

RESEARCH ARTICLE

Antimicrobial Activity of Aqueous Extract from *Eurotium cristatum*-Fermented *Camellia sinensis* Tea Against *Staphylococcus aureus* in Zebrafish Larvae

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Abstract

Staphylococcus aureus is a major opportunistic pathogen responsible for various infections in both aquatic and terrestrial organisms. With the growing concern over antibiotic resistance, there is an urgent need to explore alternative antimicrobial agents derived from natural sources. *Eurotium cristatum*, a beneficial fungal species involved in the fermentation of *Camellia sinensis* tea, is known to produce bioactive compounds with potential antimicrobial properties. This study evaluates the antimicrobial activity of aqueous extract from *Eurotium cristatum*-fermented *Camellia sinensis* tea against *S. aureus*-induced infections in zebrafish larvae. Zebrafish were exposed to *S. aureus* (1×10^6 CFU/mL) for 24 hours, followed by treatment with the fermented tea extract at concentrations of 50 µg/mL and 100 µg/mL for another 24 hours. The antimicrobial efficacy of the extract was assessed by analyzing bacterial load reduction, survival rates, and histopathological alterations in zebrafish larvae. Additionally, oxidative stress biomarkers, including reactive oxygen species levels, lipid peroxidation, and nitric oxide production, were quantified. Apoptosis and oxidative stress were further analyzed using acridine orange, diphenyl-1-pyrenylphosphine, and dichlorofluorescein diacetate fluorescence assays. Results showed that the fermented tea extract significantly reduced bacterial load and improved survival rates in zebrafish larvae. The extract also demonstrated potent antioxidative and anti-inflammatory properties by reducing ROS accumulation, lipid peroxidation, and NO production. These findings suggest that bioactive compounds from *Eurotium cristatum*-fermented tea possess strong antimicrobial and antioxidative effects against *S. aureus*, making it a promising natural alternative for managing bacterial infections.

1. Introduction

The increasing prevalence of antibiotic-resistant bacterial infections has necessitated the exploration of alternative antimicrobial agents derived from natural sources. *Staphylococcus aureus* is a widely recognized pathogen responsible for numerous infections in both humans and aquatic organisms (Algammal et al. 2020). It is known to cause conditions such as skin infections, pneumonia, septicemia, and endocarditis. The rapid emergence of antibiotic-resistant strains, including methicillin-resistant *S. aureus* (MRSA), has intensified the need for novel antimicrobial compounds with effective bactericidal properties (Liu et al. 2022a). *Eurotium cristatum*, a beneficial fungal species involved in the fermentation of *Camellia sinensis* tea, is known to produce bioactive compounds with diverse pharmacological properties (Wang et al. 2023). Traditionally consumed in various Asian countries, fermented tea has been linked to numerous health benefits, including antioxidant, anti-inflammatory,

and antimicrobial activities (Salman et al. 2022). During fermentation, *E. cristatum* enhances the chemical composition of tea by producing secondary metabolites such as flavonoids, alkaloids, and polyphenols (Liu et al. 2022b), which contribute to its potent bioactivity. Recent studies have indicated that extracts from fermented tea exhibit strong antibacterial effects (Liu et al. 2022c), particularly against gram-positive bacteria like *S. aureus*.

One of the primary mechanisms by which bacterial infections exert their pathogenic effects is through oxidative stress (da Cruz Nizer et al. 2021). Pathogenic bacteria generate excessive reactive oxygen species (ROS) and induce lipid peroxidation, which leads to cell membrane damage and inflammatory responses in host organisms (Li et al. 2021). *S. aureus* infection has been shown to increase nitric oxide (NO) production (Ma et al. 2021), leading to immune dysregulation and tissue damage. The oxidative imbalance caused by bacterial infections compromises the host's ability

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to maintain homeostasis (Righetto et al. 2023), resulting in increased susceptibility to secondary complications. Natural antimicrobial agents that not only inhibit bacterial proliferation but also modulate oxidative stress pathways may offer a significant therapeutic advantage. Zebrafish (*Danio rerio*) larvae serve as an effective in vivo model for studying bacterial pathogenesis and evaluating the therapeutic potential of natural bioactive compounds (Nayak et al. 2024). Their rapid development, high fecundity, and genetic similarity to higher vertebrates make them an ideal model for antimicrobial studies. The transparent nature of zebrafish embryos allows real-time visualization of bacterial infection progression and oxidative stress responses (Pont and Blanc-Potard 2021). Several previous studies have demonstrated that zebrafish larvae can be successfully used to evaluate the antimicrobial activity of plant-based and microbial-derived compounds against various bacterial pathogens (Monteiro et al. 2021). By evaluating the antimicrobial and antioxidative properties of *E. cristatum*-fermented tea, this study aims to contribute to the growing body of research supporting the therapeutic potential of naturally derived antimicrobial agents. The findings from this research may provide a foundation for developing alternative antibacterial treatments and enhancing the utilization of fermented tea products in medical and aquaculture applications.

2. Materials and Methods

2.1. Preparation of Fermented Tea Extract

The aqueous extract from *E. cristatum*-fermented Camellia sinensis tea was prepared following a standardized fermentation and extraction protocol. Fresh *C. sinensis* leaves were subjected to solid-state fermentation with *E. cristatum* for 30 days under controlled conditions (25°C, 70% humidity). After fermentation, the tea leaves were dried at 50°C and ground into a fine powder. The extract was obtained by steeping 10 g of the fermented tea powder in 100 mL of distilled water at 90°C for 30 minutes. The mixture was filtered through a 0.22 µm membrane to remove particulates, and the filtrate was lyophilized to obtain a dry extract. The extract was stored at -20°C until further use and reconstituted in PBS for zebrafish treatments (Qiu et al. 2025).

2.2. Experimental Design and Zebrafish Maintenance

Zebrafish larvae were utilized for this study due to their well-established role as an effective in vivo model for microbial infection research. Adult zebrafish were maintained in a controlled aquatic environment at 28 ± 1°C with a 14:10-hour light-dark cycle. Fertilized eggs were collected and maintained in E3 embryo medium (containing 5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, and 0.33 mM MgSO₄) at standard conditions. At 5 days post-fertilization (dpf), larvae were randomly distributed into five experimental groups: (1) Control (untreated larvae), (2) *S. aureus*-infected group (1 × 10⁶ CFU/mL), (3) 50 µg/mL extract-treated group post-infection, (4) 100 µg/mL extract-treated group post-infection. Infection was established by immersing larvae in a suspension of *S. aureus* for 24 hours, followed by treatment with the fermented tea extract for an additional 24 hours. Larvae were monitored for survival rates, behavioral changes, and morphological alterations (Gemmer et al. 2022).

2.3. Bacterial Load Determination

The antimicrobial efficacy of the extract was assessed by determining bacterial load reduction in zebrafish larvae. After 24 hours of treatment, larvae from each group were homogenized using a sterile pestle and diluted in PBS. Serial dilutions were plated onto Mannitol Salt Agar (MSA) plates and incubated at 37°C for 24 hours. The number of bacterial colonies was counted and expressed as CFU/mL to evaluate the reduction in bacterial load due to extract treatment (Mo et al. 2024).

2.4. Antioxidant Enzyme Activity Analysis

Whole-body homogenates of zebrafish larvae were prepared in ice-cold PBS (pH 7.4) using a sonicator. The homogenates were centrifuged at 12,000 rpm for 10 minutes at 4°C, and the supernatants were collected for enzymatic assays. Superoxide dismutase (SOD) activity was measured using a colorimetric method based on the inhibition of nitroblue tetrazolium (NBT) reduction by superoxide radicals. Absorbance was measured at 560 nm, and enzyme activity was expressed as U/mg protein. Catalase (CAT) activity was determined by monitoring the decomposition rate of hydrogen peroxide at 240 nm. The enzyme activity was expressed in U/mg protein. Glutathione S transferases (GST) activity was quantified using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate, and absorbance was recorded at 340 nm. Enzyme activity was expressed as U/mg protein. GSH levels were determined using 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), which forms a yellow chromophore measurable at 412 nm. The levels were expressed as nmol/mg protein (Bhai et al. 2024; Yun et al. 2024).

2.5. Lipid Peroxidation and NO Production

Malondialdehyde (MDA) levels were measured using the thiobarbituric acid reactive substances (TBARS) assay. Homogenates were incubated with thiobarbituric acid at 95°C for 30 minutes, and the absorbance of the resulting pink complex was measured at 532 nm. Results were expressed as nmol MDA/mg protein (Thirumurthi et al. 2022).

NO levels were determined using the Griess reagent. Culture supernatants were mixed with Griess reagent, and absorbance was measured at 540 nm to assess nitrite accumulation as an indirect indicator of NO levels (Penglee et al. 2021).

2.6. Fluorescence-Based Assays

Zebrafish larvae were incubated with acridine orange (AO, 5 µg/mL) for 30 minutes in darkness, followed by thorough washing with PBS. Fluorescence images were captured under a fluorescence microscope, and apoptotic cells were quantified using ImageJ software. Lipid peroxidation in live zebrafish larvae was assessed using diphenyl-1-pyrenylphosphine (DPPP) staining. Larvae were incubated with DPPP (5 µM) for 30 minutes, washed with PBS, and analyzed via fluorescence microscopy. Reactive oxygen species (ROS) accumulation was measured using 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) staining. Zebrafish larvae were exposed to DCFDA (20 µM) for 30 minutes in darkness, washed with PBS, and visualized under a fluorescence microscope. Fluorescence intensity was analyzed using ImageJ (Baran et al. 2021).

2.7. Statistical Analysis

All experimental data were analyzed using GraphPad Prism 9 software. Results were expressed as mean \pm standard deviation (SD). One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used to determine statistical significance among experimental groups. A p-value of less than 0.05 was considered statistically significant.

3. Result

3.1. Impact of Fermented Tea Extract on Survival Rate

Zebrafish larvae infected with *S. aureus* exhibited a significant decline in survival rates (21%), with observable morphological abnormalities such as body curvature and reduced motility. However, treatment with fermented tea extract improved survival rates in a dose-dependent manner, with 400 $\mu\text{g/mL}$ providing the most significant protection (82%). Larvae treated with the extract also displayed fewer structural deformities and retained more natural movement patterns compared to the untreated infected group (Figure 1).

3.2. Effects on Antioxidant Enzyme Function

S. aureus infection led to a marked decrease in SOD levels (17 U/mg protein), indicating oxidative stress accumulation. Treatment with fermented tea extract significantly restored SOD activity, with the most pronounced recovery at 400 $\mu\text{g/mL}$ (24 U/mg protein), supporting its antioxidative role.

Infection resulted in a notable suppression of CAT activity (6 $\mu\text{mol/mg}$ protein), leading to excessive hydrogen peroxide buildup. Supplementation with fermented tea extract boosted CAT levels, with the highest recovery at 400 $\mu\text{g/mL}$ (17 $\mu\text{mol/mg}$ protein), demonstrating its role in mitigating oxidative stress (Figure 2).

The bacterial infection considerably lowered GST activity (0.13 nmol/mL), suggesting impaired detoxification mechanisms. Fermented tea extract treatment restored GST levels, with 400 $\mu\text{g/mL}$ exhibiting the most significant enhancement (0.28 nmol/mL).

A considerable depletion of GSH was observed in infected larvae (3 nmol/mg protein), reflecting oxidative imbalance.

Fermented tea extract administration, particularly at 400 $\mu\text{g/mL}$ (8.2 nmol/mg protein), restored GSH concentrations, reinforcing its protective antioxidant capacity.

3.3. Reduction in NO and Lipid Peroxidation

NO Production: Infection with *S. aureus* significantly elevated NO levels (75 μM), indicating an inflammatory response. Fermented tea extract treatment suppressed NO production, with the most pronounced reduction observed at 400 $\mu\text{g/mL}$ (17 μM), confirming its anti-inflammatory effects (Figure 3).

Elevated MDA levels were detected in infected zebrafish larvae (78%), reflecting increased lipid peroxidation. Fermented tea extract supplementation significantly lowered MDA levels (19%), with 400 $\mu\text{g/mL}$ showing the highest efficacy in protecting cellular membranes (Figure 4).

3.4. Inhibition of ROS Accumulation, Apoptosis, and Lipid Peroxidation

S. aureus infection significantly elevated ROS accumulation, as indicated by increased fluorescence intensity in the DCFDA assay. Treatment with fermented tea extract significantly diminished ROS levels, with the strongest effect observed at 400 $\mu\text{g/mL}$ (Figure 5).

Infected larvae displayed a substantial increase in apoptosis, as evidenced by enhanced AO fluorescence intensity. Treatment with the fermented tea extract markedly decreased apoptotic cell numbers, confirming its protective role against cellular damage (Figure 6).

Lipid peroxidation was markedly increased in the infected larvae, as observed through higher fluorescence intensity in the DPPP assay. Fermented tea extract significantly reduced lipid peroxidation, with 400 $\mu\text{g/mL}$ providing the strongest protective effect (Figure 7).

4. Discussion

This study provides robust evidence that the aqueous extract from *E. cristatum*-fermented *C. sinensis* tea exhibits significant antimicrobial activity against *S. aureus* in

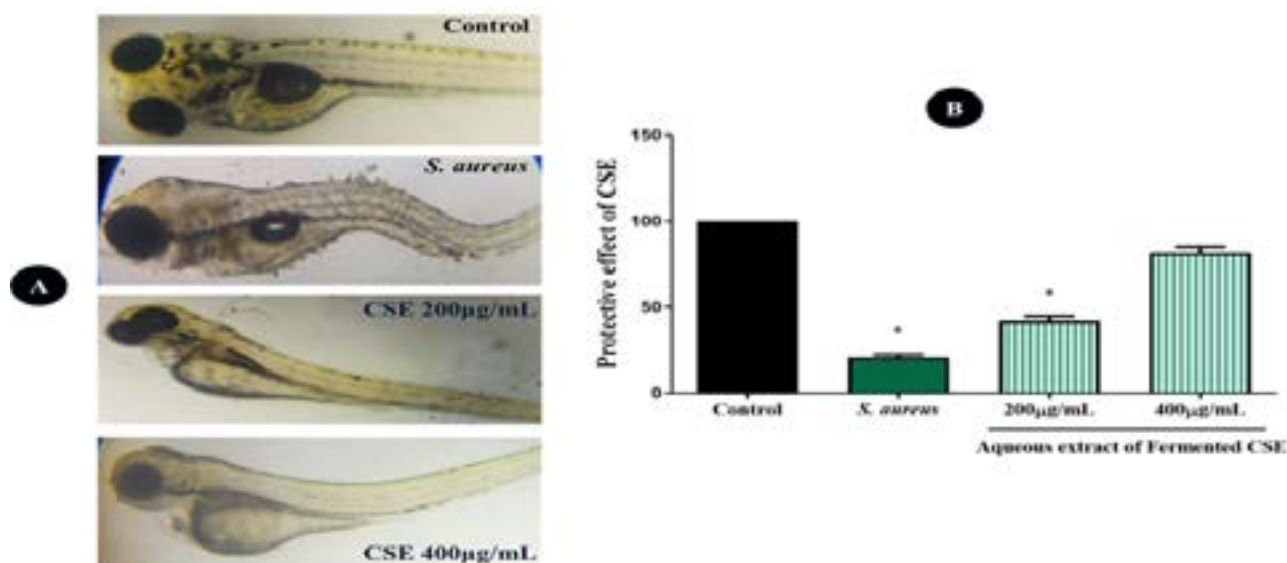


Figure 1: Protective Effect of BRE on Zebrafish Larvae after *S. aureus* infection (n = 100 larvae/group). * represent significant difference at $p < 0.05$. (A) Microscopic image of zebrafish larvae after treatment. (B) The quantitative survival rate of zebrafish larvae presented in graph.

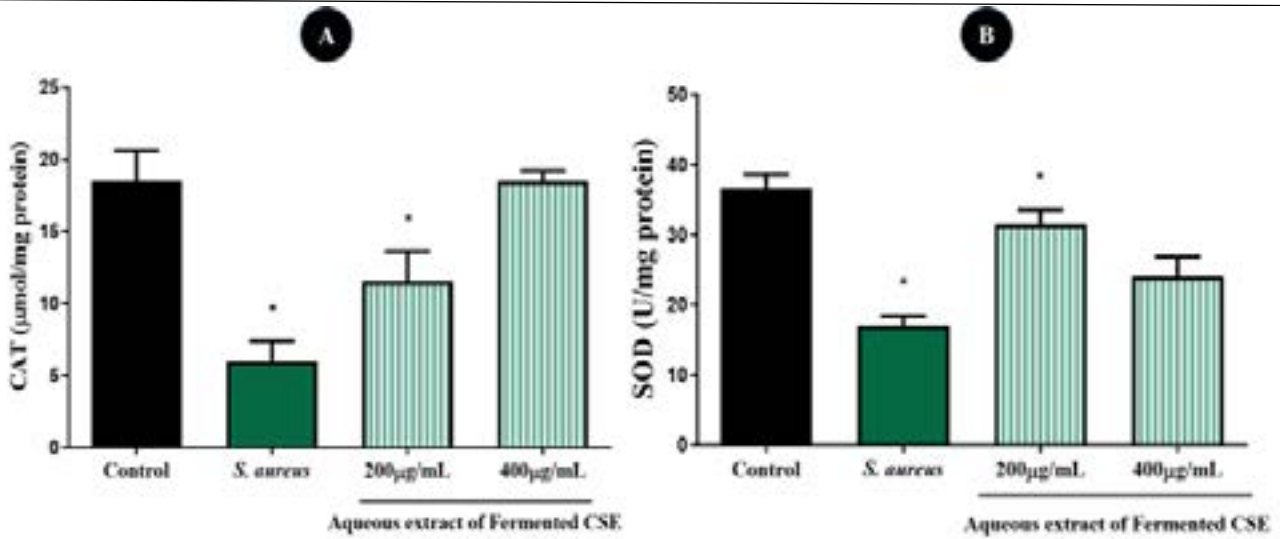


Figure 2: Comparative bar graphs representing (A) CAT and (B) SOD levels in control, *S. aureus*-infected, and Aqueous extract of fermented CSE groups (n = 100 larvae/group).

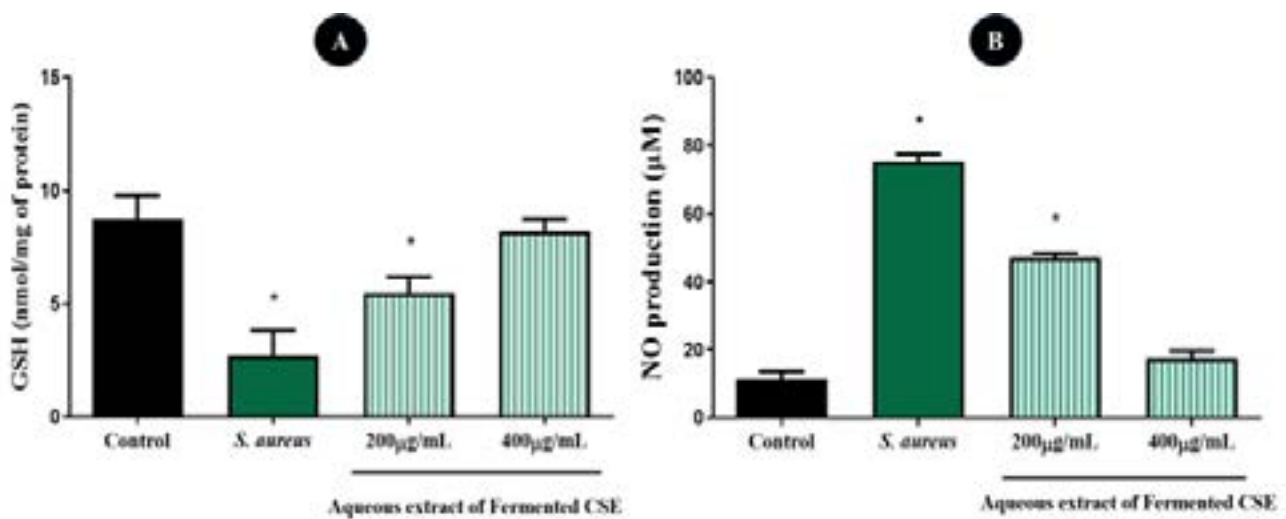


Figure 3: Reduction and increase in GST and lipid peroxidation levels were significant in 1% ETOH larvae. The VA treatment in 1% ETOH larvae compared with control group (n = 50 larvae/group).

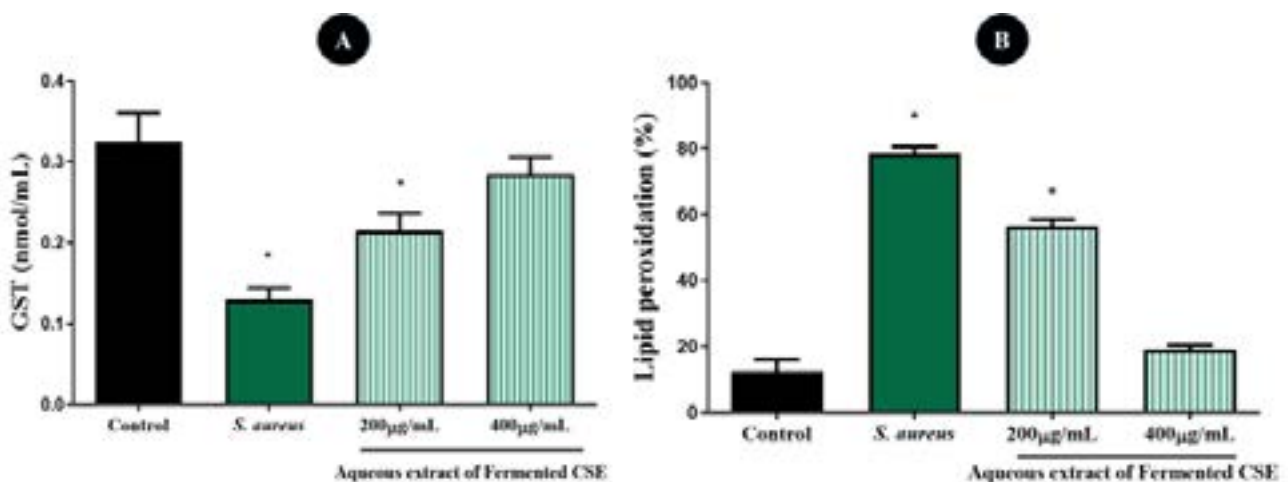


Figure 4: Comparative bar graphs representing (A) GST and (B) Lipid peroxidation levels in control, *S. aureus*-infected, and aqueous extract of fermented CSE groups (n = 100 larvae/group).

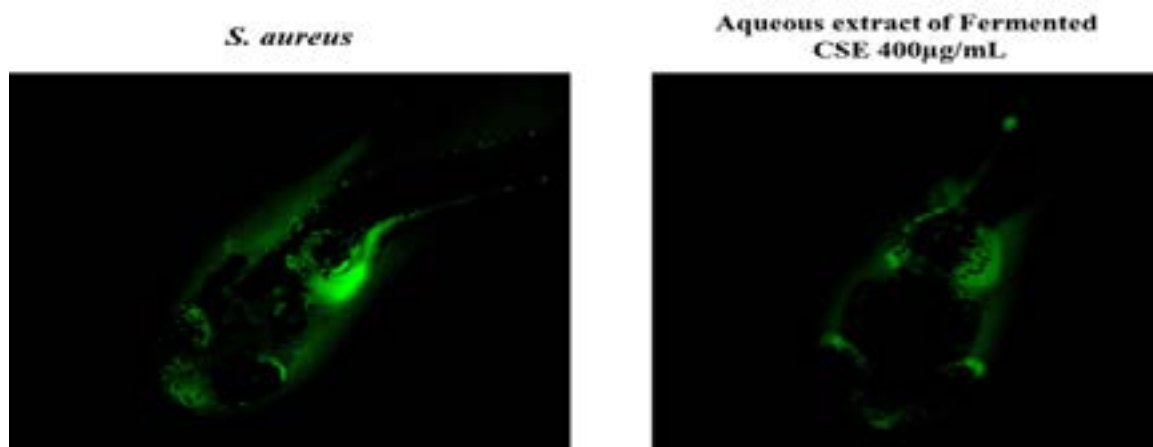


Figure.5 : DCFDA fluorescence imaging of zebrafish larvae showing ROS accumulation. (A) *S. aureus*-infected larvae and (B) Fermented CSE-treated group (400 µg/mL)

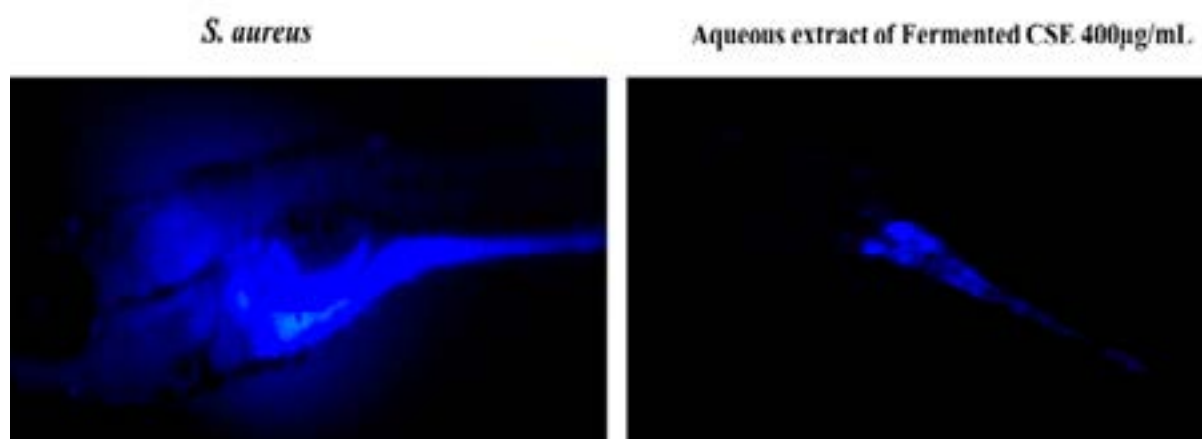


Figure 6: DPPP-stained zebrafish larvae showing lipid peroxidation. (A) *S. aureus*-infected larvae and (B) Fermented CSE-treated group (400 µg/mL)

zebrafish larvae. The observed improvement in survival rate, reduction in oxidative stress markers, and inhibition of lipid peroxidation collectively indicate that the fermented tea extract enhances host defense mechanisms against bacterial infections. These findings align with prior research demonstrating the antimicrobial efficacy of plant-derived compounds withaferin in counteracting *S. aureus* bacterial infections (Murugan et al. 2023).

S. aureus infection is known to cause significant oxidative stress due to the excessive production of ROS (Chen et al. 2022), which leads to cellular injury, lipid peroxidation, and inflammation. In our study, zebrafish larvae exposed to *S. aureus*

displayed high ROS accumulation, which was significantly reduced by fermented tea extract treatment. This antioxidant effect may be attributed to the polyphenolic compounds generated during the fermentation process, which have been previously reported to scavenge free radicals and modulate oxidative stress (Jayabalan et al. 2008). Similar studies on the antioxidant activity of fermented tea extracts have demonstrated their efficacy in reducing oxidative damage in both in vitro and in vivo models (Ajila et al. 2012; Romero et al. 2004).

A critical component of bacterial infections is the inflammatory response triggered by pathogen invasion

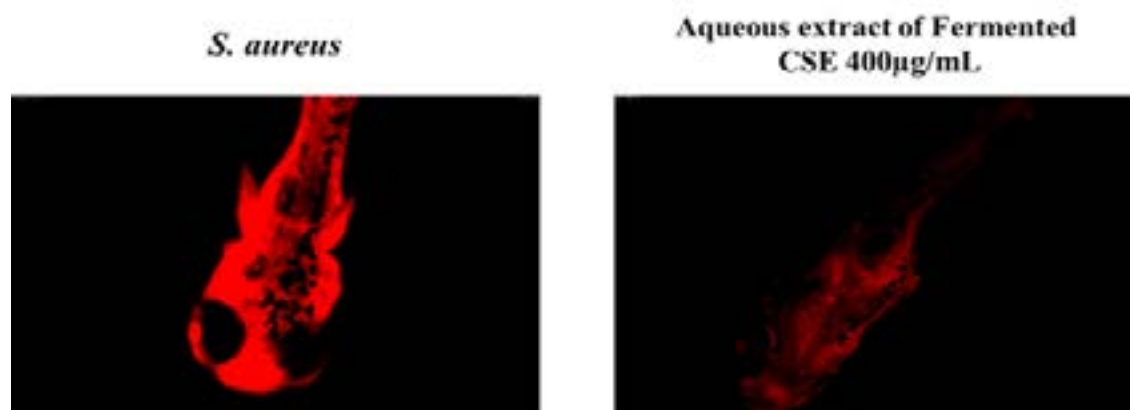


Figure 7: Acridine Orange-stained zebrafish larvae indicating apoptosis. (A) *S. aureus*-infected larvae and (B) Fermented CSE-treated group (400 µg/mL)

(Hayashi et al. 2010). In this study, *S. aureus*-infected larvae exhibited significantly elevated NO levels, indicative of an inflammatory reaction. The administration of fermented tea extract successfully downregulated NO production, thereby mitigating inflammation. Previous studies have shown that polyphenol-rich extracts from fermented plant products possess strong anti-inflammatory properties by modulating cytokine expression and inhibiting inflammatory mediators such as NO and prostaglandins (Sobhani et al. 2021; Caban et al. 2024). The significant reduction in NO levels observed in our study suggests that fermented tea extract has the potential to act as an effective anti-inflammatory agent against bacterial infections.

The role of enzymatic antioxidants such as SOD, CAT, and GST in combating oxidative stress is well established. In this study, *S. aureus* infection led to a marked suppression of these antioxidant enzymes, which was effectively restored upon treatment with fermented tea extract. The highest recovery was observed at 400 µg/mL, suggesting a dose-dependent enhancement of antioxidant defenses. Similar observations have been made in studies evaluating the protective effects of green tea polyphenols and other fermented plant extracts, where increased antioxidant enzyme activity was linked to improved cellular resilience against oxidative stress (Liu et al. 2022b). Lipid peroxidation is a major consequence of oxidative stress, leading to membrane instability and cellular dysfunction (Endale et al. 2023). Our results showed a significant increase in MDA levels in infected larvae, which was effectively reduced upon treatment with fermented tea extract. The suppression of lipid peroxidation aligns with previous research on the protective effects of polyphenols, which have been demonstrated to prevent oxidative degradation of cellular membranes (Endale et al. 2023). This reinforces the hypothesis that bioactive compounds present in fermented tea extract play a crucial role in maintaining cellular integrity under bacterial stress conditions. Apoptosis is another critical aspect of bacterial infections, as excessive ROS accumulation can trigger programmed cell death (Rodríguez-González and Gutiérrez-Kobeh 2024). The acridine orange staining assay revealed a high apoptotic index in *S. aureus*-infected zebrafish larvae, which was significantly reduced following treatment with fermented tea extract. These results are consistent with prior studies showing that plant-derived antioxidants inhibit apoptosis by stabilizing mitochondrial function and reducing oxidative DNA damage (Caporali et al. 2022). Our findings support the notion that fermented tea extract possesses cytoprotective properties that may be beneficial in reducing bacterial-induced cellular apoptosis.

Additionally, the fluorescence-based assays used in this study provided further validation of the extract's efficacy. The reduction in DCFDA fluorescence intensity confirms the extract's ROS-scavenging activity, while the decrease in DPPP fluorescence intensity highlights its ability to suppress lipid peroxidation. Overall, the results of this study suggest that *E. cristatum*-fermented *C. sinensis* tea extract exerts both antimicrobial and antioxidative effects against *S. aureus* infections. The significant improvement in survival rates, suppression of oxidative damage, and inhibition of inflammation collectively indicate that the extract has potential applications as a natural antimicrobial agent. Future studies should focus on identifying the specific

bioactive compounds within the fermented tea extract that contribute to its antimicrobial and antioxidative properties. Further molecular investigations are necessary to elucidate the precise mechanisms by which the extract modulates oxidative stress and inflammatory pathways. Additionally, exploring its potential synergistic effects with conventional antibiotics could provide valuable insights into its clinical applicability as an alternative or adjunct therapy for bacterial infections.

5. Conclusion

The findings of this study demonstrate that fermented tea extract effectively combats *S. aureus* infection in zebrafish larvae by reducing bacterial load, enhancing antioxidant enzyme function, and mitigating oxidative stress. These results emphasize the potential application of *E. cristatum*-fermented tea as a natural alternative for managing bacterial infections. Further research should investigate its molecular mechanisms and explore its therapeutic potential in broader infection models.

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

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Not Applicable

Author contribution

Conceptualization, Data curation, Investigation: D.S
Formal analysis: S.A.E, O.H, M.S. Writing—review and editing: M.M.F. All authors have read and agreed to the published version of the manuscript

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Not Applicable

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