

Investigating the Effects of *Echium amoenum* on Nkx2-5, ApoE, and Tbx5 Gene Expression in Zebrafish Cardiovascular Embryo

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Abstract

The increasing prevalence of bacteria that resist antibiotics has had a significant influence on the way we treat urinary tract infections (UTIs). This problem has increased Cardiovascular diseases (CVDs) remain the leading cause of death globally and on average take 30% of all deaths around the world. Such alarming statistics mandate that an effort be made to find appropriate preventive and therapeutic strategies. The present study characterized the effects of *Echium amoenum* extract, a traditional plant with known antioxidant properties, in developing the cardiovascular system and investigated these effects on expression levels of Nkx2-5, ApoE, and Tbx5 genes responsible for heart development using zebrafish as an animal model. In the present work, dry *Echium amoenum* extract was obtained using an alcoholic extraction procedure, and different concentrations of this mixture were used to treat zebrafish embryos at 24 hpf (hours post-fertilization), 48 hpf, as well as 72 hpf stages. Morphological assessments revealed that concentrations above 62.5 µg/mL led to significant abnormalities and increased mortality rates, with an LD50 determined at 101 µg/mL.

Gene expression analysis indicated that the lower concentrations of the extract significantly stimulated Nkx2-5, ApoE, and Tbx5 gene expressions as candidates involved in cardiac differentiation and lipid metabolism. In contrast, expression levels of these genes were reduced at higher concentrations suggesting a dose-dependent effect. The LD50 concentration was not associated with any observable heart toxicity, and histological evaluation revealed no signs of injury in exposed hearts when compared to controls; cellular architecture remained intact and all standard features were present throughout the subendocardial region (comprised mainly by cardiomyocytes, or endocardium/substrate) except for some cells demonstrating vacuolization.

These findings highlight the therapeutic efficacy of *Echium amoenum* as a natural antioxidant and its cardio-protective effects. This study emphasizes the requirement of mechanistic studies supporting its effects and the development of safe dose for future clinical trials in cardiovascular diseases control.

1. Introduction

Cardiovascular diseases (CVDs) are the foremost reason of mortality worldwide. In 2008, approximately 17.3 million people died from CVDs, accounting for 30% of total global

deaths. It is estimated that by 2030, there will be 23.3 million deaths resulting from CVDs. Physical inactivity, high blood pressure, obesity, and unhealthy diets are recognized as major contributing factors to the development of cardiovascular diseases (1).

Previous studies have shown that antioxidants can enhance endothelial dysfunction in various environments such as atherosclerosis, hypertension, diabetes, and dyslipidemia (2). Antioxidants play a crucial role in inhibiting atherosclerosis by reducing LDL oxidation (3). Moreover, plant-derived antioxidants, including polyphenols, have successfully prevented apoptosis of endothelial cells after exposure to oxidative agents like hydrogen peroxide (4). Various studies have demonstrated the role of polyphenols in improving endothelium-dependent vasodilatory responses and modulating endothelial-leukocyte interactions. Furthermore, they have been shown to balance pro-thrombotic and anti-thrombotic properties (5).

Herbal medicine, with cultural and traditional sources that have developed over the history of many developing countries, continues prevalent today. It is estimated that 80% of the population in some Asian and African countries currently relies on herbal remedies as the primary basis for their healthcare needs. In Africa, up to 90% and in India, 70% of the population depend on traditional medicine (6). Newly, due to evidence of better patient tolerance and a holistic treatment approach, herbal medicine has gained renewed consideration in the Western world. Unlike synthetic drugs, herbal remedies have been consumed by humans for centuries, and their safety has been recognized over time. However, assessing the toxicology of herbal medicine to identify adverse effects is crucial for protecting human health (7). Toxicity assessment during clinical and preclinical drug discovery facilitates the identification of toxic substances that can be rejected or modified into safer alternatives (8). Herbal medicine is an extraordinary tapestry of traditional wisdom and modern scientific inquiry. As research continues to unfold a variety of plants for their therapeutic applications, there is great scope for integrating these remedies within the modern practice of healthcare. Bridging the gap between traditional uses and modern evidence, herbal medicine can be of vital importance in promoting health and well-being among diverse populations (9).

Echium amoenum is from the Boraginaceae family (10), observed in northern Iran and Mediterranean regions of Europe. This plant characteristics bright blue, star-shaped flowers and is recognized as a traditional solution, possessing antioxidant, analgesic, antibacterial, anxiolytic, antidepressant, and

immune-modulating properties. It is a strong source of anthocyanins, including cyanidin and delphinidin (11). In addition to its antioxidant and anti-inflammatory properties, beneficial cardiovascular effects such as anti-atherosclerotic and anti-hypertensive effects have been recognized in anthocyanins (12). Animal models, such as zebrafish, are utilized to investigate the effect of *Echium amoenum* on the development of the cardiovascular system.

Zebrafish (*Danio rerio*) is a tropical freshwater fish appropriate to the Cyprinidae family. Over the past 30 years, it has become an extremely popular model organism for studies on embryonic development and human diseases. This species was chosen due to its small size (approximately 3-5 cm), transparency during development, and high fertility. These characteristics were first discovered by George Streisinger, the founding father of zebrafish research (13).

The heart of an adult zebrafish grows to a size of about 1 ml or bigger in ventricular length. Its atrium is internally covered with pectinate muscle, while the ventricle consists of a compact layer that encompasses internal trabeculae, which, unlike in mammalian hearts, remain throughout adulthood (14).

The development of the zebrafish heart progresses through stages very similar to those of amniotes. The specification of precardiac cells occurs in the mesoderm of the anterior lateral plate, followed by the fusion of bilateral heart fields at the embryonic midline. The mechanism of this seemingly simple morphogenetic event in the early stages of embryonic development is better understood due to the powerful genetic capabilities and high-resolution imaging of zebrafish embryos. Transcription factors involved in cardiac growth have been identified through genetic studies using various animal model systems (15). Members of the *NKX*, *GATA*, and *T-box* families form the main regulatory network responsible for normal cardiogenesis and are implicated in congenital heart disease (CHD).

systems (15). Members of the *NKX*, *GATA*, and T-box families form the main regulatory network responsible for normal cardiogenesis and are implicated in congenital heart disease (CHD).

The T-box family consists of valuable transcription factors involved in cardiac development. *TBX5* and *TBX1* are implicated in the etiology of Holt-Oram syndrome and 22q11.2 deletion syndrome, respectively (16). In addition to their association with syndromic congenital heart disease (CHD), mutations in *TBX5* and *TBX1* have also been identified in non-syndromic CHD, such as tetralogy of Fallot (TOF) and septal defects (17). An alternative member of the family, *TBX20*, has subsequently been related to non-syndromic CHD, including septal defects, mitral valve stenosis, dilated cardiomyopathy, and TOF (18).

Nkx2.5 is a homeodomain transcription factor and one of the primary markers of cardiac lineage. Genetic studies in *Drosophila*, zebrafish, and mice have demonstrated the distinct functions of *Nkx2.5* during early heart development. *Nkx2.5* activity is essential for cardiac specification in flies, and the *tinman/nkx2.5* mutant completely lacks dorsal vessels (19,20). In contrast, a heart tube typically forms in zebrafish and mouse embryos lacking *nkx2.5*, however, the subsequent differentiation and maturation of their chambers are disrupted (21,22).

Disruption of both *Nkx2.5* and *Nkx2.7*, the zebrafish analogs of mammalian *Nkx2.5*, results in the formation of an abnormal heart tube. This defect is marked by a hyperplastic and enlarged atrial chamber alongside a reduced ventricular chamber containing fewer cardiomyocytes (22). The restoration of *Nkx2.5* expression using a heat shock-induced transgene during cardiomyocyte differentiation restores chamber-specific cardiomyocytes in *nkx2.5* mutant embryos, suggesting that early *Nkx2.5* expression may initiate cardiomyocyte identity for their respective chambers (23).

Moreover, transient overexpression of *Nkx2.5* in *nkx2.5* mutants during embryonic stages allows for normal heart function in adulthood, representative that, different the consistent requirement for

nkx2.5 in adult mouse cardiomyocytes, *Nkx2.5* is replaceable for the cardiac function of adult zebrafish. However, given the further roles of *Nkx2.5* and *Nkx2.7* during early cardiac development, further studies are needed to fully determine the tasks of *nkx* genes in the adult zebrafish heart (23).

The *APOE* gene is located on chromosome 19 and exists in several allelic forms, with three major isoforms: *APOE2*, *APOE3*, and *APOE4*. Each isoform has different affinities for lipoprotein receptors and varying impacts on lipid metabolism and cardiovascular health. Role in Cardiovascular Development (24).

Throughout embryonic development, appropriate lipid metabolism is critical for the construction of cell membranes and the synthesis of signaling molecules. *APOE* plays a main role in providing lipids to developing tissues, containing the heart and blood vessels (25).

APOE is implicated in the differentiation of cardiomyocytes (heart muscle cells) and the development of cardiac organization. It supports the formation of the cardiac outflow tract and the maturation of the heart during embryogenesis (26).

The protein aids in the remodeling of blood vessels and the development of the vascular system. By regulating lipid levels in the embryonic environment, *APOE* controls endothelial cell function, which is important for angiogenesis (27).

This paper, therefore, seeks to explore, through induction with *Echium amoenum* extract, the expression of *Nkx2-5*, *ApoE*, and *Tbx5* genes in the cardiovascular embryonic development of zebrafish. Expression levels of the identified genes will be quantified in the embryos exposed to the

the extract, providing the research with valuable insights into the potential use of *Echium amoenum* for the enhancement of cardiovascular development and functionality.

2 Materials and Methods

2.1 Extraction by Alcoholic Method

The plant sample of *Echium amoenum* was obtained from an herbal shop in Tehran. This sample was then registered with an herbarium code at the Herbarium of the School of Pharmaceutical Sciences at Islamic Azad University in Tehran (Voucher NO: 347_PMP/A). After obtaining the sample, the plant was dried in an oven at 45 degrees Celsius for 48 hours. Once dried, the leaves were ground into 2-millimeter particles using a cyclone mill. The resulting plant powder was stored at -15 degrees Celsius until the extraction of the extract. 20 g mass and packed down in Buchner funnel. A solution of 500 ml made up by 70% alcohol to the volume. Next, the crude substance was just allowed diffuse out of Funnel into which solution collected. It was done three times for better extraction yield.

2.2 Care and Maintenance of Zebrafish Embryos

The maintenance of zebrafish was conducted according to the guidelines provided in the zebrafish reference book.(6). Wild-type adult zebrafish (over 6 months old) were purchased from a local supplier and were acclimated to dechlorinated tap water for a minimum of 4 weeks. Initial spawning of the adult zebrafish occurred in a 200-liter aquarium equipped with a continuous water flow system, maintaining a maximum density of 1 gram of fish per liter of dechlorinated tap water at a temperature of 26 ± 1 degrees Celsius. The light cycle was set to a constant 14:10 hours of light and darkness, with a pH range of 6.8-7.2. The zebrafish were fed in the morning with Artemia and Fluval.

2.3 Preparation of Embryos from Adult Zebrafish

Wild-type zebrafish (over 6 months old) with high potential for producing fertilized eggs were selected for spawning. Male and female zebrafish were kept separately in aquariums with a volume of 1 liter of water per fish, under a light cycle of 10 hours of light and 14 hours of darkness. During the

spawning period, no additional water filtration or feeding was performed, and water quality was regularly monitored. The breeding method [87] was adopted before toxicity testing on the embryos. The ratio of spawning between the groups was 2:3. Egg collection and mating occurred at 8:30 AM under room lighting. Subsequently, the parent fish were returned to their tanks, and the fertilized embryos were selected, washed three times in embryonic medium (E3M), and placed at 28 degrees Celsius for 6 hours of growth.

2.4 Treatment of Zebrafish Embryos with *Echium amoenum*

Preparation and Dilution of *Echium amoenum* Extracts

The concentration of the *Echium amoenum* extract was prepared separately in plate wells to investigate its toxic effects on zebrafish embryos. For this, 50 microliters of the stock extract were diluted with 49,950 microliters of embryonic medium (E3M) to achieve a concentration of 125 micrograms in 0.1% DMSO. This dilution was then repeated with a two-fold serial dilution across the wells to obtain concentrations of 125, 62.5, 31.25, 15.63, and 7.8 micrograms per milliliter of E3M containing 0.1% DMSO. DMSO is the most commonly used solvent for delivering extracts in zebrafish-based assays, and it has been reported that a concentration of 2.5% is tolerable for the embryos and larvae of this fish.

2.5 Testing the Effectiveness of *Echium amoenum* on Embryos

The placement of zebrafish embryos in a 24-well plate was conducted according to the method outlined in [OECD] Test No. 236 in 2006 [88]. After 6 hours post-fertilization (6 hpf), healthy embryos were selected, washed, and examined under a microscope. Fertilized embryos were then chosen for further testing. Twelve fertilized embryos ($n=12$) at 6 hpf were treated with different concentrations of *Echium amoenum* extract. The experiment was conducted in three independent replicates in a 24-well plate, with each well containing 2 milliliters of embryonic medium with 0.1% DMSO and 125 micrograms of *Echium amoenum* extract. Different extract concentrations were prepared through successive two-fold dilutions. The control group was exposed to 5 milliliters of E3M containing 0.1%

-well plate, with each well containing 2 milliliters of embryonic medium with 0.1% DMSO and 125 micrograms of Echium amoenum extract. Different extract concentrations were prepared through successive two-fold dilutions. The control group was exposed to 5 milliliters of E3M containing 0.1% DMSO. After 5 days of exposure, growth endpoints such as egg coagulation, embryo mortality, somite formation, tail separation, otolith development, eye formation, and skeletal anomalies were recorded. Larvae and embryos were examined under an inverted microscope, and digital images of the morphological anomalies at each concentration were captured.

2.6 Histological Examination

Initially, the fish were anesthetized using clove oil to facilitate the easy removal of the heart. The hearts were isolated from both the control (untreated) fish and the treated fish. The heart tissues were fixed in 4% paraformaldehyde for 24 to 48 hours to preserve cellular structure. Subsequently, the fixed tissues were dehydrated with 80% ethanol. In the next step, the tissues were cleared with xylene and embedded in paraffin to allow for the preparation of thin sections. Thin sections (5 micrometers) of the paraffin-embedded tissues were then placed on microscope slides. The sections were stained with hematoxylin and eosin (H&E) to visualize the structure of the cells and tissues. Finally, the stained sections were examined under a light microscope to assess morphological changes in the heart tissue, such as muscle fibers, blood vessels, and signs of inflammation or damage.

2.7 Genes expression evaluation

Initially heart tissue is collected and minced on sterile foil using a scalpel, then transferred to a 1.5 mL tube. Next, 1 mL of TRIzol is added to the minced tissue, which is then inverted several times and vortexed 2-3 times until a homogeneous mixture is achieved. Following this, 200 μ L of chloroform is added, and the mixture is inverted 2-3 times before centrifuging at 12,000 g for 15 minutes at 4°C. This process separates the mixture into three phases, with the upper phase containing RNA.

The upper phase is carefully transferred to a new tube, and 400 μ L of cold isopropanol is added. Af-

ter inverting the tube 2-3 times, it is frozen at -20°C for over 12 hours, followed by incubation at room temperature for 10 minutes. The next step involves centrifuging the mixture at 12,000 g for 10 minutes at 4°C to isolate the RNA pellet, after which the supernatant is removed.

To wash the RNA pellet, 1 mL of 75% ethanol is added, and the mixture is centrifuged at 7,500 g for 5 minutes at 4°C. This washing step is repeated 2-3 times, and the pellet is allowed to dry at room temperature, ensuring it does not dry completely to maintain solubility. Finally, the RNA pellet is dissolved in 50 μ L of DEPC-treated water and stored at -20°C for short-term use or -80°C for long-term storage. It is important to perform all steps under a laminar flow hood to minimize RNase activity, keeping all materials on ice throughout the process.

Quantitative and Qualitative Assessment of Extracted RNA

To determine the quantity and purity of the extracted RNA, the optical density method using a NanoDrop device was employed. This device measures the concentration and purity of the sample based on absorbance at wavelengths of 260 nm (RNA) and 280 nm (protein). An absorbance ratio of 260/280 between 1.8 and 2.1 indicates pure RNA, while ratios below 1.8 suggest contamination. After RNA extraction and amplification of the target DNA, electrophoresis is performed to identify the components of the DNA molecule. The size of each band is determined by comparing it with standard bands run simultaneously.

cDNA Synthesis

Following the synthesis of cDNA from the extracted RNA samples, RT-PCR was conducted using control primers (ACTB and GAPDH) to ensure the proper functioning of the cDNA synthesis kit and to estimate the quality of the extracted RNAs.

Primer Design

To investigate the expression of the target genes, specific primers for the cDNA sequence of each gene were designed using Oligo Analyzer and Primer 3

Table 1: Primer Sequences for the FGF19 Gen and Reference Genes ACTB and GAPDH

Gene	Primer Sequences	Length of the Amplified Fragment
<i>NKx2-5</i>	Forward: `5 ATGCCATCCGGATCCTCTCT `3	582
	Reverse: `5 CGCAGGGTAGGTGTTGTAGG `3	
<i>Tbx5</i>	Forward: `5 CAAATGGTCTGTGACGGGGA `3	592
	Reverse: `5 TGTGTGTTTCGTGGTAGGAGC `3	
<i>GAPDH</i>	Forward: `5 TCGGAGTCAACGGATTG `3	219
	Reverse: `5 CCTGGAAGATGGTGATGG `3	
<i>ApoE</i>	Forward: `5 TGCTGTACACACAGCAGAGG `3	120
	Reverse: `5 TGGAAGGCGGTTTTGGTCTT `3	

To investigate the expression of the target genes, specific primers for the cDNA sequence of each gene were designed using Oligo Analyzer and Primer 3 Plus software. Their characteristics were analyzed for optimization using BLAST. The primers used for the target genes in this study are listed in Table 1.

2.7 Real-Time PCR Reaction

The SYBR Green method was used to determine the expression of the *Nkx2-5*, *ApoE*, and *Tbx5* genes in zebrafish heart tissue. The *GAPDH* gene was used as an internal control. The final volume of each reaction was set to 25 microliters, consisting of 12.5 microliters of SYBR Green Master Mix, 1 microliter of synthesized cDNA, 1 microliter of forward and reverse primers for each *Nkx2-5*, *ApoE*, and *Tbx5* (10 mM), and 9.5 μ L of DEPC-treated water. Real-time PCR was performed using the Qiagen Rotor-Gene according to the following protocol.

2.8 Statistical Analysis

For statistical analysis of multiple groups, one-way ANOVA followed by the Dunnett test will be used. All experimental data results will be plotted using GraphPad 7.0, and the Student's t-test will be employed for the analysis of two-group data.

3. Result

3.1 Morphological Features

At 24 hours post-fertilization (hpf), the embryos were incubated with *Echium amoenum* extract at various concentrations. At this time, no visible effects were observed, and no hatching of the embryos was noted. At 48 hpf, hatching of the embryos was observed, with no visible effects at concentrations of 7.80, 15.63, 31.25, and 62.50 μ g/mL. Unhatched embryos were seen at a concentration of 125.0 μ g/mL. At 72 hpf, dead embryos were observed at 62.5 and 125.0 μ g/mL, along with morphological deformities such as stunted growth and body curvature (Fig 1). For all tested concentrations of the extract, the efficacy of *Echium amoenum* on each fish was dependent on concentration and time.

3.2 LD50 After Treatment with *Echium amoenum* in Zebrafish Embryos

LD50 refers to the lethal dose that kills 50% of the test subjects. In this study, we investigated the effect of *Echium amoenum* extract on zebrafish embryos. To determine the LD50, the embryos were exposed to various doses of the extract, and their mortality was recorded. At 24 and 48 hours, the mortality rate was low, and no LD50 was calculated. However, at 72 hours, the LD50 was found to be 101 μ g/mL (Fig 2).



Figure 1. Morphological Features as a Measure of the Efficacy of Echium amoenum Extract at 24 and 48 Hours: A) Control after 24 hours, B) Treatment with a concentration of 62.5 µg/mL (24 hours), and C) Treatment with a concentration of 62.5 µg/mL after 48 hours.

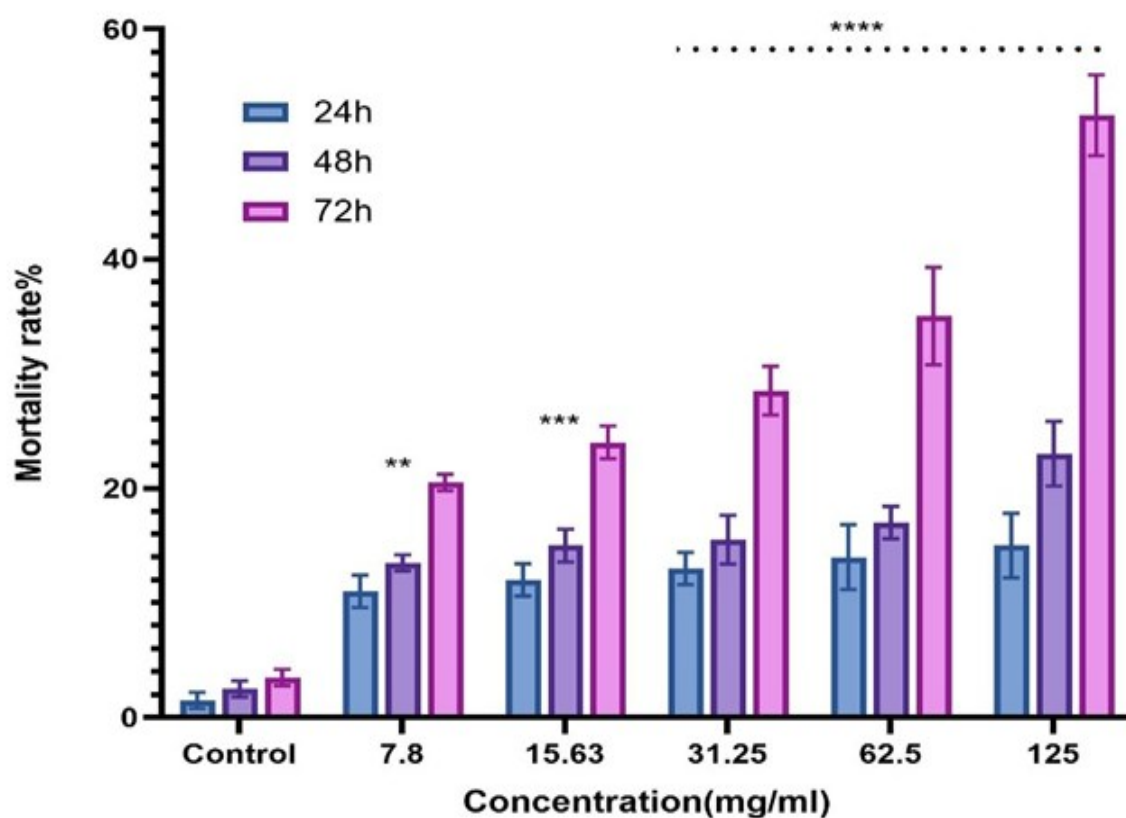


Figure 2. Mortality Rate in Zebrafish Embryos After Treatment at Various Concentrations. At lower concentrations of 7.8 and 15.63 mg/mL, the mortality rate remains relatively low. However, as the concentration increases to 31.25 mg/mL and beyond, the mortality rate rises significantly. Notably, at the highest concentration of 125 mg/mL, the mortality rate exceeds 50% ($P < 0.05^*$, $P < 0.01^{**}$, $P < 0.001^{***}$).

3.3 Histological Examination

To investigate the effect of *Echium amoenum* extract on heart tissue, embryos exposed to the calculated LD50 (101 $\mu\text{g/mL}$) after 72 hours were selected, and their heart tissue was prepared. The tissue was then examined using histopathological methods to identify any tissue changes resulting from the extract administration (Fig 3).

Figure 4-2 shows the various layers of the heart, including the endocardium, myocardium, and pericardium. Additionally, different types of heart cells, such as cardiomyocytes, endothelial cells, and smooth muscle cells, can be observed in the heart tissue. The image displays the heart tissue and its various components, including blood vessels. Different types of heart cells, such as muscle cells, are also visible under the microscope. Since the image pertains to the treatment with *Echium amoenum*, it appears that this plant has no toxicity on the heart tissue of zebrafish embryos.

3.4 Expression of the genes *Nkx2-5*, *ApoE*, and *Tbx5* in zebrafish heart tissue

To assess the expression levels of the genes *Nkx2-5*, *ApoE*, and *Tbx5* in the heart tissue of zebrafish embryos, the expression of these genes was examined at both the LD50 concentration (101 $\mu\text{g/ml}$ for 72 hours) and a concentration of 62.5 $\mu\text{g/ml}$. The Real-Time PCR method was used to analyze the expression of the genes *Nkx2-5*, *ApoE*, and *Tbx5*. In the group treated with LD50 of *Echium amoenum*, the expression level of the *Tbx5* gene was measured between 0.748 and 0.757, which showed a significant decrease (DOWN) compared to the control group ($P(H1)=0.000$). Therefore, the analysis indicates that treatment with LD50 of *Echium amoenum* significantly reduced *Tbx5* expression compared to the control group. In the group treated with 62.5 $\mu\text{g/ml}$ of *Echium amoenum*, the expression level of *Tbx5* ranged from 1.403 to 1.517, which showed a significant increase compared to the control group ($P(H1)=0.000$) (Fig 4).

In the group treated with LD50 of *Echium amoenum*, the expression level of the *NKX-2-5* gene in the control group was recorded between 0.803 and 1.359, showing a significant decrease compared to the control group ($P(H1)=0.000$). Therefore, it can be concluded that treatment with LD50 concentra-

tion significantly reduced *NKX-2-5* expression compared to the control group. In the group treated with 62.5 $\mu\text{g/ml}$ of *Echium amoenum*, the expression level of *NKX-2-5* ranged from 1.117 to 1.189, indicating a significant increase compared to the control group ($P(H1)=0.000$). Thus, it can be concluded that treatment with this concentration significantly increased *NKX-2-5* expression compared to the control group (Fig 5).

In the LD50 treatment group, the expression level of the *APOE* gene ranged from 0.750 to 0.777, showing a decrease compared to the control group; however, this decrease was not significant ($P(H1)=0.326$). In the unnormalized section, it was also observed that the expression of the *APOE* gene in the treatment group decreased compared to the control, but this decrease was not significant ($P(H1)=0.154$). Therefore, treatment with LD50 concentration resulted in a non-significant reduction in *APOE* expression. In the group treated with concentrations below 62.5 $\mu\text{g/ml}$, the expression level of *APOE* ranged from 2.647 to 2.879, indicating a significant increase compared to the control group ($P(H1)=0.000$). Thus, treatment with this concentration significantly increased *APOE* expression (Fig 6).

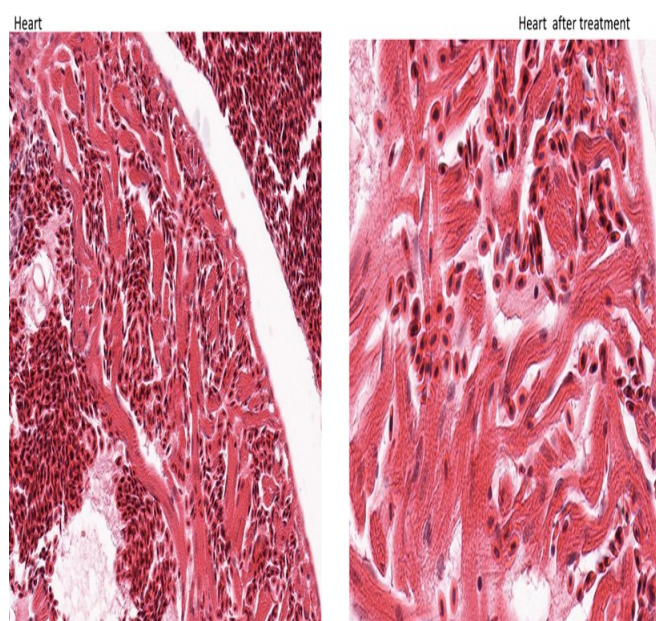


Figure 3. Histology of heart tissue after treatment with *Echium amoenum* at 40x magnification

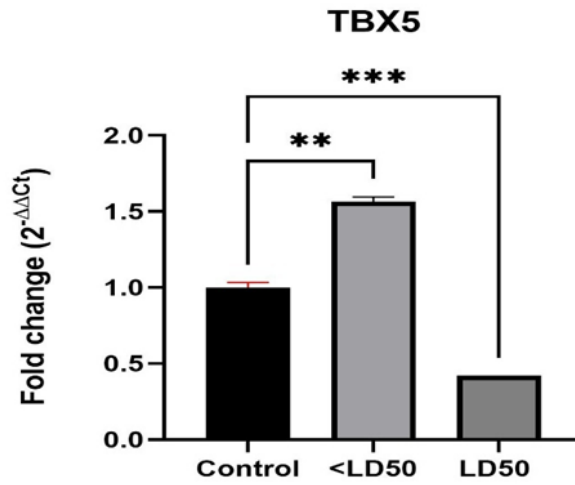


Figure 4. Investigation of Tbx5 expression after treatment with LD50(A) and 62.5 μ g/ml (B) of *Echium amoenum*($P<0.05^*$, $P<0.01^{**}$, $P<0.001^{***}$).

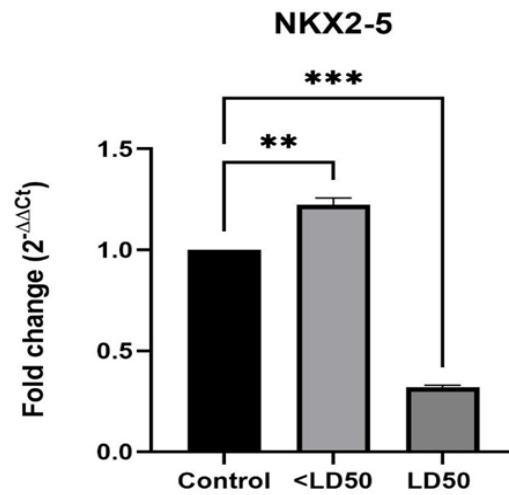


Figure 5. Investigation of NKX-2-5 expression after treatment with LD50 (A) and 62.5 μ g/ml (B) of *Echium amoenum*($P<0.05^*$, $P<0.01^{**}$, $P<0.001^{***}$).

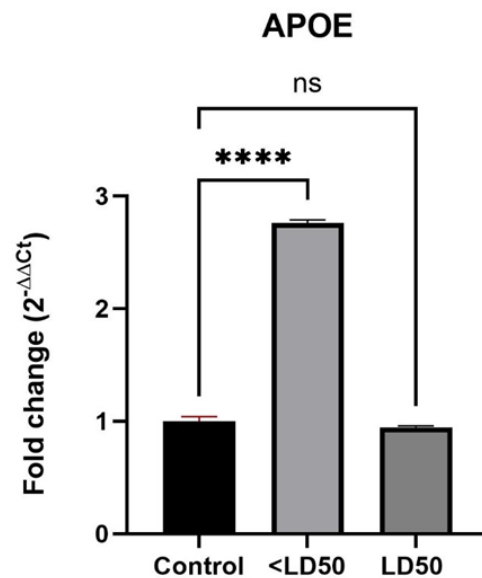


Figure 6. Investigation of APOE expression after treatment with LD50 (A) and 62.5 μ g/ml (A) of *Echium amoenum*($P<0.05^*$, $P<0.01^{**}$, $P<0.001^{***}$).

4. Discussion

Herbal medicines have been consumed by humans for centuries, and their safety has been established over time. However, evaluating the toxicology of herbal medicine is crucial for identifying adverse effects to protect human health. Toxicity measurements during clinical and preclinical drug discovery facilitate the recognition of toxic substances that can be discarded or modified into safer alternatives (91,91)

Echium amoenum is recognized as a traditional therapy and influences antioxidant, analgesic, antibacterial, anxiolytic, antidepressant, and immunomodulating properties. It is a rich source of anthocyanins, including cyanidin and delphinidin(102). In addition to its antioxidant and anti-inflammatory properties, useful cardiovascular effects such as anti-atherosclerotic and antihypertensive effects have been attributed to anthocyanins(103).

Toxicity screening is conducted using both animal and non-animal models before clinical application. Animal models are studied as the gold standard in toxicological testing, as the whole organism typically has a close relationship with human toxicity, incorporating pharmacokinetics, absorption, distribution, and metabolism. The zebrafish model is widely used for these purposes(92). *Danio rerio*, commonly known as zebrafish, is a small freshwater fish measuring around 2 to 4 centimeters in length. Its small size, short life cycle, and high fecundity make it beneficial for laboratory applications as a model organism. Zebrafish are extensively used in genetic development, transgenic studies, and toxicology research.(28).

This study investigates the effect of *E. amoenum* extract on the development of the cardiovascular system in zebrafish embryos, concentrating on the expression of *Nkx2-5*, *ApoE*, and *Tbx5* genes. Real-time PCR results indicated that *E. amoenum* extract significantly affects the expression of *Nkx2-5* and *Tbx5* genes. The findings suggest that *E. amoenum* extract has a profound impact on the cardiovascular development of zebrafish embryos. The *Nkx2-5* gene is a crucial transcription factor involved in cardiac development, and its increased

expression may show enhanced cardiac differentiation. The *Tbx5* gene is also essential for heart development and is associated with congenital heart defects in humans. An increase in *Tbx5* expression may reflect the potential role of *E. amoenum* extract in modulating cardiac growth. The *ApoE* gene is involved in lipid metabolism and is linked to cardiovascular diseases. An increase in *ApoE* expression may indicate the potential role of *E. amoenum* extract in modifying lipid metabolism. In line with our study, Tássia Flávia Dias Castro and colleagues assessed the impact of various concentrations of *Eryngium foetidum* extracts on zebrafish embryo development. Their results showed that depending on the extraction solvent, concentration used, and exposure time, *E. foetidum* extract caused mortality, altered hatching time, and promoted changes in enzymatic activities. *Eryngium foetidum* extracts exhibit antioxidant, anti-inflammatory, antidiabetic, and antimicrobial properties due to compounds such as flavonoids and phenols (29). In our study, we also observed progress interruption at concentrations of 62.5 and 125.0 µg/ml after 72 hours.

On the other hand, in a study by Chune Zhou and colleagues, transcriptome sequencing of zebrafish embryos at three key stages of cardiovascular development showed thousands of differentially expressed genes (DEGs). This study confirmed the significance of these stages during gene expression analysis and highlighted key biological processes and pathways involved in cardiovascular development.(30). We also identified three genes associated with zebrafish cardiac development in our study.

Many studies have been conducted on *E. amoenum*. A recent study examined the protective and antioxidant effects of anthocyanin-rich *E. amoenum*

num extract on healthy human vascular endothelial cells (HUVECs) under oxidative stress. (31). The results show the antioxidant and protective effects of the anthocyanin-rich extract of *E. amoenum* petals against oxidative stress induced by H₂O₂ in HUVECs. However, they called for further research to understand the specific mechanisms of the protective effects of this traditional herbal medicine. Hence, we utilized zebrafish embryos in this study as a model that closely looks like human physiology (86% similarity). Our results confirmed those of previous studies, confirming that *E. amoenum* has no toxicity to healthy cells. In another study, the safety of *Syzygium jambolanum* was evaluated on zebrafish embryos.(32). Similar to our study, the results indicated the potential protective effects of *Syzygium jambolanum* on zebrafish embryos, highlighting the need for further investigation.

5. Conclusion

Our results strongly support the beneficial effect of *Echium amoenum* extract on cardiovascular development in zebrafish embryos. Therefore, the increase of Nkx2-5, ApoE as well as Tbx5 just in lower concentrations may indicate improved cardiac differentiation and lipid metabolism indicating a beneficial effect on cardiovascular health. In contrast, the negative effects seen at concentrations higher than those achieved in patients have made substantiation of an effective dose necessary. Given the increasing prevalence of cardiovascular diseases and plant-based antioxidants, *E. amoenum* likely represents an exciting field for future study. Further research is needed to investigate the molecular mechanisms underlying its action as well as in clinical settings for AS, which might help promote translation into therapeutic strategies for cardiovascular disease management.

Reference

- 1.Ghorpade AG, Shrivastava SR, Kar SS, Sarkar S, Majgi SM, Roy G. Estimation of the cardiovascular risk using World Health Organization/International Society of Hypertension (WHO/ISH) risk prediction charts in a rural population of South India. *Int J Health Policy Manag.* 2015 Apr 21;4(8):531–6.

- 2.Carr A, Frei B. The role of natural antioxidants in preserving the biological activity of endothelium-derived nitric oxide. *Free Radic Biol Med.* 2000 Jun;28(12):1806–14.
- 3.Diaz MN, Frei B, Vita JA, Keaney JF. Antioxidants and Atherosclerotic Heart Disease. *New England Journal of Medicine.* 1997 Aug 7;337(6):408–16.
- 4.Touyz RM, Briones AM. Reactive oxygen species and vascular biology: implications in human hypertension. *Hypertension Research.* 2011 Jan 28;34(1):5–14.
- 5.Praticò D. Antioxidants and endothelium protection. *Atherosclerosis.* 2005 Aug;181(2):215–24.
- 6.Bodeker GOCKGCBGSK. WHO Centre for Health Development. <https://iris.who.int/handle/10665/43108>. 2005. WHO global atlas of traditional, complementary and alternative medicine.
- 7.Vinardell MP, Mitjans M. Alternative Methods for Eye and Skin Irritation Tests: An Overview. *J Pharm Sci.* 2008 Jan;97(1):46–59.
- 8.Van Wyk BE. A review of commercially important African medicinal plants. *J Ethnopharmacol.* 2015 Dec;176:118–34.
- 9.Masoud Ghorbani ASMMMFMNSNM reza nourani. A Review of 50 Herbs for Wound Healing: Traditional Use and Modern Evidence. *The Journal of Applied Tissue Engineering.* 2023;9(1):12–34.
- 10.Patocka J. Bioactivity of *Echium Amoenum*: A Mini Review. *Biomed J Sci Tech Res.* 2019 Aug 2;20(2).
- 11.Alireza Abed GV et al. *Echium Amoenum* Fisch. Et Mey: A Review on its Pharmacological and Medicinal Properties. *Asian Journal of Medical and Pharmaceutical Researches.* 2014;4(1):21–3.
- 12.Ojeda D, Jiménez-Ferrer E, Zamilpa A, Herrera-Arellano A, Tortoriello J, Alvarez L. Inhibition of angiotensin converting enzyme (ACE) activity by the anthocyanins delphinidin and cyanidin-3-O-sambubiosides from *Hibiscus sabdariffa*. *J Ethnopharmacol.* 2010 Jan;127(1):7–10.
- 13.Grunwald DJ, Streisinger G. Induction of recessive lethal and specific locus mutations in the zebrafish with ethyl nitrosourea. *Genet Res.* 1992 Apr 14;59(2):103–16.
- 14.Vikas Gupta KDP. Clonally dominant cardiomyocytes direct heart morphogenesis. *Nature.* 2012 May;25:479–84.
- 15.Poon KL, Brand T. The zebrafish model system in cardiovascular research: A tiny fish with mighty prospects. *Glob Cardiol Sci Pract.* 2013 Mar;2013(1):4.
- 16.Lindsay EA, Vitelli F, Su H, Morishima M, Huynh T, Pramparo T, et al. Tbx1 haploinsufficiency in the DiGeorge syndrome region causes aortic arch defects in mice. *Nature.* 2001 Mar;410(6824):97–101

- 17.Griffin HR, Topf A, Glen E, Zweier C, Stuart AG, Parsons J, et al. Systematic survey of variants in TBX1 in non-syndromic tetralogy of Fallot identifies a novel 57 base pair deletion that reduces transcriptional activity but finds no evidence for association with common variants. *Heart*. 2010 Oct 15;96(20):1651–5.
- 18.Chowdhury R, Ashraf H, Melanson M, Tanada Y, Nguyen M, Silberbach M, et al. Mouse Model of Human Congenital Heart Disease. *Circ Arrhythm Electrophysiol*. 2015 Oct;8(5):1255–64.
- 19.Azpiazu N, Frasch M. tinman and bagpipe: two homeobox genes that determine cell fates in the dorsal mesoderm of *Drosophila*. *Genes Dev*. 1993 Jul 1;7(7b):1325–40.
- 20.Bodmer R. The gene tinman is required for specification of the heart and visceral muscles in *Drosophila*. *Development*. 1993 Jul 1;118(3):719–29.
- 21.Tanaka M, Wechsler SB, Lee IW, Yamasaki N, Lawitts JA, Izumo S. Complex modular cis -acting elements regulate expression of the cardiac specifying homeobox gene *Csx/Nkx2.5*. *Development*. 1999 Apr 1;126(7):1439–50.
- 22.Targoff KL, Schell T, Yelon D. Nkx genes regulate heart tube extension and exert differential effects on ventricular and atrial cell number. *Dev Biol*. 2008 Oct;322(2):314–21.
- 23.George V, Colombo S, Targoff KL. An early requirement for *nkx2.5* ensures the first and second heart field ventricular identity and cardiac function into adulthood. *Dev Biol*. 2015 Apr;400(1):10–22.
- 24.Ismail AB, Balcıoğlu Ö, Özceci B, Ergoren MÇ. APOE Gene Variation's Impact on Cardiovascular Health: A Case-Control Study. *Biomedicine*. 2024 Mar 21;12(3):695.
- 25.Husain MA, Laurent B, Plourde M. APOE and Alzheimer's Disease: From Lipid Transport to Physiopathology and Therapeutics. *Front Neurosci*. 2021 Feb 17;15.
- 26.Sugishita Y, Watanabe M, Fisher SA. The Development of the Embryonic Outflow Tract Provides Novel Insights into Cardiac Differentiation and Remodeling. *Trends Cardiovasc Med*. 2004 Aug;14(6):235–41.
- 27.Yamazaki Y, Shinohara M, Yamazaki A, Ren Y, Asmann YW, Kanekiyo T, et al. ApoE (Apolipoprotein E) in Brain Pericytes Regulates Endothelial Function in an Isoform-Dependent Manner by Modulating Basement Membrane Components. *Arterioscler Thromb Vasc Biol*. 2020 Jan;40(1):128–44.
- 28.Ifeoma O, Oluwakanyinsol S. Screening of Herbal Medicines for Potential Toxicities. In: *New Insights into Toxicity and Drug Testing*. InTech; 2013.
- 29.Castro TFD, Carneiro WF, Reichel T, Fabem SL, Machado MRF, de Souza KKC, et al. The toxicological effects of *Eryngium foetidum* extracts on zebrafish embryos and larvae depend on the type of extract, dose, and exposure time. *Toxicol Res (Camb)*. 2022 Oct 29;11(5):891–9.
- 30.Zhou C, Zhao W, Zhang S, Ma J, Sultan Y, Li X. High-throughput transcriptome sequencing reveals the key stages of cardiovascular development in zebrafish embryos. *BMC Genomics*. 2022 Aug 13;23(1):587.