a marked increase in the expression of TNFα and iNOS genes and decreased at 100 µg/ml. β-Actin gene was used as a housekeeping gene as an internal loading control for comparison purposes (Fig. 3).

DISCUSSION

The present study was intended to check the efficacy of nutraceutical and toxicological activities of *P. tinctorum* lichen extracts against the adult zebrafishes. The abnormalities in the behavior pattern of *P. tinctorum*-treated zebrafishes were taken as a characteristic biological activity due to toxicological effect and antidepressant actions. The shallow breathing behavioral pattern of zebrafishes treated with various lichen extracts was observed in the previous literature [31]. This is the major sign for the presence of the defense mechanism of zebrafish in a stressful environment. The solvent extracts of *P. tinctorum* were observed to show a lack of biological activity and therefore the behavior abnormalities proved that lichen extract might not have an effective *in vivo* anti-inflammatory activity. Chemical analysis of *P. tinctorum* thallus extract revealed the presence of lecanoric acid in the solvent extract as a major phytochemical compound.

Several studies revealed that zebrafish spent more time in the upper region of the novel aquarium tank due to dwelling behavior [32]. Similarly, zebrafishes tend to have natural state affinity to darker sections in the novel tank which is due to a high degree of thigmotaxis and an inherent property of fishes in the water environment. Benneh *et al*. [8] conducted on an experiment to check the antidepression activity of *Maerua angolensis* stem bark extract using a robust zebrafish model. They reported that *M. angolensis* extract showed a promising activity in amelioration of anxiety depression 2 months of age, 3.5–4.0 cm in length and weigh on an average of 650 mg.

Discouraged by these results, the histopathological study was conducted by standard methods after the behavioral analysis. The previous study with the other lichen extracts has suggested the possession of the immune system boosting ingredients in the lichen extract. The enhancement of immune system might be due to the presence of cellular level differences in the targeted organ [33]. The results of Harvie and Huttenlocher [34] suggested the presence of neutrophils at the injury or at the site of infection. The results of Matthias *et al*. and Woodfin *et*

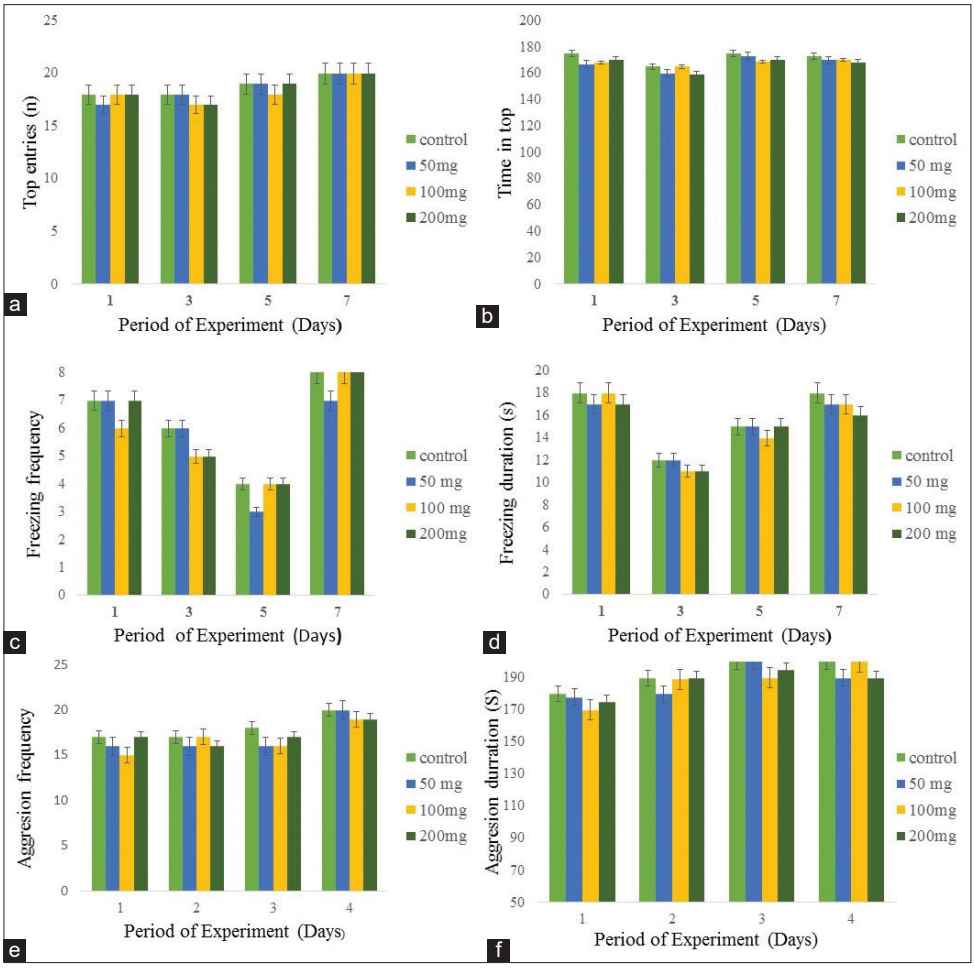

Fig. 1: Behavior analysis of zebrafishes treated with different concentrations of *Parmotrema tinctorum* extract in the aquarium tank ([a] Top entry by zebrafishes at water level, [b] Time spent to reach at top entry by zebrafishes at water level, [c] Freezing frequency of zebrafishes, [d] Freezing duration of zebrafishes, [e] Aggression frequency of zebrafishes, and [f] Aggression duration of zebrafishes)

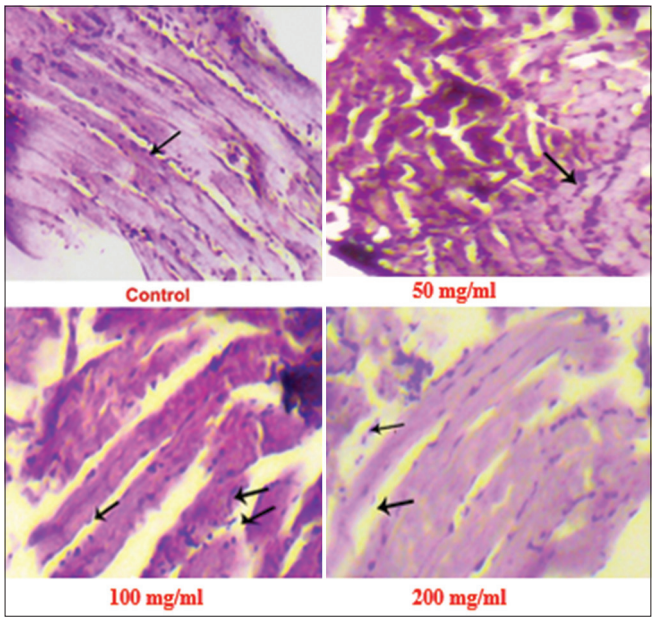

Fig. 2: Histopathological images of muscle tissues of zebrafishes treated with different concentrations of *Parmotrema tinctoram* extract (The black arrows indicated the neutrophil cells)

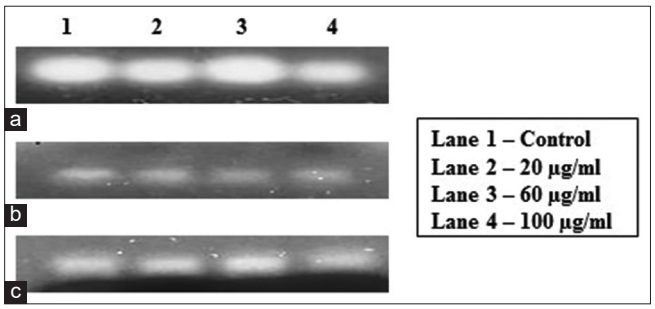

Fig. 3: Inflammatory marker gene expression in zebrafishes treated with different concentrations of *Parmotrema tinctorum* extract ([a] β-Actin gene expression, [b] iNOS gene expression and [c] TNFα gene expression)

al. supported the traffic clearance activity of neutrophils at the site of tissue damage by reverse migration process [35,36].

The skeletal muscles in the adult zebrafishes are composed of 60% body mass and studies on toxic effects of high relevance [37]. Especially, in zebrafish, optical transparency of the larvae could help to elucidate the novel mechanisms of neutrophil behaviour in response to tissue damage and infection. It is said that reversal of neutrophils might serve as a mechanism of systematic action of immune response. Examining

the changes that appear in muscle tissues of zebrafish which are made of fibers and muscle cells held together by connective tissues would provide factual data regarding tissue damage. In the present work, neutrophil profiling in the mussel tissues of zebrafishes treated with *P. tinctorum* extract was done (Fig. 2). The cells examined suggested the absence of biological activity in the lichen extract. The results of the histopathological investigation recorded a decreased number of neutrophil cells, suggesting that the lichen extract exhibited anti-inflammatory activity. Hence, the inflammatory marker gene expression due to *P. tinctorum* extract treatment in zebrafish test was carried out, and the results suggested the presence of dose-dependent anti-inflammatory induction activity.

According to Hong *et al*. [38], usnic acids of *Umbilicaria antarctica* extract possess dose-dependent survival rate efficiency in zebrafishes. They have demonstrated the presence of decreased survival rate in the *U. antarctica* extract fed zebra fish at a concentration of 200 and 400 µg/mL at 24 and 48 hpf. The extract of *P. tinctorum* decreased the gene expression of the tumor necrosis factor, TNF-α which suggested the suppression of cytokine induction (Fig. 3). Since, tumor necrosis factor is found to be present in the suppression of cell proliferation and inflammation, the same was examined using the standard PCR procedure. It was found that the extract had the dose-dependent suppression activity. This is in accordance with the report given by Hong *et al*. [38] who showed the dose-dependent suppression of TNF-α gene by usnic acid.

CONCLUSION

A few toxicological researches in the lichen extracts have been conducted extensively. A lichen species, less explored in the *Parmotrema* genus is *P. tincotrum*. This species is widespread in the study area and biological activities of such species are found in the literature. The lichen compounds of *P. tinctorum* are less studied and were considered to investigate for the present study. Zebrafishes treated with 200 mg/ml of *P. tinctorum* extract concentration showed an increase in the number of neutrophil cells in the muscle bundles. The supportive studies helped to carry out inflammatory marker gene expression studies through PCR. The results suggested the presence of dose-dependent anti-inflammatory activity in the extract of *P. tinctorum*. Thus, this may be concluded that the *P. tinctorum* extract may be an alternative drug molecule as well as nutraceutical food supplement. Extensive research is required further to acknowledge their anticancer-related biological activities.

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AUTHORS CONTRIBUTIONS

Ponnuchamy Ponmurugan performed the study design, Muthu Shenbagam, Murugan Mariraj - Data collection and drafting of manuscript, Rajendran Kalidoss, Machampalayam Arumugam Deepa - editing of the manuscript, All Authors revised and approved the final article.

CONFLICT OF INTEREST

The authors have no conflict of interest.

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