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In vivo Assessment of *Ocimum sanctum* and Ciprofloxacin Against *Escherichia coli* in *Oryzias celebensis* Embryos Using Heart Rate as a Non-Destructive Biomarker

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ABSTRACT

Oryzias celebensis, a freshwater fish endemic to Sulawesi, is increasingly recognized as a promising model organism in tropical ecotoxicological studies due to its high sensitivity to local pollutants and strong ecological relevance. Among the physiological parameters used to assess embryonic developmental disruption caused by toxic exposure, heart rate is considered one of the most reliable and non-destructive biomarkers. This study evaluated changes in the embryonic heart rate of *O. celebensis* following exposure to the bacterium *Escherichia coli*, the antibiotic ciprofloxacin, and ethanol extract of basil leaves (*Ocimum sanctum* L.), either individually or in combination. Embryos were observed from stage 36 until hatching and divided into five treatment groups: control, *E. coli* (EC), *E. coli* + ciprofloxacin (EC+AB), *E. coli* + basil extract (EC+BE), and *E. coli* + basil extract + ciprofloxacin (EC+BE+AB). Heart rate was measured under a microscope, and data were analyzed using the Kruskal–Wallis test followed by a post hoc Bonferroni test. Results showed that the median heart rates at stage 36 in the EC, EC+AB, EC+BE, and EC+BE+AB groups were 163, 168, 159, and 136 bpm, respectively, compared to 175 bpm in the control group. Of these, only the EC+BE+AB group showed a statistically significant difference ($P < 0.001$). This pronounced cardiac depression suggests synergistic effects between the bacterial pathogen, ciprofloxacin, and basil-derived compounds such as eugenol and flavonoids, likely acting through neurodepressive pathways and immune modulation. These findings reinforce the embryonic heart rate of *O. celebensis* as a valid and sensitive physiological biomarker for assessing complex, multifactorial exposures. Furthermore, they support the ecological suitability of *O. celebensis* as an *in vivo* test model and underscore the strategic value of integrating eco-medical approaches within localized ecotoxicology to address emerging environmental challenges in tropical aquatic ecosystems.

INTRODCTION

Medaka fish have long been recognized as prominent model organisms in the fields of ecotoxicology and ecophysiology, similar to the well-established zebrafish (*Danio rerio*). Both species share numerous biological characteristics, making them comparable in experimental settings **(Yaqin *et al.*, 2022; Wu & Chen, 2025)**. Medaka are particularly favored for laboratory testing because of their rapid developmental rate, short life span, clearly defined life cycle, ease of observation, and broad geographic distribution **(Yaqin *et al.*, 2024; Qin *et al.*, 2025)**. Their small body size makes them ideal vertebrate models, especially for aquatic research, as they are considered among the most practical species for experimental applications in marine and freshwater systems **(Chen *et al.*, 2024; Yaqin *et al.*, 2024)**.

In addition to adult fish, medaka embryos are widely used in ecotoxicological testing **(Yaqin *et al.*, 2024; Zhou *et al.*, 2024)**. These embryos are of significant value in the field because of their high sensitivity to a range of environmental pollutants **(Qin *et al.*, 2025)**. The transparent chorion of *Oryzias* embryos allows for the direct microscopic observation of embryonic development, thereby enhancing their biological relevance as a model organism. This transparency, coupled with their sensitivity, makes medaka embryos superior candidates for assessing the impact of pollutants during the early life stages of aquatic organisms **(Yaqin *et al.*, 2021; Kai *et al.*, 2023)**.

Escherichia coli is a microscopic, Gram-negative bacterium that, under certain conditions, can cause severe infections posing serious risks to human health **(Ramos *et al.*, 2020)**. Although some strains are commensal, *E. coli* is generally not regarded as a beneficial component of the intestinal microbiota. Its presence in aquatic environments is widely recognized as a key indicator of fecal contamination **(Nowicki *et al.*, 2021)**. Although often harmless in small numbers, *E. coli* can become an opportunistic pathogen when it proliferates excessively in the gastrointestinal tract or translocates to the extraintestinal tissues, potentially leading to systemic infections **(Sora *et al.*, 2021)**. The detection of *E. coli* in water indicates that the microbial quality of water does not meet acceptable safety standards. Notably, *E. coli* has limited growth capacity in chlorinated water; the higher the chlorine concentration in the medium, the more inhibited its growth becomes **(Cheswick *et al.*, 2020; Clayton *et al.*, 2021)**.

Antimicrobial resistance (AMR) is a critical global health concern, often referred to as a "silent pandemic." Ciprofloxacin, a broad-spectrum antibiotic belonging to the fluoroquinolone class, is commonly used to treat a wide range of infections caused by both Gram-positive and gram-negative bacteria, including *Escherichia coli*, *Shigella*, *Salmonella*, *Enterobacter*, *Staphylococcus*, *Clostridium*, *Brucella*, and *Mycobacterium* spp. **(Shaygh *et al.*, 2021)**. This bactericidal agent works by inhibiting DNA gyrase, an

essential enzyme involved in bacterial DNA replication, thereby halting bacterial cell division and growth **(Spencer & Panda, 2023)**.

However, the growing prevalence of resistance to conventional antibiotics, such as ciprofloxacin, has highlighted the urgent need for alternative approaches that are both sustainable and environmentally friendly. One strategy that has gained increasing attention is the use of natural antimicrobial compounds derived from medicinal plants. Herbal plants are known to contain a wide array of secondary metabolites with antibacterial, anti-inflammatory, and antioxidant properties **(Maran *et al.*, 2022; Abdallah *et al.*, 2023)**. One such medicinal plant is basil (*Ocimum basilicum*), which has shown promising potential.

Basil (*Ocimum basilicum*) is a plant commonly found throughout Indonesia, growing either wild or cultivated. It contains a diverse range of bioactive compounds, including essential oils, alkaloids, saponins, flavonoids, triterpenoids, steroids, tannins, and phenols, all of which contribute to its potential antibacterial properties. These antibacterial effects may act in two primary ways: by inhibiting the growth and reproduction of bacteria (bacteriostatic) or by directly destroying bacterial cells (bactericidal) **(Zhakipbekov *et al.*, 2024)**.

Several studies have provided compelling evidence that flavonoids, essential oils, and tannins found in the leaves of *Ocimum sanctum* exhibit significant antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*. Flavonoids isolated from *O. sanctum* have been shown to effectively inhibit the growth of both bacterial species, with greater efficacy observed when used in combination, demonstrating a synergistic effect **(Ali & Dixit, 2012)**. Additionally, the essential oil of *Ocimum tenuiflorum* (a synonym of *O. sanctum*) contains active compounds such as eugenol, which are known to exert strong antibacterial effects against *S. aureus*, including resistant strains such as MRSA and *E. coli* **(Yamani *et al.*, 2016)**.

Furthermore, the combination of bioactive compounds such as phenols and flavonoids in *Ocimum sanctum* extracts has also been investigated by **Hussain *et al.* (2017)**, who demonstrated the effectiveness of the extract in inhibiting the growth of *E. coli* and *S. aureus*. These findings are supported by a study conducted by **Joshi (2013)**, who highlighted the antimicrobial activity of *O. sanctum* essential oil, which is rich in eugenol, flavonoids, and tannins. Additionally, **Dharsono *et al.* (2022)** affirmed that the synergistic presence of flavonoids, tannins, and essential oils in *Ocimum* species holds strong antibacterial potential and is highly relevant for the development of plant-based therapeutic applications.

These findings not only highlight the *in vitro* antibacterial potential of *Ocimum sanctum* but also pave the way for exploring its effectiveness in more complex biological systems through *in vivo* studies. Building on this background, the present study aimed to evaluate the efficacy of an ethanol extract from basil (*O. sanctum*) leaves on the heart rate of medaka fish embryos (*Oryzias celebensis*) exposed to *Escherichia coli in vivo*. This study also sought to assess the potential synergistic effects of basil leaf extract and

conventional antibiotics. Embryonic heart rate is widely recognized as a sensitive, non-destructive biomarker in toxicological research, particularly for assessing physiological responses to toxic exposure. Previous studies have demonstrated the reliability of detecting the effects of UV light exposure **(Yaqin *et al.*, 2025)**, pollutants from the Tallo River **(Yaqin *et al.*, 2024)**, 17α-ethinylestradiol (EE2) **(Qin *et al.*, 2025)**, and metals such as nickel **(Liu *et al.*, 2021)**. Therefore, the use of embryonic heart rate as a parameter in this study is considered highly appropriate for investigating both the pharmacological and toxicological effects of plant-based interventions against bacterial infections.

MATERIALS AND METHODS

1. Materials

Fresh basil (*Ocimum sanctum* L.) leaves were obtained from a local market in Makassar, Indonesia. Ethanol (99.5%) was purchased from Sigma–Aldrich, and ciprofloxacin tablets (500mg) were obtained from a licensed pharmacy.

2. Fertilization of *Oryzias celebensis*

The broodstock used in this study consisted of fish maintained at the Aquatic Animal Physiology Laboratory, Faculty of Marine and Fisheries Sciences, Hasanuddin University, Indonesia. To induce successful fertilization, broodstock were kept in aquaria under a controlled photoperiod of 12h light and 12h dark (12L:12D), with a male-to-female ratio of 1:1. Following fertilization, eggs—initially clustered and attached to the abdominal region of the female—were carefully transferred to Petri dishes containing embryo-rearing medium (ERM).

To obtain individual eggs for experimental use, clusters were gently separated by rotating them with the index finger, taking care to avoid physical damage. Eggs were then inspected, and those meeting the selection criteria were retained for subsequent procedures.

3. Embryo selection

Separated eggs were examined under a light microscope at 40× magnification to determine fertilization status. Eggs lacking a discernible perivitelline space were classified as unfertilized and excluded. The presence of a perivitelline space was taken as evidence of successful fertilization **(González-Doncel *et al.*, 2005)**. Fertilization criteria for *Oryzias* species followed the guidelines of **Iwamatsu (2004)**.

4. Research design and exposure procedures

4.1 Ciprofloxacin solution preparation

A single 500mg ciprofloxacin tablet was finely ground using a mortar and pestle. The resulting powder was dissolved in 500mL of 0.9% physiological saline and was homogenized with a vortex mixer for approximately one minute to ensure complete

dissolution. This stock solution had a concentration of 1mg/ mL (1000µg/ mL) and was diluted to a final concentration of 40µg/ L for experimental use.

4.2 Basil leaf extract preparation

Fresh basil leaves were washed thoroughly, air-dried to minimize moisture content, and ground into a coarse powder. The powder was placed in a sealed container and soaked in 96% ethanol at a ratio of 1:10 (w/v, plant material to solvent). The mixture was left at room temperature for 72h with occasional stirring to enhance extraction efficiency **(Srivastava *et al.*, 2023)**. After maceration, the mixture was filtered through filter paper, and the filtrate was concentrated using a rotary evaporator to obtain a thick basil leaf extract.

4.3 Escherichia coli suspension preparation

E. coli colonies were collected aseptically from agar plates using a sterile loop and transferred to 0.9% NaCl solution. Turbidity was adjusted to the 0.5 McFarland standard (~10^8 CFU/mL). This suspension was diluted to 10^6 CFU/mL by transferring 0.1mL of the original suspension into 9.9mL of sterile saline and mixing gently.

4.4 Treatment mixtures

Treatment solutions were prepared according to the following composition:

1. 5mL of 10% ethanolic basil leaf extract (*O. sanctum*)
2. 5mL ciprofloxacin (40 µg/L)
3. 10mL *E. coli* suspension (10^6 CFU/mL)

4.5 Treatment groups

The experimental design consisted of five groups:

1. Control (*E. coli* only)
2. *E. coli* + ciprofloxacin (EC+AB)
3. *E. coli* + 10% ethanolic basil leaf extract (EC+BE)
4. *E. coli* + ciprofloxacin + basil extract (EC+BE+AB)
5. Basil extract only (BE)

Each group had six replicates (n= 24 embryos total). Sample size was calculated using the Federer formula: $(n - 1)(t - 1) \geq 15$ **(Federer, 1955)**.

4.6 Exposure

For exposure, each prepared solution was dispensed into microplate wells (2.5mL per well), and *O. celebensis* embryos were carefully transferred into the corresponding wells. All animal experiments were approved by the Ethics Committee of Universitas Muslim Indonesia (clearance number UMI012412835).

5. Embryonic heart rate measurement

Heart rate was determined by recording the time (T, in seconds) required for 30 consecutive heartbeats under a microscope. The rate in beats per minute (N) was calculated using the formula **(Chen *et al.*, 2020; Wang *et al.*, 2023)**:

$$N=30/T * 60$$

This method provided a reliable and reproducible estimation of embryonic cardiac activity for comparison across treatments and developmental stages.

6. Data analysis

Heart rate data were analyzed using the Mann–Whitney U test and Kruskal–Wallis test to detect significant differences between groups. When significant differences were found, post hoc Bonferroni tests were applied. Time-course heart rate data were also analyzed descriptively to identify patterns across embryonic development.

RESULTS AND DISCUSSIONS

Embryonic heart rate

Embryonic heart rate is an important physiological parameter in developmental toxicology studies, particularly within the genus *Oryzias*, which is widely recognized as a model organism in biomedical and ecotoxicological research. Among the most extensively studied species is *Oryzias latipes* (Japanese medaka), which has been used to evaluate the toxicity of chemical compounds, heavy metals, pesticides, and pharmaceutical substances by monitoring fluctuations in embryonic heart rate. **Anderson *et al.* (2020)** demonstrated that exposure to ethinylestradiol (EE2) in *O. latipes* embryos significantly reduced heart rate, indicating endocrine disruption during early development. Similarly, *Oryzias melastigma*, a marine species, has been employed to investigate the effects of microplastic exposure on cardiac activity. **Chen *et al.* (2020)** reported tachycardia (increased heart rate) in embryos exposed to microplastics at 1×10^3 particles/ mL, and bradycardia (decreased heart rate) at 1×10^6 particles/ mL.

With increasing demand for ecologically relevant local species in ecotoxicity testing, *Oryzias celebensis*, endemic to Sulawesi, has gained prominence in *in vivo* toxicological studies. This species exhibits unique sensitivity and specific biological responses to local pollutants, making it highly suitable for monitoring contaminant impacts in tropical regions such as Indonesia **(Yaqin *et al.*, 2024, 2025)**.

The present study is among the few that use a simple yet effective biomarker—the embryonic heart rate of *O. celebensis*—to assess physiological responses to *Escherichia coli*, an antibiotic, and a natural herbal extract (*Ocimum sanctum*). Heart rate measurements taken at developmental stage 36 revealed distinct fluctuation patterns between treatment groups. Stage 36 was reached on day seven post-fertilization in the control, *E. coli* (EC), and *E. coli* + antibiotic (EC+AB) groups, but on day nine in *E. coli* + basil extract (EC+BE) and *E. coli* + basil extract + antibiotic (EC+BE+AB) groups, suggesting developmental delay in treatments involving basil extract.

Heart rate was recorded starting from stage 36, a point at which *O. celebensis* cardiac rhythm typically stabilizes and is especially useful for detecting cumulative cardiac

damage **(Yaqin *et al.*, 2024)**. Median heart rates were 175.0 bpm (control), 163.0 bpm (EC), 168.0 bpm (EC+AB), 159.0 bpm (EC+BE), and 136.0 bpm (EC+BE+AB) (Fig. 1). The reduction observed in the EC group suggests that *E. coli* exposure alone can depress embryonic heart rate. In the zebrafish (*Danio rerio*) larvae, *E. coli* exposure in a sepsis-induced myocarditis model produced bradycardia ($P< 0.01$) and pericardial edema, while co-treatment with metformin significantly increased heart rate ($P< 0.05$) **(Stones *et al.*, 2017; Zhang *et al.*, 2020)**.

Conversely, embryos in the EC+AB group showed a slight increase in heart rate compared with EC alone. Ciprofloxacin, a second-generation fluoroquinolone, contains 1-cyclopropyl-6-fluoro-4-oxo-7-(1-piperazinyl)-quinoline-3-carboxylic acid and exerts bactericidal action by inhibiting bacterial DNA gyrase and topoisomerase IV, preventing DNA replication and inducing bacterial cell death **(Collins *et al.*, 2024)**.

Exposure to basil (*O. sanctum*) was associated with decreased embryonic heart rate, likely due to bioactive compounds such as eugenol and linalool. Both are major constituents of basil essential oil and are known for sedative and anesthetic effects. Eugenol acts as a partial agonist of GABA_A receptors, enhancing parasympathetic activity and modulating central cardiovascular inhibition pathways, while linalool reduces neuronal excitability in brainstem regions regulating cardiac rhythm **(Capparucci *et al.*, 2022)**.

In *D. rerio* embryos, exposure to *Ocimum basilicum* extract at 100– 200µL/ L results in a dose-dependent decrease in heart rate, indicating a cardiodepressive effect **(Capparucci *et al.*, 2022)**. Basil also contains flavonoids and polyphenols, such as apigenin and rosmarinic acid, which can further suppress central nervous system activity and contribute to reduced cardiac function **(Malakar & Mandal, 2024)**. Although studies in *O. celebensis* are limited, the physiological regulation of the embryonic heart is highly conserved among freshwater fish, making zebrafish data a valid reference.

Combined exposure to *E. coli*, ciprofloxacin, and basil extract produced the most pronounced bradycardia. This synergistic effect may result from interactions between bacterial challenge, antibiotic activity, and basil-derived neuroactive compounds such as eugenol and linalool, which can lower heart rate through opioid pathway activation and central nervous system modulation **(Capparucci *et al.*, 2022)**.

Additionally, synergistic interactions between ciprofloxacin and phenolic compounds—such as flavonoids and eugenol—have been shown to enhance antibiotic efficacy and may influence physiological processes like heart rate via quorum-sensing inhibition **(Shamshad & Rajagopal, 2023)**. Further evidence from studies on freshwater fish, including zebrafish, indicates that flavonoids and rosmarinic acid can reduce physiological activity such as heart rate, particularly when combined with pharmacological stressors like antibiotics **(Steinberg, 2024)**.

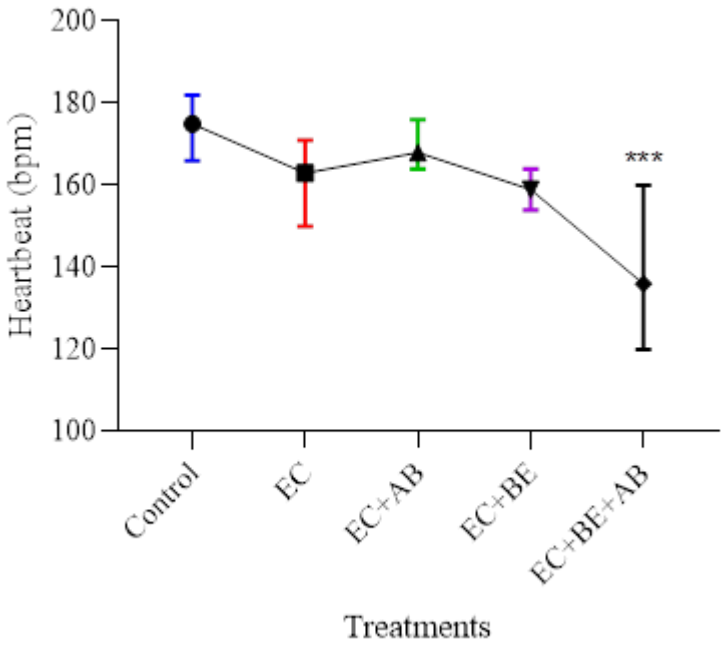

Fig. 1. Embryonic heart rate of *O. celebensis* following exposure to *E. coli*, antibiotics, and basil extract. Heart rate (bpm) of *Oryzias celebensis* embryos was monitored at 36 stages under five different treatment groups: Control, *E. coli* (EC), *E. coli* + antibiotic (EC+AB), *E. coli* + Basil Extract (EC+BE), and *E. coli* + Basil Extract + Antibiotic (EC+BE+AB). Bars represent the median with 95% CI

Statistical analysis using the Kruskal–Wallis test followed by a *post hoc* Bonferroni test revealed that, at stage 36, only the treatment group receiving a combination of *Escherichia coli*, basil (*Ocimum sanctum*) leaf extract, and the antibiotic ciprofloxacin (EC+BE+AB) showed a statistically significant difference in embryonic heart rate of *Oryzias celebensis* compared with the control ($P < 0.001$) (Fig. 1). In contrast, the other treatment groups—EC alone, EC+BE, and EC+AB—did not exhibit significant differences from the control ($P > 0.05$).

This finding suggests a unique synergistic effect of combined exposure to all three agents on the embryonic cardiovascular system. The interaction between basil-derived compounds (flavonoids, eugenol, tannins) and ciprofloxacin may induce metabolic or immunological stress that exceeds the embryo's adaptive threshold, triggering significant alterations in heart rate. Possible mechanisms include disruption of the embryonic

autonomic nervous system, osmotic pressure imbalances, or heightened pro-oxidant activity **(Azizah *et al.*, 2023; Dhama *et al.*, 2023; Sakure *et al.*, 2023)**.

Conversely, the absence of significant changes in the EC or dual-combination groups suggests that *O. celebensis* embryos can maintain cardiovascular homeostasis under single or non-synergistic dual stress conditions. These results underscore the value of embryonic heart rate in *O. celebensis* as a sensitive, nondestructive biomarker for detecting the combined toxic effects of biological and chemical contaminants. They also provide a foundation for developing ecologically relevant, locally adapted ecotoxicity assays in tropical regions.

Temporal profiling of embryonic cardiac activity in *Oryzias celebensis* under different treatment conditions

As shown in Fig. (2), the heart rate of *O. celebensis* embryos in control medium was approximately 175 bpm on day 7 post-fertilization (stage 36) and declined slightly to 171 bpm on day 8, after which hatching occurred. **Yaqin *et al.* (2024)** reported that embryos maintained in ERM exhibit heart rates around 190.5 bpm.

In the EC group, heart rate did not differ markedly from controls on day 7 (stage 36) but declined noticeably on days 8, 9, and 10 post-fertilization. These decreases coincided with the final stages of development immediately prior to hatching, which occurred primarily on day 11.

In the EC+AB group (*E. coli* plus ciprofloxacin), heart rate on days 7–9 post-fertilization remained consistent with controls. However, a pronounced decline was observed starting on day 10, continuing progressively through days 11–13.

Exposure to *E. coli*—whether as live bacterial cells or lipopolysaccharide (LPS) extracts—has been shown in the zebrafish embryos to activate the innate immune system, eliciting systemic physiological responses that include bradycardia as part of the host defense against infection. Such bradycardic effects are most pronounced during the late stages of embryogenesis, when metabolic demands are elevated and embryos are more vulnerable to physiological stress **(Shiau *et al.*, 2013)**. Similar patterns in *O. celebensis* indicate a comparable biological response to pathogenic exposure during critical developmental phases.

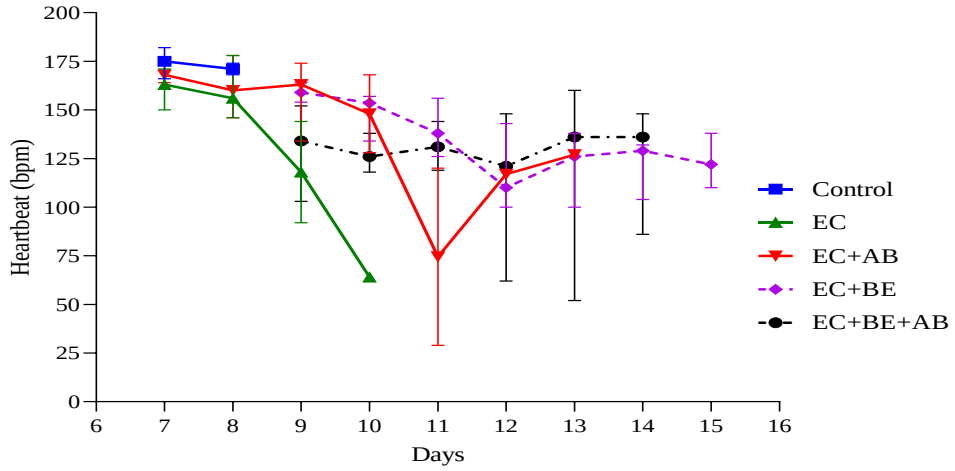

Fig. 2. Time course of embryonic heart rate in *Oryzias celebensis* under different Treatment conditions. Heart rate (bpm) of *Oryzias celebensis* embryos was monitored over a 9-day period in five different treatment groups: Control, *E. coli* (EC), *E. coli* + antibiotic (EC+AB), *E. coli* + Basil Extract (EC+BE), and *E. coli* + Basil Extract + Antibiotic (EC+BE+AB). Bars represent the median with 95% CI

In the treatment group exposed to *E. coli* and basil extract (EC+BE), the heart rate of *Oryzias celebensis* embryos showed a steady decline relative to the control group, beginning at the onset of stage 36 (day 9 post-fertilization). This downward trend continued through day 15, suggesting a potential cardiodepressive effect of the combined exposure on embryonic development.

Embryos in the group treated with *E. coli*, antibiotics, and basil extract (EC+BE+AB) exhibited bradycardia as early as stage 36, which persisted until hatching. These findings indicate a synergistic effect among the pathogenic bacteria, basil-derived bioactive compounds (e.g., eugenol and flavonoids), and antibiotics in suppressing embryonic cardiac activity **(Langeveld *et al.*, 2014; Wang *et al.*, 2018)**.

This observation was further supported by statistical analysis at stage 36, which revealed significant differences in heart rate among treatment groups, supporting the hypothesis that combined biological and pharmacological interventions can disrupt cardiac physiology.

CONCLUSION

This study demonstrated that the embryonic heart rate of *Oryzias celebensis* is a statistically robust and sensitive, nondestructive biomarker for assessing physiological responses to combined biological and chemical exposures. Statistical analysis using the Kruskal–Wallis test followed by a *post hoc* Bonferroni test revealed that only the group

exposed to the combination of *Escherichia coli*, ciprofloxacin, and *Ocimum sanctum* extract (EC+AB+BE) exhibited a statistically significant reduction in heart rate compared with the control ($P< 0.001$), whereas the other treatment groups (*E. coli* alone, EC+AB, and EC+BE) showed no significant differences ($P> 0.05$).

These results indicate a unique synergistic interaction among the three agents, likely exceeding the embryos' physiological tolerance threshold and resulting in marked bradycardia. The findings emphasize that combined exposure to microbial pathogens, antibiotics, and plant-derived bioactive compounds can substantially disrupt embryonic cardiac function.

Overall, this study not only validates the heart rate of *O. celebensis* as a reliable endpoint for *in vivo* ecotoxicological research but also provides valuable insights into medical ecology, highlighting the complex interplay between environmental stressors and physiological health outcomes in aquatic organisms—particularly within tropical ecosystems where such combined exposures are ecologically relevant.

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