

## Original Article

# A preliminary investigation on the effects of the echinoderm *Archaster typicus* extract on caudal fin regeneration and heart rate of zebrafish, *Danio rerio*

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**Abstract:** This study investigated the effect of the different concentrations of the crude hexanoic extract of the echinoderm, *Archaster typicus*, on the inherent ability of the zebrafish, *Danio rerio*, to regenerate its caudal fin after amputation, as well as on its heart function. The 100-ppm extract concentration showed the longest regenerated fin of 0.147 mm at 24h, 0.234 mm at 48h, and 0.598 mm at 72h. The fastest regeneration rate was at 100 ppm from 24 to 72. At 72h, the average length of fin regeneration in the negative control, 0.0 ppm (water only), and the concentrations 25 and 50 ppm were highly equivalent at 0.41 mm. The regenerated fin was longest in 100 ppm (0.6 mm) and shortest in 200 ppm (0.35 mm). After exposure to the positive control, benzaldehyde, for 72 h, the length of the regenerated fin was 0.66 mm at 25 ppm and 0.88 mm at 200 ppm. Increasing the benzaldehyde extract concentration also increased the regeneration length, with a maximum of 0.88 mm at 200 ppm. ANOVA showed significant differences in the length of the new fin among replicates, concentrations, and exposure durations. The effect of the echinoderm extract was a decrease in heart rate with increasing extract concentration and exposure duration (bradycardia).

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## Introduction

Marine natural products are a valuable medicinal resource for humans. Research on compounds isolated from starfish with pharmacological properties has expanded rapidly over the years (Sumitha et al., 2019). They are a promising source of diverse chemical metabolites, attracting significant interest from chemists and pharmacologists worldwide due to their unique chemical composition and intriguing pharmacological properties (Peng et al., 2019). Echinoderms, especially starfish, possess remarkable natural regenerative abilities, attracting significant attention from researchers and pharmacologists worldwide. Their exceptional regenerative capabilities serve diverse biological functions and have attracted interest for potential therapeutic applications (Ben Khadra et al., 2018). Although only a few were identified and isolated, echinoderms are an exceptional source of polar steroids, with vast structural diversity and a wide range of bioactivities

(Sumitha et al., 2017). Various secondary metabolites, including steroids, steroidal glycosides, anthraquinones, alkaloids, phospholipids, peptides, and fatty acids, were reported from starfish. These chemical constituents exhibit cytotoxic, hemolytic, antiviral, antifungal, and antimicrobial activities (Dong et al., 2011).

*Archaster typicus* (Asteroidea: Archasteridae) is listed as Not Evaluated (NE) on the IUCN Red List, and is one of the most popular echinoderm samples for numerous studies, ranging from the examination of its reproductive biology to the characterization of its metabolites (Yang et al., 2011). *Archaster typicus* is a five-limbed star with long, slightly tapering arms with pointed tips. Occasionally, three, four, or even six-armed individuals occur. Adults grow to 12-15 cm in diameter, with males often smaller than females (Bos et al., 2011). This starfish is adapted to life on the sandy seabed, where it buries itself in the sediment during high tides and moves across the sediment

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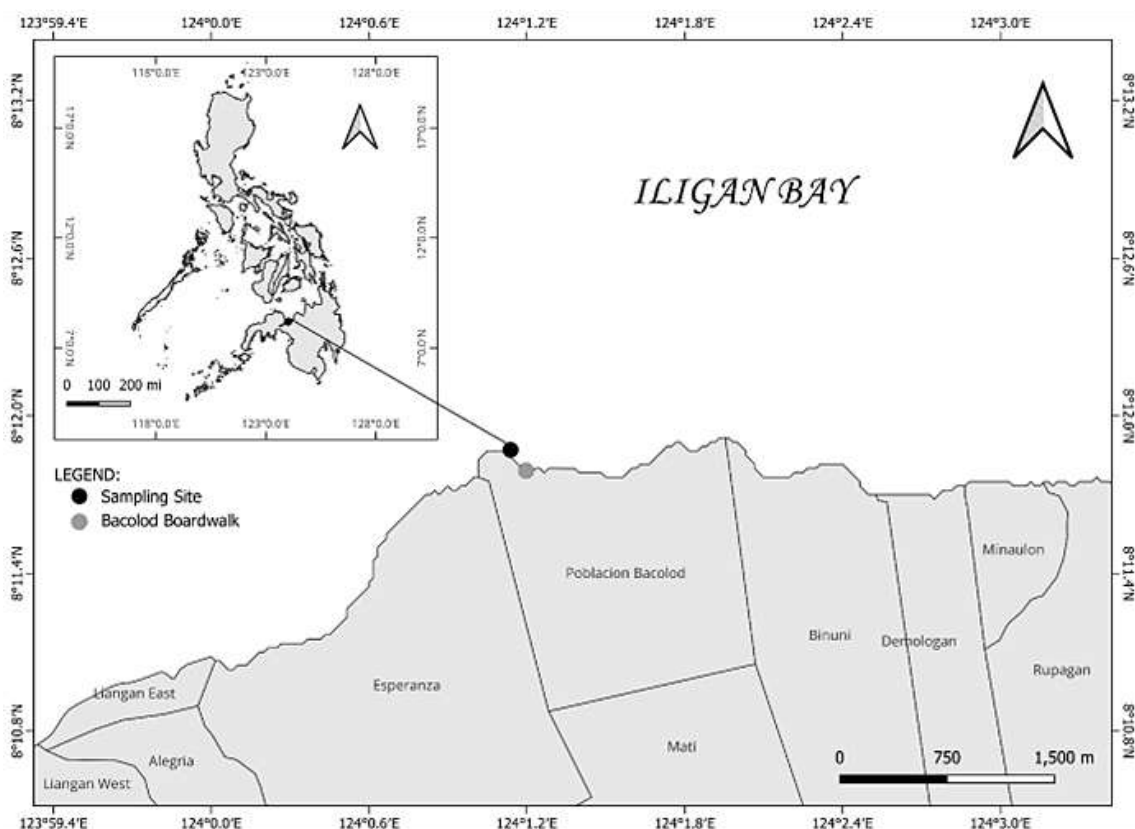


Figure 1. Location map of the sampling area in Poblacion, municipality of Bacolod, Lanao del Norte, Mindanao, Philippines. (Source: QGIS v. 3.34.0).

surface during low tides (Bos et al., 2011). It is found in areas of the seabed with soft sediments, including sand, silt, and seagrass meadows (Mah et al., 2010).

Zebrafish (*Danio rerio*, Hamilton, 1822) is a small tropical fish, native to Southeast Asia, and belongs to the minnow family (Danionidae) (Bournele et al., 2016). Zebrafish are used in diverse phases of drug discovery and have proven to be a constructive and cost-effective alternative to various mammalian models. They have proven to be a dynamic *in vivo* model for assessing the toxicity of some compounds and, with further testing and characterization, may help us evolve them into effective bioactive compounds (Sumitha et al., 2019).

Zebrafish can completely regenerate an amputated caudal fin. A regenerating fin forms a blastema, and cells of the blastema proliferate actively and re-differentiate to provide new fin rays. The zebrafish embryotoxicity model is at the forefront of toxicology research due to its short analysis time, transparency of embryos, short life cycle, high fertility, and genetic

data similarity (Modarresi Chahardehi et al., 2020). Results of a study using a sterol isolate from the echinoderm *Stellaster equestris* suggest that the extent of regeneration in the caudal fin of *D. rerio* is marginally affected by increasing the concentration from 50 to 400 ppm (Sumitha et al., 2019).

Literature on the effect of *A. typicus* extract on caudal fin regeneration and cardiotoxicity in zebrafish larvae is scarce. The bulk of investigations involving *A. typicus* focus on isolating and structurally characterizing compounds from the starfish and on gene-related cellular responses to extract fractions. Therefore, this study aimed to evaluate the effect of the different concentrations of the crude hexanoic extract of the echinoderm, *A. typicus* to the regeneration of the amputated caudal fin of the adult zebrafish, *Danio rerio*, using the fin regeneration assay and to evaluate the effect of the same concentrations of the extract on the heart rate of the zebra fish larvae 120 hpf using the cardiotoxicity assay.

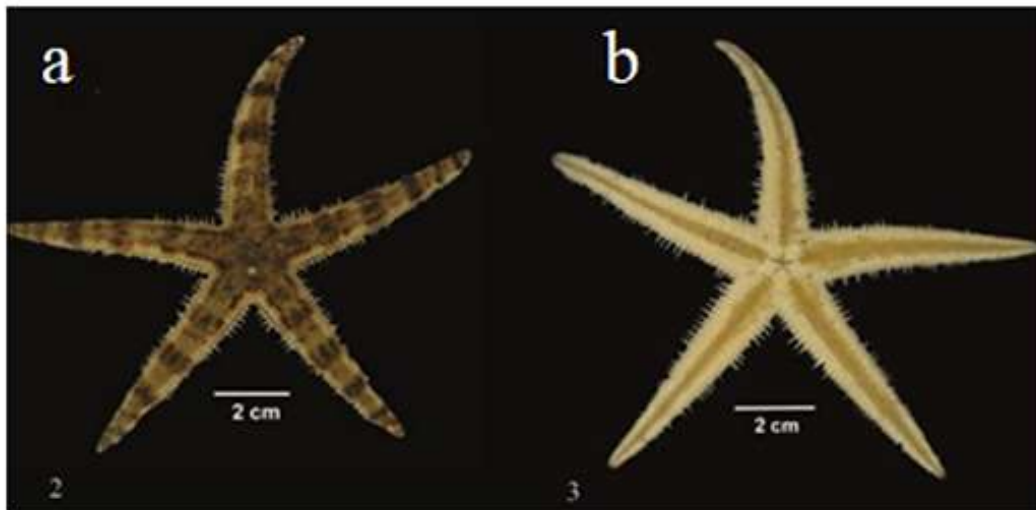


Figure 2. *Archaster typicus*. A-Dorsal view; b-Ventral view of the echinoderm.

### Materials and Methods

**Study area:** Echinoderms were collected from the coastal barangay of Poblacion, Bacolod, Lanao del Norte, Mindanao, Philippines. The coordinates of the collection area were  $8^{\circ}11'52.26''\text{N}$  and  $124^{\circ}01'08.31''\text{E}$ . The prominent landmark was the Bacolod Boardwalk, which was located near the sampling area, with coordinates  $8^{\circ}11'47.51''\text{N}$  and  $124^{\circ}01'11.88''\text{E}$  (Fig. 1).

**Permits and echinoderm collection:** The permit to use zebrafish as the animal model in the caudal fin regeneration assay was issued by the Institutional Animal Care and Use Committee (IACUC) of MSU-Iligan Institute of Technology, Iligan City. After the permit to collect echinoderms was issued by the local officials of the Bacolod Municipality, the researchers began collecting echinoderms by random hand-picking at the fish landing area in Barangay Poblacion, Bacolod, Lanao del Norte. The common sea star, *A. typicus* (Müller and Troschel, 1840) (Fig. 2), was easily collected as the starfish were mostly found in exposed sandy intertidal areas as well as in the shallow waters of the lower intertidal zone during low tide. Twenty adult echinoderms were stored in a cold-storage, temperature-resistant box containing seawater and immediately transported to the MSU-Iligan Institute of Technology Marine Science Laboratory for processing.

**Processing and extraction:** The whole body of *A. typicus* was chopped into small, thin slices using a

sharp knife. The finely-chopped materials were placed in a clear glass bottle, soaked in hexane, and stored at room temperature. After two weeks, the liquid was filtered through Whatman No. 2 filter paper. Extraction was performed using rotary evaporation at the Natural Products Laboratory in the Chemistry Department. The stock solution at 300 ppm was prepared by dissolving 0.045 g of the extract in 150 ml of distilled water. Test concentrations of 200, 100, 50, and 25 ppm were prepared by diluting the stock solution with distilled water. The concentrations were selected based on the study of Sumitha et al. (2019).

**Breeding and maintenance of zebrafish:** Healthy adult pink zebrafish were purchased from local pet fish sellers in Iligan City and maintained in 45 L tanks at  $28^{\circ}\text{C}$  under a 14 h/10 h light/dark cycle (Fig. 3). Fishes were fed regularly, provided with aeration, and the water was monitored daily and changed every 3 days. The study protocol was approved by the university's Institutional Animal Care and Use Committee (IACUC). The animal study was carried out in strict compliance with the established institutional guidelines.

**Caudal fin regeneration assay:** The protocol followed that of Sumitha et al. (2019) with modifications. After 2 weeks, the fish were anesthetized by immersing them in an anesthetic tub (25 mg of J-pro cream in 1 L of water) for 3-5 minutes. When the fish appeared to swim slowly in a random direction, the anesthetized fish was carefully laid flat



Figure 2. The 45-L aquarium with a culture of 5-day-old zebrafish, *Danio rerio* larvae.

on the left hand between the index and middle fingers with the caudal fin pointing outward. While immersed in the anesthetic tub, the tip of the caudal fin (~2 mm) was cut using a sterile, sharp scissor. The amputated fish was immediately transferred to a revival bath (freshwater) provided with aeration. When the amputated fish started to swim normally after approximately 5 minutes, five fish were added to labelled containers with five hundred milliliters of the different concentrations (25, 50, 100, and 200 ppm) of the hexanoic extract of *A. typicus* with water and benzaldehyde as negative and positive controls, respectively, with 3 replicates for each concentration. Moderate aeration was provided to each container.

Measurement of the length of caudal fin regeneration was done at 12, 24, 48, and 72 hrs after caudal fin amputation. At 12h, five fish, selected at random from any container, were again anaesthetized and examined under a laboratory Trinocular Compound Microscope equipped with a 10 MP digital camera (UB1031 Model) to measure caudal fin regeneration length using a micrometer eyepiece. The mean length measurement from the five fish was used to calibrate the ImageJ application on the computer. All the remaining fish were anaesthetized, and the fins were photographed under the same Trinocular Compound Microscope at the same focal distance. The fin images were saved to the computer and processed in ImageJ to measure the length of the regenerated fin accurately.

**Cardiotoxicity assay:** The protocol for the

embryotoxicity assay was adapted from Sumitha et al. (2019) with modifications. Healthy adult pink zebrafish were purchased from local pet fish sellers in Iligan City, cultured in a 45-liter aquarium, provided with adequate aeration and food, and the water was changed every 3 days. Dissolved oxygen, salinity, and pH were monitored daily. The fish were bred in the aquarium. When the eggs were laid, the adult fish were removed and transferred to another aquarium. After 120 hpf, 30 larvae were collected and transferred to a labelled container containing 500 ml of the extract at 25, 50, 100, and 200 ppm, along with the negative control (freshwater) and the positive control (benzaldehyde). Each concentration was replicated three times. Each replicate container was provided with moderate aeration.

After 24 h, the larvae were pipetted from each container and carefully transferred to a Petri dish containing 200 ml of the solution for counting heartbeats using video microscopy. The larva was focused under the HPO of a laboratory Trinocular Compound Microscope provided with a 10 MP digital camera (UB1031 Model). The microscope is equipped with a cable that connects to the computer for monitoring. Three three-minute high-definition videos were recorded to capture the heartbeat of each fish. The video recordings were saved on the computer and backed up with a high-resolution recording from a mobile camera. The videos were played several times on a computer in slow motion to count the number of heartbeats per minute in the 3-minute recording. Heartbeat counts were recorded manually as the frequency of ventricular contractions per minute, as displayed on the laptop monitor. The larvae were returned to their containers for the second monitoring at 48h. In both assays, a Completely Randomized Design (CRD) was used to minimize bias in assessing the effects of different concentrations and exposure durations of the hexanoic extract on the regenerative potential and heart rate of *D. rerio*.

**Statistical analysis:** To test if there was a significant difference in the effects of the various concentrations of the hexanoic extract on the heart rate as well as on the regenerative capacity of the zebrafish, Two-Way

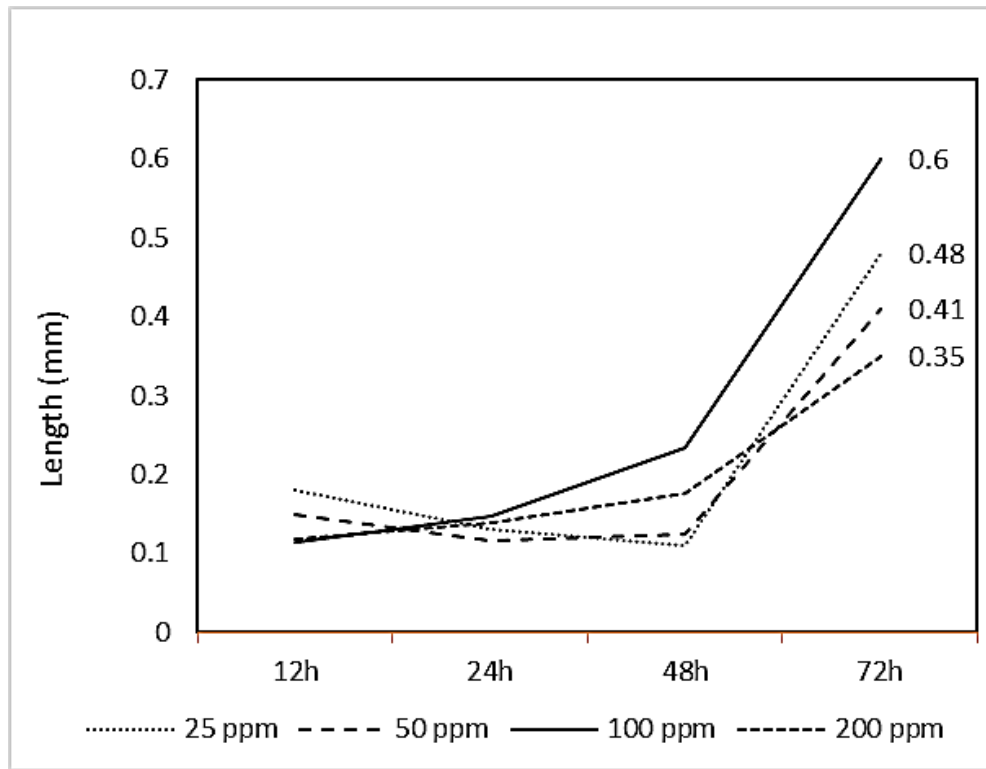


Figure 4. Length of zebrafish’s regenerated fin at various concentrations of the extract as a function of time.

ANOVA with replications was used using JAMOV Software (version 1.2.5). *P*-values <0.05 were considered statistically significant.

**Results and Discussions**

**Caudal fin regeneration assay:** Fish exposed to 25 ppm showed the longest regenerated fin of 0.180 mm in the first 12 hours after amputation. At 24 hours, lengths were almost identical in all concentrations. Growth decreased at 48 hours, with the highest value of 0.234 mm in 100 ppm. Growth accelerated and peaked at 72 h across all concentrations, with the highest value of 0.598 mm at 100 ppm, followed by 25 and 50 ppm; the shortest fin regeneration was observed at 200 ppm (Fig. 4). Zebrafishes exposed to 100 ppm showed the fastest progression in length over time, and thus the longest regenerated fin, from 48 h (0.234 mm) to 72 h (0.60 mm).

Figure 5 showed a significant increase in the length of the regenerated fin with time. During the first 12h, the length ranged from 0.00 to 0.25 mm. Lengths gradually increased to more than 0.125 mm at 24h. After 48h, a significant increase in length of

approximately 0.50 was recorded, and the length range was 0.25 to 0.50 mm. At 72h the lengths range from 0.60 to 0.75 mm. At 72h, the length of the regenerated fin was longest (0.6 mm) in 100 ppm (Fig. 6c) and shortest (0.35 mm) in 200 ppm (Fig. 6d). This result suggested that 100 ppm was the optimum concentration to effect maximum regeneration rate of 0.6 mm in 72h. Furthermore, the decrease in fin length from 0.6 mm at 100 ppm to 0.35 mm at 200 ppm suggested that increasing the extract concentration above 100 ppm diminished regeneration potential.

With exposure to benzaldehyde for 72h, the length of the regenerated fin was 0.66 mm in 25 ppm (Fig. 6e) and 0.88 mm in 200 ppm (Fig. 6h). Increasing the concentration of benzaldehyde also increased the regeneration length, with a maximum of 0.88 mm in 72h in 200 ppm. The maximum positive effect of the extract at 100 ppm was comparable to that of the lowest benzaldehyde concentration, which served as the positive control. The various concentrations of benzaldehyde resulted in a greater increase in the length of regenerated fin compared to the four experimental groups (Fig. 7). Benzaldehyde was used

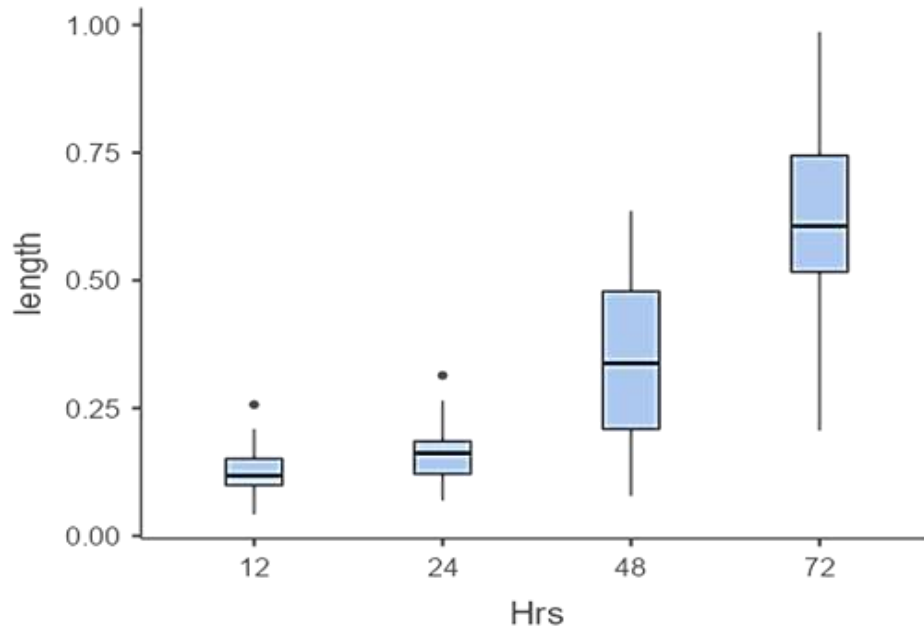


Figure 5. Length (in mm) distribution of regenerated caudal fin at various exposure times (hours) to the extract.

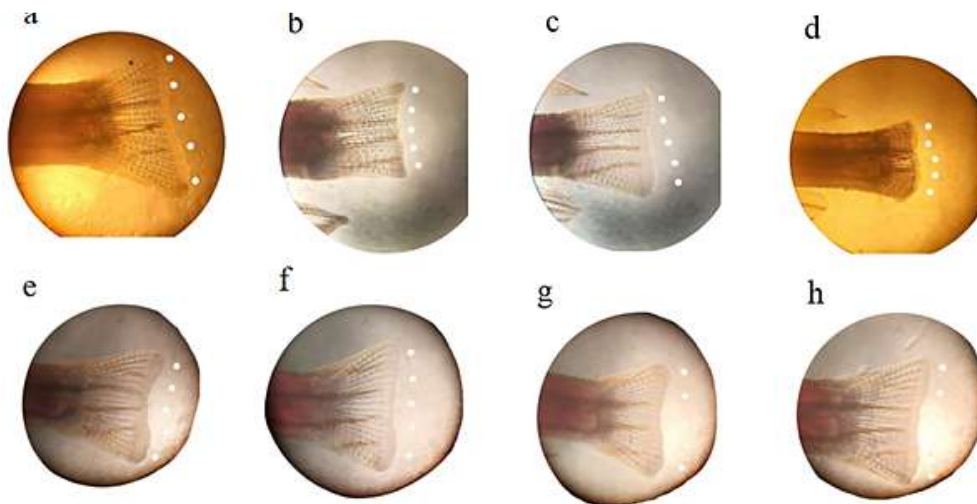


Figure 6. Fin regeneration at various concentrations of *Archaster typicus* extract and Benzaldehyde at 72h after amputation. a- E25 ppm, b-E50 ppm, c-E100 ppm, d-E200 ppm, e-B25 ppm, f-B50 ppm, g-B100 ppm, and h-B200 ppm. E- *A. typicus* extract and B-Benzaldehyde.

as a positive control drug because it promotes regeneration of the caudal fin in zebrafish experiments. The length of fin regeneration was proportional to the concentration of the control drug. Benzaldehyde produced longer fins than the equivalent-concentration extract (Fig. 7). The results, however, indicated that there was no significant difference in the lengths of the regenerated fins between benzaldehyde concentrations and with the different concentrations of the extract of *A. typicus*.

The 100 ppm extract concentration yielded a more diverse group than the other concentrations, with the

longest regenerated fin at this concentration (Fig. 8). The difference between 25 and 100 ppm was almost exactly half as large. The variations in fin lengths at 50 and 200 ppm were almost identical. The results showed significant differences in the length of the regenerated fin across replicates ( $P < 0.001$ ), concentrations ( $P < 0.001$ ), and exposure durations ( $P < 0.001$ ). The results showed that the associations between the variables were statistically significant ( $P < 0.05$ ).

The average length of regenerated fin in the negative control (0.0 ppm, water only) after 72h was

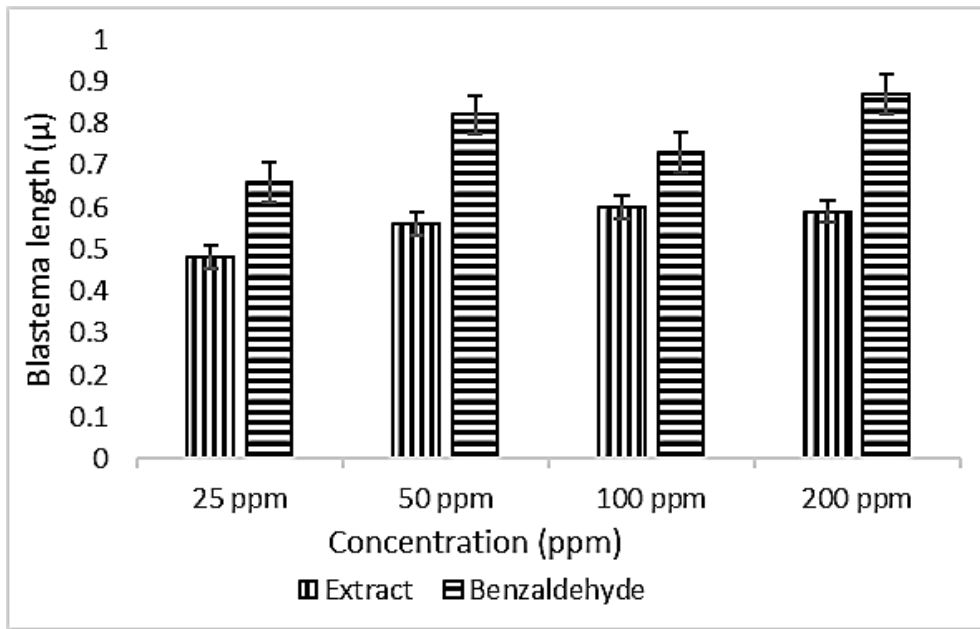


Figure 7. Regenerated fin length (in mm) induced by varying concentrations of *Archaster typicus* extract with Benzaldehyde as a positive control.

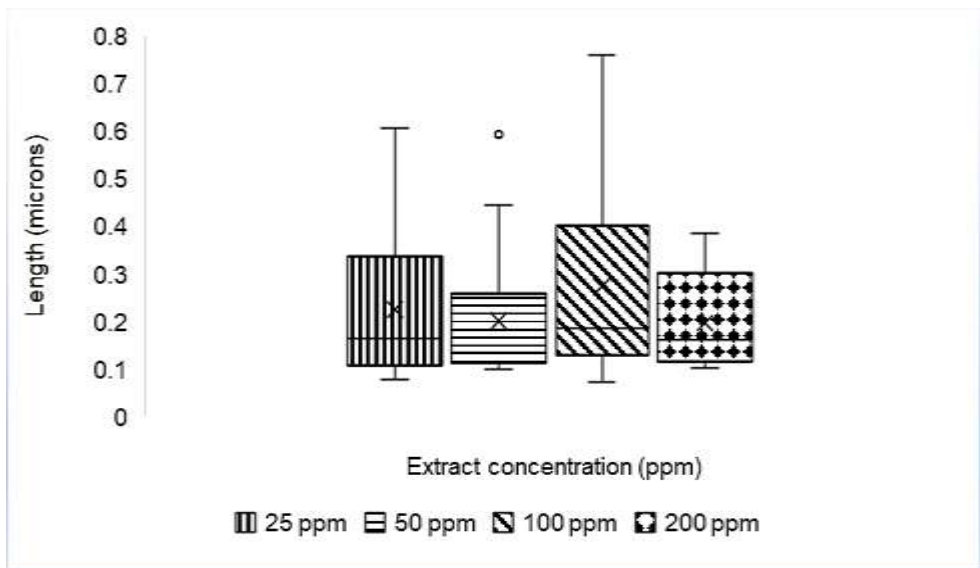


Figure 8. Length distribution in various hexanoic extract concentrations.

0.41 mm (Fig. 9). This implied that zebrafish has the inherent, normal capacity to regenerate the amputated caudal fin in its natural freshwater environment. From 0.48 mm in 25 ppm, the length reached a maximum of 0.6 mm in 100 ppm after 72h. At 72h, the fin length in 25 ppm (0.4 mm) was comparable to the negative control (0.4 mm), clearly suggesting that the lowest extract concentration of 25 ppm has no significant effect on regeneration. The 100-ppm extract and 25 ppm benzaldehyde produced the same regenerated length of 0.66 mm.

Post hoc comparisons across extract concentrations (in ppm) using Tukey’s test indicated that the difference between 25 and 100 ppm was significant. There was no difference between 25 and 50 ppm, nor between 50 and 200 ppm. All remaining associations showed significantly different measurements. The results of exposure duration to the extract indicated that the difference in fin regeneration length between 12h and 24h was not significant. The remaining periods demonstrate a highly significant difference. There was a significant increase in the length of the

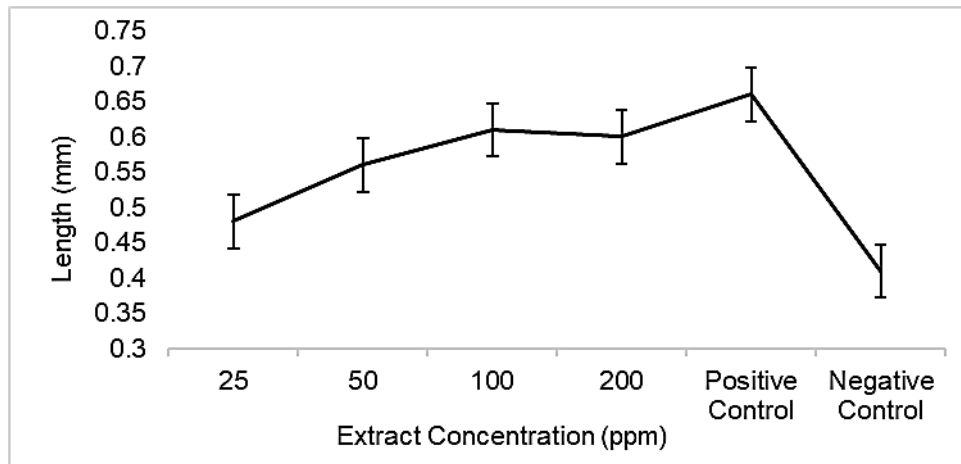


Figure 9. Regenerated fin length (in mm) induced by varying concentrations of *Archaster typicus* extract at 72h with Benzaldehyde and water as positive and negative controls, respectively.

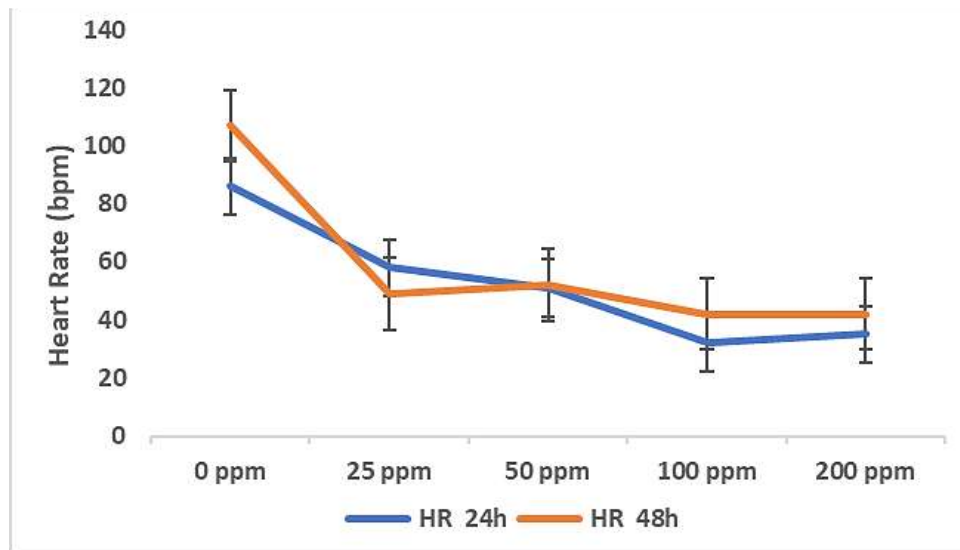


Figure 10. Average heart rate of the 5-day-old zebrafish larvae exposed to different concentrations of the *Archaster typicus* extract for 24h and 48h.

regenerated fin of the zebrafish from 24h to 72h as a result of exposure to the various concentrations of the hexanoic extract from *A. typicus*. Survival rate of the larvae exposed to the echinoderm extract from 25 to 200 ppm for a maximum period of 72 hours was 100%.

**Cardiotoxicity assay:** Heartbeat measurement is important in assessing cardiac function because variations in heart rhythm can be both a cause and an effect of underlying cardiac pathology. Zebrafish has emerged as one of the most useful model organisms for cardiac research (De Luca et al., 2014). There were five concentrations of the *A. typicus* extract used, namely, the negative control (0.0 ppm; water only), 25, 50, 100, and 200 ppm, with three replicates per

concentration, and monitoring periods of 24h and 48h. Considering the control heart rate (HR) as the baseline, the effect of the extract at 25 ppm is a 67% reduction in the larvae's normal HR during the first 24 h. Heart rate decreased (bradycardia) with increasing concentrations of *A. typicus* extract, except at 200 ppm, which was elevated by 3 units. In the first 24 hours, HR decreased from 58 beats per minute (bpm) in 25 ppm to 51 bpm in 50 ppm, and 32 bpm in 100 ppm. At 48h, HR decreased from 49 bpm in 25 ppm to 42 bpm in both 100 ppm and 200 ppm (Fig. 10). The average HR at 24h in the control was 86 bpm. After 48 h, the average HR increased to 107 bpm, a 44 percent increase. Zebrafish heart rate was reported at 140-180 bpm, which is much closer to the human fetal

heart rate of 130-170 bpm (Sarmah et al., 2016).

In the experimental groups, the effect of the extract across the period from 24h to 48h was decreased HR from 58 to 49 bpm in 25 ppm; stable HR in 50 ppm; increased HR from 32 to 42 bpm in 100 ppm; and increased HR from 35 to 42 bpm in 200 ppm. Although the high concentrations of 100 and 200 ppm increased HR over 48 h (from 32 to 42 bpm), the increase was only 39.25% of the control HR (107 bpm). Therefore, the general effect of the *A. typicus* hexanoic extract is decreased HR with increasing concentration from 25 to 200 ppm, as well as a reduced HR with exposure to the extract from 24h to 48h in each concentration of the extract.

The results indicated a significant difference in HR among zebrafish larvae exposed to different concentrations of *A. typicus* extract ( $P=0.001$ ). The effect of exposure duration on HR was also significant ( $P=0.017$ ). Furthermore, the combined effect of concentration and time was significant ( $P=0.024$ ). The difference in HR is highly significant between the control and all concentrations of *A. typicus* extract. Post hoc comparisons between periods based on estimated marginal means indicated a significant difference in HR between 24 hours and 48 hours ( $P=0.017$ ). The results suggest that the fish exhibit a change in heart rate after 24h and 48h exposure to the echinoderm extract. Survival rate of the larvae exposed to the echinoderm extract from 25 to 200 ppm for a maximum period of 48 hours was 100%. Zebrafish heart rate was reported at 140 -180 bpm, where it is much closer to the human fetal heart rate of 130-170 bpm (Sarmah et al., 2016).

## Conclusion

The crude hexanoic extract from *A. typicus* has the potential to increase the regeneration rate in zebrafish faster than it would normally occur in its freshwater environment. The optimal concentration for the fastest regeneration was 100 ppm; above this concentration, the regeneration rate declined. Regeneration was discernible starting 12h after amputation, and the length of the regenerated caudal fin increased to a maximum with time until 72h. The heart rate of 5-day-

old zebrafish larvae decreased with increasing concentration of *A. typicus* extract from the first 24h until 72h. The general effect of the echinoderm hexanoic extract from 25 to 200 ppm on the larvae was bradycardia in 24-48 hours.

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