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ZEBRAFISH AS A MODEL FOR ANALYZING PROTECTIVE EFFECT OF *ACTINOMYCETES* EXTRACT FROM *ESCHERICHIA COLI*

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Abstract

This study investigates the protective potential of an actinomycetes extract derived from Escherichia coli against its own toxicity using zebrafish larvae as the model organism. Actinomycetes are known for producing bioactive compounds with diverse therapeutic applications. The study aims to assess the anti-inflammatory properties of the extract by analyzing pro-inflammatory cytokine expression levels. A nutrient-rich media is prepared for actinomycetes growth, and bioactive compounds are extracted. Antibacterial activity is evaluated using the agar well plate method. Results show the extract protects against E. coli-induced toxicity in zebrafish larvae, highlighting its therapeutic potential in mitigating E. coli-associated toxicity and inflammation. This research emphasizes the importance of exploring actinomycetes as natural sources for novel therapeutic interventions against microbial-induced ailments. In conclusion, the study reveals promising protective and anti-inflammatory properties of actinomycetes extracts, warranting further investigation into their therapeutic applications for E. coli-related health complications.

Keywords: Zebra Fish, Actinomycetes Extract, Escherichia Coli, Biofilm Inhibition Assay, Mortality Index, Hatchability Index

INTRODUCTION

Zebrafish (*Danio rerio*) has emerged as a powerful model organism in biomedical research, offering unique advantages for studying various aspects of human health and disease(Choi, Choi et al. 2021). Its genetic similarity to humans, rapid development, and optical transparency during embryonic stages have propelled it to the forefront of research in fields such as toxicology, drug discovery, and developmental biology. Particularly noteworthy is its relevance in understanding infectious diseases and host-pathogen interactions, making it an invaluable tool in disease modeling and drug research(Zhang, Chromy et al. 2005, BABU and MOHANRAJ 2020).

Zebrafish boasts the most extensive collection of sequenced genes among vertebrates, with approximately 70 percent of human genes having at least one evident orthologue in zebrafish. This genetic similarity underpins its utility as a model for investigating human diseases, including bacterial infections. Among the myriad of bacterial pathogens, *Escherichia coli (E. coli)* stands out as a significant threat, capable of causing a range of infections from urinary tract infections to life-threatening conditions like sepsis(Nourani, Khunjush et al. 2015).

The emergence of antibiotic-resistant strains of *E. coli* has further compounded the challenge of treating these infections, necessitating alternative therapeutic strategies(Mohanraj, Varshini et al. 2021, Streicher 2021). In this context, the exploration of novel sources for potential therapeutic agents has gained traction, with actinomycetes garnering significant attention.

Actinomycetes, filamentous bacteria ubiquitous in natural habitats like soils and composts, are renowned for their ability to produce a diverse array of bioactive compounds, including antibiotics and anti-inflammatory drugs(Dilip, Mulaje et al. 2013, Palaniappan, Mohanraj et al. 2021). These bioactive compounds hold promise as alternative therapeutic agents against bacterial infections, offering potential solutions to the growing crisis of antibiotic resistance.

Zebrafish larvae offer a unique experimental platform for evaluating the effects of various chemicals on growth, toxicity, and disease progression(Jia, Zhu et al. 2019, Akshaya and Ganesh 2022). Their well-studied immune system and amenability to genetic manipulation make them particularly well-suited for studying host-pathogen interactions and assessing the efficacy of potential therapeutic agents. Previous studies have demonstrated the utility of zebrafish in evaluating the toxic effects of different compounds and investigating their protective effects against bacterial infections(USHANTHIKA and MOHANRAJ 2020, Abbate, Maugeri et al. 2021).

However, limited research has specifically focused on actinomycetes extracts derived from E. coli and their potential protective properties. Against this backdrop, this study aims to investigate the protective effect of an actinomycetes extract derived from *E. coli* against its own toxicity using zebrafish as a model organism. By leveraging the optical transparency of zebrafish embryos, real-time visualization of pathogen-host interactions can be achieved, providing insights into the protective mechanisms of the *actinomycetes* extract(Chakraborty, Sharma et al. 2016, Yuvaraj, Sangeetha et al. 2020).

Through a combination of survival analysis, assessment of developmental abnormalities, and analysis of pro-inflammatory cytokine expression, the study seeks to elucidate the potential of these extracts as protective agents against E. coli-induced toxicity.

The findings from this study hold implications for the development of novel therapeutic strategies to combat *E.coli i*nfections and address the challenge of antibiotic resistance(Brooks and Brooks 2014, Karthik and Priya 2021).

Furthermore, the identification of specific bioactive components within the actinomycetes extract could pave the way for further research into their modes of action and potential applications in fighting bacterial infections. Overall, this study contributes to our understanding of the protective mechanisms of actinomycetes extracts and lays the groundwork for future investigations in this area.

MATERIALS AND METHODS

Actinomycetes agar preparation:

The preparation of actinomycetes agar commenced by accurately weighing agar granules, nutrient sources, salts, and other necessary ingredients according to the specified recipe(Tindall, Sikorski et al. 2007). These components were meticulously combined in a container.

Distilled water was gradually added to the mixture while stirring to achieve a homogeneous suspension. The liquid was then heated while stirring to ensure complete dissolution of the contents. pH adjustment was performed by determining the pH of the agar mixture using a pH meter or indicator paper.

Sodium hydroxide (NaOH) was utilized to raise the pH or hydrochloric acid (HCl) to lower the pH to the desired level. Once the agar mixture reached the appropriate pH, it underwent sterilization by autoclaving at high pressure and temperature to eliminate any existing bacteria.

Following autoclaving, the mixture was allowed to cool slightly before being poured into sterilized petri dishes. Care was taken to leave a small amount of air space around the dishes during cooling. After pouring, the agar was allowed to cool and solidify in the petri dishes.

The prepared agar was then immunized by inoculating it with sterile procedures using actinomycetes cultures. The inoculated petri dishes were placed in an incubator set to a temperature conducive for actinomycetes growth, typically between 28 and 30 °C. The agar plates were then left to incubate for the specified duration to allow for the development of actinomycetes.

Agar well plate method:

In the agar well plate method, the assessment of antibacterial activity of extracts or bioactive compounds was performed(Seanego and Ndip 2012). Three experimental groups were prepared: the control group consisted of saliva, the positive group included streptomycin, a well-known antibiotic with established antibacterial properties, and the experimental group comprised the actinomycetes extract.

The agar plates were prepared by pouring and allowing the agar medium to solidify. Subsequently, microbial suspensions were prepared and adjusted to the desired level of inoculation(Tanaka, Kawasaki et al. 2014). Inoculated plates were then placed in an inoculation chamber for 24 to 48 hours to establish appropriate growth conditions.

Following incubation, the plates were examined for zones of inhibition around the wells, indicating antibacterial activity. This assessment provided insight into the efficacy of the experimental actinomycetes extract in inhibiting bacterial growth, compared to the positive and negative control groups(Lee, Zhang et al. 2004).

Zebrafish embryos toxicity assessment:

Before advancing to further experiments, it's crucial to assess the potential toxicity of the actinomycetes extract to ensure the biosafety of zebrafish embryos. To accomplish this, various concentrations of the test substance were prepared in suitable solvents or medium(Handy, Cornelis et al. 2012). Dose-response relationships were determined by utilizing a range of concentrations. Zebrafish embryos were carefully transferred into Petri dishes or multi-well plates containing the prepared test solutions.

Each well or plate contained the test material at a specific concentration. Throughout the experiment, the embryos were regularly observed under a microscope. Comprehensive monitoring was conducted to track all aspects of embryonic development, including hatching, mortality, toxicity, and other critical milestones.

This meticulous approach allowed for the thorough evaluation of the potential effects of the actinomycetes extract on zebrafish embryo development, ensuring the safety and integrity of subsequent research endeavors(Dalisay, Tenebro et al. 2024).

RESULTS

Biofilm inhibition assay:

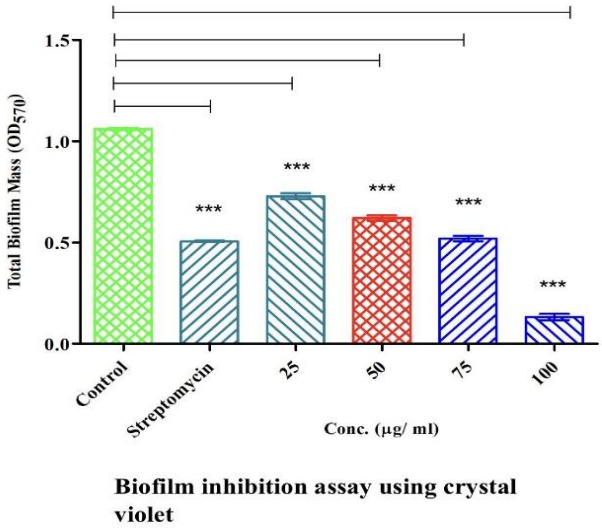

Figure 1: The statistical analysis of total biofilm mass among the experimental groups, control, streptomycin and actinomycetes extract at 25 ,50,75 and 100 ug/ml concentration

A biofilm inhibition assay is a clinician examination technique used to evaluate the ability of different substances to prevent or disrupt the formation of biofilms. Biofilms are colonies of microorganisms that adhere to surfaces and are encapsulated in a protective matrix. These structures can be found in various environments and can lead to persistent infections and they also cause dental issues like dental plaque and dental calculus which could result in dental caries which is a more prevalent oral disease.

Mortality index of zebrafish embryos:

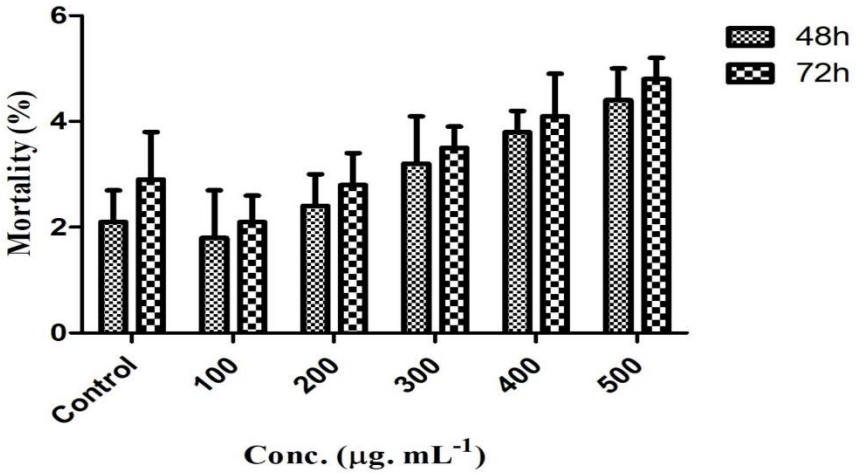

Figure 2: the statistical analysis of mortality index of zebrafish embryos exposed to actinomycetes extract in 48 hours and 72 hours are examined

Hatchability inhibition index of zebrafish embryos

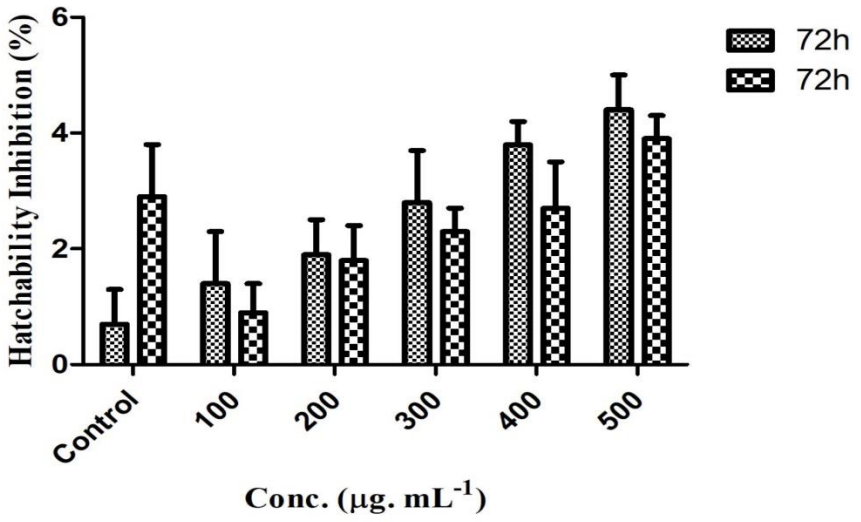

Figure 3: The statistical analysis of hatchability index of zebrafish embryos exposed to actinomycetes extract in 72 hours are examined

The hatchability inhibition index is typically calculated using the following formula:

Hatchability Inhibition Index = (Control Hatching Rate −Treatment Hatching Rate / control hatching rate) x 100

Control Hatching Rate: The hatching rate of the control group (without the test substance).

Treatment Hatching Rate: The hatching rate of the group exposed to the test substance. A quantifiable indicator of the test substance's impact on the hatching success rate is the hatchability inhibition index. A more detrimental effect on hatching is indicated by a higher inhibition index. It is assumed that the test chemical had no influence on hatching if the inhibition index is zero (0%). If the index is 100%, it means that the test chemical entirely prevented hatching.

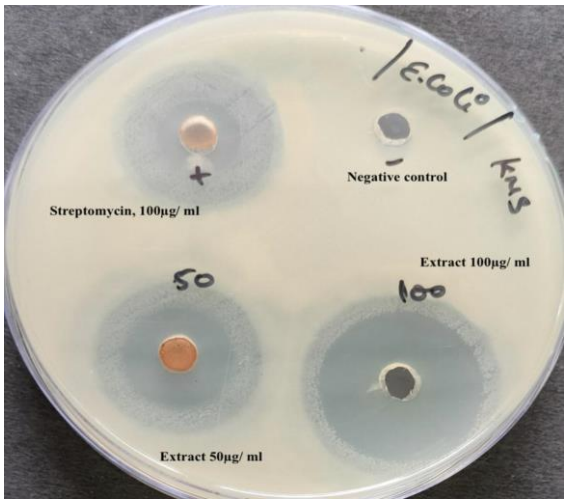

Figure 4: Agar well plate method results showing, Zone of inhibition of control group A: Positive group - streptomycin B, Negative control - actinomycetes extract C

DISCUSSION

Using a green synthesis method with a good yield, silver chloride nanoparticles were produced from the supernatant of a cell-free culture of the *Streptomyces* strain. Utilizing UV-Vis, IR, SEM, AFM, and XRD techniques, the nanoparticles were identified. These nanoparticles demonstrated broad-spectrum antibacterial action against methicillin-resistant *Staphylococcus aureus, methicillin-sensitive S. aureus, Escherichia coli, Pseudomonas aeruginosa,* and *Klebsiella pneumonia* at concentrations below 2 g/ml, the lowest inhibitory concentration. on vivo bioassays on nanoparticle-treated zebrafish embryos showed that a dose of 16 g/ml was the maximum cardiac safety concentration, and that concentration increases resulted in unfavorable effects like pericardial bulging, mouth protrusion, bleeding, and yolk sac elongation. In terms of cardiac assessment and lethality study, the embryos treated with nanoparticles were found to be less harmful. Below 5 g/ml, the dose is considered an in vitro dose. The researchers wanted to isolate and identify potential medications from the marine actinomycete *Nocardiopsis exhalans* and test them for biological uses. Using thin-layer chromatography, the solvent extract from the cell-free culture of *N. exhalans* revealed six components(Peltola, Andersson et al. 2001). In order to purify the fractions and test their efficacy against human clinical infections, they were submitted to column chromatography. Spectral investigations of fraction 4 revealed that it was N-(2-hydroxyphenyl)-2-phenazinamine (NHP), which was the result of the considerable activity it displayed. Also, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*—human clinical pathogens—were effectively inhibited by NHP's biofilm-inhibitory properties. The scavenging of the compounds that increase oxidative stress by NHP was validated by its in vitro antioxidant activity(Wang, Jin et al. 2021). When used in concentrations of 300 g/ml or less, NHP showed strong anti-proliferative effect against human breast cancer cells and less cytotoxic activity. One of the most significant pathogenic bacteria today, *Staphylococcus aureus* causes biofilm to grow on indwelling medical devices and is a major cause of bloodstream nosocomial infections. A hurdle for the creation of new antimicrobial drugs is its growing resistance to current antibiotics, which is partially related to its capacity to build biofilms. In light of this, the objective of this work was to assess the impact of a coral associated actinomycete (CAA) - 3 on S. aureus biofilms both in vitro and in vivo. At a biofilm inhibitory concentration (BIC) of 0.1 mg ml-1, methanolic extracts of CAA-3 prevented the growth of methicillin-resistant *S. aureus* ATCC 33591, *S. aureus* ATCC 11632, and clinical isolates of *S. aureus* in in vitro biofilms. Furthermore, research using the confocal laser scanning microscope (CLSM) shows that CAA-3 prevents the nematode *Caenorhabditis elegans* from harboring *S. aureus* in its digestive tract. For the first time, this study identifies CAA as a possible source of anti-biofilm chemicals for the creation of innovative medications to combat highly resistant staphylococcal biofilms(Seethalakshmi, Rajeev et al. 2020).

A total of 54 actinomycetes, including three phytopathogens, were isolated and tested for antibacterial activity against 15 test species. A polyphasic identification investigation was conducted on nine physically unique and physiologically active isolates. While one sample only showed a 93.57% resemblance to *Streptomyces albogriseolus* NRRL B-1305T, 16 s rDNA sequencing revealed eight isolates to show the highest similarity to the genus *Streptomyces*. Their metabolic relatedness was discovered by a utilization assay of twenty-three chemical sources and seventy-one carbon sources. Three particular strains out of these nine isolates were discovered to

have a noticeably higher level of antibacterial capability and to be effective against a wider spectrum of microorganisms, including phyto-pathogenic fungi. In the end, the strain SMS_SU21 was identified, which demonstrated antibacterial activity with a MIC value of 0.05 mg ml1 and antioxidant activity with an IC50 value of 0.242 0.33 mg ml1 was shown to be the one with the greatest promise. The bioactive component responsible for this strain's antibacterial action was isolated, and the true potential of this strain was assessed using GC-MS. *Streptomyces sannanensis* SU118 was discovered in an actinomycete strain that had been isolated from Phoomdi soil in Loktak Lake, Manipur, India. It is a filamentous bacterium with a gram-positive phenotype that only exhibits antibacterial activity against other Gram-positive bacteria, with no effect on Gram-negative species. Glucose Soybean Meal Broth was discovered to be the ideal medium for antibiotic synthesis at 28°C for seven days of incubation. Thin layer chromatography was used to extract and purify the antibacterial ingredient that the strain generated. The culture filtrate's ethyl acetate extract was subjected to screening and bioassay-guided fractionation, which resulted in the isolation of an active potential compound with a R f value of 0.56 and a maximum wavelength of 275.0 nm that has the lowest minimum inhibitory concentration (0.5 g/ml) against *Staphylococcus aureus*. the highest (3.0 g/ml) was reported against *Mycobacterium smegmatis* MTCC 6 and *Bacillus circulans* MTCC 8074. MTCC 96 and *Staphylococcus aureus* (clinical isolate).

CONCLUSION

As a result, this study examined the protective effects of an actinomycetes extract obtained from Escherichia coli using zebrafish as a model organism. The results showed the extract's potential to reduce E. coli-caused toxicity and enhance zebrafish larvae health in general. As a result of exposure to E. coli, treatment with the actinomycetes extract greatly improved zebrafish larval survival rates and decreased the aberrant developmental outcomes. A decrease in the expression of pro-inflammatory cytokines further demonstrated the extract's anti-inflammatory effects. These findings demonstrate how actinomycetes extracts can be used therapeutically to reduce inflammation and toxicity brought on by E. coli. In-depth investigation of the extract's broader applications in preclinical and clinical settings should concentrate on identifying the precise bioactive chemicals inside it as well as clarifying underlying mechanisms and its various biological effects.

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