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*Full Length Research Paper*

# Developmental effects of extracts produced by some newly isolated *Actinobacteria* on the heart of Zebra fish (*Danio rerio*) embryos

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**Most actinomycete species have the capability to synthesize many different biologically active secondary metabolites such as antibiotics, herbicides, pesticides, anti-parasitic and enzyme inhibitors. The main focus of the present study was to isolate some new actinobacterial strains from the Saudi habitats and study the biological effects of their secondary metabolites on the heart development of the zebra fish embryos as a drug discovery screening model. For this purpose, 6 soil samples were collected and 100 strains were isolated. Out of the isolated strains, 5 isolates were selected for extraction and screening based on differences in morphological appearance. The selected strains were characterized morphologically to confirm that they belong to actinobacteria. All of them were Gram-positive and rod or spherical in shape with variable lengths or diameters and arrangements. Most of the extracts induced abnormalities in the developing heart in zebra fish embryos on a dose dependent manner if exposed to sublethal concentrations (less than 20 µg/ml) of these extracts. The embryos exposed to the extracts had cardiac edema with enlarged cardiac chamber (cardiac hypertrophy). Some extracts also induced teratogenecity in developing brain. While on the other hand, some extracts even at higher concentration did not induce any significant abnormalities in developing zebra fish embryos. The current study highlights the significance of the biomedical applications of the actinobacteria isolated from the Saudi habitats.**

**Keywords:** Actinobacteria, extracts, developmental effects, heart, zebra fish.

## INTRODUCTION

The search for new drugs will continue for a long time to overcome resistance, to discover safer and broad spectrum compounds and to improve the biological activities against viruses, tumors, protozoa, insects and herbs. In this regard, microbial natural products still appear as the most promising source for drug discovery (Luzhetskyy et al., 2007). Actinobacteria are of special biotechnological interest since they are known to produce chemically diverse compounds with a wide range of biological activity (Ballav et al., 2012; Omura et al., 2001).

The intact zebra fish embryo provides diverse opportunities to examine the biological effects of embryonic morphogenesis. Its relatively small size, transparency and exceedingly rapid rate of development make it ideal for *in vivo* studies. The zebra fish, *Danio rerio*, offers several advantages for toxicity testing including economic husbandry requirements, high fecundity and rapid *ex utero* development (Nagel, 2002; Hill et al., 2005; Crawford et al., 2008). As it is closely related to human genome it is employed for *in vitro* assays in drug/ pharmaceutical research (Alestrom et al., 2006).

Zebra fish development has been well characterized (Kimmel et al., 1995). The eggs remain transparent from fertilization up to and beyond pharyngulation when the

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tissues become dense and pigmentation is initiated. This allows unobstructed observation of the main morphological changes during earlier developmental stages. Furthermore, zebra fish embryos that are malformed, lack organs, or display organ dysfunction can usually survive well beyond the time at which those organs normally start to function in healthy individuals. In addition, the fish is sensitive to chemical exposure during early development (Peterson et al., 1993). These characteristics make the zebra fish an attractive drug discovery screening model for screening of toxicants and the elucidation of mechanisms thereof. In this study, the zebra fish embryos were treated with various extracts obtained from some actinobacterial strains newly isolated from soil samples collected from Jobail Industrial City, Saudi Arabia.

## MATERIALS AND METHODS

### Collection of samples

A total of 6 soil samples were collected from Jobail Industrial City, the Eastern Province, Saudi Arabia. Field collection of samples was made during January 2012. Soil samples were taken at a depth of 10- 20 cm below the soil surface with a collecting spatula in clean sterile plastic bags. These samples were transported to the laboratory by keeping them in ice box and then used for isolation.

### Isolation of actinobacteria

Two different agar media were used for isolation of actinobacteria, namely, M1 medium (Mincer et al., 2002) and the minimal medium (MM) (Hozzein et al., 2008). Ten fold serial dilution of the soil samples were made up to $10^{-4}$. Inocula of 0.1 ml aliquots of these dilutions were spread over the surface of the isolation plates (Johnson et al., 1959). Three replicates were used for each dilution and the plates were then incubated for 2 weeks at 30°C. Some colonies were selected and purified by streaking twice on the same isolation medium.

### Maintenance of the isolates

Pure cultures were subcultured and grown on slants of the same isolation medium at 30°C for 7 days. The isolates were then maintained in the refrigerator at 4°C and as suspensions in 20% (v/v) glycerol at -80°C for long-term maintenance.

### Cultural and morphological characteristics of the actinobacterial strains

The cultural characteristics, colony shape, elevation and margin, of the selected strains were observed as on the agar plates after incubation at 30°C for 7 days. The Gram reaction and cell morphology were examined using light microscopy according to Smibert and Krieg (1981).

### Preparation of the actinobacterial extracts

The selected purified strains were cultivated in MM broth at 30°C and 150 rpm for 2 days on a shaking incubator. The growing strains were then inoculated into ISP2 broth medium (Shirling and Gottlieb, 1966) and incubated at 30°C and 150 rpm for 5 days on a shaking incubator. The culture was extracted with the same volume of ethyl acetate twice. The organic phase was concentrated on a rotary evaporator till complete dryness and then weighed and dissolved in the least amount of methanol. These methanol stock solutions were used to prepare a range of working solutions from 1 to 100 µg for the next experiments.

### Methodology for treating zebra fish embryos

#### *Animals*

Wild type zebra fish (*AB/Tuebingen TAB-14)* were obtained from Zebra fish International Resource Center (ZIRC University of Oregon, Oregon, USA) and maintained by us in the laboratory of the Bioproducts Research Chair, Department of Zoology, College of Science, King Saud University. All experiments were carried out in accordance with the national and international animal use guidelines.

### Animal treatment

The zebra fish (*D. rerio*) embryos were obtained by natural pair wise mating. Embryos were staged following Kimmel et al. (1995). Synchronized embryos were raised to shield stage (6 h post fertilization). Around thirty (30) embryos were transferred to each sterile 35 mm Petri dishes and exposed to the desired concentration of the extracts (1, 10, 20, 50 and 100 µg) on vehicle (methanol, 0.5%) in 10 ml embryo medium (5.03 mM $NaCl_2$, 0.17 mM $KCl_2$, 0.33 mM $CaCl_2$ and 0.33 mM $MgSO_4$) for 12 h (Ponrasu et al., 2012). As the extracts were dissolved in methanol, so, 0.5% methanol treated embryos served as control. The embryos were incubated at 28.5°C overnight. The mortality in control and treated embryos was recorded next day and subsequently the live embryos were raised in embryo medium without extract up to end point either three days or five days post fertilization (5 dpf) with replacement of embryo medium daily. Any change in the phenotype and development of the embryos was monitored by microscopy. The mortality rate is expressed as the total number of dead embryos after 5 dpf as compared to control.

### Microscopy and photography

Images were acquired using a Nikon Eclipse E600 binocular microscope, fitted with Nikon digital camera model DXM1200F, Japan.

## RESULTS

From about 100 colonies appeared on the plates, 5 dissimilar actinobacterial strains were purified and selected for further work. Colonies of the isolates under investigation showed moderate to good growth and were mainly circular or irregular in shape, white, creamy white, yellow to orange in color with flat, raised to convex elevation. They were non-transparent on solid media and had entire to irregular margins, mucoid consistency and shiny surfaces. Morphologically, all the selected isolates were Gram-positive and their cells were found to be rod-shaped with variable lengths or spherical with variable diameters and arrangements (Figure 1). The cultural and morphological characteristics are summarized in Table 1.

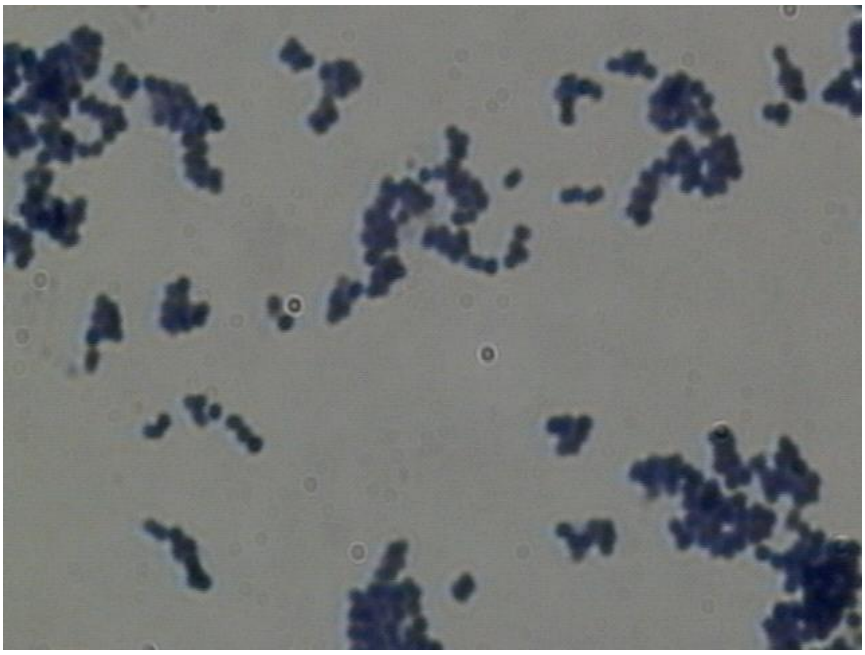

**Figure 1.** A light micrograph showing the cell morphology of strain S11, one of the isolated actinobacterial strains.

**Table 1.** Cultural characteristics and cell morphology of the selected actinobacterial isolates on MM medium.

| Characteristic | Strains | | | | |
|---|---|---|---|---|---|
| | **S11** | **S12** | **S15** | **S31** | **S33** |
| Growth | Good | Moderate | Moderate | Good | Good |
| Colony shape | Circular | Circular | Irregular | Irregular-wrinkled | Circular |
| Colony color | Yellow | White | Creamy white | Creamy | Orange |
| Colony elevation | Convex | Flat | Raised | Raised | Covex |
| Colony margin | Entire | Entire | Irregular | Irregular | Entire |
| Gram reaction | +ve | +ve | +ve | +ve | +ve |
| Cell morphology | Spherical | Rod | Rod | Rod | Spherical |

The selected actinobacterial cultures were grown in liquid media, extracted and tested on the zebra fish embryos for their developmental effects on the heart. As presented in Table 2, all the extracts were toxic and lethal to zebra fish embryos used at higher concentration (more than 20 µg/ml). The zebra fish embryos exposed to sublethal concentrations of these extracts showed various levels of developmental abnormalities. The most prominent of those were the severe effect on the circulation system.

The embryos exposed to extract number 1, showed cardiac edema and cardiac hypertrophy on a dose dependent manner. The embryos exposed to a dose between 1 to 5 µg/ml of the extract had milder cardiac edema with normal circulation (Figure 2, compare A and B). While, this edema increased with increasing concentration of the extract. The heart formation was also affected in zebra fish embryos exposed to 20 µg/ml of the extract. The heart was malformed and chambers of the heart were dilated showing typical hypertrophy phenotype (Figure 2, C and D). The circulation was also severely affected in 10 and 20 µg/ml treated embryos. The brain formation in 20 µg/ml treated embryos was also affected. In the embryos exposed to 20 µg/ml, the growth was also retarded and there was severe developmental delay. The second extract also induced similar kind of developmental toxicity as extract number 1 but at lesser extent. The embryos exposed to 20 µg/ml showed milder cardiac edema and cardiac hypertrophy as compared to extract number 1 (Figure 2, G and H). The blood circulation was also severely affected the embryos. Surprisingly, the growth was not retarded in the embryos even at 20 µg/ml, which means that this extract very specifically target only the developing heart and circulation.

Zebra fish embryos exposed to extract 3 did not show any obvious abnormality up to 10 µg/ml dose. At double concentration (20 µg/ml), there was overall reduction in

**Table 2.** The mortality percentage of the zebra fish embryos in response to different concentrations of the actinobacterial extracts.

| Extract no. | 1 ug/ml | 5 ug/ml | 10 ug/ml | 20 ug/ml | 50 ug/ml | 100 ug/ml |
|---|---|---|---|---|---|---|
| Control | 1% | 1% | 1% | 1% | 1% | 1% |
| Extract 1 | 20% | 20% | 40% | 60% | 100% | 100% |
| Extract 2 | 5% | 10% | 20% | 50% | 100% | 100% |
| Extract 3 | 5% | 25% | 33% | 40% | 100% | 100% |
| Extract 4 | 5% | 25% | 33% | 60% | 100% | 100% |
| Extract 5 | 5% | 15% | 20% | 40% | 100% | 100% |

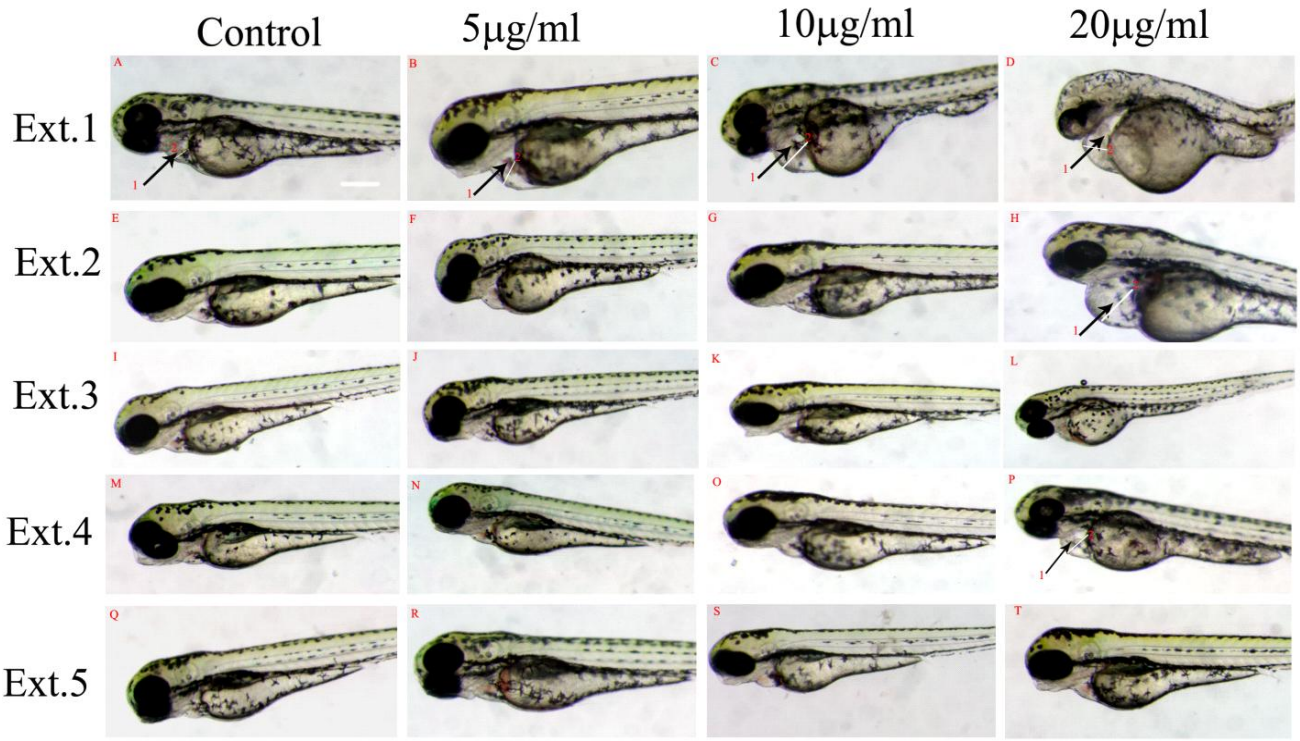


**Figure 2.** The representative images of live zebra fish embryos at 48 hpf of mock (0.5 % methanol V/V) treated (A, E, I, M, Q) and different concentrations of the five extracts from actinobacteria. Extract 1 (B- D), extract 2 (F- H), extract 3 (J- L), extract 4 (N- P) and extract 5 (R- T) treated embryos. The mock treated embryos developed normally, with normal size of the heart and heart chamber as represented by black arrow (1) and white thick line (2). Whereas, the extracts from actinobacteria induced server cardiac hypertrophy (enlarged cardiac chambers) as shown by black arrow (1) by extracts 1, 2 and 4 (20 µg/ml, D, H, P). A cardiac edema which is represented by thick white line (2, compare the length of line in mock treated embryos with extract treated embryos) is also clearly visible in those embryos having the cardiac hypertrophy (D, H, P). All the images are on lateral side with anterior facing front. All the images are taken at same magnification, (white bar at the bottom, reprsents 100 µm).

the size of the embryos without any significant developmental delay (Figure 2, J, K and L). Extract 4 induced very specific cardiac hypertrophy at higher concentration with very mild cardiac edema. The cardiac hypertrophy was very much obvious in the treated zebra fish embryos at 20 µg/ml (Figure 2P). The zebra fish embryos exposed to extract 5 did not produce any abnormal phenotype of toxicity up to 20 µg/ml, a concentration at which other extracts had induced sever toxicity or teratogenecity. The embryos exposed to higher concentration (50 µg/ml) had all died, so it was difficult to judge any specific phenotype associated with this extract.

## DISCUSSION

Saudi Arabia has diverse habitats that can be considered a good source for the isolation of actinobacteria, which

are well known for their biotechnological and medical applications (Bérdy, 2005). Therefore, our main objective was to isolate some new actinobacterial strains from the Saudi ecosystems and study their developmental effects on the zebra fish embryos. Very little knowledge about the biological activities and chemical constituents of the secondary metabolites from actinobacteria is available and essentially no studies were done to check the anti-angiogenic potential of these bacteria from the Saudi habitats so far. Therefore, this study has been designed to screen the secondary metabolites produced by actinobacteria for anti-angiogenic activity. The biotechnological significance and applications of some actinobacteria was recently reviewed (Ballav et al., 2012).

Drug discovery involves a complex iterative process of biochemical and cellular assays, with final validation in animal models, and ultimately in humans. Mammalian models of absorption, distribution, metabolism and excretion, pharmacokinetics and efficacy are expensive, laborious and consume large quantities of precious compounds. There is also increasing pressure to limit animal use to situations in which they are absolutely necessary, such as in preclinical toxicity and safety assessment (Zon and Peterson, 2005). Zebra fish (*D. rerio*) is beginning to be used at different stages of the drug discovery process and is useful and cost-effective alternative to some mammalian models.

The zebra fish embryos are transparent and development occurs externally, enabling an easy and thorough assessment of drug's effects on internal organs in the live organism. This is a great advantage over mammalian model organisms where embryonic development occurs *in utero* (Peterson et al., 2000; Parng et al., 2002). Zebra fish embryogenesis is very rapid, with entire body plan established by 24 h post fertilization (hpf). Most of the internal organ-including heart, liver, intestine and kidney are fully developed by 96 hpf. Recently, Crawford et al. (2011) developed an *in vivo* bioassay-guided isolation approach for natural product discovery that combines bioactivity screening in zebra fish embryos with rapid fractionation by analytical thin-layer chromatography (TLC) and initial structural elucidation by high-resolution electrospray mass spectrometry (HRESIMS).

In the current study, 5 actinobacterial isolates were selected and characterized. Their cultural and morphological characteristics are consistent with the affiliation of these strains to the Gram-positive actinobacteria group (Holt et al., 1996; Stackebrandt et al., 1997). Their metabolites were extracted and screened for their developmental effects on the zebra fish embryos. It was clear that all the extracts were toxic and lethal to zebra fish embryos used at higher concentration (more than 20 µg/ml) and the mortality percentage of the zebra fish embryos in response to different concentrations of the actinobacterial extracts is represented in Table 2. The zebra fish embryos exposed to sublethal concentrations of these extracts showed some developmental abnormalities.

Most of the extracts induced abnormalities in developing heart on a dose dependent manner. The embryos exposed to the extracts had cardiac edema with enlarged cardiac chamber (cardiac hypertrophy) comparing to the control. While, some extracts even at higher concentration did not induce any significant phenotypic abnormalities in the developing heart of the zebra fish embryos. It was interesting also to observe that some extracts induced teratogenecity in the developing brain of the embryos. Recently, Kannan el al. (2011) studied the biomedical effects of a small molecule produced by a *Streptomyces* strain (a genus of the actinobacteria) on the zebra fish embryos, which was not toxic and did not show abnormal phenotypes.

In conclusion, the results draw the attention to the significance of the actinobacteria isolated from the Saudi habitats and their possible biomedical applications. The results are encouraging to screen other actinobacteria for drug discovery, biotechnological and medical applications. The identification of the isolated strains, purification and chemical characterization of the active secondary metabolites are under investigation.

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