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Cardioprotective Activity of the Ethanolic Leaf Extract of *Amaranthus viridis* on Verapamil-Induced Heart Failure in Zebrafish (*Danio rerio*)

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Abstract: Cardiovascular disease (CVD), a major global health concern, is characterized by cardiac complications that can lead to death. The commonly used treatments for this condition are synthetic drugs, but these often come with risky side effects. A potential alternative is the use of traditional medicinal plants, such as *Amaranthus viridis*, which is rich in bioactive compounds. This study aimed to determine the non-toxic concentration of *A. viridis* ethanolic extract, investigate its cardioprotective effects on zebrafish (*Danio rerio*) heart rate and cardiac phenotype, qualitatively assess the presence of phytochemicals, and assess its free radical scavenging activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH). Zebrafish larvae at 72 hours post-fertilization (hpf) were used to evaluate mortality and optimize dosing. Physio-morphological screening was conducted by pre-treating zebrafish larvae with the extract 4 hours prior to administering a heart failure inducer, verapamil. The maximum non-toxic concentration was found to be 25 µg/mL, as all zebrafish survived after 24 hours. Mortality began at 50 µg/mL, and concentrations from 100 µg/mL to 400 µg/mL resulted in 100% mortality. All tested concentrations of *A. viridis* leaf extract showed cardioprotective activity in the physio-morphological analysis. Phytochemical analysis detected the presence of alkaloids, flavonoids, and saponins. Furthermore, *A. viridis* exhibited free radical scavenging activity from all tested concentrations. Based on the results, *A. viridis* exhibited cardioprotective effects against verapamil-induced cardiotoxicity, as evidenced by the recovery of heart rate and cardiac phenotype in the zebrafish model.

Keywords: cardiovascular disease; *Amaranthus viridis; Danio rerio*; 72-hour post fertilization; verapamil

Introduction

Cardiovascular disease (CVD) is a significant global health concern, consistently impacting human life and overall well-being. It is the leading cause of mortality worldwide, affecting millions of individuals of all ages and demographics. According to the World Health Organization (WHO), CVD is responsible for a staggering 9.6 million deaths annually in men and 8.9 million in women, collectively constituting nearly one-third of all deaths attributed to diseases. In the Philippines, the primary causes of death in 2021 were coronary heart disease (CHD), other cerebrovascular diseases or stroke, and various forms of cancer. Notably, there were over half a million reported cases of CHD in 2021, making up approximately 19% of the total deaths in the country (Bray *et al*., 2021; Corpuz, 2023; Daniels *et al*., 2014; Nguyen & Cheng-Lai, 2013; Timmis *et al*., 2020; Timmis *et al*., 2022).

Conventional treatments for heart failure involve a combination of medications such as angiotensin converting enzyme inhibitors (ACEIs), beta-blockers, diuretics, corticosteroid receptor antagonists, sodium-glucose cotransporter two inhibitors, and others. These drugs aim to reduce the heart's workload, enhance efficiency, alleviate symptoms, and target specific aspects of heart failure. Despite the extensive pharmaceutical options, heart failure remains a significant medical challenge,

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with persistently high rates of illness and mortality suggesting that current treatments, while valuable, often fall short in completely managing the condition (Chia *et al*., 2016; Krum *et al*., 2011). Amid this ongoing challenge, interest in herbal medicines as complementary treatments has grown. *Amaranthus viridis,* commonly referred to as "Spinach Tagalog" or "Kolitis," is a locally available vegetable in the Philippines and widely distributed in Asia. Abundant in bioactive compounds like flavonoids, alkaloids, saponins, and polyphenols, this plant has been linked to various pharmacological activities. Significantly, the bioactive compounds present in *Amaranthus viridis* demonstrates noteworthy properties including antioxidants, anti-inflammatory, antithrombotic, antiarrhytmic, and anti-hypertensive effect which collectively contribute to cardiovascular health (Bachheti *et al*., 2022; Pirdhankar *et al*., 2023; Saravanan & Ponmurugan, 2012; Shah *et al*., 2019).

The zebrafish (*Danio rerio*) is a versatile model organism in cardiovascular research. Native to Southeast Asia, this small tropical freshwater fish is particularly valuable for studying cardiac function and disease. A key feature is the transparency of zebrafish embryos, enabling non-invasive, real-time visualization of internal organs, including the developing heart. This transparency facilitates detailed observations of cardiac morphogenesis and function during various developmental stages, as early as 24 hours post-fertilization (Irion & Nüsslein-Volhard, 2022; Shen & Zuo, 2020; Tavares & Lopes, 2013).

Furthermore, zebrafish exhibit external development, and their embryos undergo rapid and synchronous growth. This characteristic makes them particularly suitable for studies involving developmental biology and the effects of various compounds on embryonic development, including potential therapeutic agents. The external development also allows for easy manipulation and observation, making zebrafish a cost-effective and time-efficient model for cardiovascular studies (Eimon and Ashkenazi, 2010). However, to date, no studies have evaluated *Amaranthus viridis* using zebrafish larvae model. Hence, this study was conducted to evaluate the cardioprotective activity of *Amaranthus viridis* in verapamil-induced heart failure in zebrafish model. The findings from this research are expected to offer valuable insights that can contribute to further exploration and understanding within the field of cardiovascular health.

This study aims to determine the maximum non-toxic concentration of the ethanolic leaf extract of *Amaranthus viridis* in 72-hours post-fertilization (hpf) zebrafish larvae. Additionally, it seeks to identify the lowest effective concentration and evaluate the cardioprotective activity of *A. viridis* against verapamil-induced heart failure through physio-morphological assessments. The study also qualitatively examines the presence of phytochemicals, including saponins, flavonoids, phenols, and alkaloids, which may contribute to cardioprotective effects. Furthermore, the free radical scavenging activity of the leaf extract is assessed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay.

Materials and Methods

Collection and Authentication of Plant Material

Approximately 2 kilograms of fresh young leaves from the actively growing tops of *Amaranthus viridis* were collected at Barangay Tabunan, Cebu City as depicted in Figure 1. Subsequently, a whole plant sample was transported to the Department of Biology at the University of San Carlos for botanical authentication by Mr. Val Salares. A voucher specimen was submitted to the University of San Carlos Herbarium.

Plant Extraction

The leaves were washed with distilled water to remove any external impurities and will be air-dried in the shade at 21-27°C for 2 weeks to remove excess moisture. The leaves were grounded to a fine powder using an Osterizer blender for 5 minutes to achieve 68-200 µm particle size. After grinding, straining was done to obtain a finely powdered particle using a 100 µm sieve strain (Alsaud and Farid, 2020). The finely powdered leaves were stored in Erlenmeyer flasks and covered with

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wooden cork to prevent contamination from moisture and other pollutants. Twenty grams of dried powder were macerated in 200 mL of ethanol solvent at room temperature for 72 hours. The extract was filtered using a Whatman filter paper number 1. Subsequently, the filtrate was transferred to a Heidolph rotary evaporator for evaporation, resulting in the collection of a solid concentrate (Gahlot *et al*., 2018). Following this, the extract was placed in amber vials and stored at a temperature of 4°C.

Preparations were made for various concentrations of 25, 50, 100, 200, and 400 µg/mL. The final crude extracts were resuspended to dimethyl sulfoxide (DMSO) and subjected to sonication to ensure homogeneity. They were stored in appropriately labeled amber vials for safety and future use (Lee and Yang, 2021).

Preparation of Egg Water

Throughout the experiment, the medium for zebrafish embryos was consisted of egg water. The egg water was prepared by thoroughly mixing 40 g of sea salt (Mars Fishcare Europe, UK) with 1 L of distilled water. Following this, 1.5 mL from the stock salts were dissolved in another 1 L of distilled water to attain a final concentration of 60 µg/ml (Westerfield, 2007).

Ethics Declaration

All protocols were subjected to approval by the Institutional Animal Care and Use Committee (IACUC) at the University of San Carlos. Furthermore, future experiments related to zebrafish were taken place at the Medical Biology Laboratory of the Department of Biology at the University of the Visayas.

Establishment of Zebrafish Aquarium and Husbandry

An aquarium with a divider in the center, measuring 30 cm in length, 20 cm in height, and 20 cm in width, was constructed using acrylic glass containing 5 liters of dechlorinated water. The tank was equipped with Precision aquarium air pumps (PR-7500 and PR-2500, China), Xinyou aquarium sponge filters (China), thermometers, and Zetlight control units (China). The water temperature in the tank was maintained between 24-29 ℃. Light conditions, following a 10-hour dark and 14-hour light cycle (10:14), were regulated using a timer. Tap water with a pH range of 6.5 to 8 was utilized for water quality. Monitoring and daily documentation of pH, total hardness, nitrate, nitrite, free chloride, and carbonate were carried out using 6in1 Aquarium freshwater test strips (China). To ensure water quality, a 25% water change was performed daily using AquaCare aquarium water conditioner (Seachem, Philippines), ParaGuard Parasite control (Seachem, Philippines), and anti-chlorine (SeaQuest). Weekly testing of ammonia levels was also conducted with Ammonia aquarium freshwater test kits (China). Zebrafish was housed at a density of 4-10 adult fish per liter and fed twice daily with dry feeds dispensed by a food timer (Warmtone, China), supplemented by one feeding of decapsulated brine shrimp. Routine cleaning, including the cleaning of sponge filters, was conducted weekly on the fish tanks. (Avdesh *et al*., 2012; Evidente *et al*., 2021).

Procurement of Zebrafish

A total of 420 at 72hpf zebrafish larvae were used for the entire study. To facilitate the breeding, 2 males and 2 females adult wild type zebrafish (> 6 months old with 3-4 cm in length) were purchased from a local breeder since a 1:1 ratio of male and female will give the highest production of embryos around 200 - 300 fertilized eggs and lesser aggression in the tank (Ruhl *et al*., 2009). The zebrafish was further identified by Mr. Dave Valles of the University of San Carlos. The identification of male and female zebrafish will be based on specific characteristics, as illustrated in Figure 2. Upon acquisition, the differentiation between males and females were based on specific characteristics. Females were identified by their larger underbelly, while males were recognized for their slimmer and darker coloration compared to females. Subsequently, male and female zebrafish were separated and individually placed in containers.

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For transportation, the zebrafish were carefully placed inside a container within a box and transported using an air-conditioned delivery car. Upon acquisition, a thorough inspection of the zebrafish was conducted to identify potential signs of sickness, with a focus on behaviors such as decreased movement, lethargy, low food intake, and anxiety. Daily records were diligently maintained to monitor their food intake. Additionally, the zebrafish's anxiety behavior was observed as they initially dive to the bottom of a novel environment, followed by monitoring their exploration of the upper portion after six minutes, as indicated by Kirsten *et al*. (2018). The zebrafish were maintained according to standard protocols outlined by Alestrom *et al*. (2019) and Avdesh *et al*. (2012), with confirmation from the Institutional Animal Care and Use Committee (IACUC). During transportation, the protocol involved placing two adult zebrafish per 500 mL and maintaining a 1:1 ratio of air to water in the vessel. Additionally, adult zebrafish underwend a 24-hour period of fasting before transportation to reduce excretion and fouling in the transportation vessel.

Breeding and Embryo Isolation

The process of zebrafish breeding and isolation were conducted according to the protocol of outlined by the Zebrafish Information Network (ZFIN). Before spawning, a tank with a central divider housed a 1:2 ratio of male and female adult zebrafish. Separate breeding boxes, each holding less than 1 L of water, was arranged in a separate container filled with fresh tap water. In the evening, the zebrafish were introduced into the tank, and the following morning, when spawning occurs, fertilized eggs were collected on the enclosure floor. Zebrafish typically lay eggs within the first two hours after lights are turned on.

Post-spawning, the upper compartment, along with the adult fish, were transferred to a new tank or returned using a fishnet. The water containing settled eggs were siphoned and passed through a 0.10 mm strainer. The collected fertilized eggs were washed 3 times with egg water to remove debris. Subsequently, all fertilized eggs were transferred into each 1000 mL beakers filled with 100 mL egg water. The beakers were maintained at in a room temperature, providing an environment for the embryos to develop over a period of 72 hours. (Avdesh *et al*., 2012; Mason, 2016). Selection of embryos for analysis were based on symmetrical appearance in the one-cell stage or the presence of well-defined dark masses within a chorion, indicating fertility. Only embryos without morphological defects were chosen for further analysis.

Preliminary Mortality Test of A. viridis Extracts Against Zebrafish Larvae

Seventy zebrafish larvae at 72 hours post-fertilization were carefully distributed into a 96-well plate, with each well accommodating one larva. The larvae were subjected to 100 µL *A. viridis* leaf extracts at 25, 50, 100, 200, and 400 µg/mL concentration as a soaking drug, along with a vehicle control containing the highest concentration of DMSO used in the experimental treatments and a control group treated with egg water without any leaf extract. Each treatment concentration was replicated 10 times to ensure statistical reliability. The zebrafish larvae were left in a room temperature and were inspected after 24 hours. The concentration exhibiting the least number of dead larvae were recorded and then discarded. Death was judged by the coagulation of larvae, the absence of the heartbeat, or the lack of movement observed for 20 seconds. The extract associated with the zero-mortality rate or did not lead to any observable side effect under stereomicroscope was selected as the best extract for further investigation into its cardioprotective activity (Li *et al*., 2021).

Treatment Protocol for Assessing Cardioprotective Activity

A total of 240 zebrafish larvae (72 hpf) were used across 3 trials with 10 replicates per treatment. Each larva was placed in a well of a 96-well plate. The first treatment group received 100 µL of A. viridis extract at concentrations of 3.125, 6.25, 12.5, 25, and 50 µg/mL. The second group (positive control) received 100 µL of 200 µM eplerenone, the third group (negative control) received 100 µL of

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200 µM verapamil, and the fourth group was exposed to egg water. After 4 hours of treatment, all groups were exposed to 100 µL of 200 µM verapamil for 30 minutes to induce heart failure. (Kossack *et al*., 2017; Maciag *et al*., 2022).

Heart Rate Assessment

The heart rate of each zebrafish larva per treatment were assessed. The zebrafish larvae have easily observable hearts, which can be seen through a stereomicroscope. A 15-second video of each specimen were recorded using a camera attached to the stereomicroscope. These videos were played in slow motion to accurately count the number of heartbeats. The heart rate was calculated using the formula (Heideman *et al*., 2005; Hoage *et al*., 2012):

$$\text{heart rate} = \frac{\text{Number of heart beats}}{\text{15 seconds}} \text{ x } 4$$

Scoring on Cardiac Phenotypes

After the treatment, the zebrafish larvae were analyzed using a dissecting microscope. For imaging purposes, photographs were captured using a Nikon D750 camera. The acute heart failure (AHF) phenotypes were classified into 4 groups as shown in Figure 7. Category I end stage of AHF: heart with no contraction; Category II severe AHF: heart with contraction only in the atrium and no circulation; Category III mild AHF: heart with a distinct contraction in both cardiac chambers but morphological abnormalities, including edema, accumulated blood cells in front of the heart, stretched heart; Category IV normal heart. Larvae were tallied into the four AHF categories and the heart failure attenuation was determined by the percentage of each category (Haege *et al*., 2021; Hoyberghs *et al*., 2020; Maciag *et al*., 2022).

Disposal of Zebrafish Carcass

Disposal of the dead zebrafish followed the NIH guidelines (2013) by placing them into 1 part sodium hypochlorite and five parts water for 5 minutes. Then, the larvae were set into a tightly sealed bag and disposing of them in a yellow bin as clinical waste.

Qualitative Phytochemical Analysis

The qualitative phytochemicals analyses were involved in determining the primary group of bioactive compounds such as saponins, flavonoids, phenols, and alkaloids which are known to have cardioprotective properties. The most cardioprotective concentration were used for phytochemical analyses. All experiments were conducted in triplicate to ensure the reliability of results.

Statistical Analyses

The analysis began with descriptive statistics to summarize mortality rates at various concentrations. This involves calculating the mean mortality rate and standard deviation for each concentration of the verapamil and ethanolic leaf extract. Additionally, Kruskal-Wallis Test (Minitab 21.1.1) was applied to describe the phenotypic differences in zebrafish hearts, followed by pairwise comparison. To compare the means of cardiac function indicators between the control group and the group treated with the candidate concentration extract, one-way analysis of variance (ANOVA), followed by Dunnett's t-test will be utilized. A p-value of <0.05 were considered statistically significant.

Results and Discussion

In vivo maximum non-toxic concentration of the ethanolic leaf extracts of *A. viridis* against 72hpf zebrafish *A. viridis* leaf extract exhibited mortality and toxicity at concentrations starting from 50 µg/mL. As shown in Figure 7 and Table 4, concentrations ranging from 100 µg/mL to 400 µg/mL,

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100% mortality occurred with distinct morphological abnormalities such as hemorrhage, bent tails, pericardial edema, yolk sac edema, coagulation and a cloudy appearance. In contrast, all larvae exposed to 25 µg/mL remained alive, indicating this as the non-toxic concentration of *A. viridis* leaf extract.

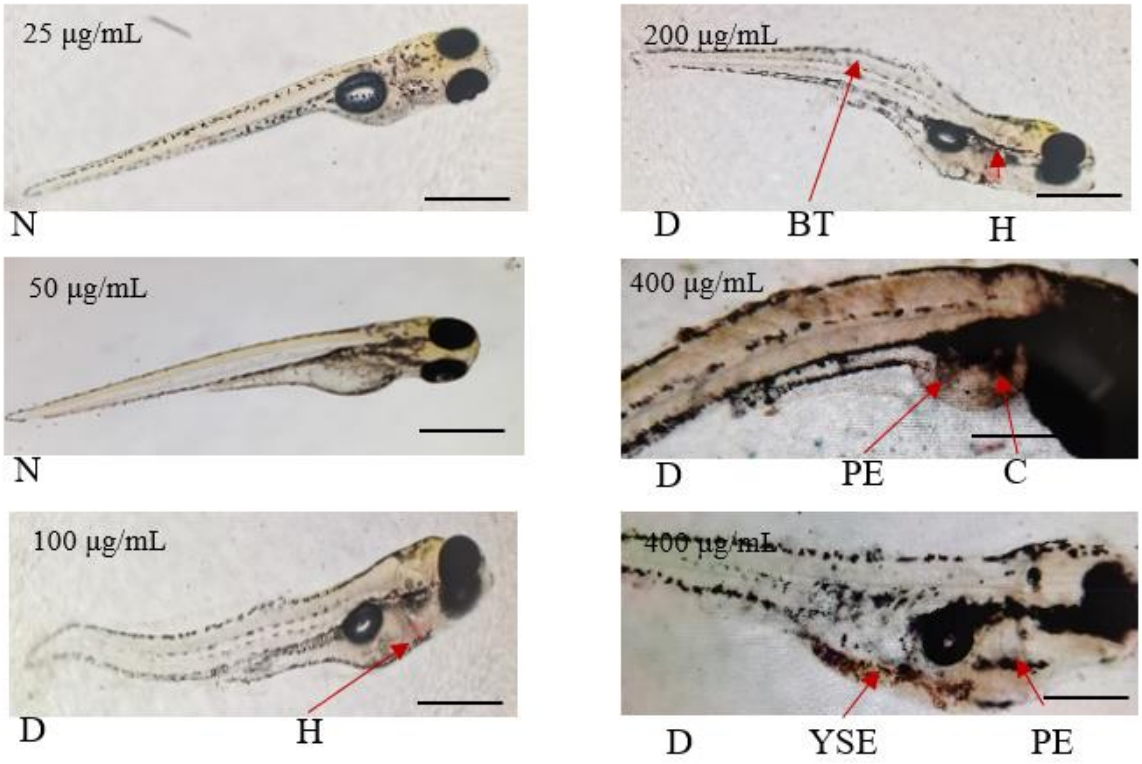

Figure 7. Representative visual images of zebrafish larvae at 72hpf treated with *A. viridis* ethanolic leaf extracts for survival and mortality. Images were captured at 400x magnification using stereomicroscope. N: Normal, H: Hemorrhage, BT: Bent Tail, PE: Pericardial Edema, C: Coagulation, YSE: Yolk Sack Edema, D: Dead. Scale bar: 3mm.

Table 2. Percentage mortality of zebrafish treated with *A. viridis* leaf extract.

Concentrations (µg/mL)	*A. viridis* (%)
0.5% DMSO	0
0	0
25	0
50	13.33%
100	100%
200	100%
400	100%

The results demonstrated a direct proportional relationship between the concentration of *A. viridis* extract and mortality rates among zebrafish larvae. Specifically, higher concentrations of the extract were associated with increased mortality, while lower concentrations resulted in fewer to zero mortality. This observation aligns with the findings of Zahir et al. (2021), who reported that higher concentrations of *Aervasan guinolenta*, *Amaranthus viridis*, and *Cynodon dactylon* reduced the survival rates of zebrafish embryos, particularly with prolonged exposure. *Aervasan guinolenta* and

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Amaranthus viridis, both belonging to the family Amaranthaceae, and *Cynodon dactylon* belonging to the family of Poaceae caused mortality in zebrafish embryos at various concentrations (100, 200, 300, 400, and 600 µg/mL). The study demonstrated that mortality rates increased with longer exposure durations. Prolonged exposure may result in the accumulation of bioactive compounds, which, at specific concentrations, can induce toxicity (Alafiatayo *et al*., 2019).

Similarly, Chen et al. (2018) observed toxicity in zebrafish embryos exposed to ethanol and aqueous extracts of *Sutherlandia frutescens* at concentrations of 20, 30, 50, 100, and 200 µg/mL. Chronic teratogenic effects, including pericardial edema, yolk sac swelling, and other developmental abnormalities, were documented.

Furthermore, these findings suggest that the compounds present in the leaf extract of *A. viridis* may interfere cellular processes within the heart of zebrafish, potentially inducing toxicity by affecting mechanisms such as cell proliferation, differentiation, and migration. For instance, the observed hemorrhages could be linked to damage to endothelial cells, which from the lining of blood vessels (Rajendran *et al*., 2013). Pericardial edema may arise from increased vascular permeability or impaired cardiac function (Wiegand *et al*., 2023). The bent tail phenotype observed suggests additional neurotoxic effects, as the development of the spinal cord and nervous system is closely linked to heart formation (Zhao *et al*., 2024). Coagulation abnormalities could result from direct damage to blood cells or interference with the coagulation cascade (Kretz *et al*., 2015). Yolk sac edema likely reflects impaired fluid balance and potential to the circulatory system (Sant *et al*., 2018).

Heart Rate and Cardiac Phenotype Response of Zebrafish larvae Protected by Amaranthus viridis Leaf Ethanolic Extracts Against Verapamil-Induced Heart Failure

As shown in Figure 8, the heart rate of zebrafish larvae in egg water (control) was within the normal range of 120–180 bpm (De Luca *et al*., 2014), recorded at 151.57 ± 3.86 bpm. Verapamil treatment significantly reduced the heart rate to 71.60 ± 6.74 bpm, confirming heart failure (Li *et al*., 2024). However, eplerenone provided protection from heart damage, resulting in a moderate heart rate recovery to 126.37 ± 3.65 bpm. The cardioprotective effect of *A. viridis* leaf extract was evident across all tested concentrations, from 50 µg/mL to 3.125 µg/mL, with heart rates of 111.47 ± 6.85 bpm, 118.20 ± 5.37 bpm, 121.20 ± 2.75 bpm, 120.30 ± 6.09 bpm, and 117.60 ± 6.16 bpm, respectively. These values indicate an improvement in heart rate, suggesting cardioprotective properties at each of these concentrations. However, the heart rate at 50 µg/mL was not within the normal range, as mortality began at this concentration. The study initiated testing at 50 µg/mL to identify the lowest effective concentration, using it as a baseline for subsequent evaluations. The results suggest that concentrations below 50 µg/mL not only improved heart rate but also minimized mortality, highlighting the efficacy of lower doses of the extract.

Identifying lower effective doses is critical in drug discovery and development research as it ensures both efficacy and safety. Lower concentrations reduce the risk of adverse effects, minimize toxicity, and are often more sustainable for large-scale production. Additionally, determining the minimum effective dose allows for the optimization of therapeutic regimens, improving patient outcomes while reducing the likelihood of drug resistance or cumulative toxicity. This approach is particularly important when developing natural product-based therapies, where the balance between bioactivity and safety is essential for clinical applicability (Mohs & Greig, 2017).

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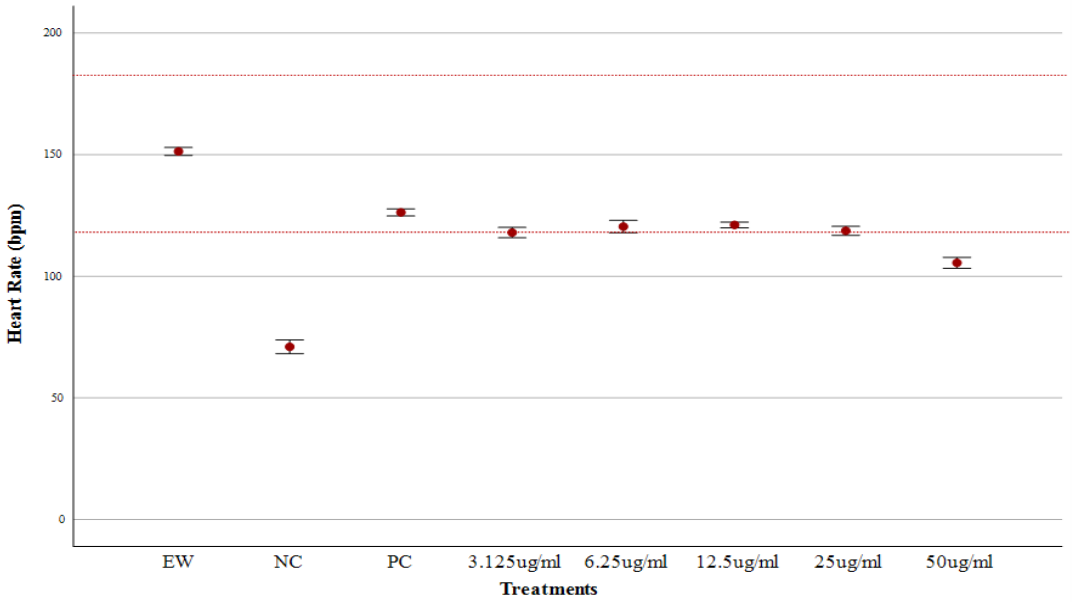

Figure 8. Heart rate response of 72 hpf zebrafish treated with *A. viridis* leaf extract in verapamil-induced heart failure, expressed as mean ± SD. Means without 'A' differ significantly from the control ($p < 0.001$). Means without 'a' differ significantly from the EW ($p < 0.001$). '---' indicates the normal heart rate range of 120–180 bpm.

For the cardiac phenotypes, the verapamil-induced zebrafish larvae (negative control) had lower scores between 1 and 2, indicating heart failure in this group (Li *et al*., 2024). In contrast, the eplerenone-treated larvae (positive control) scored higher, between 3 and 4, suggesting protection from verapamil-induced damage. As shown in Figure 10, which highlights the observed cardiac phenotypes in zebrafish across various treatments, Table 3, which summarizes the cardiac functions and phenotypes, and in Figure 11, which highlights the cardiac scores of zebrafish, *A. viridis* demonstrated promising results, consistently scoring in the higher range across all tested concentrations (3.125, 6.25, 12.5, 25, and 50 µg/mL). Some larvae showed lower scores in the extract treatments, indicating individual variations in tolerance (Meyer *et al*., 2013). The Kruskal-Wallis test and pairwise comparisons revealed a highly significant difference between the negative control and the treatments ($p < 0.001$). The positive control did not differ significantly from any treatments. These results suggest that the treatments provide cardiac protection for the larvae against verapamil-induced heart failure.

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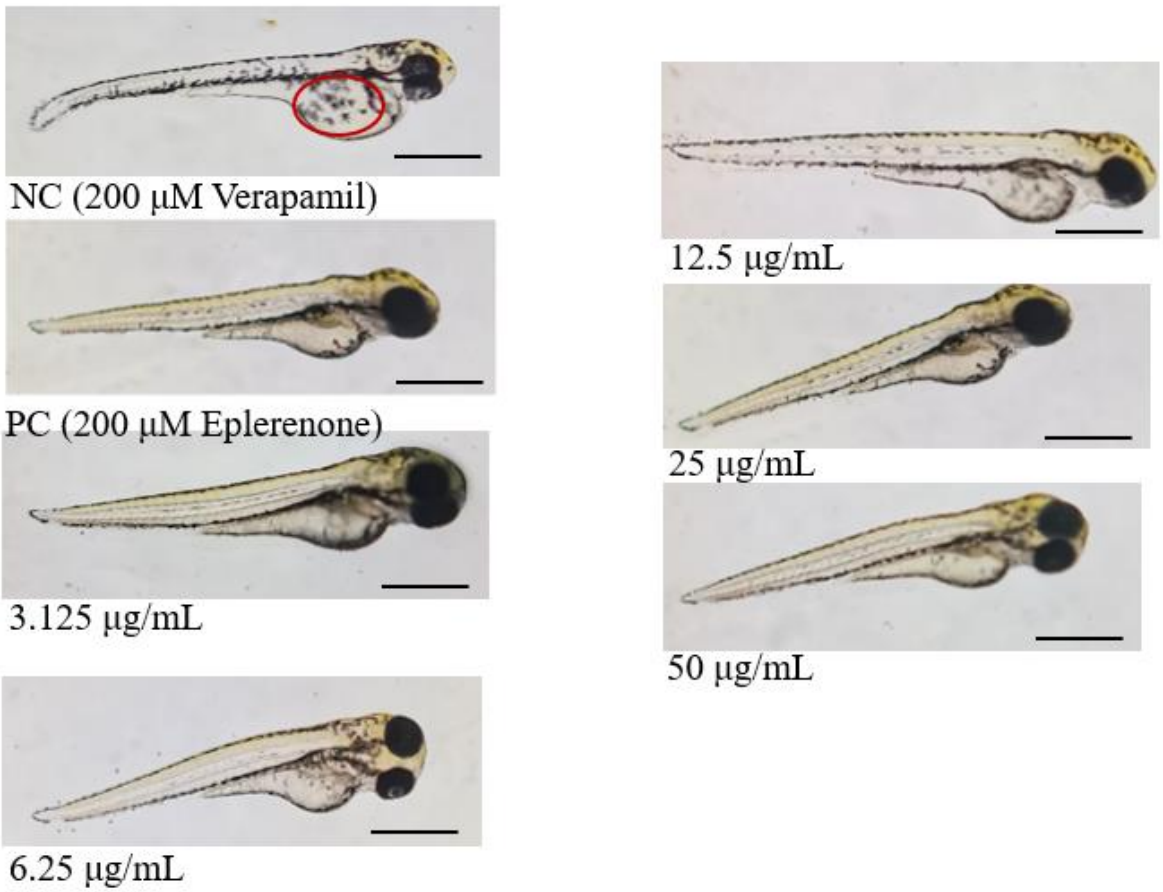

Figure 9. Representative images of 72 hpf zebrafish larve treated with various concentrations of *A. viridis* leaf extract for cardioprotective response, captured at 400x magnification using a stereomicroscope. **---** indicates pericardial edema. Scale bar: 3mm.

Table 3. Cardiac function and phenotypes of 72hpf zebrafish in different treatments.

Treatments	Cardiac functions and phenotypes
Egg water	Normal function and no visible abnormality
NC – Verapamil	No contraction in the heart; Weak contraction in the atria; No circulation; Pericardial edema
PC – Eplerenone + Verapamil	Some showed slow circulation, while most had no visible abnormalities
3.125 µg/mL *A. viridis* + Verapamil	Some showed slow circulation, while most had no visible abnormalities
6.25 µg/mL *A. viridis* + Verapamil	Some showed slow circulation, while most had no visible abnormalities
12.5 µg/mL *A. viridis* + Verapamil	Some showed slow circulation, while most had no visible abnormalities
25 µg/mL *A. viridis* + Verapamil	Some showed slow circulation, while most had no visible abnormalities
50 µg/mL *A. viridis* + Verapamil	No contraction in the heart; Weak contraction in the atria; No circulation;

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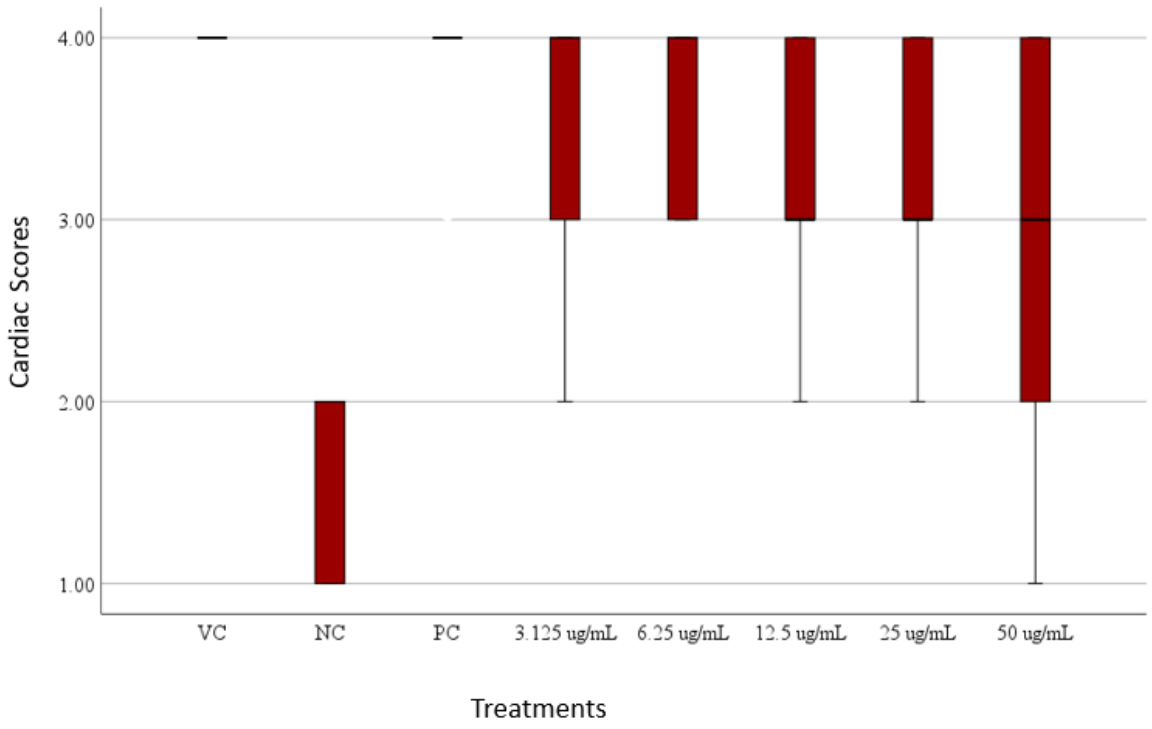

Figure 10. Cardiac scores of 72hpf zebrafish treated with *A. viridis* leaf extract in verapamil-induced heart failure model. Kruskal-Wallis test, followed by pairwise comparison with Dunn Bonferroni corrections were used in cardiac scoring for the cardiac phenotypes of the larvae. VC – Vehicle Control (0.5% DMSO), NC – Negative Control (Verapamil), PC – Positive Control (Eplerenone). n = 80 larvae per treatment, divided into three independent experiments.

Verapamil is a calcium channel blocker used in humans to manage hypertension and certain heart conditions. In zebrafish larvae, verapamil induces heart damage by disrupting calcium ion influx into cardiac cells. Calcium is crucial for maintaining normal heart contractions and overall cardiac health, so this disruption impairs cardiac function and causes structural damage (Horng *et al*., 2024). The damage is further increased by oxidative stress, the downregulation of cardiomyocyte biomarkers, and the obstruction of protein synthesis (Chatterjee *et al*., 2010; Abbate, 2021). Prior studies indicated that the verapamil (200 μM)-induced larval zebrafish heart failure model is a useful tool for whole animal heart failure studies and for screening safe agents of heart failure. Zebrafish treated with 200uM verapamil exhibited obvious heart failure phenotypes and weakened cardiac function. These data indicated that the heart failure zebrafish model was successfully established and can be utilized as an alternative, novel and simple experimental animal model of heart failure (Li *et al*., 2022; Li *et al*., 2023; Narumanchi *et al*., 2021; Zhu *et al*., 2018).

Cardiac damage or abnormalities are directly linked to deviations in heart rate, as the heart's rhythm and rate are critical indicators of its functional integrity. An elevated heart rate, often indicative of stress or pathological conditions, can lead to increased myocardial workload and oxygen demand, potentially causing structural and functional damage over time. Conversely, bradycardia, or abnormally slow heart rate, can impair adequate blood flow, resulting in tissue hypoxia and systemic complications (Fatisson *et al*., 2016; Sessa *et al*., 2018). In zebrafish models, alterations in heart rate often reflect underlying cardiac phenotypes, such as edema, chamber malformations, or disrupted contractility, which are common indicators of cardiotoxicity or cardiac dysfunction (Wang *et al*., 2017).

Sedighi et al. (2019) demonstrated that different concentrations of *Melissa officinalis* L. (25, 50, and 100 mg/kg) significantly improved ischemia/reperfusion-induced arrhythmia and heart injury in an in vitro rat model. The treatment, administered for five days following reperfusion, effectively stabilized heart rate and mitigated cardiac damage. Similarly, Zhou *et al*. (2020) found that 100 μg/mL

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of timosaponin B-II extracted from *Anemarrhena* improved heart function in rats due to its anti-inflammatory properties. Additionally, Chen *et al*. (2021) reported that 5 μg/mL of *Gardenia jasminoides* extract reduced inflammation in a zebrafish embryo model. Likewise, Rafacho et al. (2017) demonstrated that a 0.02% concentration of *Rosmarinus officinalis* L. effectively mitigated cardiac damage in male Wistar rats. The study revealed that this concentration improved cardiac metabolism and significantly reduced oxidative stress. Furthermore, it enhanced diastolic function and decreased cardiac hypertrophy following myocardial infarction.

These studies highlight the potential of natural plant extracts and their active compounds to protect the heart by reducing inflammation, minimizing oxidative stress, and improving overall heart function. Their ability to prevent cardiac damage offers promising insights for developing effective treatments for heart-related conditions.

Based on the results of this study, cardiac analysis revealed significant improvements in both heart rate and cardiac phenotypes, in all tested concentrations of *A. viridis* leaf ethanolic extract.

Phytochemical Composition of A. viridis Crude Leaf Extracts

For the phytochemical screening, qualitative tests were conducted to detect the presence of alkaloids, flavonoids, phenols, and saponins in *A. viridis*. As shown in Table 4, the results revealed that *A. viridis* contains alkaloids, flavonoids, and saponins, while no phenols were detected.

Table 4. Qualitative phytochemical composition of *A. viridis*. ('+' indicates presence and '-' indicates absence).

A. viridis	
Alkaloids	+
Flavonoids	+
Phenols	-
Saponins	+

A. viridis was evaluated for its cardioprotective potential due to its bioactive compounds, which contribute to its medicinal properties. It is important to note that the phytochemical composition of *A. viridis* can vary due to environmental factors such as climate, seasonal changes, soil conditions, and geographical location (Kumar, 2017).

Phytochemical analysis is essential for identifying the specific bioactive compounds responsible for the observed cardioprotective effects. In this study, alkaloids, flavonoids, and saponins were detected in the *A. viridis* leaf ethanolic extracts, which align with findings from Fouad *et al*. (2024) who reported the presence of alkaloids, flavonoids, phenols, and saponins in *A. viridis* extracts and highlighted their role in various therapeutic activities. Furthermore, Kumari *et al*., (2018) confirmed the presence of various phytochemicals in *A. viridis* leaf extract across different solvents, including aqueous, methanol, chloroform, and hexane. These phytochemicals included flavonoids, alkaloids, phenolics, steroids, terpenoids, saponins, cardiac glycosides, and tannins.

The cardioprotective activity of *A. viridis* leaf extracts were notably linked to its alkaloid, flavonoid, and saponin content. Alkaloids are bioactive compounds known for their cardiovascular benefits, including the regulation of heart rate and promotion of vasodilation, which help maintain cardiac stability and reduce the risk of heart dysfunction. Flavonoids, on the other hand, are well-documented for their antioxidant properties, which play a critical role in neutralizing reactive oxygen species (ROS), preserving cardiac cell integrity, and reducing inflammation within the heart. Additionally, flavonoids enhance endothelial function, promoting vascular relaxation and lowering blood pressure (Ullah *et al*., 2020). This mechanism aligns with the findings of Kumar et al. (2013), who demonstrated that flavonoids from *A. viridis* significantly reduced oxidative stress and inflammation in rat models, thereby mitigating cardiotoxicity.

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Saponins, another key component of *A. viridis* leaf extract, further support cardioprotection through their anti-inflammatory and antihypertensive properties. They modulate immune responses and suppress the production of pro-inflammatory cytokines, alleviating inflammation in cardiovascular tissues. Saponins also enhance vascular function by reducing blood vessel constriction, thereby aiding in blood pressure regulation. Saravanan et al. (2013) observed that saponins from *A. viridis* mitigated inflammation and improved cardiac function in isoproterenol (ISO)-induced heart failure in rats. The combined effects of alkaloids, flavonoids, and saponins underscore the potential of *A. viridis* as a natural cardioprotective agent.

The mechanisms underlying these effects involve several biochemical processes. Alkaloids play a vital role by promoting vasodilation and regulating heart rate, which contribute to cardiovascular stability. Flavonoids reduce oxidative stress by scavenging reactive oxygen species (ROS) and inhibiting inflammatory pathways, thereby maintaining cardiovascular health. Saponins further enhance this protective effect by reducing inflammation and improving vascular health (Li et al., 2020). Together, alkaloids, flavonoids, and saponins synergistically support heart function and protection.

Supporting these observations, Krishna *et al*. (2023) reported that alkaloids, flavonoids, and saponins from *A. viridis* alleviated verapamil-induced cardiotoxicity in rat models through their antioxidant and anti-inflammatory actions.

In Vitro Free Radical Scavenging Activity of A. viridis Ethanolic Leaf Extracts

The DPPH radical scavenging activity of *A. viridis* ethanolic leaf extracts at various concentrations is presented in Figure 11. The extract exhibited the highest scavenging activity at 50 µg/mL, achieving an inhibition rate of 45.06% ± 10.90%. A concentration-dependent decline in scavenging activity was observed at lower concentrations, with inhibition rates of 34.95% ± 4.16% at 25 µg/mL, 24.36% ± 2.69% at 12.5 µg/mL, 9.92% ± 4.31% at 6.25 µg/mL, and 6.04% ± 4.66% at 3.125 µg/mL, respectively.

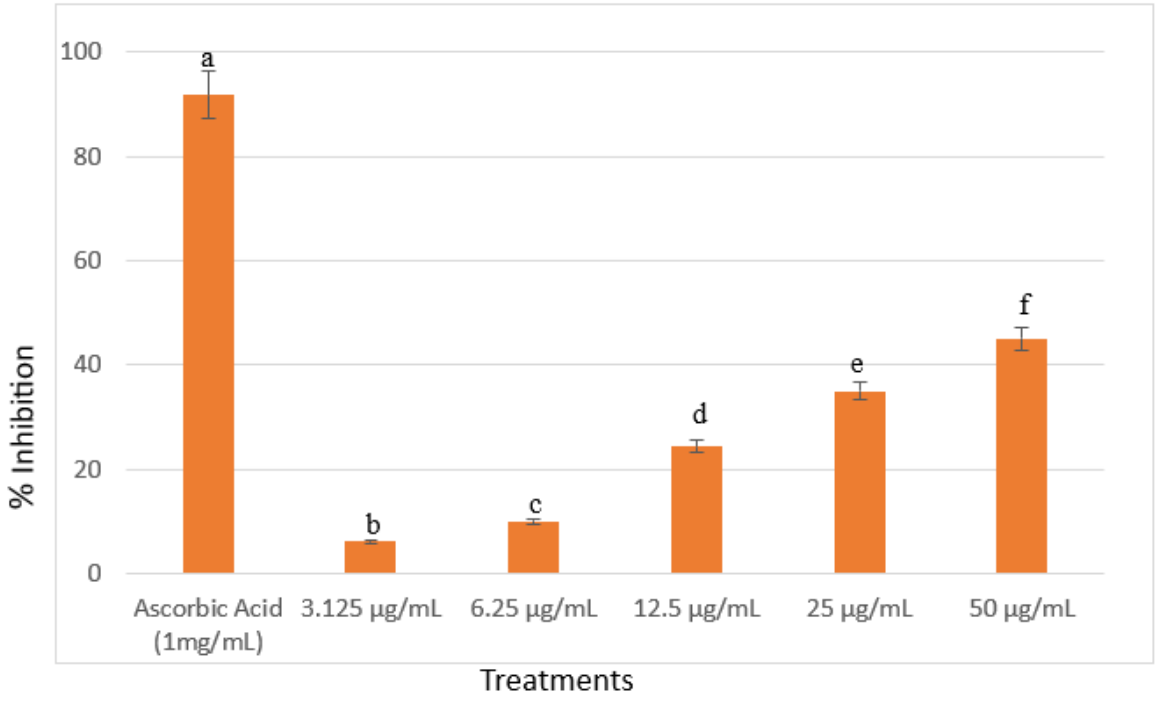

Figure 11. Percentage inhibition of DPPH radical scavenging activity of various concentrations. Data were expressed as mean ± standard deviation. Means with different letters indicate a significant difference, $p = <0.001$.

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This suggests that as the concentration of *A. viridis* decreases, the free radical scavenging activity also diminishes, indicating that higher concentrations of the extract possess greater free radical scavenging potential. This trend underscores the importance of bioactive compounds, such as alkaloids, flavonoids, and saponins, in neutralizing reactive oxygen species (ROS) and protecting cells from oxidative damage (Soni & Sosa, 2013). The observed reduction in antioxidant activity at lower concentrations highlights the importance of determining optimal dosages for therapeutic applications to maximize the efficacy of *A. viridis* in combating oxidative stress.

The results demonstrated significant free radical scavenging activity of *A. viridis* ethanolic leaf extract in all concentrations tested, which contributes to its cardioprotective properties. This free radical scavenging potential plays a crucial role in preventing oxidative stress-induced cardiac damage and supports the traditional use of *A. viridis* in treating cardiovascular diseases (Valaei *et al*., 2021).

The antioxidant property of *A. viridis* can be primarily attributed to its phytochemical composition such as alkaloids, flavonoids, and saponins. These compounds are known for their ability to chelate metal ions and inhibit lipid peroxidation, which are crucial mechanisms in preventing oxidative stress-induced cardiac damage. The presence of these bioactive compounds supports the extract's observed cardioprotective effects in verapamil-induced heart failure (Kumari *et al*., 2018).

Furthermore, the ability of *A. viridis* leaf extract to scavenge free radicals correlates with its protective effects against verapamil-induced heart failure in zebrafish. This correlation is supported by the study of Nowak *et al*., (2018), who demonstrated that natural antioxidants could effectively protect cardiac damage from oxidative stress.

The observed free radical scavenging activity supports the traditional use of *A. viridis* in managing various cardiovascular conditions. As highlighted by Lalhminghlui and Jagetia (2023), natural antioxidants from plant sources often provide additional benefits beyond their primary antioxidant activity, including anti-inflammatory and membrane-stabilizing properties, which enhance their overall therapeutic efficacy.

The crude preparation of the extract likely contributed to its strong free radical scavenging activity, with 50 µg/mL demonstrating the highest inhibition rate of 45.06% ± 10.90%. However, the presence of other bioactive compounds in the crude extract may also explain its observed toxicity to zebrafish larvae at higher concentrations. While 50 µg/mL emerged as the most effective concentration for antioxidant activity, the potential toxicity associated with the crude extract highlights the importance of isolating and identifying specific bioactive compounds to optimize therapeutic applications while minimizing adverse effects (Cordero-Maldonado *et al*., 2013; Mohamad-Shariff *et al*., 2020).

Summary, Conclusion, and Recommendation

Cardiovascular disease (CVD) remains one of the most pressing health challenges worldwide, requiring immediate and effective management. With the incidence of CVDs continuing to rise, there is an increasing need for alternative treatments to complement existing therapies. Natural products, which are abundant in bioactive compounds, offer promising potential in addressing this issue. These bioactive compounds, derived from plants and other natural sources, have shown potential for therapeutic use, making them a valuable resource in the search for new, effective treatments for cardiovascular conditions.

The 72-hpf zebrafish larvae were able to tolerate concentration of up to 25 µg/mL of *A. viridis* ethanolic leaf extract, indicating that their tolerance to the extract is dose-dependent. All tested concentrations exhibited cardioprotective activity, as shown through heart rate and cardiac phenotype analyses in zebrafish larvae exposed to verapamil-induced heart damage.

Additional improvements for future research are advised to enrich the pharmacological application of *A. viridis* on a verapamil-induced zebrafish research model. It is encouraged to isolate and identify the specific cardioprotective compounds of the whole plant extracts. Since only the end-

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stage (72 hpf) is applied in both survival and physiomorphological screening, future studies can increase the time frame and include observations during 96, 120, and 144 hpf. It is also recommended to incorporate blood flow in addition to the heart rate of the treated zebrafish larvae. Histopathology analysis of the heart s advised to provide a deeper understanding of tissue-level effects. Lastly, quantitative analysis on the levels of Reactive Oxygen Species (ROS) could also be performed to measure the oxidative stress and inflammatory pathways.

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