Chemical genetic effects of *Sargassum wightii* during embryonic development in zebrafish

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**ABSTRACT**

**Objective:** Phenotype based small molecule discovery is a category of chemical genetic study. The aim of this study was to observe the phytochemical based genetic effects of *Sargassum wightii* during organogenesis in embryonic zebrafish.

**Materials and Methods:** The phytomolecules from *S. wightii* were extracted using organic solvents and treated with the 24 h old developing zebrafish embryos. The active extract was partially purified by column chromatography, C₁₈ Sep-Pak column and reversed-phase high-performance liquid chromatography.

**Results:** Initially, cardiac bulging was found in 2 dpf to 3 dpf (days post fertilization), then bradycardia and tubular heart were observed in the next 8 h, which also showed the reduction in the heart beat rates. The phenotypic mutation effects of *bre*, *has*, *dou yan*, *heg* and *you* were observed in the 3 dpf and 4 dpf of the extract treated zebrafish embryos.

**Conclusions:** This study demonstrated that the phytomolecules from *S. wightii* exhibited potential molecular switches on the developmental process, which might have significant role in understanding the development based chemical genetic studies in zebrafish.

**KEY WORDS:** Chemical genetics, phytomolecules, small molecules screening, tubular heart, zebrafish

**Introduction**

Zebrafish based chemical genetic studies have identified a wealth of mutations to understand the developmental biology and functions. Chemical screens in the zebrafish model have identified small molecules which can modulate specific functions in developmental physiology and processes. Chemical genetics can achieve both forward and reverse approaches. Several prospective studies have shown the chemical genetic screening and phenotypic comparisons, manifesting chemical-specific endpoints of toxicity in a defined biological system.

Generally, zebrafish embryos are transparent which permits imaging of internal organs. The heart, which is the first organ system to develop with the similarity of the human heart by 3 weeks suitable for the organism-based small molecule discovery. This model system has attracted attention during the completion of large scale-screens for mutations affecting numerous aspects of embryonic development.

The brown seaweed *Sargassum* spp. has been used in traditional Chinese medicine to treat a variety of diseases and exhibits anticancer, anti-inflammatory, antimicrobial and antiviral activities. The present study was undertaken to investigate the phytochemical based genetic effects of *Sargassum wightii* during the organogenesis of embryonic zebrafish.

**Materials and Methods**

**Sample Preparation and Zebrafish Maintenance**

The seaweed, *S. wightii* was collected from Puttom coast of Arabian Sea, India and the sample was processed. 50 g of powderised sea weed was extracted based on the increasing polarity of solvents (hexane, chloroform, acetone, and methanol) using Soxhlet method. Zebrafishes were bred and maintained according to Westerfield in Fish Culture facility of International Centre for Nanobiotechnology, Centre for Marine Science and Technology, Manonmaniam Sundaranar University.

**Partial Purification of the Phytomolecules**

The hexane extract of *S. wightii* having cardio activity was fractioned by normal phase Silica gel (60–120 mesh) column chromatography and eluted with gradients of solvents from 10:1%
of benzene: Ethyl acetate to 1:10% of benzene: Ethyl acetate. The fractions with similar absorption maxima were pooled and eluted with C$_{18}$ Sep-Pak column using methanol and evaporated by vacuum concentrator (Eppendorf 5301). The elution was analyzed in ultraviolet-visible spectroscopy and reversed-phase high-performance liquid chromatography (RP-HPLC). 25 μL of the Sep-Pak column fraction (CF) was injected in RP-HPLC using acetonitrile: Water (6:4 ratio) as mobile phase for 1 mL/min flow rate at 220 nm detection using C$_{18}$ isocratic elution.[10]

Chemical Genetic and Phenotypic Evaluation

For the chemical genetic screening the crude and partially purified phytochemicals were treated to the 24 well plates containing four embryos per well in 1% dimethyl sulfoxide vehicle from 24 hpf (hours post fertilization) and incubated at 28°C. Chemical genetic effect was observed between 2 and 5 dpf under light microscope (Motic). The ventricular contractions and the heart beat rate (HBR)[8] were analyzed for quantitative physiological parameters of cardiovascular performance. Approval number for animal usage: MSU/Ethical/2009/5. Fish embryo toxicity test was carried out according to OECD[11] and the LC$_{50}$ values were determined using four parameter logistic curve analysis. Statistical analyses were carried out using SPSS software (SPSS Inc., Chicago).

Results

Phytochemicals mediated phenotypic characteristics in the developing embryos were observed in the crude extract and the partially purified phytochemicals with major peaks in the HPLC retention time of 2.12 and 2.27 respectively [Figure 1]. The phytochemicals generated a series of phenotypic changes resulting in massive pericardial bulging [Figure 2a-e] at 14 μg, and decrease in HBR was confirmed by several treatment assays and phenocopies. Exposure of the CF showed the rhythmicity as the beating rate of atrium and ventricle as 2:1 ratio (AV ratio). The CF treatment generated the phenotypic mutations, which shows phenocopies of the genetic zebrafish mutant you,[12] bre,[13] has[14] and heg[15] are shown in Figure 2 and Table 1. The atrial and ventricular regions were observed breakdance

Figure 1: High-performance liquid chromatography profile of partially purified extract column fraction from Sargassum wightii. The retention time of 2.12 and 2.27 shows the active component of the present study

Figure 2: Chemical genetic effect of Sargassum wightii in zebrafish embryos. (a) has mutation (curved body axis) and heg mutation (tubular heart formation) at 3 dpf. (b) Black arrow showing has mutation and white arrows shows dou yan mutation (reduction of eye size) at 4 dpf. (c) 3 dpf embryonic heart with pericardial bulging. (d) heg mutation, has mutation (black arrow), mouth protrudation and eye mutation at 4 dpf. (e) Magnified view of the 2c shows the tubular heart (white arrow) as single-cell – layered myocardium at 4 dpf. Black arrow shows the mouth protrudation in the 4 dpf embryos. (f) Control embryo at 3 dpf
mutant of zebrafish has been described as cardiac arrhythmia in which only every second atrial contraction is followed by a ventricular one.

After a 24-h treatment at 10 μg CF, a curvature of the body axis was observed and shown in Figure 2a, b, and d. It seems to be the disruption of the body axis patterning which is similar to heart and soul (has) mutation. However also visible defects in many tissues, including the eye, trunk, mouth and brain [Figure 2a, b, and d] were observed. Figure 2b shows the phenocopies of the reduction in the eye size in the embryo which is evident as dou yan genetic mutation. Tubular heart phenotype was observed in the GF treated embryos and a failure of the myocardium thickening was noticed in the developing embryo as like the heart of glass (heg) mutant and shown in Figure 2d and e, resulting the decrease of HBR with pericardial bulging and tubular heart.

Pericardial edema affected the HBR in the developing embryos in which the time duration for one cardiac cycle was found to be 2.6852 ± 0.160/s in the control and 1.6319 ± 0.060/s for the hexane extract of S. wightii at 10 μg/mL with the highly significant P = 0.0002. The HPLC fraction F3 showed 1.5972 ± 0.0601/s with highly significant P = 0.0002. Reduced heart rate leads to a visible reduction in blood flow was observed, which showed the property of you mutant. These findings suggest that during cardiogenesis of CF/phytochemicals affects the rhythmicity in blood flow at higher concentration with a determined LC50 value, 56.404 μg/mL (with 95% confident limit) showing 48.956 μg/mL lower limit and 63.657 μg/mL for upper limit.

Discussion

The larval zebrafish is a powerful genetic model system for organogenesis, whole organism phenotypic assays and high-throughput screening techniques. Small molecule (s) have proven to be valuable tools for cell biological studies, but their use in studies of development biology has been limited. The crude extract exhibited cardio genetic chemical effects in developing embryos at 3 dpf to 6 dpf, supporting these studies. Significance of cardiac physiology research revealed that drugs can cause depolarization abnormalities in humans were shown consistently to cause bradycardia and AV block in the zebrafish.[17] Evidencing by the study, following the phytochemical induced phenocopies of genetic mutation were reported in the present study.

Peterson et al.[18] screened 1,100 selected compounds in 96-well plates for small molecules that caused developmental phenotypes during the first 3 days of development. This was an important proof-of-concept study showing that small molecule screening in zebrafish could recognize chemicals that, like genetic mutations, interrupt specific developmental processes. Zebrafish heart consists of two major cell types (myocardium and endocardium) and two chambers (atrium and ventricle), and has faster development when compared with other vertebrate models.[19] In the present study, breakdance (bre) mutant[12] was observed as cardiac arrhythmia in which only every second atrial contraction is followed by a ventricular one.

Tubular heart phenotype evidence the previous studies by Chen et al.[13] which represent the end product of abnormal ion-channel function, which can result from chemical genetic mutations.[20] Similar effects were also observed for an anti-methicillin resistant Staphylococcus aureus molecule from a mangrove symbiont but did not affect the HBR and blood cell counting.[18] The observed CF induced heart of glass (heg) mutation in zebrafish cardiac muscle development forms the pericardial bulging. It supports the earlier study in which a single-cell – layered myocardium and a failure of the myocardium to thicken, hence the chambers dilate resulting in a massively enlarged heart.[13]

Conclusion

The above study on chemical genetics will help in the identification of any molecular switches during organogenesis for developmental biology research and therapeutic applications in any regenerative medicine and further studies on functional chemical genomic approaches in the model. This could be used as a tool for the small molecule based developmental reprogramming studies.

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