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RESEARCH ARTICLE

Evaluation of the Protective Effects of Beetroot Extract Against *Aeromonas hydrophila*-Induced Toxicity in Zebrafish Larvae

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Abstract

Aeromonas hydrophila is a gram-negative bacterium responsible for severe infections in aquatic organisms, including zebrafish. The present study evaluates the protective effects of beetroot extract (BRE) against *A. hydrophila*-induced toxicity in zebrafish larvae. Zebrafish were exposed to *A. hydrophila* (1×10^6 CFU/mL) and subsequently treated with BRE at concentrations of 100 and 200 µg/mL. The antioxidant enzyme activities of superoxide dismutase, and catalase, glutathione levels, nitric oxide inhibition, lipid peroxidation, and glutathione S-transferase activities were assessed. Additionally, apoptosis and oxidative stress were analyzed using acridine orange, diphenyl-1-pyrenylphosphine, and dichlorofluorescin diacetate assays. Results demonstrated a significant reduction in oxidative stress and apoptosis in BRE-treated groups compared to *A. hydrophila*-infected groups. BRE significantly restored the antioxidant enzyme activities, reduced lipid peroxidation, and mitigated ROS production. The findings indicate that BRE offers substantial protection against *A. hydrophila*-induced toxicity by modulating oxidative stress and apoptosis in zebrafish larvae.

1. Introduction

Aquatic organisms, particularly fish, are highly susceptible to bacterial infections due to their constant exposure to waterborne pathogens (Haenen et al., 2023). *Aeromonas hydrophila*, a gram-negative opportunistic pathogen, is one of the most common causes of infectious diseases in fish, leading to high mortality rates and severe economic losses in aquaculture (Semwal et al., 2023). This bacterium is known to cause hemorrhagic septicemia, ulcerative lesions, and systemic infections in fish species, including zebrafish (*Danio rerio*). The virulence factors of *A. hydrophila* include exotoxins, hemolysins, lipases, and proteases, which contribute to its pathogenicity (Majeed et al., 2023). The infection process is further exacerbated by the production of reactive oxygen species (ROS), which disrupt cellular integrity, induce apoptosis, and lead to extensive oxidative stress. Oxidative stress is a major consequence of bacterial infections, wherein an imbalance occurs between the production of ROS and the antioxidant defense mechanisms of the host (S. Kumar et al., 2022). Excessive ROS production can damage lipids, proteins, and DNA, resulting in cell death and inflammation. The primary antioxidant defense system in organisms comprises enzymatic and non-enzymatic components. Superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) play crucial roles in neutralizing ROS and protecting cells from oxidative damage (Aribisala and Sabiu, 2022; Sahu et al., 2022). However, during bacterial infections, the overwhelming increase in ROS production can lead to the depletion of these antioxidant enzymes, further exacerbating cellular damage.

Natural plant-derived compounds have garnered significant attention as potential therapeutic agents due to their antioxidant, anti-inflammatory, and antimicrobial properties. Beetroot (Beta vulgaris) is a rich source of bioactive compounds, including betalains, flavonoids, polyphenols, and nitrates (Thiruvengadam et al., 2024). These compounds have been extensively studied for their health benefits, such as reducing oxidative stress, modulating inflammation, and improving metabolic health. Betalains, the red and yellow pigments found in beetroot, exhibit strong free radical-scavenging abilities, making them effective in mitigating oxidative stress-induced damage (Bashir et al., 2024; de Oliveira et al., 2021). Several studies have demonstrated the protective effects of plant extracts in modulating oxidative stress and inflammatory responses in various biological models. For instance, curcumin, resveratrol, and quercetin have been reported to alleviate oxidative stress-related

apoptosis in zebrafish larvae (Vicidomini et al., 2024; J. Wang et al., 2023). Similarly, beetroot extract has been shown to possess cardioprotective, neuroprotective, and hepatoprotective properties, making it a promising candidate for mitigating bacterial-induced toxicity. The nitrate content in beetroot further contributes to its protective effects by enhancing nitric oxide (NO) production, which plays a role in vascular function and cellular signaling (Brzezińska-Rojek et al., 2023).

Zebrafish have emerged as a powerful model organism in biomedical and toxicological research due to their genetic similarity to humans, rapid development, and transparent embryos, which facilitate in vivo imaging. Zebrafish larvae provide an excellent platform for studying host-pathogen interactions, oxidative stress responses, and the efficacy of therapeutic agents (Franza et al., 2024; He et al., 2024). Their small size and high fecundity make them suitable for high-throughput screening of drug candidates and natural compounds. Additionally, zebrafish share key components of immune and antioxidant defense systems with higher vertebrates, making them an ideal model for evaluating the protective effects of beetroot extract against *A. hydrophila*-induced toxicity. This study aims to investigate the potential of aqueous beetroot extract (BRE) in mitigating oxidative stress, apoptosis, and lipid peroxidation in zebrafish larvae exposed to *A. hydrophila* infection. By assessing key oxidative stress biomarkers, antioxidant enzyme activities, and apoptotic markers, we seek to elucidate the protective mechanisms of beetroot extract in combating bacterial-induced toxicity. The findings from this study could provide valuable insights into the use of natural plant-based therapeutics in aquaculture and human health applications.

2. Materials and Methods

2.1. Zebrafish toxicity

Zebrafish larvae at 5 days post-fertilization (dpf) were used for the study. The larvae were randomly divided into five groups: (1) Control (untreated larvae), (2) A. hydrophila-infected group, (3) BRE 100 µg/mL-treated group, (4) BRE 200 µg/mL-treated group. The bacterial infection was induced by exposing larvae to A. hydrophila at a concentration of 1×10^6 CFU/mL for 24 hours. Following infection, larvae were treated with BRE at the specified concentrations for another 24 hours. Larvae were observed under a stereomicroscope for mortality rates. Survival percentages were calculated, and images of larvae were captured for comparative analysis (Hariharan et al., 2024).

2.2. SOD and CAT Enzyme Activity

Whole-body homogenates of zebrafish larvae were prepared using ice-cold phosphate-buffered saline (PBS, pH 7.4). The homogenates were centrifuged at 12,000 rpm for 10 minutes at 4°C, and the supernatants were collected for enzyme assays. SOD activity was determined using a colorimetric assay, where the inhibition of nitroblue tetrazolium reduction by superoxide radicals was measured at 560 nm. CAT activity was assessed by measuring the decomposition of hydrogen peroxide at 240 nm. Enzyme activities were expressed as units per milligram of protein (U/mg protein), and protein concentration was determined using the Bradford assay (Lanzarin et al., 2021; Zhao et al., 2022).

2.3. GSH Enzyme Activity and NO Production Inhibition

GSH levels were measured using Ellman's reagent (5,5'-dithiobis(2-nitrobenzoic acid), DTNB). Briefly, the reaction mixture contained homogenate supernatant, DTNB, and sodium phosphate buffer, and the absorbance was read at 412 nm. The levels of GSH were expressed as nmol/mg protein. NO production was quantified using the Griess reagent, which detects nitrite accumulation in the culture supernatant. The absorbance was measured at 540 nm, and the NO levels were compared between the infected and treated groups (Zhang et al., 2023; Zhou et al., 2022).

2.4. Lipid Peroxidation Inhibition and GST Enzyme Activity

Lipid peroxidation was evaluated by measuring malondialdehyde (MDA) levels using the thiobarbituric acid reactive substances assay. Homogenate supernatants were incubated with thiobarbituric acid at 95°C for 30 minutes, and the absorbance of the pink chromophore was measured at 532 nm. The results were expressed as nmol MDA/mg protein. GST activity was measured using 1-chloro-2,4-dinitrobenzene as a substrate, and the formation of a conjugate with GSH was recorded at 340 nm. GST activity was expressed in U/mg protein (Mukherjee et al., 2022; Yan et al., 2022).

2.5. Acridine Orange Assay for Apoptosis Detection

Acridine orange staining was performed to detect apoptotic cells in live zebrafish larvae. Larvae were incubated with acridine orange (5 µg/mL) for 30 minutes in the dark and then washed with PBS. Fluorescence images were captured under a fluorescence microscope using the FITC filter. The intensity of green fluorescence, indicative of apoptotic cells, was quantified using ImageJ software (Wang et al., 2022).

2.6. Detection of Lipid Peroxidation

The diphenyl-1-pyrenylphosphine (DPPP) assay was used to detect lipid peroxidation in live zebrafish larvae. Larvae were incubated with DPPP (5 µM) for 30 minutes at room temperature, followed by washing with PBS. Fluorescence images were acquired using a fluorescence microscope, and lipid peroxidation levels were analyzed based on fluorescence intensity (Priya et al., 2023).

2.7. Assay for ROS Measurement

ROS levels were assessed using 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) staining. Zebrafish larvae were exposed to DCFDA (20 µM) for 30 minutes in the dark, washed with PBS, and analyzed under a fluorescence microscope. The fluorescence intensity was quantified to determine intracellular ROS levels. All assays were performed in triplicates to ensure reproducibility (Xia et al., 2021).

2.8 Statisitcal analysis:

All experiments were conducted in triplicate, and data were expressed as mean ± standard deviation (SD). Statistical analyses were performed using GraphPad Prism

9.0 (GraphPad Software, USA). One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used to compare differences between multiple groups.

3. Result

3.1. Protective Effect of BRE against *A. hydrophila*

Infection with *A. hydrophila* significantly reduced the survival rate (21%) of zebrafish larvae, with notable morphological deformities observed. However, treatment with BRE at both 100 µg/mL (34%) and 200 µg/mL (80%) significantly improved survival rates and mitigated morphological abnormalities (Figure 1). The highest protective effect was observed at 200 µg/mL, where larvae showed improved movement and structural integrity.

3.2. Antioxidant Enzyme Activity

A. hydrophila infection led to a significant decline in SOD activity (17 U/mg of protein). BRE treatment restored SOD levels, with the 200 µg/mL (31 U/mg protein) concentration exhibiting a higher increase compared to 100 µg/mL (24 U/mg protein). This indicates the potential of BRE in enhancing superoxide dismutation and mitigating oxidative stress. Also, the infection resulted in a drastic reduction in CAT activity (7 µmol/mg of protein), but treatment with BRE significantly increased CAT levels. The 200 µg/mL (20 µmol/mg of protein) treatment group showed a more pronounced effect, demonstrating the ability of BRE to improve hydrogen peroxide breakdown (Figure 2). GST activity was significantly suppressed in infected larvae (0.16 nmol/mg of protein) but was restored upon BRE treatment. The increase in GST levels in the 200 µg/mL group (0.30 nmol/ mg of protein) suggests that BRE enhances detoxification mechanisms and oxidative damage repair. Meanwhile the infection significantly depleted GSH levels (4.3 nmol/ mg of protein), indicating increased oxidative stress. BRE treatment at both concentrations restored GSH levels, with 200 µg/mL (9.7 nmol/mg of protein) exhibiting a more substantial effect. This suggests that BRE enhances cellular antioxidant defenses.

3.3. Inhibition of NO and Lipid Peroxidation

NO levels were significantly elevated in *A. hydrophila*-infected larvae (75 µM), indicating inflammation and oxidative stress (Figure 3). BRE treatment effectively reduced NO levels, with 200 µg/mL (17 µM) showing a more significant decrease, highlighting its anti-inflammatory potential. The increased MDA levels, indicating heightened lipid peroxidation (Figure 4). BRE treatment significantly reduced MDA levels, with the higher concentration offering greater protection against oxidative damage.

3.4. Inhibition of ROS, Apoptosis, and Lipid Peroxidation

A. hydrophila infection increased intracellular ROS levels, as indicated by strong fluorescence signals. BRE treatment significantly reduced ROS accumulation, demonstrating its antioxidant efficacy (Figure 5). Infected larvae showed a high level of apoptosis, indicated by intense AO staining. BRE treatment significantly reduced apoptosis, with the 200 µg/mL treatment group showing the most considerable reduction (Figure 6). DPPP fluorescence was significantly higher in infected larvae, suggesting increased lipid peroxidation (Figure 7). BRE treatment markedly reduced fluorescence intensity, indicating reduced oxidative damage to lipid membranes.

4. Discussion

The findings of this study demonstrate the significant protective effects of BRE in mitigating *A. hydrophila*-induced toxicity in zebrafish larvae. The protective effects were evident through the improvement in survival rates, restoration of antioxidant enzyme activities, reduction in oxidative stress markers, and inhibition of apoptosis. These findings align with previous studies indicating the role of plant-derived antioxidants in counteracting oxidative stress and bacterial toxicity (Murugan et al., 2023). One of the primary mechanisms by which *A. hydrophila* exerts its pathogenicity is through excessive ROS generation (M. Kumar et al., 2022). ROS accumulation can lead to lipid peroxidation, protein oxidation, and DNA damage,

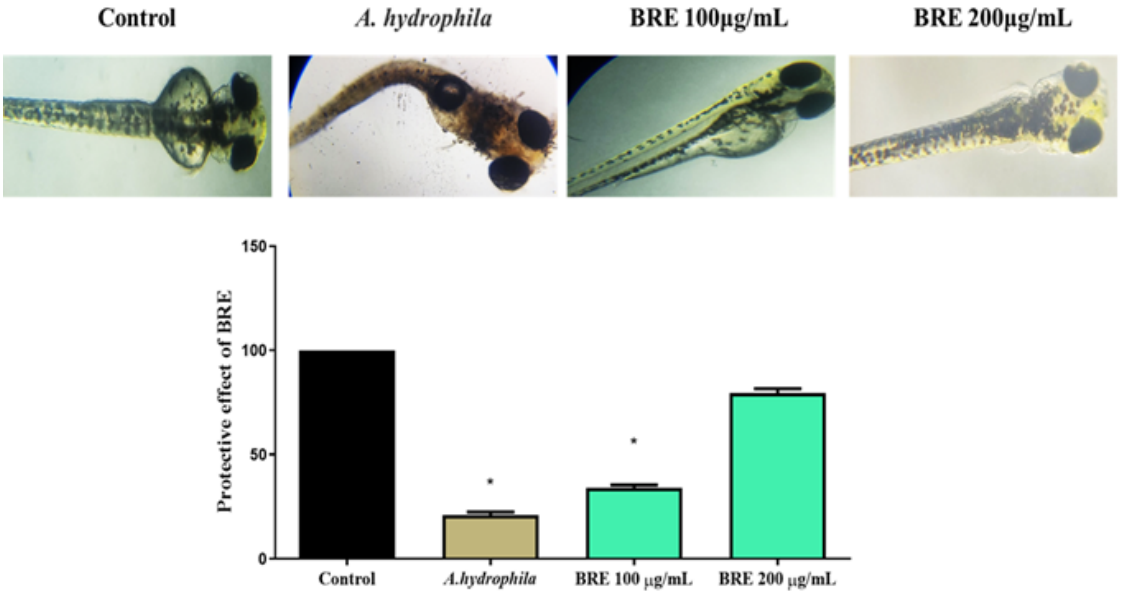

Figure 1: Survival rate of zebrafish larvae following *A. hydrophila* infection and treatment with BRE at 100 µg/mL and 200 µg/mL. The infected and treatment group was compared with control (n = 20 larvae/group). * represent significant difference at $p < 0.05$.

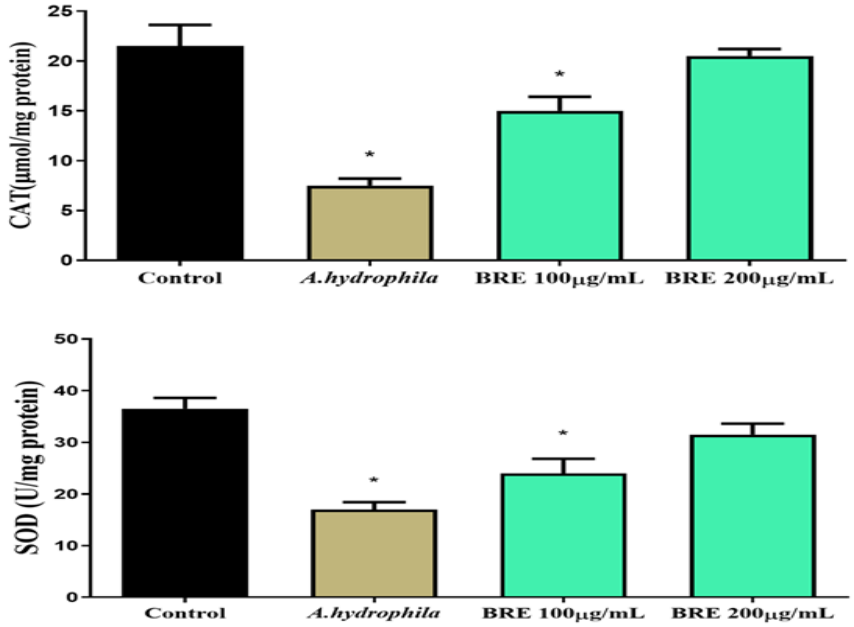

Figure 2: Effect of BRE on SOD and CAT levels in *A. hydrophila*-infected Zebrafish Larvae. The untreated and infected zebrafish larvae used as control and model group (n = 20 larvae/group).

ultimately resulting in apoptosis and tissue injury (B. Wang et al., 2023). The present study observed that infection significantly increased ROS levels in zebrafish larvae, as evidenced by elevated fluorescence intensities in the DCFDA assay. However, BRE treatment effectively reduced ROS accumulation, with the 200 µg/mL concentration exhibiting a more pronounced effect than 100 µg/mL. These results corroborate previous research indicating that plant antioxidants, such as flavonoids and betalains present in beetroot, serve as potent ROS scavengers (Nirmal et al., 2024).

Antioxidant enzymes play a crucial role in mitigating oxidative stress by neutralizing free radicals (Pisoschi et al., 2021). The observed decline in SOD and CAT activities in infected zebrafish larvae highlights the detrimental effects of *A. hydrophila* infection on the antioxidant defense system. The post-treatment increases in these enzyme activities following BRE administration suggests that BRE enhances the endogenous antioxidant defense. Similar findings were reported in a study on the effects of curcumin in fish models, where treatment restored SOD and CAT activity in bacterial-infected fish, thereby reducing oxidative stress (Borgo et al., 2024). GSH is a key non-enzymatic antioxidant that regulates cellular redox homeostasis (He et al., 2017). The significant depletion of GSH levels in infected larvae reflects oxidative damage caused by *A. hydrophila* infection. The administration of BRE significantly restored GSH levels, indicating its role in maintaining redox balance and reducing cellular oxidative stress. A similar increase in GSH levels has been observed in studies involving polyphenol-

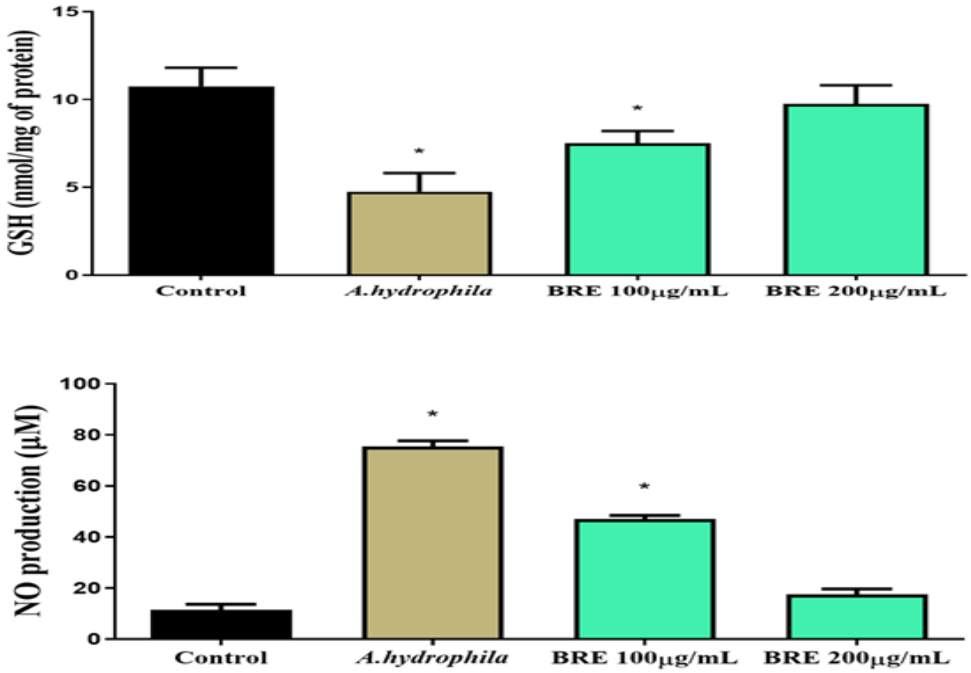

Figure 3: Effect of BRE on NO and GSH levels in *A. hydrophila*-infected Zebrafish Larvae. The untreated and infected zebrafish larvae used as control and model group (n = 20 larvae/group).

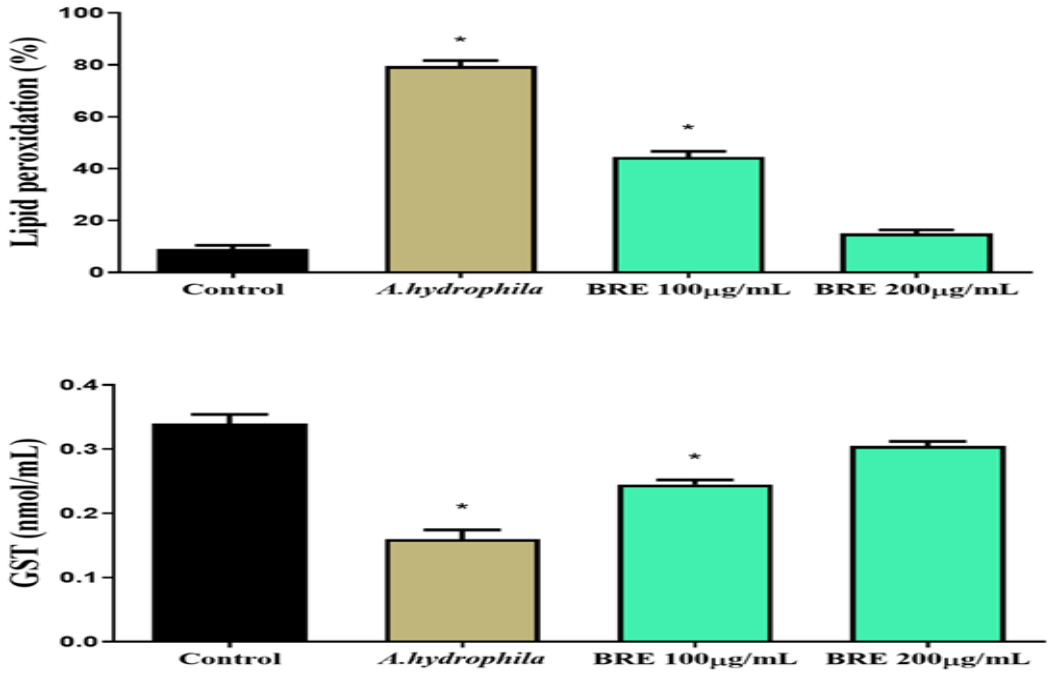

Figure 4: Effect of BRE on GST and Lipid peroxidation levels in *A. hydrophila*-infected Zebrafish Larvae. The untreated and infected zebrafish larvae used as control and model group (n = 20 larvae/group).

rich plant extracts (Mężyńska and Brzóska, 2019), suggesting that beetroot's bioactive components effectively replenish cellular antioxidant stores.

The inhibition of NO production in BRE-treated larvae further supports the anti-inflammatory role of BRE. *A. hydrophila* infection resulted in a significant increase in NO levels, indicative of an inflammatory response. BRE administration significantly reduced NO levels, with 200 µg/mL showing greater efficacy. This finding is consistent with previous research that demonstrated the anti-inflammatory effects of red pepper extract in reducing NO-mediated inflammation in bacterial infections (Allemand et al., 2016). Lipid peroxidation, a hallmark of oxidative stress, was also significantly elevated in infected larvae, as indicated by increased MDA levels. The reduction of MDA levels following BRE treatment suggests that beetroot extract effectively inhibits lipid peroxidation and protects cell membranes from oxidative damage. Previous studies on natural antioxidants, Rubus fairholmianus root extract have reported similar reductions in lipid peroxidation in bacterial-infected models (George et al., 2014).

Apoptosis is a critical factor in host responses to bacterial infections, and excessive apoptotic activity can contribute to tissue damage (Zhang et al., 2022). The acridine orange assay revealed that *A. hydrophila* infection significantly induced apoptosis in zebrafish larvae. BRE treatment markedly reduced apoptotic cell numbers, indicating its protective effect against bacterial-induced cell death. The observed decrease in AO fluorescence intensity aligns with studies on other plant-derived antioxidants that prevent apoptosis by modulating mitochondrial pathways and reducing oxidative stress. The DPPP assay further confirmed the role of BRE in reducing lipid peroxidation in live zebrafish larvae. Infected larvae exhibited significantly higher fluorescence intensity due to elevated lipid peroxidation levels. However, BRE treatment markedly decreased fluorescence, confirming its membrane-protective properties. Comparing these results with previous literature, multiple studies have demonstrated the protective effects of plant extracts against bacterial infections in aquatic models. For example, studies on the use of curcumin, green tea polyphenols, and resveratrol in fish have shown similar antioxidant and anti-inflammatory effects in counteracting bacterial toxicity (González-Renteria et al., 2020). These studies provide additional evidence supporting the efficacy of natural

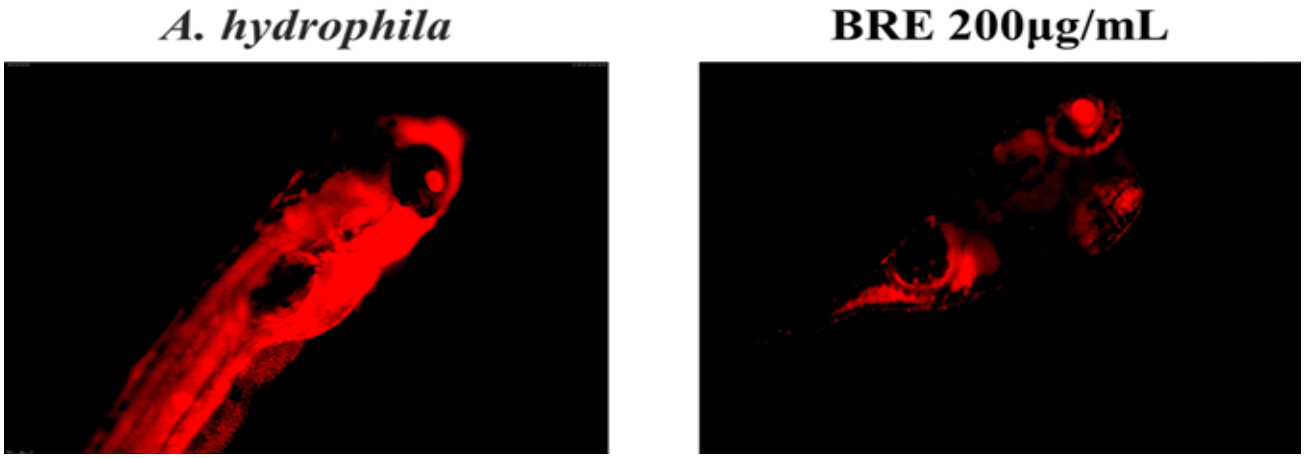

Figure 5: Acridine orange red fluorescence imaging of zebrafish larvae showing apoptosis. (A) *A.hydrophila*-infected larvae and (B) Fermented BSE-treated group (200 µg/mL) (n = 20 larvae/group).

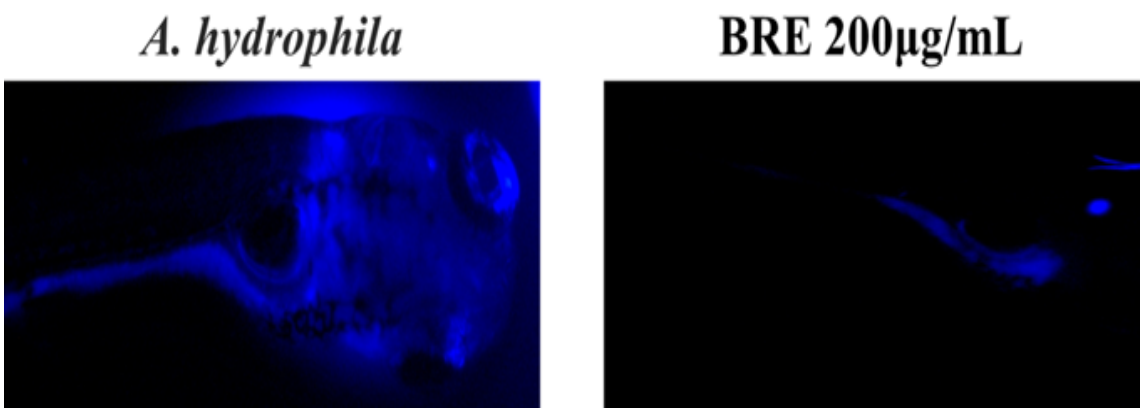

Figure 6: DPPP blue fluorescence imaging of zebrafish larvae showing lipid peroxidation. (A) A.*hydrophila*-infected larvae and (B) Fermented BSE-treated group (200 µg/mL) (n = 20 larvae/group).

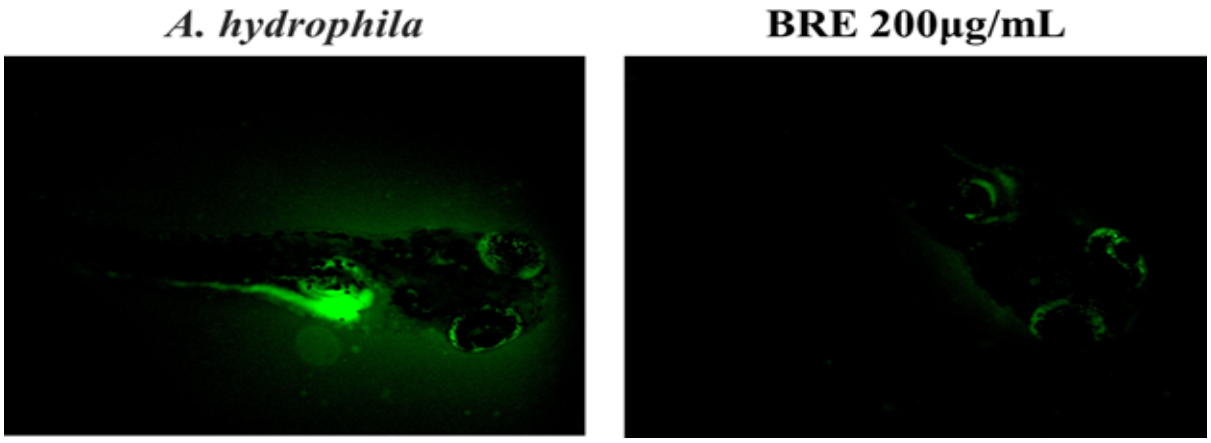

Figure 7: DCFDA green fluorescence imaging of zebrafish larvae showing ROS. (A) *A.hydrophila*-infected larvae and (B) Fermented BSE-treated group (200 µg/mL) (n = 20 larvae/group).

compounds in mitigating bacterial-induced oxidative stress.

In summary, the protective effects of BRE against *A. hydrophila* toxicity can be attributed to its potent antioxidant, anti-inflammatory, and anti-apoptotic properties. The ability of BRE to restore antioxidant enzyme activities, reduce ROS levels, inhibit NO production, and prevent apoptosis underscores its potential as a therapeutic agent for managing bacterial infections in aquaculture. Further studies should focus on elucidating the molecular mechanisms underlying its bioactivity and exploring its applications in other infection models. Additionally, future research could investigate the synergistic effects of beetroot extract with other natural antioxidants to enhance its protective efficacy.

5. Conclusion

The study confirms the protective role of BRE against *A. hydrophila*-induced toxicity in zebrafish larvae. BRE effectively modulated oxidative stress markers, reduced apoptosis, and restored antioxidant enzyme activities. These findings provide a strong foundation for further investigations into the therapeutic applications of BRE in aquaculture and human medicine.

Declarations

Ethics approval statement

No ethical approval was required for the current study as it did not deal with any human or animal samples.

Consent to participate

Not applicable

Consent to publish

Not applicable

Data Availability Statement

The data are available from the corresponding author upon reasonable request

Competing Interests

The authors declare that they have no conflict of interest

Funding

Not Applicable

Author contribution

Conceptualization, Data curation, Investigation, Formal analysis: M.M.M Writing—review and editing: M.M.M All authors have read and agreed to the published version of the manuscript

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