Protective Effects of Rosmarinic Acid Against Microplastic-Induced Oxidative Stress in RTgill-W1 Fish Gill Cell Culture: A Pharmacological *In Vitro* Study

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Abstract

Microplastic contamination has emerged as a significant environmental threat to aquatic ecosystems, contributing to oxidative stress, inflammation, and cellular toxicity in aquatic organisms. This study investigated the protective effects of rosmarinic acid (RA) against microplastic-induced cytotoxicity and oxidative stress using RTgill-W1 fish gill cells as an *in vitro* aquatic toxicology model. Cells were exposed to polystyrene microplastics (PS-MPs; 100 µg/mL) either alone or in combination with low-dose (25 µM) or high-dose (50 µM) RA for 24 h. Cell viability was determined using the MTT assay, while oxidative stress biomarkers including reactive oxygen species (ROS), malondialdehyde (MDA), glutathione (GSH), and catalase activity were evaluated. Inflammatory responses were assessed through quantitative PCR analysis of TNF-α and IL-1β expression. PS-MP exposure significantly reduced cell viability from 100.0 ± 2.4% to 52.6 ± 4.1% ($p < 0.05$), accompanied by marked increases in ROS production (248 ± 12%), MDA levels (6.8 ± 0.5 nmol/mg protein), TNF-α expression (4.86 ± 0.31-fold), and IL-1β expression (5.42 ± 0.40-fold). Simultaneously, antioxidant defenses were impaired, as evidenced by reductions in catalase activity (21.4 ± 1.8 U/mg protein) and GSH content (3.1 ± 0.3 µmol/g protein). Co-treatment with RA significantly attenuated these effects in a dose-dependent manner. The high-dose RA treatment restored cell viability to 88.4 ± 3.2%, reduced ROS and MDA levels to 122 ± 7% and 3.1 ± 0.3 nmol/mg protein, respectively, and increased catalase activity and GSH content to 43.7 ± 1.9 U/mg protein and 7.0 ± 0.3 µmol/g protein. Furthermore, RA markedly suppressed TNF-α and IL-1β expression to 1.42 ± 0.11-fold and 1.66 ± 0.13-fold, respectively. These findings demonstrate that RA effectively protects RTgill-W1 cells against microplastic-induced oxidative and inflammatory injury and highlight its potential as a natural pharmacological strategy for mitigating aquatic pollutant toxicity.

Keywords: Rosmarinic acid; Microplastics; RTgill-W1; Oxidative stress; Aquatic toxicology; Fish cell culture; Antioxidant; Inflammation

How to cite this article: Younis JSK, Alabdulwahed A, Manap ASA, Albokhadaim I. Protective Effects of Rosmarinic Acid Against Microplastic-Induced Oxidative Stress in RTgill-W1 Fish Gill Cell Culture: A Pharmacological In Vitro Study. Int J Drug Deliv Technol. 2026;16(64s):1653-1663. DOI: 10.25258/ijddt.16.64s.181

1. Introduction

Microplastic pollution has become a major global environmental concern due to its persistence, bioaccumulation potential, and toxicological effects on aquatic organisms (1,2). Microplastics, generally defined as plastic particles smaller than 5 mm, originate from industrial products, cosmetic materials, textile fibers, and degradation of larger plastic debris (3). Their widespread presence in marine and freshwater environments has raised increasing concerns regarding aquatic ecosystem health and food chain contamination (4).

Accumulating evidence suggests that microplastics can induce substantial cellular toxicity through oxidative stress mechanisms (5,6). Previous studies have demonstrated that exposure to polystyrene microplastics (PS-MPs) promotes excessive reactive oxygen species (ROS) production, lipid peroxidation, mitochondrial dysfunction, inflammatory responses, and apoptosis in aquatic organisms (7–9). Oxidative stress is considered one of the primary molecular

pathways underlying microplastic-mediated toxicity (5). Elevated ROS generation disrupts antioxidant defense systems, leading to cellular membrane damage, protein oxidation, and DNA injury (10,11).

In recent years, natural antioxidant compounds have gained considerable attention as potential protective agents against environmental toxicants (12). Rosmarinic acid (RA), a naturally occurring polyphenolic compound abundantly found in rosemary, basil, mint, and other medicinal plants, possesses potent antioxidant, anti-inflammatory, and cytoprotective properties (13,14). Several pharmacological studies have demonstrated that RA effectively scavenges free radicals, modulates antioxidant enzymes, and suppresses inflammatory cytokine production through regulation of oxidative stress pathways (15,16). However, despite extensive investigations on RA in mammalian systems, limited information is available regarding its protective effects against microplastic-induced toxicity in aquatic cell models.

The RTgill-W1 fish cell line derived from rainbow trout gill tissue is widely recognized as a reliable *in vitro* model for aquatic toxicology and environmental risk assessment (17). This cell line provides an ethically favorable alternative to live fish experimentation while maintaining high sensitivity toward aquatic pollutants and toxic compounds (18).

Therefore, the present study aimed to evaluate the protective pharmacological effects of rosmarinic acid against polystyrene microplastic-induced oxidative stress and inflammatory injury using RTgill-W1 fish cell culture. The study specifically investigated changes in cell viability, oxidative stress biomarkers, antioxidant enzyme activities, and inflammatory cytokine expression following microplastic exposure and RA treatment.

2. Materials and Methods

The overall experimental workflow illustrating RTgill-W1 cell culture preparation, microplastic exposure, rosmarinic acid treatment, oxidative stress evaluation, and inflammatory biomarker analysis is presented in Figure 1.

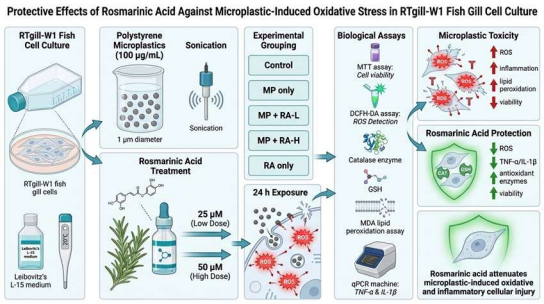

Figure 1. Schematic illustration of the experimental workflow investigating the protective effects of rosmarinic acid against microplastic-induced oxidative stress in RTgill-W1 fish cell culture. RTgill-W1 cells were cultured in Leibovitz's L-15 medium and exposed to polystyrene microplastics (PS-MPs; 100 µg/mL) following sonication. Cells were subsequently treated with low-dose (25 µM) and high-dose (50 µM) rosmarinic acid (RA). Experimental groups included control, microplastic-only, microplastic plus low-dose RA (MP + RA-L), microplastic plus high-dose RA (MP + RA-H), and RA-only groups. Following 24 h exposure, multiple biological assays were performed, including MTT assay for cell viability, DCFH-DA assay for intracellular reactive oxygen species (ROS) detection, catalase enzyme activity, glutathione (GSH) analysis, malondialdehyde (MDA) lipid peroxidation assay, and quantitative PCR analysis of inflammatory cytokines (TNF-α and IL-1β). The workflow highlights the oxidative and inflammatory cellular injury induced by microplastic exposure and the protective antioxidant effects mediated by rosmarinic acid treatment.

2.1 Chemicals and Reagents

Rosmarinic acid (purity ≥98%), polystyrene microplastics (1 µm diameter), dimethyl sulfoxide (DMSO), and MTT reagent were purchased from Sigma-Aldrich (St. Louis, MO, USA). DCFH-DA ROS assay kits, catalase activity assay kits, glutathione assay kits, and lipid peroxidation (MDA) assay kits were obtained from Abcam (Cambridge, UK). Cell culture reagents including Leibovitz's L-15 medium, fetal bovine serum (FBS), and penicillin-streptomycin were purchased from Gibco (Thermo Fisher Scientific, USA).

2.2 Cell Culture

RTgill-W1 fish gill cell lines derived from rainbow trout (*Oncorhynchus mykiss*) were obtained from the American Type Culture Collection (ATCC, USA). Cells were cultured in Leibovitz's L-15 medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin under humidified conditions at 20°C without CO_2 supplementation. Cells were subcultured upon reaching approximately 80–90% confluence.

2.3 Preparation of Microplastic Suspension

Commercial fluorescent-free polystyrene microplastics (PS-MPs) with an average particle diameter of 1 µm were purchased from Sigma-Aldrich (St. Louis, MO, USA). Prior to experimental use, PS-MPs were sterilized under ultraviolet (UV) light exposure for 30 min inside a biosafety cabinet to minimize microbial contamination. A primary stock suspension of PS-MPs was prepared by dispersing the particles in sterile distilled water at a concentration of 1 mg/mL. The suspension was vortexed vigorously for 5 min followed by ultrasonication using a bath sonicator for 15 min at room temperature to minimize particle aggregation and ensure homogeneous dispersion.

To further improve suspension stability, the stock solution was intermittently mixed during sonication. The prepared stock suspension was freshly diluted in Leibovitz's L-15 culture medium to obtain the final working concentration of 100 µg/mL immediately before cell treatment. The selected concentration was based on preliminary cytotoxicity optimization and previously published aquatic toxicology studies reporting significant oxidative stress induction without causing excessive irreversible cell death in fish cell culture models.

To maintain consistency and minimize sedimentation, PS-MP suspensions were freshly prepared before each experiment and gently vortexed immediately prior to application onto RTgill-W1 cells. All experimental procedures involving microplastic preparation were conducted under sterile conditions to prevent external contamination.

2.4 Experimental Design

Cells were seeded into 96-well plates at a density of 1×10^4 cells/well and allowed to attach for 24 h before treatment. The experimental groups were divided as the Table 1 below.

Table 1. Experimental grouping and treatment conditions for RTgill-W1 fish cell culture exposed to polystyrene microplastics (PS-MPs) and rosmarinic acid (RA). Cells were treated with PS-MPs at a concentration of 100 µg/mL either alone or in combination with low-dose (25 µM) and high-dose (50 µM) RA for 24 h exposure. The concentrations of RA were selected based on preliminary non-cytotoxic optimization and previously reported antioxidant-active ranges in *in vitro* models.

Group	Treatment
Control	Untreated cells
MP	PS-MPs (100 µg/mL)
MP + RA-L	PS-MPs + Rosmarinic acid (25 µM)
MP + RA-H	PS-MPs + Rosmarinic acid (50 µM)
RA only	Rosmarinic acid (50 µM)

Cells were treated for 24 h prior to subsequent analyses.

2.5 Cell Viability Assay

Cell viability was determined using the MTT assay. Following treatment, 20 µL of MTT solution (5 mg/mL) was added into each well and incubated for 4 h. Formazan crystals formed were dissolved using DMSO, and absorbance was measured at 570 nm using a microplate reader.

2.6 Reactive Oxygen Species (ROS) Assay

Intracellular ROS production was measured using DCFH-DA fluorescent dye. Treated cells were incubated with DCFH-DA solution (10 µM) for 30 min in the dark. Fluorescence intensity was measured using a fluorescence microplate reader at excitation/emission wavelengths of 485/530 nm.

2.7 Determination of Oxidative Stress Biomarkers

Catalase activity, glutathione (GSH) levels, and malondialdehyde (MDA) concentrations were analyzed according to manufacturer instructions using commercial assay kits. Absorbance values were recorded using a microplate spectrophotometer.

2.8 Quantitative PCR Analysis

Total RNA was extracted from treated RTgill-W1 cells using TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol. RNA purity and concentration were determined spectrophotometrically using a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, USA). Complementary DNA (cDNA) was synthesized from 1 µg of total RNA using a reverse transcription kit (Thermo Fisher Scientific, USA).

Quantitative real-time PCR (qPCR) was performed using SYBR Green Master Mix (Applied Biosystems, USA) on a QuantStudio™ 5 Real-Time PCR System (Applied Biosystems, USA). The amplification conditions consisted of an initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min.

The expression levels of tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β) were evaluated as inflammatory biomarkers. β-actin was used as the housekeeping gene for normalization. Relative gene expression was calculated using the 2^-ΔΔCt method.

The primer sequences used in this study are presented in Table 2.

Table 2. Primer sequences used for quantitative PCR analysis

Gene	Primer Sequence (5′–3′)	Product Size (bp)
TNF-α	F: AGGCTGCTGTAACCGAAAGT	152
	R: TGGTGTGGGTGAGGAGAGTA	
IL-1β	F: TGTGTGTTTGGGAATCTGGC	145
	R: AGGTCGTCATCATCCCACAC	
β-actin	F: CCTTCCTTCCTGGGTATGGA	138
	R: GGTCCTTACGGATGTCCACG	

2.9 Statistical Analysis

All data were expressed as mean ± standard deviation (SD) from three independent experiments. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test using GraphPad Prism version 9.0 (GraphPad Software Inc., USA). Statistical significance was considered at $p < 0.05$.

3. Results

3.1 Effect of Rosmarinic Acid on Cell Viability

Exposure to PS-MPs significantly reduced RTgill-W1 cell viability compared to the control group ($p < 0.05$) (Table 3, Figure 2). However, co-treatment with rosmarinic acid significantly restored cell viability in a dose-dependent manner (Table 3, Figure 2).

Table 3. Effect of Rosmarinic Acid on RTgill-W1 Cell Viability

Group	Cell Viability (%)
Control	100.0 ± 2.4
MP	52.6 ± 4.1*
MP + RA-L	71.2 ± 3.8#
MP + RA-H	88.4 ± 3.2#δ
RA only	98.1 ± 2.0

*Significantly different from control group ($p < 0.05$). #Significantly different from MP group ($p < 0.05$). δSignificantly different from MP + RA-L group ($p < 0.05$).

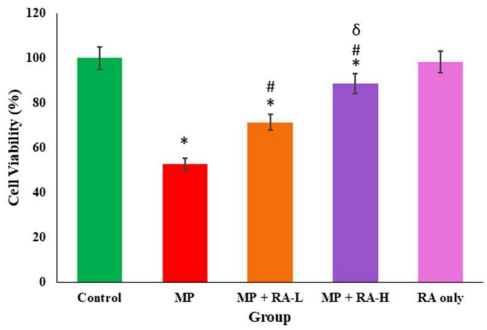

Figure 2. Effects of rosmarinic acid on RTgill-W1 cell viability following exposure to polystyrene microplastics (PS-MPs). RTgill-W1 cells were exposed to PS-MPs (100 µg/mL) alone or in combination with low-dose rosmarinic acid (RA-L, 25 µM) and high-dose rosmarinic acid (RA-H, 50 µM) for 24 h. Exposure to PS-MPs significantly reduced cell viability compared with the control group. Co-treatment with rosmarinic acid significantly restored cell viability in a dose-dependent manner, with the high-dose treatment exhibiting greater cytoprotective effects than the low-dose treatment. Data are presented as mean ± SD (n = 3 independent experiments).

**Significantly different from the control group ($p < 0.05$).*
#Significantly different from the MP group ($p < 0.05$).
δSignificantly different from the MP + RA-L group ($p < 0.05$).

Abbreviations: MP, polystyrene microplastics; RA-L, low-dose rosmarinic acid (25 µM); RA-H, high-dose rosmarinic acid (50 µM).

3.2 Effects on Oxidative Stress Biomarkers

Exposure to PS-MPs significantly increased ROS production and lipid peroxidation while reducing antioxidant enzyme activity and GSH levels compared with the control group. Co-treatment with rosmarinic acid significantly attenuated oxidative stress and restored antioxidant defenses in a dose-dependent manner, with the high-dose treatment exhibiting greater protective effects than the low-dose treatment. (Table 4, Figure 3).

Table 4. Effects of Rosmarinic Acid on Oxidative Stress Biomarkers in RTgill-W1 Cells

Group	ROS (%)	MDA (nmol/mg protein)	Catalase (U/mg protein)	GSH (µmol/g protein)
Control	100 ± 5	2.1 ± 0.2	48.6 ± 2.3	7.8 ± 0.4
MP	248 ± 12*	6.8 ± 0.5*	21.4 ± 1.8*	3.1 ± 0.3*
MP + RA-L	176 ± 10#	4.5 ± 0.4#	34.8 ± 2.0#	5.6 ± 0.4#
MP + RA-H	122 ± 7#δ	3.1 ± 0.3#δ	43.7 ± 1.9#δ	7.0 ± 0.3#δ
RA only	98 ± 4	2.0 ± 0.1	49.5 ± 2.1	7.9 ± 0.2

*Significantly different from control group ($p < 0.05$). #Significantly different from MP group ($p < 0.05$). δ Significantly different from MP + RA-L group ($p < 0.05$).

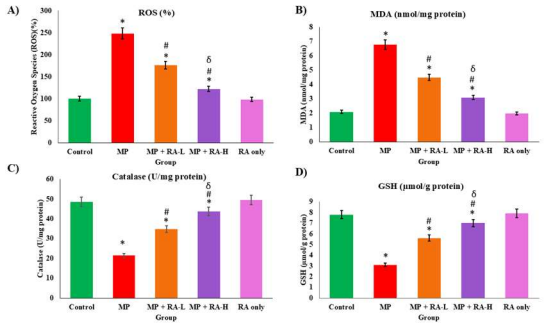

Figure 3. Effects of rosmarinic acid on oxidative stress biomarkers in RTgill-W1 cells exposed to polystyrene microplastics (PS-MPs). RTgill-W1 cells were treated with PS-MPs (100 µg/mL) alone or in combination with low-dose rosmarinic acid (RA-L, 25 µM) and high-dose rosmarinic acid (RA-H, 50 µM) for 24 h. **(A)** Intracellular reactive oxygen species (ROS) levels, **(B)** malondialdehyde (MDA) concentrations, **(C)** catalase activity, and **(D)** glutathione (GSH) content. Data are presented as mean ± SD (n = 3 independent experiments).

**Significantly different from the control group ($p < 0.05$).*
#Significantly different from the MP group ($p < 0.05$).
δSignificantly different from the MP + RA-L group ($p < 0.05$).

Abbreviations: MP, polystyrene microplastics; RA-L, low-dose rosmarinic acid (25 µM); RA-H, high-dose rosmarinic acid (50 µM); ROS, reactive oxygen species; MDA, malondialdehyde; GSH, reduced glutathione.

3.3 Effects on Inflammatory Cytokine Expression

PS-MPs significantly upregulated TNF-α and IL-1β expression compared to control cells. Rosmarinic acid markedly suppressed inflammatory gene expression (Table 5, Figure 4).

Table 5. Relative Gene Expression of Inflammatory Cytokines

Group	TNF-α Fold Change	IL-1β Fold Change
Control	1.00 ± 0.08	1.00 ± 0.07
MP	4.86 ± 0.31*	5.42 ± 0.40*
MP + RA-L	2.91 ± 0.22#	3.10 ± 0.28#
MP + RA-H	1.42 ± 0.11# δ	1.66 ± 0.13# δ
RA only	0.96 ± 0.06	0.94 ± 0.05

*Significantly different from control group ($p < 0.05$).
#Significantly different from MP group ($p < 0.05$).
δ Significantly different from MP + RA-L group ($p < 0.05$).

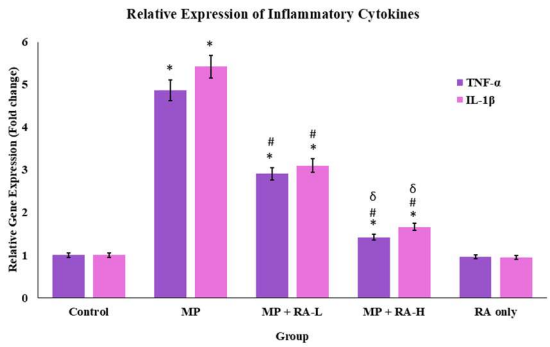

Figure 4. Effects of rosmarinic acid on inflammatory cytokine expression in RTgill-W1 cells exposed to polystyrene microplastics (PS-MPs). Relative mRNA expression levels of TNF-α and IL-1β were quantified by quantitative real-time PCR (qPCR) following 24 h exposure to PS-MPs (100 µg/mL) in the presence or absence of rosmarinic acid (RA). Exposure to PS-MPs significantly upregulated TNF-α and IL-1β expression compared with the control group, indicating activation of inflammatory responses. Co-treatment with rosmarinic acid significantly reduced cytokine expression in a dose-dependent manner, with the high-dose treatment (50 µM) exhibiting greater anti-inflammatory effects than the low-dose treatment (25 µM). Data are presented as mean ± SD (n = 3 independent experiments).

**Significantly different from the control group ($p < 0.05$).*
#Significantly different from the MP group ($p < 0.05$).
δSignificantly different from the MP + RA-L group ($p < 0.05$).

Abbreviations: MP, polystyrene microplastics; RA-L, low-dose rosmarinic acid (25 µM); RA-H, high-dose rosmarinic acid (50 µM); TNF-α, tumor necrosis factor-alpha; IL-1β, interleukin-1 beta.

4. Discussion

The present study demonstrated that exposure to polystyrene microplastics (PS-MPs) induced significant cytotoxicity, oxidative stress, and inflammatory responses in RTgill-W1 fish cell cultures, whereas rosmarinic acid (RA) effectively attenuated these adverse effects. Specifically, PS-MP exposure resulted in reduced cell viability, elevated intracellular reactive oxygen species (ROS) generation, increased lipid peroxidation, impaired

antioxidant defenses, and enhanced expression of pro-inflammatory cytokines. In contrast, co-treatment with RA significantly restored cellular homeostasis in a dose-dependent manner, highlighting its potential as a pharmacological intervention against microplastic-induced toxicity in aquatic systems.

The observed reduction in cell viability following microplastic exposure is consistent with previous studies reporting that PS-MPs impair cellular integrity and metabolic activity in aquatic organisms (19,20). Several investigations involving zebrafish, rainbow trout, and fish-derived cell lines have demonstrated that microplastics disrupt membrane function, mitochondrial activity, and redox balance, ultimately leading to cellular injury (21). In the present study, exposure to 100 µg/mL PS-MPs reduced cell viability to approximately 53%, demonstrating that this concentration was sufficient to induce measurable toxicity while preserving an adequate number of viable cells for evaluating protective interventions. The selected PS-MP concentration was based on previous aquatic toxicology studies and preliminary optimization (22), ensuring a balance between biological relevance and experimental sensitivity. In contrast, certain studies have demonstrated minimal cytotoxic effects. Wu et al. (2020) reported that exposure of Caco-2 cells to 5 µm microplastics at concentrations up to 100 µg/mL did not affect cell viability after 24 h, whereas a slight 10% decrease was observed following 48 h of exposure (23).

Oxidative stress appeared to be a major mechanism underlying microplastic toxicity in RTgill-W1 cells (24). The significant increase in ROS and MDA levels observed in the MP-treated group indicates excessive free radical production and membrane lipid peroxidation. Elevated ROS generation has been widely recognized as one of the earliest cellular responses following microplastic exposure. Similar increases in oxidative stress markers have been reported in zebrafish liver tissues (25), fish gill cells (26), and marine organisms (27) exposed to polystyrene microplastics. Excessive ROS accumulation can damage cellular proteins, lipids, and nucleic acids, thereby contributing to cellular dysfunction and inflammatory activation (28).

Importantly, RA treatment markedly reduced ROS generation and MDA accumulation while restoring endogenous antioxidant defenses, including catalase activity and GSH content. These findings are in agreement with previous pharmacological studies demonstrating the potent antioxidant properties of RA (15). The compound possesses multiple hydroxyl groups capable of directly scavenging reactive oxygen species while simultaneously enhancing endogenous antioxidant enzyme systems (29). Earlier studies in mammalian neuronal, hepatic, and epithelial cell models have shown that RA activates antioxidant signaling pathways, particularly the Nrf2/ARE pathway, resulting in increased expression of antioxidant enzymes and enhanced cellular resistance against oxidative insults (30–32). Although the present study did not investigate Nrf2 signaling directly, the restoration of catalase and GSH strongly suggests involvement of antioxidant regulatory mechanisms.

One of the most significant findings of this study was the clear dose-dependent protective effect of RA. Both concentrations tested (25 µM and 50 µM) significantly improved cellular outcomes compared with the MP group; however, the higher concentration consistently produced superior protection across all measured parameters. The high-dose RA group demonstrated significantly lower ROS and MDA levels, higher catalase activity and GSH content, and greater restoration of cell viability compared with the low-dose group. Furthermore, statistical comparisons between the two treatment groups confirmed that the protective efficacy of 50 µM RA was significantly greater than that of 25 µM RA. These findings suggest that RA-mediated cytoprotection is concentration-dependent within the tested range.

The selection of 25 µM and 50 µM RA concentrations was based on previous reports demonstrating effective antioxidant and anti-inflammatory activities without inducing cytotoxicity in cultured cells (33,34). The lower concentration was chosen to evaluate moderate protective activity, whereas the higher concentration represented an upper non-toxic dose commonly reported in cellular pharmacology studies. Notably, the 50 µM concentration restored several biomarkers to levels approaching those of the control group, suggesting that this dose may represent an optimal protective concentration for future investigations

involving aquatic cell models. Nevertheless, additional dose-response studies involving broader concentration ranges are warranted to establish the maximum effective concentration and therapeutic window of RA in aquatic toxicology applications.

Inflammatory responses also contributed substantially to microplastic-induced cellular injury. Exposure to PS-MPs significantly increased TNF-α and IL-1β expression, indicating activation of pro-inflammatory pathways. Similar findings have been reported in zebrafish and fish-derived cell models, where microplastics stimulate inflammatory signaling cascades through oxidative stress-mediated activation of transcription factors such as NF-κB (7). The observed increase in cytokine expression likely reflects a cellular attempt to respond to oxidative damage and foreign particle exposure. Importantly, RA significantly suppressed TNF-α and IL-1β expression in a dose-dependent manner, demonstrating potent anti-inflammatory activity. Previous studies have reported that RA inhibits NF-κB activation and reduces cytokine production in various inflammatory models (35,36), supporting the findings obtained in the present study.

The present work possesses several notable strengths. First, the study integrates environmental toxicology with pharmacological intervention, an area that remains relatively underexplored despite increasing concerns regarding microplastic pollution. Most microplastic studies focus primarily on toxicological characterization, environmental monitoring, or ecological risk assessment. In contrast, the current study investigates a potential mitigation strategy using a naturally occurring bioactive compound. Second, the use of RTgill-W1 cells provides an ethically favorable and cost-effective alternative to animal experimentation while maintaining biological relevance to aquatic toxicology. Third, the demonstration of dose-dependent protection strengthens the pharmacological significance of RA and supports its potential development as a protective agent against emerging aquatic pollutants.

To our knowledge, this study represents one of the first investigations evaluating the protective effects of rosmarinic acid against microplastic-induced oxidative and inflammatory injury in RTgill-W1 fish cells. This novel combination of aquatic toxicology, environmental pollution research, and natural-product pharmacology provides valuable mechanistic evidence supporting the use of antioxidant-based interventions in aquatic health management. The findings also expand the current understanding of how natural polyphenolic compounds may counteract cellular damage induced by emerging environmental contaminants.

Despite these promising findings, several limitations should be acknowledged. The study was conducted using a single cell line and one microplastic type, which may not fully represent the complexity of environmental exposure scenarios. Additionally, mechanistic pathways such as Nrf2, NF-κB, MAPK, and apoptosis-related signaling were not examined. Future studies should investigate these molecular pathways and validate the protective effects of RA in additional fish cell models and in vivo aquatic systems. Furthermore, the efficacy of RA against environmentally aged microplastics, mixed polymer types, and chronic exposure conditions warrants further investigation.

Overall, the present findings suggest that rosmarinic acid possesses significant potential as a natural pharmacological agent for mitigating microplastic-induced oxidative and inflammatory damage in aquatic organisms. These results may contribute to the future development of antioxidant-based strategies for protecting aquatic species from the growing threat of microplastic pollution and provide a foundation for further translational research in environmental pharmacology and aquatic health.

5. Conclusion

This study demonstrated that polystyrene microplastics induce significant oxidative stress, inflammatory responses, and cytotoxic effects in RTgill-W1 fish cell culture. Rosmarinic acid effectively attenuated these toxicological effects by restoring antioxidant enzyme activity, reducing ROS accumulation and lipid peroxidation, and suppressing inflammatory cytokine expression. The findings establish rosmarinic acid as a promising natural pharmacological agent against microplastic-induced aquatic cellular toxicity. Moreover, the study provides an ethically favorable *in vitro* framework for

investigating environmental pollutant toxicity and pharmacological mitigation strategies in aquatic systems. These results contribute important mechanistic evidence supporting the future development of antioxidant-based interventions for protecting aquatic organisms against emerging environmental contaminants.

Acknowledgments

The author would like to thank the Deanship of Scientific Research, Vice Presidency for Graduate Studies and Scientific Research, King Faisal University, Saudi Arabia for supporting this project.

Funding Sources

The author declares financial support was received for the research, authorship, and/or publication of this article. This work was supported by the Deanship of Scientific Research, Vice Presidency for Graduate Studies and Scientific Research, King Faisal University, Saudi Arabia (Grant No. KFU262981)

Competing Interest

The authors declare no conflict of interest.

Authors' Contributions

A.S.A.M. and I.A. conceived and designed the study. J.S.K.Y. and A.A. performed the experiments and data collection. A.S.AM., J.S.K.Y, and A.A. generated the data. A.S.A.M. and I.A. analyzed the data. A.S.A.M., J.S.K.Y., and A.A. wrote the paper. All authors discussed the data and read and approved the final version of the manuscript.

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